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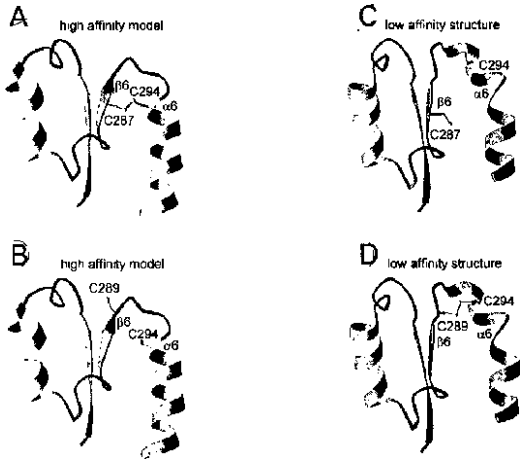
(54)【発明の名称】所望のコンホメーションで安定させた改変ポリペプチド及び該ポリペプチドの作製方法

(57)【要約】
本発明は、少なくとも一つのジスルフィド結合をポリペプチドに導入することにより、タンパク質を所望のコンホメーションで安定させる方法を提供する。コンピュータ・デザインを用いて、どの位置にシステイン残基を導入すれば、ある一種のタンパク質コンホメーションのみでジスルフィドが形成され、ひいてはこのタンパク質を一定のコンホメーションで固定できるかを、特定する。従って、所望のタンパク質コンホメーションに特異的な抗体及び小分子治療薬が選択される。さらに本発明は、所望のコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドも提供する。さらに本発明は、本発明の改変インテグリンＩ－ドメインを利用したスクリーニング検定及び治療法も提供する。



【特許請求の範囲】
【請求項１】
改変Ｉ－ドメインポリペプチドが所望のコンホメーションで安定するよう、少なくとも一つのジスルフィド結合を含有する改変インテグリンＩ－ドメインポリペプチド。
【請求項２】
開いたコンホメーションで安定させた、請求項１に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項３】
閉じたコンホメーションで安定させた、請求項１に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項４】
リガンドに高親和結合する、請求項２に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項５】
野生型配列に比較して少なくとも一つのシステイン置換を含有するアミノ酸配列にコードされた、請求項１に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項６】
システインに置換する残基のＣβカーボン同士間の距離が、３．００乃至８．０９オングストロームである、請求項２に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項７】
α１、α２、α１０、α１１、αＤ、αＥ、αＬ（ＣＤ１１ａ）、αＭ（ＣＤ１１ｂ）及びαＸ（ＣＤ１１ｃ）からなる群より選択されるインテグリンαサブユニットのＩ－ドメインから誘導される、請求項１に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項８】
ＬＦＡ－１のαＬサブユニットのＩ－ドメインから誘導される、請求項２に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項９】
ＬＦＡ－１のαＬサブユニットのＩ－ドメインから誘導される、請求項３に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１０】
Ｋ２８７Ｃ／Ｋ２９４Ｃ、Ｅ２８４Ｃ／Ｅ３０１Ｃ、Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃからなる群より選択されるアミノ酸置換を含有する、請求項７に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１１】
アミノ酸置換Ｌ２８９Ｃ／Ｋ２９４Ｃを含有する、請求項８に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１２】
Ｍａｃ－１のαＭサブユニットのＩ－ドメインから誘導される、請求項２に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１３】
Ｍａｃ－１のαＭサブユニットのＩ－ドメインから誘導される、請求項３に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１４】
Ｑ１６３Ｃ／Ｑ３０９Ｃ 及びＤ２９４Ｃ／Ｑ３１１Ｃからなる群より選択されるアミノ酸置換を含有する、請求項１２に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１５】
アミノ酸置換Ｑ１６３Ｃ／Ｒ３１３Ｃを含有する、請求項１３に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１６】
インテグリンαサブユニット内に包含される、請求項１に記載の改変インテグリンＩ－ド

メインポリペプチド。
【請求項１７】
インテグリンβサブユニットにさらに結合された、請求項１６に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１８】
可溶性のポリペプチドである、請求項１に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項１９】
異種のポリペプチドに作動的に連結された、請求項１に記載の改変インテグリンＩ－ドメインポリペプチド。
【請求項２０】
請求項１乃至１５のいずれかに定義された改変インテグリンＩ－ドメインポリペプチドをコードするヌクレオチド配列を含んで成る単離された核酸分子。
【請求項２１】
請求項２、３、４、５、７、８、９、１０、１１、１２、１３、１４、及び１５のいずれかに定義された改変インテグリンＩ－ドメインポリペプチドと、薬学的に許容可能な担体とを含んで成る組成物。
【請求項２２】
前記改変インテグリンＩ－ドメインポリペプチドが可溶性のポリペプチドである、請求項２０に記載の組成物。
【請求項２３】
抗炎症性もしくは免疫抑制性作用薬をさらに含んで成る、請求項２１に記載の組成物。
【請求項２４】
開いたコンホメーションのインテグリンＩ－ドメインに選択的に結合する抗体を生成する免疫原としての、請求項２に記載の改変インテグリンＩ－ドメインポリペプチドの使用。
【請求項２５】
開いたコンホメーションの改変インテグリンＩ－ドメインに選択的に結合する、抗体又はその抗原結合フラグメント。
【請求項２６】
インテグリンＩ－ドメイン上の活性化特異的エピトープに結合する、請求項２５に記載の抗体。
【請求項２７】
インテグリンとコグネイト・リガンドとの間の相互作用を遮断する、請求項２５に記載の抗体。
【請求項２８】
医薬組成物及び薬学的に許容可能な担体をさらに含んで成る、請求項２５に記載の抗体又はその抗原結合フラグメント。
【請求項２９】
前記抗体がＬＦＡ－１抗体である、請求項２５に記載の抗体又はその抗原結合フラグメント。
【請求項３０】
開いたコンホメーションのＬＦＡ－１　Ｉ－ドメインに選択的に結合する、抗ＬＦＡ－１抗体又はその抗原結合フラグメント。
【請求項３１】
前記抗ＬＦＡ－１抗体又はその抗原結合フラグメントが、改変ＬＦＡ－１　Ｉ－ドメインに選択的に結合する、請求項３０に記載のＬＦＡ－１抗体。
【請求項３２】
改変Ｉ－様ドメインポリペプチドが所望のコンホメーションで安定するよう、少なくとも一つのジスルフィド結合を含有する、改変インテグリンＩ－様ドメインポリペプチド。
【請求項３３】

開いたコンホメーションで安定させた、請求項３０に記載の改変インテグリンＩ－様ドメインポリペプチド。
【請求項３４】
リガンドに高親和結合する、請求項３１に記載の改変インテグリンＩ－様ドメインポリペプチド。
【請求項３５】
野生型配列に比較して少なくとも一つのシステイン置換を含有するアミノ酸配列にコードされた、請求項３０に記載の改変インテグリンＩ－様ドメインポリペプチド。
【請求項３６】
インテグリンβサブユニットのＩ－様ドメインから誘導される、請求項３０に記載の改変インテグリンＩ－様ドメインポリペプチド。
【請求項３７】
インテグリンβサブユニット内に包含される、請求項３０に記載の改変インテグリンＩ－様ドメインポリペプチド。
【請求項３８】
ポリペプチドが所望のコンホメーションで安定するよう、ポリペプチド内に少なくとも一つのジスルフィド結合を導入するステップを含む、ポリペプチドを所望のコンホメーションで安定させる方法。
【請求項３９】
前記ジスルフィド結合が、ポリペプチドのアミノ酸配列内に少なくとも一つのシステイン置換を導入することにより形成される、請求項３８に記載の方法。
【請求項４０】
システインに置換する残基中のＣβカーボン同士の間の距離が、３．００乃至８．０９オングストロームである、請求項３８に記載の方法。
【請求項４１】
前記ポリペプチドが、ある一個のタンパク質の機能的ドメインを含んで成る、請求項３８に記載の方法。
【請求項４２】
前記ポリペプチドがインテグリンＩ－ドメインを含んで成る、請求項４１に記載の方法。
【請求項４３】
前記ポリペプチドが、：インテグリンサブユニット、小型Ｇタンパク質、ヘテロ三量体Ｇタンパク質αサブユニット、チロシンキナーゼ、Ｇタンパク質共役受容体、アロステリックな調節を受ける酵素、チモーゲン、補体Ｃ３、補体Ｃ４、及びフィブリノーゲンからなるポリペプチド群より選択される、請求項３８に記載の方法。
【請求項４４】
（ａ）請求項２に記載の改変インテグリンＩ－ドメインポリペプチドを提供するステップと、
（ｂ）前記改変インテグリンＩ－ドメインポリペプチドをテスト化合物に接触させるステップと、
（ｃ）改変インテグリンＩ－ドメインポリペプチドへの、テスト化合物の結合能を検定することで、インテグリン活性のモジュレータを同定するステップと
を含む、インテグリン活性のモジュレータを同定する方法。
【請求項４５】
（ａ）請求項２に記載の改変インテグリンＩ－ドメインポリペプチドを提供するステップと、
（ｂ）テスト化合物の存在下及び非存在下で、インテグリンのリガンドに、前記改変インテグリンＩ－ドメインポリペプチドを接触させるステップと、
（ｃ）前記改変インテグリンＩ－ドメインポリペプチドと前記リガンドとの間の結合を検出することで、インテグリンとコグネイト・リガンドとの間の相互作用を修飾することのできる化合物を同定するステップと

を含む、インテグリンとコグネイト・リガンドとの間の相互作用を修飾することのできる化合物を同定する方法。

【請求項４６】

開いたコンホメーションで安定させた、治療上有効量の改変インテグリンＩ－ドメインポリペプチドを対象に投与することにより、対象のインテグリン関連疾患を治療又は予防するステップを含む、対象のインテグリン媒介疾患を治療又は予防する方法。

【請求項４７】

前記インテグリン媒介疾患が炎症性疾患である、請求項４６に記載の方法。

【請求項４８】

前記インテグリン媒介疾患が自己免疫疾患である、請求項４６に記載の方法。

【請求項４９】

開いたコンホメーションで安定させた、有効量の改変インテグリンＩ－ドメインポリペプチドを対象に投与することにより、対象におけるインテグリンとコグネイト・リガンドとの結合を阻害するステップを含む、対象においてインテグリンとコグネイト・リガンドとの結合を阻害する方法。

【請求項５０】

前記改変インテグリンＩ－ドメインポリペプチドがリガンドに高親和結合する、請求項４６及び４９のいずれか一方に記載の方法。

【請求項５１】

前記改変インテグリンＩ－ドメインポリペプチドが可溶性ポリペプチドである、請求項４６及び４９のいずれか一方に記載の方法。

【請求項５２】

前記改変インテグリンＩ－ドメインポリペプチドが、異種ポリペプチドに作動的に連結されている、請求項５０に記載の方法。

【請求項５３】

前記改変インテグリンＩ－ドメインポリペプチドが、αＬ Ｋ２８７Ｃ／Ｋ２９４Ｃ、αＬ Ｅ２８４Ｃ／Ｅ３０１Ｃ、αＬ Ｌ１６１Ｃ／Ｆ２９９Ｃ、αＬ Ｋ１６０Ｃ／Ｆ２９９Ｃ、αＬ Ｌ１６１Ｃ／Ｙ３００Ｃ、αＭ Ｑ１６３Ｃ／Ｑ３０９Ｃ 及びαＭ Ｄ２９４Ｃ／Ｑ３１１Ｃからなる群より選択される、請求項４６及び４９のいずれか一方に記載の方法。

【請求項５４】

開いたコンホメーションのインテグリンＩ－ドメインに選択的に結合する、治療上有効量の抗体又はその抗原結合フラグメントを対象に投与することにより、前記対象のインテグリン関連疾患を治療又は予防するステップを含む、対象においてインテグリン媒介疾患を治療又は予防する方法。

【請求項５５】

前記抗体が、改変インテグリンＩ－ドメイン又はその抗原結合フラグメントに結合する、請求項５４に記載の方法。

【請求項５６】

前記抗体がＬＦＡ－１抗体又はその抗原結合フラグメントである、請求項５４に記載の方法。

【請求項５７】

前記インテグリン媒介疾患が炎症性疾患である、請求項５４に記載の方法。

【請求項５８】

前記インテグリン媒介疾患が自己免疫疾患である、請求項５４に記載の方法。

【請求項５９】

開いたコンホメーションのインテグリンＩ－ドメインに選択的に結合する、治療上有効量の抗ＬＦＡ－１抗体又はその抗原結合フラグメントを対象に投与することにより、前記対象のインテグリン関連疾患を治療又は予防するステップを含む、対象においてインテグリン媒介疾患を治療する方法。

【請求項６０】
前記抗ＬＦＡ－１抗体が、改変ＬＦＡ－１　Ｉ－ドメイン又はその抗原結合フラグメントに結合する、請求項５９に記載の方法。
【請求項６１】
前記インテグリン媒介疾患が炎症性疾患である、請求項５９に記載の方法。
【請求項６２】
開いたコンホメーションのインテグリンＩ－ドメインに選択的に結合する、有効量の抗体又はその抗原結合フラグメントを対象に投与することにより、前記対象におけるインテグリンとコグネイト・リガンドとの間の結合を阻害するステップを含む、対象においてインテグリンとコグネイト・リガンドとの間の結合を阻害する方法。
【請求項６３】
前記抗体がＬＦＡ－１抗体又はその抗原結合フラグメントである、請求項６２に記載の方法。
【請求項６４】
前記抗体又はその抗原結合フラグメントが、インテグリンＩ－ドメイン上の活性化特異的エピトープに結合する、請求項５４、５９、又は６２のいずれかに記載の方法。
【請求項６５】
改変インテグリンＩ－ドメインポリペプチド又はその活性フラグメントをコードする核酸を有効量含んで成る、炎症性疾患の予防的もしくは治療的処置のためのワクチン製剤。
【請求項６６】
抗原性成分をさらに含んで成る、請求項６５に記載のワクチン製剤。
【請求項６７】
薬学的に許容可能な担体をさらに含んで成る、請求項６５に記載のワクチン製剤。
【請求項６８】
ベクタに挿入された、改変インテグリンＩ－ドメインポリペプチド又はその活性フラグメントをコードする核酸分子を対象に投与するステップを含む、前記対象のインテグリン媒介疾患を治療する方法。
【請求項６９】
前記核酸分子が静脈注射により対象に投与される、請求項６８に記載の方法。
【請求項７０】
前記核酸分子が抗原性成分をさらに含んで成る、請求項６８に記載の方法。
【請求項７１】
改変インテグリンＩ－ドメインポリペプチドをコードする核酸分子を含んで成る非ヒトトランスジェニック動物。
【請求項７２】
前記動物がマウスである、請求項７１に記載のトランスジェニック動物。
【発明の詳細な説明】
【０００１】
関連出願
本出願は、引用によってその全文をここに援用することとする、２０００年９月１日出願の米国暫定特許出願第６０／２２９，７００号に基づく優先権を主張するものである。
【０００２】
発明の背景
インテグリン・ファミリーの接着分子は非共有結合的に結合したα／βヘテロ二量体である。今日までのところ、少なくとも１４種類の異なるインテグリンαサブユニットと８種類の異なるβサブユニットが報告されている（Ｈｙｎｅｓ，ＲＯ（１９９２）Ｃｅｌｌ ６９：１－２５）。リンパ球機能関連抗原１（ＬＦＡ－１）は、白血球インテグリンサブファミリーのメンバーの一つである。白血球インテグリンサブファミリのメンバー間では、β２サブユニット（ＣＤ１８）は共通であるが、αサブユニットは異なっており、ＬＦＡ－１、Ｍａｃ－１、ｐ１５０．９５及びαｄ／β２はそれぞれ、αＬ（ＣＤ１１

ａ）、αＭ（ＣＤ１１ｂ）、αＸ（ＣＤ１１ｃ）及びαｄを有する（Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９０）Ｎａｔｕｒｅ３４６：４２５－４３３；Ｌａｒｓｏｎ，ＲＳ ａｎｄ Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９０）Ｉｍｍｕｎｏｌ Ｒｅｖ １１４：１８１－２１７；Ｖａｎ ｄｅｒ Ｖｉｅｒｅｎ，Ｍｅｔ ａｌ．（１９９５）Ｉｍｍｕｎｉｔｙ ３：６８３－６９０）。これら白血球インテグリンは正常な免疫性及び炎症性応答に必須な幅広い接着上の相互作用を媒介する。

【０００３】

インテグリンα及びβサブユニットは両者とも、タイプＩ膜貫通型糖タンパク質であり、それぞれ大きな細胞外ドメインと、一回膜貫通領域と、細胞質側の短い尾とを持つ。α及びβサブユニットの細胞外ドメインでは、構造上異なるドメインがいくつか、同定又は予測されてきた。

【０００４】

インテグリンαサブユニットのＮ末端領域は、それぞれ約６０個のアミノ酸が７回繰り返された部分を含有するが、これが折り畳まれて７枚の羽根のあるβ－プロペラドメインを形成していると予測されている（Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９７）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９４：６５－７２）。白血球インテグリンαサブユニットであるα１、α２、α１０、α１１、及びαＥサブユニットは、約２００個のアミノ酸から成る挿入ドメイン即ちＩ－ドメインを含有する（Ｌａｒｓｏｎ，ＲＳ ｅｔ ａｌ．（１９８９）Ｊ Ｃｅｌｌ Ｂｉｏｌ １０８：７０３－７１２；Ｔａｋａｄａ，Ｙ ｅｔ ａｌ．（１９８９）ＥＭＢＯ Ｊ ８：１３６１－１３６８；Ｂｒｉｅｓｅｗｉｔｚ，Ｒ ｅｔ ａｌ．（１９９３）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２６８：２９８９－２９９６；Ｓｈａｗ，ＳＫ ｅｔ ａｌ．（１９９４）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２６９：６０１６－６０２５；Ｃａｍｐｅｒ，Ｌ ｅｔ ａｌ．（１９９８）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７３：２０３８３－２０３８９）。このＩ－ドメインは、β－プロペラドメインのβシート２と３との間に挿入されていると予測されている。αＭ、αＬ、α１及びα２のＩ－ドメインの三次元構造が解明されており、これが、金属イオン依存性接着部位（ＭＩＤＡＳ）と呼ばれる特有の二価陽イオン配位部位を持つジヌクレオチド結合折り畳み構造を採っていることが示されている（Ｌｅｅ，Ｊ－Ｏ，ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３：１３３３－１３４０；Ｌｅｅ，Ｊ－Ｏ，ｅｔ ａｌ．（１９９Ｓ）Ｃｅｌｌ ８０：６３１－６３８；Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９５）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９２：１０２７７－１０２８１；Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９６）Ｓｔｒｕｃｔｕｒｅ ４：９３１－９４２；Ｅｍｓｌｅｙ，Ｊ ｅｔ ａｌ．（１９９７）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７２：２８５１２－２８５１７；Ｂａｌｄｗｉｎ，ＥＴ ｅｔ ａｌ．（１９９８）Ｓｔｒｕｃｔｕｒｅ ６：９２３－９３５；Ｋａｌｌｅｎ，Ｊ ｅｔ ａｌ．（１９９９）Ｊ Ｍｏｌ Ｂｉｏｌ ２９２：１－９）。αＭサブユニットのＣ末端領域は、βサンドイッチ構造に折り畳まれていると予測されている（Ｌｕ，Ｃ ｅｔ ａｌ．（１９９８）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７３：１５１３８－１５１４７）。

【０００５】

インテグリンβサブユニットは、約２５０個のアミノ酸から成る保存ドメインをＮ末端部分に、そしてシステイン－リッチな領域をＣ末端部分に、含有する。β保存ドメイン、又はＩ様ドメインは、「Ｉドメインに似た」折り畳み構造を有していると予測されている（Ｐｕｚｏｎ－ＭｃＬａｕｇｈｌｉｎ，Ｗ ａｎｄ Ｔａｋａｄａ，Ｙ（１９９６）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７１：２０４３８－２０４４３；Ｔｕｃｋｗｅｌｌ，ＤＳ ａｎｄ Ｈｕｍｐｈｒｉｅｓ，ＭＪ（１９９７）ＦＥＢＳ Ｌｅｔｔ ４００：２９７－３０３；Ｈｕａｎｇ，Ｃ ｅｔ ａｌ．（２０００）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７５：２１５１４－２４）。βサブユニットのＣ末端にあるシステイン・リッチな領域は、β１、β２及びβ３サブユニットに対する数多くの活性化抗体がこの領域に結合することから考えても、インテグリンの機能調節に重要であると思われる（Ｐｅｔｒｕｚｚｅｌｌｉ，Ｌ ｅｔ ａｌ．（１９９５）Ｊ Ｉｍｍｕｎｏｌ １５５：８５４－８６６；Ｒｏｂｉ

nson, MK et al.（１９９２）J Immunol １４８：１０８０－１０８５；Faull, RJ et al.（１９９６）J Biol Chem ２７１：２５０９９－２５１０６；Shih, DT et al.（１９９３）J Cell Biol １２２：１３６１－１３７１；Du, X et al.（１９９３）J Biol Chem ２６８：２３０８７－２３０９２）。

【０００６】

インテグリンの電子顕微鏡像からは、α及びβサブユニットのＮ末端部分同士が折り畳まれて球形の頭部を形成し、この頭部が、α及びβ細胞外ドメインのＣ末端部分に相当する長さ約１６ｎｍの二本の棹状の尾によって、膜に繋がれていることが分かる（Ｎermut, MV et al.（１９８８），EMBO J ７：４０９３－４０９９；Weisel, JW et al.（１９９２）J Biol Chem ２６７：１６６３７－１６６４３；Wippler, J et al.（１９９４）J Biol Chem ２６９：８７５４－８７６１）。

【０００７】

ＬＦＡ－１はあらゆる白血球上に発現し、三種類のＩｇスーパーファミリーのメンバーである細胞間接着分子１、２及び３の受容体である（Marlin, SD et al.（１９８７）Cell ５１：８１３－８１９；Staunton, DE et al.（１９８９）Nature ３３９：６１－６４；de Fougerolles, et al.（１９９１）J Exp Med １７４：２５３－２６７）。多くのデータが、ＬＦＡ－１のＩ－ドメインがリガンドとの相互作用にとって重要であることを示している。変異誘発実験では、Ｉ－ドメイン内の残基Ｍ１４０、Ｅ１４６、Ｔ１７５、Ｌ２０５、Ｅ２４１，Ｔ２４３、Ｓ２４５及びＫ２６３が、リガンド結合にとって重要であることが示唆された（Huang, C et al.（１９９５）J Biol Chem ２７０：１９００８－１９０１６；Edwards, CP et al.（１９９８）J Biol Chem ２７３：２８９３７－２８９４４）。これらの残基はＩ－ドメイン表面上に位置してMg^{2+}イオンを取り囲み、Ｉ－ドメインの上側表面上でリガンド結合界面を規定している。リガンド結合におけるこのようなＩ－ドメインの重要性は、さらにｍＡｂ遮断実験でも明らかにされている。ＬＦＡ－１の、そのリガンドとの相互作用を阻害するｍＡｂの多くはＩ－ドメインの位置に来る（Randi, AM et al.（１９９４）J Biol Chem ２６９：１２３９５－１２３９８；Champe, M et al.（１９９５）J Biol Chem ２７０：１３８８－１３９４；Huang, C et al.（１９９５）J Biol Chem ２７０：１９００８－１９０１６；Edwards, CP et al.（１９９８）J Biol Chem ２７３：２８９３７－２８９４４）。二つのグループが最近、Ｉ－ドメインを削除したＬＦＡ－１はリガンド認識能及び結合能を欠いていることを示しており、ＬＦＡ－１機能におけるＩ－ドメインの役割がさらに実証された（Leitinger, B et al.（２０００）Mol Biol Cell １１，６７７－６９０；Yalamanchili, P et al.（２０００）J Biol Chem ２７５：２１８７７－８２）。Ｉ－ドメインを含有する他のインテグリンのＩ－ドメインも、リガンド結合における関与が指摘されている（Diamond, MS（１９９３）J Cell Biol １２０：５４５５５６；Michishita, M et al.（１９９３）Cell ７２：８５７－８６７；Muchowski, PJ et al.（１９９４）J Biol Chem ２６９：２６４１９－２６４２３；Zhou, L et al.（１９９４）J Biol Chem ２６９：１７０７５－１７０７９；Ueda, T et al.（１９９４）Proc Natl Acad Sci USA ９１：１０６８０－１０６８４；Kamata, T et al.（１９９４）J Biol Chem ２６９：９６５９９６６３；Kern, A et al.（１９９４）J Biol Chem ２６９：２２８１１－２２８１６）。

【０００８】

ＬＦＡ－１がＩＣＡＭに結合するには、ＬＦＡ－１の活性化が必要である。ＬＦＡ－１は

、「インサイド・アウト」シグナリングと呼ばれる細胞質からのシグナルによって活性化する（Ｄｉａｍｏｎｄ，ＭＳ ｅｔ ａｌ．（１９９４）Ｃｕｒｒｅｎｔ Ｂｉｏｌｏｇｙ ４：５０６－５１７）。二価の陽イオンMn^{2+}、Mg^{2+}及びCa^{2+}は、ＬＦＡ－１のリガンド結合機能を直接修飾することができる（Ｄｒａｎｓｆｉｅｌｄ，Ｉ ｅｔ ａｌ．（１９８９）ＥＭＢＯ Ｊ ８：３７５９－３７６５；Ｄｒａｎｓｆｉｅｌｄ，Ｉ ｅｔ ａｌ．（１９９２）．Ｊ Ｃｅｌｌ Ｂｉｏｌ １１６：２１９－２２６；Ｓｔｅｗａｒｔ，ＭＰ ｅｔ ａｌ．（１９９６）Ｊ Ｉｍｍｕｎｏｌ １５６：１８１０－１８１７）。加えて、ＬＦＡ－１ は、αＬ又はβ２サブユニットの細胞外ドメインに結合するいくつかのｍＡｂによっても活性化する（Ｋｅｉｚｅｒ，ＧＤ ｅｔ ａｌ．（１９８８）Ｊ Ｉｍｍｕｎｏｌ １４０：１３９３－ １４００；Ｒｏｂｉｎｓｏｎ，ＭＫ ｅｔ ａｌ．（１９９２）Ｊ Ｉｍｍｕｎｏｌ １４８：１０８０－１０８５；Ａｎｄｒｅｗ，Ｄ ｅｔ ａｌ．（１９９３）Ｅｕｒ Ｊ Ｉｍｍｕｎｏｌ ２３：２２１７－２２２２；Ｐｅｔｒｕｚｚｅｌｌｉ，Ｌ ｅｔ ａｌ．（１９９５）Ｊ Ｉｍｍｕｎｏｌ １５５：８５４－８６６）。インテグリン活性化の分子機序はまだ余り理解が進んではいない。インテグリン活性化を伴うような分子内のコンホメーション上の変化が起きると、リガンドに対するインテグリンの親和性が増すことが提唱されているが、この考えは、活性化後のインテグリンしか認識しない抗体が存在することで裏付けられている（Ｄｒａｎｓｆｉｅｌｄ，Ｉ ｅｔ ａｌ．（１９８９）ＥＭＢＯ Ｊ ８：３７５９－３７６５；Ｄｉａｍｏｎｄ，ＭＳ ｅｔ ａｌ．（１９９３）Ｊ Ｃｅｌｌ Ｂｉｏｌ １２０：５４５－５５６；Ｓｈａｔｔｉｌ，ＳＪ ｅｔ ａｌ．（１９８５）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２６０：１１１０７－１１１１４）。このような抗体の一つであるＣＢＲＬＦＡ－１／５ は、リガンド結合部位に非常に近いＭａｃ－１ Ｉ－ドメインに結合する（Ｏｘｖｉｇ，Ｃ ｅｔ ａｌ．（１９９９）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９６：２２１５－２２２０）が、このことは、Ｉ－ドメイン自体が、活性化によってコンホメーション上の変化を起こすことの更なる証拠である。

【０００９】

異なる二つの形の結晶型Ｍａｃ－１ Ｉ－ドメインが得られており、これら二つの構造はそれぞれ「活性」及び「不活性」コンホメーションではないかとの仮説が立てられた（Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３，１３３３－１３４０；Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｃｅｌｌ ８０：６３１－６３８）。Mg^{2+}と一緒に結晶化した「活性」型では、隣のＩ－ドメイン由来のグルタミン酸が６番目の金属配位部位となっているが、Mn^{2+}と錯体形成した「不活性」型コンホメーションでは、一個の水分子が金属配位領域を完成させる。このような金属配位の変化は、Ｃ末端αヘリックスの大幅なシフトに関係がある。即ち、推定「活性」コンホメーションでは、Ｃ末端ヘリックスはＩ－ドメイン本体の１０オングストローム下に移動する（Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３：１３３３－１３４０）。活性化Ｍａｃ－１しか認識しないｍＡｂ ＣＢＲＭ－１／５をエピトープ・マッピングした結果、このコンホメーション上の違いは生理学的なものであることが示唆された（Ｏｘｖｉｇ，Ｃ ｅｔ ａｌ．（１９９９）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９６：２２１５－２２２０）。ＬＦＡ－１ Ｉ－ドメインの結晶構造及びＮＭＲ構造の有するコンホメーションは、Ｍａｃ１ Ｉ－ドメインの推定「不活性」コンホメーションに類似である（Ｑｕ，Ａ ｅｔ ａｌ．（１９９５）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９２：１０２７７－１０２８１；Ｑｕ，Ａ（１９９６）Ｓｔｒｕｃｔｕｒｅ ４：，９３１－９４２；Ｋａｌｌｅｎ，Ｊ ｅｔ ａｌ．（１９９９）Ｊ Ｍｏｌ Ｂｉｏｌ ２９２：１－９；Ｌｅｇｇｅ，ＧＢ ｅｔ ａｌ．（２０００）Ｊ Ｍｏｌ Ｂｉｏｌ ２９５：１２５１－１２６４）。

【００１０】

インテグリンに加え、薬学的に重要な多くのタンパク質は、コンホメーション又はコンホーマと呼ばれる二つの代替的な三次元構造で存在する。これらのタンパク質は、しばしば、例えば小型Ｇタンパク質、三量体Ｇタンパク質αサブユニット、チロシンキナーゼ、及

びＧタンパク質共役受容体など、重要なシグナリング機能を有する。典型的には、これらのコンホメーションのうちの一つに酵素活性又はエフェクター機能があって、他のものにはない。従って、例えば活性コンホメーションなど、ある特定のコンホメーションになったタンパク質に特異的な抗体又は小分子治療薬は、非選択的なものに比べて、大きな長所を有するであろう。

【００１１】

発明の概要

コンピュータ・デザインを利用して、あるタンパク質又はポリペプチドに、その分子が所望のコンホメーションで安定するようジスルフィド結合を導入することができる。従って、「開いた」もしくは活性コンホメーション、又は、「閉じた」もしくは不活性コンホメーションなど、ある所望のタンパク質コンホメーションに特異的な、抗ＬＦＡ－１抗体などの抗体又は小分子治療薬を同定することができる。

【００１２】

本発明は、あるタンパク質の機能ドメインを含むポリペプチドなどのポリペプチドを、所望のコンホメーションで安定させる方法に関するものである。本方法は、ポリペプチドが所望のコンホメーションで安定するよう、少なくとも一つのジスルフィド結合を当該ポリペプチドに導入するステップを含む。一の好適な実施態様では、前記ジスルフィド結合は、ポリペプチドのアミノ酸配列に少なくとも一つのシステイン置換を導入することにより、形成される。別の実施態様では、前記ジスルフィド結合のＣβ炭素間の距離が、３．００乃至８．０９オングストロームの範囲内である。別の実施態様では、前記ジスルフィド結合のＣβ炭素間の距離が、３．４１乃至７．０８オングストロームの範囲内である。

【００１３】

コンピュータ・デザインを利用して、あるタンパク質又はポリペプチドに、その分子が所望のコンホメーションで安定するようジスルフィド結合を導入することができる。従って、ある所望のタンパク質コンホメーションに特異的な抗体又は小分子治療薬を同定することができる。

【００１４】

本発明の方法は、インテグリンサブユニット、小型Ｇタンパク質、ヘテロ三量体Ｇタンパク質アルファサブユニット、チロシンキナーゼ、Ｇタンパク質共役受容体、アロステリック調節を受ける酵素、チモーゲン、補体Ｃ３、補体Ｃ４、及びフィブリノーゲンを含め、二つの異なる三次元コンホメーションで存在する多種の生物学的かつ薬学的に重要なタンパク質に幅広く応用できる。ある好適な実施態様では、当該ポリペプチドはインテグリンＩ－ドメインポリペプチドである。

【００１５】

別の局面では、本発明は、少なくとも一つのジスルフィド結合を導入することによって所望のコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドを提供する。実施態様の一つでは、改変インテグリンＩ－ドメインは、ジスルフィド結合が形成されるよう野生型配列に比較して少なくとも一つのシステイン置換を含有したアミノ酸配列にコードされている。別の実施態様では、システインに置換する残基のＣβ炭素間の距離は、３．００乃至８．０９オングストロームの範囲内である。さらに別の実施態様では、ジスルフィド結合中のＣβ炭素間の距離は、３．４１乃至７．０８オングストロームの範囲内である。

【００１６】

一の実施態様では、本発明の改変インテグリンＩ－ドメインポリペプチドは、例えばα１、α２、α１０、α１１、αＤ、αＥ、αＬ（ＣＤ１１ａ）、αＭ（ＣＤ１１ｂ）、及びαＸ（ＣＤ１１ｃ）など、インテグリンαサブユニットのＩ－ドメインから誘導される。例えば、本発明の一実施態様では、改変インテグリンＩ－ドメインポリペプチドは、ヒトαＬサブユニットのＩ－ドメインから誘導され、アミノ酸置換Ｋ２８７Ｃ／Ｋ２９４Ｃ、Ｅ２８４Ｃ／Ｅ３０１Ｃ、Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、Ｌ１６１Ｃ／Ｔ３００Ｃ、又はＬ２８９Ｃ／Ｋ２９４Ｃを含有する。本発明の別の実施態様では、

改変インテグリンＩ－ドメインポリペプチドは、ヒトαＭサブユニットのＩ－ドメインから誘導され、アミノ酸置換 Ｑ１６３Ｃ／Ｑ３０９Ｃ、Ｄ２９４Ｃ／Ｑ３１１Ｃ、又はＱ１６３Ｃ／Ｒ３１３Ｃを含有する。

【００１７】

一の好適な実施態様では、本発明の改変インテグリンＩ－ドメインポリペプチドは、開いたコンホメーションで安定している。別の実施態様では、本発明の改変インテグリンＩ－ドメインポリペプチドは、閉じたコンホメーションで安定している。別の実施態様では、改変インテグリンＩ－ドメインは、リガンドに高親和結合する。さらに別の実施態様では、改変インテグリンＩ－ドメインポリペプチドは、異種ポリペプチドに作動的に連結している。

【００１８】

関連する一の局面では、本発明は、本発明の改変インテグリンＩ－ドメインポリペプチドをコードする単離された核酸分子を提供する。

【００１９】

本改変インテグリンＩ－ドメインポリペプチド、及び／又は、その生物学的活性もしくは抗原性を有するフラグメントは、例えば、インテグリンが媒介する疾患の治療及び／又は診断に利用できる検定用試薬又は標的などとして、有用である。

【００２０】

従って、ある局面では、本発明は、開いたコンホメーションの改変インテグリンＩ－ドメインに選択的に結合する抗体又はその抗原結合フラグメントを提供する。別の局面では、本発明は、開いたコンホメーションのインテグリンＩ－ドメインポリペプチド、閉じたコンホメーションのインテグリンＩ－ドメインポリペプチド、又は、改変インテグリンＩ－ドメインポリペプチド、に選択的に結合する抗体又はその抗原結合フラグメントを提供する。一の実施態様では、本抗体は、インテグリンＩ－ドメイン上にある活性化特異的エピトープに結合する。別の実施態様では、本抗体はインテグリンとコグネイト・リガンドとの間の相互作用を阻害するものである。実施態様の一つでは、本抗体は、開いたもしくは閉じたコンホメーションのＬＦＡ－１ポリペプチド、又は改変ＬＦＡ－１ Ｉ－ドメインインテグリンポリペプチド、又はそのフラグメント、と反応又は結合する抗ＬＦＡ－１抗体など、抗ＬＦＡ－１抗体又はその抗原結合フラグメントである。

【００２１】

別の局面では、本発明は、開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドに対するテスト化合物の結合能を検定するステップを含む、インテグリン活性のモジュレータを同定する方法を提供するものである。別の実施態様では、本発明は、インテグリンとコグネイト・リガンドとの間の相互作用を修飾することのできる化合物を同定する方法を提供し、この方法では、開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドに対するリガンドの結合が、テスト化合物の存在下及び非存在下で検定される。

【００２２】

別の局面では、本発明は、改変インテグリンＩ－ドメインポリペプチド又は抗インテグリンＩ－ドメイン抗体（又はその抗原結合フラグメント）を含んで成る組成物を提供するものであり、このような組成物には、さらに薬学的に許容可能な担体を含めることができる。

【００２３】

さらに別の局面では、本発明は、対象においてインテグリン媒介疾患（例えば炎症性又は自己免疫疾患）を治療又は予防する方法、又は、対象においてコグネイト・リガンドに対するインテグリンの結合を阻害する方法、に関するものであり、このとき本方法は、開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチド、又は、開いたコンホメーションのインテグリンＩ－ドメインに選択的に結合する抗体（又はその抗原結合フラグメント）を、対象に治療上有効量、投与するステップを含む。一実施態様では、本抗体は、開いたコンホメーションのＬＦＡ－１ Ｉ－ドメインと特異的に反応もし

くは結合する、又は、改変ＬＦＡ－１　Ｉ－ドメインポリペプチドと特異的に反応もしくは結合する、ＬＦＡ－１抗体又はその抗原結合フラグメントである。ある好適な実施態様では、本改変インテグリンＩ－ドメインポリペプチドは、リガンドに高親和結合する。別の好適な実施態様では、治療用の本改変インテグリンＩ－ドメインポリペプチドは、例えば融合タンパク質などの可溶性ポリペプチドである。

【００２４】

本発明の他の特徴及び長所は、以下の詳細な説明及び請求項から明白となるであろう。

【００２５】

発明の詳細な説明

本発明は、少なくとも部分的には、あるポリペプチドに少なくとも一つのジスルフィド結合を導入することにより、前記ポリペプチドを所望のコンホメーションで安定させる方法に基づくものである。一実施態様では、特定のタンパク質コンホメーションのＮＭＲ又は結晶構造に基づき、コンピュータ・デザインを利用して、前記タンパク質を特定のコンホメーションで固定するようなジスルフィド結合を導入する。ここで用いる「コンホメーション」又は「コンホーマ」とは、タンパク質の三次元構造を言う。「所望の」コンホメーションには、当該ポリペプチドの特定の使用に役立つようなタンパク質コンホメーションがあり、例えば、特定の生物学的機能及び／又は活性、又は治療効果を支えるようなコンホメーションである。ここで用いる用語「ポリペプチド」及び「タンパク質」は全体的に交換可能に用いられている。

【００２６】

ある実施態様では、所望のコンホメーションは、例えば開いたもしくは活性なコンホメーションなど、生物学的機能及び／又は活性を促進又は活性化するようなタンパク質コンホメーションである。別の実施態様では、所望のコンホメーションとは、例えば閉じた又は不活性のコンホメーションなど、生物学的機能及び／又は活性を阻害又は抑制するようなタンパク質コンホメーションである。

【００２７】

具体的には、本発明の方法は、あるタンパク質又はその機能ドメインを、前記タンパク質の所望の三次元構造のテンプレート上でモデル化するステップと、所望のコンホメーションの前記タンパク質上でのみ、ジスルフィド結合を形成できるシステインを導入することで、その特定のコンホメーションで前記タンパク質を安定させるステップと、を含む。ここでのタンパク質は、三次元構造が公知であるか、又は生成できれば、いかなるタンパク質又はそのドメインでもよいが、好ましくは、二つの異なるコンホメーションで存在するタンパク質であるとよい。タンパク質コンホメーションをデザイン及び／又はモデル化するためのコンピュータ・アルゴリズムは、例えばＷＯ　９８／４７０８９に記載されている。ＳＳＢＯＮＤ プログラム（Ｈａｚｅｓ，Ｂ ａｎｄ Ｄｉｊｋｓｔｒａ，ＢＷ（１９８８）Ｐｒｏｔｅｉｎ Ｅｎｇｉｎｅｅｒｉｎｇ ２：１１９－１２５）を用いると、適当な位置にある残基対をシステインに変異させることで、あるタンパク質構造にジスルフィド結合を導入できる位置を特定することができる。

【００２８】

ジスルフィド結合の形成は、あるポリペプチドの三次元構造内で適当な位置にある二つのシステイン残基同士の間で起きる。本発明の一実施態様では、ジスルフィド結合が形成されるよう、少なくとも一つのシステイン置換をアミノ酸配列に導入することにより、ポリペプチドを所望のコンホメーションで安定させる。ポリペプチドの天然アミノ酸配列中に、ジスルフィド結合を形成するのに適当な位置に既に一つのシステイン残基が存在している場合には、一箇所においてシステイン置換が行われる。好適な一実施態様では、ポリペプチドのアミノ酸配列中、ジスルフィド結合の形成が可能な位置で、二箇所のシステイン置換を導入して、このポリペプチドを所望のコンホメーションで安定させる。別の実施態様では、システインに置換する残基のＣβ炭素間の距離は３．００乃至８．０９オングストロームである。さらに別の実施態様では、当該ジスルフィド結合中のＣβ炭素間の距離は３．４１乃至７．０８オングストロームの範囲内である。

【００２９】
本発明の一実施態様では、タンパク質が特定のコンホメーションで安定するようにジスルフィド結合の形成が一のタンパク質コンホメーションでのみ優先的に起きるよう、システイン置換を導入する。
【００３０】
システイン置換の導入による本発明の改変ポリペプチドの調製は、好ましくは、目的のポリペプチド（例えばインテグリンポリペプチド）をコードするＤＮＡの変異誘発によって行われる。例えば、コードされたタンパク質に、一箇所又はそれ以上のアミノ酸置換、例えばシステイン置換、が導入されるよう、インテグリン遺伝子のヌクレオチド配列に一箇所又はそれ以上のヌクレオチド置換を導入することで、改変インテグリンＩ－ドメインポリペプチドをコードする単離された核酸分子を作製することができる。例えば部位指定変異誘発法及びＰＣＲ媒介変異誘発法など、標準的な技術により、変異を核酸配列に導入することができる。
【００３１】
適したタンパク質には、限定はしないが、産業上及び治療上重要なタンパク質、例えば１）小型Ｇタンパク質、三量体Ｇタンパク質アルファサブユニット、チロシンキナーゼ、及びＧタンパク質共役受容体などのシグナリング分子、２）アロステリック調節を受ける酵素、３）タンパク質開裂による活性化後にコンホメーションが変化するチモーゲン、例えば補体及び凝固カスケードのプロテアーゼ（コンバターゼ及び因子）及び４）補体成分Ｃ３及びＣ４やフィブリノーゲンなどのタンパク質分解により活性化するエフェクター分子、がある。一の実施態様では、例えば「開いた」コンホメーションなど、酵素活性があるか、又は、リガンド結合能及び／又はエフェクター機能を有するようなコンホメーションなど、生物学的に活性なコンホメーションでタンパク質を安定させることに本発明の方法を利用できる。別の実施態様では、 例えば「閉じた」コンホメーションなど、酵素として不活性であるか、又は、リガンド結合能及び／又はエフェクター機能を持たないようなコンホメーションなど、生物学的に不活性なコンホメーションでタンパク質を安定させることに本発明の方法を利用できる。
【００３２】
特定のコンホメーションで安定させたタンパク質は、例えば、プロテオーム・スクリーニング・テクノロジーなどに、用途があるであろう。組織及び疾患状態のプロテオーム・スクリーニングでは、活性なタンパク質コンホーマ又は不活性なタンパク質コンホーマなどに特異的な抗体、ポリペプチド及び／又は小分子を、様々な細胞シグナリング経路、代謝経路及び接着経路の活動を評価するのに利用できる。こうして、特定の疾患と、特定の生化学的経路及びシグナリング経路との間の関連性を見出すことができる。さらに本発明は、ここに解説した方法を用いて同定されたポリペプチド、抗体、及び小分子や、炎症性疾患などを治療するなど、同ポリペプチド、抗体、及び小分子の用途にも関するものである。コンホーマ特異的な試薬をチップに載せて、組織抽出物をスクリーニングするのに用いたり、又は組織切片を染色するのにも利用できる。さらに、開いたコンホーマ又は閉じたコンホーマなどの特定のコンホーマに対して選択的な薬物又は抗体、例えば改変ＬＦＡ－１ Ｉ－ドメインポリペプチドを特異的に認識する抗ＬＦＡ－１抗体などの改変インテグリンＩ－ドメインポリペプチドを特異的に認識する抗インテグリン抗体など、は、示差的な治療効果をもたらすであろう。従って、特定のコンホーマで安定させたタンパク質を用いた選択的スクリーニングアッセイを利用すれば、所望の活性を持つ化合物を合理的に得ることができる。
【００３３】
インテグリン
インテグリンは、リガンドを結合させない不活性なコンホメーションで細胞表面上に存在する。細胞が活性化すると、インテグリンは形（コンホメーション）を変えてリガンドを結合させるようになる。体内のすべての細胞で、選択的に発現する様々なインテグリンヘテロ二量体（α及びβサブユニットの組合せが異なる）は２０種を越える。活性化後、イ

ンテグリンは、細胞外マトリックス中の、又は、凝固もしくは補体カスケード中で凝集した他の細胞表面上のタンパク質リガンドに特異的に結合する。白血球上のインテグリンは、白血球の遊出や炎症及び免疫応答において中心的な重要性を持つ。 白血球インテグリンＭａｃ－１ （αＭβ２）のリガンドには、炎症関連細胞表面分子ＩＣＡＭ－１、補体成分ｉＣ３ｂ、及び凝固成分フィブリノーゲン、がある。白血球インテグリンＬＦＡ－１ （αＬβ２）のリガンドには、ＩＣＡＭ－１、ＩＣＡＭ－２、及びＩＣＡＭ－３、がある。白血球インテグリンに対する抗体は、細胞対細胞の相互作用や細胞対細胞外マトリックスの相互作用を、阻害するなど修飾するなどにより、多種の炎症性疾患及び自己免疫疾患を遮断することができる。血小板上のインテグリンは凝固及び心疾患で重要であり、認可薬には、抗体アブシキシマブ（Ｒｅｏｐｒｏ^{TM}） 及びペプチド様アンタゴニスト、エプティフィバチド（Ｉｎｔｅｇｒｉｌｉｎ^{TM}）がある。結合組織細胞、上皮細胞及び内皮細胞上のインテグリンは、これらの細胞に影響する疾患状態で重要である。これらは細胞成長、分化、創傷治癒、線維症、アポトーシス、及び脈管形成を調節する。癌細胞上のインテグリンは、浸潤及び転移を調節する。

【００３４】

インテグリンに拮抗するには、活性化したリガンド結合性コンホメーションに結合する薬物が必要である。大半の抗体は活性及び不活性のコンホメーションの両方に結合する。なぜなら、インテグリン分子の表面上のごく小さな部分しか形が変わらないからである。不活性なコンホメーションに結合すると、副作用が起きたり、抗イディオタイプ抗体が生成されたり、抗体のクリアランスが起きて、投与量を増やしたりしなければならないため、「開いた」コンホメーションなどの活性なインテグリンコンホメーションにのみ、抗体が結合するのが好ましい。

【００３５】

ここに記載した方法は、ジスルフィド結合を、ＬＦＡ－１及びＭａｃ－１などのインテグリンのＩドメインに導入するのに用いられ、成功を収めている。従って、別の局面では、本発明は、改変Ｉ－ドメインポリペプチドが所望のコンホメーションで安定するように少なくとも一つのジスルフィド結合を含有する改変インテグリンＩ－ドメインポリペプチドを提供するものである。本発明の改変インテグリンＩ－ドメインポリペプチドは、α１、α２、α１０、α１１、αＤ、αＥ、αＬ（ＣＤ１１ａ）、αＭ（ＣＤ１１ｂ）及びαＸ（ＣＤ１１ｃ）などのインテグリンαサブユニットのＩ－ドメインから誘導することができる。

【００３６】

ここで用いる「改変インテグリンＩ－ドメインポリペプチド」又は「改変インテグリンポリペプチド」には、少なくとも一つのジスルフィド結合が当該ポリペプチドに導入されたことで、当該Ｉ－ドメインが所望のコンホメーションで安定しているように、野生型配列、即ち天然状態に比べて変更されたインテグリンＩ－ドメインポリペプチドが含まれる。

【００３７】

ここで交換可能に用いられた用語「誘導された」又は「誘導体」とは、ある配列が、天然に存在する配列などの別の配列と同一であるか、又は、このような別の配列から改変されていることを意味することを意図している。本発明の範囲内の誘導体には、ポリヌクレオチド誘導体及びポリペプチド誘導体が含まれる。ポリペプチド誘導体又はタンパク質誘導体には、アミノ酸配列が記述済みもしくは公知であるか、又は配列に関係しない方法で記述済もしくは公知である、あるいはこれらの両方である、ような配列とは異なるが、当該ポリペプチド又はタンパク質の活性が残っているようなポリペプチド又はタンパク質配列が含まれる。一つ又はそれ以上のアミノ酸を異なる天然アミノ酸、アミノ酸誘導体又は非天然アミノ酸に置換した場合、アミノ酸配列での誘導体が生成される。いくつかの実施態様では、タンパク質誘導体には、一つ又はそれ以上の保存的アミノ酸置換がある点で、野生型配列とは配列が異なるが、当該タンパク質又はペプチドの二次構造又は疎水性に与えた影響は概ね最小限であるような天然発生型のポリペプチドもしくはタンパク質又はその生物学的に活性なフラグメントが包含される。さらに誘導体は、当該ポリペプチド又はタ

ンパク質の生物学的活性を損なわないような一つ又はそれ以上の非保存的アミノ酸置換、欠失又は挿入がある点で異なる配列を含んでいてもよい。

【００３８】

保存的置換（置換基）には、典型的に、類似の特徴（例えば電荷、大きさ、形状、及び他の生物学的性質）を持つ別のアミノ酸に一個のアミノ酸を置換することが含まれる。例えば以下のグループ：バリン、グリシン；グリシン、アラニン；バリン、イソロイシン；アスパラギン酸、グルタミン酸；アスパラギン、グルタミン；セリン、スレオニン；リシン、アルギニン；及びフェニルアラニン、チロシン；同士の置換である。非極性（疎水性）のアミノ酸には、アラニン、ロイシン、イソロイシン、バリン、プロリン、フェニルアラニン、トリプトファン及びメチオニンがある。極性の中性のアミノ酸にはグリシン、セリン、スレオニン、システイン、チロシン、アスパラギン及びグルタミンがある。正に耐電した（塩基性）アミノ酸には、アルギニン、リシン及びヒスチジンが含まれる。負に耐電した（酸性）アミノ酸にはアスパラギン酸及びグルタミン酸が含まれる。

【００３９】

他の実施態様では、比較的保存的でないアミノ酸置換を持つ誘導体により、例えば電荷、コンホメーション及び他の生物学的性質に変化を起こすなどにより、所望の誘導体が得られることもある。このような置換には、例えば、親水性残基を疎水性残基に換える置換、システイン又はプロリンを別の残基に換える置換、小さな側鎖を有する残基を大型の側鎖を有する残基に換える置換、又は、実効正電荷を有する残基を実効負電荷を有する残基に換える置換、があるであろう。ある置換を行った結果を確実に予測できない場合、ここに開示した方法により、所望の特徴の有無を調べれば、その誘導体を容易に検定できよう。さらに本発明のポリペプチド及びタンパク質に、保存的又は非保存的のいずれにしろ、例えば挿入、欠失及び置換などの多種の変更を行うとそれらの使用時に有利である場合、このような改変を行ってもよい。

【００４０】

ある好適な実施態様では、改変インテグリンＩ－ドメインポリペプチドは開いたコンホメーションで安定させてあり、高い親和性でリガンドを結合させる。

【００４１】

一実施態様では、本発明の改変インテグリンＩ－ドメインポリペプチドは、野生型配列に比較して少なくとも一つのシステイン置換、そして好ましくは二つのシステイン置換、を含有するアミノ酸配列にコードされている。別の実施態様では、システインに置換する残基のＣβ炭素間の距離は、例えばタンパク質モデリングで予測したときなどに、３．００乃至８．０９オングストロームの範囲内である。更なる実施態様では、ジスルフィド結合中のＣβ炭素間の距離は、３．４１乃至７．０８オングストロームの範囲内である。

【００４２】

Ｉ－ドメインポリペプチドのアミノ酸配列中の適当な位置にシステイン残基を導入すると、例えば活性な「開いた」コンホメーションや、又は、不活性な「閉じた」コンホメーションなど、特定のコンホメーションでこのドメインを安定させるジスルフィド結合を形成させることができる。例えば、αＬ Ｋ２８７Ｃ／Ｋ２９４Ｃ、Ｅ２８４Ｃ／Ｅ３０１Ｃ、Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、Ｌ１６１Ｃ／Ｔ３００Ｃ、及びＬ２８９Ｃ／Ｋ２９４Ｃ 変異型、及びαＭ Ｑ１６３Ｃ／Ｑ３０９Ｃ 及びＤ２９４Ｃ／Ｑ３１１Ｃ 変異型は、高いもしくは中程度の親和性でリガンドを結合させる「開いた」コンホメーションで安定しているが、αＬ Ｌ２８９Ｃ／Ｋ２９４Ｃ 変異型及びαＭ Ｑ１６３Ｃ／Ｒ３１３Ｃ 変異型は、リガンドを結合させない不活性即ち「閉じた」コンホメーションで安定し、一方でＥ２８４Ｃ／Ｅ３０１Ｃの親和性は、高親和性など、Ｋ２８７Ｃ／Ｋ２９４Ｃのそれにほぼ匹敵する。Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃの親和性は、野生型よりも有意に高いが、高親和性αＬ Ｉ－ドメイン、Ｋ２８７Ｃ／Ｋ２９４Ｃの２０乃至３０分の１である。Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃをここでは中間親和性αＬ Ｉ－ドメインと呼ぶ。

【００４３】
一の実施態様では、本発明は、インテグリンαサブユニット内に構成され、さらにインテグリンβサブユニットと結合され得る改変インテグリンＩ－ドメインを提供するものである。別の実施態様では、本発明の改変インテグリンＩ－ドメインポリペプチドは可溶性ポリペプチドである。さらに本発明は、異種ポリペプチドに作動的に連結された改変インテグリンＩ－ドメインポリペプチドを提供する。
【００４４】
実験データ（Ｈｕａｎｇ，Ｃ ｅｔ ａｌ．（２０００）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７５：２１５１４－２４）で裏付けられたインテグリンβ－サブユニットのＩ－様ドメインのモデルも作製されている。このデータは、Ｉドメイン中で劇的な１０オングストロームのコンホメーション上の移動を行う重要なＣ末端αヘリックスの位置を確認している。Ｉドメイン及びＩ－様ドメインのこの領域でのアライメントは良好である。従って、別の局面では、本発明は、改変Ｉ－様ドメインポリペプチドを所望のコンホメーションで安定させるように、少なくとも一つのジスルフィド結合を含有させた改変インテグリンＩ－様ドメインポリペプチドを提供する。
【００４５】
ある好適な実施態様では、改変インテグリンＩ－様ドメインポリペプチドは、開いたコンホメーションで安定しており、高い親和性でリガンドを結合させる。実施態様の一つでは、本発明の改変インテグリンＩ－様ドメインポリペプチドは、野生型配列に比較して少なくとも一つのシステイン置換、そして好ましくは二つのシステイン置換、を含有するアミノ酸配列にコードされている。
【００４６】
ある実施態様では、本発明は、インテグリンβサブユニット内に構成され、さらにインテグリンαサブユニットと結合され得る改変インテグリンＩ－様ドメインを提供するものである。別の実施態様では、本発明の改変インテグリンＩ－様ドメインポリペプチドは可溶性ポリペプチドである。さらに本発明は、異種ポリペプチドに作動的に連結された改変インテグリンＩ－様ドメインポリペプチドを提供する。
【００４７】
インテグリンは数多くの疾患で鍵となるターゲットである。よって、本発明における分離された高親和Ｉ－ドメインや、活性化した白血球インテグリンに対して選択的な抗体又は小分子アンタゴニストを利用すると、自己免疫及び炎症性疾患、移植片拒絶、及び、血液量減少性ショック、心筋梗塞及び脳ショックの場合の虚血性／再潅流傷害を、阻害又は防止するなど、修飾することができる。さらに、天然のリガンドに結合させた高親和Ｉドメインの共結晶、及び／又は、小分子アンタゴニストを容易に生成でき、またこのような再結晶及び小分子アンタゴニストは、コンピュータ・ドラッグ・デザインを可能にし、薬物開発候補の改良及び向上に進歩をもたらすであろう。
【００４８】
従って、本発明は、開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドに対するテスト化合物の結合能を検定するステップを含む、インテグリン活性のモジュレータを同定する方法を提供する。別の実施態様では、本発明は、インテグリンとコグネイト・リガンドとの間の相互作用を修飾することのできる化合物を同定する方法を提供し、当該方法では、開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドに対するリガンドの結合を、テスト化合物の存在下及び非存在下で検定する。
【００４９】
さらに本発明は、改変インテグリンＩ－ドメインポリペプチドか、又は、開いたコンホメーションのＩ－ドメインなどの改変インテグリンＩ－ドメインに選択的に結合する高ＬＦＡ－１抗体（又はその抗原結合フラグメント）などの抗インテグリン抗体と、薬学的に許容可能な担体と、を含んで成る組成物を提供する。本発明の組成物は、本発明の治療法に用いられる。例えば本発明は、対象においてインテグリンが媒介する疾患（例えば炎症性

又は自己免疫性疾患）を治療又は予防する方法、又は、対象においてコグネイト・リガンドに対するインテグリンの結合を阻害する方法を提供し、当該方法は、開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドか、又は、開いたコンホメーションのインテグリンＩ－ドメインに選択的に結合する抗インテグリン抗体（又はその抗原結合フラグメント）、を、治療上有効量、投与するステップを含む。ある好適な実施態様では、本改変インテグリンＩ－ドメインポリペプチドは、リガンドに高親和結合する。別の好適な実施態様では、治療用の本改変インテグリンＩ－ドメインポリペプチドは、融合タンパク質などの可溶性ポリペプチドである。

【００５０】

ここで用いる場合の、インテグリンが媒介する疾患には、例えば炎症性もしくは免疫系疾患、及び／又は、細胞増殖性疾患がある。インテグリンが媒介する疾患の例には、心筋梗塞、卒中、再狭窄、移植片拒絶、移植片対宿主疾患又は宿主対移植片疾患、及び再潅流傷害、がある。炎症性又は免疫系疾患には、限定はしないが、成人呼吸窮迫症候群（ＡＲＤＳ）、敗血症又は外傷に続発する多臓器傷害症候群、ウィルス感染、炎症性腸疾患、潰瘍性大腸炎、クローン病、白血球接着欠陥ＩＩ症候群、熱損傷、血液透析、白血球搬出、腹膜炎、慢性閉塞性肺疾患、肺炎、喘息、急性虫垂炎、急性炎症成分を伴う皮膚病、創傷治癒、敗血性ショック、急性腎炎、腎炎、アミロイド症、反応性関節炎、リウマチ性関節炎、慢性気管支炎、シェーグレン症候群、サルコイドーシス、強皮症、狼瘡、多発性筋炎、ライター症候群、乾癬、皮膚炎、骨盤炎症疾患、炎症性乳房疾患、眼窩炎症性疾患、免疫不全疾患（例えばＨＩＶ、後天性免疫不全、先天性Ｘ染色体小児性低ガンマグロブリン血症、一過性低ガンマグロブリン血症、選択性免疫グロブリンＡ欠損症、壊死性小腸大腸炎、顆粒球輸血随伴性症候群、サイトカイン誘導性傷害、慢性粘膜皮膚カンジダ症、重症複合型免疫不全）、自己免疫疾患、及び急性化膿性髄膜炎又は他の中枢神経系炎症疾患、がある。

【００５１】

「細胞増殖性疾患」には、細胞の増殖、活性化、接着、成長、分化、又は移動のプロセスに影響を与える疾患が含まれる。ここで用いる場合の「細胞の増殖、活性化、接着、成長、分化、又は移動のプロセス」とは、細胞が数、大きさ、活性化状態、又は内容量を増すプロセス、細胞が他の細胞のそれとは異なる一組の特化した特徴を生ずるプロセス、又は、細胞が特定の位置又は刺激に近寄る又は遠ざかるプロセス、である。疾患は、成長、活性化、接着、分化、又は移動の調節異常を特徴とする。このような疾患には、癌、例えば癌腫、肉腫、リンパ腫又は白血病があるが、その例には、限定はしないが、例えば乳房、子宮内膜、卵巣、子宮、肝臓、胃腸管、前立腺、結腸直腸、及び肺の癌、黒色腫、神経線維腫、腺腫様多発結腸ポリープ、ウィルムス腫瘍、腎芽細胞腫、奇形腫、横紋筋肉腫；腫瘍の浸襲、脈管形成及び転移；骨格形成異常；造血系及び／又は骨髄増殖性疾患、がある。

【００５２】

本発明の多様な局面を、以下の小節でさらに詳述することとする。

【００５３】

改変インテグリンＩ－ドメインポリペプチド及び抗インテグリンＩ－ドメイン抗体

本発明の方法は、分離された改変インテグリンポリペプチド、及びその生物学的活性部分の使用を包む。ここで用いる場合の改変インテグリンポリペプチドは、改変Ｉ－ドメインポリペプチド及び改変Ｉ－様ドメインポリペプチドを包含する。本発明の改変インテグリンポリペプチドは、それぞれインテグリンα又はβサブユニットポリペプチド内に構成された改変インテグリンＩ－ドメイン及びＩ－様ドメインポリペプチド；可溶性の改変インテグリンＩ－ドメイン及びＩ－様ドメインポリペプチド；及び、融合タンパク質など、異種ポリペプチドに作動的に連結された改変インテグリンＩ－ドメイン及びＩ－様ドメインポリペプチド、を包含するものである。

【００５４】

多数のヒトインテグリンα及びβサブユニットポリペプチドのｃＤＮＡのクローンが作製

され、配列決定されて、そのポリペプチド配列が決定されている（例えばジェンバンク登録番号：ＮＭ＿００２２０３（α２）、ＡＦ１１２３４５（α１０）、ＮＭ＿０１２２１１（α１１）、ＮＭ＿００５３５３（αＤ）、ＮＭ＿００２２０８（αＥ）、ＮＭ＿０００８８７（αＸ）、ＮＭ＿０００６３２（αＭ）、ＮＭ＿００２２０９（αＬ）、Ｘ６８７４２及びＰ５６１９９（α１）、ＮＭ＿０００２１１（β２）、ＮＭ＿０００２１２（β３）、ＮＭ＿００２２１４（β８）を参照されたい）。具体的には、ヒトαＬ及びαＭをコードするポリペプチド配列を、それぞれＳＥＱ ＩＤ ＮＯ：２（ジェンバンク登録番号Ｐ２０７０１）及びＳＥＱ ＩＤ ＮＯ：４（ジェンバンク登録番号Ｐ１１２１５）に記載してある。加えて、他の種由来のインテグリンα及びβサブユニットポリペプチドをコードする配列も、当該技術分野において入手できる。さらに、前述したように、αＭ、αＬ、α１及びα２のＩ－ドメインの三次元構造が解明されている（Ｌｅｅ，Ｊ－Ｏ，ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３：１３３３－１３４０；Ｌｅｅ，Ｊ－Ｏ，ｅｔ ａｌ．（１９９Ｓ）Ｃｅｌｌ ８０：６３１－６３８；Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９５）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９２：１０２７７－１０２８１；Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９６）Ｓｔｒｕｃｔｕｒｅ ４：９３１－９４２；Ｅｍｓｌｅｙ，Ｊ ｅｔ ａｌ．（１９９７）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７２：２８５１２－２８５１７；Ｂａｌｄｗｉｎ，ＥＴ ｅｔ ａｌ．（１９９８）Ｓｔｒｕｃｔｕｒｅ ６：９２３－９３５；Ｋａｌｌｅｎ，Ｊ ｅｔ ａｌ．（１９９９）Ｊ Ｍｏｌ Ｂｉｏｌ ２９２：１－９）。

【００５５】

本発明の分離された改変インテグリンポリペプチドは、好ましくは、天然インテグリンポリペプチドのアミノ酸配列に充分同一であるが、当該ポリペプチドを所望のコンホメーションで安定させるジスルフィド結合が形成されるよう、少なくとも一つ、そして好ましくは二つのシステイン置換を含むようなアミノ酸配列を有するとよい。ここで用いる用語「充分同一である」とは、あるアミノ酸（又はヌクレオチド）配列が、インテグリンアミノ酸（又はヌクレオチド）配列と同一又は同等（例えば類似の側鎖を有するアミノ酸残基など）アミノ酸残基（又はヌクレオチド）を充分もしくは最小限の数、含有する結果、これらポリペプチドが天然インテグリンポリペプチドと共通の構造ドメイン又はモチーフ、及び／又は共通の機能的活性を有することを言う。例えば、少なくとも３０％、４０％、又は５０％、好ましくは６０％、より好ましくは７０％、７５％、８０％、８５％又は９０％、９１％、９２％、９３％、９４％、９５％又はそれより大きい同一性を有し、（例えばここに解説した通りの改変インテグリンＩ－ドメイン又はＩ－様ドメインの活性など）共通の機能的活性を有するアミノ酸又はヌクレオチド配列が、充分に同一である、とここに定義しておく。インテグリンＩ－ドメインポリペプチドは、天然のアレルの違い又は変異誘発のために、ここに開示したインテグリンポリペプチドとはアミノ酸配列が異なることもある。

【００５６】

二つのアミノ酸配列又は二つの核酸配列のパーセント同一性を決定するには、最適な比較が行えるように、これら配列をアライメントする（最適なアライメントを行うには第一及び第二のアミノ酸又は核酸配列の一方又は両方にギャップを導入してもよく、同一でない配列は、比較を目的にしたときは無視してもよい）。ある好適な実施態様では、基準配列のうちで比較用にアライメントする長さは、基準配列の長さの少なくとも３０％、好ましくは少なくとも４０％、より好ましくは少なくとも５０％、さらにより好ましくは少なくとも６０％、そしてさらにより好ましくは少なくとも７０％、８０％、又は９０％である。次に、対応するアミノ酸位置又はヌクレオチド位置にあるアミノ酸残基又はヌクレオチドを比較する。第一の配列中のある一つの位置に、第二の配列中の対応する位置にあるのと同じアミノ酸残基又はヌクレオチドが来ていれば、それら分子はその位置において同一であることになる（ここで用いる場合のアミノ酸又は核酸の「同一性」は、アミノ酸又は核酸の「ホモロジー」と同等である）。二つの配列間のパーセント同一性は、これら二つの配列を最適にアライメントするのに導入せねばならないギャップの数、及び各ギャップ

の長さを考慮に入れたときの、これら配列に共通の同一位置の数の関数である。

【００５７】

二つの配列間の配列の比較及びパーセント同一性の決定は、数学的アルゴリズムを用いて行うことができる。ある好適な実施態様では、二つのアミノ酸配列間のパーセント同一性を、ＧＣＧソフトウェア・パッケージ（ｈｔｔｐ：／／ｗｗｗ．ｇｃｇ．ｃｏｍで入手できる）のＧＡＰプログラムに組み込まれたニードルマン及びワンシュ（Ｊ．Ｍｏｌ．Ｂｉｏｌ．（４８）：４４４－４５３（１９７０））のアルゴリズムを用い、Ｂｌｏｓｓｏｍ６２行列又はＰＡＭ２５０行列を用いて、ギャップ・ウェイトを１６、１４、１２、１０、８、６、又は４にし、レングス・ウェイトを１、２、３、４、５、又は６にして、決定する。さらに別の好適な実施態様では、二つのヌクレオチド配列間のパーセント同一性を、ＧＣＧソフトウェア・パッケージ（ｈｔｔｐ：／／ｗｗｗ．ｇｃｇ．ｃｏｍで入手できる）のＧＡＰプログラムを用い、ＮＷＳｇａｐｄｎａ．ＣＭＰ行列を用いて、ギャップ・ウェイトを４０、５０、６０、７０、又は８０にし、そしてレングス・ウェイトを１、２、３、４、５、又は６にして決定する。別の実施態様では、二つのアミノ酸又はヌクレオチド配列間のパーセント同一性を、ＡＬＩＧＮプログラム（バージョン２．０）に組み込まれたＥ．マイヤース及びＷ．ミラーのアルゴリズム（Ｃｏｍｐｕｔ．Ａｐｐｌ．Ｂｉｏｓｃｉ．，４：１１－１７（１９８８））を用い、ＰＡＭ１２０ウェイト残基表を用いて、ギャップ・レングス・ペナルティを１２、そしてギャップ・ペナルティを４にして、決定する。

【００５８】

ここで用いる場合の、ある改変インテグリンポリペプチド（例えば改変インテグリンＩ－ドメインポリペプチド）の「生物学的活性部分」とは、改変インテグリンポリペプチドの活性を保持した改変インテグリンポリペプチドの一フラグメントを包含するものである。典型的には、改変インテグリンポリペプチドの生物学的活性部分は、リガンド結合など、改変インテグリンポリペプチドの少なくとも一つの活性を持つ少なくとも一つのドメイン又はモチーフを含んで成るものである。ある好適な実施態様では、改変インテグリンポリペプチドの生物学的活性部分には、改変インテグリンＩ－ドメインポリペプチドが含まれる。改変インテグリンポリペプチドの生物学的活性部分は、改変インテグリンポリペプチドのアミノ酸配列と充分同一もしくはそれに由来するアミノ酸配列を含んで成り、完全長改変インテグリンポリペプチドよりも少ないアミノ酸を含有し、改変インテグリンポリペプチドの少なくとも一つの活性を呈するであろう。改変Ｉ－ドメイン又はＩ－様ドメインなど、改変インテグリンポリペプチドの生物学的活性部分は、リガンド結合、細胞間接着や、細胞と細胞外マトリックスの接着などの接着、及び／又は、シグナリング活性などのインテグリンポリペプチド活性を修飾する薬剤を開発するためのターゲットとして利用できる。改変インテグリンポリペプチドの生物学的活性部分は、遺伝子組換え技術で調製でき、かつ、改変インテグリンポリペプチドの機能的活性の一つ以上について評価できる一ポリペプチドを含んで成る。

【００５９】

ある好適な実施態様では、改変インテグリンポリペプチドは、組換えＤＮＡ技術で調製される。例えば、改変インテグリンポリペプチド（例えばＩ－ドメインポリペプチド又は可溶性Ｉ－ドメイン融合たんぱくなど）をコードするポリヌクレオチド配列をトランスフェクトしたホスト細胞から、標準的なタンパク質精製技術を用いた、適当な精製スキームを利用して改変インテグリンポリペプチドを単離することができる。組換え発現の代わりに、標準的なペプチド合成技術を利用して改変インテグリンポリペプチドを化学合成することもできる。

【００６０】

「単離された」または「精製された」ポリペプチド又はタンパク質、あるいはその生物学的活性部分は、改変インテグリンＩ－ドメインポリペプチドが由来する細胞源などのソースにある細胞性物質又は他の夾雑タンパク質を実質的に含まないか、あるいは、化学合成した場合には前駆化学物質又は他の化学物質を実質的に含まないものである。「細胞性物

質を実質的に含まない」という文言は、当該タンパク質が、それが単離もしくは組換え生産された基の細胞の細胞成分から分離されているような、改変インテグリンポリペプチドの調製品を包含するものである。一実施態様では、「細胞性物質を実質的に含まない」という文言は、（未改変のインテグリンポリペプチドここでは「夾雑タンパク質」とも言及された）を約３０％未満（乾燥重量で）、より好ましくは、未改変のインテグリンポリペプチドを約２０％未満、さらにより好ましくは未改変のインテグリンポリペプチドを約１０％未満、そして最も好ましくは未改変のインテグリンポリペプチドを約５％有するような、改変インテグリンポリペプチドの調製品を包含する。改変インテグリンポリペプチド又はその生物学的活性部分を組換え生産した場合、培地を実質的に含まないことが好ましく、即ち、タンパク質調製品の体積のうちで培地の占める割合が、約２０％未満、より好ましくは約１０％未満、そして最も好ましくは約５％未満であるとよい。

【００６１】

「前駆化学物質又は他の化学物質を実質的に含まない」という文言は、当該タンパク質の合成に関与する前駆化学物質又は他の化学物質から当該タンパク質が分離されている、改変インテグリンポリペプチド調製品を包含する。一実施態様では、文言「前駆化学物質又は他の化学物質を実質的に含まない」は、約３０％未満（乾燥重量で）の前駆化学物質又は未改変インテグリンポリペプチド化学物質、より好ましくは約２０％未満の前駆化学物質又は未改変インテグリンポリペプチド化学物質、さらにより好ましくは約１０％未満の前駆化学物質又は未改変インテグリンポリペプチド化学物質、そして最も好ましくは約５％未満の前駆化学物質又は未改変インテグリンポリペプチド化学物質、を有する改変インテグリンポリペプチドの調製品を包含する。

【００６２】

本発明の方法では、さらにキメラタンパク質又は融合タンパク質である改変インテグリンポリペプチドを使用してもよい。ここで用いる場合の改変インテグリン「キメラタンパク質」又は「融合タンパク質」は、異種ポリペプチドなどの未改変のインテグリンポリペプチドに作動的に連結させた改変インテグリンポリペプチドを含んで成る。ある好適な実施態様では、改変インテグリン融合タンパク質は、少なくとも一つのＩ－ドメイン又は一つのＩ－様ドメインを含んで成る。融合タンパク質内で、「作動的に連結させた」とは、当該改変インテグリンポリペプチドと、異種ポリペプチド配列とが、相互にインフレームで融合されていることを指すことを意図している。異種ポリペプチドは、改変インテグリンポリペプチドのＮ末端に融合させても、Ｃ末端に融合させてもよい。

【００６３】

例えば、ある好適な実施態様では、前記融合タンパク質は、例えば免疫グロブリン（例えばＩｇＧ１）のヒンジ、Ｃ１及びＣ２配列などのＦｃ領域を、改変インテグリン配列のＣ末端に融合してある改変インテグリンＩ－ドメイン融合タンパク質である。インテグリン免疫グロブリンキメラは、基本的にはＷＯ ９１／０８２９８に解説された通りに構築することができる。このような融合タンパク質があると、組換え改変インテグリンポリペプチドの精製が簡便となる。別の実施態様では、前記融合タンパク質は、当該融合タンパク質が細胞表面上に発現するよう、異種の膜貫通ドメインに融合してある改変インテグリンＩ－ドメインポリペプチドである。

【００６４】

本発明の改変インテグリンポリペプチド及び融合タンパク質を医薬組成物中に組み込み、対象にインビボで投与することもできる。ある実施態様では、開いた、リガンド結合型のコンホメーションで安定させた可溶性の改変インテグリンＩ－ドメインポリペプチドか、又はその融合タンパク質を、対象のインテグリン活性（例えばコグネイト・リガンドに対するインテグリンの結合）を修飾するのに利用され得る。別の実施態様では、可溶性の改変インテグリンＩ－ドメインポリペプチド又は融合タンパク質を、炎症性疾患や、自己免疫疾患などの免疫系の疾患を治療するのに利用できよう。別の実施態様では、可溶性改変インテグリンポリペプチド又は融合タンパク質を、細胞増殖性疾患を治療するのに利用できよう。さらに、可溶性改変インテグリンポリペプチド及び融合タンパク質の使用を利用

して、ＩＣＡＭなどインテグリンリガンドの生物学的利用能に影響を与えることもできる。

【００６５】

さらに、本発明の改変インテグリンポリペプチド及び融合タンパク質を、対象において抗ＬＦＡ－１抗体などの抗インテグリン抗体を産生させる免疫原として用いたり、インテグリン活性を修飾する分子、及び／又は、インテグリンリガンド又は受容体とのインテグリンポリペプチドの相互作用を修飾する分子、を同定するスクリーニング検定に用いることもできる。

【００６６】

好ましくは、本発明の改変インテグリン融合タンパク質は、標準的な組換えＤＮＡ技術で作製される。例えば、連結に平滑末端又は付着末端を利用すること、制限酵素消化を行って適した末端にすること、付着末端を適宜充填すること、アルカリホスファターゼ処理して不要な接合を防ぐこと、酵素による連結を行うことなどの常法で異なるポリペプチド配列をコードするＤＮＡ断片を相互にインフレームで連結する。別の実施態様では、自動化ＤＮＡ合成装置を含む従来技術により、融合遺伝子を合成することもできる。代替的には、二本の連続した遺伝子断片の間に相補的な張り出し部を生ずるようなアンカー・プライマーを用いて遺伝子断片のＰＣＲ増幅を行い、その後これら断片をアニールし、再増幅してキメラ遺伝子配列を作製することもできる（例えばＣｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ，ｅｄｓ．Ａｕｓｕｂｅｌ ｅｔ ａｌ．Ｊｏｈｎ Ｗｉｌｅｙ ＆ Ｓｏｎｓ：１９９２を参照されたい）。さらに、最初から一融合成分（例えばＧＳＴポリペプチド）をコードしている多くの発現ベクタが市販されている。このような融合成分が改変インテグリンポリペプチドにインフレームで連結されるよう、改変インテグリンポリペプチドをコードする核酸をこのような発現ベクタ内にクローンすることもできる。

【００６７】

さらに本発明の方法には、インテグリン・アゴニスト（ミメティック）又はインテグリン・アンタゴニストのいずれかとして機能する改変インテグリンポリペプチドの使用を含み得る。インテグリンポリペプチドのアゴニストには、天然発生型のインテグリンポリペプチドの生物学的活性と実質的に同じ活性を維持していても、又は、その一部分を維持していてもよい。インテグリンポリペプチドのアンタゴニストは、例えばインテグリン活性を競合的に修飾するなどにより、天然型のインテグリンポリペプチドの活性のうちの一つ又はそれ以上を阻害することができる。このように、所望のコンホメーションで安定させた改変インテグリンポリペプチドで処理すると、特定の生物学的効果を引き出すことができる。

【００６８】

改変ＬＦＡ－１ポリペプチドなどの分離された改変インテグリンポリペプチド、又はその一部分もしくはフラグメントを免疫原として用い、ポリクローナル及びモノクローナル抗体作製のための標準的な技術を利用すると、インテグリンＩ－ドメインなどの特定のコンホメーションのインテグリンに結合する抗体を生成させることができる（概略的には、Ｒ．Ｈ．Ｋｅｎｎｅｔｈ，ｉｎ Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ：Ａ Ｎｅｗ Ｄｉｍｅｎｓｉｏｎ Ｉｎ Ｂｉｏｌｏｇｉｃａｌ Ａｎａｌｙｓｅｓ，Ｐｌｅｎｕｍ Ｐｕｂｌｉｓｈｉｎｇ Ｃｏｒｐ．，Ｎｅｗ Ｙｏｒｋ，Ｎｅｗ Ｙｏｒｋ（１９８０）；Ｅ．Ａ．Ｌｅｒｎｅｒ（１９８１）Ｙａｌｅ Ｊ．Ｂｉｏｌ．Ｍｅｄ．，５４：３８７－４０２；Ｍ．Ｌ．Ｇｅｆｔｅｒ ｅｔ ａｌ．（１９７７）Ｓｏｍａｔｉｃ Ｃｅｌｌ Ｇｅｎｅｔ．３：２３１－３６を参照されたい）。さらに、当業者であれば、このような方法の変更例を数多く知るところであり、このような変法も有用であろう。抗ＬＦＡ－１の調製は、例えば、その内容全文を引用をもってここに援用することとする米国特許第５，６２２，７００号に解説されている。

【００６９】

ここで用いる用語「抗体」とは、免疫グロブリン分子や、免疫グロブリン分子の免疫学的

活性部分、即ち、開いた又は閉じたコンホメーションのインテグリンＩ－ドメイン、又は改変インテグリンＩ－ドメイン、例えばＬＦＡ－１ Ｉ－ドメイン、例えば開いた又は閉じたＬＦＡ－１ Ｉ－ドメイン、又は、ＬＦＡ－１の改変インテグリンＩ－ドメイン、などの抗原に特異的に結合する（免疫反応する）抗原結合部位を含有する分子、を言う。免疫グロブリン分子の免疫学的活性部分の例には、抗体をペプシンなどの酵素で消化して生成できる Ｆ（ａｂ）及びＦ（ａｂ）’２フラグメントがある。本発明は、改変ＬＦＡ－１ポリペプチド又はその一部分もしくはフラグメントなどの改変インテグリンポリペプチドに結合するポリクローナル及びモノクローナル抗体を提供する。ここで用いる用語「モノクローナル抗体」又は「モノクローナル抗体組成物」とは、改変ＬＦＡ－１ポリペプチド又はその一部分もしくはフラグメントなどの改変インテグリンポリペプチドの特定のエピトープと免疫反応できる抗原結合部位を一種しか含まない一集団の抗体分子を言う。従って、モノクローナル抗体組成物は、典型的には、免疫反応する相手である特定の改変インテグリンポリペプチド又はその一部分もしくはフラグメントに対して、単一の結合親和性を呈する。

【００７０】

モノクローナル抗体産生ハイブリドーマを調製する代わりに、組換えコンビナトリアル免疫グロブリン・ライブラリ（例えば抗体ファージディスプレイライブラリ）を、例えば開いたコンホメーションで安定させた改変インテグリンＩ－ドメインなど、改変インテグリンポリペプチドでスクリーニングすれば、モノクローナル抗インテグリン抗体を同定及び単離でき、ひいては開いたコンホメーションなどのインテグリンポリペプチド上のコンホメーション特異的エピトープに結合する免疫グロブリンライブラリメンバを単離することができる。ファージディスプレイライブラリを作製及びスクリーニングするキットは市販されている（例えばファルマシア・リコンビナント・ファージ・アンティボディ・システム、カタログ番号２７－９４００－０１；及びストラタジーンＳｕｒｆＺＡＰ^{TM}ファージ・ディスプレイ・キット、カタログ番号２４０６１２）。ジスルフィド結合によって高親和性コンホメーションで固定されたインテグリンＩ－ドメインを持つファージ・ライブラリをスクリーニングする際は、ジスルフィドを破壊してＩ－ドメインの高親和性コンホメーションを無くす還元試薬を加えたときに、特定のファージを溶離できなければならないことに留意されたい。

【００７１】

加えて、抗体ディスプレイライブラリを作製及びスクリーニングするための使用に特に適する方法及び試薬の例は、例えばラドナー氏らの米国特許第５，２２３，４０９号；カン氏らのＰＣＴ 国際公報Ｎｏ． ＷＯ ９２／１８６１９；ダワー氏らのＰＣＴ国際公報Ｎｏ． ＷＯ ９１／１７２７１；ウィンター氏らのＰＣＴ国際公報ＷＯ ９２／２０７９１；マークランド氏らのＰＣＴ国際公報Ｎｏ． ＷＯ ９２／１５６７９；ブレイトリング氏らのＰＣＴ国際公報ＷＯ ９３／０１２８８；マッカファーティ氏らの ＰＣＴ国際公報Ｎｏ． ＷＯ ９２／０１０４７；ジェラード氏らのＰＣＴ国際公報Ｎｏ． ＷＯ ９２／０９６９０；ラドナー氏らのＰＣＴ 国際公報Ｎｏ． ＷＯ ９０／０２８０９；Ｆｕｃｈｓ ｅｔ ａｌ．（１９９１）Ｂｉｏ／Ｔｅｃｈｎｏｌｏｇｙ ９：１３７０－１３７２；Ｈａｙ ｅｔ ａｌ．（１９９２）Ｈｕｍ．Ａｎｔｉｂｏｄ．Ｈｙｂｒｉｄｏｍａｓ ３：８１－８５；Ｈｕｓｅ ｅｔ ａｌ．（１９８９）Ｓｃｉｅｎｃｅ ２４６：１２７５－１２８１；Ｇｒｉｆｆｉｔｈｓ ｅｔ ａｌ．（１９９３）ＥＭＢＯ Ｊ １２：７２５－７３４；Ｈａｗｋｉｎｓ ｅｔ ａｌ．（１９９２）Ｊ．Ｍｏｌ．Ｂｉｏｌ．２２６：８８９－８９６；Ｃｌａｒｋｓｏｎ ｅｔ ａｌ．（１９９１）Ｎａｔｕｒｅ ３５２：６２４－６２８；Ｇｒａｍ ｅｔ ａｌ．（１９９２）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８９：３５７６－３５８０；Ｇａｒｒａｄ ｅｔ ａｌ．（１９９１）Ｂｉｏ／Ｔｅｃｈｎｏｌｏｇｙ ９：１３７３－１３７７；Ｈｏｏｇｅｎｂｏｏｍ ｅｔ ａｌ．（１９９１）Ｎｕｃ．Ａｃｉｄ Ｒｅｓ．１９：４１３３－４１３７；Ｂａｒｂａｓ ｅｔ ａｌ．（１９９１）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８８：７９７８－７９８２；及びＭｃＣａｆｆｅｒｔｙ ｅｔ ａｌ．

Ｎａｔｕｒｅ（１９９０）３４８：５５２－５５４に見ることができる。

【００７２】

さらに、標準的な組換えＤＮＡ技術を用いて作製できる、ヒト及び非ヒト部分の両方を含んで成る、キメラ及びヒト化モノクローナル抗体などの組換え抗インテグリン抗体も、本発明の方法に利用できる。このようなキメラ及びヒト化モノクローナル抗体は、例えばロビンソン氏らの国際出願Ｎｏ．ＰＣＴ／ＵＳ８６／０２２６９；アキラ氏らのヨーロッパ特許出願１８４，１８７；タニグチ．Ｍ氏らのヨーロッパ特許出願１７１，４９６；モリソン氏らのヨーロッパ特許出願１７３，４９４；ノイベルガー氏らのＰＣＴ国際公報Ｎｏ．ＷＯ８６／０１５３３；キャビリー氏らの米国特許第４，８１６，５６７号；キャビリー氏らのヨーロッパ特許出願１２５，０２３；Ｂｅｔｔｅｒ ｅｔ ａｌ．（１９８８）Ｓｃｉｅｎｃｅ ２４０：１０４１－１０４３；Ｌｉｕ ｅｔ ａｌ．（１９８７）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８４：３４３９－３４４３；Ｌｉｕ ｅｔ ａｌ．（１９８７）Ｊ．Ｉｍｍｕｎｏｌ．１３９：３５２１－３５２６；Ｓｕｎ ｅｔ ａｌ．（１９８７）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８４：２１４－２１８；Ｎｉｓｈｉｍｕｒａ ｅｔ ａｌ．（１９８７）Ｃａｎｃ．Ｒｅｓ．４７：９９９－１００５；Ｗｏｏｄ ｅｔ ａｌ．（１９８５）Ｎａｔｕｒｅ ３１４：４４６－４４９；ａｎｄ Ｓｈａｗ ｅｔ ａｌ．（１９８８）Ｊ．Ｎａｔｌ．Ｃａｎｃｅｒ Ｉｎｓｔ．８０：１５５３－１５５９）；Ｍｏｒｒｉｓｏｎ，Ｓ．Ｌ．（１９８５）Ｓｃｉｅｎｃｅ ２２９：１２０２－１２０７；Ｏｉ ｅｔ ａｌ．（１９８６）ＢｉｏＴｅｃｈｎｉｑｕｅｓ ４：２１４；ウィンター氏らの米国特許第５，２２５，５３９号；Ｊｏｎｅｓ ｅｔ ａｌ．（１９８６）Ｎａｔｕｒｅ ３２１：５５２－５２５；Ｖｅｒｈｏｅｙａｎ ｅｔ ａｌ．（１９８８）Ｓｃｉｅｎｃｅ ２３９：１５３４；及びＢｅｉｄｌｅｒ ｅｔ ａｌ．（１９８８）Ｊ．Ｉｍｍｕｎｏｌ．１４１：４０５３－４０６０に解説された方法を用いるなど、当該分野で公知の組換えＤＮＡ技術によって作製できる。

【００７３】

ある好適な実施態様では、本発明の抗インテグリン抗体は、活性化したインテグリンに固有のエピトープ（ここでは活性化特異的エピトープとも呼ばれる）など、開いた、高親和性コンホメーションのインテグリンＩ－ドメインに選択的に結合する。ある好適な実施態様では、本発明の抗インテグリン抗体は、活性化インテグリンとそのコグネイト・リガンドとの間の結合相互作用を修飾する（例えば阻害するなど）。別の実施態様では、抗インテグリン抗体は、白血球接着及び／又は凝集を阻害する。別の実施態様では、本発明の抗インテグリン抗体は、開いたコンホメーションのＬＦＡ－１ Ｉ－ドメインなどの開いたインテグリンＩ－ドメインや、又は、ＬＦＡ－１分子の改変Ｉ－ドメインなどの改変インテグリンＩ－ドメインに、選択的に結合する。

【００７４】

抗インテグリン抗体（例えばモノクローナル抗体など）は、本発明の方法において、インテグリン又はインテグリンＩ－ドメインポリペプチドの発現及び／又は活性を修飾することにも、利用することができる。また抗インテグリン抗体は、アフィニティクロマトグラフィ又は免疫沈降などの標準的な技術を用いて、改変ＬＦＡ－１ポリペプチド、又は融合タンパク質などの改変インテグリン又はインテグリンＩ－ドメインポリペプチドを単離することにも、利用できる。別の実施態様では、抗インテグリン抗体を用いて、活性化インテグリンを発現している細胞を除去する及び／又は死滅させることもできる。さらに、抗インテグリン抗体を用いて、特定のコンホメーション（活性化インテグリンなど）のインテグリンポリペプチドを検出すると、被刺激白血球及び／又は活性化白血球の局在定位などを行うことができる。さらに、開いたコンホメーションのインテグリンＩ－ドメイン又は改変インテグリンＩ－ドメインと反応もしくは結合する抗体などの抗インテグリン抗体を、ここに解説するように治療上利用することもできる。従って、抗インテグリン抗体を診断に用いて、炎症を検出するなど、臨床検査法の一部として血中のタンパク質レベルを観察することもできる。検出は、抗体を、検出可能な物質に共役（即ち物理的に連結）す

ることで、簡便化できる。検出可能な物質の例には、多様な酵素、補欠分子団、蛍光物質、発光物質、生物発光物質、及び放射性物質がある。適した酵素の例には、西洋わさびペルオキシダーゼ、アルカリホスファターゼ、β－ガラクトシダーゼ、又はアセチルコリンエステラーゼがある。適した補欠分子団複合体の例には、ストレプトアビジン／ビオチン及びアビジン／ビオチンがある。適した蛍光物質の例には、ウンベリフェロン、フルオレセイン、フルオレセインイソチオシアネート、ローダミン、ジクロロトリアジニルアミンフルオレセイン、塩化ダンジル又はフィコエリトリンがある。発光物質の例にはルミノールがある。生物発光物質の例には、ルシフェラーゼ、ルシフェリン、及びエクオリンがある。適した放射性物質の例には１２５Ｉ、１３１Ｉ、３５Ｓ又は３Ｈがある。

【００７５】

単離された核酸分子

本発明は、インテグリンポリペプチド（例えば改変インテグリンＩ－ドメインポリペプチド、例えば改変インテグリンＩ－ドメイン又はＩ－様ドメインポリペプチド）又はその生物学的活性部分をコードする、単離された核酸分子の使用を包含する。

【００７６】

ここで用いる場合の用語「核酸分子」には、ＤＮＡ分子（例えばｃＤＮＡ又はゲノムＤＮＡ）及びＲＮＡ分子（例えばｍＲＮＡ）や、ヌクレオチド類似体を用いて作製したＤＮＡもしくはＲＮＡの類似体が含まれることを、意図している。当該核酸分子は一本鎖でも、二本鎖でもよいが、好ましくは二本鎖ＤＮＡである。野生型ヒトαＬ及びαＭポリペプチドをコードするヌクレオチド配列を、それぞれＳＥＱ ＩＤ ＮＯ：１（ジェンバンク登録番号ＮＭ＿００２２０９）及びＳＥＱ ＩＤ ＮＯ：３（ジェンバンク登録番号Ｎｏ．Ｊ０３９２５）に記載する。本発明の単離された核酸分子は、例えば以下の表９に特定したものなど、ここに解説したαＬ及びαＭ変異型の改変後アミノ酸配列をコードするＳＥＱ ＩＤ ＮＯ：１ 及びＳＥＱ ＩＤ ＮＯ：３のヌクレオチド配列を包含する。表９は、ここに解説した改変αＬ及びαＭ変異型になるように変更された特定のヌクレオチド残基を示す。例えば、αＬＫ２８７Ｃ／Ｋ２９４Ｃ 変異型は改変αＬポリペプチドであり、この場合αＬ（ＳＥＱ ＩＤ ＮＯ：２） のアミノ酸配列に変更を行った結果、アミノ酸残基２８７位及び２９４位がシステイン残基に置換されている。対応する野生型ヌクレオチド配列ＳＥＱ ＩＤ ＮＯ：１は、それぞれヌクレオチド残基１０２２－１０２４ 及び１１４３－１１４５で改変がある。従って、表９に示すように、αＬ Ｋ２８７Ｃ／Ｋ２９４Ｃ変異型のアミノ酸Ｋ２８７位では、野生型αＬ核酸配列（ＳＥＱ ＩＤ ＮＯ：１）の対応ヌクレオチド残基であるヌクレオチド残基１０２２－１０２４が「ａａａ」から「ｔｇｔ」に改変されている。

【００７７】

【表９】

表９．

変異型				ヌクレオチド配列	
αM or αL	変異	#アミノ酸	#ヌクレオチド	WT	変異体
αL	K287C/K294C	K287	1022-1024	aaa	tgt
		K294	1043-1045	aag	tgt
	E284C/E301C	E284	1013-1015	gag	tgt
		E301	1064-1066	gag	tgt
	L161C/F299C	L161	644-646	ctc	tgt
		F299	1058-1060	ttc	tgt
	K160C/F299C	K160	641-643	aaa	tgt
		F299	1058-1060	ctc	tgt
	L161C/T300C	L161	644-646	ctc	tgt
		T300	1061-1063	act	tgt
	L289C/K294C	L289	1028-1030	ctg	tgt
		K294	1043-1045	aag	tgt
αM	Q163C/Q309C	Q163	607-609	caa	tgt
		Q309	1045-1047	cag	tgt
	D294C/Q311C	D294	1000-1002	gat	tgt
		Q311	1051-1053	cag	tgt
	Q163C/R313C	Q163	607-609	caa	tgt
		R313	1057-1059	cgg	tgt

【００７８】
αＬ；ジェンバンクＮＭ　００２２０９
αＭ；ジェンバンク　Ｊ０３９２５
【００７９】
用語「単離された核酸分子」には、当該核酸の天然源に存在する他の核酸分子から分離された核酸分子が含まれる。例えばゲノムＤＮＡの場合、用語「単離された」には、ゲノムＤＮＡが天然で結合している先の染色体から分離された核酸分子が含まれる。好ましくは「単離された」核酸分子が、当該核酸の由来である生物のゲノムＤＮＡ中で、天然で当該核酸の両側にある配列（即ち当該核酸の５末端及び３末端にある配列）を含まないとよい。例えば、多様な実施態様では、改変インテグリンＩ－ドメインポリペプチドをコードする単離核酸分子が、当該核酸の由来である細胞のゲノムＤＮＡ中で当該核酸分子の両側に天然で存在するヌクレオチド配列を、約５ｋｂ、４ｋｂ、３ｋｂ、２ｋｂ、１ｋｂ、０．５ｋｂ又は０．１ｋｂ未満、含有していてもよい。さらに、ｃＤＮＡ分子などの「単離された」核酸分子は、組換え技術で作製した場合に他の細胞性物質、又は培地を実質的に含まない状態であり得、あるいは、化学合成した場合には前駆化学物質又は他の化学物質を実質的に含まない状態であり得る。
【００８０】
当業者であれば、さらに、改変インテグリンポリペプチドをコードするヌクレオチド配列に更なる変更を変異により加えて、コードされた改変インテグリンポリペプチドのアミノ酸配列を、当該改変インテグリンポリペプチドの構造上の特徴又は機能的能力をそれ以上変えることなく、変更できることは認識されるであろう。例えば、「重要でない」アミノ酸残基位置でアミノ酸置換が起きるようなヌクレオチド置換を、改変インテグリンポリペプチドをコードする配列中で行うことができる。「重要でない」アミノ酸残基とは、当該構造及び／又は生物学的活性をそれ以上変化させることなく、改変インテグリンポリペプチドの配列から変更できる残基である。本発明の方法に基づいて、コンピュータ・デザイン及びモデリングを用いて、所望のタンパク質コンホメーションを得るために、いずれのアミノ酸残基が変更に適するかが決定される。
【００８１】
従って、本発明の方法には、活性にとって重要でないアミノ酸残基の変更を含有する改変

インテグリンポリペプチドをコードする核酸分子の使用が含まれよう。

【００８２】

好ましくは、保存的アミノ酸置換は、重要でないと予測される一つ又はそれ以上のアミノ酸残基で行うとよい。「保存的アミノ酸置換」とは、類似の側鎖を有する一アミノ酸残基に、当該アミノ酸残基が置換されるものである。類似の側鎖を有するアミノ酸残基の系統群が当該分野で定義されている。これらの系統群には、塩基性の側鎖（例えばリシン、アルギニン、ヒスチジン）、酸性の側鎖（例えばアスパラギン酸、グルタミン酸）、無電荷の極性側鎖（例えばグリシン、アスパラギン、グルタミン、セリン、スレオニン、チロシン、システイン）、非極性の側鎖（例えばアラニン、バリン、ロイシン、イソロイシン、プロリン、フェニルアラニン、メチオニン、トリプトファン）、ベータ分岐側鎖（例えばスレオニン、バリン、イソロイシン）及び芳香族の側鎖（例えばチロシン、フェニルアラニン、トリプトファン、ヒスチジン）、を持つアミノ酸がある。このように、改変インテグリンポリペプチド中で重要でないと予測されるアミノ酸残基を、同じ側鎖系統群の別のアミノ酸残基に置換することが好ましい。

【００８３】

組換え発現ベクタ及びホスト細胞

本発明の別の局面は、インテグリンＩ－ドメインもしくはＩ－様ドメインポリペプチドなどの改変インテグリンポリペプチド（又はその一部分）又は融合タンパク質をコードする核酸を含有する組換え発現ベクタなどのベクタに関するものである。ここで用いる用語「ベクタ」とは、連結された相手である別の核酸を輸送できる核酸分子を言う。ベクタの一つの形は、別のＤＮＡセグメントを途中に連結できる環状の二本鎖ＤＮＡループを言う「プラスミド」である。もう一つの種類のベクタは、別のＤＮＡセグメントをウィルスゲノム中に連結できるウィルスベクタである。いくつかのベクタは、導入された先のホスト細胞で自律的複製が可能である（例えば、細菌由来の複製開始点を有する細菌ベクタ及びエピソームほ乳類ベクタなど）。他のベクタ（例えば非エピソームほ乳類ベクタなど）は、ホスト細胞に導入されると、ホスト細胞中のゲノムに組み込まれて、ホストゲノムと一緒に複製される。さらに、いくつかのベクタは、作動的に連結された先の遺伝子の発現を命令することができる。このようなベクタをここでは「発現ベクタ」と呼ぶ。一般に、組換えＤＮＡ技術で実用性のある発現ベクタは、プラスミドの形であることが多い。本明細書では、プラスミドが最もよく用いられている形のベクタであるため、「プラスミド」及び「ベクタ」を交換可能に用いている場合がある。しかし、本発明の方法には、例えばウィルスベクタ（複製能欠損レトロウィルス、アデノウィルス及びアデノ随伴ウィルスなど）など、同等の機能を果たす他の形の発現ベクタも含まれよう。

【００８４】

本発明の組換え発現ベクタは、本発明の核酸を、当該核酸がホスト細胞内で発現するのに適した形で含むが、このことは、この組換え発現ベクタには、発現に用いるホスト細胞に基づいて選択され、発現させようとする核酸配列に作動的に連結させた一つ又はそれ以上の調節配列が含まれることを意味する。組換え発現ベクタ内で、「作動的に連結されている」とは、当該ヌクレオチド配列の発現が可能な態様で（例えばインビトロ転写／翻訳系で、又は、ベクタをホスト細胞内に導入した場合にはホスト細胞内で）、目的のヌクレオチド配列が調節配列に連結されていることを意味することを、意図している。用語「調節配列」には、プロモータ、エンハンサ及び他の発現調節配列（例えばポリアデニレーション・シグナル）が包含されることを、意図している。このような調節配列は、例えばＧｏｅｄｄｅｌ；Ｇｅｎｅ Ｅｘｐｒｅｓｓｉｏｎ Ｔｅｃｈｎｏｌｏｇｙ：Ｍｅｔｈｏｄｓ ｉｎ Ｅｎｚｙｍｏｌｏｇｙ １８５，Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ，Ｓａｎ Ｄｉｅｇｏ，ＣＡ（１９９０）に解説がある。調節配列には、多種のホスト細胞でヌクレオチド配列の構成的発現を命令するものや、特定のホスト細胞でのみ、ヌクレオチド配列の発現を命令するもの（例えば組織特異的調節配列）、がある。発現ベクタのデザインは、例えば、形質転換させようとするホスト細胞の選択、所望のタンパク質の発現レベル、等々の因子に応じて様々であろうことは、当業者であれば理解されよう。本発明の発現ベク

タをホスト細胞に導入することで、融合タンパク質又はペプチドを含め、ここに解説したように核酸がコードするタンパク質又はペプチド（例えば改変インテグリンＩ－ドメインポリペプチド、融合タンパク質等）を産生させることができる。

【００８５】

従って本発明は、当該タンパク質が産生するよう組換え発現ベクタを含有する本発明のホスト細胞（例えば原核ホスト細胞又は真核ホスト細胞）を適した培地で培養することで、改変インテグリンＩ－ドメインポリペプチドなどの改変インテグリンポリペプチドを生成する方法を提供する。

【００８６】

本発明の組換え発現ベクタは、例えば本発明の方法に用いるためなど、原核細胞又は真核細胞における改変インテグリンポリペプチド又は融合タンパク質の発現用として設計することができる。例えば、Ｅ．ｃｏｌｉなどの細菌細胞、（バキュロウィルス発現ベクタを用いて）昆虫細胞、酵母細胞又は哺乳動物細胞などで、改変インテグリンＩ－ドメインポリペプチド又は融合タンパク質を発現させることができる。適したホスト細胞はさらにＧｏｅｄｄｅｌ，Ｇｅｎｅ Ｅｘｐｒｅｓｓｉｏｎ Ｔｅｃｈｎｏｌｏｇｙ：Ｍｅｔｈｏｄｓ ｉｎ Ｅｎｚｙｍｏｌｏｇｙ １８５，Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ，Ｓａｎ Ｄｉｅｇｏ，ＣＡ（１９９０）に解説されている。あるいは、組換え発現ベクタは、Ｔ７プロモータ調節配列及びＴ７ポリメラーゼを用いるなどして、インビトロで転写及び翻訳させることもできる。

【００８７】

原核細胞によるタンパク質の発現は、多くの場合、Ｅ．ｃｏｌｉで、融合タンパク質又は非融合タンパク質の発現を命令する構成的もしくは誘導性プロモータを含有するベクタを用いて行われる。融合ベクタは、数多くのアミノ酸を、それにコードされたタンパク質、通常は組換えタンパク質のアミノ末端、に付加する。このような融合ベクタは、典型的には三つの目的：即ち１）組換えタンパク質の発現を増加させる；２）組換えタンパク質の可溶性及び／又は安定性を増加させる；及び３）アフィニティ精製の際にリガンドとして働くことで、組換えタンパク質の精製を助ける、に役立つ。融合発現ベクタの場合、しばしば、タンパク質分解開裂部位を融合部分と組換えタンパク質との間の接合部位に導入して、融合タンパク質の精製後に、融合部分から組換えタンパク質を切り離せるようにする。このような酵素、及びそれらのコグネイト認識配列には、因子Ｘａ、スロンビン、及びエンテロキナーゼ、がある。典型的な融合発現ベクタには、それぞれグルタチオンＳ－トランスフェラーゼ（ＧＳＴ）、マルトースＥ結合たんぱく、又はプロテインＡを標的組換えたんぱくに融合させるｐＧＥＸ（ファルマシア・バイオテック社製；Ｓｍｉｔｈ，Ｄ．Ｂ．ａｎｄ Ｊｏｈｎｓｏｎ，Ｋ．Ｓ．（１９８８）Ｇｅｎｅ ６７：３１－４０）、ｐＭＡＬ（ニューイングランド・バイオラブズ社、マサチューセッツ州ビバリー）及びｐＲＩＴ５（ファルマシア社、ニュージャージー州ピスカタウェイ）がある。精製された改変インテグリンＩ－ドメイン融合タンパク質（例えば可溶性Ｉ－ドメイン－Ｉｇ）は、ここに解説するようにインテグリン活性を修飾するのに利用できる。

【００８８】

適した誘導性非融合Ｅ．ｃｏｌｉ発現ベクタの例には、ｐＴｒｃ（Ａｍａｎｎ ｅｔ ａｌ．，（１９８８）Ｇｅｎｅ ６９：３０１－３１５）及びｐＥＴ １１ｄ（Ｓｔｕｄｉｅｒ ｅｔ ａｌ．，Ｇｅｎｅ Ｅｘｐｒｅｓｓｉｏｎ Ｔｅｃｈｎｏｌｏｇｙ：Ｍｅｔｈｏｄｓ ｉｎ Ｅｎｚｙｍｏｌｏｇｙ １８５，Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ，Ｓａｎ Ｄｉｅｇｏ，Ｃａｌｉｆｏｒｎｉａ（１９９０）６０－８９）がある。ｐＴｒｃベクタからの標的遺伝子の発現は、ハイブリッドｔｒｐ－ｌａｃ融合プロモータからのホストＲＮＡポリメラーゼ転写に依拠するものである。ｐＥＴ １１ｄベクタからの標的遺伝子の発現は、共発現するウィルスＲＮＡポリメラーゼ（Ｔ７ ｇｎ１）が媒介する、Ｔ７ ｇｎ１０－ｌａｃ融合プロモータからの転写に依拠するものである。このウィルスポリメラーゼは、ホスト株ＢＬ２１（ＤＥ３）又はＨＭＳ１７４（ＤＥ３）により、Ｔ７ ｇｎ１遺伝子をｌａｃＵＶ ５プロモータの転写制御下に持つ定住プロファージから提供される

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【００８９】

Ｅ．ｃｏｌｉ内での組換えタンパク質の発現を最大にする戦略の一つは、タンパク質分解により組換えタンパク質を開裂する能力を欠損したホスト細菌で当該タンパク質を発現させることである（Ｇｏｔｔｅｓｍａｎ，Ｓ．，Ｇｅｎｅ Ｅｘｐｒｅｓｓｉｏｎ Ｔｅｃｈｎｏｌｏｇｙ：Ｍｅｔｈｏｄｓ ｉｎ Ｅｎｚｙｍｏｌｏｇｙ １８５，Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ，Ｓａｎ Ｄｉｅｇｏ，Ｃａｌｉｆｏｒｎｉａ（１９９０）１１９－１２８）。もう一つの戦略は、各アミノ酸に対応する個々のコドンがＥ．ｃｏｌｉ内で優先的に利用されるものとなるよう、発現ベクタに挿入しようとする核酸の核酸配列を変更することである（Ｗａｄａ ｅｔ ａｌ．，（１９９２）Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．２０：２１１１－２１１８）。本発明の核酸配列のこのような変更は、標準的なＤＮＡ合成技術で行うことができる。

【００９０】

別の実施態様では、前記発現ベクタは酵母発現ベクタである。酵母Ｓ．セレビジエで発現させるためのベクタの例には、ｐＹｅｐＳｅｃ１（Ｂａｌｄａｒｉ，ｅｔ ａｌ．，（１９８７）ＥＭＢＯ Ｊ．６：２２９－２３４）、ｐＭＦａ（Ｋｕｒｊａｎ ａｎｄ Ｈｅｒｓｋｏｗｉｔｚ，（１９８２）Ｃｅｌｌ ３０：９３３－９４３）、ｐＪＲＹ８８（Ｓｃｈｕｌｔｚ ｅｔ ａｌ．，（１９８７）Ｇｅｎｅ ５４：１１３－１２３）、ｐＹＥＳ２（カリフォルニア州サンジエゴ、インビトロジェン社）及びｐｉｃＺ（カリフォルニア州サンジエゴ、インビトロジェン社）、がある。

【００９１】

あるいは、改変インテグリンポリペプチドは、バキュロウィルス発現ベクタを用いて昆虫細胞で発現させることができる。培養昆虫細胞（例えばＳｆ９細胞）でタンパク質を発現させるのに利用できるバキュロウィルスベクタには、ｐＡｃシリーズ（Ｓｍｉｔｈ ｅｔ ａｌ．（１９８３）Ｍｏｌ．Ｃｅｌｌ Ｂｉｏｌ．３：２１５６－２１６５）及びｐＶＬシリーズ（Ｌｕｃｋｌｏｗ ａｎｄ Ｓｕｍｍｅｒｓ（１９８９）Ｖｉｒｏｌｏｇｙ １７０：３１－３９）がある。

【００９２】

さらに別の実施態様では、本発明の核酸を、哺乳動物発現ベクタを用いて哺乳動物細胞で発現させる。哺乳動物発現ベクタの例には、ｐＣＤＭ８（Ｓｅｅｄ，Ｂ．（１９８７）Ｎａｔｕｒｅ ３２９：８４０）及びｐＭＴ２ＰＣ（Ｋａｕｆｍａｎ ｅｔ ａｌ．（１９８７）ＥＭＢＯ Ｊ．６：１８７－１９５）がある。哺乳動物細胞で用いる場合、発現ベクタの制御機能は、しばしばウィルス調節配列によって提供される。例えば、よく用いられるプロモータは、ポリオーマ、アデノウィルス２、サイトメガロウィルス及びシミアン・ウィルス４０由来のものである。原核細胞及び真核細胞の両方にとって適した他の発現系については、Ｓａｍｂｒｏｏｋ，Ｊ．，Ｆｒｉｔｓｈ，Ｅ．Ｆ．，ａｎｄ Ｍａｎｉａｔｉｓ，Ｔ．Ｍｏｌｅｃｕｌａｒ Ｃｌｏｎｉｎｇ：Ａ Ｌａｂｏｒａｔｏｒｙ Ｍａｎｕａｌ．２ｎｄ，ｅｄ．，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ Ｐｒｅｓｓ，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ，ＮＹ，１９８９の第１６章及び第１７章を参照されたい。

【００９３】

別の実施態様では、前記組換え哺乳動物発現ベクタは、特定の細胞種で優先的に核酸の発現を命令することができる（例えば組織特異的調節配列を用いて核酸を発現させるなど）。組織特異的調節配列は当業で公知である。適した組織特異的プロモータの非限定的な例には、アルブミンプロモータ（肝臓特異的；Ｐｉｎｋｅｒｔ ｅｔ ａｌ．（１９８７）Ｇｅｎｅｓ Ｄｅｖ．１：２６８－２７７）、リンパ球特異的プロモータ（Ｃａｌａｍｅ ａｎｄ Ｅａｔｏｎ（１９８８）Ａｄｖ．Ｉｍｍｕｎｏｌ．４３：２３５－２７５）、特にＴ細胞受容体のプロモータ（Ｗｉｎｏｔｏ ａｎｄ Ｂａｌｔｉｍｏｒｅ（１９８９）ＥＭＢＯ Ｊ．８：７２９－７３３）及び免疫グロブリンのプロモータ（Ｂａｎｅ

ｒｊｉ ｅｔ ａｌ．（１９８３）Ｃｅｌｌ ３３：７２９－７４０；Ｑｕｅｅｎ ａｎｄ Ｂａｌｔｉｍｏｒｅ（１９８３）Ｃｅｌｌ ３３：７４１－７４８）、神経細胞特異的プロモータ（例えば神経フィラメントプロモータ；Ｂｙｒｎｅ ａｎｄ Ｒｕｄｄｌｅ（１９８９）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８６：５４７３－５４７７）、内皮細胞特異的プロモータ（例えばＫＤＲ／ｆｌｋ プロモータ；米国特許第５，８８８，７６５号）、膵臓特異的プロモータ（Ｅｄｌｕｎｄ ｅｔ ａｌ．（１９８５）Ｓｃｉｅｎｃｅ ２３０：９１２－９１６）、及び乳腺特異的プロモータ（例えば乳清プロモータ；米国特許第４，８７３，３１６号及びヨーロッパ特許出願公報第２６４，１６６号）、がある。例えば、マウスホックスプロモータ（Ｋｅｓｓｅｌ ａｎｄ Ｇｒｕｓｓ（１９９０）Ｓｃｉｅｎｃｅ ２４９：３７４－３７９）及びα－フェトタンパク質プロモータ（Ｃａｍｐｅｓ ａｎｄ Ｔｉｌｇｈｍａｎ（１９８９）Ｇｅｎｅｓ Ｄｅｖ．３：５３７－５４６）などの発生調節性プロモータも、包含するところである。

【００９４】

本発明の他の局面は、例えば改変インテグリンＩ－ドメイン核酸分子を含有させた組換え発現ベクタや、又は、ホスト細胞のゲノムの特定の部位で相同組換えが可能なような配列を含有する改変インテグリンＩ－ドメイン核酸分子などの本発明の改変インテグリンポリペプチドをコードする核酸分子を導入する先のホスト細胞に関する。用語「ホスト細胞」及び「組換えホスト細胞」はここでは交換可能に用いられている。このような用語は特定の対象細胞だけでなく、このような細胞の後代又は潜在的な後代の細胞も言うと理解されたい。突然変異又は環境の影響により、何らかの改変が後の世代に起きることがあるため、このような後代は実際のところ、親細胞と同一でないかも知れないが、それでもなお、ここで用いるこの用語の範囲内に包含される。

【００９５】

ホスト細胞はいかなる原核もしくは真核細胞でもよい。例えば、改変インテグリンポリペプチド又は融合タンパク質を、Ｅ．ｃｏｌｉなどの細菌細胞、昆虫細胞、酵母もしくは哺乳動物細胞（例えば造血細胞、白血球、Ｋ５６２細胞、２９３Ｔ細胞、ヒト臍帯静脈内皮細胞（ＨＵＶＥＣ）、ヒト微小血管内皮細胞（ＨＭＶＥＣ）、チャイニーズ・ハムスター卵巣細胞（ＣＨＯ）もしくはＣＯＳ細胞など）で、発現させることができる。他の適したホスト細胞は当業者に公知である。

【００９６】

ベクタＤＮＡは、原核もしくは真核細胞に、常法である形質転換もしくはトランスフェクション技術を通じて導入することができる。ここで用いる場合の用語「形質転換」及び「トランスフェクション」とは、リン酸カルシウムもしくは塩化カルシウム共沈殿法、ＤＥＡＥ－デキストラン－媒介トランスフェクション、リポフェクション、又は電気穿孔法を含め、ホスト細胞に外来の核酸（例えばＤＮＡ）を導入するための、多種の当業者に公知の技術を言うと、意図している。ホスト細胞を形質転換もしくはトランスフェクトする適した方法は、サムブルック氏らの文献（Ｍｏｌｅｃｕｌａｒ Ｃｌｏｎｉｎｇ：Ａ Ｌａｂｏｒａｔｏｒｙ Ｍａｎｕａｌ．２ｎｄ，ｅｄ．，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ Ｐｒｅｓｓ，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ，ＮＹ，１９８９）、及び他の実験用マニュアルに見ることができる。

【００９７】

哺乳動物細胞の安定なトランスフェクトに関しては、用いる発現ベクタ及びトランスフェクション技術によっては、細胞の一部しか、外来のＤＮＡをそのゲノムに組み込まないことがあることが知られている。これらの組み込み体を同定及び選別するには、選択マーカ（例えば抗生物質に対する耐性）をコードする遺伝子を目的の遺伝子と一緒にホスト細胞に導入することが多い。好適な選択マーカには、例えばＧ４１８、ヒグロマイシン及びメトトレキセートなどの薬物に対する耐性をもたらすものがある。選択マーカをコードする核酸をホスト細胞に導入するには、改変インテグリンポリペプチドをコードするのと同じベクタに載せて導入しても、又は別のベクタに載せて導入してもよい。導入した核酸が安

定にトランスフェクトした細胞は、薬物選択（例えば選択マーカ遺伝子を取り込んだ細胞は生き残り、他のものは死滅するなど）で判定できる。

【００９８】

培養液中の原核もしくは真核ホスト細胞などの、本発明のホスト細胞を利用すると、本発明の方法で使用できる改変インテグリンポリペプチド、例えば改変インテグリンＩ－ドメインポリペプチド又は融合タンパク質など、を産生（即ち発現）させることができる。一実施態様では、ホスト細胞を（改変インテグリンＩ－ドメインポリペプチド又は融合タンパク質をコードする組換え発現ベクタを導入した）、改変インテグリンＩ－ドメインポリペプチド又は融合タンパク質が産生されるように、適した培地で培養する。別の実施態様では、改変インテグリンＩ－ドメインポリペプチド又は融合タンパク質を培地又はホスト細胞から単離する。さらに、インテグリン活性を修飾するために、改変インテグリンポリペプチド又は融合タンパク質を発現している組換え細胞を対象に投与してもよい。

【００９９】

さらに本発明のホスト細胞は、非ヒトトランスジェニック動物を作製するのにも利用できる。例えば、ある実施態様では、本発明のホスト細胞は、改変インテグリンＩ－ドメインポリペプチドをコードする配列が導入してある受精卵母細胞又は胚性幹細胞である。次に、このようなホスト細胞を用いて、外因性の改変インテグリンＩ－ドメイン配列がそれらのゲノムに導入された非ヒトトランスジェニック動物や、又は、内因性のインテグリンＩ－ドメイン配列が変更された相同組換え動物を作製することができる。このような動物は、改変インテグリンＩ－ドメイン分子の機能及び／又は活性を研究したり、また、改変インテグリンＩ－ドメインポリペプチド活性のモジュレータを同定及び／又は評価するのに、有用である。ここで用いる「トランスジェニック動物」とは、当該動物の一つ又はそれ以上の細胞が導入遺伝子を含有する、非ヒト動物、好ましくは哺乳動物、より好ましくはラットもしくはマウスなどのげっ歯類、である。トランスジェニック動物の他の例には、非ヒト霊長類、ヒツジ、イヌ、ウシ、ヤギ、ニワトリ、両生類等がある。導入遺伝子とは、トランスジェニック動物が発生する元となる細胞のゲノム中に組み込まれ、成熟動物のゲノム中に留まって、そのトランスジェニック動物の一つ又はそれ以上の細胞種又は組織で、コードされた遺伝子産物の発現を命令する外因性のＤＮＡである。ここで用いる「相同組換え動物」とは、当該動物の発生前に、当該動物の胚細胞など、動物の細胞中に導入された外因性ＤＮＡ分子と内因性遺伝子との間で相同組換えが起きることで、内因性インテグリンＩ－ドメイン遺伝子が変更されている非ヒト動物、好ましくは哺乳動物、より好ましくはマウス、である。

【０１００】

本発明のトランスジェニック動物は、改変インテグリンＩ－ドメインをコードする核酸を、受精卵母細胞の雄前核に、マイクロインジェクション、レトロウィルス感染などで導入し、この卵母細胞を偽妊娠の借り腹動物で発生させることで、作製できる。導入遺伝子には、導入遺伝子の発現効率を高めるために、イントロン配列及びポリアデニレーションシグナルも含めてもよい。組織特異的調節配列を改変インテグリンＩ－ドメイン導入遺伝子に作動的に連結させると、特定の細胞に改変インテグリンＩ－ドメインタンパク質の発現を命令することができる。胚の操作及びマイクロインジェクションを通じてトランスジェニック動物、特にマウスなどの動物を作製する方法は従来技術となっており、例えば、両者ともレダー氏らの米国特許第４，７３６，８６６号及び第４，８７０，００９号、ワグナー氏らの米国特許第４，８７３，１９１号、及びＨｏｇａｎ，Ｂ．，Ｍａｎｉｐｕｌａｔｉｎｇ ｔｈｅ Ｍｏｕｓｅ Ｅｍｂｒｙｏ，（Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ Ｐｒｅｓｓ，Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ，Ｎ．Ｙ．，１９８６）に解説がある。

【０１０１】

相同組換え動物を作製するには、欠失、付加又は置換を導入することで、改変インテグリンＩ－ドメイン遺伝子を機能的に破壊するなど変更してあるような改変インテグリンＩ－ドメイン遺伝子を少なくとも一部分を含有するベクタを作製する。改変インテグリンＩ－

ドメイン遺伝子は、ヒト遺伝子でもよいが、より好ましくはヒト改変インテグリンＩ－ドメイン遺伝子の非ヒト相同体である。例えばマウス改変インテグリンＩ－ドメイン遺伝子を用いて、マウスゲノム中の内因性改変インテグリンＩ－ドメイン遺伝子を改変するのに適した、ベクタなどの相同組換え核酸分子を構築することができる。ある好適な実施態様では、相同組換えが起きると、当該内因性改変インテグリンＩ－ドメイン遺伝子が機能的に破壊される（即ち機能タンパク質をもはやコードしていない；ここでは「ノックアウト」ベクタとも呼ぶ）よう、相同組換え核酸分子をデザインする。代替的に、相同組換えが起きると、内因性改変インテグリンＩ－ドメイン遺伝子が変異もしくは変化するが、以前として機能たんぱくをコードしているよう、相同組換え核酸分子をデザインすることができる（例えば、上流の調節領域を変えることで、内因性改変インテグリンＩ－ドメインタンパク質の発現を変えることができる）。相同組換え核酸分子において、改変インテグリンＩ－ドメイン遺伝子のうちで変更された部分は、その５末端及び３末端で、改変インテグリンＩ－ドメイン遺伝子の追加の核酸配列部分でフランクされているため、胚性幹細胞中などの細胞中で、相同組換え核酸分子が持つ外因性改変インテグリンＩ－ドメイン遺伝子と、内因性改変インテグリンＩ－ドメイン遺伝子との間で、相同組換えが起きる。この追加のフランキング改変インテグリンＩ－ドメイン核酸配列は、内因性遺伝子との間の相同組換えが成功するよう、充分な長さのものである。典型的には、数キロベースの（５末端及び３末端の両側の）フランキングＤＮＡが相同組換え核酸分子に含まれる（相同組換えベクタの解説については、例えばＴｈｏｍａｓ，Ｋ．Ｒ．ａｎｄ Ｃａｐｅｃｃｈｉ，Ｍ．Ｒ．（１９８７）Ｃｅｌｌ ５１：５０３ を参照されたい）。相同組換え核酸分子を胚性幹細胞株などの細胞中に（例えばエレクトロポレーションで）導入し、導入された改変インテグリンＩ－ドメイン遺伝子が、内因性の改変インテグリンＩ－ドメイン遺伝子と相同組換えを起こした細胞を選択する（例えばＬｉ，Ｅ．ｅｔ ａｌ．（１９９２）Ｃｅｌｌ ６９：９１５を参照されたい）。こうして選択された細胞を、動物（マウスなど）の胚盤胞に注射して凝集キメラを形成することができる（例えばＢｒａｄｌｅｙ，Ａ．ｉｎ Ｔｅｒａｔｏｃａｒｃｉｎｏｍａｓ ａｎｄ Ｅｍｂｒｙｏｎｉｃ Ｓｔｅｍ Ｃｅｌｌｓ：Ａ Ｐｒａｃｔｉｃａｌ Ａｐｐｒｏａｃｈ，Ｅ．Ｊ．Ｒｏｂｅｒｔｓｏｎ，ｅｄ．（ＩＲＬ，Ｏｘｆｏｒｄ，１９８７）ｐｐ．１１３－１５２）。次に、キメラ胚を適した偽妊娠のメス借り腹動物に移植することができ、この胚を産期に至らせる。相同組換えを起こしたＤＮＡを生殖細胞中に持つ後代を用いれば、導入遺伝子の生殖細胞系伝播により、動物のすべての細胞が相同組換えを起こしたＤＮＡを含有するような動物を育種することができる。例えばベクタ、又は、相同組換え動物など、相同組換え核酸分子を構築する方法は、さらにＢｒａｄｌｅｙ，Ａ．（１９９１）Ｃｕｒｒｅｎｔ Ｏｐｉｎｉｏｎ ｉｎ Ｂｉｏｔｅｃｈｎｏｌｏｇｙ ２：８２３－８２９ 及びル・ムーレック氏らのＰＣＴ 国際公報Ｎｏｓ．：ＷＯ ９０／１１３５４ ；スミシーズ氏らのＷＯ ９１／０１１４０ ；ジズルストラ氏らのＷＯ ９２／０９６８ ；及びバーンズ氏らのＷＯ ９３／０４１６９に解説されている。

【０１０２】

別の実施態様では、導入遺伝子の発現を調節可能とする所定の系を含有するトランスジェニック非ヒト動物を作製することができる。このような系の一例は、バクテリオファージＰ１のｃｒｅ／ｌｏｘＰリコンビナーゼ系である。ｃｒｅ／ｌｏｘＰリコンビナーゼ系の解説については、例えばＬａｋｓｏ ｅｔ ａｌ．（１９９２）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ８９：６２３２－６２３６を参照されたい。リコンビナーゼ系のもう一つの例は、サッカロミセス－セレビジエのＦＬＰリコンビナーゼ系である（Ｏ’Ｇｏｒｍａｎ ｅｔ ａｌ．（１９９１）Ｓｃｉｅｎｃｅ ２５１：１３５１－１３５５）。ｃｒｅ／ｌｏｘＰ リコンビナーゼ系を用いて導入遺伝子の発現を調節する場合、Ｃｒｅリコンビナーゼ及び所定のタンパク質の両方をコードする導入遺伝子を含有する動物が必要である。このような動物は、例えば一方が所定のタンパク質をコードする導入遺伝子を含有し、他方がリコンビナーゼをコードする導入遺伝子を含有するような二種のトランスジェニック動物を交配するなどにより、「二重」トランスジェニック動物を構築

することで、提供できる。

【０１０３】

スクリーニング検定

本発明は、インテグリン活性を修飾するモジュレータ、即ち候補もしくはテスト化合物又は薬剤（例えばペプチド、抗体、ペプチドミメティック、小分子（有機もしくは無機）又は他の薬剤など）を同定する方法（ここでは「スクリーニング検定」とも呼ぶ）を提供するものである。これらの検定を、例えば活性コンホメーションのインテグリンＩ－ドメインポリペプチドなどのインテグリンＩ－ドメインポリペプチドに結合する、インテグリンＩ－ドメインポリペプチドと相互作用する他のタンパク質と結合する、インテグリンＩ－ドメインポリペプチドと他のタンパク質、例えばＩＣＡＭなどのインテグリンリガンド、との結合を誘導し、その相互作用を修飾することで、インテグリン活性を修飾する、などのような化合物を同定するよう、デザインする。

【０１０４】

ここで用いる用語「インテグリン活性のモジュレータ」には、ここに解説するように、少なくとも一つのインテグリン活性を修飾又は調節できる化合物又は作用薬が包含される。インテグリン活性のモジュレータには、限定はしないが、小型の有機もしくは無機分子、核酸分子、ペプチド、抗体等が含まれよう。インテグリン活性のモジュレータは、例えば細胞接着又はリガンド結合など、インテグリン活性の誘導物質又は阻害物質であってよい。ここで用いる「インテグリン活性の誘導物質」は、インテグリン活性を刺激、亢進、及び／又は、模倣するものである。ここで用いる「インテグリン活性の阻害物質」は、インテグリン活性を低減、遮断又は拮抗するものである。

【０１０５】

ここで交換可能に用いられるように、「インテグリン活性」又は「インテグリン媒介活性」とは、インビトロ及びインビボで、標準的手法に従って調べたときに、インテグリンポリペプチド又は核酸分子が、インテグリン応答性細胞、又はインテグリンリガンドもしくは受容体に及ぼす活性を言う。一の実施態様では、インテグリン活性は、例えば細胞間又は細胞と細胞外マトリックス間の接着などの細胞接着事象の媒介能である。別の実施態様では、インテグリン活性は、細胞シグナリング事象の伝達能である。さらに別の実施態様では、インテグリン活性は、ＩＣＡＭなどのリガンドに対する結合能である。

【０１０６】

ある好適な実施態様では、可溶性の組換え高親和インテグリンＩ－ドメインは、インテグリンリガンド結合を阻害する小分子アンタゴニストをスクリーニングするのに、利用できる。さらに、最小限のリガンド結合活性を示す野生型インテグリンＩ－ドメインに対する作用との比較に基づき、直接的／競合的及び間接的／非競合的な阻害の態様を持つ、抗体などのアンタゴニストを区別することができる。例えば、間接的な阻害物質は、活性化野生型インテグリンＩ－ドメインではリガンド結合を阻害するであろうが、ジスルフィドで固定された高親和Ｉ－ドメインではしないはずである。

【０１０７】

別の実施態様では、検定は細胞ベースの検定であり、改変インテグリンポリペプチドを細胞表面上に発現している細胞にテスト化合物を接触させるステップと、テスト化合物のインテグリン活性の修飾（誘導又は阻害など）能を調べるステップとを含む。例えば、開いたコンホメーションで安定した改変インテグリンＩ－ドメインポリペプチドを細胞表面上に発現している細胞を、テスト化合物に接触させ、このテスト化合物の、インテグリンリガンドに対する接着の修飾能を、ここに解説し、例示するように調べる。

【０１０８】

さらに別の実施態様では、例えば開いたコンホメーションで安定させた改変インテグリンＩ－ドメインポリペプチドを検出可能な標識に共役させて、改変インテグリンポリペプチドの結合を、固定化インテグリンリガンドに対する標識付インテグリンＩ－ドメインの結合量を検出することで調べられるようにしても、テスト化合物によるインテグリンリガンド結合の修飾能を調べることができる。

【０１０９】
炎症の動物モデルなど、動物ベースのモデル系を、例えばインテグリン活性のモジュレータである化合物を同定するようデザインされたスクリーニング戦略の一部として使用してもよい。このように、動物ベースのモデルを、炎症を修飾したり、インテグリン媒介疾患を治療するのに効果的であろう薬剤、薬品、治療法及び介入法を判定するのに、用いてもよい。例えば、動物モデルを、インテグリン活性の修飾能を示すと思われる化合物に暴露し、この動物のその暴露に対する応答を、処置前後の炎症性活性を評価することでモニタすることができる。ここに解説したような改変インテグリンＩ－ドメインポリペプチドを発現するトランスジェニックマウスなどのトランスジェニック動物を用いても、炎症を修飾したり、インテグリン媒介疾患を治療するのに効果的であろう薬剤、薬品、治療法及び介入法を判定することができる。

【０１１０】
別の局面では、本発明は、ここに解説した検定法の二つ又はそれ以上の組合せに関する。例えば、インテグリン活性のモジュレータは、細胞ベースの検定法を使用して同定することができ、作用薬のインテグリン活性修飾能を、炎症の動物モデルなどの動物などで、ｉｎ　ｖｉｖｏで確認してもよい。

【０１１１】
さらに、スクリーニング検定法を用いてインテグリン活性の誘導物質を同定することができ、例えばインテグリンのリガンド又は受容体への結合などのインテグリンポリペプチド活性を模倣したり、インテグリンのインテグリン応答細胞に対する活性を模倣したりするような誘導物質を同定することができる。このような化合物には、限定はしないが、ペプチド、抗体、又は小有機もしくは無機化合物が含まれるであろう。ある実施態様では、開いた活性化コンホーマに選択的に結合する本発明の抗ＬＦＡ－１抗体など、抗インテグリン抗体を用いても、テスト化合物のインテグリン活性化能を評価することができる。

【０１１２】
テスト化合物は、生物学的ライブラリ；空間指定可能なパラレル固相又は液相ライブラリ；逆重畳積分が必要な合成ライブラリ法；「ワン・ビーズ・ワン・コンパウンドライブラリ法；及びアフィニティ・クロマトグラフィ選別法を用いた合成ライブラリ法を含め、当該分野で公知のコンビナトリアル・ライブラリ法での数多くのアプローチのいずれを用いて得てもよい。生物学的ライブラリ法はペプチド・ライブラリに限られるが、他の四つの方法は、、化合物のペプチド、非ペプチド・オリゴマー又は小分子ライブラリに応用できる（Ｌａｍ，Ｋ．Ｓ．（１９９７）Ａｎｔｉｃａｎｃｅｒ Ｄｒｕｇ Ｄｅｓ．１２：１４５）。

【０１１３】
分子ライブラリの合成法の例は、例えばＤｅＷｉｔｔ ｅｔ ａｌ．（１９９３）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．Ｕ．Ｓ．Ａ．９０：６９０９；Ｅｒｂ ｅｔ ａｌ．（１９９４）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９１：１１４２２；Ｚｕｃｋｅｒｍａｎｎ ｅｔ ａｌ．（１９９４）．Ｊ．Ｍｅｄ．Ｃｈｅｍ．３７：２６７８；Ｃｈｏ ｅｔ ａｌ．（１９９３）Ｓｃｉｅｎｃｅ ２６１：１３０３；Ｃａｒｒｅｌｌ ｅｔ ａｌ．（１９９４）Ａｎｇｅｗ．Ｃｈｅｍ．Ｉｎｔ．Ｅｄ．Ｅｎｇｌ．３３：２０５９；Ｃａｒｅｌｌ ｅｔ ａｌ．（１９９４）Ａｎｇｅｗ．Ｃｈｅｍ．Ｉｎｔ．Ｅｄ．Ｅｎｇｌ．３３：２０６１；及びＧａｌｌｏｐ ｅｔ ａｌ．（１９９４）Ｊ．Ｍｅｄ．Ｃｈｅｍ．３７：１２３３に見ることができる。

【０１１４】
化合物のライブラリは、溶液中（例えばＨｏｕｇｈｔｅｎ（１９９２）Ｂｉｏｔｅｃｈｎｉｑｕｅｓ １３：４１２－４２１）で提供されても、又はビーズ（Ｌａｍ（１９９１）Ｎａｔｕｒｅ ３５４：８２－８４）、チップ（Ｆｏｄｏｒ（１９９３）Ｎａｔｕｒｅ ３６４：５５５－５５６）、細菌（ラドナー氏の米国特許第５，２２３，４０９号）、胞子（ラドナー氏の米国特許第’４０９号）、プラスミド（Ｃｕｌｌ ｅｔ ａｌ．（１９９２）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ８９：１８６５－１８６９）又

はファージ（Ｓｃｏｔｔ ａｎｄ Ｓｍｉｔｈ（１９９０）Ｓｃｉｅｎｃｅ ２４９：３８６－３９０）；（Ｄｅｖｌｉｎ（１９９０）Ｓｃｉｅｎｃｅ ２４９：４０４－４０６）；（Ｃｗｉｒｌａ ｅｔ ａｌ．（１９９０）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．８７：６３７８－６３８２）；（Ｆｅｌｉｃｉ（１９９１）Ｊ．Ｍｏｌ．Ｂｉｏｌ．２２２：３０１－３１０）；（ラドナー氏の上記文献．）上でも提供され得る。

【０１１５】

さらに本発明は、上記のスクリーニング検定法で同定された新規な作用薬に関するものである。介入法に関しては、インテグリン活性及び／又は炎症性活性を修飾するあらゆる処置を、ヒトの治療的介入法候補とみなすべきである。

【０１１６】

医薬組成物

本発明の改変インテグリンポリペプチドをコードする核酸分子、改変インテグリンポリペプチド（例えば改変Ｉ－ドメインポリペプチド及び融合タンパク質）、及びそれらの活性フラグメント、抗インテグリンＩ－ドメイン抗体、及びインテグリンモジュレータ（ここでは「有効化合物」ともよぶ）ＤＮＡワクチン、又はＤＮＡベクタは、投与に適した医薬組成物中に組み込むことができる。ここで用いる、インテグリン活性の「モジュレータ」、例えば阻害物質及び誘導物質など、には、例えばインテグリン媒介シグナリング事象、インテグリン媒介接着事象、又は、コグネイト・リガンドに対するインテグリン結合などのインテグリン活性を修飾する化合物が含まれる。インテグリンモジュレータには、本発明の改変インテグリンＩ－ドメイン又はＩ－様ドメインポリペプチド、抗インテグリンＩ－ドメインポリペプチドや、ここで解説したスクリーニング検定で同定された化合物がある。このような組成物は典型的に、当該化合物、核酸分子、ベクタ、タンパク質又は抗体と、薬学的に許容可能な担体とを含んで成る。ここで用いる用語「薬学的に許容可能な担体」は、薬学的投与に適合性ある、あらゆる溶媒、分散媒、コーティング、抗菌剤及び抗カビ剤、等張剤及び吸収遅延剤等が含まれることを意図する。薬学的に有効な物質のためのこのような媒質及び薬剤の使用は当該分野で公知である。従来の媒質又は薬剤が有効化合物に対して適合性がない場合を除き、組成物中へのその使用が考慮される。補助的な有効化合物も本組成物中に取り入れてよい。

【０１１７】

本発明の医薬組成物は、意図した投与経路にとって適合性あるように調合される。投与経路の例には、疾患部位への直接的設置を含め、非経口、例えば静脈内、皮内、皮下、経口（例えば吸入）、経皮（局所）、経粘膜、眼内、及び直腸投与がある。非経口、皮内、又は皮下投与用に用いられる溶液又は懸濁液には、以下の成分：注射用の水、生理食塩水、不揮発性油、ポリエチレングリコール、グリセリン、プロピレングリコール又は他の合成溶媒などの無菌の希釈剤；ベンジルアルコール又はメチルパラベンなどの抗菌剤；アスコルビン酸又は亜硫酸水素ナトリウムなどの抗酸化剤；エチレンジアミン四酢酸などのキレート剤；酢酸、クエン酸又はリン酸などの緩衝剤や、塩化ナトリウム又はデキストロースなどの張性調節剤、がある。ｐＨは、塩酸又は水酸化ナトリウムなどの酸又は塩基で調節できる。非経口用製剤は、ガラス製又はプラスチック製のアンプル、使い捨ての注射筒又は複数容量用バイアルに封入することができる。

【０１１８】

注射用途に適した医薬組成物には、無菌の水溶液（水溶性の場合）又は分散液や、無菌の注射用溶液又は分散液の即時調合用の無菌粉末がある。静脈内投与の場合、適した担体には、生理食塩水、静菌水、クレモフォールＥＬ^{TM}（ＢＡＳＦ社製、ニュージャージー州パーシパニー）又はリン酸緩衝生理食塩水（ＰＢＳ）がある。いずれの場合も、組成物は無菌でなければならず、注射筒への注入が容易な程度に流動的でなければならない。また、製造及び保管の条件下で安定でなくてはならず、細菌及びカビなどの微生物の汚染作用から守られていなければならない。担体は、例えば水、エタノール、ポリオール（例えばグリセロール、プロピレングリコール、及び液体ポリエチレングリコール等や、これらの

適した混合物を含有する溶媒又は分散媒であってよい。適当な流動性は、例えばレシチンなどのコーティングを用いたり、分散液の場合には必要な粒子の大きさを維持したり、そして界面活性剤を利用するなどにより、維持できる。微生物の活動を防ぐには、多種の抗菌剤及び抗カビ剤、例えばパラベン、クロロブタノール、フェノール、アスコルビン酸、チメロサール等により、可能である。多くの場合、糖類、マンニトール、ソルビトールなどの多価アルコールや、塩化ナトリウムなどの等張剤を組成物中に含めるのが好ましいであろう。注射用組成物の吸収を長引かせるには、モノステアリン酸アルミニウム及びゼラチンなど、吸収を遅らせる薬剤を組成物中に含めると可能である。

【０１１９】

無菌の注射用溶液は、有効化合物（例えば可溶性の改変インテグリンＩ－ドメイン融合タンパク質）を必要量、適した溶媒中に、必要に応じて上に列挙した成分のうちの一つ又は組合せと一緒に取り入れた後、濾過滅菌して調製することができる。一般的には、分散液は、塩基性分散媒と、上に列挙したうちで必要な他の成分とを含有する無菌の賦形剤に有効化合物を取り入れることにより、調製されている。無菌の注射用溶液の調製用の無菌粉末の場合、好適な調製法は真空乾燥及び凍結乾燥であり、その結果、有効成分と、予め無菌濾過したその溶液から出た更なる所望の成分とから成る粉末が生じる。

【０１２０】

経口用組成物には、一般に不活性の希釈剤又は食用の担体が含まれる。これらをゼラチンカプセルに封入することができ、又は圧縮して錠剤にしてもよい。経口による治療用投与の場合、有効化合物は医薬品添加物と一緒に組み込み、錠剤、トローチ、又はカプセルの形で用いることができる。経口用組成物はさらに、含嗽剤として利用するために流動性担体を用いて調製してもよく、この場合この流動性担体中の化合物は経口投与され、スイッシュ（原語：ｓｗｉｓｈ）、喀出、又は嚥下される。薬学的に適合性ある結合剤、及び／又はアジュバント材料を、組成物の一部として含めてもよい。錠剤、丸剤、カプセル、トローチ等は、以下の成分：即ち、例えば微小結晶セルロース、トラガカントゴム又はゼラチンなどの結合剤；でんぷん又は乳糖などの医薬品添加剤、アルギン酸、プリモゲル、又はコーン・スターチなどの崩壊剤；ステアリン酸マグネシウム又はステロートなどの潤滑剤；コロイド状二酸化珪素などの推進剤；ショ糖又はサッカリンなどの甘味料；ペパーミント、サリチル酸メチル、又はオレンジ甘味料などの着香料、か、又は同様な性質の化合物のいずれを含有してもよい。

【０１２１】

吸入による投与の場合、二酸化炭素などの気体などの適した噴射剤を含有する加圧容器又はディスペンサ、又はネブライザから、エーロゾル・スプレーの形で化合物を送達する。

【０１２２】

全身投与は経粘膜もしくは経皮手段によってもよい。経粘膜もしくは経皮投与の場合、透過させようとする障壁に適当な浸透剤を、製剤中に用いる。このような浸透剤は一般に当該分野で公知であるが、例えば経粘膜投与用には、界面活性剤、胆汁酸塩、及びフシジン酸誘導体がある。経粘膜投与は鼻孔用スプレー又は座薬の利用を通じて行うことができる。経皮投与には、有効化合物を当該分野で広く公知の軟膏、軟膏剤、ゲル又はクリームに調合する。

【０１２３】

また化合物を、直腸送達用の座薬（例えばココア・バター及び他のグリセリドなどの従来の座薬用基剤を用いて）又は停留浣腸剤の形で調製することもできる。

【０１２４】

一の実施態様では、インプラント及びマイクロ封入送達系を含め、制御放出製剤など、身体から化合物が急速に失われないようにする担体と一緒に、有効化合物を調製する。エチレン酢酸ビニル、ポリ無水物、ポリグリコール酸、コラーゲン、ポリオルトエステル、及びポリ乳酸など、生分解性で生体適合性のあるポリマーを利用してもよい。このような製剤の調製法は当業者に明白である。材料はアルザ・コーポレーション及びノバ・ファーマシューティカルズ社から市販のものを利用できる。リポソーム懸濁液（感染細胞を狙うよ

うウィルス抗原に対するモノクローナル抗体で標的決めしたリポソームを含む）も、薬学的に許容可能な担体として利用できる。これらは、例えば米国特許第４，５２２，８１１号に解説されているように、当業者に公知の方法に基づいて調製できる。

【０１２５】

投与が簡単なよう、かつ投薬量が均一になるように、単位剤形で経口もしくは非経口組成物を調合すると、特に有利である。ここで用いる単位剤形とは、治療しようとする対象の一回ごとの投薬量として合わせた物理的に個別の単位を言う。このとき各単位は、必要な薬剤担体との関連から所望の治療効果を生じるよう計算された所定量の有効化合物を含有する。本発明の単位剤形の詳細は、有効化合物に固有の特徴や、達成しようとする特定の治療効果、及び、個人を治療するためのこのような有効化合物を配合する当業に内在する限界の制約を受け、またこれらに直接依存する。

【０１２６】

本発明の有効化合物の投与は、予防目的又は治療目的のいずれであってもよい。従って、一実施態様では、「治療上有効な用量」とは、投与を受ける患者において生理学的に検出可能な変化をもたらすのに充分な量の有効化合物を言う。一実施態様では、治療上有効な用量とは、炎症及び／又は免疫応答の修飾を得るのに充分な有効化合物量を言う。別の実施態様では、治療上有効な用量とは、炎症及び／又は免疫系疾患の症状を寛解させるのに充分な有効化合物量を言う。別の実施態様では、治療上有効な用量とは、炎症及び／又は免疫系応答を妨げるのに充分な有効化合物量を言う。さらに別の実施態様では、治療上有効な用量とは、ここに解説したインテグリン活性（例えばシグナリング活性、接着活性又はリガンド結合活性）を修飾するのに充分な有効化合物量を言う。

【０１２７】

このような化合物の毒性及び治療効果は、例えばＬＤ５０（集団の５０％にとって致命的な用量）及びＥＤ５０（集団の５０％において治療上有効な用量）を調べるなどの、細胞培養又は実験動物による標準的な薬学的手法により決定することができる。毒性効果対治療効果の用量比が治療指数であり、比ＬＤ／ＥＤ５０で表すことができる。大きな治療指数を呈する化合物が好適である。毒性の副作用を呈する化合物を用いてもよいが、非感染細胞へのダメージを抑え、ひいては副作用を軽減するためには、感染組織部位へこのような化合物をターゲティングする送達系をデザインするよう、配慮が必要である。

【０１２８】

細胞培養アッセイ及び動物実験で得られるデータは、ヒトで用いる投薬量範囲を設定する上で利用することができる。このような化合物の用量は、好適には、毒性が小さいかもしくはないような、ＥＤ５０を含む血中濃度範囲内であるとよい。投薬量は、用いる剤形及び利用する投与経路に応じて、この範囲内で様々であってよい。本発明の方法で用いる化合物の場合、まず細胞培養アッセイで治療上の有効量を推測することができる。動物モデルで用量を設定して、細胞培養で調べたときにＩＣ５０（即ち、症状の半分－最大を抑制するようなテスト化合物濃度）を含む血中血漿濃度範囲が得られるようにしてもよい。このような情報は、ヒトに応用できる用量をより精確に決定するのに、利用できる。血漿中のレベルは、例えば高速液体クロマトグラフィーで測定してもよい。

【０１２９】

ここで定義するように、抗体、タンパク質又はポリペプチドの治療上有効な量（即ち有効量）は、約０．００１乃至３０ｍｇ／ｋｇ体重、好ましくは約０．０１乃至２５ｍｇ／体重１ｋｇ、より好ましくは約０．１乃至２０ｍｇ／体重１ｋｇ、そしてさらにより好ましくは約１乃至１０ｍｇ／ｋｇ、２乃至９ｍｇ／ｋｇ、３乃至８ｍｇ／ｋｇ、４乃至７ｍｇ／ｋｇ、又は５乃至６ｍｇ／体重１ｋｇの範囲である。上記の値の中間の範囲も、本発明の一部として意図されたところである。例えば、上に挙げた値のいずれかを、上限及び／又は下限値として組み合わせて用いた範囲も包含されるものと、意図する。

【０１３０】

当業者であれば、疾患又は障害の重症度、治療歴、対象の全身の健康及び／又は年齢、及び現在の他の疾患、を含め、しかしこれらに限らず、いくつかの因子が、対象を効果的に

治療するのに必要な投薬量を左右するであろうことを、認識するであろう。さらに、あるタンパク質、ポリペプチド、又は抗体の治療上有効な量による対象の治療には、一回の処置を含めることができるが、又は好ましくは一連の処置を含めることもできる。

【０１３１】

ある好適な実施態様では、約０．１乃至２０ｍｇ／体重１ｋｇの範囲の抗体、タンパク質、又はポリペプチドで、１週当たり１回を約１乃至１０週間、好ましくは２乃至８週間、より好ましくは約３乃至７週間、そしてさらにより好ましくは約４、５、又は６週間、対象を処置する。さらに、処置に用いる抗体、タンパク質、又はポリペプチドの有効量は、特定の処置経過にわたって増減させてもよいことも理解されよう。投薬量を変更してもよく、投薬量の変更は、ここに解説する診断検定の結果から明白となるであろう。

【０１３２】

別の好適な実施態様では例えば、開いたもしくは活性コンホメーションのインテグリンのＩ－ドメインと反応又は結合する抗ＬＦＡ－１抗体などの抗インテグリン抗体か、又は、改変ＬＦＡ－１　Ｉ－ドメインと反応もしくは結合する抗ＬＦＡ－１抗体などの抗インテグリン抗体を、治療上有効量、初回投与して対象を処置し、次に、前記抗体の初回投薬量の１００％未満の治療上有効量の抗体を、日毎で計算して間欠的に投与する。このとき、前記抗体は、後続の投薬中、１週間当たり１回を越えて投与されない。別の実施態様では、後続の投薬は１週間当たり２回以上である。別の実施態様では、後続の投薬は２週間毎に１回以上である。別の実施態様では、後続の投薬は３週間毎に１回以上である。別の実施態様では、後続の投薬は４週間毎に１回以上である。一の実施態様では、後続の投薬は、一日単位で計算して、当該抗体の初回の投薬量の約５０％、４５％、４０％、３５％、３０％、２５％、２０％、１５％、１０％、９％、８％、７％、６％、５％、４％、３％、２％、又は１％未満である。一の実施態様では、初回の投薬量は、０．００１　乃至３０　ｍｇ／体重１ｋｇ、好ましくは約０．０１　乃至２５　ｍｇ／体重１ｋｇ、より好ましくは約０．１乃至２０　ｍｇ／体重１ｋｇ、そしてさらにより好ましくは約１乃至１０　ｍｇ／ｋｇ、２　乃至９　ｍｇ／ｋｇ、３　乃至８　ｍｇ／ｋｇ、４　乃至７　ｍｇ／ｋｇ、又は５　乃至６　ｍｇ／体重１ｋｇ、の間である。一の好適な実施態様では、初回の投薬量は、０．３　ｍｇ／体重１ｋｇ未満、例えば０．００１　乃至０．３０の間、例えば０．１、０．１２５、０．１５、０．１７５、０．２、０．２２５、０．２５、及び０．２７５など、である。上記の値の中間の範囲も、本発明の一部と意図する。

【０１３３】

さらに別の実施態様では、例えば開いたもしくは活性コンホメーションのインテグリンのＩ－ドメインと反応又は結合する、抗ＬＦＡ－１抗体などの抗インテグリン抗体か、又は、改変ＬＦＡ－１　Ｉ－ドメインと反応もしくは結合する抗ＬＦＡ－１抗体などの抗インテグリン抗体を、治療上有効量、初回投与して対象を処置し、次に、前記抗体の初回投薬量の１００％を越える治療上有効量の抗体を、日毎で計算して間欠的に投与する。このとき、前記抗体は、後続の投薬中、１週間当たり１回を越えて当該哺乳動物に投与されない。別の実施態様では、後続の投薬は１週間当たり２回以上である。別の実施態様では、後続の投薬は２週間毎に１回以上である。さらに別の実施態様では、後続の投薬は３週間毎に１回以上である。さらに別の実施態様では、後続の投薬は４週間毎に１回以上である。ある実施態様では、初回の投薬量は、０．００１　乃至３０　ｍｇ／体重１ｋｇ、好ましくは約０．０１　乃至２５　ｍｇ／体重１ｋｇ、より好ましくは約０．１乃至２０　ｍｇ／体重１ｋｇ、そしてさらにより好ましくは約１乃至１０　ｍｇ／ｋｇ、２　乃至９　ｍｇ／ｋｇ、３　乃至８　ｍｇ／ｋｇ、４　乃至７　ｍｇ／ｋｇ、又は５　乃至６　ｍｇ／体重１ｋｇ、の間である。ある好適な実施態様では、初回の投薬量は、０．３　ｍｇ／体重１ｋｇ未満、例えば０．００１　乃至０．３の間であり、例えば０．１、０．１２５、０．１５、０．１７５、０．２、０．２２５、０．２５、及び０．２７５、である。上記の値の中間の範囲も、本発明の一部と意図する。抗ＬＦＡ－１などの抗インテグリン抗体の投薬量は、例えば米国特許第５，６２２，７００号に解説されている。

【０１３４】

さらに別の実施態様では、初回の投薬量の次に、同じ投薬量を、例えば後続の投薬中、１週間毎に１回を越えずに行う。別の実施態様では、後続の投薬は一週間当たり２回以上である。別の実施態様では、後続の投薬は２週間毎に１回以上である。さらに別の実施態様では、後続の投薬は、３週間毎に１回以上である。さらに別の実施態様では、後続の投薬は、４週間毎に１回以上である。

【０１３５】

抗ＬＦＡ－１などの抗インテグリン抗体の投薬量は、例えば米国特許第５，６２２，７００号に解説されている。

【０１３６】

別の実施態様では、有効量の抗炎症性又は免疫抑制性の薬剤を、哺乳動物に対し、同時又は異なる時点で、本抗体と組み合わせて投与する。

【０１３７】

本発明は、インテグリン活性を修飾する有効な作用薬を包含するものである。作用薬は例えば小分子であるかも知れない。例えば、このような小分子には、限定はしないが、ペプチド、ペプチドミメティック、アミノ酸、アミノ酸類似体、ポリヌクレオチド、ポリヌクレオチド類似体、ヌクレオチド、ヌクレオチド類似体、分子量が１モル当たり約１０，０００グラム未満の有機もしくは無機の（即ちヘテロ有機及び有機金属化合物を含む）化合物、分子量が１モル当たり約５，０００グラム未満の有機もしくは無機の化合物、分子量が１モル当たり約１，０００グラム未満の有機もしくは無機の化合物、分子量が１モル当たり約５００グラム未満の有機もしくは無機の化合物、並びにこのような化合物の塩、エステル、及び薬学的に許容可能な他の形、がある。小分子作用薬の適当な用量は、当業の担当医、獣医、又は研究者の知る範囲の数多くの因子に依存すると、思われる。小分子の用量は、例えば、治療しようとする対象又はサンプルのアイデンティティ、大きさ、及び状態に依存し、またさらに該当すれば当該組成物を投与する経路に応じて、さらに本発明の核酸又はポリペプチドに対して当該小分子が有するとよいと担当医が思う作用に応じて変動するであろう。

【０１３８】

用量の例には、対象もしくはサンプルの体重１キログラム当たりの小分子の量ミリグラム又はマイクログラム量（例えば１キログラムあたり約１マイクログラムから、１キログラム当たり約５００ミリグラム、１キログラム当たり約１００マイクログラムから１キログラム当たり約５ミリグラム、又は、１キログラムあたり約１マイクログラムから１キログラム当たり約５０マイクログラム）がある。さらに、小分子の適当な用量は、修飾しようとする発現又は活性に対する、小分子の効力に依存するとも考えられる。このような適当な用量は、ここに解説した検定を用いて調べることができる。本発明のポリペプチド又は核酸の発現又は活性を修飾するために、これらの小分子の一つ又はそれ以上を動物（例えばヒト）に投与する場合、担当医、獣医又は研究者は、例えば、まず比較的に低い用量を処方し、その後適当な応答が得られるまでこの用量を増加させていってもよい。加えて、特定の対象動物にとっての特定の用量レベルは、用いる特定の化合物の活性、対象の年齢、体重、全身の健康状態、性別、及び食事、投与期間、投与経路、排出速度、薬剤の組合せ、及び、修飾しようとする発現又は活性の程度、を含む多様な因子に依存することであろうと理解される。

【０１３９】

本発明のいくつかの実施態様では、インテグリン活性のモジュレータを、他の薬剤（例えば小分子）と組み合わせて、又は別の補足的な治療計画と連携させて、投与する。例えばある実施態様では、インテグリン活性の阻害物質を用いて、炎症性もしくは免疫系疾患を治療する。従って、対象をインテグリン活性の阻害物質で処置し、さらに抗炎症性薬剤又は免疫抑制剤で処置してもよい。

【０１４０】

さらに、例えば抗ＬＦＡ－１抗体などの抗体（又はそのフラグメント）を、細胞毒、治療薬又は放射性金属イオンなどの治療部分に結合させてもよい。本発明の複合体は、任意の

生物学的応答を調節するのに利用でき、また当該薬剤部分は、伝統的な化学治療薬に限られるとみなしてはならない。例えば、当該薬剤部分は、所望の生物活性を持つタンパク質又はポリペプチドであってもよい。このようなタンパク質には、例えば、組織因子などの凝固因子；血管内皮細胞成長因子（「ＶＥＧＦ」）、血小板由来成長因子、及び組織プラスミノーゲン活性化因子などのタンパク質、例えばリンホカイン、サイトカイン及び成長因子などの生物学的応答調節因子；又はトキシンなどが含まれよう。

【０１４１】

このような治療部分を抗体に結合させる技術は公知であり、例えばＡｒｎｏｎ ｅｔ ａｌ．，”Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ Ｆｏｒ Ｉｍｍｕｎｏｔａｒｇｅｔｉｎｇ Ｏｆ Ｄｒｕｇｓ Ｉｎ Ｃａｎｃｅｒ Ｔｈｅｒａｐｙ”，ｉｎ Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ Ａｎｄ Ｃａｎｃｅｒ Ｔｈｅｒａｐｙ，Ｒｅｉｓｆｅｌｄ ｅｔ ａｌ．（ｅｄｓ．），ｐｐ．２４３－５６（Ａｌａｎ Ｒ．Ｌｉｓｓ，Ｉｎｃ．１９８５）；Ｈｅｌｌｓｔｒｏｍ ｅｔ ａｌ．，”Ａｎｔｉｂｏｄｉｅｓ Ｆｏｒ Ｄｒｕｇ Ｄｅｌｉｖｅｒｙ”，ｉｎ Ｃｏｎｔｒｏｌｌｅｄ Ｄｒｕｇ Ｄｅｌｉｖｅｒｙ（２ｎｄ Ｅｄ．），Ｒｏｂｉｎｓｏｎ ｅｔ ａｌ．（ｅｄｓ．），ｐｐ．６２３－５３（Ｍａｒｃｅｌ Ｄｅｋｋｅｒ，Ｉｎｃ．１９８７）；Ｔｈｏｒｐｅ，”Ａｎｔｉｂｏｄｙ Ｃａｒｒｉｅｒｓ Ｏｆ Ｃｙｔｏｔｏｘｉｃ Ａｇｅｎｔｓ Ｉｎ Ｃａｎｃｅｒ Ｔｈｅｒａｐｙ：Ａ Ｒｅｖｉｅｗ”，ｉｎ Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ ’８４：Ｂｉｏｌｏｇｉｃａｌ Ａｎｄ Ｃｌｉｎｉｃａｌ Ａｐｐｌｉｃａｔｉｏｎｓ，Ｐｉｎｃｈｅｒａ ｅｔ ａｌ．（ｅｄｓ．），ｐｐ．４７５－５０６（１９８５）；”Ａｎａｌｙｓｉｓ，Ｒｅｓｕｌｔｓ，Ａｎｄ Ｆｕｔｕｒｅ Ｐｒｏｓｐｅｃｔｉｖｅ Ｏｆ Ｔｈｅ Ｔｈｅｒａｐｅｕｔｉｃ Ｕｓｅ Ｏｆ Ｒａｄｉｏｌａｂｅｌｅｄ Ａｎｔｉｂｏｄｙ Ｉｎ Ｃａｎｃｅｒ Ｔｈｅｒａｐｙ”，ｉｎ Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ Ｆｏｒ Ｃａｎｃｅｒ Ｄｅｔｅｃｔｉｏｎ Ａｎｄ Ｔｈｅｒａｐｙ，Ｂａｌｄｗｉｎ ｅｔ ａｌ．（ｅｄｓ．），ｐｐ．３０３－１６（Ａｃａｄｅｍｉｃ Ｐｒｅｓｓ １９８５），ａｎｄ Ｔｈｏｒｐｅ ｅｔ ａｌ．，”Ｔｈｅ Ｐｒｅｐａｒａｔｉｏｎ Ａｎｄ Ｃｙｔｏｔｏｘｉｃ Ｐｒｏｐｅｒｔｉｅｓ Ｏｆ Ａｎｔｉｂｏｄｙ－Ｔｏｘｉｎ Ｃｏｎｊｕｇａｔｅｓ”，Ｉｍｍｕｎｏｌ．Ｒｅｖ．，６２：１１９－５８（１９８２）を参照されたい。あるいは、抗体を二次抗体に結合させると、シーガル氏が米国特許第４，６７６，９８０号に解説した抗体ヘテロ結合抗体を形成することができる。

【０１４２】

高親和改変インテグリンＩ－ドメインポリペプチド又はその活性フラグメントなどをコードする核酸分子など、本発明の核酸分子を単独で遺伝子ベースの治療に用いることもできるが、又は、ベクターに挿入して遺伝子治療ベクターとして用いてもよい。遺伝子治療は、患者の細胞に機能遺伝子を挿入して、（ｉ）代謝の先天異常を補正したり、又は（ｉｉ）細胞に新しい機能を提供する、ことである（Ｋｕｌｖｅｒ，Ｋ．Ｗ．，”Ｇｅｎｅ Ｔｈｅｒａｐｙ”，１９９４，ｐ．ｘｉｉ，Ｍａｒｙ Ａｎｎ Ｌｉｅｂｅｒｔ，Ｉｎｃ．，Ｐｕｂｌｉｓｈｅｒｓ，Ｎｅｗ Ｙｏｒｋ，Ｎ．Ｙ．）。ウィルスベクタなどのベクタを用いると、当該遺伝子が天然では存在しないような体内位置などに、哺乳動物で通常発現している遺伝子を導入し、安定に発現させることができよう。遺伝子治療ベクタは、例えば静脈注射、局所投与（例えば米国特許第５，３２８，４７０号を参照されたい）又は定位注射（例えばＣｈｅｎ ｅｔ ａｌ．（１９９４）Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９１：３０５４－３０５７）などにより、対象に送達することができる。遺伝子治療ベクタには、例えば、対象でｉｎ ｖｉｖｏで免疫応答を誘導するための目的抗原をコードするＤＮＡなどを含めることができる。従って、高親和改変インテグリンＩ－ドメインポリペプチド又はその活性フラグメントなどの改変インテグリンＩ－ドメインポリペプチドは、抗原に対する亢進した抗体反応を生ずるアジュバントとして働く。遺伝子治療ベクタの医薬製剤には、許容可能な希釈剤に入れた遺伝子治療ベクタを含めることができ、あるいは、遺伝子送達媒体を包埋した徐放型マトリックスを含め

ることもできる。あるいは、完全な遺伝子送達ベクタをレトロウィルスベクタなどの組換え細胞からインタクトで作製する場合、当該医薬製剤には、遺伝子送達系を形成する一つ又はそれ以上の細胞を含めてもよい。

【０１４３】

また本発明の核酸分子は、炎症性疾患などのインテグリン媒介疾患の治療的もしくは予防的処置のためのＤＮＡワクチン製剤中にも、利用できる。一の実施態様では、本ＤＮＡワクチン製剤は、改変インテグリンＩ－ドメインポリペプチド又はそのフラグメントなど、改変インテグリンポリペプチドをコードする核酸分子を、抗原性成分をコードするＤＮＡなどの抗原性成分に共役させたものを含んで成る。ここで用いる抗原性成分とは、特異抗体に充分な高親和性で結合することで検出可能な抗原－抗体複合体を形成することのできる部分である。別の実施態様では、本ＤＮＡワクチンは薬学的に許容可能な担体をさらに含んで成る。

【０１４４】

本医薬組成物を、容器、パック、又はディスペンサに、投与の際の注意書きと一緒に封入してもよい。

【０１４５】

処置の方法

本発明は、炎症性もしくは免疫疾患、及び／又は、細胞増殖性疾患など、インテグリン媒介疾患の危険性があるか、又は、インテグリン媒介疾患を有する対象を処置する予防法及び治療法の両方を提供するものである。ここで用いる「処置」とは、疾患もしくは異常、疾患もしくは異常の症状、又は疾患又は異常への素因を有する患者に対し、前記疾患もしくは異常、疾患もしくは異常の症状、又は疾患又は異常への素因を治癒させる、直す、寛解させる、軽減する、変化させる、治療する、改善させる、向上させるもしくは影響を与える目的で、治療薬を適用もしくは投与すること、又は、患者の摘出組織もしくは細胞株へ治療薬を適用もしくは投与すること、と定義される。治療薬には、限定はしないが、核酸分子、ＤＮＡワクチン、遺伝子ベースの治療法、改変Ｉ－ドメインポリペプチドと反応もしくは結合する小分子、ペプチド、抗ＬＦＡ－１抗体などの抗体、リボザイム及びアンチセンスオリゴヌクレオチド、が包含される。

【０１４６】

予防的処置法及び治療的処置法の両方に関しては、このような処置を、ファーマコゲノミクスの分野で得られた知見に基づき、特異的にテーラー・メードにしたり、又は改良してもよい。ここで用いる「ファーマコゲノミクス」とは、遺伝子配列決定、統計学的遺伝学、及び臨床開発中及び市場にある薬剤に対する遺伝子発現解析、などのゲノミクス・テクノロジーの応用を言う。より具体的には、この用語は、ある患者の遺伝子が、薬物に対してどのような応答をするか（例えば、患者の「薬物応答表現型」、又は「薬物応答遺伝子型」など）を調べる研究を言う。このように、本発明の別の局面では、個人の薬物応答遺伝子型に基づいて、本発明のインテグリンＩ－ドメインポリペプチド又はそのモジュレータのいずれかを用いた個人の予防的処置法又は治療的処置法をテーラーする方法を提供する。ファーマコゲノミクスにより、臨床医又は担当医は、当該処置から最も大きな利益を得るであろう患者に予防的もしくは治療的処置のターゲットを決め、そして薬物の関連する毒性の副作用が出るであろう患者の処置を避けることができる。

【０１４７】

１．　予防の方法

一の局面では、本発明は、本発明のインテグリンＩ－ドメインポリペプチド又はそのモジュレータを一種又はそれ以上、対象に投与することにより、対象におけるインテグリン媒介異常に関連する疾患又は状態を防ぐ方法を提供するものである。インテグリン媒介異常の危険性のある対象は、例えば、ここに解説する診断検定又は予後検定のいずれか又は組み合わせで、特定することができる。予防的作用薬の投与は、インテグリン媒介疾患の特徴を示す症状が発現する前に行って、疾患又は異常が防がれるか、又は、その進行が遅れるようにしてもよい。インテグリン媒介疾患の種類に応じて、例えば、本発明の適当なイ

ンテグリンＩ－ドメインポリペプチド又はそのモジュレータを、対象を治療するのに利用することができる。この適当な作用薬は、ここに解説するスクリーニング検定に基づいて決定することができる。

【０１４８】

２．治療の方法

本発明の別の局面は、治療を目的として、インテグリンＩ－ドメインポリペプチドの発現又はそれらの活性を修飾する（例えば、炎症性もしくは免疫疾患、及び／又は細胞増殖性疾患などのインテグリン媒介疾患の危険性があるか、又は、インテグリン媒介疾患を有する対象を治療するなど）方法に関するものである。従って、一実施態様では、本発明の修飾法は、本発明の一種又はそれ以上のインテグリンＩ－ドメインポリペプチド、又はその一種又はそれ以上のモジュレータ、例えば、開いたコンホメーションのインテグリンＩ－ドメインと反応又は結合する抗体など、や、開いたコンホメーションのＬＦＡ－１　Ｉ－ドメインもしくは改変ＬＦＡ－１　Ｉ－ドメインポリペプチドに特異的な抗ＬＦＡ－１抗体などの改変インテグリンＩ－ドメインポリペプチドなど、に細胞を接触させるステップを含む。インテグリンＩ－ドメインポリペプチド活性を修飾する作用薬は、例えば核酸又はタンパク質、インテグリンＩ－ドメインポリペプチドの標的分子（例えば基質）、改変インテグリンＩ－ドメインポリペプチドと反応もしくは結合する抗体、インテグリンＩ－ドメインポリペプチドアゴニストもしくはアンタゴニスト、インテグリンＩ－ドメインポリペプチドアゴニストもしくはアンタゴニストのペプチドミメティック、又は他の小分子など、ここに解説した作用薬とすることができる。一実施態様では、本作用薬は一種又はそれ以上のインテグリンＩ－ドメインポリペプチド活性を刺激するものである。このような刺激性作用薬の例には、活性インテグリンＩ－ドメインポリペプチドタンパク質や、細胞に導入してある、インテグリンＩ－ドメインポリペプチドをコードする核酸分子がある。別の実施態様では、本作用薬は、一種又はそれ以上のインテグリンＩ－ドメインポリペプチド活性を阻害するものである。このような阻害性作用薬の例には、アンチセンスインテグリンＩ－ドメインポリペプチド核酸分子、遺伝子治療ベクタ、ＤＮＡワクチン、抗インテグリンＩ－ドメインポリペプチド抗体、及びインテグリンＩ－ドメインポリペプチド阻害剤、がある。これらの修飾法はインビトロで（例えば細胞を当該作用薬と一緒に培養するなどにより）行っても、又はその代わりにインビボで（例えば当該作用薬を対象に投与するなどにより）行ってもよい。従って、本発明は、インテグリン媒介疾患に関連する特徴とする疾患又は異常に罹患した個体を処置する方法を提供するものである。ある実施態様では、本方法は、インテグリンＩ－ドメインポリペプチド発現又は活性を修飾（例えば上方調節又は下方調節）する作用薬（例えばここに解説するスクリーニング検定で同定した作用薬など）又は作用薬の組合せを投与することを含む。

【０１４９】

３．　ファーマコゲノミクス

本発明のインテグリンＩ－ドメインポリペプチド分子や、インテグリンＩ－ドメインポリペプチド活性（例えばインテグリンＩ－ドメインポリペプチドの遺伝子発現）に対する刺激性もしくは阻害性作用を有することが、ここに解説するスクリーニング検定で判明した作用薬、又はモジュレータは、炎症性もしくは免疫疾患、及び／又は、細胞増殖性疾患などのインテグリン媒介疾患を処置（予防的もしくは治療的に）するために、個体に投与することができる。このような処置と関連して、ファーマコゲノミクス（即ち、個体の遺伝子型と、その個体の外来化合物又は薬物に対する応答との間の関係の研究）を考慮してもよい。治療薬の代謝の違いが原因で、薬理学的に活性な薬物の用量と血中濃度との関係が変化して、重篤な毒性もしくは治療上の失敗が起きる可能性がある。このように、担当医又は臨床医は、インテグリンＩ－ドメインポリペプチド分子（及び／又はそのモジュレータ）を投与するかどうかを決定したり、このような分子及び／又はモジュレータによる処置の投薬量及び／又は治療計画を調整する上で、関連するファーマコゲノミクス研究で得られた知見を応用することを考慮してもよい。

【０１５０】

ファーマコゲノミクスでは、薬物の性質が変化したり、患者で異常な働きをすることを原因とする、薬物に対する応答の臨床上有意な遺伝的ばらつきを扱う。例えばＥｉｃｈｅｌｂａｕｍ，Ｍ．ｅｔａｌ．（１９９６）Ｃｌｉｎ．Ｅｘｐ．Ｐｈａｒｍａｃｏｌ．Ｐｈｙｓｉｏｌ．２３（１０－１１）：９８３－９８５ａｎｄＬｉｎｄｅｒ，Ｍ．Ｗ．ｅｔａｌ．（１９９７）Ｃｌｉｎ．Ｃｈｅｍ．４３（２）：２５４－２６６を参照されたい。一般的には、二種類の薬理遺伝学的条件を区別できる。即ち、薬物が身体に作用する態様の違いを生ずる（薬物作用の違い）単一の因子として伝えられる遺伝的条件、又は、身体が薬物に働きかける態様の違いを生ずる（薬物代謝の違い）単一の因子として伝えられる遺伝的条件、である。これらの薬理遺伝学的条件は、まれな遺伝的欠陥として起きたり、又は、天然発生型の多型現象として起きたりする。例えば、グルコース－６－リン酸アミノペプチダーゼ欠損症（Ｇ６ＰＤ）は、よくある遺伝性酵素欠損症であり、その主な臨床上の合併症は、酸化剤薬物（抗マラリア剤、スルホンアミド、鎮痛剤、ニトロフラン）の摂取後及びソラマメ摂取後の溶血である。

【０１５１】

「ゲノム－ワイド－アソシエーション（原語：ｇｅｎｏｍｅ－ｗｉｄｅ ａｓｓｏｃｉａｔｉｏｎ）として公知の、薬物応答を予測する遺伝子を同定するファーマコゲノミクス的アプローチの一つは、既知の遺伝子関連マーカ（例えば、それぞれが二つのバリアントを有する、ヒトゲノム上で６０，０００乃至１００，０００箇所の多型性もしくは可変部位から成る「バイ－アレリック」遺伝子マーカマップなど）から成るヒトゲノムの高解像度マップに主に依拠するものである。このような高解像度遺伝子マップを、第２相／３相薬物治験に参加する統計上有意な数の患者のそれぞれのゲノムのマップに比較すると、観察された特定の薬物応答又は副作用に伴うマーカを特定することができる。あるいは、このような高解像度マップは、ヒトゲノム中の数千万の公知の単一塩基多型（ＳＮＰ）を組み合わせて作製することもできる。ここで用いる「ＳＮＰ」とは、ある範囲のＤＮＡ中の一個のヌクレオチド塩基に起き得るごく普通の変化である。例えば、ＳＮＰはＤＮＡの１０００塩基毎に一回、あると思われる。ＳＮＰは疾患のプロセスに関与していることもあるが、大半は疾患に関係ないであろう。このようなＳＮＰの存在に基づいた遺伝子マップを基に、個々のゲノム中のＳＮＰの特定のパターンに応じて、個体を遺伝子カテゴリーに分類することができる。このような方法で、このような遺伝子上似た個体間で共通であろう形質を考慮に入れながら、遺伝子的に似た個体群に合わせて、治療計画をテーラーメードすることができる。

【０１５２】

実例として、薬物代謝酵素の活性は、薬物作用の強さ及び持続性の両方の主要な決定因子である。薬物代謝酵素（例えばＮ－アセチルトランスフェラーゼ２（ＮＡＴ２）及びチトクロームＰ４５０酵素ＣＹＰ２Ｄ６及びＣＹＰ２Ｃ１９）の遺伝子多型の発見により、標準的かつ安全な用量の薬物を摂取した後の患者の中に、予測通りの薬物効果が得られなかったり、又は、過剰な薬物応答が起きて重篤な毒性を呈するような者がなぜいるかが、解明された。これらの多型は、集団中で、高代謝群（ＥＭ）及び低代謝群（ＰＭ）という二つの表現型で表される。ＰＭの頻度は集団によって異なる。例えば、ＣＹＰ２Ｄ６をコードする遺伝子は多型性が高く、いくつかの変異がＰＭで判明しているが、そのいずれも機能的ＣＹＰ２Ｄ６の欠損を起こす。ＣＹＰ２Ｄ６及びＣＹＰ２Ｃ１９ による低代謝群は、標準的用量を摂取しても、過剰な薬物応答及び副作用を頻繁に起こす。代謝産物が有効治療成分の場合、ＰＭは、ＣＹＰ２Ｄ６がコデインから形成する代謝産物モルヒネの鎮痛効果で実証されるような、治療上の応答を示さない。反対の極端な例は、標準的用量では応答を生じないいわゆる超高代謝群である。最近、超高代謝群の分子的機序が解明されて、ＣＹＰ２Ｄ６遺伝子の増幅が原因であることが分かった。

【０１５３】

あるいは、「遺伝子発現プロファイリング」と呼ばれる方法を用いて、薬物応答を予測する遺伝子を同定することもできる。例えば、ある薬物（例えばインテグリンＩ－ドメインポリペプチド分子又はインテグリンＩ－ドメインポリペプチドモジュレータ）を投薬した

動物の遺伝子発現が、毒性に関係する遺伝子経路のスイッチが入ったかどうかを示す指標となることがある。

【０１５４】

上のファーマコゲノミクス的アプローチのうちの二つ以上から得られた情報を、個人の予防的もしくは治療的処置に適当な投薬量及び処置計画を決定するのに利用してもよい。この知見を、投薬量又は薬物の選択に利用すると、インテグリンＩ－ドメインポリペプチド分子や、ここで解説したスクリーニング検定例の一つで同定されたモジュレータなどのそのモジュレータで対象を処置したときの薬害反応又は治療の失敗を防ぎ、ひいては、治療もしくは予防効率を高めることができる。

【０１５５】

以下の実施態様では本発明をさらに解説するが、以下の例を限定的なものと捉えられてはならない。本出願を通じて引用された全参考文献、特許及び公開済み特許出願の内容や、図面及び配列表を、引用をもってここに援用することとする。

【０１５６】

実施例

実施例１　開いたもしくは閉じたコンホメーションで固定したＬＦＡ－１ 及び Ｍａｃ－１ 変異型のデザイン

ＬＦＡ－１ Ｉドメインの現在の結晶及びＮＭＲ構造（Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９５）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９２：１０２７７－１０２８１；Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９６）Ｓｔｒｕｃｔｕｒｅ ４：９３１－９４２；Ｋａｌｌｅｎ，Ｊ ｅｔ ａｌ．（１９９９）Ｊ Ｍｏｌ Ｂｉｏｌ ２９２：１－９）は、Ｍａｃ－１Ｉドメインの低親和性の閉じたコンホーマ（１ｊｌｍ）と類似のコンホメーションを有している（Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｃｅｌｌ ８０：６３１－６３８）。従って、Ｍａｃ－１ Ｉ ドメインの高親和性の開いたコンホーマ（１ｉｄｏ）（Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３：１３３３－１３４０）を用いて、高親和性の開いたＬＦＡ－１ Ｉ ドメインのモデルを作製した。このモデルのテンプレートは、Ｃα骨格が１ｊｌｍ構造とは著しく異なる領域の１ｉｄｏ構造部分と、１ｉｄｏ 及び１ｊｌｍ が類似である領域の１ｌｆａ構造部分とから成る。

【０１５７】

簡単に説明すると、以下のタンパク質データバンク（ＰＤＢ）同定記号：Ｍａｃ－１、１ｉｄｏ 及び１ｊｌｍ（Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３：１３３３－１３４０；Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｃｅｌｌ ８０：６３１－６３８）；ＬＦＡ－１、１ｌｆａ 分子Ａ 及び Ｂ（Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９５）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９２：１０２７７－１０２８１）、１ｚｏｎ 及び １ｚｏｐ（Ｑｕ，Ａ ａｎｄ Ｌｅａｈｙ，ＤＪ（１９９６）Ｓｔｒｕｃｔｕｒｅ ４：９３１－９４２）；及びＶＬＡ－２、１ａｏｘ（Ｅｍｓｌｅｙ，Ｊ ｅｔ ａｌ．（１９９７）Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７２：２８５１２－２８５１７）を持つＩドメインを、Ｃαカーボン、ＭＯＤＥＬＬＥＲ ４のＣＤ ＭＡＬＩＧＮ アルゴリズム（Ｓａｌｉ，Ａ ａｎｄ Ｂｌｕｎｄｅｌｌ，ＴＬ（１９９３）Ｊ Ｍｏｌ Ｂｉｏｌ ２３４：７７９－８１５）、１オングストロームのギャップ伸長ペナルティを用いて構造を重層した。このアルゴリズムでは、重層に用いられた１２１のフレームワーク残基が見つかった。次に配列のアラインメントを行った。１ｉｄｏ 及び１ｊｌｍ 構造をそれらの配列でアラインメントし、そして１ｌｆａ 分子 Ａ 及び １ｚｏｎ は１ｊｌｍに対する構造上の類似性でアライメントした。構造の重層と配列のアラインメントを利用して、対応するの配列位置にあるすべてのＣαカーボン同士の距離を、マイクロソフト・エクセル・スプレッドシートを用いて計算した。これは、１ｊｌｍと １ｉｄｏ を比較するのと同等の意味になるが（Ｌｅｅ，Ｊ－Ｏ ｅｔ ａｌ．（１９９５）Ｓｔｒｕｃｔｕｒｅ ３：１３３３－１３４０）、例外としてＬＦＡ－１ Ｉドメイン構造も含めた。高親和性の開いたＬＦＡ－１ Ｉドメインモデルのテンプレート

として用いるために、１ｌｆａ 分子Ａのうちで、四つのＩドメイン同士の間の違いが小さいか、又は、１ｌｆａ及び１ｊｌｍ（低親和性の閉じたＬＦＡ－１及びＭａｃ－１ Ｉドメイン）間の違いが１ｉｄｏ及び１ｊｌｍ間（開いた及び閉じたＭａｃ－１ Ｉドメイン）よりも大きいような、いくつかのセグメントを選び出した。１ｉｄｏからは、１ｉｄｏと１ｊｌｍとの違いが１ｌｆａと１ｊｌｍとの違いよりも大きいいくつかのセグメントを選び出した。これらのセグメントを、骨格ができるだけ同じになるような領域で一緒にスプライスした。このように、当該テンプレートは、１ｌｆａのセグメントＧ１２８ からＦ１３６、Ｍ１５４ から Ｌ２０３、Ｆ２０９からＬ２３４、Ｔ２４３ からＩ２５５、及びＥ２７２ からＡ２８２ ；及び１ｉｄｏのセグメント Ｄ１４０ からＦ１５６、Ｇ２０７ からＴ２１１、Ｖ２３８から Ｋ２４５、Ｒ２６６ からＲ２８１、及びＲ２９３ からＫ３１５を用いていた。ＬＯＯＫ™（カリフォルニア州パロアルト、モラキュラー・アプリケーション・グループ）では、ｌｆａ－ｍａｃと呼ぶスプライス後のテンプレートに、鎖のちぎれは検出されなかった。高親和性の開いた形のＬＦＡ－１ のモデルを、ＭＯＤＥＬＬＥＲ ４™で、このテンプレートと、１ｉｄｏのMg^{2+}、水分子４０３及び ４０４ を用い、ヘテロ原子、水、及び水素の添加を「入り」に、そしてクーロン力を「入り」にして、作製した。その結果得られたモデル（ｌｆａ＿ｈｉ．０６３）はテンプレートＣα座標に近似していた （ＲＭＳ ＝ ０．１２オングストローム）。ＱＵＡＣＨＫスコア （Ｖｒｉｅｎｄ，Ｇ （１９９０） Ｊ Ｍｏｌ Ｇｒａｐｈ ８：５２－５６） は優秀 （ｌｆａ－ｍａｃ テンプレートでは－０．２１５、１ｉｄｏでは－０．０８、そして１ｌｆａでは０．０とは対照的に、－０．１３５）だった。

【０１５８】

二つの適当な位置にある対の残基をシステインに変異させれば、ジスルフィド結合を導入できそうな位置を、ＳＳＢＯＮＤ プログラム（Ｈａｚｅｓ，Ｂ ａｎｄ Ｄｉｊｋｓｔｒａ，ＢＷ （１９８８） Ｐｒｏｔｅｉｎ Ｅｎｇｉｎｅｅｒｉｎｇ ２：１１９－１２５） を用いて特定した。ＬＦＡ－１ Ｉドメインを開いたもしくは閉じたコンホメーションのいずれかに固定するのに、ジスルフィド結合を利用できるのではないかと、仮説を立てた。

【０１５９】

高親和性の開いたＬＦＡ－１ Ｉ ドメインモデル（１ｆａ＿ｈｉ．０６３ モデル）と、二つの低親和性の閉じたＬＦＡ－１ Ｉ ドメイン構造である１ｌｆａ 及び１ｚｏｎを、ＳＳＢＯＮＤ で調べ、１４乃至１９対のこのような残基を各構造に見出した。これらのうち、高親和性の開いたモデルの一対の残基と、低親和性の閉じた構造中の一対の残基とが、二つのコンホーマのうちで大規模な移動を行った結果、ジスルフィド結合の形成は一方のコンホーマでしか起きなかった（図１）。これらのジスルフィドは、βストランド６をＣ末端αヘリックスα６に架橋する。βストランド及びαヘリックスの番号は、Ｉドメイン間で異なる。我々は統一した命名法を用いている（Ｈｕａｎｇ，Ｃ ｅｔ ａｌ．（２０００） Ｊ Ｂｉｏｌ Ｃｈｅｍ，２７５：２１５１４－２４）。ヘリックスα６はその軸方向に、高親和性の開いた構造のＩドメイン本体を下へ１０オングストローム移動する。この移動には、β６及びα６間のループの完全なリモデリング及び下向きのシフトが伴う。Ｋ２８７及びＫ２９４の代わりに導入されるシステインは、高親和性の開いたコンホーマでのみジスルフィドを形成するため、Ｉドメインを高親和性の開いた状態に固定するだろうと予測した（図２）。Ｋ２８７ 及びＫ２９４のＣβカーボンは、高親和性の開いたモデル （１ｆａ＿ｈｉ．０６３）では、ジスルフィド結合にとって理想的な３．４１乃至４．２５オングストロームの範囲内である３．８オングストローム、離れていると予測でき、Ｃβ－Ｓγ及びＳγ－Ｓγ距離を調べると、四つの好ましい側鎖ジスルフィドコンホメーションを有することが見出された。対照的に、低親和性の閉じたコンホーマ１ｌｆａ 及び１ｚｏｎでは、これらの残基のＣβ原子同士は８．９ 乃至９．２オングストローム、離れている（図２）。

【０１６０】

Ｌ２８９及びＫ２９４ の代わりに導入するシステインは、親和性の低い閉じたコンホー

マ（図２）のみでジスルフィドを形成して、このＩドメインを低親和性の閉じた状態に固定すると予測された。Ｌ２８９ 及びＫ２９４ のＣβカーボン同士は、低親和性の閉じた１ｌｆａ及び１ｚｏｎコンホーマでは、好ましい範囲内である３．９乃至４．０オングストローム離れているが、好ましいシステイン側鎖コンホメーションは見つからなかった。しかしながら、残基２９４が存在するαヘリックスは、１ｌｆａ、１ｚｏｎ、及び最近のＮＭＲ 構造（Ｑｕ，Ａａｎｄ Ｌｅａｈｙ，ＤＪ（１９９５）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ９２：１０２７７－１０２８１；Ｑｕ，Ａａｎｄ Ｌｅａｈｙ，ＤＪ（１９９６）Ｓｔｒｕｃｔｕｒｅ ４：９３１－９４２；Ｋａｌｌｅｎ，Ｊ ｅｔ ａｌ．（１９９９）Ｊ Ｍｏｌ Ｂｉｏｌ ２９２：１－９）間で小さな変位を示し、αヘリックスを微調節すればジスルフィドが形成されるのではないかと予測された。対照的に、高親和性の開いたモデルでは、これらの残基のＣβ原子は、８．０オングストローム離れていると予測できる（図２）。

【０１６１】

予測上のシステインが存在し、適当な場合にはジスルフィド結合が形成されているようなモデルも、ＭＯＤＥＬＬＥＲ ４のＰＡＴＣＨ ＤＩＳＵＬＦＩＤＥ ルーチンを用いて構築した（図２）。しかし、ここで報告されているすべてのＣβ原子距離は、ジスルフィドを導入していないモデル又は構造に基づくものであることに注意されたい。

【０１６２】

コンホメーション特異的なジスルフィド架橋を形成するための対のシステイン置換を探すコンピュータ検索に加え、構造を基にした手動的方法（又は視覚的検査）も用いた。開いたコンホメーションと閉じたコンホメーションというコンホメーション間で異なるＩドメインの領域を検査して、一方のコンホメーションが他方のコンホメーションに比べてジスルフィドが形成され易いような、対のシステインを導入できそうな位置を探した。このように、コンホメーション上可動のＣ末端αヘリックスの領域と、それに先行するループを調べて、一個のシステインを導入できそうな位置を探し、また構造上隣接する領域を調べて、ジスルフィド結合を形成するであろう第二のシステインを導入できそうな位置を探した。側鎖が互いに向かい合う対の残基を選んだ。これらの対のそれぞれのＣα及びＣβ原子間の距離を、ソフトウェアＬＯＯＫ^{TM}で、開いた及び閉じたコンホメーションの両方で測定した。ジスルフィド結合が形成されるのに理想的なシステインＣβカーボンの分離は３．４１乃至４．２５オングストロームであると報告されている。しかしながら、これらを測定した結晶構造又はモデルは、平均的なスナップショットの位置を示すものであり、他方、タンパク質は動的であり、その原子は可動性である。さらに、ジスルフィド結合を取り入れるよう、構造調節も可能である。βシートよりもループ及びαヘリックスでの方が、はるかに多くの調節が可能であると期待できる。従って、残基の一つがループ内又はヘリックス内にあると、ジスルフィド形成に、より長い距離を持たせることができると予測した。

【０１６３】

αＬの場合、Ｃα－Ｃα及びＣβ－Ｃβ距離が、開いたコンホメーションの方が閉じたコンホメーションよりもジスルフィド形成にとって、より好適になっている４対のシステイン置換が見つかった；Ｅ２８４Ｃ／Ｅ３０１Ｃ、Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃ（表１）。

【０１６４】

αＭの場合、Ｃα－Ｃα及びＣβ－Ｃβ距離が、開いたコンホメーションの方が閉じたコンホメーションよりもジスルフィド形成にとって、より好適になっている４対のシステイン置換が見つかった；Ｑ１６３Ｃ／Ｑ３０９Ｃ、Ｑ２９８Ｃ／Ｎ３０１Ｃ、Ｄ２９４Ｃ／Ｔ３０７Ｃ、及びＤ２９４Ｃ／Ｑ３１１ｃ（表７）、そしてＣα－Ｃα及びＣβ－Ｃβの距離が、開いたコンホメーションより閉じたコンホメーションの方がジスルフィド形成にとって好適になっている１対のシステイン置換が見られた：Ｑ１６３Ｃ／Ｒ３１３Ｃ。加えて、αＬでのＫ２８７Ｃ／Ｋ２９４Ｃの変異に相似のＦ２９７Ｃ／Ａ３０４Ｃも含めた。

【０１６５】
実施例２　ＬＦＡ－１システイン置換変異型の構築及び発現
５種の開いたαＬ　Ｉ－ドメイン変異型を作製した。高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃを作製するために、αＬサブユニットのＩ－ドメインのＫ２８７及びＫ２９４をシステインに置換した。高親和性の開いた変異型Ｅ２８４Ｃ／Ｅ３０１Ｃを作製するには、αＬサブユニットのＩ－ドメインのＥ２８４及びＥ３０１をシステインに置換した。加えて、３種類の中間の親和性の開いたαＬ　Ｉ－ドメイン変異型を作製したが、ここで以下：Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃと特定しておく。Ｌ１６１Ｃ／Ｆ２９９Ｃは、Ｌ１６１及びＦ２９９をシステインに置換して作製した。Ｋ１６０Ｃ／Ｆ２９９Ｃは、Ｋ１６０及びＦ２９９をシステインに置換して作製した。Ｌ１６１Ｃ／Ｔ３００Ｃは、Ｌ１６１及びＴ３００をシステインに置換して作製した。低親和性の閉じた変異型　Ｌ２８９Ｃ／Ｋ２９４Ｃは、Ｌ２８９　及びＫ２９４をシステインに置換して作製した。これらの６種類の変異型の変異させた残基同士の距離を下の表１に示す。また、一箇所だけシステインを置換した変異型Ｋ２８７Ｃ、Ｌ２８９Ｃ及びＫ２９４Ｃも作製した。
【０１６６】
【表１】

表１．開いた又は閉じたコンホメーションでの変異残基間のＣα及びＣβ

αL I-domain	開いたコンホメーション Cα (A)	開いたコンホメーション Cβ (A)	閉じたコンホメーション Cα (A)	閉じたコンホメーション Cβ (A)
開いた状態に固定				
K287C/K294C	6.32	3.75	10.72	9.08
E284C/E301C	9.12	6.96	12.88	12.52
L161C/F299C	9.16	8.09	11.87	11.38
K160C/F299C	9.97	7.75	9.83	7.96
L161C/T300C	12.30	13.00	13.50	14.87
閉じた状態に固定				
L289C/K294C	7.90	7.96	6.19	3.86

開いたコンホメーション又は閉じたコンホメーションにおける野生型残基間の距離はＬｏｏｋ^{TM}ソフトウエアで測定した。
【０１６７】
ヒトαＬ　ｃＤＮＡをＣＤＭ８の誘導体であるベクタＡｐｒＭ８に入れた（Ｓｅｅｄ，　Ｂａｎｄ　Ａｒｕｆｆｏ，　Ａ（１９８７）　Ｐｒｏｃ　Ｎａｔｌ　Ａｃａｄ　Ｓｃｉ　ＵＳＡ　８４：３３６５－３３６９）。重複伸長ＰＣＲ法を用いてシステイン置換変異をαＬ　Ｉ－ドメインに起こした（Ｈｏ，　ＳＮ　ｅｔ　ａｌ．（１９８９）　Ｇｅｎｅ　７７：５１－５９；　Ｈｏｒｔｏｎ，　ＲＭ　ｅｔ　ａｌ．（１９９０）　ＢｉｏＴｅｃｈｎｉｑｕｅｓ　８：５２８）。ＰＣＲ伸長反応用の外側左側のプライマは、ベクタの配列に、１８２６位でＥｃｏＲＩ部位の５’末端に相補的であり、外側右側のプライマは、αＬ　ｃＤＮＡのＥｃｏＲＩ部位の３’末端にあった。内側のプライマは、それぞれ個々の変異用にデザインされており、重複配列を含有していた。ＡｐｒＭ８中の野生型αＬ　ｃＤＮＡを、一回

目のＰＣＲ反応のテンプレートとして用いた。二回目のＰＣＲ産物をＥｃｏＲＩで消化し、ＡｐｒＭ８中の野生型αＬ ｃＤＮＡの同じ部位に連結した。インサートの方向が正しいかを、制限酵素消化で確認した。変異はすべて、ＤＮＡ配列決定法で確認した。

【０１６８】

安定に発現させるために、αＬ野生型及び変異型のｃＤＮＡのＸｂａＩ断片を、安定発現ベクタｐＥＦｐｕｒｏの同じ部位にサブクローンした（Ｌｕ，Ｃ ａｎｄ Ｓｐｒｉｎｇｅｒ，ＴＡ．（１９９７）Ｊ Ｉｍｍｕｎｏｌ １５９：２６８－２７８）。

【０１６９】

変異型のαＬサブユニットを、β２サブユニットと一緒に２９３Ｔ細胞で一過的に共発現させ、αＬ／β２複合体の細胞表面上の発現を、αＬ／β２複合体中のαＬサブユニットに対するモノクローナル抗体ＴＳ２／４を使ったフローサイトメトリで調べた。

【０１７０】

簡単に説明すると、ヒト胚性腎２９３Ｔ細胞（ＳＶ４０で形質転換させたもの）を、１０％ウシ胎児血清（ＦＢＳ）、２ｍＭグルタミン及び５０μｇ／ｍｌゲンタマイシンを添加したＤＭＥＭ培地で培養した。２９３Ｔ細胞をリン酸カルシウム法を用いて一過的にトランスフェクトした（ＤｕＢｒｉｄｇｅ，ＲＢ ｅｔ ａｌ．（１９８７）Ｍｏｌ Ｃｅｌｌ Ｂｉｏｌ ７：３７９－３８７；Ｈｅｉｎｚｅｌ，ＳＳ ｅｔ ａｌ．（１９８８）Ｊ Ｖｉｒｏｌ ６２：３７３８－３７４６）。簡単に説明すると、プラスミドＡｐｒＭ８に入れた７．５μｇの野生型もしくは変異型αＬ ｃＤＮＡと、ＡｐｒＭ８に入れた７．５μｇのβ２ ｃＤＮＡを用いて、７０乃至８０％コンフルエントの細胞の１枚の６ｃｍプレートを同時トランスフェクトした。トランスフェクト後２日目に、５ｍＭのＥＤＴＡを含有するハンクス液（ＨＢＳＳ）を用いてプレートから細胞を剥がし、ＬＦＡ－１の発現を調べ、機能解析を行った。

【０１７１】

既述（Ｌｕ，Ｃ ａｎｄ Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９７）Ｊ Ｉｍｍｕｎｏｌ １５９：２６８－２７８）のようにフローサイトメトリ解析を行った。簡単に説明すると、細胞を洗浄し、２．５％ＦＢＳを添加したＬ１５培地（シグマ社製）（Ｌ１５／ＦＢＳ）中に再懸濁させた。１×１０×１０５細胞を、１００μｌのＬ１５／ＦＢＳ内の一次抗体と一緒に氷上で３０分間、インキュベートした。モノクローナル抗体を、１：２０のハイブリドーマ上清、１：２００の腹水、又は１０μｇ／ｍｌ精製ＩｇＧの最終濃度になるように用いた。次に細胞をＬ１５／ＦＢＳで２回、洗浄し、ＦＩＴＣ結合ヤギ抗マウスＩｇＧ（重鎖及び軽鎖、カリフォルニア州サンフランシスコ、ザイメッド・ラボラトリーズ社製）と一緒に３０分間、氷上でインキュベートした。洗浄後、細胞を低温のＰＢＳ中に再懸濁させ、ＦＡＣＳｃａｎ（カリフォルニア州サンホセ、ベクトン・ディッキンソン社製）で解析した。

【０１７２】

図３Ａに示すように、推定上の高及び低親和性の変異型と、システイン置換を一箇所のみした変異型とでは、発現した細胞表面αＬ／β２複合体は、同様なレベルであった。

【０１７３】

システインの導入が、Ｉ－ドメインの全体的なコンホメーションに影響を与えるかどうかを調べるために、Ｉ－ドメインの様々な領域に対する一パネルのモノクローナル抗体を、Ｉ－ドメイン変異型との各々の反応性についてテストした。これらの研究で用いたモノクローナル抗体は以下の通りである：

【０１７４】

マウス抗ヒトαＬ（ＣＤ１１ａ）モノクローナル抗体ＴＳ１／１１、ＴＳ１／１２、ＴＳ１／２２、ＴＳ２／４、ＴＳ２／６ 及びＴＳ２／１４；抗－β２（ＣＤ１８）モノクローナル抗体ＴＳ１／１８、ＣＢＲＬＦＡ－１／２、及びＣＢＲＬＦＡ－１／７；ｍＡｂ ＹＦＣ５１；及び非結合性のｍＡｂ Ｘ６３が以前に解説されている（Ｓａｎｃｈｅｚ－Ｍａｄｒｉｄ，Ｆ ｅｔ ａｌ．（１９８２）Ｐｒｏｃ Ｎａｔｌ Ａｃａｄ Ｓｃｉ ＵＳＡ ７９：７４８９－７４９３；Ｈａｌｅ，ＬＰ ｅｔ ａｌ．（１９８９）Ａｒｔ

ｈｒｉｔｉｓ Ｒｈｅｕｍ ３２：２２－３０； Ｐｅｔｒｕｚｚｅｌｌｉ, Ｌ ｅｔ ａｌ. （１９９５） Ｊ Ｉｍｍｕｎｏｌ １５５：８５４－８６６）。モノクローナル抗体ＢＬ５、Ｆ８．８、２５－３－１、Ｍａｙ．０３５、ＣＢＲＬＦＡ－１／９、ＣＢＲＬＦＡ－１／１、Ｓ６Ｆ、及びＭａｙ．０１７ は、Ｌｅｕｋｏｃｙｔｅ Ｔｙｐｅ Ｖ に解説されており、フィフス・インターナショナル・ロイコサイト・ワークショップスから入手した。

【０１７５】

モノクローナル抗体Ｘ６３ 及びＴＳ１／１１を、ハイブリドーマ上清の形で、１：２０の希釈度で用い、モノクローナル抗体ＴＳ１／１２、ＤＢＲＬＦＡ－１／２、ＣＢＲＬＦＡ－１／７ 及びＹＦＣ５１を、精製済みＩｇＧとして、１０μｇ／ｍｌ用い、モノクローナル抗体ＴＳ１／２、ＴＳ２／１４、ＴＳ１／１８ 及びＴＳ２／４は腹水の形で、１：２００の希釈度で用い、そしてフィフス・インターナショナル・ロイコサイト・ワークショップスから得たモノクローナル抗体はすべて、１：２００の希釈度で用いた。ＣＢＲＬＦＡ－１／１を除き、抗体はすべて、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ及びＬ２８９Ｃ／Ｋ２９４Ｃ と野生型 ＬＦＡ－１に同等に良好に結合した（表２）。このことは、当該システイン置換が、Ｉ－ドメイン構造を破壊しなかったことを示唆している。モノクローナル抗体ＣＢＲＬＦＡ－１／１ の、高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ への結合は、野生型の４０乃至５０％に低下していたが、この抗体は変異型Ｌ２８９Ｃ／Ｋ２９４Ｃや、一箇所のみシステイン置換した変異型Ｋ２８７Ｃ、Ｌ２８９Ｃ及びＫ２９４Ｃとも、野生型とも反応した。抗体ＣＢＲＬＦＡ－１／１は残基３０１位～３５９位に位置し（Ｈｕａｎｇ, Ｃ ａｎｄ Ｓｐｒｉｎｇｅｒ, ＴＡ （１９９５） Ｊ Ｂｉｏｌ Ｃｈｅｍ ２７０：１９００８－１９０１６）、Ｋ２８７及びＫ２９４の一箇所のみのシステイン置換は、この抗体の結合に影響しなかったため、ＣＢＲＬＦＡ－１／１の変異型Ｋ２８７Ｃ／Ｋ２９４Ｃへの結合の低下は間接的な効果であると思われる。従って、変異型Ｋ２８７Ｃ／Ｋ２９４ＣのＩ－ドメインとβ－プロペラドメインとの間の界面におけるコンホメーションは、野生型ＬＦＡ－１のそれとは異なるのであろう。

【０１７６】

αＬのβ－プロペラドメインやβ２サブユニットに対する抗体の、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ及びＬ２８９Ｃ／Ｋ２９４Ｃとの反応性は、野生型ＬＦＡ－１のそれと同様であり、ＬＦＡ－１分子の他のドメインの構造が変異の影響を受けていないことが確認できた。

【０１７７】

【表２】

表２．LFA-1システイン置換変異型に対する抗体反応性(%対野生型の結合)

		K287C/K294C		L298C/K294C		K287C	L289C	K294C
Mab	エピトープ	293T	K562	293T	K562	293T	293T	293T
	I-ドメイン							
BL5	119-153, 185-215	92.4±11.29	92.39	85.79±16.4	97.61	93.35	92.44	88.31
F8.8	119-153, 185-215	93.70	102.15	83.56	93.88	95.86	99.63	95.47
CBRLFA-1/9	119-153, 185-215	ND	84.7	ND	ND	ND	ND	ND
TS2/6	154-183	84.88±5.64	89.24	78.59±2.62	95.89	91.39	88.24	91.67
May.035	185-215	92.61±8.4	92.59	82.14±14.15	101.10	95.8	95.4	106.39
TS1/11	185-215	94.36	95.96	93.67	104.54	ND	ND	ND
TS1/12	185-215	88.66	87.32	101.98	105.63	99.32	103.89	93.68
TS1/22	185-302	95.85±12.04	93.06	90.96±8.11	110.49	102.99	96.21	92.24
TS2/14	250-303	85.54±9.38	95.41	83.31±10.59	102.85	102.6	100.4	102.83
25-3-1	250-303	93.06	88.48	90.93	85.66	ND	ND	ND
CBRLFA-1/1	I及びβプロペラ	43.59±0.58	55.53	95.89±7.74	118.44	86.11	93.32	89.41
S6F1	β-プロペラ	89.39	97.38	95.32	85.69	98.3	86.39	92.34
	β2サブユニット							
TS1/18	I様ドメイン	99.82±10.47	97.42	95.72±4.67	105.71	87.88	87.35	107.58
YFC51	I様ドメイン	102.63	100.73	95.09	110.96	ND	ND	ND
CLBLFA-1/1	I様ドメイン	ND	96.48	ND	100.50	ND	ND	ND
CBRLFA-1/7	C末端領域	95.32	95.25	91.68	97.19	ND	ND	ND

【０１７８】

野生型ＬＦＡ－１及びＬＦＡ－１変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ、Ｌ２８９Ｃ／Ｋ２９４Ｃ、Ｋ２８７Ｃ、Ｌ２８９Ｃ、及びＫ２９４Ｃを、２９３Ｔ細胞の表面上に一過的に発現させるか、又はＫ５６２トランスフェクタント上で安定に発現させた。これらトランスフェクタントとの抗体の反応性をフローサイトメトリで調べた。各抗体の結合の平均蛍光を

ウェルで正規化した。結果を、野生型結合に対するパーセントで表す。データは少なくとも二つの独立したＦＣＡＳ実験の平均±ＳＤである。いくつかの抗体では、実験を一回のみ行った。ＮＤは決定できずを示す。

【０１７９】

実施例３　ＬＦＡ－１システイン置換変異型のリガンド結合活性

ＬＦＡ－１システイン置換変異型のＬＦＡ－１リガンドＩＣＡＭ－１に対する結合能を調べた。野生型ＬＦＡ－１と、推定上の高親和性の開いたＩ－ドメイン変異型Ｋ２８７Ｃ／Ｋ２９４Ｃを発現する２９３Ｔ細胞トランスフェクタントは、固定化ＩＣＡＭ－１に対して構成的に強い結合を示した（図４Ａ）。対照的に、低親和性の閉じた変異型Ｌ２８９Ｃ／Ｋ２９４ＣはＩＣＡＭ－１に結合しなかった。システイン置換が一箇所である変異型Ｋ２８７Ｃ及びＬ２８９Ｃの呈するＩＣＡＭ－１への結合は低いが、変異型Ｋ２９４Ｃの結合は野生型のそれに匹敵した。変異型Ｋ２８７Ｃ及びＬ２８９Ｃの結合は、活性化モノクローナル抗体ＣＢＲＬＦＡ－１／２により、野生型の結合と同様のレベルまで上昇した。しかしながら、ＣＢＲＬＦＡ－１／２は、低親和性の閉じた変異型Ｌ２８９Ｃ／Ｋ２９４ＣのＩＣＡＭ－１への結合を活性化できなかった（図４Ａ）。同様な結果は、２種の他のＬＦＡ－１活性化モノクローナル抗体Ｋｉｍ１２７及びＫｉｍ１８５でも得られた。推定上の高親和性の変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ及び低親和性の閉じた変異型Ｌ２８９Ｃ／Ｋ２９４Ｃの機能をさらに研究するために、これらの変異型を発現する安定なＫ５６２トランスフェクタントを作製した。

【０１８０】

簡単に説明すると、ヒト赤白血病細胞株Ｋ５６２をＲＰＭＩ　１６４０、１０％ ＦＢＳ及び５０μｇ／ｍｌ ゲンタマイシン中で培養した。安定なＫ５６２細胞株を作製するために、αＬサブユニットｃＤＮＡを含有する２μｇのＰｖｕＩ－直線化ｐＥＦｐｕｒｏに、β２サブユニットｃＤＮＡを含有する４０μｇのＳｆｉＩ－直線化ＡｐｒＭ８を、２５０Ｖ及び９６０μＦでのエレクトロポレーションで同時トランスフェクトした。４μｇ／ｍｌのプロマイシン（シグマ社製）に対する耐性でトランスフェクタントを選択し、限界希釈でサブクローンした。安定な細胞株をすべて、４μｇ／ｍｌプロマイシンを添加したＲＰＭＩ　１６４０、１０％ ＦＢＳ中に維持した。

【０１８１】

モノクローナル抗体ＴＳ２／４を用いたフローサイトメトリで調べたときに同様なレベルの細胞表面ＬＦＡ－１を発現していたトランスフェクタントのクローン（図３Ｂ）を既述（Ｌｕ，Ｃ ａｎｄ Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９７）Ｊ Ｉｍｍｕｎｏｌ １５９：２６８－２７８）のように、固定化ＩＣＡＭ－１への結合能についてテストした。

【０１８２】

簡単に説明すると、既述（Ｌｕ，Ｃ ａｎｄ Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９７）Ｊ Ｉｍｍｕｎｏｌ １５９：２６８－２７８）のように、ＩＣＡＭ－１をヒト口蓋扁桃から精製し、９６穴プレートにコートした。細胞に蛍光染料２’，７’－ビス－（カルボキシエチル）－５（及び－６）－カルボキシフルオレセイン、アセトキシメチルエステル（ＢＣＥＣＦ－ＡＭ）で標識し、１×１０^{6}／ｍｌ になるようにＬ１５／ＦＢＳ中に再懸濁させた。５０μｌの細胞懸濁液を、ＩＣＡＭ－１でコートしたたウェルの中で、モノクローナル抗体（ＣＢＲＬＦＡ－１／２、１０μｇ／ｍｌ）を加えてもしくは加えずに、等量のＬ１５／ＦＢＳと混合した。モノクローナル抗体は、最終濃度で、１：２０のハイブリドーマ上清、１：２００の腹水又は１０μｇ／ｍｌの精製済ＩｇＧを用いた。２価陽イオンの効果をテストするために、ＢＣＥＣＦ－ＡＭで標識した細胞を、５ｍＭのＥＤＴＡを含有するＴＳ緩衝液、ｐＨ７．５（２０ｍＭ トリス、ｐＨ ７．５、１５０ｍＭ ＮａＣｌ）で２回、洗浄した後、ＴＳ 緩衝液、ｐＨ７．５で２回、洗浄した。次に細胞を、１ｍＭ $MgCl_2$／$CaCl_2$、$MgCl_2$、$MnCl_2$ 又は５ｍＭ ＥＤＴＡを添加した ＴＳ 緩衝液、ｐＨ７．５中に５×１０^{5}／ｍｌ になるように、再懸濁させ、１００μｌの細胞懸濁液を、ＩＣＡＭ－１でコートしたウェルに加えた。３７℃で３０分間インキュベートした後、結合しなかった細胞をマイクロプレート・オートワッシャ（ヴァ

ーモント州ウィヌースキ、バイオーテック・インスツルメンツ社製）で洗い落とした。各ウェル中の総投入細胞及び結合した細胞の蛍光量を、フルオレセント・コンセントレーション・アナライザ（メーン州ウェストブルック、ＩＤＥＸＸ社製）で定量した。結合した細胞は、サンプルウェル当たりの総投入細胞のパーセンテージで表した。

【０１８３】

野生型ＬＦＡ－１を発現するＫ５６２トランスフェクタントが示したＩＣＡＭ－１への基礎結合量は低かったが、この結合は、活性化モノクローナル抗体 ＣＢＲＬＦＡ－１／２により大きく上昇した（図４Ｂ）。対照的に、推定上の高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃを発現している細胞は、ＩＣＡＭ－１に強く結合し、モノクローナル抗体ＣＢＲＬＦＡ－１／２ によっても、この変異型の結合がそれ以上、高められることはなかったが、一方、推定上の低親和性の閉じた変異型 Ｌ２８９Ｃ／Ｋ２９４Ｃは、活性化抗体が存在した場合でも、ＩＣＡＭ－１に結合しなかった。

【０１８４】

Ｋ５６２トランスフェクタントのＩＣＡＭ－１への結合に二価陽イオンが及ぼす作用も調べた。図４Ｃに示すように、変異型Ｋ２８７Ｃ／Ｋ２９４ＣのＩＣＡＭ－１への結合はＥＤＴＡがあると妨げられたことから、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃのリガンド結合は二価イオン依存的であることが裏付けられた。他方、野生型ＬＦＡ－１の結合はMn^{2+}があると大きく高まり、またMg^{2+}があっても程度は劣るが高まったが、低親和性の閉じた変異型Ｌ２８９Ｃ／Ｋ２９４Ｃのリガンドへの結合は、Mn^{2+}及びMg^{2+}があっても増加しなかった。

【０１８５】

野生型ＬＦＡ－１、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ、又は変異型Ｌ２８９Ｃ／Ｋ２９４Ｃを発現したＫ５６２トランスフェクタントへの可溶性ＩＣＡＭ－１の結合も評価した。簡単に説明すると、既述（Ｍａｒｔｉｎ，Ｓｅｔａｌ．（１９９３）Ｊ Ｖｉｒｏｌ ６７：３５６１－３５６８）のように、ヒトＩＣＡＭ－１の５つのＩｇドメインを含有する可溶性ＩＣＡＭ－１－ＩｇＡキメラを、モノクローナル抗体Ｒ６．５アフィニティ・クロマトグラフィにより、安定なＣＨＯトランスフェクタントの培養上清から精製した。Ｋ５６２トランスフェクタントをＬ１５／ＦＢＳで一回、洗浄し、同じ緩衝液中に1×10^{7}／ｍｌになるように再懸濁させた。２５μｌの細胞懸濁液を、抗体ＣＢＲＬＦＡ－１／２（１０μｇ／ｍｌ）を加えたもしくは加えない、ＩＣＡＭ－１－ＩｇＡ融合タンパク質を最終濃度１００μｇ／ｍｌで含有する２５μｌのＬ１５／ＦＢＳに混合し、３７℃で３０分間、インキュベートした。インキュベート後、細胞をＬ１５／ＦＢＳで一回、洗浄し、ＦＩＴＣ結合抗ヒトＩｇＡ （シグマ社製）と一緒に室温で２０分間、インキュベートした。２回洗浄後、細胞をＰＢＳ中に再懸濁させ、ＦＡＣＳｃａｎ （カリフォルニア州サンホセ、ベクトン・ディッキンソン社製）で解析した。

【０１８６】

図５に示すように、可溶性のＩＣＡＭ－１－ＩｇＡ融合タンパク質は、高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃを発現している細胞に結合し、活性化モノクローナル抗体ＣＢＲＬＦＡ－１／２の存在下では、結合は更に増加した。しかしながら、ＩＣＡＭ－１融合タンパク質は、野生型ＬＦＡ－１を発現していたトランスフェクタントにも、あるいは低親和性の閉じた変異型Ｌ２８９Ｃ／Ｋ２９４Ｃを発現していたトランスフェクタントにも、モノクローナル抗体ＣＢＲＬＦＡ－１／２が存在してもしなくとも結合しなかった。またＩＣＡＭ－１融合タンパク質濃度を高く（３００μｇ／ｍｌ）しても、結合は検出されなかった。

【０１８７】

これらのデータをまとめると、高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ は、構成的に活性であるが、他方、低親和性の閉じた変異型Ｌ２８９Ｃ／Ｋ２９４Ｃは、不活性の状態で固定されており、リガンド結合能に欠けると思われる。

【０１８８】

別の研究では、αＬ及びβ２サブユニットの様々なドメインに対する一パネルのモノクロ

ーナル抗体を、野生型ＬＦＡ－１及び変異型Ｋ２８７Ｃ／Ｋ２９４Ｃのリガンド結合に及ぼすそれらの阻害作用について調べた。２９３Ｔ一過性トランスフェクタント及びＫ５６２安定トランスフェクタントで得られた結果は同様であり、表３に要約する。ＣＢＲＬＦＡ－１／１を除くすべての抗体は、高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃや野生型と反応した（表２）が、野生型ＬＦＡ－１及び変異型Ｋ２８７Ｃ／Ｋ２９４Ｃのリガンド結合に対してこれらが示した阻害作用は異なった。

【０１８９】

図３に示すように、Ｉ－ドメイン抗体は、野生型ＬＦＡ－１及び高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４ＣのＩＣＡＭ－１への結合を示差的に阻害した。モノクローナル抗体ＢＬ５、Ｆ８．８、ＣＢＲＬＦＡ－１／９、Ｍａｙ．０３５、ＴＳ１／２２及びＴＳ２／６ は、野生型及び変異型Ｋ２８７Ｃ／Ｋ２９４Ｃの両方の結合を強く阻害し、野生型ＬＦＡ－１と変異型とに対する阻害のレベルは同様であった。モノクローナル抗体ＴＳ１／１１ 及びＴＳ１／１２は、野生型ＬＦＡ－１を発現するトランスフェクタントの結合の９０％を越える結合を阻害した。一方、これらの抗体が、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃに対して示す結合阻害作用はそれより低かった（４０～６０％）。野生型の結合に対して９０％を越える阻害を示したモノクローナル抗体ＴＳ２／１４、２５－３－１ 及びＣＢＲＬＦＡ－１／１ は、変異型Ｋ２８７Ｃ／Ｋ２９４ＣのＩＣＡＭ－１への結合はほとんど阻害しなかった。β－プロペラドメイン抗体Ｓ６Ｆ１ 及びＴＳ２／４ と、β２サブユニットのＣ末端領域に対する抗体ＣＢＲＬＦＡ－１／７ は、野生型及び変異型Ｋ２８７Ｃ／Ｋ２９４Ｃの両方で結合を阻害しなかったが、β２保存ドメインに対する５種の抗体ＴＳ１／１８、ＹＦＣ５１、ＣＬＢＬＦＡ－１／１、Ｍａｙ．０１７、及び６．５Ｅはすべて、野生型ＬＦＡ－１の結合は阻害した（９０％を越える阻害）が、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃの結合は阻害しなかった。

【０１９０】

β－プロペラドメインに対する抗体と、β２のＣ末端領域に対する抗体は、野生型ＬＦＡ－１の結合も、又は変異型Ｋ２８７／Ｋ２９４Ｃの結合も阻害しなかった。βサブユニットのＩ－様ドメインに対する抗体は、野生型ＬＦＡ－１のＩＣＡＭ－１への結合を遮断したが、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃは遮断しなかった。

【０１９１】

【表３】

表３．固定化ICAM-1への野生型LFA-1及び変異型K287C/K294Cの結合に対する抗体阻害の差異

		% 阻害			
		野生型 LFA-1		K287C/K294C	
Mab	エピトープ	293T	K562 (+CBRLFA-1/2)	293T	K562
RR1/1	I-CAM-1 I-domain	95.98	ND	97.89	ND
BL5	119-153, 185-215	97.01±1.63	97.54	91.06±3.8	90.68±6.23
F8.8	119-153, 185-215	94.51	97.61	91.94	98.18
CBRLFA-1/9	119-153, 185-215	ND	97.83	ND	3.60
TS2/6	154-183	96.84±1.73	91.76±4.67	79.09±10.06	88.12±7.40
May.035	185-215	96.20±0.57	95.80±1.66	97.43±1.52	93.33±2.54
TS1/11	185-215	94.12	96.55	45.18	41.30
TS1/12	185-215	95.68±3.92	97.46±0.66	48.96±9.52	63.67±8.13
TS1/22	250-303	95.77	96.94±0.79	95.07	93.56±4.79
TS2/14	250-303	94.47±2.34	96.24±1.70	2.95±9.87	8.55±0.66
25-3-1	250-303	90.49	92.01±0.36	3.71	2.53±4.10
CBRLFA-1/1	I及びβプロペラ	92.52±1.68	94.69±5.22	9.03	2.85±4.90
S6F1	β-プロペラ	ND	6.19	ND	9.70
TS2/4	β-プロペラ β2サブユニット	ND	6.99	ND	2.82
TS1/18	I様ドメイン	ND	98.48	ND	5.90
YFC51	I様ドメイン	ND	98.43	ND	0.08
CLBLFA-1/1	I様ドメイン	ND	94.63	ND	6.69
May.017	I様ドメイン	ND	97.76	ND	2.98
6.5E	I様ドメイン	ND	98.36	ND	5.79
CBRLFA-1/7	C末端領域	ND	5.04	ND	5.77

【０１９２】
野生型ＬＦＡ－１及びＬＦＡ－１変異型Ｋ２８７Ｃ／Ｋ２９４Ｃを、２９３Ｔ細胞表面上で一過的に発現させるか、又は、Ｋ５６２トランスフェクタントで安定に発現させた。このトランスフェクタントの固定化ＩＣＡＭ－１への結合を、図示した抗体の存在下で調べた。野生型ＬＦＡ－１を発現するＫ５６２トランスフェクタントの結合については、細胞を、１０μｇ／ｍｌの活性化ｍＡｂ ＣＢＲＬＦＡ－１／２と一緒に３０分間、プレインキュベートした。図示のデータは、少なくとも二つの独立した実験の％阻害±ＳＤである。％阻害は、図示のｍＡｂの存在時に結合した細胞の％／非結合性ｍＡｂＸ６３の存在時に結合した細胞の％×１００、であると定義しておく。いくつかの抗体では、実験を一回のみ行った。しかし、各実験において、各抗体は三重反復試験し、三重試験試料の標準偏差は５％未満であった。ＮＤは決定できずを表す。

【０１９３】
まとめると、これらの結果は、Ｉ－ドメイン抗体の一部と、β２保存ドメインに対する抗体は、ＬＦＡ－１のＩＣＡＭ－１への結合を直接的には遮断しないこと、そして、高親和性の開いた変異型Ｋ２８７Ｃ／Ｋ２９４Ｃは、高親和性の開いた状態でコンホメーション上固定されていると思われ、従って、間接的な機序でリガンド結合を遮断するような抗体は、変異型Ｋ２８７Ｃ／Ｋ２９４ＣのＩＣＡＭ－１への結合を遮断しないであろうこと、を示唆している。

【０１９４】
本発明の高親和性の開いたＩ－ドメインは、ＬＦＡ－１の直接的／競合的な阻害と、間接的／非競合的な阻害との間を区別するのに利用できる。例えば、ＬＦＡ－１阻害剤であるロバスタチンは、Ｉ－ドメインに、βシートとＣ末端αヘリックスが形成する疎水性のポケットで結合する（Ｋａｌｌｅｎ，Ｊ ｅｔ ａｌ．（１９９９）Ｊ Ｍｏｌ Ｂｉｏｌ ２９２：１－９）という、間接的な機序でＬＦＡ－１を阻害する。従って、高親和性Ｉ－ドメイン（Ｋ２８７Ｃ／Ｋ２９４Ｃ）のリガンド結合を阻害する上でのロバスタチンの阻害能を評価した。ＤＭＳＯに溶解させた５０ｍＭのロバスタチンを検定緩衝液中に希釈した。ＢＣＥＣＦ－ＡＭで標識した細胞（10^6／ｍｌ）を、ロバスタチン（０乃至５０μＭ）と一緒に３７℃で１５分間プレインキュベートした後、ＩＣＡＭ－１でコートした９６ウェルプレートに移して、さらに３７℃で３０分間、活性化モノクローナル抗体（ＣＢＲ ＬＦＡ１／２）又は$MnCl_2$の存在下又は不在下でインキュベートした。Ca^{2+}及びMg^{2+}を含有するウシ胎児血清（Ｌ１５／ＦＢＳ）を添加したＬ１５培地を、抗体ＣＢＲ ＬＦＡ１／２で活性化させた野生型αＬβ２に用い、そして２０ ｍＭ ＨＥＰＥＳ ｐＨ７．４、１４０ ｍＭ ＮａＣｌ、１ｍＭ $MnCl_2$、２ ｍｇ／ｍｌ グルコース、１％ ＢＳＡ を、Mn^{2+}による活性化に用いた。
【０１９５】
図６に示すように、ロバスタチンは、野生型ＬＦＡ－１を発現しており、かつMn^{2+}又は抗体（ＣＢＲ ＬＦＡ１／２）で刺激を受けた細胞へのＩＣＡＭ－１の結合は阻害するが、高親和性の開いたＫ２８７Ｃ／Ｋ２９４Ｃ変異型（ＨＡ／ａＬｂ２）へのリガンド結合には干渉しない。
【０１９６】
実施例４　分離型の野生型及び変異型ＬＦＡ－１ Ｉ－ドメインの発現及び機能
推定上の高親和性及び低親和性変異型の機能をさらに調べるために、野生型Ｉ－ドメインと、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ及びＬ２８９Ｃ／Ｋ２９４ＣのＩ－ドメインの残基Ｖ１３０からＡ３３８までを、Ｋ５６２細胞の表面上で、ＰＤＧＦ受容体の膜貫通ドメインに発現させた。
【０１９７】
分離型の、細胞表面発現型Ｉドメインを構築するために、シグナルペプチドと、それに続くαＬの反復配列ＩＩの５’末端側から６個のアミノ酸とをコードするＤＮＡ配列を、Ｉドメインを含有する残基Ｖ１３０－Ａ３３８をコードする配列に連結した。ＨｉｎｄＩＩＩ及びＳａｌＩ部位をこの断片のそれぞれ５’末端及び３’末端のすぐ隣に導入した。このＨｉｎｄＩＩＩ－ＳａｌＩ断片を、インフレームで、ｃ－ｍｙｃ ｔａｇ及びＰＤＧＦ受容体（ＰＤＧＦＲ）膜貫通ドメインの５’側にベクタｐＤｉｓｐｌａｙ™（インビトロジェン社製）でサブクローンし、さらに、ｐｃＤＮＡ３．１／Ｈｙｇｒｏに、ＨｉｎｄＩＩＩ 及びＮｏｔＩを用いてサブクローンした。ＤＮＡの増幅はすべて、Ｐｆｕ ＤＮＡ ポリメラーゼ（ストラタジーン社製）を用いて行い、最終的なコンストラクトはＤＮＡ配列決定で確認した。
【０１９８】
Ｉ－ドメインを表面上に発現する安定なＫ５６２トランスフェクタントを作製するために、ＩドメインとＰＤＧＦＲ膜貫通ドメインとをコードする配列を含有する２０μｇのＳｓｐＩ－直線化ｐｃＤＮＡ３．１／Ｈｙｇｒｏ（＋）を用いて、Ｋ５６２細胞を上述のようにエレクトロポレーションでトランスフェクトした。１００μｇ／ｍｌのヒグロマイシンＢに対する耐性でトランスフェクタントを選択し、さらに細胞ソーティング及び限界希釈法でサブクローンした。同様なレベルの表面野生型及び変異型Ｉ－ドメインを発現していたクローンを、機能研究に用いるべく、選び出した。１０％ＦＢＳ及び１００μｇ／ｍｌヒグロマイシンＢを添加したＲＰＭＩ培地１６４０中に安定な細胞株を維持した。分離型のＩ－ドメインの細胞表面発現を、フローサイトメトリにより、Ｉ－ドメインに対する抗体ＴＳ１／２２を用いて調べた（図７）。各トランスフェクタントからクローンを２つ選び出し、固定化ＩＣＡＭ－１に対する結合についてテストしたところ、同様な

結果がこれら二つのクローンのそれぞれについて得られた（図８Ａ）。インタクト野生型ＬＦＡ－１を発現していたトランスフェクタントはＩＣＡＭ－１に対して低い基礎結合を見せた。しかし、分離型の野生型Ｉ－ドメイン及び変異型Ｌ２８９Ｃ／Ｋ２９４Ｃ Ｉ－ドメインを発現した細胞は、ＩＣＡＭ－１に結合しなかった。このことは、分離型の野生型Ｉ－ドメインのみでは、リガンドとの強力かつ安定な相互作用を媒介するには充分でないことを示唆している（Ｋｎｏｒｒ，Ｒａｎｄ Ｄｕｓｔｉｎ，ＭＬ（１９９７）Ｊ Ｅｘｐ Ｍｅｄ １８６：７１９－７３０）。対照的に、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインを発現した細胞は、ＩＣＡＭ－１に対して強い結合を示した。

【０１９９】

変異型Ｋ２８７Ｃ／Ｋ２９４Ｃの構成的リガンド結合活性が、導入されたＣ２８７及びＣ２９４間のジスルフィド結合の形成によるものだとすれば、還元剤でこのジスルフィド結合を破壊すれば、この変異型のリガンド結合能を破壊できるはずである。よって、Mg^{2+}及びCa^{2+}を含有するＬ１５／ＦＢＳに還元剤ＤＴＴ（１０ｍＭ）を加えたものでトランスフェクタントを処理して、ＩＣＡＭ－１に対するトランスフェクタントの結合能を評価した。図８Ａに示すように、細胞表面に発現した変異型Ｋ２８７Ｃ／Ｋ２９４ＣＩ－ドメインのＩＣＡＭ－１への結合は、ＤＴＴ処理で破壊された。対照的に、インタクト野生型ＬＦＡ－１の結合はＤＴＴにより増加し、同様の結果はインタクトαＩＩｂβ３インテグリンでも観察された。ＤＴＴ処理はおそらく、インテグリンを不活性なコンホメーションに拘束している、インタクト分子中のジスルフィド結合を破壊するのであろう。しかしながら、ＤＴＴ処理では、分離型の野生型Ｉ－ドメインの結合にも、又は、変異型Ｌ２８９Ｃ／Ｋ２９４ＣＩ－ドメインの結合にも、影響を与えなかった。このＩ－ドメイン構造に見られるようなジスルフィド結合は、ＬＦＡ－１ Ｉ－ドメインには他にないため、これらのデータは、導入されたＣｙｓ２８７及びＣｙｓ２９４が、高親和状態にＩ－ドメインを拘束するジスルフィド架橋を形成したことを、強く示唆するものである。

【０２００】

さらに、二価陽イオンが、Ｋ５６２トランスフェクタントの表面上に発現した分離型のＩ－ドメインのリガンド結合に及ぼす作用についても、テストした。結合反応は、１ ｍＭ Mn^{2+}、１ ｍＭ Mg^{2+}、又は１ ｍＭ ＥＤＴＡを添加したＨＥＰＥＳ／ＮａＣｌ／グルコース（２０ ｍＭ ＨＥＰＥＳ、ｐＨ ７．５、１４０ ｍＭ ＮａＣｌ、２ｍｇ／ｍｌ グルコース） 中で行わせた。図８Ｂに示すように、Ｋ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインのＩＣＡＭ－１への結合は、ＥＤＴＡ処理により結合が破壊されたように、二価陽イオン依存的であった。インタクト野生型ＬＦＡ－１とは対照的に、Mn^{2+}又はMg^{2+}は、分離型の野生型Ｉ－ドメインのリガンド結合も、又は、変異型Ｌ２８９Ｃ／Ｋ２９４Ｃ Ｉ－ドメインのリガンド結合も、活性化しなかった。

【０２０１】

Ｉ－ドメイン抗体が、分離型のＫ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインのリガンド結合に及ぼす作用も調べた。インタクトＬＦＡ－１を発現しているトランスフェクタントを活性化抗体ＣＢＲＬＦＡ－１／２と一緒にプレインキュベートし、この細胞のＩＣＡＭ－１への結合を、Ｉ－ドメイン抗体ＴＳ１／２２、ＴＳ２／６、ＴＳ１／１１、ＴＳ１／１２、ＣＢＲＬＦＡ－１／９、ＣＢＲＬＦＡ－１／１、２５．３．１、ＴＳ２／１４か、又は非結合性抗体Ｘ６３の存在下で、図示のように行わせた。モノクローナル抗体ＴＳ１／２２、ＴＳ２／６、ＴＳ１／１１、ＴＳ１／１２ 及びＣＢＲＬＦＡ－１／９ は、分離型のＫ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインのＩＣＡＭ－１への結合を阻害したが、抗体２５－３－１、ＴＳ２１４ 及びＣＢＲＬＦＡ－１／１ はしなかった（図８Ｃ）。ＣＢＲＬＦＡ－１／１を除き、抗体はすべて、変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインや野生型Ｉ－ドメインに、フローサイトメトリで調べたところ、結合した。ＣＢＲＬＦＡ－１／１の変異型Ｉ－ドメインへの結合は、野生型Ｉ－ドメインの８０％まで減少していた。これらの結果は、インタクトＬＦＡ－１ Ｋ２８７Ｃ／Ｋ２９４Ｃ 変異型で観察されたもの（表２及び３）と一致し、分離型のＫ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインが、インタクト分子と同じような構造上の一体性を留めていることを示唆するものである。

【０２０２】
実施例５　可溶性Ｉ－ドメイン変異型によるＬＦＡ－１機能のインビトロ及びｉｎ　ｖｉｖｏでの阻害
ジスルフィド結合（Ｋ２８７Ｃ／Ｋ２９４Ｃ）で開いたコンホメーションに安定させた可溶性のαＬ　Ｉ－ドメイン変異型をＥ．ｃｏｌｉで作製した。
【０２０３】
簡単に説明すると、開いたコンホメーション（Ｋ２８７Ｃ／Ｋ２９４Ｃ）で安定させた組換え変異型αＬ　Ｉ－ドメインか、又は、アミノ酸残基Ｇ１２８からＹ３０７までの組換え野生型αＬ　Ｉ－ドメインを、ｐＥＴ１１ｂ（ノバジェン社製）内にクローンし、１ｍＭ　ＩＰＴＧ　で４時間誘導したＥ．ｃｏｌｉで発現させた。封入体を６Ｍグアニジン　ＨＣＬ中に可溶化させることで、封入体からこれら組換えタンパク質を精製し、０．１ｍＭ　Cu^{2+}／フェナントロリンの存在下で希釈してリフォールディングを行わせて、ジスルフィド結合の形成を促した。硫安沈殿法でタンパク質を濃縮し、透析し、モノＱイオン交換カラムで精製した。ジスルフィド結合が形成されていない物質を取り除くために、遊離スルフヒドリルを、活性化ビオチンに反応させ、ストレプトアビジンカラムを通した。次にこれら組換えタンパク質をゲル濾過で精製し、セントリプレップで濃縮した。ＢＩＡｃｏｒｅ^{TM}解析には、組換えＩＣＡＭ－１、ＩＣＡＭ－２及びＩＣＡＭ－３　Ｆｃ　キメラをＢＩＡｃｏｒｅ^{TM}　センサチップ上に、アミン共役法で固定した。組換えαＬ　Ｉ－ドメインを流し入れ、ＢＩＡｃｏｒｅ^{TM}検定を、１ｍＭの$MgCl_2$又は２ｍＭのＥＤＴＡを添加したＴｒｉｓ緩衝生理食塩水で、流速を１０μｌ／分にし、２５℃で行った。
【０２０４】
ＢＩＡｃｏｒｅ^{TM}解析で評価したところ、精製済みの開いたＩ－ドメインは、そのリガンドＩＣＡＭ－１、－２、及び－３に対し、１ｍＭの$MgCl_2$の存在下で高い親和性を示した。他方、可溶性の野生型Ｉドメインの結合は、検出不能であった（図９、パネルＡ、Ｃ及びＥ；表４）。開いたＩ－ドメインとリガンドとの相互作用は二価陽イオン依存的であり、２ｍＭのＥＤＴＡの存在下では失われたことから、この相互作用はＭＩＤＡＳに依存することが示された。野生型Ｉ－ドメインはリガンドと何ら相互作用を示さなかったため、その開いたＩ－ドメインにより、ＬＦＡ－１とそのリガンドとの結合動態を詳細に解析できた。結合動態を解析するために、様々な濃度の開いたＩ－ドメインを、リガンド結合についてテストした（図９、パネルＢ、Ｄ及びＦ）。動態解析の結果、内皮細胞上への主要なリガンドであるＩＣＡＭ－１について、会合速度が高く（１．２８ｘ１０^5 $M^{-1}s^{-1}$）、解離速度が中間（０．０２３０ s^{-1}）であることが示された（表４）。ＩＣＡＭ－１　のＫＤはナノモルの範囲であり、ＩＣＡＭ－１が最も高い親和性を示し、次いでＩＣＡＭ－２　及びＩＣＡＭ－３であった。開いたＩ－ドメインはまた、マウスＩＣＡＭ－１に対してもナノモル範囲の親和性を示した。
【０２０５】
【表４】

表４．ICAMS に結合する開いたI-ドメインの動態

リガンド	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM^{-1})
ICAM-1	1.28 x 10^5	0.0230	180
ICAM-2	0.23 x 10^5	0.0118	513
ICAM-3	0.19 x 10^5	0.0749	3942

【０２０６】
k_{on}、k_{off}、及びK_Dは、ＢＩＡエバリュエーション^{TM}ソフトウェアを用いて１

：１の相互作用モデルに基づいて計算した。

【０２０７】

別の研究で、組換え体である可溶性の高親和性αＬ Ｉドメインの、そのリガンドＩＣＡＭ－１に対する親和性を測定したところ、ＢＩＡｃｏｒｅによる評価では２００ｎＭというＫｄを示す。このように、分離型のαＬ Ｉドメインの高親和性コンホーマは、最も活性化したαＬβ２ヘテロ二量体と同じくらい、活性なのである。

【０２０８】

可溶性の開いたＩ－ドメインが、ＬＦＡ－１依存的接着を阻害する活性をテストした。ある研究では、野生型ＬＦＡ－１を安定に発現しているＫ５６２細胞をＢＣＥＣＦで蛍光標識し、この細胞表面上のＬＦＡ－１を、ＦＣＳを添加したＬ１５培地中に入れた活性化モノクローナル抗体ＣＢＲＬＦＡ－１／２で活性化させた。次にこの細胞を、Ｉ－ドメインの存在下又は不在下で、ＩＣＡＭ－１でコートしたプラスチック製９６ウェルプレート内でインキュベートした。３７℃で４０分間インキュベートした後、結合しなかった細胞をマイクロプレート・オートワッシャで洗い落とした。各ウェル中で、総投入細胞の蛍光含有量と、結合した細胞を、フルオレセント・コンセントレーション・アナライザで定量した。結合した細胞は、試料ウェル一つ当たりの総投入細胞のパーセントで表した。野生型Ｉ－ドメインとは対照的に、開いたＩ－ドメイン変異型は、固定化ＩＣＡＭ－１へのＬＦＡ－１発現細胞の接着を強く阻害した（図１０Ａ）。

【０２０９】

別の研究では、マウスＬＦＡ－１と、マウスＩＣＡＭ－１を含むそのリガンドとの両方を発現すると共に、ＰＭＡで活性化したときにＬＦＡ－１依存的同型凝集を示すマウスＴリンパ腫細胞株ＥＬ－４ を用いた。細胞を、９６ウェルプレートで、５０ｎｇ／ｍｌのＰＭＡと、様々な量の可溶性Ｉ－ドメインとの存在下でインキュベートした。３７℃で２時間、５％ CO_2 でインキュベートした後、凝集の程度を、以下のように顕微鏡下で採点した：０は、本質的にどの細胞も凝集しなかったことを示した；１は、細胞の１０％未満が凝集したことを示した；２ は、５０％未満の凝集を示した；３は、細胞の最高１００％が小型であり、緩やかな凝集体となったことを示した；４ は、細胞のほぼ１００％がより大きな凝集体となったことを示した；そして５は、細胞のほぼ１００％が大変大型の密な凝集体になったことを示した。図１０Ｂに示すように、可溶性の開いたＩ－ドメインも、マウスＴ細胞株ＥＬ－４のＰＭＡ誘導性ＬＦＡ－１依存的同型凝集を阻害した。

【０２１０】

さらに、末梢リンパ節（ＬＮ）の微小循環を生体内顕微鏡法で観察することにより、開いたＩ－ドメイン変異型によるＬＦＡ－１機能のｉｎ ｖｉｖｏでの阻害能をテストした。簡単に説明すると、Ｔ－ＧＦＰマウスのＬＮ細胞懸濁液を少量（２０乃至５０μｌ）、大腿動脈カテーテルを通じて逆行注射し、ビデオトリガー式セノン－アーク・ストロボスコープから蛍光落射照射により、腸骨下部ＬＮを観察した。Ｉ－ドメインがない状態でのコントロール T^{GFP} 細胞挙動を記録した後、マウスにＩ－ドメイン（１０μｇ／体重１ｇ）を動脈内注射して予備処置してから、５分後に T^{GFP} 細胞を注射した。すべての場面をビデオテープに記録し、オフライン解析を行った。回転する細胞量、対、細静脈に進入した T^{GFP} 細胞の総数のパーセンテージとして、回転画分を計算した。付着している（接着が強固な）画分は、細静脈中で回転する T^{GFP} 細胞の数のうちで、２０秒を越えて強固に接着したままの T^{GFP} 細胞のパーセンテージとして、決定した。結果を準定量的に以下のように採点した－：０％、±： ０－５％、＋： ５－２０％、＋＋： ２０－４０％、＋＋＋： ４０－６０％、＋＋＋＋： ６０－８０％、＋＋＋＋＋：８０－１００％。

【０２１１】

下の表５に示すように、野生型Ｉ－ドメインでなく、開いたＩ－ドメインを注射すると、高内皮細静脈へのＴリンパ球の強固な接着が効果的に遮断され、この遮断はＬＦＡ－１依存的であった。Ｌ－セレクチン及びＰＮＡｄが媒介するリンパ球の回転は損なわれなかったことから、開いたＩ－ドメインの阻害作用はＬＦＡ－１特異的であることが分かる。

【０２１２】
【表５】

表５．In vivoにおいて、末端リンパ節内のリンパ球の高内皮細静脈への強固な接着が開いたI-ドメインによって阻害され、野生型I-ドメインでは阻害されなかった

	画分		
I-ドメイン	回転	強接着	移動
なし	+++	++	±
野生型	+++	++	±
開	++++	±	-

【０２１３】
αＬ変異型Ｉ－ドメインのＩＣＡＭ－１への結合の動態
αＬ Ｉ－ドメインのＩＣＡＭ－１との相互作用の動態をさらに調査するために、組換え可溶性αＬ Ｉ－ドメインを Ｅ．ｃｏｌｉ内で発現させ、リフォールディングさせ、精製した。下の表６に示すように、Ｅ２８４Ｃ／Ｅ３０１Ｃの親和性はＫ２８７Ｃ／Ｋ２９４Ｃにほぼ等しい。Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃ の親和性は、野生型よりも有意に高いが、高親和性の開いたαＬ Ｉ－ドメインである Ｋ２８７Ｃ／Ｋ２９４Ｃの２０乃至３０分の１である。Ｌ１６１Ｃ／Ｆ２９９Ｃ、Ｋ１６０Ｃ／Ｆ２９９Ｃ、及びＬ１６１Ｃ／Ｔ３００Ｃを中親和性αＬ Ｉ－ドメインと呼ぶ。
【０２１４】
【表６】

表６．αLI-ドメインとICAM-1との相互作用の動態

αLI-ドメイン	Kon (1/Ms)	Koff (1/s)	KD (μM)
開いた状態に固定			
K287C/K294C	1.28 X 10^5	0.0230	0.180
E284C/E301C	1.28 X 10^5	0.0459	0.360
L161C/F299C	1.36 X 10^5	0.513	3.76
K160C/F299C	1.53 X 10^5	0.67	4.39
L161C/T300C	1.35 X 10^5	0.65	4.8
WT	2.22 X 10^3	3.00	1350
閉じた状態に固定			
L289C/K294C	2.11 X 10^3	2.84	1760

【０２１５】
組換え可溶性αＬ Ｉ－ドメインをＥ．ｃｏｌｉ内で発現させ、リフォールディングさせ、精製した。Ｉ－ドメインのＩＣＡＭ－１への結合の動態は ＢＩＡｃｏｒｅ^{TM}装置を用いて測定した。動態はＢＩＡエバリュエーション^{TM}ソフトウェアを用いて解析した。ＫＤ はスカッチャード・プロットにより、定常期のデータを用いて計算した。Ｋｏｆｆは、１：１結合モデルを用い、解離相の曲線適合で得た。Ｋｏｎ はＫｏｆｆ／ＫＤで計

算した。

【０２１６】

実施例６　Ｍａｃ－１システイン置換変異型の構築及び活性

同様のアプローチを用いて、ジスルフィド結合をＩ－ドメインに導入することにより、開いた高親和性のコンホメーションのＭａｃ－１をデザインした。Ｍａｃ－１システイン置換変異型のデザインは実施例１で説明した。

【０２１７】

【表７】

表７．開いたまたは閉じたコンホメーションでの変異残基間のＣα及びＣβ

	ido (開いたｺﾝﾎﾒｰｼｮﾝ)		jlm (閉じたｺﾝﾎﾒｰｼｮﾝ)	
変異型	Cα	Cβ	Cα	Cβ
開いた状態に固定				
Q163C/Q309C	8.37	6.36	9.11	7.16
Q298C/N301C	5.31	4.21	9.05	10.91
D294C/T307C	9.21	8.67	16.01	17.52
D294C/Q311C	9.02	7.08	9.79	10.02
F297C/A304C	6.31	3.78	11.18	10.17
閉じた状態に固定				
Q163C/R313C	13.8	13.33	7.36	5.15

【０２１８】

野生型残基同士の間の距離を、開いたコンホメーション（１ｉｄｏ）又は閉じた（コンホメーション（１ｊｌｍ）で、Ｌｏｏｋ^{TM}ソフトウェアで測定した。

【０２１９】

ジスルフィド結合を形成する可能性のある対のシステインを、αＭβ２のＩ－ドメインに導入すると、ＣＢＲＭ１／５活性化依存的エピトープの発現及びリガンド結合にどのような影響が出るかを評価するために、野生型又は変異型αＭサブユニット及びβ２サブユニットをコードするプラスミドを２９３Ｔ細胞及びＫ５６２細胞に共トランスフェクトした。αβヘテロ二量体の形成は、β２サブユニットと結合して初めてαＭサブユニットの推定β－プロペラドメイン中のエピトープを認識するようになるモノクローナル抗体ＣＢＲＭ１／３２を用いて確認し、さらに抗体ＣＢＲＭ１／５を用いてインテグリン活性化を検出した。

【０２２０】

Ｑ１６３Ｃ／Ｑ３０９Ｃ の対を変異させると良好な結果が得られた（図１１Ｂ、図１２Ｂ及びＣ）。この変異型は、推定上のジスルフィド結合を、Ｉ－ドメインの低部前側近傍の、最後のαヘリックスの下側３分の１にある残基と、最初のαヘリックスとの間に導入しており、６．３６オングストローム離れたＣβカーボンを１ｉｄｏ構造に有する。対照的に、Ｄ２９４Ｃ／Ｔ３０７Ｃ 及びＤ２９４Ｃ／Ｎ３１１Ｃ置換のＣβカーボンは、それぞれ８．６７オングストローム及び７．０８オングストローム離れている。Ｑ２９８Ｃ／Ｎ３０１Ｃ 及びＦ２９７Ｃ／Ａ３０４Ｃ 置換のＣβカーボンは、理想的な範囲内にあるが、これらの置換は、最後のβストランドとαヘリックスとの間のループにより近いため、リガンド結合部位を歪めるなどの望ましくない影響があるに違いない。

【０２２１】
一過的にトランスフェクトされた２９３Ｔ細胞内でインタクト二量体内に発現させた場合、このＱ１６３Ｃ／Ｑ３０９Ｃ 変異型は、ＣＢＲＭ１／３２抗体で測定したときに野生型の半分しか、発現されないが、 ＣＢＲＭ１／５ 活性化依存的エピトープ、対、ＣＢＲＭ１／３２ 発現、の比は、著しく高い（図１１Ａ）。加えて、Ｍａｃ－１ Ｑ１６３Ｃ／Ｑ３０９Ｃ 変異型を発現している２９３Ｔ細胞の、プラスチック上にコートしたｉＣ３ｂへの接着は、室温でＬ１５／ＦＢＳ培地内で検定したところ、その発現が低いにもかかわらず、野生型よりも高かった（図１１Ｂ）。

【０２２２】
代わりに、分離型のＭａｃ－１変異型Ｉ－ドメインを、ＰＤＧＦ受容体の人工シグナル配列及び膜貫通ドメインと一緒に、細胞表面上に発現させた。接着はＬ１５／ＦＢＳ／ＭｎＣｌ２ 中で３７℃で検定した。分離型の野生型Ｉ－ドメインは、ｉＣ３ｂへの結合を全く見せなかったが、他方、コンピュータで再デザインした疎水性のコア、ｉｄｏ１ｒ及びｉｄｏ２ｒを持つ、前述の変異型は活性を有していた（図１１Ｃ）（Ｓｈｉｍａｏｋａ，Ｍｅｔａｌ．（２０００）Ｎａｔｕｒｅ Ｓｔｒｕｃｔｕｒａｌ Ｂｉｏｌｏｇｙ ７：６７４－６７８）。Ｑ１６３Ｃ／Ｑ３０９Ｃ 変異型Ｉ－ドメインは、強力な特異的リガンド結合を示したが、この結合は、阻害性のＩ－ドメインモノクローナル抗体 ＣＢＲＭ１／５で完全に遮断された（図１２Ｃ）。

【０２２３】
更なる研究では、開いたＩ－ドメイン変異型Ｑ１６３Ｃ／Ｑ３０９Ｃ及びＤ２９４Ｃ／Ｑ３１１Ｃ をＫ５６２細胞で安定に発現させ、同じレベルの受容体を発現しているクローンを選び出した。固定化ｉＣ３ｂに対する接着検定を、Ｌ１５／ＦＢＳで、３７℃で行った。２９３Ｔ細胞とは対照的に、野生型Ｍａｃ－１ はこれらの細胞ではリガンド結合についてほとんど基礎活性を持たない（図１２Ａ及び１２Ｂ）。Ｑ１６３Ｃ／Ｑ３０９Ｃ及びＤ２９４Ｃ／Ｑ３１１Ｃ の両者ともで、インタクトαＭβ２ヘテロ二量体で発現させたとき、野生型に比べてＣＢＲＭ１／５ 活性化依存的エピトープ発現が増加し、またリガンド結合も増加していた（図１２Ａ及び１２Ｂ）。さらに、分離型の開いたＩ－ドメイン変異型を細胞表面上に発現していたＫ５６２細胞は、野生型に比べ、ｉＣ３ｂに対して強い特異的結合を示した（図１２Ｃ）。

【０２２４】
開いたＩ－ドメイン変異型のリガンド結合活性の上昇が、ジスルフィド結合の形成により誘導されることを確認するために、還元剤ＤＴＴの影響をテストした。変異型Ｉ－ドメインを含有するαＭβ２トランスフェクタントの、プラスチック上に固定したｉＣ３ｂへの結合を、ＤＴＴの存在下及び非存在下で調べた。下の表８に要約するように、固定された開いたαＭ Ｉ－ドメイン、 （Ｑ１６３Ｃ／Ｑ３０９Ｃ） 及び （Ｄ２９４Ｃ／Ｑ３１１ｃ）は、活性化がない時には活性であり、これらの活性は、ＤＴＴによるジスルフィド還元により部分的に低下した。対照的に、固定された閉じたαＭ Ｉ－ドメインＱ１６３Ｃ／Ｒ３１３Ｃ は不活性であり、活性化に対して耐性であるが、ＤＴＴによるジスルフィド還元後には活性化可能となる。

【０２２５】
図１２Ｃに示すようにＤＴＴ処理により、分離型の固定された開いたＩ－ドメインのリガンド結合はなくなった。対照的に、ＤＴＴにより、インタクト野生型αＭβ２の結合は増した（図２Ｂ）ことから、この実験で用いたＤＴＴは毒性でなく、開いたＩ－ドメイン変異型のリガンド結合が失われたことは、ＤＴＴの非特異的な作用が原因ではないことが示唆された。まとめると、これらのデータは、システインの導入により、ジスルフィド架橋が形成され、Ｍａｃ－１ Ｉ－ドメインが開いたもしくは閉じたコンホメーションに固定されることを示すものである。

【０２２６】
【表８】

表８．変異型I-ドメインを有するαMβ2トランスフェクタントの接着アセイのまとめ

	-DTT	-DTT	+DTT	+DTT
変異型	－活性化	＋活性化	－活性化	＋活性化
野生型	±	++++	++	++++
開いた状態に固定				
Q163C/Q309C	++++	++++	++	++++
Q298C/N301C	±	+	NT	NT
D294C/T307C	±	+	NT	NT
D294C/Q311C	++++	++++	++	++++
F297C/A304C	±	++	NT	NT
閉じた状態に固定				
Q163C/R313C	±	±	++	+++

【０２２７】
変異型Ｉ－ドメインを含有するαＭβ２トランスフェクタントの、プラスチック上に固定されたｉＣ３ｂへの結合をテストした。結果は以下のように準定量的に採点した：±：活性化した野生型トランスフェクタントの結合の０－５％、＋：５－２５％、＋＋２５－５０％、＋＋＋：５０－７５％、＋＋＋＋：７５－１００％。
ＮＴ：テストせず
ＤＴＴ：ＤＴＴ処理によるジスルフィド還元
＋活性化：活性化ｍＡＢ　ＣＢＲ　ＬＦＡ－１／２による活性化
【０２２８】
同等物
当業者であれば、ごく慣例的な実験を用いれば、ここに記載した本発明の具体的な実施例の同等物を数多く認識され、又は確認できることであろう。このような同等物は以下の請求の範囲の包含するところと意図されている。
【０２２９】
【配列表】

SEQUENCE LISTING

〈110〉 The Center for Blood Research, Inc.
Timothy Springer
Motomu Shimaoka
Chafen Lu

〈120〉 MODIFIED POLYPEPTIDES STABILIZED IN A
DESIRED CONFORMATION AND METHODS FOR PRODUCING SAME

〈130〉 CBN-002CPPC
〈150〉 US 60/229,700
〈151〉 2000-09-01
〈160〉 4
〈170〉 FastSEQ for Windows Version 4.0
〈210〉 1
〈211〉 5133
〈212〉 DNA
〈213〉 Homo sapiens
〈400〉 1

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〈211〉 1170
〈212〉 PRT
〈213〉 Homo sapiens
〈400〉 2

Met Lys Asp Ser Cys Ile Thr Val Met Ala Met Ala Leu Leu Ser Gly
1 5 10 15
Phe Phe Phe Phe Ala Pro Ala Ser Ser Tyr Asn Leu Asp Val Arg Gly
20 25 30
Ala Arg Ser Phe Ser Pro Pro Arg Ala Gly Arg His Phe Gly Tyr Arg
35 40 45
Val Leu Gln Val Gly Asn Gly Val Ile Val Gly Ala Pro Gly Glu Gly

```
    50                  55                  60
Asn Ser Thr Gly Ser Leu Tyr Gln Cys Gln Ser Gly Thr Gly His Cys
65                  70                  75                  80
Leu Pro Val Thr Leu Arg Gly Ser Asn Tyr Thr Ser Lys Tyr Leu Gly
                85                  90                  95
Met Thr Leu Ala Thr Asp Pro Thr Asp Gly Ser Ile Leu Ala Cys Asp
            100                 105                 110
Pro Gly Leu Ser Arg Thr Cys Asp Gln Asn Thr Tyr Leu Ser Gly Leu
        115                 120                 125
Cys Tyr Leu Phe Arg Gln Asn Leu Gln Gly Pro Met Leu Gln Gly Arg
    130                 135                 140
Pro Gly Phe Gln Glu Cys Ile Lys Gly Asn Val Asp Leu Val Phe Leu
145                 150                 155                 160
Phe Asp Gly Ser Met Ser Leu Gln Pro Asp Glu Phe Gln Lys Ile Leu
                165                 170                 175
Asp Phe Met Lys Asp Val Met Lys Lys Leu Ser Asn Thr Ser Tyr Gln
            180                 185                 190
Phe Ala Ala Val Gln Phe Ser Thr Ser Tyr Lys Thr Glu Phe Asp Phe
        195                 200                 205
Ser Asp Tyr Val Lys Arg Lys Asp Pro Asp Ala Leu Leu Lys His Val
    210                 215                 220
Lys His Met Leu Leu Leu Thr Asn Thr Phe Gly Ala Ile Asn Tyr Val
225                 230                 235                 240
Ala Thr Glu Val Phe Arg Glu Glu Leu Gly Ala Arg Pro Asp Ala Thr
                245                 250                 255
Lys Val Leu Ile Ile Ile Thr Asp Gly Glu Ala Thr Asp Ser Gly Asn
            260                 265                 270
Ile Asp Ala Ala Lys Asp Ile Ile Arg Tyr Ile Ile Gly Ile Gly Lys
        275                 280                 285
```

```
His Phe Gln Thr Lys Glu Ser Gln Glu Thr Leu His Lys Phe Ala Ser
    290                 295                 300
Lys Pro Ala Ser Glu Phe Val Lys Ile Leu Asp Thr Phe Glu Lys Leu
305                 310                 315                 320
Lys Asp Leu Phe Thr Glu Leu Gln Lys Lys Ile Tyr Val Ile Glu Gly
                325                 330                 335
Thr Ser Lys Gln Asp Leu Thr Ser Phe Asn Met Glu Leu Ser Ser Ser
            340                 345                 350
Gly Ile Ser Ala Asp Leu Ser Arg Gly His Ala Val Val Gly Ala Val
        355                 360                 365
Gly Ala Lys Asp Trp Ala Gly Gly Phe Leu Asp Leu Lys Ala Asp Leu
    370                 375                 380
Gln Asp Asp Thr Phe Ile Gly Asn Glu Pro Leu Thr Pro Glu Val Arg
385                 390                 395                 400
Ala Gly Tyr Leu Gly Tyr Thr Val Thr Trp Leu Pro Ser Arg Gln Lys
                405                 410                 415
Thr Ser Leu Leu Ala Ser Gly Ala Pro Arg Tyr Gln His Met Gly Arg
            420                 425                 430
Val Leu Leu Phe Gln Glu Pro Gln Gly Gly Gly His Trp Ser Gln Val
        435                 440                 445
Gln Thr Ile His Gly Thr Gln Ile Gly Ser Tyr Phe Gly Gly Glu Leu
    450                 455                 460
Cys Gly Val Asp Val Asp Gln Asp Gly Glu Thr Glu Leu Leu Leu Ile
465                 470                 475                 480
Gly Ala Pro Leu Phe Tyr Gly Glu Gln Arg Gly Gly Arg Val Phe Ile
                485                 490                 495
Tyr Gln Arg Arg Gln Leu Gly Phe Glu Glu Val Ser Glu Leu Gln Gly
            500                 505                 510
Asp Pro Gly Tyr Pro Leu Gly Arg Phe Gly Glu Ala Ile Thr Ala Leu
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515 520 525
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530 535 540
Leu Glu Glu Gln Gly Ala Val Tyr Ile Phe Asn Gly Arg His Gly Gly
545 550 555 560
Leu Ser Pro Gln Pro Ser Gln Arg Ile Glu Gly Thr Gln Val Leu Ser
565 570 575
Gly Ile Gln Trp Phe Gly Arg Ser Ile His Gly Val Lys Asp Leu Glu
580 585 590
Gly Asp Gly Leu Ala Asp Val Ala Val Gly Ala Glu Ser Gln Met Ile
595 600 605
Val Leu Ser Ser Arg Pro Val Val Asp Met Val Thr Leu Met Ser Phe
610 615 620
Ser Pro Ala Glu Ile Pro Val His Glu Val Glu Cys Ser Tyr Ser Thr
625 630 635 640
Ser Asn Lys Met Lys Glu Gly Val Asn Ile Thr Ile Cys Phe Gln Ile
645 650 655
Lys Ser Leu Tyr Pro Gln Phe Gln Gly Arg Leu Val Ala Asn Leu Thr
660 665 670
Tyr Thr Leu Gln Leu Asp Gly His Arg Thr Arg Arg Arg Gly Leu Phe
675 680 685
Pro Gly Gly Arg His Glu Leu Arg Arg Asn Ile Ala Val Thr Thr Ser
690 695 700
Met Ser Cys Thr Asp Phe Ser Phe His Phe Pro Val Cys Val Gln Asp
705 710 715 720
Leu Ile Ser Pro Ile Asn Val Ser Leu Asn Phe Ser Leu Trp Glu Glu
725 730 735
Glu Gly Thr Pro Arg Asp Gln Arg Ala Gln Gly Lys Asp Ile Pro Pro
740 745 750

```
Ile Leu Arg Pro Ser Leu His Ser Glu Thr Trp Glu Ile Pro Phe Glu
        755                 760                 765
Lys Asn Cys Gly Glu Asp Lys Lys Cys Glu Ala Asn Leu Arg Val Ser
    770                 775                 780
Phe Ser Pro Ala Arg Ser Arg Ala Leu Arg Leu Thr Ala Phe Ala Ser
785                 790                 795                 800
Leu Ser Val Glu Leu Ser Leu Ser Asn Leu Glu Glu Asp Ala Tyr Trp
                805                 810                 815
Val Gln Leu Asp Leu His Phe Pro Pro Gly Leu Ser Phe Arg Lys Val
            820                 825                 830
Glu Met Leu Lys Pro His Ser Gln Ile Pro Val Ser Cys Glu Glu Leu
        835                 840                 845
Pro Glu Glu Ser Arg Leu Leu Ser Arg Ala Leu Ser Cys Asn Val Ser
    850                 855                 860
Ser Pro Ile Phe Lys Ala Gly His Ser Val Ala Leu Gln Met Met Phe
865                 870                 875                 880
Asn Thr Leu Val Asn Ser Ser Trp Gly Asp Ser Val Glu Leu His Ala
                885                 890                 895
Asn Val Thr Cys Asn Asn Glu Asp Ser Asp Leu Leu Glu Asp Asn Ser
            900                 905                 910
Ala Thr Thr Ile Ile Pro Ile Leu Tyr Pro Ile Asn Ile Leu Ile Gln
        915                 920                 925
Asp Gln Glu Asp Ser Thr Leu Tyr Val Ser Phe Thr Pro Lys Gly Pro
    930                 935                 940
Lys Ile His Gln Val Lys His Met Tyr Gln Val Arg Ile Gln Pro Ser
945                 950                 955                 960
Ile His Asp His Asn Ile Pro Thr Leu Glu Ala Val Val Gly Val Pro
                965                 970                 975
Gln Pro Pro Ser Glu Gly Pro Ile Thr His Gln Trp Ser Val Gln Met
```

```
          980                 985                 990
Glu Pro Pro Val Pro Cys His Tyr Glu Asp Leu Glu Arg Leu Pro Asp
        995                1000                1005
Ala Ala Glu Pro Cys Leu Pro Gly Ala Leu Phe Arg Cys Pro Val Val
    1010                1015                1020
Phe Arg Gln Glu Ile Leu Val Gln Val Ile Gly Thr Leu Glu Leu Val
1025                1030                1035                1040
Gly Glu Ile Glu Ala Ser Ser Met Phe Ser Leu Cys Ser Ser Leu Ser
                1045                1050                1055
Ile Ser Phe Asn Ser Ser Lys His Phe His Leu Tyr Gly Ser Asn Ala
          1060                1065                1070
Ser Leu Ala Gln Val Val Met Lys Val Asp Val Val Tyr Glu Lys Gln
        1075                1080                1085
Met Leu Tyr Leu Tyr Val Leu Ser Gly Ile Gly Gly Leu Leu Leu Leu
    1090                1095                1100
Leu Leu Ile Phe Ile Val Leu Tyr Lys Val Gly Phe Phe Lys Arg Asn
1105                1110                1115                1120
Leu Lys Glu Lys Met Glu Ala Gly Arg Gly Val Pro Asn Gly Ile Pro
                1125                1130                1135
Ala Glu Asp Ser Glu Gln Leu Ala Ser Gly Gln Glu Ala Gly Asp Pro
          1140                1145                1150
Gly Cys Leu Lys Pro Leu His Glu Lys Asp Ser Glu Ser Gly Gly Gly
        1155                1160                1165
Lys Asp
    1170
```

〈210〉 3
〈211〉 4740
〈212〉 DNA

〈213〉 Homo sapiens

〈400〉 3

gaattccgtg gttcctcagt ggtgcctgca acccctggtt cacctccttc caggttctgg 60
ctccttccag ccatggctct cagagtcctt ctgttaacag ccttgacctt atgtcatggg 120
ttcaacttgg acactgaaaa cgcaatgacc ttccaagaga acgcaagggg cttcgggcag 180
agcgtggtcc agcttcaggg atccagggtg gtggttggag ccccccagga gatagtggct 240
gccaaccaaa ggggcagcct ctaccagtgc gactacagca caggctcatg cgagcccatc 300
cgcctgcagg tccccgtgga ggccgtgaac atgtccctgg gcctgtccct ggcagccacc 360
accagccccc ctcagctgct ggcctgtggt cccaccgtgc accagacttg cagtgagaac 420
acgtatgtga aagggctctg cttcctgttt ggatccaacc tacggcagca gccccagaag 480
ttcccagagg ccctccgagg gtgtcctcaa gaggatagtg acattgcctt cttgattgat 540
ggctctggta gcatcatccc acatgacttt cggcggatga aggagtttgt ctcaactgtg 600
atggagcaat taaaaaagtc caaaaccttg ttctctttga tgcagtactc tgaagaattc 660
cggattcact ttaccttcaa agagttccag aacaacccta acccaagatc actggtgaag 720
ccaataacgc agctgcttgg gcggacacac acggccacgg gcatccgcaa agtggtacga 780
gagctgttta acatcaccaa cggagcccga aagaatgcct ttaagatcct agttgtcatc 840
acggatggag aaaagtttgg cgatcccttg ggatatgagg atgtcatccc tgaggcagac 900
agagagggag tcattcgcta cgtcattggg gtgggagatg ccttccgcag tgagaaatcc 960
cgccaagagc ttaataccat cgcatccaag ccgcctcgtg atcacgtgtt ccaggtgaat 1020
aactttgagg ctctgaagac cattcagaac cagcttcggg agaagatctt tgcgatcgag 1080
ggtactcaga caggaagtag cagctccttt gagcatgaga tgtctcagga aggcttcagc 1140
gctgccatca cctctaatgg ccccttgctg agcactgtgg ggagctatga ctgggctggt 1200
ggagtctttc tatatacatc aaaggagaaa agcaccttca tcaacatgac cagagtggat 1260
tcagacatga atgatgctta cttgggttat gctgccgcca tcatcttacg gaaccgggtg 1320
caaagcctgg ttctgggggc acctcgatat cagcacatcg gcctggtagc gatgttcagg 1380
cagaacactg gcatgtggga gtccaacgct aatgtcaagg gcacccagat cggcgcctac 1440
ttcggggcct ccctctgctc cgtggacgtg gacagcaacg gcagcaccga cctggtcctc 1500
atcggggccc cccattacta cgagcagacc cgaggggggc aggtgtccgt gtgccccttg 1560
cccaggggggc agagggctcg gtggcagtgt gatgctgttc tctacgggga gcagggccaa 1620

ccctggggcc gctttggggc agccctaaca gtgctggggg acgtaaatgg ggacaagctg 1680
acggacgtgg ccattggggc cccaggagag gaggacaacc ggggtgctgt ttacctgttt 1740
cacggaacct caggatctgg catcagcccc tcccatagcc agcggatagc aggctccaag 1800
ctctctccca ggctccagta ttttggtcag tcactgagtg ggggccagga cctcacaatg 1860
gatggactgg tagacctgac tgtaggagcc caggggcacg tgctgctgct caggtcccag 1920
ccagtactga gagtcaaggc aatcatggag ttcaatccca gggaagtggc aaggaatgta 1980
tttgagtgta atgatcaggt ggtgaaaggc aaggaagccg gagaggtcag agtctgcctc 2040
catgtccaga agagcacacg ggatcggcta agagaaggac agatccagag tgttgtgact 2100
tatgacctgg ctctggactc cggccgccca cattcccgcg ccgtcttcaa tgagacaaag 2160
aacagcacac gcagacagac acaggtcttg gggctgaccc agacttgtga gaccctgaaa 2220
ctacagttgc cgaattgcat cgaggaccca gtgagcccca ttgtgctgcg cctgaacttc 2280
tctctggtgg gaacgccatt gtctgctttc gggaacctcc ggccagtgct ggcggaggat 2340
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tgccaggatg acctcagcat caccttcagt ttcatgagcc tggactgcct cgtggtgggt 2460
gggccccggg agttcaacgt gacagtgact gtgagaaatg atggtgagga ctcctacagg 2520
acacaggtca ccttcttctt cccgcttgac ctgtcctacc ggaaggtgtc cacactccag 2580
aaccagcgct cacagcgatc ctggcgcctg gcctgtgagt ctgcctcctc caccgaagtg 2640
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gaggtcacct ttaatatcac gtttgatgta gactctaagg cttcccttgg aaacaaactg 2760
ctcctcaagg ccaatgtgac cagtgagaac aacatgccca gaaccaacaa aaccgaattc 2820
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caggtcagca acctggggca gaggagcctc cccatcagcc tggtgttctt ggtgcccgtc 3000
cggctgaacc agactgtcat atgggaccgc ccccaggtca ccttctccga gaacctctcg 3060
agtacgtgcc acaccaagga gcgcttgccc tctcactccg actttctggc tgagcttcgg 3120
aaggcccccg tggtgaactg ctccatcgct gtctgccaga gaatccagtg tgacatcccg 3180
ttctttggca tccaggaaga attcaatgct accctcaaag gcaacctctc gtttgactgg 3240
tacatcaaga cctcgcataa ccacctcctg atcgtgagca cagctgagat cttgtttaac 3300
gattccgtgt tcaccctgct gccgggacag ggggcgtttg tgaggtccca gacggagacc 3360

aaagtggagc cgttcgaggt ccccaacccc ctgccgctca tcgtgggcag ctctgtcggg 3420
ggactgctgc tcctggccct catcaccgcc gcgctgtaca agctcggctt cttcaagcgg 3480
caatacaagg acatgatgag tgaaggggct ccccgggggc ccgaacccca gtagcggctc 3540
cttcccgaca gagctgcctc tcggtggcca gcaggactct gcccagacca cacgtagccc 3600
ccaggctgct ggacacgtcg gacagcgaag tatccccgac aggacgggct tgggcttcca 3660
tttgtgtgtg tgcaagtgtg tatgtgcgtg tgtgcgagtg tgtgcaagtg tctgtgtgca 3720
agtgtgtgca cgtgtgcgtg tgcgtgcatg tgcactcgca cgcccatgtg tgagtgtgtg 3780
caagtatgtg agtgtgtcca gtgtgtgtgc gtgtgtccat gtgtgtgcag tgtgtgcatg 3840
tgtgcgagtg tgtgcatgtg tgtgctcagg ggctgtggct cacgtgtgtg actcagagtg 3900
tctctggcgt gtgggtaggt gacggcagcg tagcctctcc ggcagaaggg aactgcctgg 3960
gctcccttgt gcgtgggtaa gccgctgctg ggttttcctc cgggagaggg gacggtcaat 4020
cctgtgggtg aagagagagg gaaacacagc agcatctctc cactgaaaga agtgggactt 4080
cccgtcgcct gcgagcctgc ggcctgctgg agcctgcgca gcttggatgg atactccatg 4140
agaaaagccg tgggtggaac caggagcctc ctccacacca gcgctgatgc ccaataaaga 4200
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taatttttg gatggataag cctgtctatg gtacaaaaat cacaaggcat tcaagtgtac 4320
agtgaaaagt ctccctttcc agatattcaa gtcacctcct taaaggtagt caagattgtg 4380
ttttgaggtt tccttcagac agattccagg cgatgtgcaa gtgtatgcac gtgtgcacac 4440
accacacaca tacacacaca caagcttttt tacacaaatg gtagcatact ttatattggt 4500
ctgtatcttg ctttttttca ccaatatttc tcagacatcg gttcatatta agacataaat 4560
tacttttca ttcttttata ccgctgcata gtattccatt gtgtgagtgt accataatgt 4620
atttaaccag tcttcttttg atatactatt ttcatctctt gttattgcat ctgctgagtt 4680
aataaatcaa atatatgtca aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 4740

〈210〉 4
〈211〉 1152
〈212〉 PRT
〈213〉 Homo sapiens
〈400〉 4

```
Met Ala Leu Arg Val Leu Leu Leu Thr Ala Leu Thr Leu Cys His Gly
 1               5                  10                  15
Phe Asn Leu Asp Thr Glu Asn Ala Met Thr Phe Gln Glu Asn Ala Arg
            20                  25                  30
Gly Phe Gly Gln Ser Val Val Gln Leu Gln Gly Ser Arg Val Val Val
        35                  40                  45
Gly Ala Pro Gln Glu Ile Val Ala Ala Asn Gln Arg Gly Ser Leu Tyr
    50                  55                  60
Gln Cys Asp Tyr Ser Thr Gly Ser Cys Glu Pro Ile Arg Leu Gln Val
65                  70                  75                  80
Pro Val Glu Ala Val Asn Met Ser Leu Gly Leu Ser Leu Ala Ala Thr
                85                  90                  95
Thr Ser Pro Pro Gln Leu Leu Ala Cys Gly Pro Thr Val His Gln Thr
            100                 105                 110
Cys Ser Glu Asn Thr Tyr Val Lys Gly Leu Cys Phe Leu Phe Gly Ser
        115                 120                 125
Asn Leu Arg Gln Gln Pro Gln Lys Phe Pro Glu Ala Leu Arg Gly Cys
    130                 135                 140
Pro Gln Glu Asp Ser Asp Ile Ala Phe Leu Ile Asp Gly Ser Gly Ser
145                 150                 155                 160
Ile Ile Pro His Asp Phe Arg Arg Met Lys Glu Phe Val Ser Thr Val
                165                 170                 175
Met Glu Gln Leu Lys Lys Ser Lys Thr Leu Phe Ser Leu Met Gln Tyr
            180                 185                 190
Ser Glu Glu Phe Arg Ile His Phe Thr Phe Lys Glu Phe Gln Asn Asn
        195                 200                 205
Pro Asn Pro Arg Ser Leu Val Lys Pro Ile Thr Gln Leu Leu Gly Arg
    210                 215                 220
Thr His Thr Ala Thr Gly Ile Arg Lys Val Val Arg Glu Leu Phe Asn
```

```
225                 230                 235                 240
Ile Thr Asn Gly Ala Arg Lys Asn Ala Phe Lys Ile Leu Val Val Ile
                245                 250                 255
Thr Asp Gly Glu Lys Phe Gly Asp Pro Leu Gly Tyr Glu Asp Val Ile
            260                 265                 270
Pro Glu Ala Asp Arg Glu Gly Val Ile Arg Tyr Val Ile Gly Val Gly
        275                 280                 285
Asp Ala Phe Arg Ser Glu Lys Ser Arg Gln Glu Leu Asn Thr Ile Ala
    290                 295                 300
Ser Lys Pro Pro Arg Asp His Val Phe Gln Val Asn Asn Phe Glu Ala
305                 310                 315                 320
Leu Lys Thr Ile Gln Asn Gln Leu Arg Glu Lys Ile Phe Ala Ile Glu
                325                 330                 335
Gly Thr Gln Thr Gly Ser Ser Ser Ser Phe Glu His Glu Met Ser Gln
            340                 345                 350
Glu Gly Phe Ser Ala Ala Ile Thr Ser Asn Gly Pro Leu Leu Ser Thr
        355                 360                 365
Val Gly Ser Tyr Asp Trp Ala Gly Gly Val Phe Leu Tyr Thr Ser Lys
    370                 375                 380
Glu Lys Ser Thr Phe Ile Asn Met Thr Arg Val Asp Ser Asp Met Asn
385                 390                 395                 400
Asp Ala Tyr Leu Gly Tyr Ala Ala Ala Ile Ile Leu Arg Asn Arg Val
                405                 410                 415
Gln Ser Leu Val Leu Gly Ala Pro Arg Tyr Gln His Ile Gly Leu Val
            420                 425                 430
Ala Met Phe Arg Gln Asn Thr Gly Met Trp Glu Ser Asn Ala Asn Val
        435                 440                 445
Lys Gly Thr Gln Ile Gly Ala Tyr Phe Gly Ala Ser Leu Cys Ser Val
    450                 455                 460
```

```
Asp Val Asp Ser Asn Gly Ser Thr Asp Leu Val Leu Ile Gly Ala Pro
465                 470                 475                 480
His Tyr Tyr Glu Gln Thr Arg Gly Gly Gln Val Ser Val Cys Pro Leu
                485                 490                 495
Pro Arg Gly Arg Ala Arg Trp Gln Cys Asp Ala Val Leu Tyr Gly Glu
            500                 505                 510
Gln Gly Gln Pro Trp Gly Arg Phe Gly Ala Ala Leu Thr Val Leu Gly
        515                 520                 525
Asp Val Asn Gly Asp Lys Leu Thr Asp Val Ala Ile Gly Ala Pro Gly
    530                 535                 540
Glu Glu Asp Asn Arg Gly Ala Val Tyr Leu Phe His Gly Thr Ser Gly
545                 550                 555                 560
Ser Gly Ile Ser Pro Ser His Ser Gln Arg Ile Ala Gly Ser Lys Leu
                565                 570                 575
Ser Pro Arg Leu Gln Tyr Phe Gly Gln Ser Leu Ser Gly Gly Gln Asp
            580                 585                 590
Leu Thr Met Asp Gly Leu Val Asp Leu Thr Val Gly Ala Gln Gly His
        595                 600                 605
Val Leu Leu Leu Arg Ser Gln Pro Val Leu Arg Val Lys Ala Ile Met
    610                 615                 620
Glu Phe Asn Pro Arg Glu Val Ala Arg Asn Val Phe Glu Cys Asn Asp
625                 630                 635                 640
Gln Val Val Lys Gly Lys Glu Ala Gly Glu Val Arg Val Cys Leu His
                645                 650                 655
Val Gln Lys Ser Thr Arg Asp Arg Leu Arg Glu Gly Gln Ile Gln Ser
            660                 665                 670
Val Val Thr Tyr Asp Leu Ala Leu Asp Ser Gly Arg Pro His Ser Arg
        675                 680                 685
Ala Val Phe Asn Glu Thr Lys Asn Ser Thr Arg Arg Gln Thr Gln Val
```

690 695 700
Leu Gly Leu Thr Gln Thr Cys Glu Thr Leu Lys Leu Gln Leu Pro Asn
705 710 715 720
Cys Ile Glu Asp Pro Val Ser Pro Ile Val Leu Arg Leu Asn Phe Ser
725 730 735
Leu Val Gly Thr Pro Leu Ser Ala Phe Gly Asn Leu Arg Pro Val Leu
740 745 750
Ala Glu Asp Ala Gln Arg Leu Phe Thr Ala Leu Phe Pro Phe Glu Lys
755 760 765
Asn Cys Gly Asn Asp Asn Ile Cys Gln Asp Asp Leu Ser Ile Thr Phe
770 775 780
Ser Phe Met Ser Leu Asp Cys Leu Val Val Gly Gly Pro Arg Glu Phe
785 790 795 800
Asn Val Thr Val Thr Val Arg Asn Asp Gly Glu Asp Ser Tyr Arg Thr
805 810 815
Gln Val Thr Phe Phe Phe Pro Leu Asp Leu Ser Tyr Arg Lys Val Ser
820 825 830
Thr Leu Gln Asn Gln Arg Ser Gln Arg Ser Trp Arg Leu Ala Cys Glu
835 840 845
Ser Ala Ser Ser Thr Glu Val Ser Gly Ala Leu Lys Ser Thr Ser Cys
850 855 860
Ser Ile Asn His Pro Ile Phe Pro Glu Asn Ser Glu Val Thr Phe Asn
865 870 875 880
Ile Thr Phe Asp Val Asp Ser Lys Ala Ser Leu Gly Asn Lys Leu Leu
885 890 895
Leu Lys Ala Asn Val Thr Ser Glu Asn Asn Met Pro Arg Thr Asn Lys
900 905 910
Thr Glu Phe Gln Leu Glu Leu Pro Val Lys Tyr Ala Val Tyr Met Val
915 920 925

```
Val Thr Ser His Gly Val Ser Thr Lys Tyr Leu Asn Phe Thr Ala Ser
    930                 935                 940
Glu Asn Thr Ser Arg Val Met Gln His Gln Tyr Gln Val Ser Asn Leu
945                 950                 955                 960
Gly Gln Arg Ser Leu Pro Ile Ser Leu Val Phe Leu Val Pro Val Arg
                965                 970                 975
Leu Asn Gln Thr Val Ile Trp Asp Arg Pro Gln Val Thr Phe Ser Glu
            980                 985                 990
Asn Leu Ser Ser Thr Cys His Thr Lys Glu Arg Leu Pro Ser His Ser
        995                 1000                1005
Asp Phe Leu Ala Glu Leu Arg Lys Ala Pro Val Val Asn Cys Ser Ile
    1010                1015                1020
Ala Val Cys Gln Arg Ile Gln Cys Asp Ile Pro Phe Phe Gly Ile Gln
1025                1030                1035                1040
Glu Glu Phe Asn Ala Thr Leu Lys Gly Asn Leu Ser Phe Asp Trp Tyr
                1045                1050                1055
Ile Lys Thr Ser His Asn His Leu Leu Ile Val Ser Thr Ala Glu Ile
            1060                1065                1070
Leu Phe Asn Asp Ser Val Phe Thr Leu Leu Pro Gly Gln Gly Ala Phe
        1075                1080                1085
Val Arg Ser Gln Thr Glu Thr Lys Val Glu Pro Phe Glu Val Pro Asn
    1090                1095                1100
Pro Leu Pro Leu Ile Val Gly Ser Ser Val Gly Gly Leu Leu Leu Leu
1105                1110                1115                1120
Ala Leu Ile Thr Ala Ala Leu Tyr Lys Leu Gly Phe Phe Lys Arg Gln
                1125                1130                1135
Tyr Lys Asp Met Met Ser Glu Gly Gly Pro Pro Gly Ala Glu Pro Gln
            1140                1145                1150
```

【図面の簡単な説明】

【図１】図１は、ジスルフィド結合を導入する変異を加えた、ＬＦＡ－１　Ｉドメインの高親和性モデルの立体図である。このモデルは、推定上の高親和性Ｍａｃ－１　Ｉドメイン構造の部分と、推定上の低親和性ＬＦＡ－１　Ｉドメイン構造の部分とを、テンプレートとして用いて作製した。Ｋ２８７Ｃ　及びＫ２９４Ｃ　の変異をモデルに含めた。Ｃ２８７及びＣ２９４　の側鎖及びジスルフィド結合を黄色で示した。ＭＩＤＡＳ　のMg^{2+}イオンを金色の球で示す。ＩＣＡＭ－１及びＩＣＡＭ－２への結合に重要な残基の側鎖を、ローズ・ピンクの側鎖及び黄色の硫黄、赤色の酸素及び青色の窒素原子で示す。ＩＣＡＭ－１への種特異的な結合にとって重要である（Ｈｕａｎｇ，Ｃ　ａｎｄ　Ｓｐｒｉｎｇｅｒ，ＴＡ（１９９５）Ｊ　Ｂｉｏｌ　Ｃｈｅｍ　２７０：１９００８－１９０１６）か、又は、アラニンに変異させたときにＩＣＡＭ－１又はＩＣＡＭ－２への結合が少なくとも２倍になるという点で重要である（Ｅｄｗａｒｄｓ，ＣＰ　ｅｔ　ａｌ．，（１９９８）Ｊ　Ｂｉｏｌ　Ｃｈｅｍ　２７３：２８９３７－２８９４４）と定義されたこれらの残基は、Ｍ１４０、Ｅ１４６、Ｔ１７５、Ｌ２０５、Ｅ２４１、Ｔ２４３、Ｓ２４５、及びＫ２６３である。これらの残基はMg^{2+}イオンを取り囲んでおり、ジスルフィドからは遠いことに留意されたい。ＲＩＢＢＯＮＳで作製した（Ｃａｒｓｏｎ，Ｍ（１９９７）Ｍｅｔｈｏｄｓ　ｉｎ　Ｅｎｚｙｍｏｌｏｇｙ，ＲＭ　Ｓｗｅｅｔ　ａｎｄ　ＣＷ　Ｃａｒｔｅｒ　ｅｄｓ．，Ａｃａｄｅｍｉｃ　Ｐｒｅｓｓ　ｐｐ．４９３－５０５）。

【図２】図２は、ＬＦＡ－１　Ｉドメインの高親和性もしくは低親和性コンホーマに対して選択的な推定上のジスルフィド結合を示す。Ｋ２８７Ｃ／Ｋ２９４Ｃ変異（パネルＡ、Ｃ）及びＬ２８９Ｃ／Ｋ２９４Ｃ　変異（パネルＢ、Ｄ）を、高親和性（パネルＡ、Ｂ）

及び低親和性（パネルＣ、Ｄ）Ｉドメインコンホーマの両方でモデルを作製した。このモデルの残基 ２５４位から３０５位までを示す。これら四つのモデルは、コンホメーション上のシフトに関与しない残基を用いて重ね合わされており、図面の作製には全く同じ方向で用いた。従って、パネルＣ及びＤに比較したときの、パネルＡ及びＢでのα６ヘリックスの下向きの移動が明白である。β６及びα６をつないでいるループのリモデリングに伴って、残基２８９位の側鎖の配向が逆転している（パネルＤに比較したときのパネルＢ）。ＲＩＢＢＯＮＳで作製した。

【図３】図３は、２９３Ｔ一過性トランスフェクタント（パネルＡ）、及びＫ５６２安定トランスフェクタント（パネルＢ）上でのＬＦＡ－１システイン置換変異型の細胞表面発現を、フローサイトメトリ解析で、αＬ／β２複合体中のαＬに対するモノクローナル抗体ＴＳ２／４（塗りつぶしたヒストグラム）か、又は、非結合性の抗体Ｘ６３（白抜きのヒストグラム）を用いて調べた図である。括弧内の数字は、Ｋ５６２安定トランスフェクタントのクローン数である。

【図４】図４は、固定化ＩＣＡＭ－１へのＬＦＡ－１トランスフェクタントの結合を示す。パネルＡは２９３Ｔ一過性トランスフェクタントであり、そしてパネルＢ及びＣは、Ｋ５６２安定トランスフェクタントである。パネルＡ及びＢでは、固定化ＩＣＡＭ－１へのトランスフェクタントの結合を、１０μｇ／ｍｌの活性化抗体ＣＢＲＬＦＡ－１／２の存在下又は非存在下（コントロール）で、Ca^{2+}及びMg^{2+}を含有するＬ１５培地内で調べた。パネルＣでは、結合検定を、図示のように二価陽イオン又はＥＤＴＡを添加したＴＢＳ、ｐＨ７．５内で行った。 括弧内の数字は、Ｋ５６２安定トランスフェクタントのクローン数である。結果は、三重式試料の平均±ＳＤ、少なくとも三回の実験の代表である。

【図５】図５は、野生型ＬＦＡ－１、推定上の高親和性変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ、又は変異型Ｌ２８９Ｃ／Ｋ２９４Ｃを発現するＫ５６２トランスフェクタントに対する可溶性ＩＣＡＭ－１－ＩｇＡ融合タンパク質の結合を、フローサイトメトリ解析で評価したものである。ＩＣＡＭ－１－ＩｇＡ結合の平均蛍光強度をヒストグラムの上部右隅に示す。括弧内の数字は、Ｋ５６２安定トランスフェクタントのクローン数である。結果は三回の実験の代表である。

【図６】図６は、活性化した野生型及び高親和性（Ｋ２８７Ｃ／Ｋ２９４Ｃ）ＬＦＡ－１を発現している細胞へのリガンド結合にロバスタチンが及ぼす阻害活性を示す。

【図７】図７は、単離されたＬＦＡ－１ Ｉ－ドメインの細胞表面発現を示す。野生型αＬ Ｉ－ドメイン並びに変異型Ｋ２８７Ｃ／Ｋ２９４Ｃ及びＬ２８９Ｃ／Ｋ２９４Ｃ Ｉ－ドメインを、ＰＤＧＦＲ膜貫通ドメインにより、Ｋ５６２トランスフェクタント表面上に発現させた。細胞表面の該Ｉ－ドメインのレベルを、フローサイトメトリにより、該Ｉ－ドメインに対するモノクローナル抗体ＴＳ１／２２を用いて調べた（塗りつぶしたヒストグラム）。コントロールのｍＡｂ Ｘ６３ の結合を白抜きのヒストグラムで示す。ＴＳ１／２２結合の平均蛍光強度を、ヒストグラムの上部右隅に示した。各Ｉ－ドメイントランスフェクタントのうちの二つの個々のクローン（＃１及び＃２）の結果を示す。

【図８】図８は、細胞表面に発現したＬＦＡ－１ Ｉ－ドメインのリガンド結合活性を示す。パネルＡは、ＤＴＴの存在下又は不在下における、固定化ＩＣＡＭ－１へのＫ５６２トランスフェクタントの結合を示す。結合はＤＴＴの存在下（白バー）又は不在下（黒バー）で行わせた。パネルＢは、Ｋ５６２トランスフェクタントのＩＣＡＭ－１への結合に対する二価陽イオンの影響を示す。結合はMn^{2+}（黒バー）、Mg^{2+}（網掛けバー）はＥＤＴＡの存在下（白棒）で行わせた。パネルＡ及びＢでは、野生型Ｉ－ドメイン又は変異型Ｉ－ドメインを発現しているトランスフェクタントの二つのクローン（＃１及び＃２）をテストした。パネルＣは、ＬＦＡ－１遮断抗体が、Ｋ２８７Ｃ／Ｋ２９４Ｃ Ｉ－ドメインのＩＣＡＭ－１への結合に及ぼす影響を示す。結果は三重試料の平均±ＳＤ、三回の実験の代表である。

【図９】図９は、開いた（Ｋ２８７Ｃ／Ｋ２９４Ｃ）又は野生型Ｉ－ドメインと、リガンドＩＣＡＭ－１（パネルＡ及びＢ）、ＩＣＡＭ－２（パネルＣ及びＤ）及びＩＣＡＭ－３

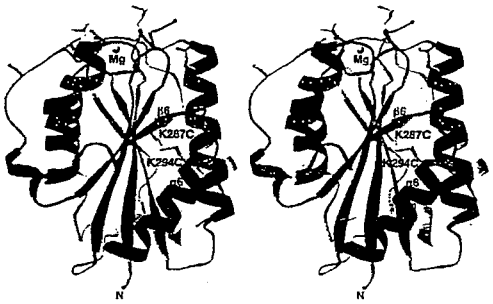
（パネルＥ及びＦ）との間の相互作用を記録したＢＩＡｃｏｒｅ^{TM}による表面プラスモン共鳴センソグラムを示す。

【図１０】図１０は、開いたαＬ Ｉ－ドメインによるＬＦＡ－１依存的接着のインビトロでの阻害を示す。パネルＡは、可溶性の野生型（塗りつぶした丸）又は開いた（Ｋ２８７Ｃ／Ｋ２９４Ｃ）Ｉ－ドメイン（白抜き丸）の存在下における、野生型ＬＦＡ－１を発現しているＫ５６２安定トランスフェクタントの固定化ＩＣＡＭ－１への接着を示す。パネルＢは、可溶性の野生型（塗りつぶした丸）又は開いた（Ｋ２８７Ｃ／Ｋ２９４Ｃ）Ｉ－ドメイン（白抜き丸）の存在下における、マウスＥＬ－４ Ｔリンパ腫細胞株の同型凝集を示す。

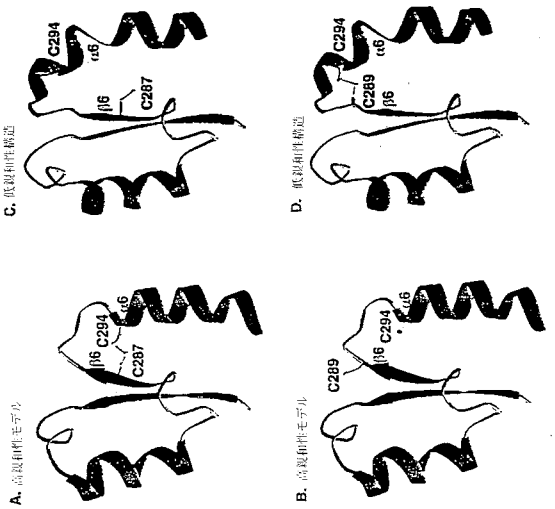
【図１１】図１１は、一過的にトランスフェクトした２９３Ｔ細胞におけるＭａｃ－１システイン置換変異型の発現及びリガンド結合活性を示す。パネルＡは、モノクローナル抗体ＣＢＲＭ１／３２ （白バー）及び ＣＢＲＭ１／５ （黒バー） のインタクト Ｍａｃ－１ Ｉ－ドメイン変異型への結合を示す。パネルＢは、インタクトＭａｃ－１システイン置換変異型を発現している２９３Ｔ一過性トランスフェクタントの、プラスチック上に被膜されたｉＣ３ｂへの接着を示す。パネルＣは、単離型Ｍａｃ－１変異型Ｉ－ドメインを発現している２９３Ｔ一過性トランスフェクタントの、ｉＣ３ｂリガンドへの、抗体ＣＢＲＭ１／５の存在下（黒バー）又は非存在下（白バー）での接着を示す。

【図１２】図１２は、Ｋ５６２安定トランスフェクタントにおけるＭａｃ－１システイン置換変異型の発現及びリガンド結合活性を示す。パネルＡは、モノクローナル抗体ＣＢＲＭ１／３２及びＣＢＲＭ１／５の、インタクトＭａｃ－１ Ｉ－ドメイン変異型への結合をフローサイトメトリで評価して示した代表的ヒストグラムである。平均蛍光強度を、ヒストグラムの上部右端に示す。パネルＢは、インタクトＭａｃ－１システイン置換変異型を発現しているＫ５６２安定トランスフェクタントの、プラスチック上にコートされたｉＣ３ｂへの接着を示す。パネルＣは、単離型Ｍａｃ－１ Ｉ－変異型Ｉ－ドメインを発現しているＫ５６２安定トランスフェクタントの、ｉＣ３ｂリガンドへの接着を示す。接着は、モノクローナル抗体 ＣＢＲＭ１／５の存在下（黒バー）もしくは非存在下（白バー）、又は１０ ｍＭ ＤＴＴの存在下 （灰色バー）で検定した。

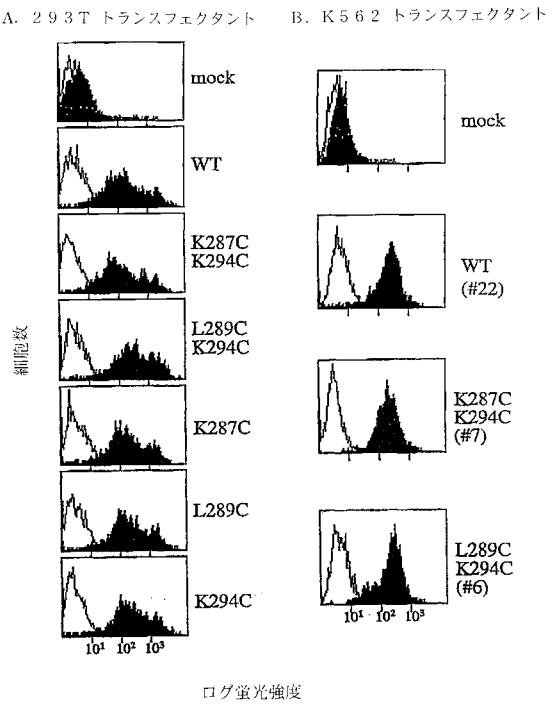
【図１】


Mg
β6
K287C
K294C
α6
N

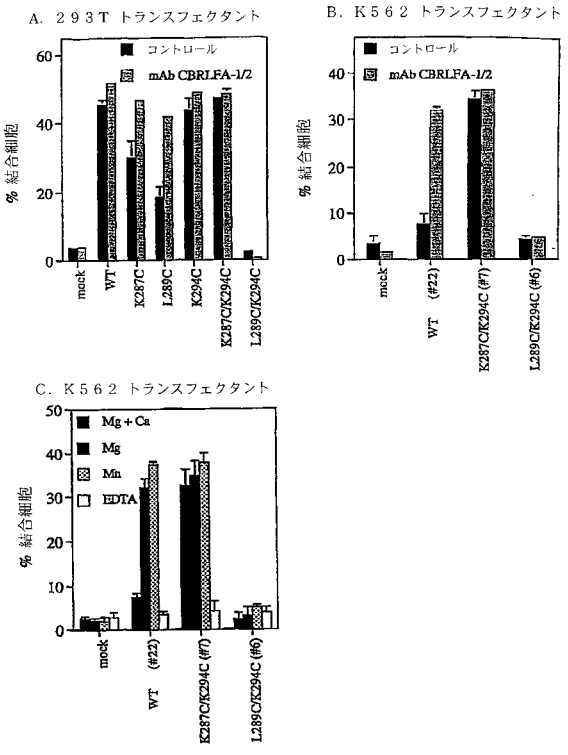
【図２】


C. 低親和性構造
D. 低親和性構造
A. 高親和性モデル
B. 高親和性モデル
C294
α6
β6
C287
C289

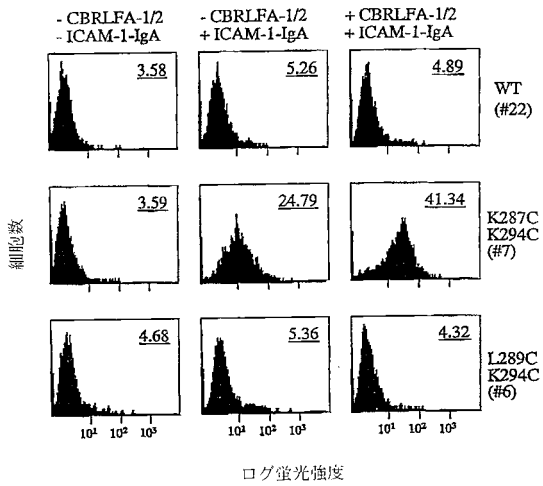
【図３】


A. 293T トランスフェクタント
B. K562 トランスフェクタント
mock
WT
K287C K294C
L289C K294C
K287C
L289C
K294C
WT (#22)
K287C K294C (#7)
L289C K294C (#6)
細胞数
10¹ 10² 10³
ログ蛍光強度

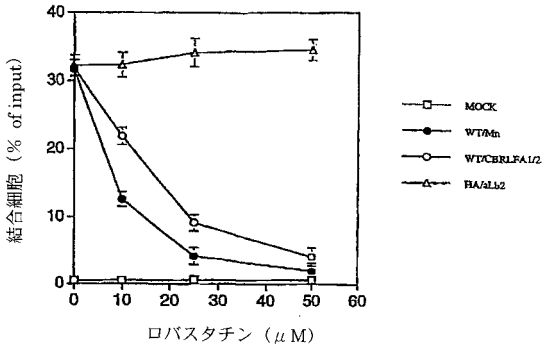
【図４】


A. 293T トランスフェクタント
コントロール
mAb CBRLFA-1/2
% 結合細胞
mock
WT
K287C
L289C
K294C
K287C/K294C
L289C/K294C
B. K562 トランスフェクタント
WT (#22)
K287C/K294C (#7)
L289C/K294C (#6)
C. K562 トランスフェクタント
Mg+Ca
Mg
Mn
EDTA

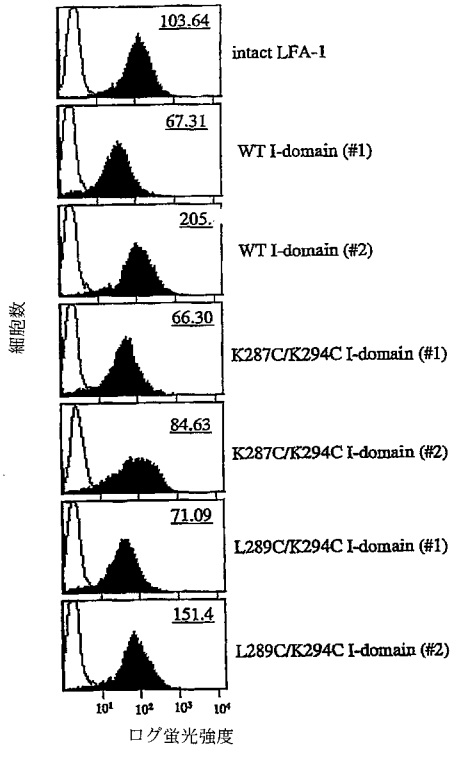
【図５】


- CBRLFA-1/2
- ICAM-1-IgA
- CBRLFA-1/2
+ ICAM-1-IgA
+ CBRLFA-1/2
+ ICAM-1-IgA
3.58
5.26
4.89
WT
(#22)
3.59
24.79
41.34
K287C
K294C
(#7)
4.68
5.36
4.32
L289C
K294C
(#6)
細胞数
ログ蛍光強度

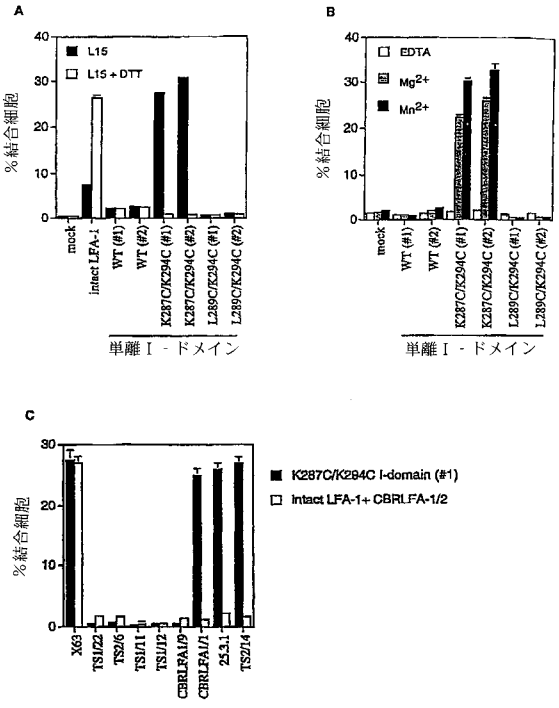
【図６】


結合細胞（% of input）
ロバスタチン（μM）
MOCK
WT/Mn
WT/CBRLFA1/2
HA/aLb2

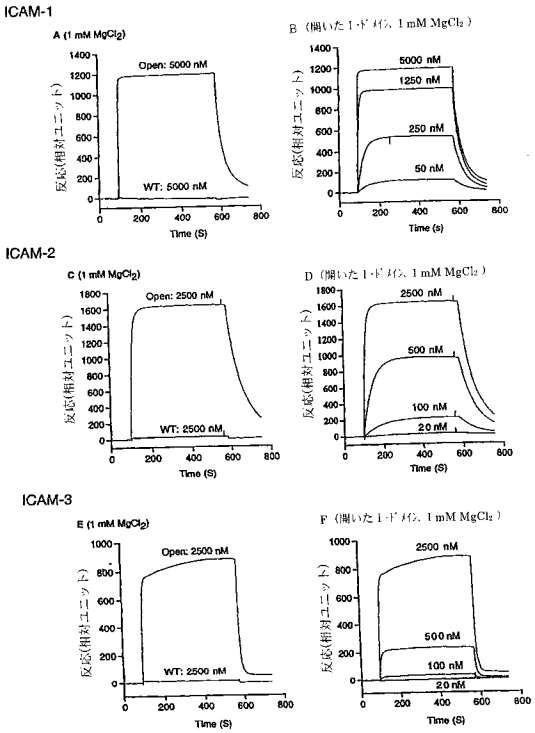
【図７】


103.64
intact LFA-1
67.31
WT I-domain (#1)
205.
WT I-domain (#2)
66.30
K287C/K294C I-domain (#1)
84.63
K287C/K294C I-domain (#2)
71.09
L289C/K294C I-domain (#1)
151.4
L289C/K294C I-domain (#2)
細胞数
ログ蛍光強度

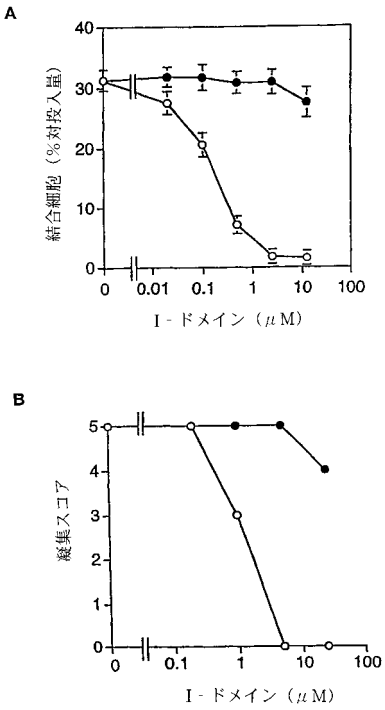
【図８】


A
L15
L15 + DTT
%結合細胞
mock
intact LFA-1
WT (#1)
WT (#2)
K287C/K294C (#1)
K287C/K294C (#2)
L289C/K294C (#1)
L289C/K294C (#2)
単離Ｉ－ドメイン
B
EDTA
Mg2+
Mn2+
%結合細胞
単離Ｉ－ドメイン
C
%結合細胞
K287C/K294C I-domain (#1)
Intact LFA-1+ CBRLFA-1/2
X63
TS1/22
TS2/6
TS1/11
TS1/12
CBRLFA1/9
CBRLFA1/1
25.3.1
TS2/14

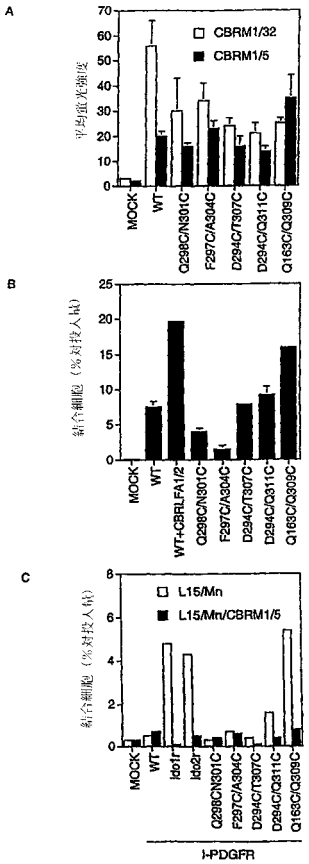
【図９】



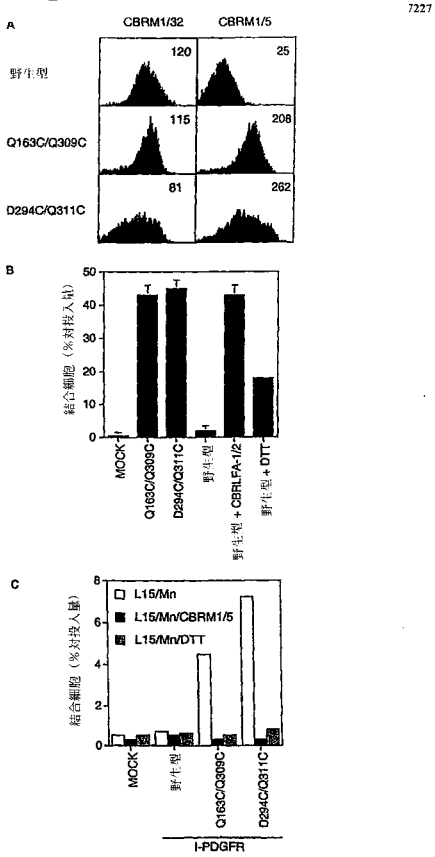
【図１０】



【図１１】



【図１２】



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(54) **Title:** MODIFIED POLYPEPTIDES STABILIZED IN A DESIRED CONFORMATION AND METHODS FOR PRODUCING SAME

(57) **Abstract:** The present invention provides a method for stabilizing a protein in a desired conformation by introducing at least one disulfide bond into the polypeptide. Computational design is used to identify positions where cysteine residues can be introduced to form a disulfide bond in only one protein conformation, and therefore lock the protein in a given conformation. Accordingly, antibody and small molecule therapeutics are selected that are specific for the desired protein conformation. The invention also provides modified integrin I-domain polypeptides that are stabilized in a desired conformation. The invention further provides screening assays and therapeutic methods utilizing the modified integrin I-domains of the invention.

MODIFIED POLYPEPTIDES STABILIZED IN A DESIRED CONFORMATION AND METHODS FOR PRODUCING SAME

Related Applications

This application claims priority to U.S. Provisional Patent Application No. 60/229,700 filed on September 1, 2000, incorporated herein in its entirety by reference.

Background of the Invention

The integrin family of adhesion molecules are noncovalently-associated α/β heterodimers. To date, at least fourteen different integrin α subunits and eight different β subunits have been reported (Hynes, RO (1992) *Cell* 69:1-25). Lymphocyte function-associated antigen-1 (LFA-1) is a member of the leukocyte integrin subfamily. Members of the leukocyte integrin subfamily share the common β2 subunit (CD18) but have distinct α subunits, αL (CD11a), αM (CD11b), αX (CD11c) and αd for LFA-1, Mac-1, p150.95 and αd/β2, respectively (Springer, TA (1990) *Nature* 346:425-433; Larson, RS and Springer, TA (1990) *Immunol Rev* 114:181-217; Van der Vieren, M *et al.* (1995) *Immunity* 3:683-690). The leukocyte integrins mediate a wide range of adhesive interactions that are essential for normal immune and inflammatory responses.

Both integrin α and β subunits are type I transmembrane glycoproteins, each with a large extracellular domain, a single transmembrane region and a short cytoplasmic tail. Several structurally distinct domains have been identified or predicted in the α and β subunit extracellular domains.

The N-terminal region of the integrin α subunits contains seven repeats of about 60 amino acids each, and has been predicted to fold into a 7-bladed β-propeller domain (Springer, TA (1997) Proc Natl Acad Sci USA 94:65-72). The leukocyte integrin α subunits, the α1, α2, α10, α11, and αE subunits contain an inserted domain or I-domain of about 200 amino acids (Larson, RS *et al.* (1989) *J Cell Biol* 108:703-712; Takada, Y *et al.* (1989) *EMBO J* 8:1361-1368; Briesewitz, R *et al.* (1993) *J Biol Chem* 268:2989-2996; Shaw, S K *et al.* (1994) *J Biol Chem* 269:6016-6025; Camper, L *et al.* (1998) *J Biol Chem* 273:20383-20389). The I-domain is predicted to be inserted between β-sheets 2 and 3 of the β-propeller domain. The three dimensional structure of the αM, αL, α1 and α2 I-domains has been solved and shows that it adopts the

dinucleotide-binding fold with a unique divalent cation coordination site designated the metal ion-dependent adhesion site (MIDAS) (Lee, J-O, *et al.* (1995) *Structure* 3:1333-1340; Lee, J-O, *et al.* (199S) *Cell* 80:631-638; Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Emsley, J *et al.* (1997) *J Biol Chem* 272:28512-28517; Baldwin, ET *et al.* (1998) *Structure* 6:923-935; Kallen, J *et al.* (1999) *J Mol Biol* 292:1-9). The C-terminal region of the αM subunit has been predicted to fold into a β-sandwich structure (Lu, C *et al.* (1998) *J Biol Chem* 273:15138-15147).

The integrin β subunits contain a conserved domain of about 250 amino acids in the N-terminal portion, and a cysteine-rich region in the C-terminal portion. The β conserved domain, or I-like domain, has been predicted to have an "I-domain-like" fold (Puzon-McLaughlin, W and Takada, Y (1996) *J Biol Chem* 271:20438-20443; Tuckwell, DS and Humphries, MJ (1997) *FEBS Lett* 400: 297-303; Huang, C *et al.* (2000) *J Biol Chem* 275:21514-24). The C-terminal Cys-rich region of the β subunits appears to be important in the regulation of integrin function, as a number of activating antibodies to the β1, β2 and β3 subunits bind to this region (Petruzzelli, L *et al.* (1995) *J Immunol* 155:854-866; Robinson, MK *et al.* (1992) *J Immunol* 148:1080-1085; Faull, RJ *et al.* (1996) *J Biol Chem* 271:25099-25106; Shih, DT *et al.* (1993) *J Cell Biol* 122:1361-1371; Du, X *et al.* (1993) *J Biol Chem* 268:23087-23092).

Electron microscopic images of integrins reveal that the N-terminal portions of the α and β subunits fold into a globular head that is connected to the membrane by two rod-like tails about 16 nm long corresponding to the C-terminal portions of the α and β extracellular domains (Nermut, MV *et al.* (1988), *EMBO J* 7:4093-4099; Weisel, JW *et al.* (1992) *J Biol Chem* 267:16637-16643; Wippler, J *et al.* (1994) *J Biol Chem* 269: 8754-8761).

LFA-1 is expressed on all leukocytes and is the receptor for three Ig superfamily members, intercellular adhesion molecule-1, -2 and –3) (Marlin, SD *et al.* (1987) *Cell 51:*813-819; Staunton, DE *et al.* (1989) *Nature* 339:61 -64; de Fougerolles, *et al.* (1991) *J Exp Med* 1 74: 253-267). Substantial data indicate that the I-domain of LFA-1 is critical for interaction with ligands. Mutagenesis studies have shown that residues M140, E146, T175, L205, E241, T243, S245 and K263 in the I-domain are important for ligand binding (Huang, C *et al.* (1995) *J Biol Chem* 270:19008-19016; Edwards, CP

et al. (1998) *J Biol Chem* 273:28937-28944). These residues are located on the surface of the I-domain surrounding the Mg^{2+} ion, defining a ligand binding interface on the upper surface of the I-domain. The importance of the I-domain in ligand binding is further underscored by mAb blocking studies. A large number mAbs that inhibit LFA-1 interaction with its ligands map to the I-domain (Randi, AM *et al.* (1994) *J Biol Chem* 269:12395-12398; Champe, M *et al.* (1995) *J Biol Chem* 270:1388-1394; Huang, C *et al.* (1995) *J Biol Chem* 270:19008-19016; Edwards, CP *et al.* (1998) *J Biol Chem* 273:28937-28944). Two groups have recently shown that I-domain deleted LFA-1 lacks ligand recognition and binding ability, further demonstrating the role of the I-domain in LFA-1 function (Leitinger, B *et al.* (2000) *Mol Biol Cell* 11, 677-690; Yalamanchili, P *et al.* (2000) *J Biol Chem* 275:21877-82). The I-domains of other I-domain containing integrins have also been implicated in ligand binding (Diamond, MS (1993) *J Cell Biol* 120:545556; Michishita, M *et al.* (1993) *Cell* 72:857-867; Muchowski, PJ *et al.* (1994) *J Biol Chem* 269:26419-26423; Zhou, L *et al.* (1994) *J Biol Chem* 269:17075-17079; Ueda, T *et al.* (1994) *Proc Natl Acad Sci USA* 91:10680- 10684; Kamata, T *et al.* (1994) *J Biol Chem* 269:96599663; Kern, A *et al.* (1994) *J Biol Chem 269:*22811-22816).

Binding of LFA-1 to ICAMs requires LFA-1 activation. LFA-1 can be activated by signals from the cytoplasm, called "inside-out" signaling (Diamond, MS *et al.* (1994) *Current Biology* 4:506-517). Divalent cations Mn^{2+}, Mg^{2+} and Ca^{2+} can directly modulate ligand-binding function of LFA-1 (Dransfield, I *et al.* (1989) *EMBO J* 8:3759-3765; Dransfield, I *et al.* (1992). *J Cell Biol* 116:219-226; Stewart, MP *et al.* (1996) *J Immunol* 156:1810-1817). In addition, LFA-1 can be activated by certain mAbs that bind the extracellular domains of the αL or β2 subunit (Keizer, GD *et al.* (1988) *J Immunol 140:*1393- 1400; Robinson, MK *et al.* (1992) *J Immunol* 148:1080-1085; Andrew, D *et al.* (1993) *Eur J Immunol* 23:2217-2222; Petruzzelli, L *et al.* (1995) *J Immunol* 155:854-866). The molecular mechanism for integrin activation is not yet well understood. It has been proposed that intramolecular conformational changes accompanying integrin activation increase integrin affinity for ligand, and this is supported by the existence of antibodies that only recognize activated integrins (Dransfield, I *et al.* (1989) *EMBO J* 8:3759-3765; Diamond, MS *et al.* (1993) *J Cell Biol* 120: 545-556; Shattil, SJ *et al.* (1985) *J Biol Chem* 260:11107-11114). One of such antibodies CBRLFA-1/5 binds to the Mac-1 I-domain very close to the ligand binding

site (Oxvig, C *et al.* (1999) *Proc Natl Acad Sci USA* 96:2215-2220), providing further evidence that the I-domain itself undergoes conformational changes with activation.

Two different crystal forms of the Mac-1 I-domain have been obtained, and it has been hypothesized that the two structures represent the "active" and "inactive" conformation, respectively (Lee, J-O *et al.* (1995) *Structure* 3, 1333-1340; Lee, J-O *et al.* (1995) *Cell* 80:631-638). In the "active" form, crystallized with Mg^{2+}, a glutamate from a neighboring I-domain provides the sixth metal coordination site, while in the "inactive" conformation, complexed with Mn^{2+}, a water molecule completes the metal coordination sphere. The change in metal coordination is linked to a large shift of the C-terminal α-helix; in the putative "active" conformation, the C-terminal helix moves 10 Å down the body of the I-domain (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340). Results from epitope mapping of mAb CBRM-1/5 that only recognizes activated Mac-1 have suggested that the conformational differences are physiologically (Oxvig, C *et al.* (1999) *Proc Natl Acad Sci USA* 96:2215-2220). The crystal and NMR structures of the LFA-1 I-domain have a conformation similar to the putative "inactive" conformation of the Mac-1 I-domain (Qu, A *et al.* (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A (1996) *Structure* 4:, 931-942; Kallen, J *et al.* (1999) *J Mol Biol* 292:1-9; Legge, GB *et al.* (2000) *J Mol Biol* 295:1251-1264).

In addition to integrins, many pharmaceutically important proteins exist in two alternative three-dimensional structures, referred to as conformations or conformers. Often these proteins have important signaling functions, such as small G proteins, trimeric G protein α subunits, tyrosine kinases, and G protein-coupled receptors. Typically, one of these conformations and not the other is enzymatically active or has effector functions. Therefore, antibody or small molecule therapeutics that are specific for a protein in a particular conformation, for example, the active conformation, would have great advantages over non-selective alternatives.

Summary of the Invention

Computational design can be used to introduce a disulfide bond into a protein or polypeptide such that the molecule is stabilized in a desired conformation. Accordingly, antibodies, *e.g.,* anti-LFA-1 antibodies, or small molecule therapeutics that are specific for a desired protein conformation, *e.g.,* an "open" or active conformation or a "closed" or inactive conformation can be identified.

The invention pertains to methods for stabilizing a polypeptide, *e.g.*, a polypeptide comprising a functional domain of a protein, in a desired conformation. The method comprises introducing at least one disulfide bond into the polypeptide such that the polypeptide is stabilized in a desired conformation. In a preferred embodiment the disulfide bond is formed by the introduction of at least one cysteine substitution into the amino acid sequence of the polypeptide. In another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.00-8.09Å. In another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

Computational design can be used to introduce a disulfide bond into a protein or polypeptide such that the molecule is stabilized in a desired conformation. Accordingly, antibody or small molecule therapeutics that are specific for a desired protein conformation can be identified.

The method of the invention is widely applicable to a variety of biologically and pharmaceutically important proteins that exist in two different three-dimensional conformations, including an integrin subunit, a small G protein, a heterotrimeric G protein alpha subunit, a tyrosine kinase, a G protein-coupled receptor, an enzyme under allosteric control, a zymogen, complement C3, complement C4, and fibrinogen. In a preferred embodiment, the polypeptide is an integrin I-domain polypeptide.

In another aspect, the invention provides a modified integrin I-domain polypeptide that is stabilized in a desired conformation by the introduction of at least one disulfide bond. In one embodiment, a modified integrin I-domain is encoded by an amino acid sequence containing at least one cysteine substitution as compared to the wild-type sequence, such that a disulfide bond is formed. In another embodiment, the distance between the Cβ carbons of the residues that are substituted for cysteines is in the range of 3.00-8.09Å. In yet another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

In one embodiment, a modified integrin I-domain polypeptide of the invention is derived from an I-domain of an integrin α subunit, for example, α1, α2, α10, α11, αD, αE, αL (CD11a), αM (CD11b), and αX (CD11c). For example, in one embodiment of the invention, a modified integrin I-domain polypeptide is derived from the I-domain of the human αL subunit and contains amino acid substitutions K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, L161C/T300C, or L289C/K294C. In

another embodiment of the invention, a modified integrin I-domain polypeptide is derived from the I-domain of the human αM subunit and contains amino acid substitutions Q163C/Q309C, D294C/Q311C, or Q163C/R313C.

In a preferred embodiment, a modified integrin I-domain polypeptide of the invention is stabilized in the open conformation. In another embodiment, a modified integrin I-domain polypeptide of the invention is stabilized in the closed conformation. In another embodiment, a modified integrin I-domain binds ligand with high affinity. In yet another embodiment, a modified integrin I-domain polypeptide is operatively linked to a heterologous polypeptide.

In a related aspect, the invention provides isolated nucleic acid molecules which encode a modified integrin I-domain polypeptide of the invention.

The modified integrin I-domain polypeptides, and/or biologically active or antigenic fragments thereof, are useful, for example, as reagents or targets in assays applicable to the treatment and/or diagnosis of integrin-mediated disorders.

Accordingly, in one aspect, the invention provides an antibody, or an antigen binding fragment thereof, which selectively binds to a modified integrin I-domain in the open conformation. In another aspect, the invention provides an antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain polypeptide in the open conformation, an integrin I-domain polypeptide in the closed conformation, or a modified integrin I-domain polypeptide. In one embodiment, the antibody binds to an activation specific epitope on the integrin I-domain. In another embodiment, the antibody blocks an interaction between an integrin and a cognate ligand. In one embodiment, the antibody is an anti-LFA-1 antibody, or an antigen binding fragment thereof, *e.g.*, an anti-LFA-1 antibody which reacts with or binds an open or closed conformation of an LFA-1 polypeptide, or a modified LFA-1 I-domain integrin polypeptide, or fragment thereof.

In another aspect the invention provides a method for identifying a modulator of integrin activity comprising assaying the ability of a test compound to bind to a modified integrin I-domain polypeptide which is stabilized in the open conformation. In another embodiment, the invention provides a method for identifying a compound capable of modulating the interaction of an integrin and a cognate ligand wherein binding of a ligand to a modified integrin I-domain polypeptide which is stabilized in the open conformation is assayed in the presence and absence of a test compound.

In another aspect, the invention provides a composition comprising a modified integrin I-domain polypeptide or an anti-integrin I-domain antibody (or an antigen binding fragment thereof), such composition can further include a pharmaceutically acceptable carrier.

In yet another aspect, the invention pertains to methods for treating or preventing an integrin-mediated disorder (*e.g.*, an inflammatory or autoimmune disorder) in a subject, or for inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to a subject a therapeutically effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation or an antibody (or antigen binding fragment thereof) which selectively binds to an integrin I-domain in the open conformation. In one embodiment, the antibody is an LFA-1 antibody, or an antigen binding fragment thereof, which specifically reacts with or binds an LFA-1 I-domain in the open conformation or specifically reacts with or binds a modified LFA-1 I-domain polypeptide. In a preferred embodiment, the modified integrin I-domain polypeptide binds ligand with high affinity. In another preferred embodiment, the modified integrin I-domain polypeptide for therapeutic use is a soluble polypeptide, *e.g.*, a fusion protein.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Figures

Figure 1 is a stereodiagram of the high affinity model of the LFA-1 I domain, with mutations to introduce a disulfide bond. The model was prepared using segments of the putative high affinity Mac-1 I domain structure and a putative low affinity LFA-1 I domain structure as templates. The K287C and K294C mutations were included in the model. The sidechains and disulfide bond of C287 and C294 are shown in yellow. The Mg^{2+} ion of the MIDAS is shown as a gold sphere. Sidechains of residues important in binding to ICAM-1 and ICAM-2 are shown with rose-pink sidechains and yellow sulfur, red oxygen, and blue nitrogen atoms. These residues, defined as important in species-specific binding to ICAM-1 (Huang, C and Springer, TA (1995) *J Biol Chem* 270:19008-19016) or by at least a 2-fold effect on binding to ICAM-1 or ICAM-2 upon mutation to alanine (Edwards, CP *et al.*, (1998) *J Biol Chem* 273:28937-28944), are

M140, E146, T175, L205, E241, T243, S245, and K263. Note that these residues surround the Mg^{2+} ion, and are distant from the disulfide. Prepared with RIBBONS (Carson, M (1997) Methods in Enzymology, RM Sweet and CW Carter eds., Academic Press pp. 493-505).

Figure 2 depicts predicted disulfide bonds that are selective for high affinity or low affinity conformers of the LFA-1 I domain. The K287C/K294C mutation (*Panels A, C*) and L289C/K294C mutation (*Panels B, D*) were modeled in both high affinity (*Panels A, B*) and low affinity (*Panels C, D*) I domain conformers. Residues 254 to 305 of the models are shown. The four models were superimposed using residues not involved in conformational shifts and were used in exactly the same orientation for figure preparation. Therefore, the downward shift in the α6 helix in panels A and B compared to panels C and D is readily apparent. The remodeling of the loop connecting β6 and α6 is accompanied by a reversal in the orientation of the sidechain of residue 289 (panel B compared to panel D). Prepared with RIBBONS.

Figure 3 depicts the cell surface expression of LFA-1 cysteine substitution mutants on 293T transient transfectants (*Panel A*), and K562 stable transfectants (*Panel B*) as determined by flow cytometric analysis using monoclonal antibody TS2/4 (shaded histogram) to αL in αL/β2 complex, or the nonbinding antibody X63 (open histogram). Numbers in the parentheses are clone numbers of the K562 stable transfectants.

Figure 4 depicts the binding of LFA-1transfectants to immobilized ICAM-1. *Panel A*, 293T transient transfectants, and *Panels B and C*, K562 stable transfectants. In Panels A and B, binding of the transfectants to immobilized ICAM-1 was determined in L15 medium containing Ca^{2+} and Mg^{2+} in the presence or absence (control) of the activating antibody CBRLFA-1/2 at 10 μg/ml. In Panel C, the binding assay was performed in TBS, pH7.5 supplemented with divalent cations or EDTA as indicated. Numbers in the parentheses are clone numbers of the K562 stable transfectants. Results are mean ± SD of triplicate samples and representative of at least three experiments.

Figure 5 depicts the binding of soluble ICAM-1-IgA fusion protein to K562 transfectants that express wild-type LFA-1, the predicted high-affinity mutant K287C/K294C, or mutant L289C/K294C as assessed by flow cytometric analysis. Mean fluorescent intensity of ICAM-1-IgA binding is indicated on the upper right corner of the histogram plot. Numbers in the parentheses are clone numbers of the K562 stable transfectants. Results are representative of three experiments.

Figure 6 depicts the inhibitory activity of lovastatin on ligand binding by cells expressing activated wild-type and high affinity (K287C/K294C) LFA-1.

Figure 7 depicts the cell surface expression of the isolated LFA-1 I-domains. The wild-type αL I-domain and the mutant K287C/K294C and L289C/K294C I-domains were expressed on the surface of the K562 transfectants by the PDGFR transmembrane domain. The level of cell surface I-domain was determined by flow cytometry using monoclonal antibody TS1/22 to the I-domain (shaded histogram). Binding of the control mAb X63 is shown as open histograms. Mean fluorescent intensity of TS1/22 binding was indicated on the upper right corner of the histogram plot. Results of two individual clones (#1 and #2) from each I-domain transfectants are shown.

Figure 8 depicts the ligand binding activity of the cell surface expressed LFA-1 I-domains. *Panel A*, Binding of K562 transfectants to immobilized ICAM-1 in the presence or absence of DTT. Binding was performed in the presence (white bar) or absence (black bar) of DTT. *Panel B,* Effect of divalent cations on binding of K562 transfectants to ICAM-1. Binding was performed in the presence of Mn^{2+} (black bar), Mg^{2+} (shaded bar) or EDTA (white bar). In Panels A and B, two clones (#1 and #2) of the transfectants expressing the wild-type I-domain or mutant I-domain were tested. *Panel C* , Effect of LFA-1 blocking antibodies on binding of the K287C/K294C I-domain to ICAM-1. Results are mean ± SD of triplicate samples and representative of 3 experiments.

Figure 9 depicts the surface plasmon resonance sensograms by BIAcore™ recording the interaction of the open (K287C/K294C) or wild-type I-domain with ligands, ICAM-1 (*Panels A and B*), ICAM-2 (*Panels C and D*), and ICAM-3 (*Panels E and F*).

Figure 10 depicts the inhibition of LFA-1-dependent adhesion *in vitro* by the open αL I-domain. *Panel A* depicts the adhesion of K562 stable transfectants expressing wild-type LFA-1 to immobilized ICAM-1 in the presence of soluble wild-type (closed circles) or open (K287C/K294C) I-domain (open circles); *Panel B* depicts the homotypic aggregation of the murine EL-4 T lymphoma cell line in the presence of soluble wild-type (closed circles) or open (K287C/K294C) I-domain (open circles).

Figure 11 depicts the expression and ligand binding activity of the Mac-1 cysteine substitution mutants in transiently transfected 293T cells. *Panel A*, binding of monoclonal antibodies CBRM1/32 (open bars) and CBRM1/5 (black bars) to intact Mac-1 I-domain mutants. *Panel B*, adhesion of 293T transient transfectants expressing intact Mac-1 cysteine substitution mutants to iC3b coated on plastic. *Panel C*, adhesion of 293T transient transfectants expressing isolated Mac-1mutant I-domains to iC3b ligand in the presence (black bars) or absence (open bars) of antibody CBRM1/5.

Figure 12 depicts the expression and ligand binding activity of the Mac-1 cysteine substitution mutants in K562 stable transfectants. *Panel A*, representative histogram showing binding of monoclonal antibodies CBRM1/32 and CBRM1/5 to intact Mac-1 I-domain mutants as assessed by flow cytometry. Mean fluorescent intensity is indicated in the upper right hand corner of the histogram plot. *Panel B*, adhesion of K562 stable transfectants expressing intact Mac-1 cysteine substitution mutants to iC3b coated on plastic. *Panel C*, adhesion of K562 stable transfectants expressing isolated Mac-1 I-mutant I-domains to iC3b ligand. Adhesion was assayed in the presence (black bars) or absence (open bars) of monoclonal antibody CBRM1/5, or in the presence of 10 mM DTT (gray bars).

Detailed Description of the Invention

The present invention is based, at least in part, on a method for stabilizing a polypeptide in a desired conformation by introducing at least one disulfide bond into the polypeptide. In one embodiment, based on NMR or crystal structures of specific protein conformations, computational design is used to introduce a disulfide bond that locks the protein in a particular conformation. As used herein, a "conformation" or "conformer" refers to the three dimensional structure of a protein. A "desired" conformation includes a protein conformation that is conducive to a particular use of the polypeptide, *e.g.*, a conformation that supports a particular biological function and/or activity, or a therapeutic effect. As used herein, the terms "polypeptide" and "protein" are used interchangeably throughout.

In one embodiment, a desired conformation is a protein conformation which promotes or activates a biological function and/or activity, *e.g.*, an open or active conformation. In another embodiment, a desired conformation is a protein conformation

which inhibits or suppresses a biological function and/or activity, *e.g.*, a closed or inactive conformation.

In particular, the method of the invention includes modeling a protein, or a functional domain thereof, on a template of the desired three-dimensional structure of the protein and introducing cysteines which are able to form a disulfide bond only in the desired conformation of the protein, thus stabilizing the protein in that particular conformation. The protein can be any protein, or domain thereof, for which a three dimensional structure is known or can be generated, and is preferably a protein that exists in two different conformations. Computational algorithms for designing and/or modeling protein conformations are described, for example, in WO 98/47089. The SSBOND program (Hazes, B and Dijkstra, BW (1988) *Protein Engineering* 2:119-125) can be used to identify positions where disulfide bonds can be introduced in a protein structure by mutating appropriately positioned pairs of residues to cysteine.

Disulfide bond formation occurs between two cysteine residues that are appropriately positioned within the three-dimensional structure of a polypeptide. In one embodiment of the invention, a polypeptide is stabilized in a desired conformation by introducing at least one cysteine substitution into the amino acid sequence such that a disulfide bond is formed. The introduction of a single cysteine substitution is performed in circumstances in which an additional cysteine residue is present in the native amino acid sequence of the polypeptide at an appropriate position such that a disulfide bond is formed. In a preferred embodiment, two cysteine substitutions are introduced into the amino acid sequence of the polypeptide at positions that allow a disulfide bond to form, thereby stabilizing the polypeptide in a desired conformation. In another embodiment, the distance between the Cβ carbons of the residues that are substituted for cysteine is 3.00-8.09Å. In yet another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

In one embodiment of the invention, cysteine substitutions are introduced such that the formation of a disulfide bond is favored only in one protein conformation, such that the protein is stabilized in that particular conformation.

Preparation of a modified polypeptide of the invention by introducing cysteine substitutions is preferably achieved by mutagenesis of DNA encoding the polypeptide of interest (*e.g.*, an integrin polypeptide). For example, an isolated nucleic acid molecule encoding a modified integrin I-domain polypeptide can be created by introducing one or

more nucleotide substitutions into the nucleotide sequence of an integrin gene such that one or more amino acid substitutions, *e.g.*, cysteine substitutions, are introduced into the encoded protein. Mutations can be introduced into a nucleic acid sequence by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

Suitable proteins include, but are not limited to, industrially and therapeutically important proteins such as: 1) signaling molecules, such as small G proteins, trimeric G protein alpha subunits, tyrosine kinases, and G protein-coupled receptors; 2) enzymes under allosteric control, 3) zymogens that undergo conformational change after activation by proteolytic cleavage, such as the proteases (convertases and factors) of the complement and clotting cascades, and 4) proteolytically activated effector molecules such as complement components C3 and C4, and fibrinogen. In one embodiment, the method of the invention can be used to stabilize a protein in a biologically active conformation, *e.g.*, a conformation that is enzymatically active or has ligand binding capacity and/or effector functions, *e.g.*, an "open" conformation. In another embodiment, the method of the invention can be used to stabilize a protein in a biologically inactive conformation, *e.g.*, a conformation that is enzymatically inactive or does not have ligand binding capacity and/or effector functions, *e.g.*, a "closed" conformation.

Proteins that are stabilized in a particular conformation may find use in, for example, in proteomic screening technologies. In proteomic screens of tissues and disease states, antibodies, polypeptide, and/or small molecules that are specific for, *e.g.*, an active protein conformer or an inactive protein conformer, can be used to assess the activity of different cellular signaling, metabolic, and adhesive pathways. Thus, associations can be made between specific diseases and the activation of specific biochemical and signaling pathways. Furthermore, the invention relates to the polypeptides, antibodies, and small molecules identified using the methods described herein and uses for same, *e.g.*, to treat, for example, inflammatory disorders. Conformer-specific reagents can also be placed on chips and used to screen tissue extracts, or used to stain tissue sections. Furthermore, drugs or antibodies, *e.g.*, anti-integrin antibodies which specifically recognize a modified integrin I-domain polypeptide, *e.g.*, an anti-LFA-1 antibody which specifically recognizes a modified LFA-1 I-domain polypeptide, that are selective for a particular conformer, *e.g.*, an open conformer or a closed conformer, may provide differential therapeutic effects. Therefore, selective screening

assays using a protein stabilized in a particular conformer can be used to rationally obtain compounds with a desired activity.

Integrins

Integrins exist on cell surfaces in an inactive conformation that does not bind ligand. Upon cell activation, integrins change shape (conformation) and can bind ligand. Over 20 different integrin heterodimers (different α and β subunit combinations) exist that are expressed in a selective fashion on all cells in the body. After activation, integrins bind in a specific manner to protein ligands on the surface of other cells, in the extracellular matrix, or that are assembled in the clotting or complement cascades. Integrins on leukocytes are of central importance in leukocyte emigration and in inflammatory and immune responses. Ligands for the leukocyte integrin Mac-1 (αMβ2) include the inflammation-associated cell surface molecule ICAM-1, the complement component iC3b, and the clotting component fibrinogen. Ligands for the leukocyte integrin LFA-1 (αLβ2) include ICAM-1, ICAM-2, and ICAM-3. Antibodies to leukocyte integrins can block many types of inflammatory and auto-immune diseases, by, *e.g.*, modulating, *e.g.*, inhibiting, for example, cell to cell interactions or cell to extracellular matrix interactions. Integrins on platelets are important in clotting and in heart disease; approved drugs include the antibody abciximab (Reopro™) and the peptide-like antagonist eptifibatide (Integrilin™). Integrins on connective tissue cells, epithelium, and endothelium are important in disease states affecting these cells. They regulate cell growth, differentiation, wound healing, fibrosis, apoptosis, and angiogenesis. Integrins on cancerous cells regulate invasion and metastasis.

To antagonize integrins, drugs are needed that bind to the active, ligand-binding conformation. Most antibodies bind to both the active and inactive conformations, since only a small portion of the surface of the integrin molecule changes shape. It is desirable that antibodies bind only to the active integrin conformation, *e.g.*, the "open" conformation, because binding to the inactive conformation can lead to side reactions, generation of anti-idiotypic antibodies, and result in clearance of the antibody and, thus, requires much higher doses to be administered.

The methods described herein have been successfully used to introduce disulfide bonds into the I domains of the integrins, *e.g.*, LFA-1 and Mac-1. Accordingly, in another aspect, the invention provides a modified integrin I-domain polypeptide containing at least one disulfide bond, such that said modified I-domain polypeptide is stabilized in a desired conformation. A modified integrin I-domain polypeptide of the invention may be derived from an I-domain of an integrin α subunit including α1, α2, α10, α11, αD, αE, αL (CD11a), αM (CD11b) and αX (CD11c).

As used herein, a "modified integrin I-domain polypeptide" or "modified integrin polypeptide" includes an integrin I-domain polypeptide that has been altered with respect to the wild-type sequence or the native state such that at least one disulfide bond has been introduced into the polypeptide thereby stabilizing the I-domain in a desired conformation.

The terms "derived from" or "derivative", as used interchangeably herein, are intended to mean that a sequence is identical to or modified from another sequence, *e.g.*, a naturally occurring sequence. Derivatives within the scope of the invention include polynucleotide and polypeptide derivatives. Polypeptide or protein derivatives include polypeptide or protein sequences that differ from the sequences described or known in amino acid sequence, or in ways that do not involve sequence, or both, and still preserve the activity of the polypeptide or protein. Derivatives in amino acid sequence are produced when one or more amino acid is substituted with a different natural amino acid, an amino acid derivative or non-native amino acid. In certain embodiments protein derivatives include naturally occurring polypeptides or proteins, or biologically active fragments thereof, whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Derivatives may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the biological activity of the polypeptide or protein.

Conservative substitutions (substituents) typically include the substitution of one amino acid for another with similar characteristics (*e.g.*, charge, size, shape, and other biological properties) such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar

(hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In other embodiments, derivatives with amino acid substitutions which are less conservative may also result in desired derivatives, *e.g.*, by causing changes in charge, conformation and other biological properties. Such substitutions would include, for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics. The polypeptides and proteins of this invention may also be modified by various changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use.

In a preferred embodiment, a modified integrin I-domain polypeptide is stabilized in the open conformation, and binds ligand with high affinity.

In one embodiment, a modified integrin I-domain polypeptide of the invention is encoded by an amino acid sequence containing at least one cysteine substitution, and preferably two cysteine substitutions, as compared to the wild-type sequence. In another embodiment, the distance between the Cβ carbons of the residues that are substituted for cysteines is in the range of 3.00-8.09Å, *e.g.*, as predicted by protein modeling. In a further embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

The introduction of cysteine residues at appropriate positions within the amino acid sequence of the I-domain polypeptide allows for the formation of a disulfide bond that stabilizes the domain in a particular conformation, e.g., an active "open" conformation, or an inactive "closed" conformation. For example, the αL K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, L161C/T300C, and L289C/K294C mutants, and the αM Q163C/Q309C and D294C/Q311C mutants are

stabilized in the "open" conformation that bind ligand with high or intermediate affinity, whereas the αL L289C/K294C mutant and the αM Q163C/R313C mutants are stabilized in an inactive or "closed" conformation that does not bind ligand. The affinity of E284C/E301C is nearly comparable to that of K287C/K294C, *e.g.*, high-affinity. The affinity of L161C/F299C, K160C/F299C, and L161C/T300C are significantly higher than wild-type, but 20-30 times lower than high-affinity αL I-domain, K287C/K294C. L161C/F299C, K160C/F299C, and L161C/T300C are referred to herein as intermediate affinity αL I-domains.

In one embodiment, the invention provides a modified integrin I-domain which is comprised within an integrin α subunit, and which may be further associated with an integrin β subunit. In another embodiment, a modified integrin I-domain polypeptide of the invention is a soluble polypeptide. Furthermore, the invention provides a modified integrin I-domain polypeptide which is operatively linked to a heterologous polypeptide.

A model of the I-like domain of the integrin β-subunit that is supported by experimental data (Huang, C *et al.* (2000) *J Biol Chem* 275:21514-24) has also been made. The data confirm the location of the key C-terminal α-helix that undergoes the dramatic 10 Å conformational movement in I domains. The I and I-like domains align well in this region. Accordingly, in another aspect, the invention provides a modified integrin I-like domain polypeptide containing at least one disulfide bond, such that said modified I-like domain polypeptide is stabilized in a desired conformation.

In a preferred embodiment, a modified integrin I-like domain polypeptide is stabilized in the open conformation, and binds ligand with high affinity. In one embodiment, a modified integrin I-like domain polypeptide of the invention is encoded by an amino acid sequence containing at least one cysteine substitution, and preferably two cysteine substitutions, as compared to the wild-type sequence.

In one embodiment, the invention provides a modified integrin I-like domain which is comprised within an integrin β subunit, and which may be further associated with an integrin α subunit. In another embodiment, a modified integrin I-like domain polypeptide of the invention is a soluble polypeptide. Furthermore, the invention provides a modified integrin I-like domain polypeptide which is operatively linked to a heterologous polypeptide.

Integrins are key targets in many diseases. Accordingly, isolated high affinity I-domains of the invention, as well as antibodies, or small molecule antagonists selective for activated leukocyte integrins can be used to modulate, *e.g.*, inhibit or prevent, autoimmune and inflammatory disease, transplant rejection, and ischemia/reperfusion injury as in hypovolemic shock, myocardial infarct, and cerebral shock. Furthermore, co-crystals of high affinity I domains bound to natural ligands and/or small molecule antagonists can readily be made, which will enable computational drug design, and advance modification and improvement of drug development candidates.

Accordingly, the invention provides a method for identifying a modulator of integrin activity comprising assaying the ability of a test compound to bind to a modified integrin I-domain polypeptide which is stabilized in the open conformation. In another embodiment, the invention provides a method for identifying a compound capable of modulating the interaction of an integrin and a cognate ligand wherein binding of a ligand to a modified integrin I-domain polypeptide which is stabilized in the open conformation is assayed in the presence and absence of a test compound.

The invention also provides a composition comprising a modified integrin I-domain polypeptide or an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody (or an antigen binding fragment thereof) which selectively binds to a modified integrin I-domain, *e.g.*, an I-domain in the open conformation, and a pharmaceutically acceptable carrier. The compositions of the invention are used in therapeutic methods of the invention. For example, the invention provides methods for treating or preventing an integrin-mediated disorder (*e.g.*, an inflammatory or autoimmune disorder) in a subject, or for inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to a therapeutically effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation or anti-integrin antibody (or an antigen binding fragment thereof) which selectively binds to an integrin I-domain in the open conformation. In a preferred embodiment, the modified integrin I-domain polypeptide binds ligand with high affinity. In another preferred embodiment, the modified integrin I-domain polypeptide for therapeutic use is a soluble polypeptide, *e.g.*, a fusion protein.

As used herein, an integrin mediated disorder includes, for example, an inflammatory or immune system disorder, and/or a cellular proliferative disorder. Examples of integrin-mediated disorders include myocardial infarction, stroke, restenosis, transplant rejection, graft versus host disease or host versus graft disease, and

reperfusion injury. An inflammatory or immune system disorder includes, but is not limited to adult respiratory distress syndrome (ARDS), multiple organ injury syndromes secondary to septicemia or trauma, viral infection, inflammatory bowel disease, ulcerative colitis, Crohn's disease, leukocyte adhesion deficiency II syndrome, thermal injury, hemodialysis, leukapheresis, peritonitis, chronic obstructive pulmonary disease, lung inflammation, asthma, acute appendicitis, dermatoses with acute inflammatory components, wound healing, septic shock, acute glomerulonephritis, nephritis, amyloidosis, reactive arthritis, rheumatoid arthritis, chronic bronchitis, Sjorgen's syndrome, sarcoidosis, scleroderma, lupus, polymyositis, Reiter's syndrome, psoriasis, dermatitis, pelvic inflammatory disease, inflammatory breast disease, orbital inflammatory disease, immune deficiency disorders (e.g., HIV, common variable immunodeficiency, congenital X-linked infantile hypogammaglobulinemia, transient hypogammaglobulinemia, selective IgA deficiency, necrotizing enterocolitis, granulocyte transfusion associated syndromes, cytokine-induced toxicity, chronic mucocutaneous candidiasis, severe combined immunodeficiency), autoimmune disorders, and acute purulent meningitis or other central nervous system inflammatory disorders.

A "cellular proliferative disorder" includes those disorders that affect cell proliferation, activation, adhesion, growth, differentiation, or migration processes. As used herein, a "cellular proliferation, activation, adhesion, growth, differentiation, or migration process" is a process by which a cell increases in number, size, activation state, or content, by which a cell develops a specialized set of characteristics which differ from that of other cells, or by which a cell moves closer to or further from a particular location or stimulus. Disorders characterized by aberrantly regulated growth, activation, adhesion, differentiation, or migration. Such disorders include cancer, *e.g.*, carcinoma, sarcoma, lymphoma or leukemia, examples of which include, but are not limited to, breast, endometrial, ovarian, uterine, hepatic, gastrointestinal, prostate, colorectal, and lung cancer, melanoma, neurofibromatosis, adenomatous polyposis of the colon, Wilms' tumor, nephroblastoma, teratoma, rhabdomyosarcoma; tumor invasion, angiogenesis and metastasis; skeletal dysplasia; hematopoietic and/or myeloproliferative disorders.

Various aspects of the invention are described in further detail in the following subsections.

Modified Integrin I-domain Polypeptides and Anti-Integrin I-domain Antibodies

The methods of the invention include the use of isolated, modified integrin polypeptides, and biologically active portions thereof. As used herein, a modified integrin polypeptide includes a modified I-domain polypeptide and a modified I-like domain polypeptide. Modified integrin polypeptides of the invention include modified integrin I-domain and I-like domain polypeptides that are comprised within an integrin α or β subunit polypeptide, respectively; soluble modified integrin I-domain and I-like domain polypeptides; and modified integrin I-domain and I-like domain polypeptides that are operatively linked to a heterologous polypeptide, *e.g.*, fusion proteins.

The cDNAs for multiple human integrin α and β subunit polypeptides have been cloned and sequenced, and the polypeptide sequences have been determined (see, for example, GenBank Accession Numbers: NM_002203 (α2), AF112345 (α10), NM_012211 (α11), NM_005353 (αD), NM_002208 (αE), NM_000887 (αX), NM_000632 (αM), NM_002209 (αL), X68742 and P56199 (α1), NM_000211 (β2), NM_000212 (β3), NM_002214 (β8)). In particular, the polypeptide sequences encoding human αL and αM are set forth as SEQ ID NO:2 (GenBank Accession No. P20701) and SEQ ID NO:4 (GenBank Accession No. P11215), respectively. In addition, the sequences encoding integrin α and β subunit polypeptides from other species are available in the art. Furthermore, as described previously, three dimensional structure of the αM, αL, α1 and α2 I-domains has been solved (Lee, J-O, *et al.* (1995) *Structure* 3:1333-1340; Lee, J-O, *et al.* (199S) *Cell* 80:631-638; Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Emsley, J *et al.* (1997) *J Biol Chem* 272:28512-28517; Baldwin, ET *et al.* (1998) *Structure* 6:923-935; Kallen, J *et al.* (1999) *J Mol Biol* 292:1-9).

Isolated modified integrin polypeptides of the present invention preferably have an amino acid sequence that is sufficiently identical to the amino acid sequence of a native integrin polypeptide, yet which comprise at least one, and preferably two cysteine substitutions, such that a disulfide bond is formed that stabilizes the polypeptide in a desired conformation. As used herein, the term "sufficiently identical" refers to an amino acid (or nucleotide) sequence which contains a sufficient or minimum number of identical

or equivalent (*e.g.*, an amino acid residue that has a similar side chain) amino acid residues (or nucleotides) to a integrin amino acid (or nucleotide) sequence such that the polypeptide shares common structural domains or motifs, and/or a common functional activity with a native integrin polypeptide. For example, amino acid or nucleotide sequences which share at least 30%, 40%, or 50%, preferably 60%, more preferably 70%, 75%, 80%, 85% or 90%, 91%, 92%, 93%, 94%, 95% or greater identity and share a common functional activity (*e.g.*, an activity of a modified integrin I-domain or I-like domain as described herein) are defined herein as sufficiently identical. An integrin I-domain polypeptide may differ in amino acid sequence from the integrin polypeptides disclosed herein due to natural allelic variation or mutagenesis.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet

another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

As used herein, a "biologically active portion" of a modified integrin polypeptide (*e.g.*, a modified integrin I-domain polypeptide) includes a fragment of a modified integrin polypeptide which retains a modified integrin polypeptide activity. Typically, a biologically active portion of a modified integrin polypeptide comprises at least one domain or motif with at least one activity of the modified integrin polypeptide, *e.g.*, ligand binding. In a preferred embodiment, biologically active portions of a modified integrin polypeptide include modified integrin I-domain polypeptides. Biologically active portions of a modified integrin polypeptide may comprise amino acid sequences sufficiently identical to or derived from the amino acid sequence of a modified integrin polypeptide, which include less amino acids than the full length modified integrin polypeptide, and exhibit at least one activity of a modified integrin polypeptide. Biologically active portions of a modified integrin polypeptide, e.g., a modified I-domain or I-like domain, can be used as targets for developing agents which modulate a integrin polypeptide activity, *e.g.*, ligand binding, adhesion, *e.g.*, cell to cell adhesion or cell to extracellular matrix adhesion, and/or signaling activity. A biologically active portion of a modified integrin polypeptide comprises a polypeptide which can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a modified integrin polypeptide.

In a preferred embodiment, modified integrin polypeptides are produced by recombinant DNA techniques. For example, a modified integrin polypeptide can be isolated from a host cell transfected with a polynucleotide sequence encoding a modified integrin polypeptide (*e.g.*, a I-domain polypeptide or a soluble I-domain fusion protein) using an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a modified integrin polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" polypeptide or protein, or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the source, *e.g.*, the cellular source, from which the modified integrin I-domain polypeptide is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of modified integrin polypeptide in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of modified integrin polypeptide having less than about 30% (by dry weight) of non-modified integrin polypeptide (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-modified integrin polypeptide, still more preferably less than about 10% of non-modified integrin polypeptide, and most preferably less than about 5% non-modified integrin polypeptide. When the modified integrin polypeptide or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of modified integrin polypeptide in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of modified integrin polypeptide having less than about 30% (by dry weight) of chemical precursors or non-modified integrin polypeptide chemicals, more preferably less than about 20% chemical precursors or non-modified integrin polypeptide chemicals, still more preferably less than about 10% chemical precursors or non-modified integrin polypeptide chemicals, and most preferably less than about 5% chemical precursors or non-modified integrin polypeptide chemicals.

The methods of the invention may also use modified integrin polypeptides that are chimeric or fusion proteins. As used herein, a modified integrin "chimeric protein" or "fusion protein" comprises a modified integrin polypeptide operatively linked to a non-modified integrin polypeptide, *e.g.*, a heterologous polypeptide. In a preferred embodiment, a modified integrin fusion protein comprises at least an I-domain or an I-like domain. Within the fusion protein, the term "operatively linked" is intended to

indicate that the modified integrin polypeptide and the heterologous polypeptide sequences are fused in-frame to each other. The heterologous polypeptide can be fused to the N-terminus or C-terminus of the modified integrin polypeptide.

For example, in a preferred embodiment, the fusion protein is a modified integrin-I-domain fusion protein in which the Fc region, *e.g.*, the hinge, C1 and C2 sequences, of an immunoglobulin, (*e.g.*, human IgG1) is fused to the C-terminus of the modified integrin sequences. Integrin immunoglobulin chimeras can be constructed essentially as described in WO 91/08298. Such fusion proteins can facilitate the purification of recombinant modified integrin polypeptides. In another embodiment, the fusion protein is a modified integrin I-domain polypeptide fused to a heterologous transmembrane domain, such that the fusion protein is expressed on the cell surface.

The modified integrin polypeptides and fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. In an exemplary embodiment, a soluble modified integrin I-domain polypeptide stabilized in an open, ligand binding conformation, or fusion protein thereof may be used to modulate integrin activity (e.g., integrin binding to a cognate ligand) in a subject. In another embodiment, a soluble modified integrin I-domain polypeptide or fusion protein may be used to treat an inflammatory or immune system disorder, *e.g.*, an autoimmune disorder. In another embodiment, a soluble modified integrin polypeptide or fusion protein may be used to treat a cellular proliferative disease. Use of soluble modified integrin polypeptides and fusion proteins can also be used to affect the bioavailability of a integrin ligand, *e.g.*, ICAM.

Moreover, the modified integrin polypeptides and fusion proteins of the invention can be used as immunogens to produce anti-integrin antibodies in a subject, *e.g.*, anti-LFA-1 antibodies, and in screening assays to identify molecules which modulate integrin activity, and/or modulate the interaction of a integrin polypeptide with a integrin ligand or receptor.

Preferably, a modified integrin fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid

undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A modified integrin polypeptide-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the modified integrin polypeptide.

The methods of the present invention may also include the use of modified integrin polypeptides which function as either integrin agonists (mimetics) or as integrin antagonists. An agonist of an integrin polypeptide can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a integrin polypeptide. An antagonist of an integrin polypeptide can inhibit one or more of the activities of a native form of the integrin polypeptide by, for example, competitively modulating an integrin activity. Thus, specific biological effects can be elicited by treatment with a modified integrin polypeptide stabilized in a desired conformation.

An isolated, modified integrin polypeptide, *e.g.*, a modified LFA-1 polypeptide, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind to a specific conformation of an integrin, *e.g.*, an integrin I-domain, using standard techniques for polyclonal and monoclonal antibody preparation (see, generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Moreover, the ordinarily skilled artisan will appreciate that there are many variations of such methods which also would be useful. Preparation of anti-LFA-1 antibodies is described in, for example, United States Patent No. 5,622,700, the entire content of which is incorporated herein by this reference.

The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, *e.g.*, an integrin I-domain in an open or closed conformation, or a modified integrin I-

domain, such as an LFA-1 I-domain, *e.g.,* an open or closed LFA-1 I-domain or a modified integrin I-domain of LFA-1. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind a modified integrin polypeptide *e.g.,* a modified LFA-1 polypeptide, or a portion or fragment thereof. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of a modified integrin polypeptide, *e.g.,* a modified LFA-1 polypeptide, or a portion or fragment thereof. A monoclonal antibody composition thus typically displays a single binding affinity for a particular modified integrin polypeptide, or a portion or fragment thereof with which it immunoreacts.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-integrin antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with a modified integrin polypeptide, *e.g.*, a modified integrin I-domain stabilized in the open conformation, to thereby isolate immunoglobulin library members that bind to an conformation specific epitope on an integrin polypeptide, *e.g.,* an open conformation. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). With regard to screening for phage libraries with integrin I-domains locked in the high affinity conformation with a disulfide bond, note that it should be possible to elute specific phage by adding a reducing agent that breaks the disulfide and abolishes the high affinity conformation of the I-domain.

Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT International Publication No. WO 92/18619; Dower *et al.* PCT International Publication No. WO 91/17271; Winter *et al.* PCT International Publication WO 92/20791; Markland *et al.* PCT International Publication No. WO 92/15679; Breitling *et al.* PCT International Publication WO 93/01288; McCafferty *et al.* PCT International Publication No. WO 92/01047; Garrard *et al.* PCT International Publication No. WO 92/09690; Ladner *et al.* PCT International

Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Additionally, recombinant anti-integrin antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, can also be used in the methods of the present invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent No. 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In a preferred embodiment, an anti-integrin antibody of the invention binds selectively to an integrin I-domain in the open, high-affinity conformation, *e.g.*, at an epitope that is unique to an activated integrin (also referred to herein as an activation specific epitope). In a preferred embodiment, an anti-integrin antibody of the invention modulates (*e.g.*, inhibits) the binding interaction between an activated integrin and its cognate ligand. In another embodiment, an anti-integrin antibody inhibits leukocyte adhesion and/or aggregation. In another embodiment, an anti-integrin antibody of the invention binds selectively to an integrin I-domain in an open conformation, *e.g.*, an

LFA-1 I-domain in an open conformation, or a modified integrin I-domain, *e.g.*, a modified I-domain of an LFA-1 molecule.

An anti-integrin antibody (*e.g.*, a monoclonal antibody) can be used in the methods of the invention to modulate the expression and/or activity of an integrin or an integrin I-domain polypeptide. An anti-integrin antibody can also be used to isolate modified integrin or integrin I-domain polypeptides, *e.g.*, a modified LFA-1 polypeptide, or fusion proteins by standard techniques, such as affinity chromatography or immunoprecipitation. In another embodiment, an anti-integrin antibody can be used to remove and/or kill cells expressing activated integrin. Moreover, an anti-integrin antibody can be used to detect integrin polypeptides in a particular conformation (*e.g.*, an activated integrin), for example, for the localization of stimulated and/or activated leukocytes. Furthermore, an anti-integrin antibody, *e.g.*, an antibody which reacts with or binds an integrin I-domain in an open conformation or a modified integrin I-domain, can be used therapeutically as described herein. Accordingly anti-integrin antibodies can be used diagnostically to monitor protein levels in blood as part of a clinical testing procedure, *e.g.*, to, for example, detect inflammation. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Isolated Nucleic Acid Molecules

The invention includes the use of isolated nucleic acid molecules that encode integrin polypeptides (*e.g.*, a modified integrin I-domain polypeptide, *e.g.*, a modified integrin I-domain or I-like domain polypeptide) or biologically active portions thereof.

As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleotide sequences encoding the wild-type human αL and αM polypeptides are set forth as SEQ ID NO:1 (GenBank Accession No. NM_002209) and SEQ ID NO:3 (Genbank Accession No. J03925), respectively. The isolated nucleic acid molecules of the present invention include the nucleotide sequences of SEQ ID NO:1 and SEQ ID NO:3, which encode the modified amino acid sequences of the αL and αM mutants described herein, *e.g.,* identified below in Table 9. Table 9 illustrates the specific nucleotide residues which are altered to result in the modified αL and αM mutants as described herein. For example, the αL K287C/K294C mutant is a modified αL polypeptide, wherein there is a change in the amino acid sequence of αL (SEQ ID NO:2) such that amino acid residues 287 and 294 are substituted with cysteine residues. The corresponding wild-type nucleotide sequence, SEQ ID NO:1, is modified at nucleotide residues 1022-1024 and 1143-1145, respectively. Therefore, as shown in Table 9, for the αL K287C/K294C mutant at amino acid K287, the corresponding nucleotide residues in the wild-type αL nucleic acid sequence (SEQ ID NO:1), nucleotide residues 1022-1024, are modified from "aaa" to "tgt."

Table 9.

Mutants				Nucleotide sequence	
αM or αL	mutations	# Amino Acid	#Nucleotide	WT	mutant
αL	K287C/K294C	K287	1022-1024	aaa	tgt
		K294	1043-1045	aag	tgt
	E284C/E301C	E284	1013-1015	gag	tgt
		E301	1064-1066	gag	tgt
	L161C/F299C	L161	644-646	ctc	tgt
		F299	1058-1060	ttc	tgt
	K160C/F299C	K160	641-643	aaa	tgt
		F299	1058-1060	ttc	tgt
	L161C/T300C	L161	644-646	ctc	tgt
		T300	1061-1063	act	tgt
	L289C/K294C	L289	1028-1030	ctg	tgt
		K294	1043-1045	aag	tgt
αM	Q163C/Q309C	Q163	607-609	caa	tgt
		Q309	1045-1047	cag	tgt
	D294C/Q311C	D294	1000-1002	gat	tgt
		Q311	1051-1053	cag	tgt
	Q163C/R313C	Q163	607-609	caa	tgt
		R313	1057-1059	cgg	tgt

αL; GenBank NM_002209
αM; GeneBank J03925

The term "isolated nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, an isolated nucleic acid molecule encoding a modified integrin I-domain polypeptide can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

The skilled artisan will further appreciate that further changes can be introduced by mutation into the nucleotide sequence encoding a modified integrin polypeptide, thereby leading to changes in the amino acid sequence of the encoded modified integrin polypeptide, without further altering the structural characteristics or functional ability of the modified integrin polypeptide. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence encoding a modified integrin polypeptide. A "non-essential" amino acid residue is a residue that can be altered from the sequence of a modified integrin polypeptide without further altering the structure and/or biological activity. In accordance with the methods of the invention, computational design and modeling are used to determine which amino acid residues are amenable to alteration in order to achieve the desired protein conformation.

Accordingly, the methods of the invention may include the use of nucleic acid molecules encoding modified integrin polypeptides that contain changes in amino acid residues that are not essential for activity.

Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a modified integrin polypeptide is preferably replaced with another amino acid residue from the same side chain family.

Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, for example, recombinant expression vectors, containing a nucleic acid encoding a modified integrin polypeptide (or a portion thereof), *e.g.*, an integrin I-domain or I-like domain polypeptide or fusion protein. As used herein, the term "vector" refers to a nucleic acid molecule capable of

transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the methods of the invention may include other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art

that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, modified integrin I-domain polypeptides, fusion proteins, and the like).

Accordingly, the invention provides a method for producing a modified integrin polypeptide, *e.g.*, a modified integrin I-domain polypeptide, by culturing in a suitable medium, a host cell of the invention (e.g., a prokaryotic or eukaryotic host cell) containing a recombinant expression vector such that the protein is produced.

The recombinant expression vectors of the invention can be designed for expression of modified integrin polypeptides or fusion proteins in prokaryotic or eukaryotic cells, *e.g.*, for use in the methods of the invention. For example, modified integrin I-domain polypeptides or fusion proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility and/or stability of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which

fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Purified modified integrin I-domain fusion proteins (*e.g.*, soluble I-domain-Ig) can be utilized to modulate integrin activity, as described herein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

Alternatively, modified integrin polypeptides can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), endothelial cell-specific promoters (*e.g.*, KDR/flk promoter; U.S. Patent No. 5,888,765), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Another aspect of the invention pertains to host cells into which a nucleic acid molecule encoding a modified integrin polypeptide of the invention is introduced, *e.g.*, a modified integrin I-domain nucleic acid molecule within a recombinant expression vector or a modified integrin I-domain nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the host cell's genome.

The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a modified integrin polypeptide or fusion protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as hematopoietic cells, leukocytes, K562 cells, 293T cells, human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells (HMVEC), Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a modified integrin polypeptide or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a modified integrin polypeptide, e.g., a modified integrin I-domain polypeptide or fusion protein, for use in the methods of the invention. In one embodiment, a host cell (into which a recombinant expression vector encoding a modified integrin I-domain polypeptide or fusion protein has been introduced) is cultured in a suitable medium such that a modified integrin I-domain polypeptide or fusion protein is produced. In another embodiment, a modified integrin I-domain polypeptide or fusion protein is isolated from the medium or the host cell. A recombinant cell expressing a modified integrin polypeptide or fusion protein can also be administered to a subject to modulate integrin activity.

The host cells of the invention can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which a modified integrin I-domain polypeptide-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous modified integrin I-domain sequences have been introduced into their genome or homologous recombinant animals in which endogenous integrin I-domain sequences have been altered. Such animals are useful for studying the function and/or activity of a modified integrin I-domain molecule and for identifying and/or evaluating modulators of modified integrin I-domain polypeptide activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, and the like. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous integrin I-domain gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing a modified integrin I-domain-encoding nucleic acid into the male pronuclei of a fertilized oocyte,

e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a modified integrin I-domain transgene to direct expression of a modified integrin I-domain protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a modified integrin I-domain gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the modified integrin I-domain gene. The modified integrin I-domain gene can be a human gene, but more preferably, is a non-human homologue of a human modified integrin I-domain gene. For example, a mouse modified integrin I-domain gene can be used to construct a homologous recombination nucleic acid molecule, *e.g.*, a vector, suitable for altering an endogenous modified integrin I-domain gene in the mouse genome. In a preferred embodiment, the homologous recombination nucleic acid molecule is designed such that, upon homologous recombination, the endogenous modified integrin I-domain gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the homologous recombination nucleic acid molecule can be designed such that, upon homologous recombination, the endogenous modified integrin I-domain gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous modified integrin I-domain protein). In the homologous recombination nucleic acid molecule, the altered portion of the modified integrin I-domain gene is flanked at its 5' and 3' ends by additional nucleic acid sequence of the modified integrin I-domain gene to allow for homologous recombination to occur between the exogenous modified integrin I-domain gene carried by the homologous recombination nucleic acid molecule and an endogenous modified integrin I-domain gene in a cell, *e.g.*, an embryonic stem cell. The additional flanking modified integrin I-

domain nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the homologous recombination nucleic acid molecule (see, *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The homologous recombination nucleic acid molecule is introduced into a cell, *e.g.*, an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced modified integrin I-domain gene has homologously recombined with the endogenous modified integrin I-domain gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells can then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells:A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination nucleic acid molecules, *e.g.*, vectors, or homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In another embodiment, transgenic non-human animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, antibodies, peptidomimetics, small molecules (organic or inorganic) or other drugs) which modulate integrin activity. These assays are designed to identify compounds, for example, that bind to an integrin I-domain polypeptide, *e.g.*, an integrin I-domain polypeptide in an active conformation, bind to other proteins that interact with an integrin I-domain polypeptide, induce binding, and modulate the interaction of an integrin I-domain polypeptide with other proteins, *e.g.*, an integrin ligand, *e.g.*, ICAM, and thus modulate integrin activity.

As used herein, the term "modulator of integrin activity" includes a compound or agent that is capable of modulating or regulating at least one integrin activity, as described herein. Modulators of integrin activity may include, but are not limited to, small organic or inorganic molecules, nucleic acid molecules, peptides, antibodies, and the like. A modulator of integrin activity can be an inducer or inhibitor of integrin activity, *e.g.*, cell adhesion or ligand binding. As used herein, an "inducer of integrin activity" stimulates, enhances, and/or mimics an integrin activity. As used herein, an "inhibitor of integrin activity" reduces, blocks or antagonizes an integrin activity.

As used interchangeably herein, an "integrin activity", or an "integrin-mediated activity" refers to an activity exerted by an integrin polypeptide or nucleic acid molecule on an integrin responsive cell, or on integrin ligand or receptor, as determined *in vitro* and *in vivo*, according to standard techniques. In one embodiment, an integrin activity is the ability to mediate cell adhesion events, *e.g*, cell to cell or cell to extracellular matrix adhesion. In another embodiment, an integrin activity is the ability to transduce cellular signaling events. In yet another embodiment, an integrin activity is the ability to bind a ligand, *e.g.*, ICAM.

In a preferred embodiment, a soluble, recombinant high affinity integrin I-domain can be used to screen for small molecule antagonists that interfere with integrin ligand binding. Furthermore, antagonists, *e.g.*, antibodies, with direct/competitive and indirect/noncompetitive modes of inhibition can be discriminated, based on comparison with effects on wild-type integrin I-domains which show minimal ligand binding activity. For example, an indirect inhibitor should inhibit ligand binding by an

activated, wild-type integrin I-domain, but not by a disulfide-locked high affinity I-domain.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a modified integrin polypeptide on the cell surface with a test compound and determining the ability of the test compound to modulate (*e.g.*, induce or inhibit) an integrin activity. For example, a cell expressing a modified integrin I-domain polypeptide stabilized in an open conformation on the cell surface is contacted with a test compound, and the ability of the test compound to modulate adhesion to an integrin ligand is determined, as described and exemplified herein.

In yet another embodiment, the ability of a test compound to modulate integrin ligand binding can also be determined, for example, by coupling a modified integrin I-domain polypeptide that is stabilized in an open conformation with a detectable label such that the binding of the modified integrin polypeptide can be determined by detecting the amount of labeled integrin I-domain binding to an immobilized integrin ligand.

Animal-based model systems, such as an animal model of inflammation, may be used, for example, as part of screening strategies designed to identify compounds which are modulators of integrin activity. Thus, the animal-based models may be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in modulating inflammation and treating integrin-mediated disorders. For example, animal models may be exposed to a compound, suspected of exhibiting an ability to modulate integrin activity, and the response of the animals to the exposure may be monitored by assessing inflammatory activity before and after treatment. Transgenic animals, *e.g.*, transgenic mice, which express modified integrin I-domain polypeptides as described herein can also be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in modulating inflammation and treating integrin-mediated disorders

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulator of integrin activity can be identified using a cell-based assay, and the ability of the agent to modulate integrin activity can be confirmed *in vivo*, *e.g.*, in an animal such as an animal model for inflammation.

Moreover, screening assays can be used to identify inducers of integrin activity, for example, that mimic the activity of a integrin polypeptide, *e.g.,* the binding of an integrin to a ligand or receptor, or the activity of an integrin towards an integrin responsive cell. Such compounds may include, but are not limited to, peptides, antibodies, or small organic or inorganic compounds. In one embodiment, an anti-integrin antibody, *e.g.,* an anti-LFA-1 antibody of the invention which selectively binds to an open, activated conformer can be used to assess the ability of a test compound to activate integrin.

The test compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.,* Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

This invention further pertains to novel agents identified by the above-described screening assays. With regard to intervention, any treatments which modulate integrin activity and/or inflammatory activity should be considered as candidates for human therapeutic intervention.

Pharmaceutical Compositions

The nucleic acid molecules encoding modified integrin polypeptides, modified integrin polypeptides (e.g., modified I-domain polypeptides and fusion proteins), and active fragments thereof, anti-integrin I-domain antibodies, and integrin modulators (also referred to herein as "active compounds") DNA vaccines, or DNA vectors of the invention can be incorporated into pharmaceutical compositions suitable for administration. As used herein, a "modulator" of integrin activity, *e.g.*, inhibitors and inducers, includes a compound that modulates an integrin activity, *e.g.*, an integrin-mediated signaling event, an integrin-mediated adhesion event, or integrin binding to a cognate ligand. Integrin modulators include modified integrin I-domain or I-like domain polypeptides of the invention, anti-integrin I-domain polypeptides, as well as compounds identified in a screening assay described herein. Such compositions typically comprise the compound, nucleic acid molecule, vector, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, ophthalmic, and rectal administration, including direct installation into a disease site. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium

chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a soluble modified integrin I-domain fusion protein) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The administration of the active compounds of the invention may be for either a prophylactic or therapeutic purpose. Accordingly, in one embodiment, a "therapeutically effective dose" refers to that amount of an active compound sufficient to result in a detectable change in the physiology of a recipient patient. In one embodiment, a therapeutically effective dose refers to an amount of an active compound sufficient to result in modulation of an inflammatory and/or immune response. In another embodiment, a therapeutically effective dose refers to an amount of an active compound sufficient to result in the amelioration of symptoms of an inflammatory and/or immune system disorder. In another embodiment, a therapeutically effective dose refers to an amount of an active compound sufficient to prevent an inflammatory and/or immune system response. In yet another embodiment, a therapeutically effective dose refers to that amount of an active compound sufficient to modulate an integrin activity (*e.g.*, a signaling activity, an adhesion activity or a ligand binding actvity) as described herein.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and

therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of antibody, protein or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. Ranges intermediate to the above recited values, also are intended to be part of this invention. For example, ranges of span values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

In a preferred example, a subject is treated with antibody, protein, or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

In another preferred example, a subject is treated with an initial dosing of a therapeutically effective amount of an anti-integrin antibody, *e.g.*, an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody, which reacts with or binds to an I-domain of an integrin in the open or active conformation, or an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody, which reacts with or binds to a modified LFA-1 I-domain, followed by a subsequent intermittent dosing of a therapeutically effective amount of the antibody that is less than 100%, calculated on a daily basis, of the initial dosing of the antibody wherein the antibody is administered not more than once per week during the subsequent dosing. In another embodiment, the subsequence dosing is two or more times per week. In another embodiment, the subsequence dosing is one or more time every two weeks. In still another embodiment, the subsequence dosing is one or more times every three weeks. In yet another embodiment, the subsequence dosing is one or more times every four weeks. In one embodiment, the subsequent dosing is less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%, calculated on a daily basis, of the initial dosing of the antibody. In one embodiment, the initial dosage is between 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. In a preferred embodiment, the initial dosage is less than 0.3 mg/kg body weight, *e.g.*, between 0.001 to 0.30, *e.g.*, 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, and 0.275. Ranges intermediate to the above recited values, also are intended to be part of this invention.

In yet another example, a subject is treated with an initial dosing of a therapeutically effective amount of an anti-integrin antibody, *e.g.*, an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody, which reacts with or binds to an I-domain of an

integrin in the open or active conformation, or an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody, which reacts with or binds to a modified LFA-1 I-domain, followed by a subsequent intermittent dosing of a therapeutically effective amount of the antibody that is greater than 100%, calculated on a daily basis, of the initial dosing of the antibody wherein the antibody is administered to the mammal not more than once per week during the subsequent dosing. In another embodiment, the subsequence dosing is two or more times per week. In another embodiment, the subsequence dosing is one or more time every two weeks. In still another embodiment, the subsequence dosing is one or more times every three weeks. In yet another embodiment, the subsequence dosing is one or more times every four weeks. In one embodiment, the initial dosage is between 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. In a preferred embodiment, the initial dosage is less than 0.3 mg/kg body weight, *e.g.*, between 0.001 to 0.3, *e.g.*, 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, and 0.275. Ranges intermediate to the above recited values, also are intended to be part of this invention. Dosages for anti-integrin antibodies, *e.g.*, anti-LFA-1 are described in, for example, U.S. Patent No. 5,622,700.

In still another example, an initial dosage is followed by the same dosage, for example, not more than once per week during the subsequent dosing. In another embodiment, the subsequence dosing is two or more times per week. In another embodiment, the subsequence dosing is one or more time every two weeks. In still another embodiment, the subsequence dosing is one or more times every three weeks. In yet another embodiment, the subsequence dosing is one or more times every four weeks.

Dosages for anti-integrin antibodies, *e.g.*, anti-LFA-1 are described in, for example, U.S. Patent No. 5,622,700.

In another embodiment, the an effective amount of an anti-inflammatory or immunosuppressive agent to the mammal in combination with the antibody, either at the same time, or at different time points.

The present invention encompasses active agents which modulate an integrin activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino

acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention.

Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

In certain embodiments of the invention, a modulator of integrin activity is administered in combination with other agents (*e.g.*, a small molecule), or in conjunction with another, complementary treatment regime. For example, in one embodiment, an inhibitor of integrin activity is used to treat an inflammatory or immune system disorder. Accordingly, the subject may be treated with an inhibitor of integrin activity, and further treated with an anti-inflammatory or immunosuppressive agent.

Further, an antibody, *e.g.,* an anti-LFA-1 antibody, (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. The conjugates of the invention can be used for modifying a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a coagulation factor such as tissue factor; a protein such as vascular endothelial growth factor ("VEGF"), platelet derived growth factor, and tissue plasminogen activator; biological response modifiers such as, for example, lymphokines, cytokines and growth factors; or a toxin.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982). Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

The nucleic acid molecules of the invention, *e.g.,* a nucleic acid molecule encoding, for example, a high-affinity modified integrin I-domain polypeptide, or active fragment thereof, can be used as a gene-based therapy alone, or, can be inserted into

vectors and used as gene therapy vectors. Gene therapy is the insertion of a functioning gene into the cells of a patient (i) to correct an inborn error of metabolism, or (ii) to provide a new function in a cell (Kulver, K. W., "Gene Therapy", 1994, p. xii, Mary Ann Liebert, Inc., Publishers, New York, N.Y.). Vectors, *e.g.,* viral vectors, may be used to introduce and stably express a gene normally expressed in mammals, for example, in a location in the body where that gene is not naturally present. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al*. (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The gene therapy vector can include, for example, DNA encoding an antigen of interest to induce an immune response in the subject *in vivo*. Therefore, the modified integrin I-domain polypeptide, *e.g.,* a high-affinity modified integrin I-domain polypeptide, or active fragment thereof, acts as an adjuvant to produce an increased antibody reaction to the antigen. The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The nucleic acid molecules of the invention can also be used in DNA vaccine formulations for therapeutic or prophylactic treatment of integrin-mediated disorders, *e.g.,* inflammatory disorders. In one embodiment, the DNA vaccine formulation comprises a nucleic acid molecule encoding a modified integrin polypeptide, *e.g.,* a modified integrin I-domain polypeptide, or fragment thereof, coupled with an antigenic component, *e.g.,* DNA encoding an antigenic component. As used herein, an antigenic component is a moiety that is capable of binding to a specific antibody with sufficiently high affinity to form a detectable antigen-antibody complex. In another embodiment, the DNA vaccine further comprises a pharmaceutically acceptable carrier.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of an integrin-mediated disorder or having an integrin-mediated disorder such as an inflammatory or immune disorder, and/or a cellular proliferative disorder. "Treatment", as used herein, is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease or disorder, a symptom of disease or disorder or a predisposition toward a disease or disorder, with the purpose of curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving or affecting the disease or disorder, the symptoms of disease or disorder or the predisposition toward a disease or disorder. A therapeutic agent includes, but is not limited to, nucleic acid molecules, DNA vaccines, gene-based therapies, small molecules, peptides, antibodies, *e.g.,* anti-LFA-1 antibodies, which react with or bind to modified I-domain polypeptides, ribozymes and antisense oligonucleotides.

With regard to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers the study of how a patient's genes determine his or her response to a drug (e.g., a patient's "drug response phenotype", or "drug response genotype"). Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the integrin I-domain polypeptides of the present invention or modulators thereof according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject a disease or condition associated with a integrin-mediated disorder by administering to the subject one or more integrin I-domain polypeptides of the present invention or

modulators thereof. Subjects at risk for an integrin-mediated disorder can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the integrin-mediated disorders, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of integrin-mediated disorder, for example, appropriate integrin I-domain polypeptides of the present invention, or modulators thereof, can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating expression of integrin I-domain polypeptides or their activity for therapeutic purposes (e.g., treating a subject at risk of an integrin-mediated disorder or having an integrin-mediated disorder such as an inflammatory or immune disorder, and/or a cellular proliferative disorder). Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell with one or more integrin I-domain polypeptides of the present invention, or one or more modulators thereof, *e.g.*, an antibody which reacts of binds to an integrin I-domain in an open conformation or a modified integrin I-domain polypeptide, *e.g.*, an anti-LFA-1 antibody specific for an LFA-1 I-domain in an open conformation or a modified LFA-1 I-domain polypeptide. An agent that modulates integrin I-domain polypeptide activity can be an agent as described herein, such as a nucleic acid or a protein, a target molecule of an integrin I-domain polypeptide (e.g., a substrate), an antibody which reacts or binds to a modified integrin I-domain polypeptide, an integrin I-domain polypeptide agonist or antagonist, a peptidomimetic of an integrin I-domain polypeptide agonist or antagonist, or other small molecule. In one embodiment, the agent stimulates one or more integrin I-domain polypeptide activities. Examples of such stimulatory agents include active integrin I-domain polypeptide protein and a nucleic acid molecule encoding integrin I-domain polypeptide that has been introduced into the cell. In another embodiment, the agent inhibits one or more integrin I-domain polypeptide activities. Examples of such inhibitory agents include antisense integrin I-domain polypeptide nucleic acid molecules, gene therapy vectors, DNA vaccines, anti-integrin I-domain polypeptide antibodies, and integrin I-domain polypeptide inhibitors. These modulatory methods can be performed in vitro (e.g., by

culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized associated with an integrin-mediated disorder. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates or downregulates) integrin I-domain polypeptide expression or activity.

3. Pharmacogenomics

The integrin I-domain polypeptide molecules of the present invention, as well as agents, or modulators which have a stimulatory or inhibitory effect on integrin I-domain polypeptide activity (e.g., integrin I-domain polypeptide gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) an integrin-mediated disorder such as an inflammatory or immune disorder, and/or a cellular proliferative disorder. In conjunction with such treatment, pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer an integrin I-domain polypeptide molecule (and/or a modulator thereof) as well as tailoring the dosage and/or therapeutic regimen of treatment with such molecule and/or modulator.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. et al. (1996) Clin. Exp.Pharmacol. Physiol. 23(10-11): 983-985 and Linder, M.W. et al. (1997) Clin. Chem. 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example,

glucose-6-phosphate aminopeptidase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association", relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (e.g., a "bi-allelic" gene marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants). Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and the cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite

frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Alternatively, a method termed the "gene expression profiling" can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (e.g., an integrin I-domain polypeptide molecule or integrin I-domain polypeptide modulator) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with an integrin I-domain polypeptide molecule or modulator thereof, such as a modulator identified by one of the exemplary screening assays described herein.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the figures and sequence listing are incorporated herein by reference.

EXAMPLES

EXAMPLE 1 DESIGN OF LFA-1 AND Mac-1 MUTANTS THAT ARE LOCKED IN OPEN OR CLOSED CONFORMATION

Current crystal and NMR structures of the LFA-1 I domain (Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Kallen, J et al. (1999) J Mol Biol 292:1-9) have a conformation that is similar to the low affinity, closed conformer of the Mac-1 I domain (1jlm) (Lee,

J-O *et al.* (1995) *Cell* 80:631-638). Therefore, the high affinity, open conformer of the Mac-1 I domain (1ido) (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340) was used to model a high affinity, open LFA-1 I domain. The template for this model consisted of segments of the 1ido structure in regions where the Cα backbone differed significantly from the 1jlm structure, and segments of the 1lfa structure in regions where 1ido and 1jlm were similar.

Briefly, I domains with the following protein data bank (PDB) identifiers were structurally superimposed using Cα carbons, the CD MALIGN algorithm of MODELLER 4 (Sali, A and Blundell, TL (1993) *J Mol Biol* 234:779-815), and a gap extension penalty of 1 Å: Mac-1, 1ido and 1jlm (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340; Lee, J-O *et al.* (1995) *Cell* 80:631-638); LFA-1, 1lfa molecules A and B (Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281), 1zon and 1zop (Qu, A and Leahy, DJ (1996) *Structure* 4:931-942); and VLA-2, 1aox (Emsley, J *et al.* (1997) *J Biol Chem* 272:28512-28517). The algorithm found 121 framework residues that were utilized for superposition. A sequence alignment was then done. The 1ido and 1jlm structures were aligned by their sequence, and 1lfa molecule A and 1zon were aligned by structural similarity to 1jlm. Using the structural superposition, and the sequence alignment, the distances between all Cα carbons at equivalent sequence positions were calculated using a Microsoft Excel spreadsheet. This was analogous to the comparison between 1jlm and 1ido (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340), except that LFA-1 I domain structures were included. For use as templates for the high affinity, open LFA-1 I domain model, segments from 1lfa molecule A were chosen where differences between all four I domains were small, or differences between 1lfa and 1jlm (low affinity, closed LFA-1 and Mac-1 I domains) were greater than between 1ido and 1jlm (open and closed Mac-1 I domains). Segments from 1ido were chosen when differences between 1ido and 1jlm were greater than between 1lfa and 1jlm. These segments were spliced together in regions where the backbones were as similar as possible. Thus, the template utilized segments G128 to F136, M154 to L203, F209 to L234, T243 to I255, and E272 to A282 of 1lfa; and segments D140 to F156, G207 to T211, V238 to K245, R266 to R281, and R293 to K315 of 1ido. No chain breaks were detected by LOOK™ (Molecular Application Group, Palo Alto, CA) in the spliced template, dubbed 1fa-mac. Models of a high affinity open form of LFA-1 were made with MODELLER 4™ using this template, the Mg^{2+} and water molecules 403 and 404 of 1ido, with heteroatom,

water, and hydrogen input turned on, and dynamic Coloumb turned on. The resulting model (1fa_hi.063) followed the template Cα coordinates closely (RMS = 0.12Å). The QUACHK score (Vriend, G (1990) *J Mol Graph* 8:52-56) is excellent (-0.135 compared to –0.215 for the 1fa-mac template, -0.08 for 1ido, and 0.0 for 1lfa).

The SSBOND program (Hazes, B and Dijkstra, BW (1988) *Protein Engineering* 2:119-125) was used to identify positions where disulfide bonds could be introduced by mutating two appropriately positioned pairs of residues to cysteine. It was hypothesized that it might be possible to use disulfide bonds to trap the LFA-1 I domain in either the open or closed conformations.

The high affinity open LFA-1 I domain model (the 1fa_hi.063 model) was examined and two low affinity closed LFA-1 I domain structures, 1lfa and 1zon, with SSBOND and found 14 to 19 pairs of such residues in each structure. Out of these, one pair of residues in the high affinity open model, and one pair of residues in the low affinity closed structures, underwent large movements between the two conformers, such that disulfide bond formation could only occur in one conformer (Figure 1). These disulfides bridge β-strand 6 to the C-terminal α-helix, α6. The numbering of β-strands and α-helices differs among I domains; we use a uniform nomenclature (Huang, C *et al.* (2000) *J Biol Chem*, 275:21514-24). Helix α6 moves 10 Å along its axis down the body of the I domain in the high affinity open structure, and this movement is accompanied by a complete remodeling and downward shift of the loop between β6 and α6. Cysteines introduced in place of K287 and K294 were predicted to form a disulfide only in the high affinity open conformer, and thus lock the I domain in the high affinity open state (Figure 2). The Cβ carbons of K287 and K294 are predicted to be 3.8 Å apart in the high affinity open model (1fa_hi.063), within the range of 3.41 to 4.25Å that is ideal for disulfide formation, and after checking for Cβ-Sγ and Sγ-Sγ distances, were found to have four favorable sidechain-disulfide conformations. By contrast, in the low affinity closed conformers 1lfa and 1zon, the Cβ atoms of these residues are 8.9 to 9.2 Å apart (Figure 2).

Cysteines introduced in place of L289 and K294 were predicted to form a disulfide only in the low affinity closed conformer (Figure 2), and thus lock the I domain in the low affinity closed state. The Cβ carbons of L289 and K294 are 3.9 to 4.0 Å apart in the low affinity closed 1lfa and 1zon conformers, within the favorable range, although favorable cysteine sidechain conformations were not found. Nonetheless, the

α-helix in which residue 294 is present shows small displacements between 1lfa, 1zon, and the recent NMR structure (Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Kallen, J et al. (1999) J Mol Biol 292:1-9), and it was expected that a disulfide could form with minor adjustment of the α-helix. By contrast, in the high affinity open model, the Cβ atoms of these residues are predicted to be 8.0 Å apart (Figure 2).

Models were also built in which the predicted cysteines were present and disulfide bonds were formed if appropriate using the PATCH DISULFIDE routine of MODELLER 4 (Figure 2); however, it should be noted that all Cβ atom distances reported here are based on models or structures without introduced disulfides.

In addition to the computational search for pairs of cysteine substitutions to form conformation-specific disulfide bridge, the structure-oriented manual approach (or visual inspection) was also used. Regions of I domains that differ in conformation between the open and closed conformations were inspected for positions in which pairs of cysteines could be introduced that would form disulfides that would favor one conformation over the other. Thus, the region of the conformationally mobile C-terminal α-helix and the preceding loop were examined for positions in which one cysteine could be introduced, and structurally adjacent regions were searched for positions where a second cysteine could be introduced that would form a disulfide bond. Pairs of residues whose side-chains face towards one another were chosen. The distance between the Cα and Cβ atoms of each of these pairs was measured by software Look™ both in the open and closed conformation. The ideal separation for cysteine Cβ carbons for formation of a disulfide bond is reported to be 3.41 to 4.25 A. However, the crystal structures or models from which these were measured represent average positions of snapshots, whereas proteins are dynamic and exhibit atomic mobility. Furthermore, structural adjustments are possible to accommodate disulfide bonds. Much more adjustment is expected to be possible in loops and a-helices than in β-sheets. Therefore greater distances were predicted to be allowable for disulfide formation when one of the residues was in a loop or helix.

For αL, 4 pairs of cysteine substitutions were found where the Cα-Cα and Cβ-Cβ distances were more favorable for disulfide formation in the open conformation than

in the closed conformation; E284C/E301C, L161C/F299C, K160C/F299C, and L161C/T300C (Table 1).

For αM, 4 pairs of cysteine substitutions were found where the Cα-Cα and Cβ-Cβ distances were more favorable for disulfide formation in the open conformation than in the closed conformation: Q163C/Q309C, Q298C/N301C, D294C/T307C, and D294C/Q311c (Table 7), and one pair of cysteine substitutions where the Cα-Cα and Cβ-Cβ distances were more favorable for disulfide formation in the closed conformation than in the open conformation: Q163C/R313C. Additionally, F297C/A304C, which is an analogous mutation to K287C/K294C in αL, was included.

EXAMPLE 2 CONSTRUCTION AND EXPRESSION OF LFA-1 CYSTEINE SUBSTITUTION MUTANTS

Five open αL I-domain mutants were generated. To generate the high affinity open mutant K287C/K294C, the K287 and K294 in the I-domain of the αL subunit were replaced by cysteines. To generate the high affinity open mutant E284C/E301C, the E284 and E301 in the I-domain of the αL subunit were replaced by cysteines. In addition, three intermediate-affinity open αL I-domain mutants were made, and are identified herein as follows: L161C/F299C, K160C/F299C, and L161C/T300C. L161C/F299C was made by substituting cysteines for the L161 and F299. K160C/F299C was made by substituting cysteines for the K160 and F299. L161C/T300C was made by substituting cysteines for the L161 and T300. The low affinity closed mutant L289C/K294C was made by substituting cysteines for the L289 and K294. The distance between mutated residues for these six mutant is shown in Table 1, below. Also, single cysteine substitution mutants K287C, L289C and K294C were generated.

Table 1. Cα and Cβ between mutated residues in either open or closed confirmation

αL I-domain	open conformation Cα (A)	open conformation Cβ (A)	closed conformation Cα (A)	closed conformation Cβ (A)
Locked open				
K287C/K294C	6.32	3.75	10.72	9.08
E284C/E301C	9.12	6.96	12.88	12.52
L161C/F299C	9.16	8.09	11.87	11.38
K160C/F299C	9.97	7.75	9.83	7.96
L161C/T300C	12.30	13.00	13.50	14.87
Locked closed				
L289C/K294C	7.90	7.96	6.19	3.86

The distance between wild-type residues was measured by Look™ software in open conformation (lfa_hi.063) or closed conformation (1lfaA).

The human αL cDNA was contained in vector AprM8, a derivative of CDM8 (Seed, B and Aruffo, A (1987) *Proc Natl Acad Sci USA* 84:3365-3369). Overlap extension PCR was used to generate cysteine substitution mutations in the αL I-domain (Ho, SN *et al.* (1989) *Gene* 77:51-59; Horton, RM *et al.* (1990) *BioTechniques* 8:528). The outer left primer for PCR extension was complementary to the vector sequence at 5' to the EcoRI site at position 1826, and the outer right primer was 3' to the EcoRI site in the αL cDNA. The inner primers were designed for each individual mutation and contained overlapping sequences. Wild-type αL CDNA in AprM8 was used as template for the first PCR reaction. The second PCR product was digested with EcoRI and ligated into the same site in the wild-type αL cDNA in AprM8. The correct orientation of the insert was confirmed by restriction enzyme digestion. All mutations were confirmed by DNA sequencing.

For stable expression, the XbaI fragment of αL wild-type and mutant cDNA was subcloned into the same site of the stable expression vector pEFpuro (Lu, C and Springer, TA. (1997) *J Immunol* 159:268-278).

The mutated αL subunit was transiently coexpressed with the β2 subunit in 293T cells, and cell surface expression of the αL/β2 complex was determined by flow cytometry with monoclonal antibody TS2/4 to the αL subunit in the αL/β2 complex.

Briefly, human embryonic kidney 293T cells (SV40 transformed) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamycin. 293T cells were transiently transfected using the calcium phosphate method (DuBridge, RB *et al.* (1987) *Mol Cell Biol* 7:379-387; Heinzel, SS *et al.* (1988) *J Virol* 62:3738-3746). Briefly, 7.5 μg of wild-type or mutant αL cDNA in plasmid AprM8 and 7.5 μg of β2 cDNA in AprM8 were used to co-transfect one 6-cm plate of 70-80% confluent cells. Two days after transfection, cells were detached from the plate with Hanks' balanced salt solution (HBSS) containing 5 mM EDTA for LFA-1 expression and functional analyses.

Flow cytometric analysis was performed as previously described (Lu, C and Springer, TA (1997) *J Immunol* 159:268-278). Briefly, cells were washed and resuspended in L15 medium (Sigma) supplemented with 2.5% FBS (L15/FBS). 1x10 x 10^5 cells were incubated with primary antibodies in 100 μl L15/FBS on ice for 30 min. Monoclonal antibodies were used at final concentration of 1:20 hybridoma supernatant, 1:200 ascites, or 10 μg/ml purified IgG. Cells were then washed twice with L15/FBS, and incubated with FITC-conjugated goat anti-mouse IgG (heavy and light chain, Zymed Laboratories, San Francisco, CA) for 30 min on ice. After washing, cells were resuspended in cold PBS and analyzed on a FACScan (Becton Dickinson, San Jose, CA).

As shown in Figure 3A, the predicted high and low affinity mutants, and the single cysteine substitution mutants expressed similar levels of cell surface αL/β2 complex.

To test whether introducing the cysteines affected the overall conformation of the I-domain, a panel of monoclonal antibodies to different regions in the I-domain were tested for their reactivity with the I-domain mutants. The monoclonal antibodies used in these studies are as follows:

The mouse anti-human αL (CD11a) monoclonal antibodies TS1/11, TS1/12, TS1/22, TS2/4, TS2/6 and TS2/14; anti-β2 (CD18) monoclonal antibodies TS1/18, CBRLFA-1/2, and CBRLFA-1/7; mAb YFC51; and the nonbinding mAb X63 have been described previously (Sanchez-Madrid, F *et al.* (1982) *Proc Natl Acad Sci USA*

79:7489-7493; Hale, LP *et al.* (1989) *Arthritis Rheum* 32:22-30; Petruzzelli, *L et al.* (1995) *J Immunol* 155:854-866). Monoclonal antibodies BL5, F8.8, 25-3-1, May.035, CBRLFA-1/9, CBRLFA-1/1, S6F, and May.017 were described in *Leukocyte Type V* and were obtained from the Fifth International Leukocyte Workshops.

Monoclonal antibodies X63 and TS1/11 were used as hybridoma supernatants at a 1:20 dilution; monoclonal antibodies TS1/12, DBRLFA-1/2, CBRLFA-1/7 and YFC51 were used as purified IgG at 10μg/ml; monoclonal antibodies TS1/2, TS2/14, TS1/18 and TS2/4 used as ascites at a 1:200 dilution; and all monoclonal antibodies from the Fifth International Leukocyte Workshops were used at a 1:200 dilution. All of the antibodies, except for CBRLFA-1/1, bound to the mutants K287C/K294C and L289C/K294C and wild-type LFA-1 equally well (Table 2), indicating that the cysteine substitutions did not disrupt the I-domain structure. Binding of monoclonal antibody CBRLFA-1/1 to the high-affinity open mutant K287C/K294C was reduced to 40-50% of wild-type, however, this antibody reacted with mutant L289C/K294C and the single cysteine substitution mutants K287C, L289C and K294C as well as wild-type. Since antibody CBRLFA-1/1 maps to residues 301-359 (Huang, C and Springer, TA (1995) *J Biol Chem* 270:19008-19016), and single Cys substitution for K287 and K294 did not affect binding of this antibody, it is likely that reduced binding of CBRLFA-1/1 to mutant K287C/K294C was an indirect effect. Therefore, the conformation at the interface between the I- and β-propeller domains in mutant K287C/K294C may be different from that in wild-type LFA-1.

The reactivity of antibody to the β-propeller domain of αL and to the β2 subunit with mutants K287C/K294C and L289C/K294C was similar to that of wild-type LFA-1, confirming that the structure of other domains of LFA-1 molecule was not affected by the mutations.

Table 2. Reactivity of antibodies with LFA-1 cysteine substitution mutants (% wild-type binding)

		K287C/K294C		L298C/K294C		K287C	L289C	K294C
Mab	epitope	293T	K562	293T	K562	293T	293T	293T
	I-domain							
BL5	119-153, 185-215	92.4±11.29	92.39	85.79±16.4	97.61	93.35	92.44	88.31
F8.8	119-153, 185-215	93.70	102.15	83.56	93.88	95.86	99.63	95.47
CBRLFA-1/9	119-153, 185-215	ND	84.7	ND	ND	ND	ND	ND
TS2/6	154-183	84.88±5.64	89.24	78.59±2.62	95.89	91.39	88.24	91.67
May.035	185-215	92.61±8.4	92.59	82.14±14.15	101.10	95.8	95.4	106.39
TS1/11	185-215	94.36	95.96	93.67	104.54	ND	ND	ND
TS1/12	185-215	88.66	87.32	101.98	105.63	99.32	103.89	93.68
TS1/22	185-302	95.85±12.04	93.06	90.96±8.11	110.49	102.99	96.21	92.24
TS2/14	250-303	85.54±9.38	95.41	83.31±10.59	102.85	102.6	100.4	102.83
25-3-1	250-303	93.06	88.48	90.93	85.66	ND	ND	ND
CBRLFA-1/1	I- and β-propeller	43.59±0.58	55.53	95.89±7.74	118.44	86.11	93.32	89.41
S6F1	β-propeller	89.39	97.38	95.32	85.69	98.3	86.39	92.34
	β2 subunit							
TS1/18	I-like domain	99.82±10.47	97.42	95.72±4.67	105.71	87.88	87.35	107.58
YFC51	I-like domain	102.63	100.73	95.09	110.96	ND	ND	ND
CLBLFA-1/1	I-like domain	ND	96.48	ND	100.50	ND	ND	ND
CBRLFA-1/7	C-terminal region	95.32	95.25	91.68	97.19	ND	ND	ND

Wild-type LFA-1 and LFA-1 mutant K287C/K294C, L289C/K294C, K287C, L289C, and K294C were transiently expressed on the surface of 293T cells or stably expressed on K562 transfectants. Reactivity of antibodies with the transfectants was determined by flow cytometry. Mean fluorescence of each antibody binding was normalized to the mean fluorescence of mAb TS2/4 binding, except for CBRLFA-1/9 that was normalized to mAb TS1/22 binding. TS2/4 bound to wild-type LFA-1 and the mutants equally well. The results are expressed as percent of wild-type binding. Data are mean ±SD of at least two independent FCAS experiments. For some antibodies, only one experiment was done. ND: not determined.

EXAMPLE 3 LIGAND BINDING ACTIVITY OF LFA-1 CYSTEINE SUBSTITUTION MUTANTS

The ability of the LFA-1 cysteine substitution mutants to bind to the LFA-1 ligand ICAM-1 was determined. 293T cell transfectants that express wild-type LFA-1 and the predicted high-affinity open I-domain mutant K287C/K294C showed constitutively strong binding to immobilized ICAM-1 (Figure 4A). By contrast, the low-affinity closed mutant L289C/K294C did not bind to ICAM-1. Whereas the single cysteine substitution mutants K287C and L289C exhibited reduced binding to ICAM-1, binding of mutant K294C was comparable to that of the wild-type. Binding of mutants K287C and L289C was increased by the activating monoclonal antibody CBRLFA-1/2 to a level similar to wild-type binding. However, CBRLFA-1/2 was not able to activate binding of the low-affinity closed mutant L289C/K294C to ICAM-1 (Figure 4A). Similar results were obtained with two other LFA-1 activating monoclonal antibodies Kim127 and Kim185.To further study the function of the predicted high affinity mutant K287C/K294C and low affinity closed mutant L289C/K294C, stable K562 transfectants that express these mutants were generated.

Briefly, the human erythroleukemia cell line K562 was cultured in RPMI 1640, 10% FBS and 50μg/ml gentamycin. For generating stable K562 cell lines, 2 μg of PvuI-linearized pEFpuro containing αL subunit cDNA was cotransfected with 40 μg of SfiI-linearized AprM8 containing the β2 subunit cDNA by electroporation at 250V and 960 μF. Transfectants were selected for resistance to 4 μg/ml puromycin (Sigma), and subcloned by limiting dilution. All stable cell lines were maintained in RPMI 1640, 10% FBS supplemented with 4 μg/ml puromycin.

Clones of the transfectants that expressed similar levels of cell surface LFA-1, as determined by flow cytometry using monoclonal antibody TS2/4 (Figure 3B), were tested for their ability to bind to immobilized ICAM-1, as previously described (Lu, C and Springer, TA (1997) *J Immunol* 159:268-278).

Briefly, ICAM-1 was purified from human tonsil, and coated to 96-well plates as described previously (Lu, C and Springer, TA (1997) *J Immunol* 159:268-278). Cells were labeled with a florescence dye 2',7'-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and resuspended to 1 x 10^6/ml in L15/FBS. 50 μl cell suspension was mixed in ICAM-1 coated wells with an equal volume of L15/FBS in the absence or presence of monoclonal antibody (CBRLFA-1/2, 10 μg/ml). Monoclonal

antibodies were used at final concentration of 1:20 hybridoma supernatant, 1:200 ascites, or 10 μg/ml purified IgG. For testing the effect of divalent cations, BCECF-AM- labeled cells were washed 2 x with TS buffer, pH7.5 (20 mM Tris, pH 7.5, 150 mM NaCl) containing 5 mM EDTA, followed by 2 washes with TS buffer, pH7.5. Cells were then resuspended to 5 x 10^5/ml in the TS buffer, pH7.5 supplemented with 1 mM $MgCl_2/CaCl_2$, $MgCl_2$, $MnCl_2$ or 5 mM EDTA, and 100 μl cell suspension was added to ICAM-1 coated wells. After incubation at 37°C for 30 minutes, unbound cells were washed off on a Microplate Autowasher (Bio-Tek Instruments, Winooski, VT). The fluorescence content of total input cells and the bound cells in each well was quantitated on a Fluorescent Concentration Analyzer (IDEXX, Westbrook, ME). The bound cells were expressed as a percentage of total input cells per sample well.

K562 transfectants that express wild-type LFA-1 showed low basal binding to ICAM-1, and binding was greatly increased by the activating monoclonal antibody CBRLFA-1/2 (Figure 4B). By contrast, cells expressing the predicted high-affinity open mutant K287C/K294C strongly bound to ICAM-1, and monoclonal antibody CBRLFA-1/2 did not further enhance binding of this mutant, whereas the predicted low-affinity closed mutant L289C/K294C did not binding to ICAM-1 even in the presence of the activating antibody.

The effect of divalent cations on binding of K562 transfectants to ICAM-1 was also examined. As shown in Figure 4C, binding of mutant K287C/K294C to ICAM-1 was abolished in the presence of EDTA, confirming that ligand binding of mutant K287C/K294C is divalent cation dependent. Whereas binding of wild-type LFA-1 was greatly enhanced by Mn^{2+}, and to a lesser degree by Mg^{2+}, the presence of Mn^{2+} and Mg^{2+} did not increase binding of the low-affinity closed mutant L289C/K294C to ligand.

The binding of soluble ICAM-1 to K562 transfectants that expressed wild-type LFA-1, mutant K287C/K294C, or mutant L289C/K294C was also assessed. Briefly, a soluble ICAM-1-IgA chimera containing the 5 Ig domains of human ICAM-1 was purified from the culture supernatant of stable CHO transfectants by monoclonal antibody R6.5 affinity chromatography as previously described (Martin, S *et al.* (1993) *J Virol* 67:3561-3568). K562 transfectants were washed once with L15/FBS, and resuspended in the same buffer to 1 x 10^7/ml. 25 μl cell suspension was mixed with 25 μl L15/FBS containing ICAM-1-IgA fusion protein at final concentration 100 μg/ml in the presence or absence of antibody CBRLFA-1/2 (10 μg/ml), and incubated at 37°C for 30 minutes. After incubation, cells were washed once in L15/FBS, and incubated with FITC-conjugated anti-human IgA (Sigma) at

room temperature for 20 minutes. After 2 washes, cells were resuspended in PBS, and analyzed on a FACScan (Becton Dickinson, San Joe, CA).

As shown in Figure 5, the soluble ICAM-1-IgA fusion protein bound to cells expressing the high-affinity open mutant K287C/K294C, and binding was further increased in the presence of the activating monoclonal antibody CBRLFA-1/2. However, the ICAM-1 fusion protein did not bind to the transfectants that expressed wild-type LFA-1 or the low affinity closed mutant L289C/K294C in the absence or presence of monoclonal antibody CBRLFA-1/2, and binding was not detected at a higher ICAM-1 fusion protein concentration (300 μg/ml).

Taken together these data indicate that the high affinity open mutant K287C/K294C is constitutively active, whereas the low-affinity closed mutant L289C/K294C appears to be locked in an inactive state and lacks ligand binding ability.

In another study, a panel of monoclonal antibodies to different domains of the αL and β2 subunits were tested for their inhibitory effect on ligand binding of wild-type LFA-1 and mutant K287C/K294C. The results obtained with the 293T transient transfectants and K562 stable transfectants were similar, and summarized in Table 3. Although all antibodies, except for CBRLFA-1/1, reacted with the high affinity open mutant K287C/K294C as well as wild-type (Table 2), they showed differential inhibition on ligand binding of wild-type LFA-1 and mutant K287C/K294C.

As shown in Table 3, the I-domain antibodies differentially inhibited binding of wild-type LFA-1 and the high affinity open mutant K287C/K294C to ICAM-1. Monoclonal antibodies BL5, F8.8, CBRLFA-1/9, May.035, TS1/22 and TS2/6 strongly inhibited binding of both wild-type and mutant K287C/K294C, and the levels of inhibition to wild-type LFA-1 and the mutant were similar. While monoclonal antibodies TS1/11 and TS1/12 inhibited >90% binding of transfectants that express wild-type LFA-1, these antibodies showed reduced inhibition on binding of mutant K287C/K294C (40-60%). Monoclonal antibodies TS2/14, 25-3-1 and CBRLFA-1/1 that showed >90% inhibition on binding of wild-type had no to little inhibition on mutant K287C/K294C binding to ICAM-1. While the β-propeller domain antibody S6F1 and TS2/4 and antibody CBRLFA-1/7 to the C-terminal region of the β2 subunit did not inhibit binding of both wild-type and mutant K287C/K294C, all five antibodies to the β2 conserved domain, TS1/18, YFC51, CLBLFA-1/1, May.017, and 6.5E, inhibited binding of wild-type LFA-1 (>90% inhibition), but did not inhibit binding of mutant K287C/K294C.

Antibodies to the β-propeller domain and to the C-terminal region of β2 did not inhibit binding of wild-type LFA-1, or mutant K287C/K294C. Antibodies to the I-like domain of the β subunit blocked binding of wild-type LFA-1 to ICAM-1, but did not block mutant K287C/K294C.

Table 3. Differential inhibition of antibodies on binding of wild-type LFA-1 and mutant K287C/K294C to immobilized ICAM-1

		% inhibition			
		wild-type LFA-1		K287C/K294C	
Mab	epitope	293T	K562 (+CBRLFA-1/2)	293T	K562
RR1/1	I-CAM-1 I-domain	95.98	ND	97.89	ND
BL5	119-153, 185-215	97.01±1.63	97.54	91.06±3.8	90.68±6.23
F8.8	119-153, 185-215	94.51	97.61	91.94	98.18
CBRLFA-1/9	119-153, 185-215	ND	97.83	ND	3.60
TS2/6	154-183	96.84±1.73	91.76±4.67	79.09±10.06	88.12±7.40
May.035	185-215	96.20±0.57	95.80±1.66	97.43±1.52	93.33±2.54
TS1/11	185-215	94.12	96.55	45.18	41.30
TS1/12	185-215	95.68±3.92	97.46±0.66	48.96±9.52	63.67±8.13
TS1/22	250-303	95.77	96.94±0.79	95.07	93.56±4.79
TS2/14	250-303	94.47±2.34	96.24±1.70	2.95±9.87	8.55±0.66
25-3-1	250-303	90.49	92.01±0.36	3.71	2.53±4.10
CBRLFA-1/1	I- and β-propeller	92.52±1.68	94.69±5.22	9.03	2.85±4.90
S6F1	β-propeller	ND	6.19	ND	9.70
TS2/4	β-propeller β2 subunit	ND	6.99	ND	2.82
TS1/18	I-like domain	ND	98.48	ND	5.90
YFC51	I-like domain	ND	98.43	ND	0.08
CLBLFA-1/1	I-like domain	ND	94.63	ND	6.69
May.017	I-like domain	ND	97.76	ND	2.98
6.5E	I-like domain	ND	98.36	ND	5.79
CBRLFA-1/7	C-terminal region	ND	5.04	ND	5.77

Wild-type LFA-1 and LFA-1 mutant K287C/K294C were transiently expressed on the surface of 293T cells or stably expressed in K562 transfectants. Binding of the transfectants to immobilized ICAM-1 was determined in the presence of the indicated antibodies. For binding of K562 transfectants that express wild-type LFA-1, the cells were preincubated with the activating mAb CBRLFA-1/2 at 10 μg/ml for 30 min. Data shown are % inhibition ± SD of at least two independent experiments. % inhibition is defined as % bound cells in the presence of the indicated mAb/% bound cells in the presence of the nonbinding mAb X63 x 100. For some antibodies, only one experiment was done. However, in each experiment, each antibody was repeated in triplicate, and the standard deviation of the triplicate samples was <5%. ND: not determined.

Taken together, these results suggest that a subset of I-domain antibodies and antibodies to the β2 conserved domain do not directly block LFA-1 binding to ICAM-1, and that the high-affinity open mutant K287C/K294C appears to be conformationally locked in a high affinity open state, and thus, antibodies that block ligand binding via indirect mechanisms could not block binding of mutant K287C/K294C to ICAM-1.

The high affinity open I-domains of the invention can be used to discriminate between direct/competitive and indirect/non-competitive modes of inhibition of LFA-1. For example, the LFA-1 inhibitor lovastatin binds to the I-domain in a hydrophobic pocket formed by the β sheet and the C-terminal α-helix (Kallen, J et al. (1999) *J Mol Biol* 292:1-9) and thus inhibits LFA-1 by an indirect mechanism. Accordingly, the ability of lovastatin to inhibit ligand binding of the high-affinity I-domain (K287C/K294C) was assessed. Lovastatin dissolved in DMSO at 50 mM was diluted in assay buffer. Cells (10^6/ml) labeled with BCECF-AM were preincubated with lovastatin (0-50μM) at 37°C for 15 minutes, then transferred to a 96 well plate coated with ICAM-1 and further incubated at 37°C for 30 minutes in the presence or absence of activating monoclonal antibody (CBR LFA1/2) or $MnCl_2$. L15 medium supplemented with fetal bovine serum (L15/FBS) which contains Ca2+ and Mg2+ was used for wild-type αLβ2 activated by antibody CBR LFA1/2. and 20 mM HEPES pH7.4, 140 mM NaCl, 1mM MnCl2, 2 mg/ml glucose, 1% BSA was used for activation by Mn2+.

As shown in Figure 6, lovastatin inhibits ICAM-1 binding by cells expressing wild-type LFA-1 and stimulated with Mn^{2+} or antibody (CBRLFA1/2), but does not interfere with ligand binding by the high affinity open K287C/K294C mutant (HA/aLb2).

EXAMPLE 4 EXPRESSION AND FUNCTION OF ISOLATED WILD-TYPE AND MUTANT LFA-1 I-DOMAINS

To further examine the function of the predicted high and low affinity mutants, the wild-type I-domain and the I-domains of mutant K287C/K294C and L289C/K294C from residues V130 to A338 were expressed on the surface of K562 cells by the transmembrane domain of the PDGF receptor.

To construct the isolated, cell-surface expressed I domains, DNA sequences that encode the signal peptide and the following 6 amino acids from the 5' end of repeat II of αL were ligated to the sequences encoding residues V130-A338 that contains the I domain. HindIII and SalI sites were introduced immediately adjacent to the 5' and 3' ends of this fragment, respectively. The HindIII-SalI fragment was subcloned in frame at the 5' to the c-myc tag and the PDGF receptor (PDGFR) transmembrane domain in vector pDisplay™ (Invitrogen), and further subcloned into pcDNA3.1/Hygro using HindIII and NotI. All DNA amplification was carried out with Pfu DNA polymerase (Stratagene), and the final constructs were verified by DNA sequencing.

For generating stable K562 transfectants that express the I-domain on the surface, 20 μg of SspI-linearized pcDNA3.1/Hygro(+) containing the sequences encoding the I domain and the PDGFR transmembrane domain was used to transfect K562 cells by electroporation as described above. Transfectants were selected for resistance to 100 μg/ml hygromycin B, and were further subcloned by cell sorting and limiting dilution; clones that expressed similar levels of surface wild-type and mutant I domain-PDGFR were selected for functional studies. Stable cell lines were maintained in RPMI medium 1640 supplemented with 10% FBS and 100 μg/ml hygromycin B. Cell surface expression of the isolated I-domains was determined by flow cytometry using antibody TS1/22 to the I-domain (Figure 7). Two clones from each transfectant were selected and tested for binding to immobilized ICAM-1, and similar results were obtained with each of the two clones (Figure 8A). Transfectants that expressed intact wild-type LFA-1 showed low basal binding to ICAM-1. However, cells that expressed the isolated wild-type I-domain and the mutant L289C/K294C I-domain did not bind to ICAM-1. This suggests that the isolated wild-type I-domain alone is not sufficient to mediate strong and stable interaction with ligand (Knorr, R and Dustin, ML (1997) *J*

Exp Med 186:719-730). By contrast, cells that expressed the mutant K287C/K294C I-domain showed strong binding to ICAM-1.

If the constitutive ligand binding activity of mutant K287C/K294C is due to the formation of a disulfide bond between the introduced C287 and C294, disruption of the disulfide bond with a reducing agent would abolish ligand binding ability of the mutant. Accordingly, the transfectants were treated with the reducing agent DTT (10 mM) in L15/FBS containing Mg^{2+} and Ca^{2+}, and the ability of transfectants to bind to ICAM-1 was assessed. As shown in Figure 8A, binding of the cell surface-expressed mutant K287C/K294C I-domain to ICAM-1 who abolished after DTT treatment. By contrast, DTT increased binding of intact wild-type LFA-1, and similar results were observed with intact αIIbβ3 integrin. DTT treatment presumably disrupts disulfide bonds in the intact molecule that constrain the integrin in an inactive conformation. However, DTT treatment did not affect binding of the isolated wild-type I-domain or the mutant L289C/K294C I-domain. Since there is no other no disulfide bond in the LFA-1 I-domain as the I-domain structure reveals, these data strongly suggest that the introduced Cys287 and Cys294 formed a disulfide bridge that constrains the I-domain in a high affinity state.

Furthermore, the effect of divalent cations on ligand binding of the isolated I-domains expressed on the surface of K562 transfectants was tested. The binding reactions were performed in HEPES/NaCl/glucose (20 mM HEPES, pH 7.5, 140 mM NaC1, 2 mg/ml glucose) supplemented with 1 mM Mn^{2+}, 1 mM Mg^{2+}, or 1 mM EDTA. As shown in Figure 8B, the binding of the K287C/K294C I-domain to ICAM-1 was divalent cation dependent, as EDTA treatment abolished the binding. In contrast to intact wild-type LFA-1, Mn^{2+} or Mg^{2+} did not activate ligand binding of the isolated wild-type I-domain or the mutant L289C/K294C I-domain.

The effect of the I-domain antibodies on ligand binding of the isolated K287C/K294C I-domain was also examined. Transfectants expressing intact LFA-1 were pre-incubated with the activating antibody CBRLFA-1/2, and binding of the cells to ICAM-1 was performed in the presence of the I-domain antibodies TS1/22, TS2/6, TS1/11, TS1/12, CBRLFA-1/9, CBRLFA-1/1, 25.3.1, TS2/14, or the nonbinding antibody X63, as indicated. Monoclonal antibodies TS1/22, TS2/6, TS1/11, TS1/12 and CBRLFA-1/9 inhibited binding of the isolated K287C/K294C I-domain to ICAM-1, whereas antibodies 25-3-1, TS214 and CBRLFA-1/1 did not (Figure 8C). All

antibodies, except for CBRLFA-1/1, bound to the mutant K287C/K294C I-domain as well as the wild-type I-domain as determined by flow cytometry. The binding of CBRLFA-1/1 to the mutant I-domain was reduced to 80% of the wild-type I-domain. These results are consistent with those obtained with the intact LFA-1 K287C/K294C mutant (Tables 2 and 3), and indicate that the isolated K287C/K294C I-domain remains structural integrity as in the intact molecule.

EXAMPLE 5 INHIBITION OF LFA-1 FUNCTION IN VITRO AND IN VIVO BY SOLUBLE I-DOMAIN MUTANTS

A soluble αL I-domain mutant stabilized in the open conformation by a disulfide bond (K287C/K294C) was made in *E. coli*.

Briefly, recombinant mutant αL I-domain stabilized in the open conformation (K287C/K294C), or recombinant wild-type αL I-domain from amino acid residue G128 to Y307, were cloned into pET11b (Novagen) and expressed in *E. coli* induced with 1 mM IPTG for 4 hours. The recombinant proteins were purified from inclusion bodies by solubilization of inclusion bodies in 6M guanidine HCl and were refolded by dilution in the presence of 0.1 mM Cu^{2+}/phenanthrolin to enhance formation of disulfide bonds. Protein was concentrated by ammonium sulfate precipitation, dialyzed, and purified over a monoQ ion-exchange column. To remove any material in which the disulfide bond did not form, free sulfhydryls were reacted with activated biotin and passed over a streptavidin column. The recombinant proteins were then purified by gel filtration and concentrated by Centriprep. For BIAcore™ analysis, recombinant ICAM-1, ICAM-2 and ICAM–3 Fc chimeras were immobilized on the BIAcore™ sensor chip by an amine-coupling method. Recombinant αL I-domains were flowed in, and BIAcore™ assays were performed with Tris-buffered saline supplemented with 1 mM $MgCl_2$ or 2 mM EDTA, at a flow rate of 10 µl/minute at 25°C.

The purified open I-domain showed high affinity to its ligands, ICAM-1, -2, and –3, in the presence of 1 mM $MgCl_2$ as assessed by BIAcore™ analysis, whereas binding of a soluble wild-type I domain was not detectable (Figure 9, Panels A, C and E; Table 4). The interaction of the open I-domain with ligands was divalent cation-dependent, and was abolished in the presence of 2 mM EDTA, suggesting that the interaction depends on MIDAS. Since the wild-type I-domain showed no interaction with ligands,

the open I-domain allowed for the detailed analysis of the binding kinetics of LFA-1 with its ligands. To analyze binding kinetics, different concentrations of open I-domain were tested for ligand binding (Figure 9, Panels B, D and F). Kinetic analysis demonstrated a fast association rate ($1.28 \times 10^5 M^{-1}s^{-1}$) and an intermediate dissociation rate ($0.0230\ s^{-1}$) for ICAM-1, the major ligand on endothelial cells (Table 4). The K_D for ICAM-1 is in the nanomolar range and ICAM-1 showed the highest affinity, followed by ICAM-2 and ICAM-3. The open I-domain also showed nanomolar range affinity for murine ICAM-1.

Table 4. Kinetics of open I-domain binding to ICAMs

Ligand	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM^{-1})
ICAM-1	1.28×10^5	0.0230	180
ICAM-2	0.23×10^5	0.0118	513
ICAM-3	0.19×10^5	0.0749	3942

k_{on}, k_{off}, and K_D were calculated based on 1:1 interaction model using BIAevaluation™ software.

In another study, measurements of the affinity of the recombinant, soluble high affinity αL I domain for its ligand ICAM-1 show a Kd of 200 nM, as assessed by BIAcore. Thus, the isolated, high affinity conformer of the αL I domain is as active as the most activated αLβ2 heterodimer.

The activity of the soluble open I-domain to inhibit LFA-1-dependent adhesion was tested. In one study, K562 cells stably expressing wild-type LFA-1 were fluorescently labeled by BCECF and LFA-1 on the cell surface was activated by the activating monoclonal antibody, CBRLFA-1/2 in L15 media supplemented with FCS. The cells were subsequently incubated in ICAM-1 coated 96-well plastic plates in the presence or absence of I-domains. After incubation for 40 minutes at 37°C, unbound cells were washed off on a Microplate Autowasher. The fluorescence content of total input cells and the bound cells in each well was quantitated on a Fluorescent Concentration Analyzer. The bound cells were expressed as a percentage of total input cells per sample well. In contrast to the wild-type I-domain, the open I-domain mutant

strongly inhibited adhesion of LFA-1 expressing cells to immobilized ICAM-1 (Figure 10A).

In another study, the murine T lymphoma cell line EL-4 which expresses both murine LFA-1 and its ligands, including murine ICAM-1, and which exhibits LFA-1-dependent homotypic aggregation upon activation by PMA was used. Cells were incubated in a 96 well plate in the presence of 50 ng/ml PMA and varying amounts of soluble I-domains. After incubation for 2 hours at 37°C, 5% CO_2, the degree of aggregation was scored under the microscope as follows: 0 indicated that essentially no cells were clustered; 1 indicated that <10% of cells were aggregated; 2 indicated clustering of <50%; 3 indicated that up to 100% of cells were in small, loose aggregates; 4 indicated that nearly 100% of cells were in larger clusters; and 5 indicated that nearly 100% of cells were in very large, tight clusters. As shown in Figure 10B, the soluble open I-domain also inhibited PMA-induced LFA-1 dependent homotypic aggregation of the murine T-cell line EL-4.

Moreover, the ability of the open I-domain mutants to inhibit LFA-1 function *in vivo* was tested by visualizing microcirculation in the peripheral lymph node (LN) with intravital microscopy. Briefly, a small bolus (20-50 μl) of LN cell suspensions from T-GFP mice were retrogradely injected through a femoral artery catheter and visualized in the subiliac LN by fluorescent epi-illumination from a video-triggered xenon arc stroboscope. After recording control T^{GFP} cell behavior in the absence of I-domain, the mouse was pretreated by intra-arterial injection of I-domain (10 μg/g of weight) 5 minutes before T^{GFP} cell injection. All scenes were recorded on videotape and off-line analysis was done. The rolling fraction was calculated as percentage of rolling cells amount the total number of T^{GFP} cells that entered a venule. The sticking (firm adhesion) fraction was determined as the percentage of T^{GFP} cells becoming firmly adherent for >20 seconds in the number of T^{GFP} cells that rolled in a venule. Results were semi-quantitatively scored as follows: -: 0%, ±: 0-5%, +: 5-20%, ++: 20-40%, +++: 40-60%, ++++: 60-80%, +++++:80-100%.

As shown in Table 5, below, injection of the open I-domain but not the wild-type I-domain effectively blocked firm adhesion of T-lymphocytes to high endothelial venules, which is LFA-1-dependent. Lymphocyte rolling that is mediated by L-selectin and PNAd was not compromised, suggesting that the inhibitory effects of the open I-domain was LFA-1 specific.

Table 5. ***In vivo*** **firm adhesion of lymphocytes under flow in peripheral lymph node high endothelial venules was inhibited by open but not wild-type I-domain**

	Fraction		
I-domain	rolling	firm adhesion	transmigration
none	+++	++	±
wild-type	+++	++	±
open	++++	±	-

Kinetics of the Binding of αL Mutant I-domains to ICAM-1

To further investigate the kinetics of the interaction of the αL I-domains with ICAM-1, recombinant soluble αL I-domains were expressed in *E. coli*, refolded and purified. As shown in Table 6, below, the affinity of E284C/E301C is nearly comparable to K287C/K294C. The affinity of L161C/F299C, K160C/F299C, and L161C/T300C are significantly higher than wild type, but 20-30 times lower than high-affinity open αL I-domain, K287C/K294C. L161C/F299C, K160C/F299C, and L161C/T300C are referred to as intermediate-affinity αL I-domains.

Table 6. Kinetics of interaction of αL I-domains with ICAM-1

αL I-domain	Kon (1/Ms)	Koff (1/s)	KD (μM)
Locked open			
K287C/K294C	1.28×10^5	0.0230	0.180
E284C/E301C	1.28×10^5	0.0459	0.360
L161C/F299C	1.36×10^5	0.513	3.76
K160C/F299C	1.53×10^5	0.67	4.39
L161C/T300C	1.35×10^5	0.65	4.8
WT	2.22×10^3	3.00	1350
Locked closed			
L289C/K294C	2.11×10^3	2.84	1760

Recombinant soluble αL I-domains were expressed in *E. coli*, refolded and purified. Kinetics of binding of the I-domains to ICAM-1 was measured by BIAcore™ instruments. Kinetics was analyzed BIAevaluation™ software. KD was calculated by Scatchard plots using data at steady states. Koff was obtained by curve fitting of the dissociation phase using 1:1 binding model. Kon was calculated by Koff/KD.

EXAMPLE 6 CONSTRUCTION AND ACTIVITY OF Mac-1 CYSTEINE SUBSTITUTION MUTANTS

A similar approach was taken to design an open, high affinity conformation of Mac-1 by introducing a disulfide bond into the I-domain. The design of Mac-1 cysteine substitution mutants was described in Example 1.

Table 7. Cα and Cβ between mutated residues in either open or closed conformation

	ido (open conformation)		jlm (closed conformation)	
mutations	Cα	Cβ	Cα	Cβ
Locked open				
Q163C/Q309C	8.37	6.36	9.11	7.16
Q298C/N301C	5.31	4.21	9.05	10.91
D294C/T307C	9.21	8.67	16.01	17.52
D294C/Q311C	9.02	7.08	9.79	10.02
F297C/A304C	6.31	3.78	11.18	10.17
Locked closed				
Q163C/R313C	13.8	13.33	7.36	5.15

The distance between wild-type residues was measured by Look™ software in open conformation (1ido) or closed conformation (1jlm).

In order to assess the effect of the introduction of pairs of potentially disulfide bond-forming cysteines into the I-domain of αMβ2 on CBRM1/5 activation-dependent epitope expression and ligand binding, plasmids encoding the wild-type or mutant αM subunits and the β2 subunit were co-transfected into 293T and K562 cells. αβ heterodimer formation was confirmed using monoclonal antibody CBRM1/32 which recognizes an epitope in the putative β-propeller domain of the αM subunit only after association with the β2 subunit, and antibody CBRM1/5 was used to detect integrin activation.

The Q163C/Q309C pair of mutations worked well (Figure 11B, Figure 12B and C). This mutant introduces a putative disulfide bond near the bottom front of the I-domain, between residues that are in the lower one-third of the last α-helix and the first α-helix, and have Cβ carbons that are 6.36Å apart in the 1ido structure. In contrast, the Cβ carbons for the D294C/T307C and D294C/N311C substitutions are 8.67Å and 7.08Å apart, respectively. The Cβ carbons for the Q298C/N301C and F297C/A304C substitutions are within the ideal range, however these substitutions are closer to the loop between the last β-strand and α-helix, and must have unfavorable effects such as distorting the ligand binding site.

When expressed within an intact heterodimer in transiently transfected 293T cells, the Q163C/Q309C mutant is expressed half as well as wild-type as measured by CBRM1/32 antibody, but the ratio of the CBRM1/5 activation-dependent epitope to CBRM1/32 expression is markedly higher (Figure 11A). In addition, the adhesion of 293T cells expressing the Mac-1 Q163C/Q309C mutant to iC3b coated on plastic, as assayed in L15/FBS medium at room temperature, was higher than wild-type, despite its lower expression (Figure 11B).

Alternatively, isolated Mac-1 mutant I-domains were expressed on the cell surface in conjunction with an artificial signal sequence and transmembrane domain of the PDGF receptor. Adhesion was assayed in L15/FBS/$MnCl_2$ at 37°C. The isolated wild-type I-domain showed no binding to iC3b, whereas the previously described mutants with computationally redesigned hydrophobic cores, ido1r and ido2r, were active (Figure 11C) (Shimaoka, M *et al.* (2000) *Nature Structural Biology* 7:674-678). The Q163C/Q309C mutant I-domain exhibited strong specific ligand binding that was completely blocked by the inhibitory I-domain monoclonal antibody CBRM1/5 (Figure 12C).

In a further study, the open I-domain mutants Q163C/Q309C and D294C/Q311C were stably expressed in K562 cells, and clones expressing the same levels of receptors were selected. Adhesion assays to immobilized iC3b were performed with L15/FBS at 37°C. In contrast to 293T cells, wild-type Mac-1 has little basal activity for ligand binding in these cells (Figure 12A and 12B). Both Q163C/Q309C and D294C/Q311C showed increased CBRM1/5 activation-dependent epitope expression and increased ligand binding when expressed in an intact αMβ2 heterodimer, as compared to wild-type (Figure 12A and 12B). Moreover, K562 cells expressing isolated open I-domain mutants on the cell surface showed strong specific binding to iC3b as compared to wild-type (Figure 12C).

In order to confirm that the increased ligand binding activity of the open I-domain mutants is induced by the formation of a disulfide bond, the effect of the reducing agent DTT was tested. Binding of αMβ2 transfectants containing mutant I-domains to immobilized iC3b on plastic was tested in the presence and absence of DTT. As summaried in Table 8, below, locked open αM I-domains, (Q163C/Q309C) and (D294C/Q311c), are active in the absence of activation and their activities are partly reduced by disulfide reduction by DTT. By contrast, locked closed αM I-domain

Q163C/R313C is inactive and resistant to activation, but becomes activatable after disulfide reduction by DTT.

As shown in Figure 12C, DTT treatment abolished ligand binding by isolated locked open I-domains. In contrast, DTT increased binding of the intact wild-type αMβ2 (Figure 2B), indicating that DTT used in this experiment was not toxic and abolishment of ligand binding by the open I-domain mutants was not due to a non-specific effect of DTT. Taken together, these data suggest that the introduced cysteines result in the formation of a disulfide bridge that constrains the Mac-1 I-domain in an open or closed conformation.

Table 8. Summary of adhesion assay of αMβ2 transfectant containing mutant I-domains

	- DTT	- DTT	+ DTT	+ DTT
mutations	- activation	+ activation	- activation	+ activation
Wild type	±	++++	++	++++
Locked open				
Q163C/Q309C	++++	++++	++	++++
Q298C/N301C	±	+	NT	NT
D294C/T307C	±	+	NT	NT
D294C/Q311C	++++	++++	++	++++
F297C/A304C	±	++	NT	NT
Locked closed				
Q163C/R313C	±	±	++	+++

Binding of αMβ2 transfectants containing mutant I-domains to immobilized iC3b on plastic was tested. Results were semi-quantitatively scored as follow; ±: 0-5%, +: 5-25%, ++25-50%, +++: 50-75%, ++++: 75-100% of binding by activated wild type transfectant.
NT: not tested
DTT: disulfide reduction by DTT treatment.
+ activation: activated by activating mAB CBR LFA-1/2

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A modified integrin I-domain polypeptide containing at least one disulfide bond, such that said modified I-domain polypeptide is stabilized in a desired conformation.

2. A modified integrin I-domain polypeptide of claim 1 which is stabilized in the open conformation.

3. A modified integrin I-domain polypeptide of claim 1 which is stabilized in the closed conformation.

4. A modified integrin I-domain polypeptide of claim 2 which binds ligand with high affinity.

5. A modified integrin I-domain polypeptide of claim 1 which is encoded by an amino acid sequence containing at least one cysteine substitution as compared to the wild-type sequence.

6. A modified integrin I-domain polypeptide of claim 2, wherein the distance between Cβ carbons of the residues that are substituted for cysteines is 3.00-8.09Å.

7. A modified integrin I-domain polypeptide of claim 1 which is derived from an I-domain of an integrin α subunit selected from the group consisting of: α1, α2, α10, α11, αD, αE, αL (CD11a), αM (CD11b) and αX (CD11c).

8. A modified integrin I-domain polypeptide of claim 2 which is derived from the I-domain of the αL subunit of LFA-1.

9. A modified integrin I-domain polypeptide of claim 3 which is derived from the I-domain of the αL subunit of LFA-1.

10. A modified integrin I-domain polypeptide of claim 7 which contains amino acid substitutions selected from the group consisting of K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, and L161C/T300C.

11. A modified integrin I-domain polypeptide of claim 8 which contains amino acid substitutions L289C/K294C.

12. A modified integrin I-domain polypeptide of claim 2 which is derived from the I-domain of the αM subunit of Mac-1.

13. A modified integrin I-domain polypeptide of claim 3 which is derived from the I-domain of the αM subunit of Mac-1.

14. A modified integrin I-domain polypeptide of claim 12 which contains amino acid substitutions selected from the group consisting of Q163C/Q309C and D294C/Q311C.

15. A modified integrin I-domain polypeptide of claim 13 which contains amino acid substitutions Q163C/R313C.

16. A modified integrin I-domain polypeptide of claim 1 which is comprised within an integrin α subunit.

17. A modified integrin I-domain polypeptide of claim 16 which is further associated with an integrin β subunit.

18. A modified integrin I-domain polypeptide of claim 1 which is a soluble polypeptide.

19. A modified integrin I-domain polypeptide of claim 1 which is operatively linked to a heterologous polypeptide.

20. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified integrin I-domain polypeptide as defined in any one of claims 1-15.

21. A composition comprising a modified integrin I-domain polypeptide as defined in any one of claims 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 15 and a pharmaceutically acceptable carrier.

22. A composition of claim 20, wherein said modified integrin I-domain polypeptide is a soluble polypeptide.

23. A composition of claim 21, further comprising an anti-inflammatory or immunosuppressive agent.

24. The use of a modified integrin I-domain polypeptide of claim 2 as an immunogen to produce antibodies that selectively bind to an integrin I-domain in the open conformation.

25. An antibody, or an antigen binding fragment thereof, which selectively binds to a modified integrin I-domain in the open conformation.

26. An antibody of claim 25 which binds to an activation specific epitope on the integrin I-domain.

27. An antibody of claim 25 which blocks an interaction between an integrin and a cognate ligand.

28. An antibody of claim 25, or an antigen binding fragment thereof, further comprising a pharmaceutical composition and a pharmaceutically acceptable carrier.

29. An antibody of claim 25, or an antigen binding fragment thereof, wherein said antibody is an LFA-1 antibody.

30. An anti-LFA-1 antibody, or an antigen binding fragment thereof, which selectively binds to an LFA-1 I-domain in the open conformation.

31. The LFA-1 antibody of claim 30, wherein said anti-LFA-1 antibody, or an antigen binding fragment thereof, selectively binds to a modified LFA-1 I-domain.

32. A modified integrin I-like domain polypeptide containing at least one disulfide bond, such that said modified I-like domain polypeptide is stabilized in a desired conformation.

33. A modified integrin I-like domain polypeptide of claim 30 which is stabilized in the open conformation.

34. A modified integrin I-like domain polypeptide of claim 31 which binds ligand with high affinity.

35. A modified integrin I-like domain polypeptide of claim 30 which is encoded by an amino acid sequence containing at least one cysteine substitution as compared to the wild-type sequence.

36. A modified integrin I-like domain polypeptide of claim 30 which is derived from an I-like domain of an integrin β subunit.

37. A modified integrin I-like domain polypeptide of claim 30 which is comprised within an integrin β subunit.

38. A method for stabilizing a polypeptide in a desired conformation, said method comprising introducing at least one disulfide bond into the polypeptide such that the polypeptide is stabilized in a desired conformation.

39. The method of claim 38, wherein the disulfide bond is formed by the introduction of at least one cysteine substitution into the amino acid sequence of the polypeptide.

40. The method of claim 38, wherein the distance between Cβ carbons in the residues that are substituted for cysteines is 3.00-8.09Å.

41. The method of claim 38, wherein said polypeptide comprises a functional domain of a protein.

42. The method of claim 41, wherein said polypeptide comprises an integrin I-domain.

43. The method of claim 38, wherein said polypeptide is selected from the group of polypeptides consisting of: an integrin subunit, a small G protein, a heterotrimeric G protein alpha subunit, a tyrosine kinases, a G protein-coupled receptor, an enzyme under allosteric control, a zymogen, complement C3, complement C4, and fibrinogen.

44. A method for identifying a modulator of integrin activity comprising:

(a) providing a modified integrin I-domain polypeptide of claim 2;

(b) contacting the modified integrin I-domain polypeptide with a test compound; and

(c) assaying the ability of the test compound to bind to the modified integrin I-domain polypeptide,

to thereby identify a modulator of integrin activity.

45. A method for identifying a compound capable of modulating the interaction of an integrin and a cognate ligand comprising the steps of:

(a) providing a modified integrin I-domain polypeptide of claim 2;

(b) contacting the modified integrin I-domain polypeptide with a ligand of the integrin in the presence and absence of a test compound; and

(c) detecting binding between the modified integrin I-domain polypeptide and said ligand,

to thereby identify a compound capable of modulating the interaction between an integrin and a cognate ligand.

46. A method for treating or preventing an integrin-mediated disorder in a subject comprising administering to said subject a therapeutically effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation, thereby treating or preventing an integrin-associated disorder in a subject.

47. The method of claim 46, wherein said integrin-mediated disorder is an inflammatory disorder.

48. The method of claim 46, wherein said integrin-mediated disorder is an autoimmune disorder.

49. A method of inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to said subject an effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation, thereby inhibiting the binding of an integrin to a cognate ligand in a subject.

50. The method of either one of claims 46 and 49, wherein said modified integrin I-domain polypeptide binds ligand with high affinity.

51. The method of either one of claims 46 and 49, wherein said modified integrin I-domain polypeptide is a soluble polypeptide.

52. The method of claim 50, wherein said modified integrin I-domain polypeptide is operatively linked to a heterologous polypeptide.

53. The method of either of claims 46 and 49, wherein said modified integrin I-domain polypeptide is selected from the group consisting of: αL K287C/K294C, αL E284C/E301C, αL L161C/F299C, αL K160C/F299C, αL L161C/Y300C, αM Q163C/Q309C and αM D294C/Q311C.

54. A method for treating or preventing an integrin-mediated disorder in a subject comprising administering to said subject a therapeutically effective amount of an antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain in the open conformation, thereby treating or preventing an integrin-associated disorder in a subject.

55. The method of claim 54, wherein the antibody binds to a modified integrin I-domain, or an antigen binding fragment thereof.

56. The method of claim 54, wherein said antibody is an LFA-1 antibody, or an antigen binding fragment thereof.

57. The method of claim 54, wherein said integrin-mediated disorder is an inflammatory disorder.

58. The method of claim 54, wherein said integrin-mediated disorder is an autoimmune disorder.

59. A method of treating an integrin-mediated disorder in a subject comprising administering to said subject a therapeutically effective amount of an anti-LFA-1 antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain in the open conformation, thereby treating or preventing an integrin-associated disorder in a subject.

60. The method of claim 59, wherein said anti-LFA-1 antibody binds to a modified LFA-1 I-domain, or an antigen binding fragment thereof.

61. The method of claim 59, wherein said integrin-mediated disorder is an inflammatory disorder.

62. A method of inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to said subject an effective amount of an antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain in the open conformation, thereby inhibiting the binding of an integrin to a cognate ligand in a subject.

63. The method of claim 62, wherein said antibody is an LFA-1 antibody, or an antigen binding fragment thereof.

64. The method of any one of claims 54, 59, or 62, wherein said antibody, or an antigen binding fragment thereof, binds to an activation specific epitope on the integrin I-domain.

65. A vaccine formulation for prophylactic or therapeutic treatment of an inflammatory disorder comprising an effective amount of a nucleic acid encoding a modified integrin I-domain polypeptide, or active fragment thereof.

66. The vaccine formulation of claim 65, further comprising an antigenic component.

67. The vaccine formulation of claim 65, further comprising a pharmaceutically acceptable carrier.

68. A method for treating an integrin-mediated disorder in a subject comprising administering to said subject a nucleic acid molecule encoding a modified integrin I-domain polypeptide, or active fragment thereof, inserted into a vector.

69. The method of claim 68, wherein said nucleic acid molecule is administered to a subject by intravenous injection.

70. The method of claim 68, wherein said nucleic acid molecule further comprises an antigenic component.

71. A non-human, transgenic animal comprising a nucleic acid molecule encoding a modified integrin I-domain polypeptide.

72. The transgenic animal of claim 71, wherein said animal is a mouse.

FIGURE 1

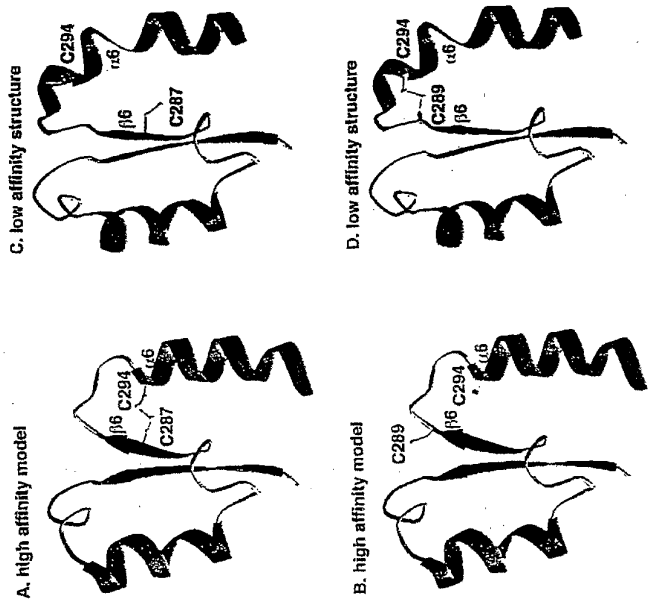

C. low affinity structure
C294
α6
β6
C287
D. low affinity structure
C294
α6
C289
β6
A. high affinity model
α6
C294
β6
C287
B. high affinity model
C289
C294
α6
β6

FIGURE 2

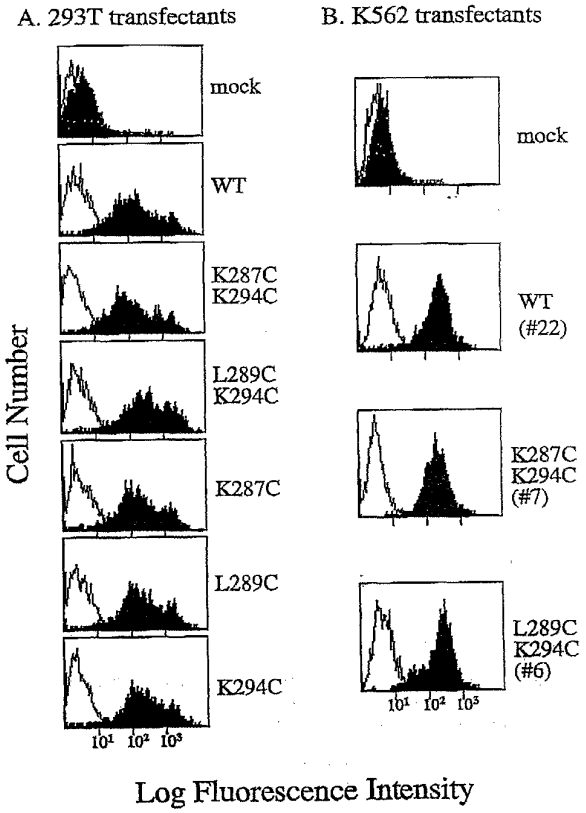

A. 293T transfectants
B. K562 transfectants
mock
WT
K287C
K294C
L289C
K294C
K287C
L289C
K294C
mock
WT
(#22)
K287C
K294C
(#7)
L289C
K294C
(#6)
Cell Number
10¹ 10² 10³
10¹ 10² 10³
Log Fluorescence Intensity

FIGURE 3

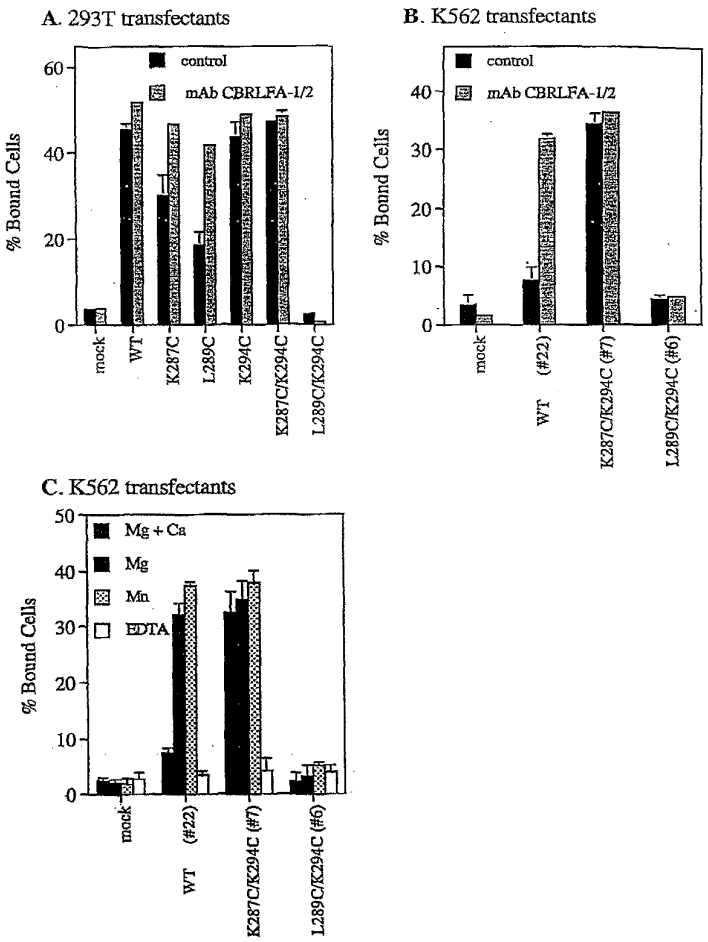

A. 293T transfectants
control
mAb CBRLFA-1/2
% Bound Cells
0
20
40
60
mock
WT
K287C
L289C
K294C
K287C/K294C
L289C/K294C
B. K562 transfectants
control
mAb CBRLFA-1/2
% Bound Cells
0
10
20
30
40
mock
WT
(#22)
K287C/K294C (#7)
L289C/K294C (#6)
C. K562 transfectants
Mg + Ca
Mg
Mn
EDTA
% Bound Cells
0
10
20
30
40
50
mock
WT
(#22)
K287C/K294C (#7)
L289C/K294C (#6)

FIGURE 4

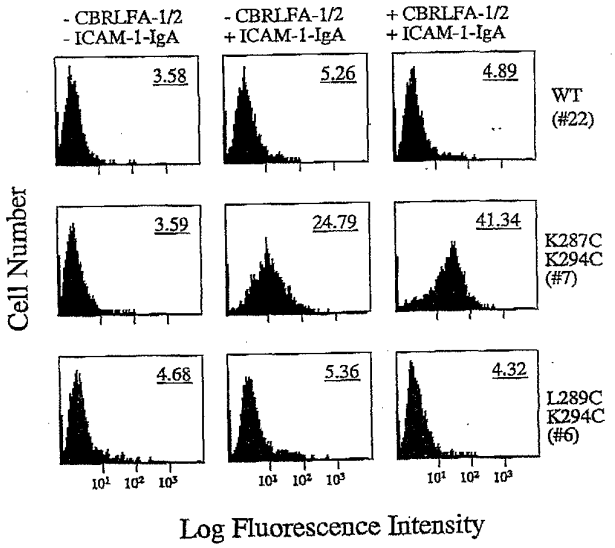
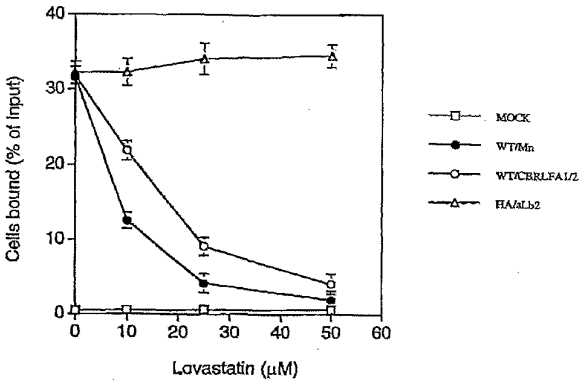

- CBRLFA-1/2
- ICAM-1-IgA
- CBRLFA-1/2
+ ICAM-1-IgA
+ CBRLFA-1/2
+ ICAM-1-IgA
3.58
5.26
4.89
WT
(#22)
3.59
24.79
41.34
K287C
K294C
(#7)
4.68
5.36
4.32
L289C
K294C
(#6)
Cell Number
Log Fluorescence Intensity

FIGURE 5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30
20
10
0
Cells bound (% of input)
0
10
20
30
40
50
60
Lovastatin (μM)
MOCK
WT/Mn
WT/CBRLFA1/2
HA/aLb2

FIGURE 6

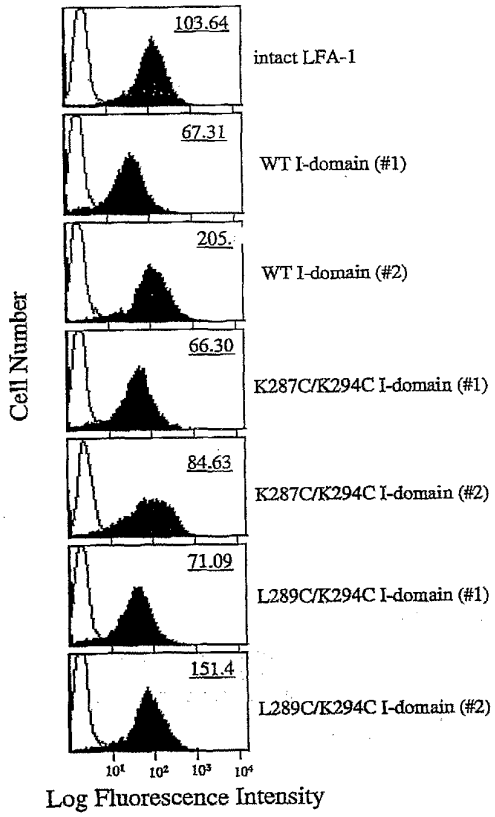

103.64
intact LFA-1
67.31
WT I-domain (#1)
205.
WT I-domain (#2)
66.30
K287C/K294C I-domain (#1)
84.63
K287C/K294C I-domain (#2)
71.09
L289C/K294C I-domain (#1)
151.4
L289C/K294C I-domain (#2)
Cell Number
10¹ 10² 10³ 10⁴
Log Fluorescence Intensity

FIGURE 7

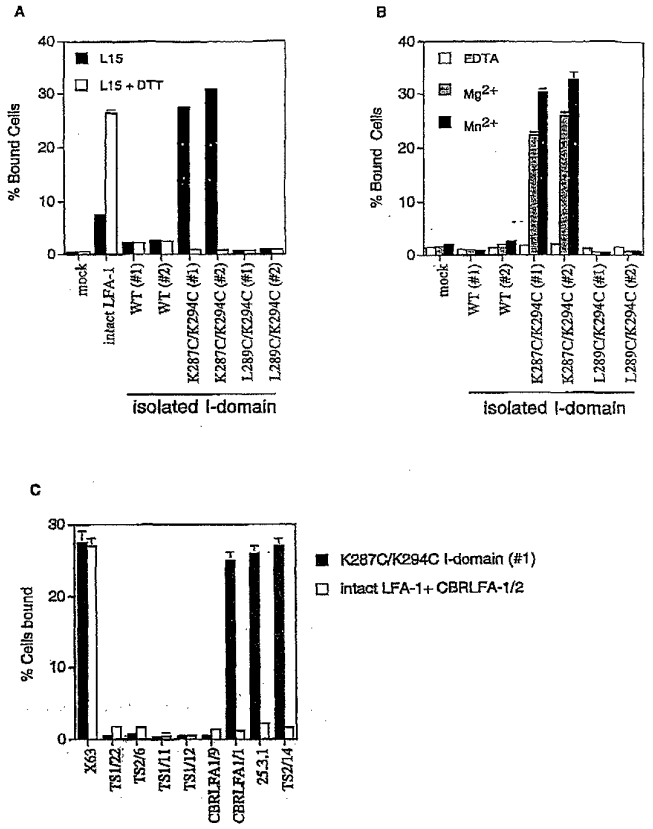

A
L15
L15 + DTT
% Bound Cells
mock
intact LFA-1
WT (#1)
WT (#2)
K287C/K294C (#1)
K287C/K294C (#2)
L289C/K294C (#1)
L289C/K294C (#2)
isolated I-domain
B
EDTA
Mg2+
Mn2+
% Bound Cells
mock
WT (#1)
WT (#2)
K287C/K294C (#1)
K287C/K294C (#2)
L289C/K294C (#1)
L289C/K294C (#2)
isolated I-domain
C
% Cells bound
K287C/K294C I-domain (#1)
intact LFA-1+ CBRLFA-1/2
X63
TS1/22
TS2/6
TS1/11
TS1/12
CBRLFA1/9
CBRLFA1/1
25.3.1
TS2/14

FIGURE 8

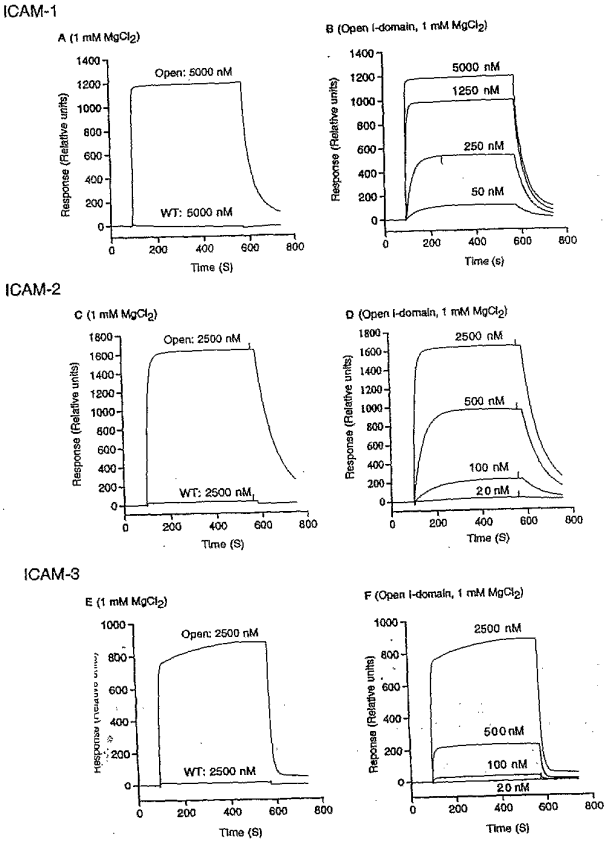
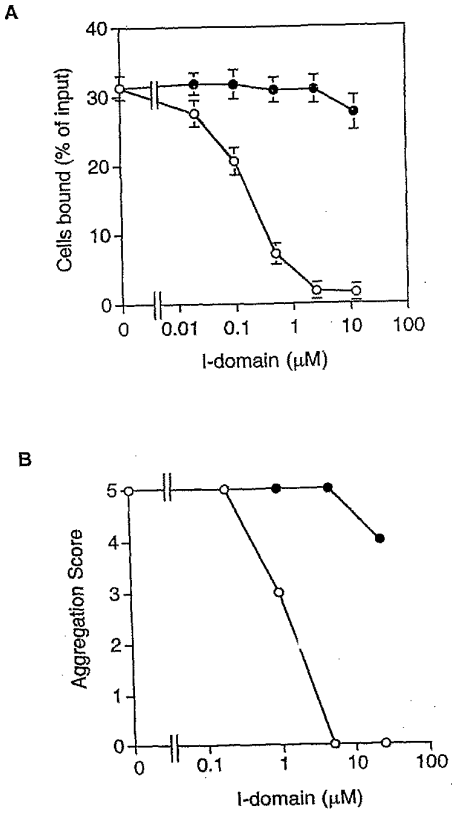

ICAM-1
A (1 mM MgCl2)
Open: 5000 nM
WT: 5000 nM
Response (Relative units)
Time (S)
B (Open I-domain, 1 mM MgCl2)
5000 nM
1250 nM
250 nM
50 nM
Response (Relative units)
Time (s)
ICAM-2
C (1 mM MgCl2)
Open: 2500 nM
WT: 2500 nM
Response (Relative units)
Time (S)
D (Open I-domain, 1 mM MgCl2)
2500 nM
500 nM
100 nM
20 nM
Response (Relative units)
Time (S)
ICAM-3
E (1 mM MgCl2)
Open: 2500 nM
WT: 2500 nM
Time (S)
F (Open I-domain, 1 mM MgCl2)
2500 nM
500 nM
100 nM
20 nM
Response (Relative units)
Time (S)

FIGURE 9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Cells bound (% of input)
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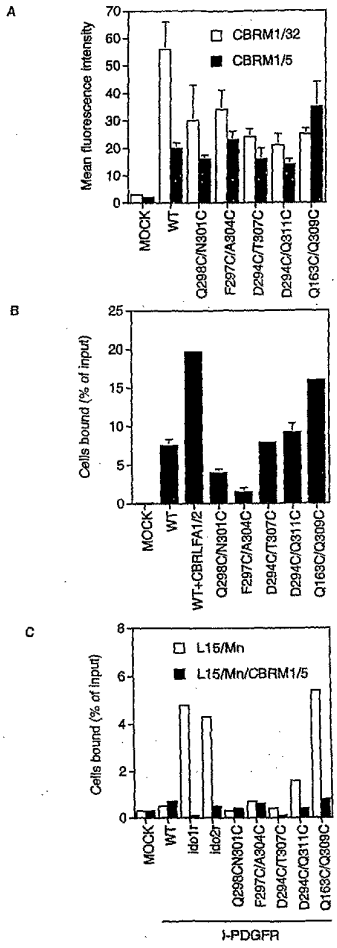
30
20
10
0
0
0.01
0.1
1
10
100
I-domain (μM)
B
Aggregation Score
5
4
3
2
1
0
0
0.1
1
10
100
I-domain (μM)

FIGURE 10


A
Mean fluorescence intensity
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CBRM1/5
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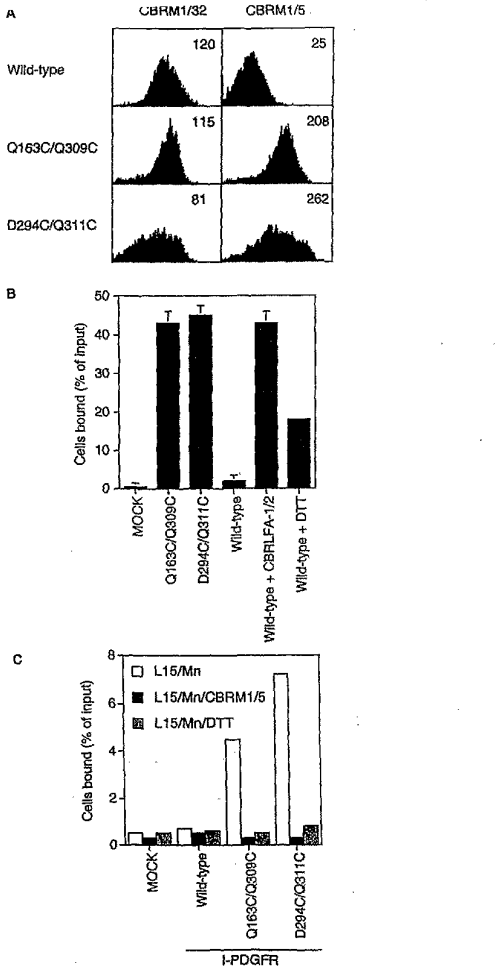
F297C/A304C
D294C/T307C
D294C/Q311C
Q163C/Q309C
B
Cells bound (% of input)
MOCK
WT
WT+CBRLFA1/2
Q298C/N301C
F297C/A304C
D294C/T307C
D294C/Q311C
Q163C/Q309C
C
Cells bound (% of input)
L15/Mn
L15/Mn/CBRM1/5
MOCK
WT
Ido1r
Ido2r
Q298CN301C
F297C/A304C
D294C/T307C
D294C/Q311C
Q163C/Q309C
I-PDGFR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CBRM1/32
CBRM1/5
Wild-type
Q163C/Q309C
D294C/Q311C
120
25
115
208
81
262
B
Cells bound (% of input)
MOCK
Q163C/Q309C
D294C/Q311C
Wild-type
Wild-type + CBRLFA-1/2
Wild-type + DTT
C
L15/Mn
L15/Mn/CBRM1/5
L15/Mn/DTT
Cells bound (% of input)
MOCK
Wild-type
Q163C/Q309C
D294C/Q311C
I-PDGFR

SEQUENCE LISTING

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Timothy Springer
Motomu Shimaoka
Chafen Lu

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65                  70                  75                  80
Leu Pro Val Thr Leu Arg Gly Ser Asn Tyr Thr Ser Lys Tyr Leu Gly
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Met Thr Leu Ala Thr Asp Pro Thr Asp Gly Ser Ile Leu Ala Cys Asp
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Pro Gly Leu Ser Arg Thr Cys Asp Gln Asn Thr Tyr Leu Ser Gly Leu
        115                 120                 125
Cys Tyr Leu Phe Arg Gln Asn Leu Gln Gly Pro Met Leu Gln Gly Arg
    130                 135                 140
Pro Gly Phe Gln Glu Cys Ile Lys Gly Asn Val Asp Leu Val Phe Leu
145                 150                 155                 160
Phe Asp Gly Ser Met Ser Leu Gln Pro Asp Glu Phe Gln Lys Ile Leu
                165                 170                 175
Asp Phe Met Lys Asp Val Met Lys Lys Leu Ser Asn Thr Ser Tyr Gln
            180                 185                 190
Phe Ala Ala Val Gln Phe Ser Thr Ser Tyr Lys Thr Glu Phe Asp Phe
        195                 200                 205
Ser Asp Tyr Val Lys Arg Lys Asp Pro Asp Ala Leu Leu Lys His Val
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Lys Val Leu Ile Ile Ile Thr Asp Gly Glu Ala Thr Asp Ser Gly Asn
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Ile Asp Ala Ala Lys Asp Ile Ile Arg Tyr Ile Ile Gly Ile Gly Lys
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Lys Pro Ala Ser Glu Phe Val Lys Ile Leu Asp Thr Phe Glu Lys Leu
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Gly Ala Pro Leu Phe Tyr Gly Glu Gln Arg Gly Gly Arg Val Phe Ile
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Ile Ile Pro His Asp Phe Arg Arg Met Lys Glu Phe Val Ser Thr Val
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Val Leu Leu Leu Arg Ser Gln Pro Val Leu Arg Val Lys Ala Ile Met
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Gln Val Val Lys Gly Lys Glu Ala Gly Glu Val Arg Val Cys Leu His
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Val Val Thr Tyr Asp Leu Ala Leu Asp Ser Gly Arg Pro His Ser Arg
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Ala Val Phe Asn Glu Thr Lys Asn Ser Thr Arg Arg Gln Thr Gln Val
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【国際公開パンフレット（コレクトバージョン）】

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(19) World Intellectual Property Organization
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WO 02/018583 A3

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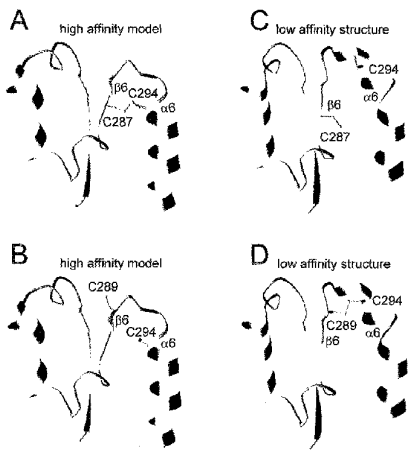
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Continued on next page]

(54) Title: MODIFIED POLYPEPTIDES STABILIZED IN A DESIRED CONFORMATION AND METHODS FOR PRODUCING SAME



(57) Abstract: The present invention provides a method for stabilizing a protein in a desired conformation by introducing at least one disulfide bond into the polypeptide. Computational design is used to identify positions where cysteine residues can be introduced to form a disulfide bond in only one protein conformation, and therefore lock the protein in a given conformation. Accordingly, antibody and small molecule therapeutics are selected that are specific for the desired protein conformation. The invention also provides modified integrin I-domain polypeptides that are stabilized in a desired conformation. The invention further provides screening assays and therapeutic methods utilizing the modified integrin I-domains of the invention.

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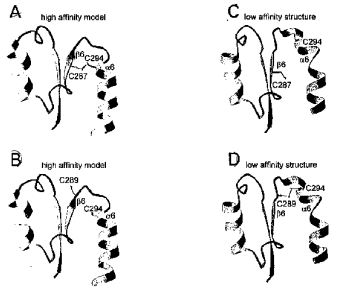
(54) **Title:** MODIFIED POLYPEPTIDES STABILIZED IN A DESIRED CONFORMATION AND METHODS FOR PRODUCING SAME



(57) **Abstract:** The present invention provides a method for stabilizing a protein in a desired conformation by introducing at least one disulfide bond into the polypeptide. Computational design is used to identify positions where cysteine residues can be introduced to form a disulfide bond in only one protein conformation, and therefore lock the protein in a given conformation. Accordingly, antibody and small molecule therapeutics are selected that are specific for the desired protein conformation. The invention also provides modified integrin I-domain polypeptides that are stabilized in a desired conformation. The invention further provides screening assays and therapeutic methods utilizing the modified integrin I-domains of the invention.

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MODIFIED POLYPEPTIDES STABILIZED IN A DESIRED CONFORMATION AND METHODS FOR PRODUCING SAME

Related Applications

This application claims priority to U.S. Provisional Patent Application No. 60/229,700 filed on September 1, 2000, incorporated herein in its entirety by reference.

Background of the Invention

The integrin family of adhesion molecules are noncovalently-associated α/β heterodimers. To date, at least fourteen different integrin α subunits and eight different β subunits have been reported (Hynes, RO (1992) *Cell* 69:1-25). Lymphocyte function-associated antigen-1 (LFA-1) is a member of the leukocyte integrin subfamily. Members of the leukocyte integrin subfamily share the common β2 subunit (CD18) but have distinct α subunits, αL (CD11a), αM (CD11b), αX (CD11c) and αd for LFA-1, Mac-1, p150.95 and αd/β2, respectively (Springer, TA (1990) *Nature* 346:425-433; Larson, RS and Springer, TA (1990) *Immunol Rev* 114:181-217; Van der Vieren, M *et al.* (1995) *Immunity* 3:683-690). The leukocyte integrins mediate a wide range of adhesive interactions that are essential for normal immune and inflammatory responses.

Both integrin α and β subunits are type I transmembrane glycoproteins, each with a large extracellular domain, a single transmembrane region and a short cytoplasmic tail. Several structurally distinct domains have been identified or predicted in the α and β subunit extracellular domains.

The N-terminal region of the integrin α subunits contains seven repeats of about 60 amino acids each, and has been predicted to fold into a 7-bladed β-propeller domain (Springer, TA (1997) Proc Natl Acad Sci USA 94:65-72). The leukocyte integrin α subunits, the α1, α2, α10, α11, and αE subunits contain an inserted domain or I-domain of about 200 amino acids (Larson, RS *et al.* (1989) *J Cell Biol* 108:703-712; Takada, Y *et al.* (1989) *EMBO J* 8:1361-1368; Briesewitz, R *et al.* (1993) *J Biol Chem* 268:2989-2996; Shaw, S K *et al.* (1994) *J Biol Chem* 269:6016-6025; Camper, L *et al.* (1998) *J Biol Chem* 273:20383-20389). The I-domain is predicted to be inserted between β-sheets 2 and 3 of the β-propeller domain. The three dimensional structure of the αM, αL, α1 and α2 I-domains has been solved and shows that it adopts the

dinucleotide-binding fold with a unique divalent cation coordination site designated the metal ion-dependent adhesion site (MIDAS) (Lee, J-O, *et al.* (1995) *Structure* 3:1333-1340; Lee, J-O, *et al.* (199S) *Cell* 80:631-638; Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Emsley, J *et al.* (1997) *J Biol Chem* 272:28512-28517; Baldwin, ET *et al.* (1998) *Structure* 6:923-935; Kallen, J *et al.* (1999) *J Mol Biol* 292:1-9). The C-terminal region of the αM subunit has been predicted to fold into a β-sandwich structure (Lu, C *et al.* (1998) *J Biol Chem* 273:15138-15147).

The integrin β subunits contain a conserved domain of about 250 amino acids in the N-terminal portion, and a cysteine-rich region in the C-terminal portion. The β conserved domain, or I-like domain, has been predicted to have an "I-domain-like" fold (Puzon-McLaughlin, W and Takada, Y (1996) *J Biol Chem* 271:20438-20443; Tuckwell, DS and Humphries, MJ (1997) *FEBS Lett* 400: 297-303; Huang, C *et al.* (2000) *J Biol Chem* 275:21514-24). The C-terminal Cys-rich region of the β subunits appears to be important in the regulation of integrin function, as a number of activating antibodies to the β1, β2 and β3 subunits bind to this region (Petruzzelli, L *et al.* (1995) *J Immunol* 155:854-866; Robinson, MK *et al.* (1992) *J Immunol* 148:1080-1085; Faull, RJ *et al.* (1996) *J Biol Chem* 271:25099-25106; Shih, DT *et al.* (1993) *J Cell Biol* 122:1361-1371; Du, X *et al.* (1993) *J Biol Chem* 268:23087-23092).

Electron microscopic images of integrins reveal that the N-terminal portions of the α and β subunits fold into a globular head that is connected to the membrane by two rod-like tails about 16 nm long corresponding to the C-terminal portions of the α and β extracellular domains (Nermut, MV *et al.* (1988), *EMBO J* 7:4093-4099; Weisel, JW *et al.* (1992) *J Biol Chem* 267:16637-16643; Wippler, J *et al.* (1994) *J Biol Chem* 269: 8754-8761).

LFA-1 is expressed on all leukocytes and is the receptor for three Ig superfamily members, intercellular adhesion molecule-1, -2 and –3) (Marlin, SD *et al.* (1987) *Cell* 51:813-819; Staunton, DE *et al.* (1989) *Nature* 339:61 -64; de Fougerolles, *et al.* (1991) *J Exp Med* 1 74: 253-267). Substantial data indicate that the I-domain of LFA-1 is critical for interaction with ligands. Mutagenesis studies have shown that residues M140, E146, T175, L205, E241, T243, S245 and K263 in the I-domain are important for ligand binding (Huang, C *et al.* (1995) *J Biol Chem* 270:19008-19016; Edwards, CP

et al. (1998) *J Biol Chem* 273:28937-28944). These residues are located on the surface of the I-domain surrounding the Mg^{2+} ion, defining a ligand binding interface on the upper surface of the I-domain. The importance of the I-domain in ligand binding is further underscored by mAb blocking studies. A large number mAbs that inhibit LFA-1 interaction with its ligands map to the I-domain (Randi, AM *et al.* (1994) *J Biol Chem* 269:12395-12398; Champe, M *et al.* (1995) *J Biol Chem* 270:1388-1394; Huang, C *et al.* (1995) *J Biol Chem* 270:19008-19016; Edwards, CP *et al.* (1998) *J Biol Chem* 273:28937-28944). Two groups have recently shown that I-domain deleted LFA-1 lacks ligand recognition and binding ability, further demonstrating the role of the I-domain in LFA-1 function (Leitinger, B *et al.* (2000) *Mol Biol Cell* 11, 677-690; Yalamanchili, P *et al.* (2000) *J Biol Chem* 275:21877-82). The I-domains of other I-domain containing integrins have also been implicated in ligand binding (Diamond, MS (1993) *J Cell Biol* 120:545556; Michishita, M *et al.* (1993) *Cell* 72:857-867; Muchowski, PJ *et al.* (1994) *J Biol Chem* 269:26419-26423; Zhou, L *et al.* (1994) *J Biol Chem* 269:17075-17079; Ueda, T *et al.* (1994) *Proc Natl Acad Sci USA* 91:10680- 10684; Kamata, T *et al.* (1994) *J Biol Chem* 269:96599663; Kern, A *et al.* (1994) *J Biol Chem 269:*22811-22816).

Binding of LFA-1 to ICAMs requires LFA-1 activation. LFA-1 can be activated by signals from the cytoplasm, called "inside-out" signaling (Diamond, MS *et al.* (1994) *Current Biology* 4:506-517). Divalent cations Mn^{2+}, Mg^{2+} and Ca^{2+} can directly modulate ligand-binding function of LFA-1 (Dransfield, I *et al.* (1989) *EMBO J* 8:3759-3765; Dransfield, I *et al.* (1992). *J Cell Biol* 116:219-226; Stewart, MP *et al.* (1996) *J Immunol* 156:1810-1817). In addition, LFA-1 can be activated by certain mAbs that bind the extracellular domains of the αL or β2 subunit (Keizer, GD *et al.* (1988) *J Immunol 140:*1393- 1400; Robinson, MK *et al.* (1992) *J Immunol* 148:1080-1085; Andrew, D *et al.* (1993) *Eur J Immunol* 23:2217-2222; Petruzzelli, L *et al.* (1995) *J Immunol* 155:854-866). The molecular mechanism for integrin activation is not yet well understood. It has been proposed that intramolecular conformational changes accompanying integrin activation increase integrin affinity for ligand, and this is supported by the existence of antibodies that only recognize activated integrins (Dransfield, I *et al.* (1989) *EMBO J* 8:3759-3765; Diamond, MS *et al.* (1993) *J Cell Biol* 120: 545-556; Shattil, SJ *et al.* (1985) *J Biol Chem* 260:11107-11114). One of such antibodies CBRLFA-1/5 binds to the Mac-1 I-domain very close to the ligand binding

site (Oxvig, C *et al.* (1999) *Proc Natl Acad Sci USA* 96:2215-2220), providing further evidence that the I-domain itself undergoes conformational changes with activation.

Two different crystal forms of the Mac-1 I-domain have been obtained, and it has been hypothesized that the two structures represent the "active" and "inactive" conformation, respectively (Lee, J-O *et al.* (1995) *Structure* 3, 1333-1340; Lee, J-O *et al.* (1995) *Cell* 80:631-638). In the "active" form, crystallized with Mg^{2+}, a glutamate from a neighboring I-domain provides the sixth metal coordination site, while in the "inactive" conformation, complexed with Mn^{2+}, a water molecule completes the metal coordination sphere. The change in metal coordination is linked to a large shift of the C-terminal α-helix; in the putative "active" conformation, the C-terminal helix moves 10 Å down the body of the I-domain (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340). Results from epitope mapping of mAb CBRM-1/5 that only recognizes activated Mac-1 have suggested that the conformational differences are physiologically (Oxvig, C *et al.* (1999) *Proc Natl Acad Sci USA* 96:2215-2220). The crystal and NMR structures of the LFA-1 I-domain have a conformation similar to the putative "inactive" conformation of the Mac-1 I-domain (Qu, A *et al.* (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A (1996) *Structure* 4:, 931-942; Kallen, J *et al.* (1999) *J Mol Biol* 292:1-9; Legge, GB *et al.* (2000) *J Mol Biol* 295:1251-1264).

In addition to integrins, many pharmaceutically important proteins exist in two alternative three-dimensional structures, referred to as conformations or conformers. Often these proteins have important signaling functions, such as small G proteins, trimeric G protein α subunits, tyrosine kinases, and G protein-coupled receptors. Typically, one of these conformations and not the other is enzymatically active or has effector functions. Therefore, antibody or small molecule therapeutics that are specific for a protein in a particular conformation, for example, the active conformation, would have great advantages over non-selective alternatives.

Summary of the Invention

Computational design can be used to introduce a disulfide bond into a protein or polypeptide such that the molecule is stabilized in a desired conformation. Accordingly, antibodies, *e.g.*, anti-LFA-1 antibodies, or small molecule therapeutics that are specific for a desired protein conformation, *e.g.*, an "open" or active conformation or a "closed" or inactive conformation can be identified.

The invention pertains to methods for stabilizing a polypeptide, *e.g.*, a polypeptide comprising a functional domain of a protein, in a desired conformation. The method comprises introducing at least one disulfide bond into the polypeptide such that the polypeptide is stabilized in a desired conformation. In a preferred embodiment the disulfide bond is formed by the introduction of at least one cysteine substitution into the amino acid sequence of the polypeptide. In another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.00-8.09Å. In another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

Computational design can be used to introduce a disulfide bond into a protein or polypeptide such that the molecule is stabilized in a desired conformation. Accordingly, antibody or small molecule therapeutics that are specific for a desired protein conformation can be identified.

The method of the invention is widely applicable to a variety of biologically and pharmaceutically important proteins that exist in two different three-dimensional conformations, including an integrin subunit, a small G protein, a heterotrimeric G protein alpha subunit, a tyrosine kinase, a G protein-coupled receptor, an enzyme under allosteric control, a zymogen, complement C3, complement C4, and fibrinogen. In a preferred embodiment, the polypeptide is an integrin I-domain polypeptide.

In another aspect, the invention provides a modified integrin I-domain polypeptide that is stabilized in a desired conformation by the introduction of at least one disulfide bond. In one embodiment, a modified integrin I-domain is encoded by an amino acid sequence containing at least one cysteine substitution as compared to the wild-type sequence, such that a disulfide bond is formed. In another embodiment, the distance between the Cβ carbons of the residues that are substituted for cysteines is in the range of 3.00-8.09Å. In yet another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

In one embodiment, a modified integrin I-domain polypeptide of the invention is derived from an I-domain of an integrin α subunit, for example, α1, α2, α10, α11, αD, αE, αL (CD11a), αM (CD11b), and αX (CD11c). For example, in one embodiment of the invention, a modified integrin I-domain polypeptide is derived from the I-domain of the human αL subunit and contains amino acid substitutions K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, L161C/T300C, or L289C/K294C. In

another embodiment of the invention, a modified integrin I-domain polypeptide is derived from the I-domain of the human αM subunit and contains amino acid substitutions Q163C/Q309C, D294C/Q311C, or Q163C/R313C.

In a preferred embodiment, a modified integrin I-domain polypeptide of the invention is stabilized in the open conformation. In another embodiment, a modified integrin I-domain polypeptide of the invention is stabilized in the closed conformation. In another embodiment, a modified integrin I-domain binds ligand with high affinity. In yet another embodiment, a modified integrin I-domain polypeptide is operatively linked to a heterologous polypeptide.

In a related aspect, the invention provides isolated nucleic acid molecules which encode a modified integrin I-domain polypeptide of the invention.

The modified integrin I-domain polypeptides, and/or biologically active or antigenic fragments thereof, are useful, for example, as reagents or targets in assays applicable to the treatment and/or diagnosis of integrin-mediated disorders.

Accordingly, in one aspect, the invention provides an antibody, or an antigen binding fragment thereof, which selectively binds to a modified integrin I-domain in the open conformation. In another aspect, the invention provides an antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain polypeptide in the open conformation, an integrin I-domain polypeptide in the closed conformation, or a modified integrin I-domain polypeptide. In one embodiment, the antibody binds to an activation specific epitope on the integrin I-domain. In another embodiment, the antibody blocks an interaction between an integrin and a cognate ligand. In one embodiment, the antibody is an anti-LFA-1 antibody, or an antigen binding fragment thereof, *e.g.*, an anti-LFA-1 antibody which reacts with or binds an open or closed conformation of an LFA-1 polypeptide, or a modified LFA-1 I-domain integrin polypeptide, or fragment thereof.

In another aspect the invention provides a method for identifying a modulator of integrin activity comprising assaying the ability of a test compound to bind to a modified integrin I-domain polypeptide which is stabilized in the open conformation. In another embodiment, the invention provides a method for identifying a compound capable of modulating the interaction of an integrin and a cognate ligand wherein binding of a ligand to a modified integrin I-domain polypeptide which is stabilized in the open conformation is assayed in the presence and absence of a test compound.

In another aspect, the invention provides a composition comprising a modified integrin I-domain polypeptide or an anti-integrin I-domain antibody (or an antigen binding fragment thereof), such composition can further include a pharmaceutically acceptable carrier.

In yet another aspect, the invention pertains to methods for treating or preventing an integrin-mediated disorder (*e.g.*, an inflammatory or autoimmune disorder) in a subject, or for inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to a subject a therapeutically effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation or an antibody (or antigen binding fragment thereof) which selectively binds to an integrin I-domain in the open conformation. In one embodiment, the antibody is an LFA-1 antibody, or an antigen binding fragment thereof, which specifically reacts with or binds an LFA-1 I-domain in the open conformation or specifically reacts with or binds a modified LFA-1 I-domain polypeptide. In a preferred embodiment, the modified integrin I-domain polypeptide binds ligand with high affinity. In another preferred embodiment, the modified integrin I-domain polypeptide for therapeutic use is a soluble polypeptide, *e.g.*, a fusion protein.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Figures

Figure 1 is a stereodiagram of the high affinity model of the LFA-1 I domain, with mutations to introduce a disulfide bond. The model was prepared using segments of the putative high affinity Mac-1 I domain structure and a putative low affinity LFA-1 I domain structure as templates. The K287C and K294C mutations were included in the model. The sidechains and disulfide bond of C287 and C294 are shown in yellow. The Mg^{2+} ion of the MIDAS is shown as a gold sphere. Sidechains of residues important in binding to ICAM-1 and ICAM-2 are shown with rose-pink sidechains and yellow sulfur, red oxygen, and blue nitrogen atoms. These residues, defined as important in species-specific binding to ICAM-1 (Huang, C and Springer, TA (1995) *J Biol Chem* 270:19008-19016) or by at least a 2-fold effect on binding to ICAM-1 or ICAM-2 upon mutation to alanine (Edwards, CP *et al.*, (1998) *J Biol Chem* 273:28937-28944), are

M140, E146, T175, L205, E241, T243, S245, and K263. Note that these residues surround the Mg^{2+} ion, and are distant from the disulfide. Prepared with RIBBONS (Carson, M (1997) Methods in Enzymology, RM Sweet and CW Carter eds., Academic Press pp. 493-505).

Figure 2 depicts predicted disulfide bonds that are selective for high affinity or low affinity conformers of the LFA-1 I domain. The K287C/K294C mutation (*Panels A, C*) and L289C/K294C mutation (*Panels B, D*) were modeled in both high affinity (*Panels A, B*) and low affinity (*Panels C, D*) I domain conformers. Residues 254 to 305 of the models are shown. The four models were superimposed using residues not involved in conformational shifts and were used in exactly the same orientation for figure preparation. Therefore, the downward shift in the α6 helix in panels A and B compared to panels C and D is readily apparent. The remodeling of the loop connecting β6 and α6 is accompanied by a reversal in the orientation of the sidechain of residue 289 (panel B compared to panel D). Prepared with RIBBONS.

Figure 3 depicts the cell surface expression of LFA-1 cysteine substitution mutants on 293T transient transfectants (*Panel A*), and K562 stable transfectants (*Panel B*) as determined by flow cytometric analysis using monoclonal antibody TS2/4 (shaded histogram) to αL in αL/β2 complex, or the nonbinding antibody X63 (open histogram). Numbers in the parentheses are clone numbers of the K562 stable transfectants.

Figure 4 depicts the binding of LFA-1transfectants to immobilized ICAM-1. *Panel A*, 293T transient transfectants, and *Panels B and C*, K562 stable transfectants. In Panels A and B, binding of the transfectants to immobilized ICAM-1 was determined in L15 medium containing Ca^{2+} and Mg^{2+} in the presence or absence (control) of the activating antibody CBRLFA-1/2 at 10 μg/ml. In Panel C, the binding assay was performed in TBS, pH7.5 supplemented with divalent cations or EDTA as indicated. Numbers in the parentheses are clone numbers of the K562 stable transfectants. Results are mean ± SD of triplicate samples and representative of at least three experiments.

Figure 5 depicts the binding of soluble ICAM-1-IgA fusion protein to K562 transfectants that express wild-type LFA-1, the predicted high-affinity mutant K287C/K294C, or mutant L289C/K294C as assessed by flow cytometric analysis. Mean fluorescent intensity of ICAM-1-IgA binding is indicated on the upper right corner of the histogram plot. Numbers in the parentheses are clone numbers of the K562 stable transfectants. Results are representative of three experiments.

Figure 6 depicts the inhibitory activity of lovastatin on ligand binding by cells expressing activated wild-type and high affinity (K287C/K294C) LFA-1.

Figure 7 depicts the cell surface expression of the isolated LFA-1 I-domains. The wild-type αL I-domain and the mutant K287C/K294C and L289C/K294C I-domains were expressed on the surface of the K562 transfectants by the PDGFR transmembrane domain. The level of cell surface I-domain was determined by flow cytometry using monoclonal antibody TS1/22 to the I-domain (shaded histogram). Binding of the control mAb X63 is shown as open histograms. Mean fluorescent intensity of TS1/22 binding was indicated on the upper right corner of the histogram plot. Results of two individual clones (#1 and #2) from each I-domain transfectants are shown.

Figure 8 depicts the ligand binding activity of the cell surface expressed LFA-1 I-domains. *Panel A*, Binding of K562 transfectants to immobilized ICAM-1 in the presence or absence of DTT. Binding was performed in the presence (white bar) or absence (black bar) of DTT. *Panel B*, Effect of divalent cations on binding of K562 transfectants to ICAM-1. Binding was performed in the presence of Mn^{2+} (black bar), Mg^{2+} (shaded bar) or EDTA (white bar). In Panels A and B, two clones (#1 and #2) of the transfectants expressing the wild-type I-domain or mutant I-domain were tested. *Panel C* , Effect of LFA-1 blocking antibodies on binding of the K287C/K294C I-domain to ICAM-1. Results are mean ± SD of triplicate samples and representative of 3 experiments.

Figure 9 depicts the surface plasmon resonance sensograms by BIAcore™ recording the interaction of the open (K287C/K294C) or wild-type I-domain with ligands, ICAM-1 (*Panels A and B*), ICAM-2 (*Panels C and D*), and ICAM-3 (*Panels E and F*).

Figure 10 depicts the inhibition of LFA-1-dependent adhesion *in vitro* by the open αL I-domain. *Panel A* depicts the adhesion of K562 stable transfectants expressing wild-type LFA-1 to immobilized ICAM-1 in the presence of soluble wild-type (closed circles) or open (K287C/K294C) I-domain (open circles); *Panel B* depicts the homotypic aggregation of the murine EL-4 T lymphoma cell line in the presence of soluble wild-type (closed circles) or open (K287C/K294C) I-domain (open circles).

Figure 11 depicts the expression and ligand binding activity of the Mac-1 cysteine substitution mutants in transiently transfected 293T cells. *Panel A*, binding of monoclonal antibodies CBRM1/32 (open bars) and CBRM1/5 (black bars) to intact Mac-1 I-domain mutants. *Panel B*, adhesion of 293T transient transfectants expressing intact Mac-1 cysteine substitution mutants to iC3b coated on plastic. *Panel C*, adhesion of 293T transient transfectants expressing isolated Mac-1mutant I-domains to iC3b ligand in the presence (black bars) or absence (open bars) of antibody CBRM1/5.

Figure 12 depicts the expression and ligand binding activity of the Mac-1 cysteine substitution mutants in K562 stable transfectants. *Panel A*, representative histogram showing binding of monoclonal antibodies CBRM1/32 and CBRM1/5 to intact Mac-1 I-domain mutants as assessed by flow cytometry. Mean fluorescent intensity is indicated in the upper right hand corner of the histogram plot. *Panel B*, adhesion of K562 stable transfectants expressing intact Mac-1 cysteine substitution mutants to iC3b coated on plastic. *Panel C*, adhesion of K562 stable transfectants expressing isolated Mac-1 I-mutant I-domains to iC3b ligand. Adhesion was assayed in the presence (black bars) or absence (open bars) of monoclonal antibody CBRM1/5, or in the presence of 10 mM DTT (gray bars).

Detailed Description of the Invention

The present invention is based, at least in part, on a method for stabilizing a polypeptide in a desired conformation by introducing at least one disulfide bond into the polypeptide. In one embodiment, based on NMR or crystal structures of specific protein conformations, computational design is used to introduce a disulfide bond that locks the protein in a particular conformation. As used herein, a "conformation" or "conformer" refers to the three dimensional structure of a protein. A "desired" conformation includes a protein conformation that is conducive to a particular use of the polypeptide, *e.g.*, a conformation that supports a particular biological function and/or activity, or a therapeutic effect. As used herein, the terms "polypeptide" and "protein" are used interchangeably throughout.

In one embodiment, a desired conformation is a protein conformation which promotes or activates a biological function and/or activity, *e.g.*, an open or active conformation. In another embodiment, a desired conformation is a protein conformation

which inhibits or suppresses a biological function and/or activity, *e.g.*, a closed or inactive conformation.

In particular, the method of the invention includes modeling a protein, or a functional domain thereof, on a template of the desired three-dimensional structure of the protein and introducing cysteines which are able to form a disulfide bond only in the desired conformation of the protein, thus stabilizing the protein in that particular conformation. The protein can be any protein, or domain thereof, for which a three dimensional structure is known or can be generated, and is preferably a protein that exists in two different conformations. Computational algorithms for designing and/or modeling protein conformations are described, for example, in WO 98/47089. The SSBOND program (Hazes, B and Dijkstra, BW (1988) *Protein Engineering* 2:119-125) can be used to identify positions where disulfide bonds can be introduced in a protein structure by mutating appropriately positioned pairs of residues to cysteine.

Disulfide bond formation occurs between two cysteine residues that are appropriately positioned within the three-dimensional structure of a polypeptide. In one embodiment of the invention, a polypeptide is stabilized in a desired conformation by introducing at least one cysteine substitution into the amino acid sequence such that a disulfide bond is formed. The introduction of a single cysteine substitution is performed in circumstances in which an additional cysteine residue is present in the native amino acid sequence of the polypeptide at an appropriate position such that a disulfide bond is formed. In a preferred embodiment, two cysteine substitutions are introduced into the amino acid sequence of the polypeptide at positions that allow a disulfide bond to form, thereby stabilizing the polypeptide in a desired conformation. In another embodiment, the distance between the Cβ carbons of the residues that are substituted for cysteine is 3.00-8.09Å. In yet another embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

In one embodiment of the invention, cysteine substitutions are introduced such that the formation of a disulfide bond is favored only in one protein conformation, such that the protein is stabilized in that particular conformation.

Preparation of a modified polypeptide of the invention by introducing cysteine substitutions is preferably achieved by mutagenesis of DNA encoding the polypeptide of interest (*e.g.*, an integrin polypeptide). For example, an isolated nucleic acid molecule encoding a modified integrin I-domain polypeptide can be created by introducing one or

more nucleotide substitutions into the nucleotide sequence of an integrin gene such that one or more amino acid substitutions, *e.g.*, cysteine substitutions, are introduced into the encoded protein. Mutations can be introduced into a nucleic acid sequence by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

Suitable proteins include, but are not limited to, industrially and therapeutically important proteins such as: 1) signaling molecules, such as small G proteins, trimeric G protein alpha subunits, tyrosine kinases, and G protein-coupled receptors; 2) enzymes under allosteric control, 3) zymogens that undergo conformational change after activation by proteolytic cleavage, such as the proteases (convertases and factors) of the complement and clotting cascades, and 4) proteolytically activated effector molecules such as complement components C3 and C4, and fibrinogen. In one embodiment, the method of the invention can be used to stabilize a protein in a biologically active conformation, *e.g.*, a conformation that is enzymatically active or has ligand binding capacity and/or effector functions, *e.g.*, an "open" conformation. In another embodiment, the method of the invention can be used to stabilize a protein in a biologically inactive conformation, *e.g.*, a conformation that is enzymatically inactive or does not have ligand binding capacity and/or effector functions, *e.g.*, a "closed" conformation.

Proteins that are stabilized in a particular conformation may find use in, for example, in proteomic screening technologies. In proteomic screens of tissues and disease states, antibodies, polypeptide, and/or small molecules that are specific for, *e.g.*, an active protein conformer or an inactive protein conformer, can be used to assess the activity of different cellular signaling, metabolic, and adhesive pathways. Thus, associations can be made between specific diseases and the activation of specific biochemical and signaling pathways. Furthermore, the invention relates to the polypeptides, antibodies, and small molecules identified using the methods described herein and uses for same, *e.g.*, to treat, for example, inflammatory disorders. Conformer-specific reagents can also be placed on chips and used to screen tissue extracts, or used to stain tissue sections. Furthermore, drugs or antibodies, *e.g.*, anti-integrin antibodies which specifically recognize a modified integrin I-domain polypeptide, *e.g.*, an anti-LFA-1 antibody which specifically recognizes a modified LFA-1 I-domain polypeptide, that are selective for a particular conformer, *e.g.*, an open conformer or a closed conformer, may provide differential therapeutic effects. Therefore, selective screening

assays using a protein stabilized in a particular conformer can be used to rationally obtain compounds with a desired activity.

Integrins

Integrins exist on cell surfaces in an inactive conformation that does not bind ligand. Upon cell activation, integrins change shape (conformation) and can bind ligand. Over 20 different integrin heterodimers (different α and β subunit combinations) exist that are expressed in a selective fashion on all cells in the body. After activation, integrins bind in a specific manner to protein ligands on the surface of other cells, in the extracellular matrix, or that are assembled in the clotting or complement cascades. Integrins on leukocytes are of central importance in leukocyte emigration and in inflammatory and immune responses. Ligands for the leukocyte integrin Mac-1 (αMβ2) include the inflammation-associated cell surface molecule ICAM-1, the complement component iC3b, and the clotting component fibrinogen. Ligands for the leukocyte integrin LFA-1 (αLβ2) include ICAM-1, ICAM-2, and ICAM-3. Antibodies to leukocyte integrins can block many types of inflammatory and auto-immune diseases, by, *e.g.,* modulating, *e.g.,* inhibiting, for example, cell to cell interactions or cell to extracellular matrix interactions. Integrins on platelets are important in clotting and in heart disease; approved drugs include the antibody abciximab (Reopro™) and the peptide-like antagonist eptifibatide (Integrilin™). Integrins on connective tissue cells, epithelium, and endothelium are important in disease states affecting these cells. They regulate cell growth, differentiation, wound healing, fibrosis, apoptosis, and angiogenesis. Integrins on cancerous cells regulate invasion and metastasis.

To antagonize integrins, drugs are needed that bind to the active, ligand-binding conformation. Most antibodies bind to both the active and inactive conformations, since only a small portion of the surface of the integrin molecule changes shape. It is desirable that antibodies bind only to the active integrin conformation, *e.g.,* the "open" conformation, because binding to the inactive conformation can lead to side reactions, generation of anti-idiotypic antibodies, and result in clearance of the antibody and, thus, requires much higher doses to be administered.

The methods described herein have been successfully used to introduce disulfide bonds into the I domains of the integrins, *e.g.*, LFA-1 and Mac-1. Accordingly, in another aspect, the invention provides a modified integrin I-domain polypeptide containing at least one disulfide bond, such that said modified I-domain polypeptide is stabilized in a desired conformation. A modified integrin I-domain polypeptide of the invention may be derived from an I-domain of an integrin α subunit including α1, α2, α10, α11, αD, αE, αL (CD11a), αM (CD11b) and αX (CD11c).

As used herein, a "modified integrin I-domain polypeptide" or "modified integrin polypeptide" includes an integrin I-domain polypeptide that has been altered with respect to the wild-type sequence or the native state such that at least one disulfide bond has been introduced into the polypeptide thereby stabilizing the I-domain in a desired conformation.

The terms "derived from" or "derivative", as used interchangeably herein, are intended to mean that a sequence is identical to or modified from another sequence, *e.g.*, a naturally occurring sequence. Derivatives within the scope of the invention include polynucleotide and polypeptide derivatives. Polypeptide or protein derivatives include polypeptide or protein sequences that differ from the sequences described or known in amino acid sequence, or in ways that do not involve sequence, or both, and still preserve the activity of the polypeptide or protein. Derivatives in amino acid sequence are produced when one or more amino acid is substituted with a different natural amino acid, an amino acid derivative or non-native amino acid. In certain embodiments protein derivatives include naturally occurring polypeptides or proteins, or biologically active fragments thereof, whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Derivatives may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the biological activity of the polypeptide or protein.

Conservative substitutions (substituents) typically include the substitution of one amino acid for another with similar characteristics (*e.g.*, charge, size, shape, and other biological properties) such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar

(hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In other embodiments, derivatives with amino acid substitutions which are less conservative may also result in desired derivatives, *e.g.*, by causing changes in charge, conformation and other biological properties. Such substitutions would include, for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics. The polypeptides and proteins of this invention may also be modified by various changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use.

In a preferred embodiment, a modified integrin I-domain polypeptide is stabilized in the open conformation, and binds ligand with high affinity.

In one embodiment, a modified integrin I-domain polypeptide of the invention is encoded by an amino acid sequence containing at least one cysteine substitution, and preferably two cysteine substitutions, as compared to the wild-type sequence. In another embodiment, the distance between the Cβ carbons of the residues that are substituted for cysteines is in the range of 3.00-8.09Å, *e.g.*, as predicted by protein modeling. In a further embodiment, the distance between the Cβ carbons in the disulfide bond is in the range of 3.41-7.08Å.

The introduction of cysteine residues at appropriate positions within the amino acid sequence of the I-domain polypeptide allows for the formation of a disulfide bond that stabilizes the domain in a particular conformation, e.g., an active "open" conformation, or an inactive "closed" conformation. For example, the αL K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, L161C/T300C, and L289C/K294C mutants, and the αM Q163C/Q309C and D294C/Q311C mutants are

stabilized in the "open" conformation that bind ligand with high or intermediate affinity, whereas the αL L289C/K294C mutant and the αM Q163C/R313C mutants are stabilized in an inactive or "closed" conformation that does not bind ligand. The affinity of E284C/E301C is nearly comparable to that of K287C/K294C, *e.g.*, high-affinity. The affinity of L161C/F299C, K160C/F299C, and L161C/T300C are significantly higher than wild-type, but 20-30 times lower than high-affinity αL I-domain, K287C/K294C. L161C/F299C, K160C/F299C, and L161C/T300C are referred to herein as intermediate affinity αL I-domains.

In one embodiment, the invention provides a modified integrin I-domain which is comprised within an integrin α subunit, and which may be further associated with an integrin β subunit. In another embodiment, a modified integrin I-domain polypeptide of the invention is a soluble polypeptide. Furthermore, the invention provides a modified integrin I-domain polypeptide which is operatively linked to a heterologous polypeptide.

A model of the I-like domain of the integrin β-subunit that is supported by experimental data (Huang, C *et al.* (2000) *J Biol Chem* 275:21514-24) has also been made. The data confirm the location of the key C-terminal α-helix that undergoes the dramatic 10 Å conformational movement in I domains. The I and I-like domains align well in this region. Accordingly, in another aspect, the invention provides a modified integrin I-like domain polypeptide containing at least one disulfide bond, such that said modified I-like domain polypeptide is stabilized in a desired conformation.

In a preferred embodiment, a modified integrin I-like domain polypeptide is stabilized in the open conformation, and binds ligand with high affinity. In one embodiment, a modified integrin I-like domain polypeptide of the invention is encoded by an amino acid sequence containing at least one cysteine substitution, and preferably two cysteine substitutions, as compared to the wild-type sequence.

In one embodiment, the invention provides a modified integrin I-like domain which is comprised within an integrin β subunit, and which may be further associated with an integrin α subunit. In another embodiment, a modified integrin I-like domain polypeptide of the invention is a soluble polypeptide. Furthermore, the invention provides a modified integrin I-like domain polypeptide which is operatively linked to a heterologous polypeptide.

Integrins are key targets in many diseases. Accordingly, isolated high affinity I-domains of the invention, as well as antibodies, or small molecule antagonists selective for activated leukocyte integrins can be used to modulate, *e.g.*, inhibit or prevent, autoimmune and inflammatory disease, transplant rejection, and ischemia/reperfusion injury as in hypovolemic shock, myocardial infarct, and cerebral shock. Furthermore, co-crystals of high affinity I domains bound to natural ligands and/or small molecule antagonists can readily be made, which will enable computational drug design, and advance modification and improvement of drug development candidates.

Accordingly, the invention provides a method for identifying a modulator of integrin activity comprising assaying the ability of a test compound to bind to a modified integrin I-domain polypeptide which is stabilized in the open conformation. In another embodiment, the invention provides a method for identifying a compound capable of modulating the interaction of an integrin and a cognate ligand wherein binding of a ligand to a modified integrin I-domain polypeptide which is stabilized in the open conformation is assayed in the presence and absence of a test compound.

The invention also provides a composition comprising a modified integrin I-domain polypeptide or an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody (or an antigen binding fragment thereof) which selectively binds to a modified integrin I-domain, *e.g.*, an I-domain in the open conformation, and a pharmaceutically acceptable carrier. The compositions of the invention are used in therapeutic methods of the invention. For example, the invention provides methods for treating or preventing an integrin-mediated disorder (*e.g.*, an inflammatory or autoimmune disorder) in a subject, or for inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to a therapeutically effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation or anti-integrin antibody (or an antigen binding fragment thereof) which selectively binds to an integrin I-domain in the open conformation. In a preferred embodiment, the modified integrin I-domain polypeptide binds ligand with high affinity. In another preferred embodiment, the modified integrin I-domain polypeptide for therapeutic use is a soluble polypeptide, *e.g.*, a fusion protein.

As used herein, an integrin mediated disorder includes, for example, an inflammatory or immune system disorder, and/or a cellular proliferative disorder. Examples of integrin-mediated disorders include myocardial infarction, stroke, restenosis, transplant rejection, graft versus host disease or host versus graft disease, and

reperfusion injury. An inflammatory or immune system disorder includes, but is not limited to adult respiratory distress syndrome (ARDS), multiple organ injury syndromes secondary to septicemia or trauma, viral infection, inflammatory bowel disease, ulcerative colitis, Crohn's disease, leukocyte adhesion deficiency II syndrome, thermal injury, hemodialysis, leukapheresis, peritonitis, chronic obstructive pulmonary disease, lung inflammation, asthma, acute appendicitis, dermatoses with acute inflammatory components, wound healing, septic shock, acute glomerulonephritis, nephritis, amyloidosis, reactive arthritis, rheumatoid arthritis, chronic bronchitis, Sjorgen's syndrome, sarcoidosis, scleroderma, lupus, polymyositis, Reiter's syndrome, psoriasis, dermatitis, pelvic inflammatory disease, inflammatory breast disease, orbital inflammatory disease, immune deficiency disorders (e.g., HIV, common variable immunodeficiency, congenital X-linked infantile hypogammaglobulinemia, transient hypogammaglobulinemia, selective IgA deficiency, necrotizing enterocolitis, granulocyte transfusion associated syndromes, cytokine-induced toxicity, chronic mucocutaneous candidiasis, severe combined immunodeficiency), autoimmune disorders, and acute purulent meningitis or other central nervous system inflammatory disorders.

A "cellular proliferative disorder" includes those disorders that affect cell proliferation, activation, adhesion, growth, differentiation, or migration processes. As used herein, a "cellular proliferation, activation, adhesion, growth, differentiation, or migration process" is a process by which a cell increases in number, size, activation state, or content, by which a cell develops a specialized set of characteristics which differ from that of other cells, or by which a cell moves closer to or further from a particular location or stimulus. Disorders characterized by aberrantly regulated growth, activation, adhesion, differentiation, or migration. Such disorders include cancer, *e.g.*, carcinoma, sarcoma, lymphoma or leukemia, examples of which include, but are not limited to, breast, endometrial, ovarian, uterine, hepatic, gastrointestinal, prostate, colorectal, and lung cancer, melanoma, neurofibromatosis, adenomatous polyposis of the colon, Wilms' tumor, nephroblastoma, teratoma, rhabdomyosarcoma; tumor invasion, angiogenesis and metastasis; skeletal dysplasia; hematopoietic and/or myeloproliferative disorders.

Various aspects of the invention are described in further detail in the following subsections.

Modified Integrin I-domain Polypeptides and Anti-Integrin I-domain Antibodies

The methods of the invention include the use of isolated, modified integrin polypeptides, and biologically active portions thereof. As used herein, a modified integrin polypeptide includes a modified I-domain polypeptide and a modified I-like domain polypeptide. Modified integrin polypeptides of the invention include modified integrin I-domain and I-like domain polypeptides that are comprised within an integrin α or β subunit polypeptide, respectively; soluble modified integrin I-domain and I-like domain polypeptides; and modified integrin I-domain and I-like domain polypeptides that are operatively linked to a heterologous polypeptide, *e.g.*, fusion proteins.

The cDNAs for multiple human integrin α and β subunit polypeptides have been cloned and sequenced, and the polypeptide sequences have been determined (see, for example, GenBank Accession Numbers: NM_002203 (α2), AF112345 (α10), NM_012211 (α11), NM_005353 (αD), NM_002208 (αE), NM_000887 (αX), NM_000632 (αM), NM_002209 (αL), X68742 and P56199 (α1), NM_000211 (β2), NM_000212 (β3), NM_002214 (β8)). In particular, the polypeptide sequences encoding human αL and αM are set forth as SEQ ID NO:2 (GenBank Accession No. P20701) and SEQ ID NO:4 (GenBank Accession No. P11215), respectively. In addition, the sequences encoding integrin α and β subunit polypeptides from other species are available in the art. Furthermore, as described previously, three dimensional structure of the αM, αL, α1 and α2 I-domains has been solved (Lee, J-O, *et al.* (1995) *Structure* 3:1333-1340; Lee, J-O, *et al.* (199S) *Cell* 80:631-638; Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Emsley, J *et al.* (1997) *J Biol Chem* 272:28512-28517; Baldwin, ET *et al.* (1998) *Structure* 6:923-935; Kallen, J *et al.* (1999) *J Mol Biol* 292:1-9).

Isolated modified integrin polypeptides of the present invention preferably have an amino acid sequence that is sufficiently identical to the amino acid sequence of a native integrin polypeptide, yet which comprise at least one, and preferably two cysteine substitutions, such that a disulfide bond is formed that stabilizes the polypeptide in a desired conformation. As used herein, the term "sufficiently identical" refers to an amino acid (or nucleotide) sequence which contains a sufficient or minimum number of identical

or equivalent (*e.g.*, an amino acid residue that has a similar side chain) amino acid residues (or nucleotides) to a integrin amino acid (or nucleotide) sequence such that the polypeptide shares common structural domains or motifs, and/or a common functional activity with a native integrin polypeptide. For example, amino acid or nucleotide sequences which share at least 30%, 40%, or 50%, preferably 60%, more preferably 70%, 75%, 80%, 85% or 90%, 91%, 92%, 93%, 94%, 95% or greater identity and share a common functional activity (*e.g.*, an activity of a modified integrin I-domain or I-like domain as described herein) are defined herein as sufficiently identical. An integrin I-domain polypeptide may differ in amino acid sequence from the integrin polypeptides disclosed herein due to natural allelic variation or mutagenesis.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet

another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

As used herein, a "biologically active portion" of a modified integrin polypeptide (*e.g.*, a modified integrin I-domain polypeptide) includes a fragment of a modified integrin polypeptide which retains a modified integrin polypeptide activity. Typically, a biologically active portion of a modified integrin polypeptide comprises at least one domain or motif with at least one activity of the modified integrin polypeptide, *e.g.*, ligand binding. In a preferred embodiment, biologically active portions of a modified integrin polypeptide include modified integrin I-domain polypeptides. Biologically active portions of a modified integrin polypeptide may comprise amino acid sequences sufficiently identical to or derived from the amino acid sequence of a modified integrin polypeptide, which include less amino acids than the full length modified integrin polypeptide, and exhibit at least one activity of a modified integrin polypeptide. Biologically active portions of a modified integrin polypeptide, e.g., a modified I-domain or I-like domain, can be used as targets for developing agents which modulate a integrin polypeptide activity, *e.g.*, ligand binding, adhesion, *e.g.*, cell to cell adhesion or cell to extracellular matrix adhesion, and/or signaling activity. A biologically active portion of a modified integrin polypeptide comprises a polypeptide which can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a modified integrin polypeptide.

In a preferred embodiment, modified integrin polypeptides are produced by recombinant DNA techniques. For example, a modified integrin polypeptide can be isolated from a host cell transfected with a polynucleotide sequence encoding a modified integrin polypeptide (*e.g.*, a I-domain polypeptide or a soluble I-domain fusion protein) using an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a modified integrin polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" polypeptide or protein, or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the source, *e.g.*, the cellular source, from which the modified integrin I-domain polypeptide is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of modified integrin polypeptide in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of modified integrin polypeptide having less than about 30% (by dry weight) of non-modified integrin polypeptide (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-modified integrin polypeptide, still more preferably less than about 10% of non-modified integrin polypeptide, and most preferably less than about 5% non-modified integrin polypeptide. When the modified integrin polypeptide or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of modified integrin polypeptide in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of modified integrin polypeptide having less than about 30% (by dry weight) of chemical precursors or non-modified integrin polypeptide chemicals, more preferably less than about 20% chemical precursors or non-modified integrin polypeptide chemicals, still more preferably less than about 10% chemical precursors or non-modified integrin polypeptide chemicals, and most preferably less than about 5% chemical precursors or non-modified integrin polypeptide chemicals.

The methods of the invention may also use modified integrin polypeptides that are chimeric or fusion proteins. As used herein, a modified integrin "chimeric protein" or "fusion protein" comprises a modified integrin polypeptide operatively linked to a non-modified integrin polypeptide, *e.g.*, a heterologous polypeptide. In a preferred embodiment, a modified integrin fusion protein comprises at least an I-domain or an I-like domain. Within the fusion protein, the term "operatively linked" is intended to

indicate that the modified integrin polypeptide and the heterologous polypeptide sequences are fused in-frame to each other. The heterologous polypeptide can be fused to the N-terminus or C-terminus of the modified integrin polypeptide.

For example, in a preferred embodiment, the fusion protein is a modified integrin-I-domain fusion protein in which the Fc region, *e.g.*, the hinge, C1 and C2 sequences, of an immunoglobulin, (*e.g.*, human IgG1) is fused to the C-terminus of the modified integrin sequences. Integrin immunoglobulin chimeras can be constructed essentially as described in WO 91/08298. Such fusion proteins can facilitate the purification of recombinant modified integrin polypeptides. In another embodiment, the fusion protein is a modified integrin I-domain polypeptide fused to a heterologous transmembrane domain, such that the fusion protein is expressed on the cell surface.

The modified integrin polypeptides and fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. In an exemplary embodiment, a soluble modified integrin I-domain polypeptide stabilized in an open, ligand binding conformation, or fusion protein thereof may be used to modulate integrin activity (e.g., integrin binding to a cognate ligand) in a subject. In another embodiment, a soluble modified integrin I-domain polypeptide or fusion protein may be used to treat an inflammatory or immune system disorder, *e.g.*, an autoimmune disorder. In another embodiment, a soluble modified integrin polypeptide or fusion protein may be used to treat a cellular proliferative disease. Use of soluble modified integrin polypeptides and fusion proteins can also be used to affect the bioavailability of a integrin ligand, *e.g.*, ICAM.

Moreover, the modified integrin polypeptides and fusion proteins of the invention can be used as immunogens to produce anti-integrin antibodies in a subject, *e.g.*, anti-LFA-1 antibodies, and in screening assays to identify molecules which modulate integrin activity, and/or modulate the interaction of a integrin polypeptide with a integrin ligand or receptor.

Preferably, a modified integrin fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid

undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A modified integrin polypeptide-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the modified integrin polypeptide.

The methods of the present invention may also include the use of modified integrin polypeptides which function as either integrin agonists (mimetics) or as integrin antagonists. An agonist of an integrin polypeptide can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of a integrin polypeptide. An antagonist of an integrin polypeptide can inhibit one or more of the activities of a native form of the integrin polypeptide by, for example, competitively modulating an integrin activity. Thus, specific biological effects can be elicited by treatment with a modified integrin polypeptide stabilized in a desired conformation.

An isolated, modified integrin polypeptide, *e.g.*, a modified LFA-1 polypeptide, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind to a specific conformation of an integrin, *e.g.*, an integrin I-domain, using standard techniques for polyclonal and monoclonal antibody preparation (see, generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Moreover, the ordinarily skilled artisan will appreciate that there are many variations of such methods which also would be useful. Preparation of anti-LFA-1 antibodies is described in, for example, United States Patent No. 5,622,700, the entire content of which is incorporated herein by this reference.

The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, *e.g.*, an integrin I-domain in an open or closed conformation, or a modified integrin I-

domain, such as an LFA-1 I-domain, *e.g.*, an open or closed LFA-1 I-domain or a modified integrin I-domain of LFA-1. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind a modified integrin polypeptide *e.g.*, a modified LFA-1 polypeptide, or a portion or fragment thereof. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of a modified integrin polypeptide, *e.g.*, a modified LFA-1 polypeptide, or a portion or fragment thereof. A monoclonal antibody composition thus typically displays a single binding affinity for a particular modified integrin polypeptide, or a portion or fragment thereof with which it immunoreacts.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-integrin antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with a modified integrin polypeptide, *e.g.*, a modified integrin I-domain stabilized in the open conformation, to thereby isolate immunoglobulin library members that bind to an conformation specific epitope on an integrin polypeptide, *e.g.*, an open conformation. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). With regard to screening for phage libraries with integrin I-domains locked in the high affinity conformation with a disulfide bond, note that it should be possible to elute specific phage by adding a reducing agent that breaks the disulfide and abolishes the high affinity conformation of the I-domain.

Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT International Publication No. WO 92/18619; Dower *et al.* PCT International Publication No. WO 91/17271; Winter *et al.* PCT International Publication WO 92/20791; Markland *et al.* PCT International Publication No. WO 92/15679; Breitling *et al.* PCT International Publication WO 93/01288; McCafferty *et al.* PCT International Publication No. WO 92/01047; Garrard *et al.* PCT International Publication No. WO 92/09690; Ladner *et al.* PCT International

Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al. Nature* (1990) 348:552-554.

Additionally, recombinant anti-integrin antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, can also be used in the methods of the present invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent No. 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In a preferred embodiment, an anti-integrin antibody of the invention binds selectively to an integrin I-domain in the open, high-affinity conformation, *e.g.*, at an epitope that is unique to an activated integrin (also referred to herein as an activation specific epitope). In a preferred embodiment, an anti-integrin antibody of the invention modulates (*e.g.*, inhibits) the binding interaction between an activated integrin and its cognate ligand. In another embodiment, an anti-integrin antibody inhibits leukocyte adhesion and/or aggregation. In another embodiment, an anti-integrin antibody of the invention binds selectively to an integrin I-domain in an open conformation, *e.g.*, an

LFA-1 I-domain in an open conformation, or a modified integrin I-domain, *e.g.*, a modified I-domain of an LFA-1 molecule.

An anti-integrin antibody (*e.g.*, a monoclonal antibody) can be used in the methods of the invention to modulate the expression and/or activity of an integrin or an integrin I-domain polypeptide. An anti-integrin antibody can also be used to isolate modified integrin or integrin I-domain polypeptides, *e.g.*, a modified LFA-1 polypeptide, or fusion proteins by standard techniques, such as affinity chromatography or immunoprecipitation. In another embodiment, an anti-integrin antibody can be used to remove and/or kill cells expressing activated integrin. Moreover, an anti-integrin antibody can be used to detect integrin polypeptides in a particular conformation (*e.g.*, an activated integrin), for example, for the localization of stimulated and/or activated leukocytes. Furthermore, an anti-integrin antibody, *e.g.*, an antibody which reacts with or binds an integrin I-domain in an open conformation or a modified integrin I-domain, can be used therapeutically as described herein. Accordingly anti-integrin antibodies can be used diagnostically to monitor protein levels in blood as part of a clinical testing procedure, *e.g.*, to, for example, detect inflammation. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Isolated Nucleic Acid Molecules

The invention includes the use of isolated nucleic acid molecules that encode integrin polypeptides (*e.g.*, a modified integrin I-domain polypeptide, *e.g.*, a modified integrin I-domain or I-like domain polypeptide) or biologically active portions thereof.

As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleotide sequences encoding the wild-type human αL and αM polypeptides are set forth as SEQ ID NO:1 (GenBank Accession No. NM_002209) and SEQ ID NO:3 (Genbank Accession No. J03925), respectively. The isolated nucleic acid molecules of the present invention include the nucleotide sequences of SEQ ID NO:1 and SEQ ID NO:3, which encode the modified amino acid sequences of the αL and αM mutants described herein, *e.g.*, identified below in Table 9. Table 9 illustrates the specific nucleotide residues which are altered to result in the modified αL and αM mutants as described herein. For example, the αL K287C/K294C mutant is a modified αL polypeptide, wherein there is a change in the amino acid sequence of αL (SEQ ID NO:2) such that amino acid residues 287 and 294 are substituted with cysteine residues. The corresponding wild-type nucleotide sequence, SEQ ID NO:1, is modified at nucleotide residues 1022-1024 and 1143-1145, respectively. Therefore, as shown in Table 9, for the αL K287C/K294C mutant at amino acid K287, the corresponding nucleotide residues in the wild-type αL nucleic acid sequence (SEQ ID NO:1), nucleotide residues 1022-1024, are modified from "aaa" to "tgt."

Table 9.

Mutants				Nucleotide sequence	
αM or αL	mutations	# Amino Acid	#Nucleotide	WT	mutant
αL	K287C/K294C	K287	1022-1024	aaa	tgt
		K294	1043-1045	aag	tgt
	E284C/E301C	E284	1013-1015	gag	tgt
		E301	1064-1066	gag	tgt
	L161C/F299C	L161	644-646	ctc	tgt
		F299	1058-1060	ttc	tgt
	K160C/F299C	K160	641-643	aaa	tgt
		F299	1058-1060	ttc	tgt
	L161C/T300C	L161	644-646	ctc	tgt
		T300	1061-1063	act	tgt
	L289C/K294C	L289	1028-1030	ctg	tgt
		K294	1043-1045	aag	tgt
αM	Q163C/Q309C	Q163	607-609	caa	tgt
		Q309	1045-1047	cag	tgt
	D294C/Q311C	D294	1000-1002	gat	tgt
		Q311	1051-1053	cag	tgt
	Q163C/R313C	Q163	607-609	caa	tgt
		R313	1057-1059	cgg	tgt

αL; GenBank NM_002209
αM; GeneBank J03925

The term "isolated nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, an isolated nucleic acid molecule encoding a modified integrin I-domain polypeptide can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

The skilled artisan will further appreciate that further changes can be introduced by mutation into the nucleotide sequence encoding a modified integrin polypeptide, thereby leading to changes in the amino acid sequence of the encoded modified integrin polypeptide, without further altering the structural characteristics or functional ability of the modified integrin polypeptide. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in the sequence encoding a modified integrin polypeptide. A "non-essential" amino acid residue is a residue that can be altered from the sequence of a modified integrin polypeptide without further altering the structure and/or biological activity. In accordance with the methods of the invention, computational design and modeling are used to determine which amino acid residues are amenable to alteration in order to achieve the desired protein conformation.

Accordingly, the methods of the invention may include the use of nucleic acid molecules encoding modified integrin polypeptides that contain changes in amino acid residues that are not essential for activity.

Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a modified integrin polypeptide is preferably replaced with another amino acid residue from the same side chain family.

Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, for example, recombinant expression vectors, containing a nucleic acid encoding a modified integrin polypeptide (or a portion thereof), *e.g.*, an integrin I-domain or I-like domain polypeptide or fusion protein. As used herein, the term "vector" refers to a nucleic acid molecule capable of

transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the methods of the invention may include other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art

that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, modified integrin I-domain polypeptides, fusion proteins, and the like).

Accordingly, the invention provides a method for producing a modified integrin polypeptide, *e.g.*, a modified integrin I-domain polypeptide, by culturing in a suitable medium, a host cell of the invention (e.g., a prokaryotic or eukaryotic host cell) containing a recombinant expression vector such that the protein is produced.

The recombinant expression vectors of the invention can be designed for expression of modified integrin polypeptides or fusion proteins in prokaryotic or eukaryotic cells, *e.g.*, for use in the methods of the invention. For example, modified integrin I-domain polypeptides or fusion proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility and/or stability of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which

fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Purified modified integrin I-domain fusion proteins (*e.g.*, soluble I-domain-Ig) can be utilized to modulate integrin activity, as described herein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

Alternatively, modified integrin polypeptides can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), endothelial cell-specific promoters (*e.g.*, KDR/flk promoter; U.S. Patent No. 5,888,765), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Another aspect of the invention pertains to host cells into which a nucleic acid molecule encoding a modified integrin polypeptide of the invention is introduced, *e.g.*, a modified integrin I-domain nucleic acid molecule within a recombinant expression vector or a modified integrin I-domain nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the host cell's genome.

The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a modified integrin polypeptide or fusion protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as hematopoietic cells, leukocytes, K562 cells, 293T cells, human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells (HMVEC), Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells *via* conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a modified integrin polypeptide or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a modified integrin polypeptide, e.g., a modified integrin I-domain polypeptide or fusion protein, for use in the methods of the invention. In one embodiment, a host cell (into which a recombinant expression vector encoding a modified integrin I-domain polypeptide or fusion protein has been introduced) is cultured in a suitable medium such that a modified integrin I-domain polypeptide or fusion protein is produced. In another embodiment, a modified integrin I-domain polypeptide or fusion protein is isolated from the medium or the host cell. A recombinant cell expressing a modified integrin polypeptide or fusion protein can also be administered to a subject to modulate integrin activity.

The host cells of the invention can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which a modified integrin I-domain polypeptide-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous modified integrin I-domain sequences have been introduced into their genome or homologous recombinant animals in which endogenous integrin I-domain sequences have been altered. Such animals are useful for studying the function and/or activity of a modified integrin I-domain molecule and for identifying and/or evaluating modulators of modified integrin I-domain polypeptide activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, and the like. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous integrin I-domain gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing a modified integrin I-domain-encoding nucleic acid into the male pronuclei of a fertilized oocyte,

e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a modified integrin I-domain transgene to direct expression of a modified integrin I-domain protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a modified integrin I-domain gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the modified integrin I-domain gene. The modified integrin I-domain gene can be a human gene, but more preferably, is a non-human homologue of a human modified integrin I-domain gene. For example, a mouse modified integrin I-domain gene can be used to construct a homologous recombination nucleic acid molecule, *e.g.*, a vector, suitable for altering an endogenous modified integrin I-domain gene in the mouse genome. In a preferred embodiment, the homologous recombination nucleic acid molecule is designed such that, upon homologous recombination, the endogenous modified integrin I-domain gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the homologous recombination nucleic acid molecule can be designed such that, upon homologous recombination, the endogenous modified integrin I-domain gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous modified integrin I-domain protein). In the homologous recombination nucleic acid molecule, the altered portion of the modified integrin I-domain gene is flanked at its 5' and 3' ends by additional nucleic acid sequence of the modified integrin I-domain gene to allow for homologous recombination to occur between the exogenous modified integrin I-domain gene carried by the homologous recombination nucleic acid molecule and an endogenous modified integrin I-domain gene in a cell, *e.g.*, an embryonic stem cell. The additional flanking modified integrin I-

domain nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the homologous recombination nucleic acid molecule (see, *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The homologous recombination nucleic acid molecule is introduced into a cell, *e.g.*, an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced modified integrin I-domain gene has homologously recombined with the endogenous modified integrin I-domain gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells can then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells:A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination nucleic acid molecules, *e.g.*, vectors, or homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In another embodiment, transgenic non-human animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, antibodies, peptidomimetics, small molecules (organic or inorganic) or other drugs) which modulate integrin activity. These assays are designed to identify compounds, for example, that bind to an integrin I-domain polypeptide, *e.g.*, an integrin I-domain polypeptide in an active conformation, bind to other proteins that interact with an integrin I-domain polypeptide, induce binding, and modulate the interaction of an integrin I-domain polypeptide with other proteins, *e.g.*, an integrin ligand, *e.g.*, ICAM, and thus modulate integrin activity.

As used herein, the term "modulator of integrin activity" includes a compound or agent that is capable of modulating or regulating at least one integrin activity, as described herein. Modulators of integrin activity may include, but are not limited to, small organic or inorganic molecules, nucleic acid molecules, peptides, antibodies, and the like. A modulator of integrin activity can be an inducer or inhibitor of integrin activity, *e.g.*, cell adhesion or ligand binding. As used herein, an "inducer of integrin activity" stimulates, enhances, and/or mimics an integrin activity. As used herein, an "inhibitor of integrin activity" reduces, blocks or antagonizes an integrin activity.

As used interchangeably herein, an "integrin activity", or an "integrin-mediated activity" refers to an activity exerted by an integrin polypeptide or nucleic acid molecule on an integrin responsive cell, or on integrin ligand or receptor, as determined *in vitro* and *in vivo*, according to standard techniques. In one embodiment, an integrin activity is the ability to mediate cell adhesion events, *e.g*, cell to cell or cell to extracellular matrix adhesion. In another embodiment, an integrin activity is the ability to transduce cellular signaling events. In yet another embodiment, an integrin activity is the ability to bind a ligand, *e.g.*, ICAM.

In a preferred embodiment, a soluble, recombinant high affinity integrin I-domain can be used to screen for small molecule antagonists that interfere with integrin ligand binding. Furthermore, antagonists, *e.g.*, antibodies, with direct/competitive and indirect/noncompetitive modes of inhibition can be discriminated, based on comparison with effects on wild-type integrin I-domains which show minimal ligand binding activity. For example, an indirect inhibitor should inhibit ligand binding by an

activated, wild-type integrin I-domain, but not by a disulfide-locked high affinity I-domain.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a modified integrin polypeptide on the cell surface with a test compound and determining the ability of the test compound to modulate (*e.g.*, induce or inhibit) an integrin activity. For example, a cell expressing a modified integrin I-domain polypeptide stabilized in an open conformation on the cell surface is contacted with a test compound, and the ability of the test compound to modulate adhesion to an integrin ligand is determined, as described and exemplified herein.

In yet another embodiment, the ability of a test compound to modulate integrin ligand binding can also be determined, for example, by coupling a modified integrin I-domain polypeptide that is stabilized in an open conformation with a detectable label such that the binding of the modified integrin polypeptide can be determined by detecting the amount of labeled integrin I-domain binding to an immobilized integrin ligand.

Animal-based model systems, such as an animal model of inflammation, may be used, for example, as part of screening strategies designed to identify compounds which are modulators of integrin activity. Thus, the animal-based models may be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in modulating inflammation and treating integrin-mediated disorders. For example, animal models may be exposed to a compound, suspected of exhibiting an ability to modulate integrin activity, and the response of the animals to the exposure may be monitored by assessing inflammatory activity before and after treatment. Transgenic animals, *e.g.*, transgenic mice, which express modified integrin I-domain polypeptides as described herein can also be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in modulating inflammation and treating integrin-mediated disorders

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulator of integrin activity can be identified using a cell-based assay, and the ability of the agent to modulate integrin activity can be confirmed *in vivo*, *e.g.*, in an animal such as an animal model for inflammation.

Moreover, screening assays can be used to identify inducers of integrin activity, for example, that mimic the activity of a integrin polypeptide, *e.g.*, the binding of an integrin to a ligand or receptor, or the activity of an integrin towards an integrin responsive cell. Such compounds may include, but are not limited to, peptides, antibodies, or small organic or inorganic compounds. In one embodiment, an anti-integrin antibody, *e.g.*, an anti-LFA-1 antibody of the invention which selectively binds to an open, activated conformer can be used to assess the ability of a test compound to activate integrin.

The test compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

This invention further pertains to novel agents identified by the above-described screening assays. With regard to intervention, any treatments which modulate integrin activity and/or inflammatory activity should be considered as candidates for human therapeutic intervention.

Pharmaceutical Compositions

The nucleic acid molecules encoding modified integrin polypeptides, modified integrin polypeptides (e.g., modified I-domain polypeptides and fusion proteins), and active fragments thereof, anti-integrin I-domain antibodies, and integrin modulators (also referred to herein as "active compounds") DNA vaccines, or DNA vectors of the invention can be incorporated into pharmaceutical compositions suitable for administration. As used herein, a "modulator" of integrin activity, *e.g.*, inhibitors and inducers, includes a compound that modulates an integrin activity, *e.g.*, an integrin-mediated signaling event, an integrin-mediated adhesion event, or integrin binding to a cognate ligand. Integrin modulators include modified integrin I-domain or I-like domain polypeptides of the invention, anti-integrin I-domain polypeptides, as well as compounds identified in a screening assay described herein. Such compositions typically comprise the compound, nucleic acid molecule, vector, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, ophthalmic, and rectal administration, including direct installation into a disease site. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium

chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a soluble modified integrin I-domain fusion protein) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an acrosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The administration of the active compounds of the invention may be for either a prophylactic or therapeutic purpose. Accordingly, in one embodiment, a "therapeutically effective dose" refers to that amount of an active compound sufficient to result in a detectable change in the physiology of a recipient patient. In one embodiment, a therapeutically effective dose refers to an amount of an active compound sufficient to result in modulation of an inflammatory and/or immune response. In another embodiment, a therapeutically effective dose refers to an amount of an active compound sufficient to result in the amelioration of symptoms of an inflammatory and/or immune system disorder. In another embodiment, a therapeutically effective dose refers to an amount of an active compound sufficient to prevent an inflammatory and/or immune system response. In yet another embodiment, a therapeutically effective dose refers to that amount of an active compound sufficient to modulate an integrin activity (*e.g.*, a signaling activity, an adhesion activity or a ligand binding actvity) as described herein.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and

therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of antibody, protein or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. Ranges intermediate to the above recited values, also are intended to be part of this invention. For example, ranges of span values using a combination of any of the above recited values as upper and/or lower limits are intended to be included.

The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

In a preferred example, a subject is treated with antibody, protein, or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

In another preferred example, a subject is treated with an initial dosing of a therapeutically effective amount of an anti-integrin antibody, *e.g.,* an anti-integrin antibody, *e.g.,* an anti-LFA-1 antibody, which reacts with or binds to an I-domain of an integrin in the open or active conformation, or an anti-integrin antibody, *e.g.,* an anti-LFA-1 antibody, which reacts with or binds to a modified LFA-1 I-domain, followed by a subsequent intermittent dosing of a therapeutically effective amount of the antibody that is less than 100%, calculated on a daily basis, of the initial dosing of the antibody wherein the antibody is administered not more than once per week during the subsequent dosing. In another embodiment, the subsequence dosing is two or more times per week. In another embodiment, the subsequence dosing is one or more time every two weeks. In still another embodiment, the subsequence dosing is one or more times every three weeks. In yet another embodiment, the subsequence dosing is one or more times every four weeks. In one embodiment, the subsequent dosing is less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%, calculated on a daily basis, of the initial dosing of the antibody. In one embodiment, the initial dosage is between 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. In a preferred embodiment, the initial dosage is less than 0.3 mg/kg body weight, *e.g.,* between 0.001 to 0.30, *e.g.,* 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, and 0.275. Ranges intermediate to the above recited values, also are intended to be part of this invention.

In yet another example, a subject is treated with an initial dosing of a therapeutically effective amount of an anti-integrin antibody, *e.g.,* an anti-integrin antibody, *e.g.,* an anti-LFA-1 antibody, which reacts with or binds to an I-domain of an

integrin in the open or active conformation, or an anti-integrin antibody, *e.g.,* an anti-LFA-1 antibody, which reacts with or binds to a modified LFA-1 I-domain, followed by a subsequent intermittent dosing of a therapeutically effective amount of the antibody that is greater than 100%, calculated on a daily basis, of the initial dosing of the antibody wherein the antibody is administered to the mammal not more than once per week during the subsequent dosing. In another embodiment, the subsequence dosing is two or more times per week. In another embodiment, the subsequence dosing is one or more time every two weeks. In still another embodiment, the subsequence dosing is one or more times every three weeks. In yet another embodiment, the subsequence dosing is one or more times every four weeks. In one embodiment, the initial dosage is between 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. In a preferred embodiment, the initial dosage is less than 0.3 mg/kg body weight, *e.g.,* between 0.001 to 0.3, *e.g.,* 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, and 0.275. Ranges intermediate to the above recited values, also are intended to be part of this invention. Dosages for anti-integrin antibodies, *e.g.,* anti-LFA-1 are described in, for example, U.S. Patent No. 5,622,700.

In still another example, an initial dosage is followed by the same dosage, for example, not more than once per week during the subsequent dosing. In another embodiment, the subsequence dosing is two or more times per week. In another embodiment, the subsequence dosing is one or more time every two weeks. In still another embodiment, the subsequence dosing is one or more times every three weeks. In yet another embodiment, the subsequence dosing is one or more times every four weeks.

Dosages for anti-integrin antibodies, *e.g.,* anti-LFA-1 are described in, for example, U.S. Patent No. 5,622,700.

In another embodiment, the an effective amount of an anti-inflammatory or immunosuppressive agent to the mammal in combination with the antibody, either at the same time, or at different time points.

The present invention encompasses active agents which modulate an integrin activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino

acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention.

Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

In certain embodiments of the invention, a modulator of integrin activity is administered in combination with other agents (*e.g.*, a small molecule), or in conjunction with another, complementary treatment regime. For example, in one embodiment, an inhibitor of integrin activity is used to treat an inflammatory or immune system disorder. Accordingly, the subject may be treated with an inhibitor of integrin activity, and further treated with an anti-inflammatory or immunosuppressive agent.

Further, an antibody, *e.g.,* an anti-LFA-1 antibody, (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. The conjugates of the invention can be used for modifying a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a coagulation factor such as tissue factor; a protein such as vascular endothelial growth factor ("VEGF"), platelet derived growth factor, and tissue plasminogen activator; biological response modifiers such as, for example, lymphokines, cytokines and growth factors; or a toxin.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982). Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

The nucleic acid molecules of the invention, *e.g.,* a nucleic acid molecule encoding, for example, a high-affinity modified integrin I-domain polypeptide, or active fragment thereof, can be used as a gene-based therapy alone, or, can be inserted into

vectors and used as gene therapy vectors. Gene therapy is the insertion of a functioning gene into the cells of a patient (i) to correct an inborn error of metabolism, or (ii) to provide a new function in a cell (Kulver, K. W., "Gene Therapy", 1994, p. xii, Mary Ann Liebert, Inc., Publishers, New York, N.Y.). Vectors, *e.g.,* viral vectors, may be used to introduce and stably express a gene normally expressed in mammals, for example, in a location in the body where that gene is not naturally present. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The gene therapy vector can include, for example, DNA encoding an antigen of interest to induce an immune response in the subject *in vivo*. Therefore, the modified integrin I-domain polypeptide, *e.g.,* a high-affinity modified integrin I-domain polypeptide, or active fragment thereof, acts as an adjuvant to produce an increased antibody reaction to the antigen. The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.,* retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The nucleic acid molecules of the invention can also be used in DNA vaccine formulations for therapeutic or prophylactic treatment of integrin-mediated disorders, *e.g.,* inflammatory disorders. In one embodiment, the DNA vaccine formulation comprises a nucleic acid molecule encoding a modified integrin polypeptide, *e.g.,* a modified integrin I-domain polypeptide, or fragment thereof, coupled with an antigenic component, *e.g.,* DNA encoding an antigenic component. As used herein, an antigenic component is a moiety that is capable of binding to a specific antibody with sufficiently high affinity to form a detectable antigen-antibody complex. In another embodiment, the DNA vaccine further comprises a pharmaceutically acceptable carrier.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of an integrin-mediated disorder or having an integrin-mediated disorder such as an inflammatory or immune disorder, and/or a cellular proliferative disorder. "Treatment", as used herein, is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease or disorder, a symptom of disease or disorder or a predisposition toward a disease or disorder, with the purpose of curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving or affecting the disease or disorder, the symptoms of disease or disorder or the predisposition toward a disease or disorder. A therapeutic agent includes, but is not limited to, nucleic acid molecules, DNA vaccines, gene-based therapies, small molecules, peptides, antibodies, *e.g.*, anti-LFA-1 antibodies, which react with or bind to modified I-domain polypeptides, ribozymes and antisense oligonucleotides.

With regard to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers the study of how a patient's genes determine his or her response to a drug (e.g., a patient's "drug response phenotype", or "drug response genotype"). Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the integrin I-domain polypeptides of the present invention or modulators thereof according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject a disease or condition associated with a integrin-mediated disorder by administering to the subject one or more integrin I-domain polypeptides of the present invention or

modulators thereof. Subjects at risk for an integrin-mediated disorder can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the integrin-mediated disorders, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of integrin-mediated disorder, for example, appropriate integrin I-domain polypeptides of the present invention, or modulators thereof, can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating expression of integrin I-domain polypeptides or their activity for therapeutic purposes (e.g., treating a subject at risk of an integrin-mediated disorder or having an integrin-mediated disorder such as an inflammatory or immune disorder, and/or a cellular proliferative disorder) . Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell with one or more integrin I-domain polypeptides of the present invention, or one or more modulators thereof, *e.g.*, an antibody which reacts of binds to an integrin I-domain in an open conformation or a modified integrin I-domain polypeptide, *e.g.*, an anti-LFA-1 antibody specific for an LFA-1 I-domain in an open conformation or a modified LFA-1 I-domain polypeptide. An agent that modulates integrin I-domain polypeptide activity can be an agent as described herein, such as a nucleic acid or a protein, a target molecule of an integrin I-domain polypeptide (e.g., a substrate), an antibody which reacts or binds to a modified integrin I-domain polypeptide, an integrin I-domain polypeptide agonist or antagonist, a peptidomimetic of an integrin I-domain polypeptide agonist or antagonist, or other small molecule. In one embodiment, the agent stimulates one or more integrin I-domain polypeptide activities. Examples of such stimulatory agents include active integrin I-domain polypeptide protein and a nucleic acid molecule encoding integrin I-domain polypeptide that has been introduced into the cell. In another embodiment, the agent inhibits one or more integrin I-domain polypeptide activities. Examples of such inhibitory agents include antisense integrin I-domain polypeptide nucleic acid molecules, gene therapy vectors, DNA vaccines, anti-integrin I-domain polypeptide antibodies, and integrin I-domain polypeptide inhibitors. These modulatory methods can be performed in vitro (e.g., by

culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized associated with an integrin-mediated disorder. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates or downregulates) integrin I-domain polypeptide expression or activity.

3. Pharmacogenomics

The integrin I-domain polypeptide molecules of the present invention, as well as agents, or modulators which have a stimulatory or inhibitory effect on integrin I-domain polypeptide activity (e.g., integrin I-domain polypeptide gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) an integrin-mediated disorder such as an inflammatory or immune disorder, and/or a cellular proliferative disorder. In conjunction with such treatment, pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer an integrin I-domain polypeptide molecule (and/or a modulator thereof) as well as tailoring the dosage and/or therapeutic regimen of treatment with such molecule and/or modulator.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. et al. (1996) Clin. Exp.Pharmacol. Physiol. 23(10-11): 983-985 and Linder, M.W. et al. (1997) Clin. Chem. 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example,

glucose-6-phosphate aminopeptidase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association", relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (e.g., a "bi-allelic" gene marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants). Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and the cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite

frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Alternatively, a method termed the "gene expression profiling" can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (e.g., an integrin I-domain polypeptide molecule or integrin I-domain polypeptide modulator) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with an integrin I-domain polypeptide molecule or modulator thereof, such as a modulator identified by one of the exemplary screening assays described herein.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the figures and sequence listing are incorporated herein by reference.

EXAMPLES

EXAMPLE 1 DESIGN OF LFA-1 AND Mac-1 MUTANTS THAT ARE LOCKED IN OPEN OR CLOSED CONFORMATION

Current crystal and NMR structures of the LFA-1 I domain (Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Kallen, J et al. (1999) J Mol Biol 292:1-9) have a conformation that is similar to the low affinity, closed conformer of the Mac-1 I domain (1jlm) (Lee,

J-O *et al.* (1995) *Cell* 80:631-638). Therefore, the high affinity, open conformer of the Mac-1 I domain (1ido) (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340) was used to model a high affinity, open LFA-1 I domain. The template for this model consisted of segments of the 1ido structure in regions where the Cα backbone differed significantly from the 1jlm structure, and segments of the 1lfa structure in regions where 1ido and 1jlm were similar.

Briefly, I domains with the following protein data bank (PDB) identifiers were structurally superimposed using Cα carbons, the CD MALIGN algorithm of MODELLER 4 (Sali, A and Blundell, TL (1993) *J Mol Biol* 234:779-815), and a gap extension penalty of 1 Å: Mac-1, 1ido and 1jlm (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340; Lee, J-O *et al.* (1995) *Cell* 80:631-638); LFA-1, 1lfa molecules A and B (Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281), 1zon and 1zop (Qu, A and Leahy, DJ (1996) *Structure* 4:931-942); and VLA-2, 1aox (Emsley, J *et al.* (1997) *J Biol Chem* 272:28512-28517). The algorithm found 121 framework residues that were utilized for superposition. A sequence alignment was then done. The 1ido and 1jlm structures were aligned by their sequence, and 1lfa molecule A and 1zon were aligned by structural similarity to 1jlm. Using the structural superposition, and the sequence alignment, the distances between all Cα carbons at equivalent sequence positions were calculated using a Microsoft Excel spreadsheet. This was analogous to the comparison between 1jlm and 1ido (Lee, J-O *et al.* (1995) *Structure* 3:1333-1340), except that LFA-1 I domain structures were included. For use as templates for the high affinity, open LFA-1 I domain model, segments from 1lfa molecule A were chosen where differences between all four I domains were small, or differences between 1lfa and 1jlm (low affinity, closed LFA-1 and Mac-1 I domains) were greater than between 1ido and 1jlm (open and closed Mac-1 I domains). Segments from 1ido were chosen when differences between 1ido and 1jlm were greater than between 1lfa and 1jlm. These segments were spliced together in regions where the backbones were as similar as possible. Thus, the template utilized segments G128 to F136, M154 to L203, F209 to L234, T243 to I255, and E272 to A282 of 1lfa; and segments D140 to F156, G207 to T211, V238 to K245, R266 to R281, and R293 to K315 of 1ido. No chain breaks were detected by LOOK™ (Molecular Application Group, Palo Alto, CA) in the spliced template, dubbed lfa-mac. Models of a high affinity open form of LFA-1 were made with MODELLER 4™ using this template, the Mg^{2+} and water molecules 403 and 404 of 1ido, with heteroatom,

water, and hydrogen input turned on, and dynamic Coloumb turned on. The resulting model (1fa_hi.063) followed the template Cα coordinates closely (RMS = 0.12Å). The QUACHK score (Vriend, G (1990) *J Mol Graph* 8:52-56) is excellent (-0.135 compared to –0.215 for the 1fa-mac template, -0.08 for 1ido, and 0.0 for 1lfa).

The SSBOND program (Hazes, B and Dijkstra, BW (1988) *Protein Engineering* 2:119-125) was used to identify positions where disulfide bonds could be introduced by mutating two appropriately positioned pairs of residues to cysteine. It was hypothesized that it might be possible to use disulfide bonds to trap the LFA-1 I domain in either the open or closed conformations.

The high affinity open LFA-1 I domain model (the 1fa_hi.063 model) was examined and two low affinity closed LFA-1 I domain structures, 1lfa and 1zon, with SSBOND and found 14 to 19 pairs of such residues in each structure. Out of these, one pair of residues in the high affinity open model, and one pair of residues in the low affinity closed structures, underwent large movements between the two conformers, such that disulfide bond formation could only occur in one conformer (Figure 1). These disulfides bridge β-strand 6 to the C-terminal α-helix, α6. The numbering of β-strands and α-helices differs among I domains; we use a uniform nomenclature (Huang, C *et al.* (2000) *J Biol Chem*, 275:21514-24). Helix α6 moves 10 Å along its axis down the body of the I domain in the high affinity open structure, and this movement is accompanied by a complete remodeling and downward shift of the loop between β6 and α6. Cysteines introduced in place of K287 and K294 were predicted to form a disulfide only in the high affinity open conformer, and thus lock the I domain in the high affinity open state (Figure 2). The Cβ carbons of K287 and K294 are predicted to be 3.8 Å apart in the high affinity open model (1fa_hi.063), within the range of 3.41 to 4.25Å that is ideal for disulfide formation, and after checking for Cβ-Sγ and Sγ-Sγ distances, were found to have four favorable sidechain-disulfide conformations. By contrast, in the low affinity closed conformers 1lfa and 1zon, the Cβ atoms of these residues are 8.9 to 9.2 Å apart (Figure 2).

Cysteines introduced in place of L289 and K294 were predicted to form a disulfide only in the low affinity closed conformer (Figure 2), and thus lock the I domain in the low affinity closed state. The Cβ carbons of L289 and K294 are 3.9 to 4.0 Å apart in the low affinity closed 1lfa and 1zon conformers, within the favorable range, although favorable cysteine sidechain conformations were not found. Nonetheless, the

α-helix in which residue 294 is present shows small displacements between 1lfa, 1zon, and the recent NMR structure (Qu, A and Leahy, DJ (1995) *Proc Natl Acad Sci USA* 92:10277-10281; Qu, A and Leahy, DJ (1996) *Structure* 4:931-942; Kallen, J et al. (1999) J Mol Biol 292:1-9), and it was expected that a disulfide could form with minor adjustment of the α-helix. By contrast, in the high affinity open model, the Cβ atoms of these residues are predicted to be 8.0 Å apart (Figure 2).

Models were also built in which the predicted cysteines were present and disulfide bonds were formed if appropriate using the PATCH DISULFIDE routine of MODELLER 4 (Figure 2); however, it should be noted that all Cβ atom distances reported here are based on models or structures without introduced disulfides.

In addition to the computational search for pairs of cysteine substitutions to form conformation-specific disulfide bridge, the structure-oriented manual approach (or visual inspection) was also used. Regions of I domains that differ in conformation between the open and closed conformations were inspected for positions in which pairs of cysteines could be introduced that would form disulfides that would favor one conformation over the other. Thus, the region of the conformationally mobile C-terminal α-helix and the preceding loop were examined for positions in which one cysteine could be introduced, and structurally adjacent regions were searched for positions where a second cysteine could be introduced that would form a disulfide bond. Pairs of residues whose side-chains face towards one another were chosen. The distance between the Cα and Cβ atoms of each of these pairs was measured by software Look™ both in the open and closed conformation. The ideal separation for cysteine Cβ carbons for formation of a disulfide bond is reported to be 3.41 to 4.25 A. However, the crystal structures or models from which these were measured represent average positions of snapshots, whereas proteins are dynamic and exhibit atomic mobility. Furthermore, structural adjustments are possible to accommodate disulfide bonds. Much more adjustment is expected to be possible in loops and a-helices than in β-sheets. Therefore greater distances were predicted to be allowable for disulfide formation when one of the residues was in a loop or helix.

For αL, 4 pairs of cysteine substitutions were found where the Cα-Cα and Cβ-Cβ distances were more favorable for disulfide formation in the open conformation than

in the closed conformation; E284C/E301C, L161C/F299C, K160C/F299C, and L161C/T300C (Table 1).

For αM, 4 pairs of cysteine substitutions were found where the Cα-Cα and Cβ-Cβ distances were more favorable for disulfide formation in the open conformation than in the closed conformation: Q163C/Q309C, Q298C/N301C, D294C/T307C, and D294C/Q311c (Table 7), and one pair of cysteine substitutions where the Cα-Cα and Cβ-Cβ distances were more favorable for disulfide formation in the closed conformation than in the open conformation: Q163C/R313C. Additionally, F297C/A304C, which is an analogous mutation to K287C/K294C in αL, was included.

EXAMPLE 2 CONSTRUCTION AND EXPRESSION OF LFA-1 CYSTEINE SUBSTITUTION MUTANTS

Five open αL I-domain mutants were generated. To generate the high affinity open mutant K287C/K294C, the K287 and K294 in the I-domain of the αL subunit were replaced by cysteines. To generate the high affinity open mutant E284C/E301C, the E284 and E301 in the I-domain of the αL subunit were replaced by cysteines. In addition, three intermediate-affinity open αL I-domain mutants were made, and are identified herein as follows: L161C/F299C, K160C/F299C, and L161C/T300C. L161C/F299C was made by substituting cysteines for the L161 and F299. K160C/F299C was made by substituting cysteines for the K160 and F299. L161C/T300C was made by substituting cysteines for the L161 and T300. The low affinity closed mutant L289C/K294C was made by substituting cysteines for the L289 and K294. The distance between mutated residues for these six mutant is shown in Table 1, below. Also, single cysteine substitution mutants K287C, L289C and K294C were generated.

Table 1. Cα and Cβ between mutated residues in either open or closed confirmation

αL I-domain	open conformation		closed conformation	
	Cα (A)	Cβ (A)	Cα (A)	Cβ (A)
Locked open				
K287C/K294C	6.32	3.75	10.72	9.08
E284C/E301C	9.12	6.96	12.88	12.52
L161C/F299C	9.16	8.09	11.87	11.38
K160C/F299C	9.97	7.75	9.83	7.96
L161C/T300C	12.30	13.00	13.50	14.87
Locked closed				
L289C/K294C	7.90	7.96	6.19	3.86

The distance between wild-type residues was measured by Look™ software in open conformation (lfa_hi.063) or closed conformation (1lfaA).

The human αL cDNA was contained in vector AprM8, a derivative of CDM8 (Seed, B and Aruffo, A (1987) *Proc Natl Acad Sci USA* 84:3365-3369). Overlap extension PCR was used to generate cysteine substitution mutations in the αL I-domain (Ho, SN *et al.* (1989) *Gene* 77:51-59; Horton, RM *et al.* (1990) *BioTechniques* 8:528). The outer left primer for PCR extension was complementary to the vector sequence at 5' to the EcoRI site at position 1826, and the outer right primer was 3' to the EcoRI site in the αL cDNA. The inner primers were designed for each individual mutation and contained overlapping sequences. Wild-type αL CDNA in AprM8 was used as template for the first PCR reaction. The second PCR product was digested with EcoRI and ligated into the same site in the wild-type αL cDNA in AprM8. The correct orientation of the insert was confirmed by restriction enzyme digestion. All mutations were confirmed by DNA sequencing.

For stable expression, the XbaI fragment of αL wild-type and mutant cDNA was subcloned into the same site of the stable expression vector pEFpuro (Lu, C and Springer, TA. (1997) *J Immunol* 159:268-278).

The mutated αL subunit was transiently coexpressed with the β2 subunit in 293T cells, and cell surface expression of the αL/β2 complex was determined by flow cytometry with monoclonal antibody TS2/4 to the αL subunit in the αL/β2 complex.

Briefly, human embryonic kidney 293T cells (SV40 transformed) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 μg/ml gentamycin. 293T cells were transiently transfected using the calcium phosphate method (DuBridge, RB *et al.* (1987) *Mol Cell Biol* 7:379-387; Heinzel, SS *et al.* (1988) *J Virol* 62:3738-3746). Briefly, 7.5 μg of wild-type or mutant αL cDNA in plasmid AprM8 and 7.5 μg of β2 cDNA in AprM8 were used to co-transfect one 6-cm plate of 70-80% confluent cells. Two days after transfection, cells were detached from the plate with Hanks' balanced salt solution (HBSS) containing 5 mM EDTA for LFA-1 expression and functional analyses.

Flow cytometric analysis was performed as previously described (Lu, C and Springer, TA (1997) *J Immunol* 159:268-278). Briefly, cells were washed and resuspended in L15 medium (Sigma) supplemented with 2.5% FBS (L15/FBS). 1x10 x 10^5 cells were incubated with primary antibodies in 100 μl L15/FBS on ice for 30 min. Monoclonal antibodies were used at final concentration of 1:20 hybridoma supernatant, 1:200 ascites, or 10 μg/ml purified IgG. Cells were then washed twice with L15/FBS, and incubated with FITC-conjugated goat anti-mouse IgG (heavy and light chain, Zymed Laboratories, San Francisco, CA) for 30 min on ice. After washing, cells were resuspended in cold PBS and analyzed on a FACScan (Becton Dickinson, San Jose, CA).

As shown in Figure 3A, the predicted high and low affinity mutants, and the single cysteine substitution mutants expressed similar levels of cell surface αL/β2 complex.

To test whether introducing the cysteines affected the overall conformation of the I-domain, a panel of monoclonal antibodies to different regions in the I-domain were tested for their reactivity with the I-domain mutants. The monoclonal antibodies used in these studies are as follows:

The mouse anti-human αL (CD11a) monoclonal antibodies TS1/11, TS1/12, TS1/22, TS2/4, TS2/6 and TS2/14; anti-β2 (CD18) monoclonal antibodies TS1/18, CBRLFA-1/2, and CBRLFA-1/7; mAb YFC51; and the nonbinding mAb X63 have been described previously (Sanchez-Madrid, F *et al.* (1982) *Proc Natl Acad Sci USA*

79:7489-7493; Hale, LP *et al.* (1989) *Arthritis Rheum* 32:22-30; Petruzzelli, *L et al.* (1995) *J Immunol* 155:854-866). Monoclonal antibodies BL5, F8.8, 25-3-1, May.035, CBRLFA-1/9, CBRLFA-1/1, S6F, and May.017 were described in *Leukocyte Type V* and were obtained from the Fifth International Leukocyte Workshops.

Monoclonal antibodies X63 and TS1/11 were used as hybridoma supernatants at a 1:20 dilution; monoclonal antibodies TS1/12, DBRLFA-1/2, CBRLFA-1/7 and YFC51 were used as purified IgG at 10μg/ml; monoclonal antibodies TS1/2, TS2/14, TS1/18 and TS2/4 used as ascites at a 1:200 dilution; and all monoclonal antibodies from the Fifth International Leukocyte Workshops were used at a 1:200 dilution.

All of the antibodies, except for CBRLFA-1/1, bound to the mutants K287C/K294C and L289C/K294C and wild-type LFA-1 equally well (Table 2), indicating that the cysteine substitutions did not disrupt the I-domain structure. Binding of monoclonal antibody CBRLFA-1/1 to the high-affinity open mutant K287C/K294C was reduced to 40-50% of wild-type, however, this antibody reacted with mutant L289C/K294C and the single cysteine substitution mutants K287C, L289C and K294C as well as wild-type. Since antibody CBRLFA-1/1 maps to residues 301-359 (Huang, C and Springer, TA (1995) *J Biol Chem* 270:19008-19016), and single Cys substitution for K287 and K294 did not affect binding of this antibody, it is likely that reduced binding of CBRLFA-1/1 to mutant K287C/K294C was an indirect effect. Therefore, the conformation at the interface between the I- and β-propeller domains in mutant K287C/K294C may be different from that in wild-type LFA-1.

The reactivity of antibody to the β-propeller domain of αL and to the β2 subunit with mutants K287C/K294C and L289C/K294C was similar to that of wild-type LFA-1, confirming that the structure of other domains of LFA-1 molecule was not affected by the mutations.

Table 2. Reactivity of antibodies with LFA-1 cysteine substitution mutants (% wild-type binding)

		K287C/K294C		L298C/K294C		K287C	L289C	K294C
Mab	epitope	293T	K562	293T	K562	293T	293T	293T
	I-domain							
BL5	119-153, 185-215	92.4±11.29	92.39	85.79±16.4	97.61	93.35	92.44	88.31
F8.8	119-153, 185-215	93.70	102.15	83.56	93.88	95.86	99.63	95.47
CBRLFA-1/9	119-153, 185-215	ND	84.7	ND	ND	ND	ND	ND
TS2/6	154-183	84.88±5.64	89.24	78.59±2.62	95.89	91.39	88.24	91.67
May.035	185-215	92.61±8.4	92.59	82.14±14.15	101.10	95.8	95.4	106.39
TS1/11	185-215	94.36	95.96	93.67	104.54	ND	ND	ND
TS1/12	185-215	88.66	87.32	101.98	105.63	99.32	103.89	93.68
TS1/22	185-302	95.85±12.04	93.06	90.96±8.11	110.49	102.99	96.21	92.24
TS2/14	250-303	85.54±9.38	95.41	83.31±10.59	102.85	102.6	100.4	102.83
25-3-1	250-303	93.06	88.48	90.93	85.66	ND	ND	ND
CBRLFA-1/1	I- and β-propeller	43.59±0.58	55.53	95.89±7.74	118.44	86.11	93.32	89.41
S6F1	β-propeller	89.39	97.38	95.32	85.69	98.3	86.39	92.34
	β2 subunit							
TS1/18	I-like domain	99.82±10.47	97.42	95.72±4.67	105.71	87.88	87.35	107.58
YFC51	I-like domain	102.63	100.73	95.09	110.96	ND	ND	ND
CLBLFA-1/1	I-like domain	ND	96.48	ND	100.50	ND	ND	ND
CBRLFA-1/7	C-terminal region	95.32	95.25	91.68	97.19	ND	ND	ND

Wild-type LFA-1 and LFA-1 mutant K287C/K294C, L289C/K294C, K287C, L289C, and K294C were transiently expressed on the surface of 293T cells or stably expressed on K562 transfectants. Reactivity of antibodies with the transfectants was determined by flow cytometry. Mean fluorescence of each antibody binding was normalized to the mean fluorescence of mAb TS2/4 binding, except for CBRLFA-1/9 that was normalized to mAb TS1/22 binding. TS2/4 bound to wild-type LFA-1 and the mutants equally well. The results are expressed as percent of wild-type binding. Data are mean ±SD of at least two independent FCAS experiments. For some antibodies, only one experiment was done. ND: not determined.

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EXAMPLE 3 LIGAND BINDING ACTIVITY OF LFA-1 CYSTEINE SUBSTITUTION MUTANTS

The ability of the LFA-1 cysteine substitution mutants to bind to the LFA-1 ligand ICAM-1 was determined. 293T cell transfectants that express wild-type LFA-1 and the predicted high-affinity open I-domain mutant K287C/K294C showed constitutively strong binding to immobilized ICAM-1 (Figure 4A). By contrast, the low-affinity closed mutant L289C/K294C did not bind to ICAM-1. Whereas the single cysteine substitution mutants K287C and L289C exhibited reduced binding to ICAM-1, binding of mutant K294C was comparable to that of the wild-type. Binding of mutants K287C and L289C was increased by the activating monoclonal antibody CBRLFA-1/2 to a level similar to wild-type binding. However, CBRLFA-1/2 was not able to activate binding of the low-affinity closed mutant L289C/K294C to ICAM-1 (Figure 4A). Similar results were obtained with two other LFA-1 activating monoclonal antibodies Kim127 and Kim185.To further study the function of the predicted high affinity mutant K287C/K294C and low affinity closed mutant L289C/K294C, stable K562 transfectants that express these mutants were generated.

Briefly, the human erythroleukemia cell line K562 was cultured in RPMI 1640, 10% FBS and 50μg/ml gentamycin. For generating stable K562 cell lines, 2 μg of PvuI-linearized pEFpuro containing αL subunit cDNA was cotransfected with 40 μg of SfiI-linearized AprM8 containing the β2 subunit cDNA by electroporation at 250V and 960 μF. Transfectants were selected for resistance to 4 μg/ml puromycin (Sigma), and subcloned by limiting dilution. All stable cell lines were maintained in RPMI 1640, 10% FBS supplemented with 4 μg/ml puromycin.

Clones of the transfectants that expressed similar levels of cell surface LFA-1, as determined by flow cytometry using monoclonal antibody TS2/4 (Figure 3B), were tested for their ability to bind to immobilized ICAM-1, as previously described (Lu, C and Springer, TA (1997) *J Immunol* 159:268-278).

Briefly, ICAM-1 was purified from human tonsil, and coated to 96-well plates as described previously (Lu, C and Springer, TA (1997) *J Immunol* 159:268-278). Cells were labeled with a florescence dye 2',7'-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM), and resuspended to 1 x 10^6/ml in L15/FBS. 50 μl cell suspension was mixed in ICAM-1 coated wells with an equal volume of L15/FBS in the absence or presence of monoclonal antibody (CBRLFA-1/2, 10 μg/ml). Monoclonal

antibodies were used at final concentration of 1:20 hybridoma supernatant, 1:200 ascites, or 10 μg/ml purified IgG. For testing the effect of divalent cations, BCECF-AM- labeled cells were washed 2 x with TS buffer, pH7.5 (20 mM Tris, pH 7.5, 150 mM NaCl) containing 5 mM EDTA, followed by 2 washes with TS buffer, pH7.5. Cells were then resuspended to 5 x 10^5/ml in the TS buffer, pH7.5 supplemented with 1 mM $MgCl_2/CaCl_2$, $MgCl_2$, $MnCl_2$ or 5 mM EDTA, and 100 μl cell suspension was added to ICAM-1 coated wells. After incubation at 37°C for 30 minutes, unbound cells were washed off on a Microplate Autowasher (Bio-Tek Instruments, Winooski, VT). The fluorescence content of total input cells and the bound cells in each well was quantitated on a Fluorescent Concentration Analyzer (IDEXX, Westbrook, ME). The bound cells were expressed as a percentage of total input cells per sample well.

K562 transfectants that express wild-type LFA-1 showed low basal binding to ICAM-1, and binding was greatly increased by the activating monoclonal antibody CBRLFA-1/2 (Figure 4B). By contrast, cells expressing the predicted high-affinity open mutant K287C/K294C strongly bound to ICAM-1, and monoclonal antibody CBRLFA-1/2 did not further enhance binding of this mutant, whereas the predicted low-affinity closed mutant L289C/K294C did not binding to ICAM-1 even in the presence of the activating antibody.

The effect of divalent cations on binding of K562 transfectants to ICAM-1 was also examined. As shown in Figure 4C, binding of mutant K287C/K294C to ICAM-1 was abolished in the presence of EDTA, confirming that ligand binding of mutant K287C/K294C is divalent cation dependent. Whereas binding of wild-type LFA-1 was greatly enhanced by Mn^{2+}, and to a lesser degree by Mg^{2+}, the presence of Mn^{2+} and Mg^{2+} did not increase binding of the low-affinity closed mutant L289C/K294C to ligand.

The binding of soluble ICAM-1 to K562 transfectants that expressed wild-type LFA-1, mutant K287C/K294C, or mutant L289C/K294C was also assessed. Briefly, a soluble ICAM-1-IgA chimera containing the 5 Ig domains of human ICAM-1 was purified from the culture supernatant of stable CHO transfectants by monoclonal antibody R6.5 affinity chromatography as previously described (Martin, S *et al.* (1993) *J Virol* 67:3561-3568). K562 transfectants were washed once with L15/FBS, and resuspended in the same buffer to 1 x 10^7/ml. 25 μl cell suspension was mixed with 25 μl L15/FBS containing ICAM-1-IgA fusion protein at final concentration 100 μg/ml in the presence or absence of antibody CBRLFA-1/2 (10 μg/ml), and incubated at 37°C for 30 minutes. After incubation, cells were washed once in L15/FBS, and incubated with FITC-conjugated anti-human IgA (Sigma) at

room temperature for 20 minutes. After 2 washes, cells were resuspended in PBS, and analyzed on a FACScan (Becton Dickinson, San Joe, CA).

As shown in Figure 5, the soluble ICAM-1-IgA fusion protein bound to cells expressing the high-affinity open mutant K287C/K294C, and binding was further increased in the presence of the activating monoclonal antibody CBRLFA-1/2. However, the ICAM-1 fusion protein did not bind to the transfectants that expressed wild-type LFA-1 or the low affinity closed mutant L289C/K294C in the absence or presence of monoclonal antibody CBRLFA-1/2, and binding was not detected at a higher ICAM-1 fusion protein concentration (300 μg/ml).

Taken together these data indicate that the high affinity open mutant K287C/K294C is constitutively active, whereas the low-affinity closed mutant L289C/K294C appears to be locked in an inactive state and lacks ligand binding ability.

In another study, a panel of monoclonal antibodies to different domains of the αL and β2 subunits were tested for their inhibitory effect on ligand binding of wild-type LFA-1 and mutant K287C/K294C. The results obtained with the 293T transient transfectants and K562 stable transfectants were similar, and summarized in Table 3. Although all antibodies, except for CBRLFA-1/1, reacted with the high affinity open mutant K287C/K294C as well as wild-type (Table 2), they showed differential inhibition on ligand binding of wild-type LFA-1 and mutant K287C/K294C.

As shown in Table 3, the I-domain antibodies differentially inhibited binding of wild-type LFA-1 and the high affinity open mutant K287C/K294C to ICAM-1. Monoclonal antibodies BL5, F8.8, CBRLFA-1/9, May.035, TS1/22 and TS2/6 strongly inhibited binding of both wild-type and mutant K287C/K294C, and the levels of inhibition to wild-type LFA-1 and the mutant were similar. While monoclonal antibodies TS1/11 and TS1/12 inhibited >90% binding of transfectants that express wild-type LFA-1, these antibodies showed reduced inhibition on binding of mutant K287C/K294C (40-60%). Monoclonal antibodies TS2/14, 25-3-1 and CBRLFA-1/1 that showed >90% inhibition on binding of wild-type had no to little inhibition on mutant K287C/K294C binding to ICAM-1. While the β-propeller domain antibody S6F1 and TS2/4 and antibody CBRLFA-1/7 to the C-terminal region of the β2 subunit did not inhibit binding of both wild-type and mutant K287C/K294C, all five antibodies to the β2 conserved domain, TS1/18, YFC51, CLBLFA-1/1, May.017, and 6.5E, inhibited binding of wild-type LFA-1 (>90% inhibition), but did not inhibit binding of mutant K287C/K294C.

Antibodies to the β-propeller domain and to the C-terminal region of β2 did not inhibit binding of wild-type LFA-1, or mutant K287C/K294C. Antibodies to the I-like domain of the β subunit blocked binding of wild-type LFA-1 to ICAM-1, but did not block mutant K287C/K294C.

Table 3. Differential inhibition of antibodies on binding of wild-type LFA-1 and mutant K287C/K294C to immobilized ICAM-1

		% inhibition			
		wild-type LFA-1		K287C/K294C	
Mab	epitope	293T	K562 (+CBRLFA-1/2)	293T	K562
RR1/1	I-CAM-1 I-domain	95.98	ND	97.89	ND
BL5	119-153, 185-215	97.01±1.63	97.54	91.06±3.8	90.68±6.23
F8.8	119-153, 185-215	94.51	97.61	91.94	98.18
CBRLFA-1/9	119-153, 185-215	ND	97.83	ND	3.60
TS2/6	154-183	96.84±1.73	91.76±4.67	79.09±10.06	88.12±7.40
May.035	185-215	96.20±0.57	95.80±1.66	97.43±1.52	93.33±2.54
TS1/11	185-215	94.12	96.55	45.18	41.30
TS1/12	185-215	95.68±3.92	97.46±0.66	48.96±9.52	63.67±8.13
TS1/22	250-303	95.77	96.94±0.79	95.07	93.56±4.79
TS2/14	250-303	94.47±2.34	96.24±1.70	2.95±9.87	8.55±0.66
25-3-1	250-303	90.49	92.01±0.36	3.71	2.53±4.10
CBRLFA-1/1	I- and β-propeller	92.52±1.68	94.69±5.22	9.03	2.85±4.90
S6F1	β-propeller	ND	6.19	ND	9.70
TS2/4	β-propeller β2 subunit	ND	6.99	ND	2.82
TS1/18	I-like domain	ND	98.48	ND	5.90
YFC51	I-like domain	ND	98.43	ND	0.08
CLBLFA-1/1	I-like domain	ND	94.63	ND	6.69
May.017	I-like domain	ND	97.76	ND	2.98
6.5E	I-like domain	ND	98.36	ND	5.79
CBRLFA-1/7	C-terminal region	ND	5.04	ND	5.77

Wild-type LFA-1 and LFA-1 mutant K287C/K294C were transiently expressed on the surface of 293T cells or stably expressed in K562 transfectants. Binding of the transfectants to immobilized ICAM-1 was determined in the presence of the indicated antibodies. For binding of K562 transfectants that express wild-type LFA-1, the cells were preincubated with the activating mAb CBRLFA-1/2 at 10 μg/ml for 30 min. Data shown are % inhibition ± SD of at least two independent experiments. % inhibition is defined as % bound cells in the presence of the indicated mAb/% bound cells in the presence of the nonbinding mAb X63 x 100. For some antibodies, only one experiment was done. However, in each experiment, each antibody was repeated in triplicate, and the standard deviation of the triplicate samples was <5%. ND: not determined.

Taken together, these results suggest that a subset of I-domain antibodies and antibodies to the β2 conserved domain do not directly block LFA-1 binding to ICAM-1, and that the high-affinity open mutant K287C/K294C appears to be conformationally locked in a high affinity open state, and thus, antibodies that block ligand binding via indirect mechanisms could not block binding of mutant K287C/K294C to ICAM-1.

The high affinity open I-domains of the invention can be used to discriminate between direct/competitive and indirect/non-competitive modes of inhibition of LFA-1. For example, the LFA-1 inhibitor lovastatin binds to the I-domain in a hydrophobic pocket formed by the β sheet and the C-terminal α-helix (Kallen, J et al. (1999) *J Mol Biol* 292:1-9) and thus inhibits LFA-1 by an indirect mechanism. Accordingly, the ability of lovastatin to inhibit ligand binding of the high-affinity I-domain (K287C/K294C) was assessed. Lovastatin dissolved in DMSO at 50 mM was diluted in assay buffer. Cells (10^6/ml) labeled with BCECF-AM were preincubated with lovastatin (0-50μM) at 37°C for 15 minutes, then transferred to a 96 well plate coated with ICAM-1 and further incubated at 37°C for 30 minutes in the presence or absence of activating monoclonal antibody (CBR LFA1/2) or $MnCl_2$. L15 medium supplemented with fetal bovine serum (L15/FBS) which contains Ca2+ and Mg2+ was used for wild-type αLβ2 activated by antibody CBR LFA1/2. and 20 mM HEPES pH7.4, 140 mM NaCl, 1mM MnCl2, 2 mg/ml glucose, 1% BSA was used for activation by Mn2+.

As shown in Figure 6, lovastatin inhibits ICAM-1 binding by cells expressing wild-type LFA-1 and stimulated with Mn^{2+} or antibody (CBRLFA1/2), but does not interfere with ligand binding by the high affinity open K287C/K294C mutant (HA/aLb2).

EXAMPLE 4 EXPRESSION AND FUNCTION OF ISOLATED WILD-TYPE AND MUTANT LFA-1 I-DOMAINS

To further examine the function of the predicted high and low affinity mutants, the wild-type I-domain and the I-domains of mutant K287C/K294C and L289C/K294C from residues V130 to A338 were expressed on the surface of K562 cells by the transmembrane domain of the PDGF receptor.

To construct the isolated, cell-surface expressed I domains, DNA sequences that encode the signal peptide and the following 6 amino acids from the 5' end of repeat II of αL were ligated to the sequences encoding residues V130-A338 that contains the I domain. HindIII and SalI sites were introduced immediately adjacent to the 5' and 3' ends of this fragment, respectively. The HindIII-SalI fragment was subcloned in frame at the 5' to the c-myc tag and the PDGF receptor (PDGFR) transmembrane domain in vector pDisplay™ (Invitrogen), and further subcloned into pcDNA3.1/Hygro using HindIII and NotI. All DNA amplification was carried out with Pfu DNA polymerase (Stratagene), and the final constructs were verified by DNA sequencing.

For generating stable K562 transfectants that express the I-domain on the surface, 20 μg of SspI-linearized pcDNA3.1/Hygro(+) containing the sequences encoding the I domain and the PDGFR transmembrane domain was used to transfect K562 cells by electroporation as described above. Transfectants were selected for resistance to 100 μg/ml hygromycin B, and were further subcloned by cell sorting and limiting dilution; clones that expressed similar levels of surface wild-type and mutant I domain-PDGFR were selected for functional studies. Stable cell lines were maintained in RPMI medium 1640 supplemented with 10% FBS and 100 μg/ml hygromycin B. Cell surface expression of the isolated I-domains was determined by flow cytometry using antibody TS1/22 to the I-domain (Figure 7). Two clones from each transfectant were selected and tested for binding to immobilized ICAM-1, and similar results were obtained with each of the two clones (Figure 8A). Transfectants that expressed intact wild-type LFA-1 showed low basal binding to ICAM-1. However, cells that expressed the isolated wild-type I-domain and the mutant L289C/K294C I-domain did not bind to ICAM-1. This suggests that the isolated wild-type I-domain alone is not sufficient to mediate strong and stable interaction with ligand (Knorr, R and Dustin, ML (1997) *J*

Exp Med 186:719-730). By contrast, cells that expressed the mutant K287C/K294C I-domain showed strong binding to ICAM-1.

If the constitutive ligand binding activity of mutant K287C/K294C is due to the formation of a disulfide bond between the introduced C287 and C294, disruption of the disulfide bond with a reducing agent would abolish ligand binding ability of the mutant. Accordingly, the transfectants were treated with the reducing agent DTT (10 mM) in L15/FBS containing Mg^{2+} and Ca^{2+}, and the ability of transfectants to bind to ICAM-1 was assessed. As shown in Figure 8A, binding of the cell surface-expressed mutant K287C/K294C I-domain to ICAM-1 who abolished after DTT treatment. By contrast, DTT increased binding of intact wild-type LFA-1, and similar results were observed with intact αIIbβ3 integrin. DTT treatment presumably disrupts disulfide bonds in the intact molecule that constrain the integrin in an inactive conformation. However, DTT treatment did not affect binding of the isolated wild-type I-domain or the mutant L289C/K294C I-domain. Since there is no other no disulfide bond in the LFA-1 I-domain as the I-domain structure reveals, these data strongly suggest that the introduced Cys287 and Cys294 formed a disulfide bridge that constrains the I-domain in a high affinity state.

Furthermore, the effect of divalent cations on ligand binding of the isolated I-domains expressed on the surface of K562 transfectants was tested. The binding reactions were performed in HEPES/NaCl/glucose (20 mM HEPES, pH 7.5, 140 mM NaC1, 2 mg/ml glucose) supplemented with 1 mM Mn^{2+}, 1 mM Mg^{2+}, or 1 mM EDTA. As shown in Figure 8B, the binding of the K287C/K294C I-domain to ICAM-1 was divalent cation dependent, as EDTA treatment abolished the binding. In contrast to intact wild-type LFA-1, Mn^{2+} or Mg^{2+} did not activate ligand binding of the isolated wild-type I-domain or the mutant L289C/K294C I-domain.

The effect of the I-domain antibodies on ligand binding of the isolated K287C/K294C I-domain was also examined. Transfectants expressing intact LFA-1 were pre-incubated with the activating antibody CBRLFA-1/2, and binding of the cells to ICAM-1 was performed in the presence of the I-domain antibodies TS1/22, TS2/6, TS1/11, TS1/12, CBRLFA-1/9, CBRLFA-1/1, 25.3.1, TS2/14, or the nonbinding antibody X63, as indicated. Monoclonal antibodies TS1/22, TS2/6, TS1/11, TS1/12 and CBRLFA-1/9 inhibited binding of the isolated K287C/K294C I-domain to ICAM-1, whereas antibodies 25-3-1, TS214 and CBRLFA-1/1 did not (Figure 8C). All

antibodies, except for CBRLFA-1/1, bound to the mutant K287C/K294C I-domain as well as the wild-type I-domain as determined by flow cytometry. The binding of CBRLFA-1/1 to the mutant I-domain was reduced to 80% of the wild-type I-domain. These results are consistent with those obtained with the intact LFA-1 K287C/K294C mutant (Tables 2 and 3), and indicate that the isolated K287C/K294C I-domain remains structural integrity as in the intact molecule.

EXAMPLE 5 INHIBITION OF LFA-1 FUNCTION IN VITRO AND IN VIVO BY SOLUBLE I-DOMAIN MUTANTS

A soluble αL I-domain mutant stabilized in the open conformation by a disulfide bond (K287C/K294C) was made in *E. coli*.

Briefly, recombinant mutant αL I-domain stabilized in the open conformation (K287C/K294C), or recombinant wild-type αL I-domain from amino acid residue G128 to Y307, were cloned into pET11b (Novagen) and expressed in *E. coli* induced with 1 mM IPTG for 4 hours. The recombinant proteins were purified from inclusion bodies by solubilization of inclusion bodies in 6M guanidine HCl and were refolded by dilution in the presence of 0.1 mM Cu^{2+}/phenanthrolin to enhance formation of disulfide bonds. Protein was concentrated by ammonium sulfate precipitation, dialyzed, and purified over a monoQ ion-exchange column. To remove any material in which the disulfide bond did not form, free sulfhydryls were reacted with activated biotin and passed over a streptavidin column. The recombinant proteins were then purified by gel filtration and concentrated by Centriprep. For BIAcore™ analysis, recombinant ICAM-1, ICAM-2 and ICAM–3 Fc chimeras were immobilized on the BIAcore™ sensor chip by an amine-coupling method. Recombinant αL I-domains were flowed in, and BIAcore™ assays were performed with Tris-buffered saline supplemented with 1 mM $MgCl_2$ or 2 mM EDTA, at a flow rate of 10 μl/minute at 25°C.

The purified open I-domain showed high affinity to its ligands, ICAM-1, -2, and –3, in the presence of 1 mM $MgCl_2$ as assessed by BIAcore™ analysis, whereas binding of a soluble wild-type I domain was not detectable (Figure 9, Panels A, C and E; Table 4). The interaction of the open I-domain with ligands was divalent cation-dependent, and was abolished in the presence of 2 mM EDTA, suggesting that the interaction depends on MIDAS. Since the wild-type I-domain showed no interaction with ligands,

the open I-domain allowed for the detailed analysis of the binding kinetics of LFA-1 with its ligands. To analyze binding kinetics, different concentrations of open I-domain were tested for ligand binding (Figure 9, Panels B, D and F). Kinetic analysis demonstrated a fast association rate ($1.28 \times 10^5 M^{-1}s^{-1}$) and an intermediate dissociation rate ($0.0230 s^{-1}$) for ICAM-1, the major ligand on endothelial cells (Table 4). The K_D for ICAM-1 is in the nanomolar range and ICAM-1 showed the highest affinity, followed by ICAM-2 and ICAM-3. The open I-domain also showed nanomolar range affinity for murine ICAM-1.

Table 4. Kinetics of open I-domain binding to ICAMs

Ligand	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM^{-1})
ICAM-1	1.28×10^5	0.0230	180
ICAM-2	0.23×10^5	0.0118	513
ICAM-3	0.19×10^5	0.0749	3942

k_{on}, k_{off}, and K_D were calculated based on 1:1 interaction model using BIAevaluation™ software.

In another study, measurements of the affinity of the recombinant, soluble high affinity αL I domain for its ligand ICAM-1 show a Kd of 200 nM, as assessed by BIAcore. Thus, the isolated, high affinity conformer of the αL I domain is as active as the most activated αLβ2 heterodimer.

The activity of the soluble open I-domain to inhibit LFA-1-dependent adhesion was tested. In one study, K562 cells stably expressing wild-type LFA-1 were fluorescently labeled by BCECF and LFA-1 on the cell surface was activated by the activating monoclonal antibody, CBRLFA-1/2 in L15 media supplemented with FCS. The cells were subsequently incubated in ICAM-1 coated 96-well plastic plates in the presence or absence of I-domains. After incubation for 40 minutes at 37°C, unbound cells were washed off on a Microplate Autowasher. The fluorescence content of total input cells and the bound cells in each well was quantitated on a Fluorescent Concentration Analyzer. The bound cells were expressed as a percentage of total input cells per sample well. In contrast to the wild-type I-domain, the open I-domain mutant

strongly inhibited adhesion of LFA-1 expressing cells to immobilized ICAM-1 (Figure 10A).

In another study, the murine T lymphoma cell line EL-4 which expresses both murine LFA-1 and its ligands, including murine ICAM-1, and which exhibits LFA-1-dependent homotypic aggregation upon activation by PMA was used. Cells were incubated in a 96 well plate in the presence of 50 ng/ml PMA and varying amounts of soluble I-domains. After incubation for 2 hours at 37°C, 5% CO_2, the degree of aggregation was scored under the microscope as follows: 0 indicated that essentially no cells were clustered; 1 indicated that <10% of cells were aggregated; 2 indicated clustering of <50%; 3 indicated that up to 100% of cells were in small, loose aggregates; 4 indicated that nearly 100% of cells were in larger clusters; and 5 indicated that nearly 100% of cells were in very large, tight clusters. As shown in Figure 10B, the soluble open I-domain also inhibited PMA-induced LFA-1 dependent homotypic aggregation of the murine T-cell line EL-4.

Moreover, the ability of the open I-domain mutants to inhibit LFA-1 function *in vivo* was tested by visualizing microcirculation in the peripheral lymph node (LN) with intravital microscopy. Briefly, a small bolus (20-50 µl) of LN cell suspensions from T-GFP mice were retrogradely injected through a femoral artery catheter and visualized in the subiliac LN by fluorescent epi-illumination from a video-triggered xenon arc stroboscope. After recording control T^{GFP} cell behavior in the absence of I-domain, the mouse was pretreated by intra-arterial injection of I-domain (10 µg/g of weight) 5 minutes before T^{GFP} cell injection. All scenes were recorded on videotape and off-line analysis was done. The rolling fraction was calculated as percentage of rolling cells amount the total number of T^{GFP} cells that entered a venule. The sticking (firm adhesion) fraction was determined as the percentage of T^{GFP} cells becoming firmly adherent for >20 seconds in the number of T^{GFP} cells that rolled in a venule. Results were semi-quantitatively scored as follows: -: 0%, ±: 0-5%, +: 5-20%, ++: 20-40%, +++: 40-60%, ++++: 60-80%, +++++:80-100%.

As shown in Table 5, below, injection of the open I-domain but not the wild-type I-domain effectively blocked firm adhesion of T-lymphocytes to high endothelial venules, which is LFA-1-dependent. Lymphocyte rolling that is mediated by L-selectin and PNAd was not compromised, suggesting that the inhibitory effects of the open I-domain was LFA-1 specific.

Table 5. *In vivo* firm adhesion of lymphocytes under flow in peripheral lymph node high endothelial venules was inhibited by open but not wild-type I-domain

	Fraction		
I-domain	rolling	firm adhesion	transmigration
none	+++	++	±
wild-type	+++	++	±
open	++++	±	-

Kinetics of the Binding of αL Mutant I-domains to ICAM-1

To further investigate the kinetics of the interaction of the αL I-domains with ICAM-1, recombinant soluble αL I-domains were expressed in *E. coli*, refolded and purified. As shown in Table 6, below, the affinity of E284C/E301C is nearly comparable to K287C/K294C. The affinity of L161C/F299C, K160C/F299C, and L161C/T300C are significantly higher than wild type, but 20-30 times lower than high-affinity open αL I-domain, K287C/K294C. L161C/F299C, K160C/F299C, and L161C/T300C are referred to as intermediate-affinity αL I-domains.

Table 6. Kinetics of interaction of αL I-domains with ICAM-1

αL I-domain	Kon (1/Ms)	Koff (1/s)	KD (μM)
Locked open			
K287C/K294C	1.28×10^5	0.0230	0.180
E284C/E301C	1.28×10^5	0.0459	0.360
L161C/F299C	1.36×10^5	0.513	3.76
K160C/F299C	1.53×10^5	0.67	4.39
L161C/T300C	1.35×10^5	0.65	4.8
WT	2.22×10^3	3.00	1350
Locked closed			
L289C/K294C	2.11×10^3	2.84	1760

Recombinant soluble αL I-domains were expressed in *E. coli*, refolded and purified. Kinetics of binding of the I-domains to ICAM-1 was measured by BIAcore™ instruments. Kinetics was analyzed BIAevaluation™ software. KD was calculated by Scatchard plots using data at steady states. Koff was obtained by curve fitting of the dissociation phase using 1:1 binding model. Kon was calculated by Koff/KD.

EXAMPLE 6 CONSTRUCTION AND ACTIVITY OF Mac-1 CYSTEINE SUBSTITUTION MUTANTS

A similar approach was taken to design an open, high affinity conformation of Mac-1 by introducing a disulfide bond into the I-domain. The design of Mac-1 cysteine substitution mutants was described in Example 1.

Table 7. Cα and Cβ between mutated residues in either open or closed conformation

	ido (open conformation)		jlm (closed conformation)	
mutations	Cα	Cβ	Cα	Cβ
Locked open				
Q163C/Q309C	8.37	6.36	9.11	7.16
Q298C/N301C	5.31	4.21	9.05	10.91
D294C/T307C	9.21	8.67	16.01	17.52
D294C/Q311C	9.02	7.08	9.79	10.02
F297C/A304C	6.31	3.78	11.18	10.17
Locked closed				
Q163C/R313C	13.8	13.33	7.36	5.15

The distance between wild-type residues was measured by Look™ software in open conformation (1ido) or closed conformation (1jlm).

In order to assess the effect of the introduction of pairs of potentially disulfide bond-forming cysteines into the I-domain of αMβ2 on CBRM1/5 activation-dependent epitope expression and ligand binding, plasmids encoding the wild-type or mutant αM subunits and the β2 subunit were co-transfected into 293T and K562 cells. αβ heterodimer formation was confirmed using monoclonal antibody CBRM1/32 which recognizes an epitope in the putative β-propeller domain of the αM subunit only after association with the β2 subunit, and antibody CBRM1/5 was used to detect integrin activation.

The Q163C/Q309C pair of mutations worked well (Figure 11B, Figure 12B and C). This mutant introduces a putative disulfide bond near the bottom front of the I-domain, between residues that are in the lower one-third of the last α-helix and the first α-helix, and have Cβ carbons that are 6.36Å apart in the 1ido structure. In contrast, the Cβ carbons for the D294C/T307C and D294C/N311C substitutions are 8.67Å and 7.08Å apart, respectively. The Cβ carbons for the Q298C/N301C and F297C/A304C substitutions are within the ideal range, however these substitutions are closer to the loop between the last β-strand and α-helix, and must have unfavorable effects such as distorting the ligand binding site.

When expressed within an intact heterodimer in transiently transfected 293T cells, the Q163C/Q309C mutant is expressed half as well as wild-type as measured by CBRM1/32 antibody, but the ratio of the CBRM1/5 activation-dependent epitope to CBRM1/32 expression is markedly higher (Figure 11A). In addition, the adhesion of 293T cells expressing the Mac-1 Q163C/Q309C mutant to iC3b coated on plastic, as assayed in L15/FBS medium at room temperature, was higher than wild-type, despite its lower expression (Figure 11B).

Alternatively, isolated Mac-1 mutant I-domains were expressed on the cell surface in conjunction with an artificial signal sequence and transmembrane domain of the PDGF receptor. Adhesion was assayed in L15/FBS/$MnCl_2$ at 37°C. The isolated wild-type I-domain showed no binding to iC3b, whereas the previously described mutants with computationally redesigned hydrophobic cores, ido1r and ido2r, were active (Figure 11C) (Shimaoka, M *et al.* (2000) *Nature Structural Biology* 7:674-678). The Q163C/Q309C mutant I-domain exhibited strong specific ligand binding that was completely blocked by the inhibitory I-domain monoclonal antibody CBRM1/5 (Figure 12C).

In a further study, the open I-domain mutants Q163C/Q309C and D294C/Q311C were stably expressed in K562 cells, and clones expressing the same levels of receptors were selected. Adhesion assays to immobilized iC3b were performed with L15/FBS at 37°C. In contrast to 293T cells, wild-type Mac-1 has little basal activity for ligand binding in these cells (Figure 12A and 12B). Both Q163C/Q309C and D294C/Q311C showed increased CBRM1/5 activation-dependent epitope expression and increased ligand binding when expressed in an intact αMβ2 heterodimer, as compared to wild-type (Figure 12A and 12B). Moreover, K562 cells expressing isolated open I-domain mutants on the cell surface showed strong specific binding to iC3b as compared to wild-type (Figure 12C).

In order to confirm that the increased ligand binding activity of the open I-domain mutants is induced by the formation of a disulfide bond, the effect of the reducing agent DTT was tested. Binding of αMβ2 transfectants containing mutant I-domains to immobilized iC3b on plastic was tested in the presence and absence of DTT. As summaried in Table 8, below, locked open αM I-domains, (Q163C/Q309C) and (D294C/Q311c), are active in the absence of activation and their activities are partly reduced by disulfide reduction by DTT. By contrast, locked closed αM I-domain

Q163C/R313C is inactive and resistant to activation, but becomes activatable after disulfide reduction by DTT.

As shown in Figure 12C, DTT treatment abolished ligand binding by isolated locked open I-domains. In contrast, DTT increased binding of the intact wild-type αMβ2 (Figure 2B), indicating that DTT used in this experiment was not toxic and abolishment of ligand binding by the open I-domain mutants was not due to a non-specific effect of DTT. Taken together, these data suggest that the introduced cysteines result in the formation of a disulfide bridge that constrains the Mac-1 I-domain in an open or closed conformation.

Table 8. Summary of adhesion assay of αMβ2 transfectant containing mutant I-domains

mutations	- DTT - activation	- DTT + activation	+ DTT - activation	+ DTT + activation
Wild type	±	++++	++	++++
Locked open				
Q163C/Q309C	++++	++++	++	++++
Q298C/N301C	±	+	NT	NT
D294C/T307C	±	+	NT	NT
D294C/Q311C	++++	++++	++	++++
F297C/A304C	±	++	NT	NT
Locked closed				
Q163C/R313C	±	±	++	+++

Binding of αMβ2 transfectants containing mutant I-domains to immobilized iC3b on plastic was tested. Results were semi-quantitatively scored as follow; ±: 0-5%, +: 5-25%, ++25-50%, +++: 50-75%, ++++: 75-100% of binding by activated wild type transfectant.
NT: not tested
DTT: disulfide reduction by DTT treatment.
+ activation: activated by activating mAB CBR LFA-1/2

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A modified integrin I-domain polypeptide containing at least one disulfide bond, such that said modified I-domain polypeptide is stabilized in a desired conformation.

2. A modified integrin I-domain polypeptide of claim 1 which is stabilized in the open conformation.

3. A modified integrin I-domain polypeptide of claim 1 which is stabilized in the closed conformation.

4. A modified integrin I-domain polypeptide of claim 2 which binds ligand with high affinity.

5. A modified integrin I-domain polypeptide of claim 1 which is encoded by an amino acid sequence containing at least one cysteine substitution as compared to the wild-type sequence.

6. A modified integrin I-domain polypeptide of claim 2, wherein the distance between Cβ carbons of the residues that are substituted for cysteines is 3.00-8.09Å.

7. A modified integrin I-domain polypeptide of claim 1 which is derived from an I-domain of an integrin α subunit selected from the group consisting of: α1, α2, α10, α11, αD, αE, αL (CD11a), αM (CD11b) and αX (CD11c).

8. A modified integrin I-domain polypeptide of claim 2 which is derived from the I-domain of the αL subunit of LFA-1.

9. A modified integrin I-domain polypeptide of claim 3 which is derived from the I-domain of the αL subunit of LFA-1.

10. A modified integrin I-domain polypeptide of claim 7 which contains amino acid substitutions selected from the group consisting of K287C/K294C, E284C/E301C, L161C/F299C, K160C/F299C, and L161C/T300C.

11. A modified integrin I-domain polypeptide of claim 8 which contains amino acid substitutions L289C/K294C.

12. A modified integrin I-domain polypeptide of claim 2 which is derived from the I-domain of the αM subunit of Mac-1.

13. A modified integrin I-domain polypeptide of claim 3 which is derived from the I-domain of the αM subunit of Mac-1.

14. A modified integrin I-domain polypeptide of claim 12 which contains amino acid substitutions selected from the group consisting of Q163C/Q309C and D294C/Q311C.

15. A modified integrin I-domain polypeptide of claim 13 which contains amino acid substitutions Q163C/R313C.

16. A modified integrin I-domain polypeptide of claim 1 which is comprised within an integrin α subunit.

17. A modified integrin I-domain polypeptide of claim 16 which is further associated with an integrin β subunit.

18. A modified integrin I-domain polypeptide of claim 1 which is a soluble polypeptide.

19. A modified integrin I-domain polypeptide of claim 1 which is operatively linked to a heterologous polypeptide.

20. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified integrin I-domain polypeptide as defined in any one of claims 1-15.

21. A composition comprising a modified integrin I-domain polypeptide as defined in any one of claims 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 15 and a pharmaceutically acceptable carrier.

22. A composition of claim 20, wherein said modified integrin I-domain polypeptide is a soluble polypeptide.

23. A composition of claim 21, further comprising an anti-inflammatory or immunosuppressive agent.

24. The use of a modified integrin I-domain polypeptide of claim 2 as an immunogen to produce antibodies that selectively bind to an integrin I-domain in the open conformation.

25. An antibody, or an antigen binding fragment thereof, which selectively binds to a modified integrin I-domain in the open conformation.

26. An antibody of claim 25 which binds to an activation specific epitope on the integrin I-domain.

27. An antibody of claim 25 which blocks an interaction between an integrin and a cognate ligand.

28. An antibody of claim 25, or an antigen binding fragment thereof, further comprising a pharmaceutical composition and a pharmaceutically acceptable carrier.

29. An antibody of claim 25, or an antigen binding fragment thereof, wherein said antibody is an LFA-1 antibody.

30. An anti-LFA-1 antibody, or an antigen binding fragment thereof, which selectively binds to an LFA-1 I-domain in the open conformation.

31. The LFA-1 antibody of claim 30, wherein said anti-LFA-1 antibody, or an antigen binding fragment thereof, selectively binds to a modified LFA-1 I-domain.

32. A modified integrin I-like domain polypeptide containing at least one disulfide bond, such that said modified I-like domain polypeptide is stabilized in a desired conformation.

33. A modified integrin I-like domain polypeptide of claim 30 which is stabilized in the open conformation.

34. A modified integrin I-like domain polypeptide of claim 31 which binds ligand with high affinity.

35. A modified integrin I-like domain polypeptide of claim 30 which is encoded by an amino acid sequence containing at least one cysteine substitution as compared to the wild-type sequence.

36. A modified integrin I-like domain polypeptide of claim 30 which is derived from an I-like domain of an integrin β subunit.

37. A modified integrin I-like domain polypeptide of claim 30 which is comprised within an integrin β subunit.

38. A method for stabilizing a polypeptide in a desired conformation, said method comprising introducing at least one disulfide bond into the polypeptide such that the polypeptide is stabilized in a desired conformation.

39. The method of claim 38, wherein the disulfide bond is formed by the introduction of at least one cysteine substitution into the amino acid sequence of the polypeptide.

40. The method of claim 38, wherein the distance between Cβ carbons in the residues that are substituted for cysteines is 3.00-8.09Å.

41. The method of claim 38, wherein said polypeptide comprises a functional domain of a protein.

42. The method of claim 41, wherein said polypeptide comprises an integrin I-domain.

43. The method of claim 38, wherein said polypeptide is selected from the group of polypeptides consisting of: an integrin subunit, a small G protein, a heterotrimeric G protein alpha subunit, a tyrosine kinases, a G protein-coupled receptor, an enzyme under allosteric control, a zymogen, complement C3, complement C4, and fibrinogen.

44. A method for identifying a modulator of integrin activity comprising:

(a) providing a modified integrin I-domain polypeptide of claim 2;

(b) contacting the modified integrin I-domain polypeptide with a test compound; and

(c) assaying the ability of the test compound to bind to the modified integrin I-domain polypeptide,

to thereby identify a modulator of integrin activity.

45. A method for identifying a compound capable of modulating the interaction of an integrin and a cognate ligand comprising the steps of:

(a) providing a modified integrin I-domain polypeptide of claim 2;

(b) contacting the modified integrin I-domain polypeptide with a ligand of the integrin in the presence and absence of a test compound; and

(c) detecting binding between the modified integrin I-domain polypeptide and said ligand,

to thereby identify a compound capable of modulating the interaction between an integrin and a cognate ligand.

46. A method for treating or preventing an integrin-mediated disorder in a subject comprising administering to said subject a therapeutically effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation, thereby treating or preventing an integrin-associated disorder in a subject.

47. The method of claim 46, wherein said integrin-mediated disorder is an inflammatory disorder.

48. The method of claim 46, wherein said integrin-mediated disorder is an autoimmune disorder.

49. A method of inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to said subject an effective amount of a modified integrin I-domain polypeptide stabilized in the open conformation, thereby inhibiting the binding of an integrin to a cognate ligand in a subject.

50. The method of either one of claims 46 and 49, wherein said modified integrin I-domain polypeptide binds ligand with high affinity.

51. The method of either one of claims 46 and 49, wherein said modified integrin I-domain polypeptide is a soluble polypeptide.

52. The method of claim 50, wherein said modified integrin I-domain polypeptide is operatively linked to a heterologous polypeptide.

53. The method of either of claims 46 and 49, wherein said modified integrin I-domain polypeptide is selected from the group consisting of: αL K287C/K294C, αL E284C/E301C, αL L161C/F299C, αL K160C/F299C, αL L161C/Y300C, αM Q163C/Q309C and αM D294C/Q311C.

54. A method for treating or preventing an integrin-mediated disorder in a subject comprising administering to said subject a therapeutically effective amount of an antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain in the open conformation, thereby treating or preventing an integrin-associated disorder in a subject.

55. The method of claim 54, wherein the antibody binds to a modified integrin I-domain, or an antigen binding fragment thereof.

56. The method of claim 54, wherein said antibody is an LFA-1 antibody, or an antigen binding fragment thereof.

57. The method of claim 54, wherein said integrin-mediated disorder is an inflammatory disorder.

58. The method of claim 54, wherein said integrin-mediated disorder is an autoimmune disorder.

59. A method of treating an integrin-mediated disorder in a subject comprising administering to said subject a therapeutically effective amount of an anti-LFA-1 antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain in the open conformation, thereby treating or preventing an integrin-associated disorder in a subject.

60. The method of claim 59, wherein said anti-LFA-1 antibody binds to a modified LFA-1 I-domain, or an antigen binding fragment thereof.

61. The method of claim 59, wherein said integrin-mediated disorder is an inflammatory disorder.

62. A method of inhibiting the binding of an integrin to a cognate ligand in a subject comprising administering to said subject an effective amount of an antibody, or an antigen binding fragment thereof, which selectively binds to an integrin I-domain in the open conformation, thereby inhibiting the binding of an integrin to a cognate ligand in a subject.

63. The method of claim 62, wherein said antibody is an LFA-1 antibody, or an antigen binding fragment thereof.

64. The method of any one of claims 54, 59, or 62, wherein said antibody, or an antigen binding fragment thereof, binds to an activation specific epitope on the integrin I-domain.

65. A vaccine formulation for prophylactic or therapeutic treatment of an inflammatory disorder comprising an effective amount of a nucleic acid encoding a modified integrin I-domain polypeptide, or active fragment thereof.

66. The vaccine formulation of claim 65, further comprising an antigenic component.

67. The vaccine formulation of claim 65, further comprising a pharmaceutically acceptable carrier.

68. A method for treating an integrin-mediated disorder in a subject comprising administering to said subject a nucleic acid molecule encoding a modified integrin I-domain polypeptide, or active fragment thereof, inserted into a vector.

69. The method of claim 68, wherein said nucleic acid molecule is administered to a subject by intravenous injection.

70. The method of claim 68, wherein said nucleic acid molecule further comprises an antigenic component.

71. A non-human, transgenic animal comprising a nucleic acid molecule encoding a modified integrin I-domain polypeptide.

72. The transgenic animal of claim 71, wherein said animal is a mouse.

FIG. 1

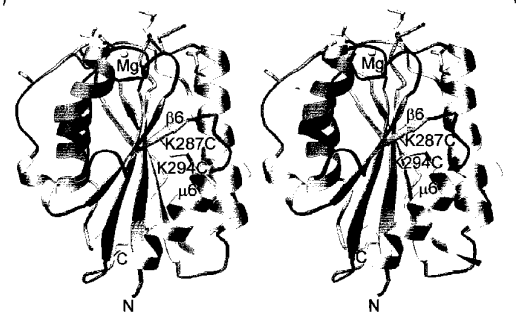


FIG.2C low affinity structure

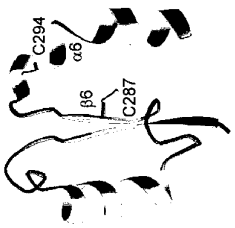


FIG.2D low affinity structure

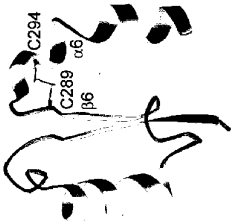


FIG.2A high affinity model

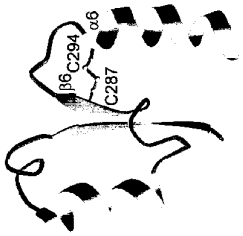


FIG.2B high affinity model

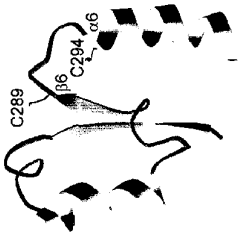


FIG. 3A

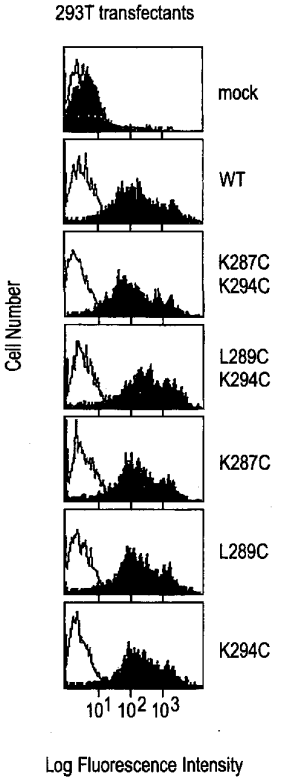

293T transfectants
mock
WT
K287C
K294C
L289C
K294C
K287C
L289C
K294C
Cell Number
10^1 10^2 10^3
Log Fluorescence Intensity

FIG. 3B

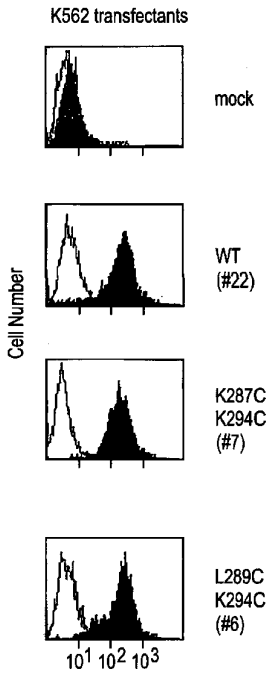

K562 transfectants
mock
WT
(#22)
K287C
K294C
(#7)
L289C
K294C
(#6)
Cell Number
10^1 10^2 10^3
Log Fluorescence Intensity

FIG. 4A

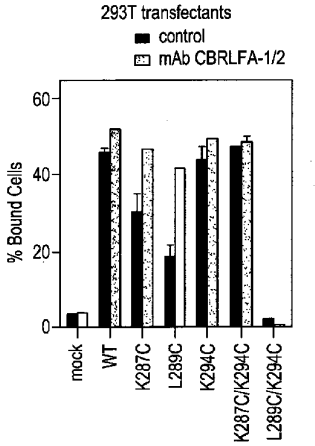


FIG. 4B

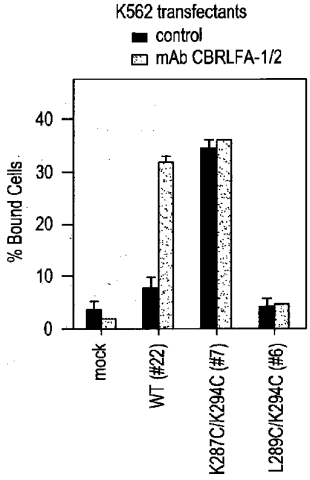


FIG. 4C

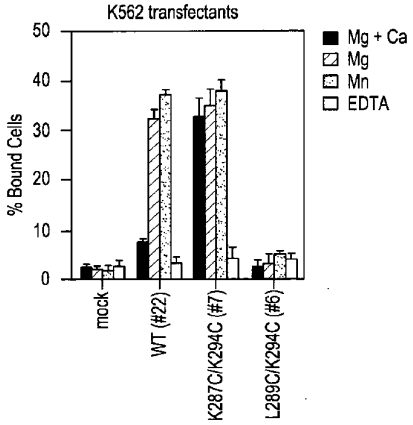


FIG. 5

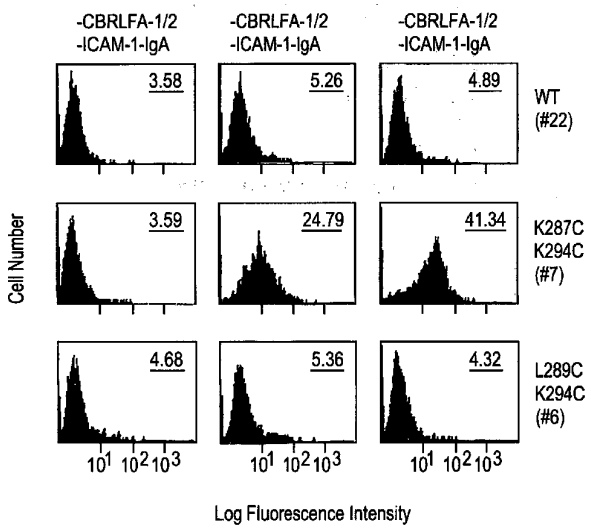

-CBRLFA-1/2
-ICAM-1-IgA
-CBRLFA-1/2
-ICAM-1-IgA
-CBRLFA-1/2
-ICAM-1-IgA
3.58
5.26
4.89
WT
(#22)
3.59
24.79
41.34
K287C
K294C
(#7)
4.68
5.36
4.32
L289C
K294C
(#6)
Cell Number
10¹ 10² 10³
10¹ 10² 10³
10¹ 10² 10³
Log Fluorescence Intensity

FIG. 6

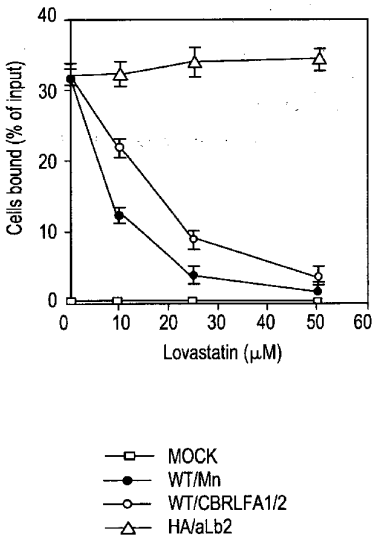

Cells bound (% of input)
40
30
20
10
0
0
10
20
30
40
50
60
Lovastatin (μM)
MOCK
WT/Mn
WT/CBRLFA1/2
HA/aLb2

FIG. 7

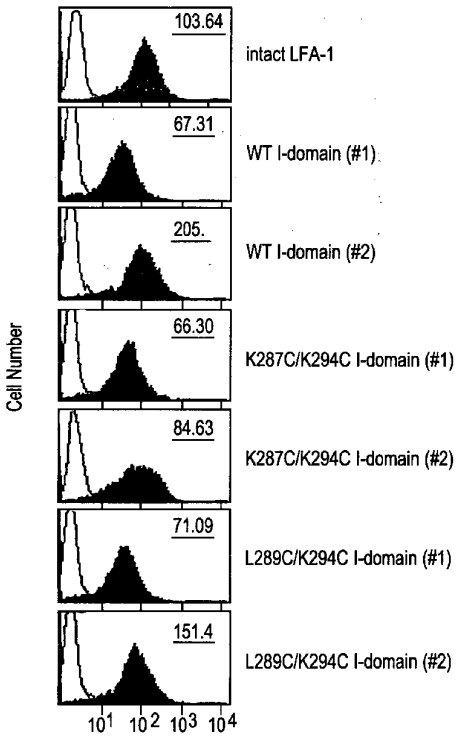

103.64
intact LFA-1
67.31
WT I-domain (#1)
205.
WT I-domain (#2)
66.30
K287C/K294C I-domain (#1)
84.63
K287C/K294C I-domain (#2)
71.09
L289C/K294C I-domain (#1)
151.4
L289C/K294C I-domain (#2)
Cell Number
10^1 10^2 10^3 10^4
Log Fluorescence Intensity

FIG. 8A

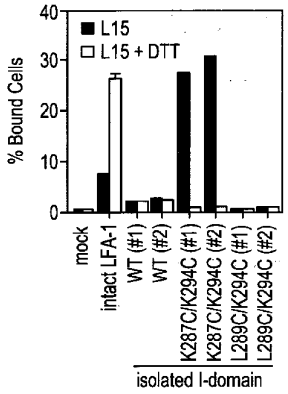


FIG. 8B

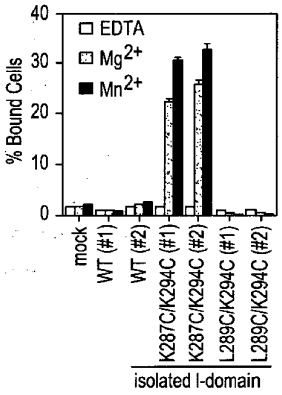


FIG. 8C

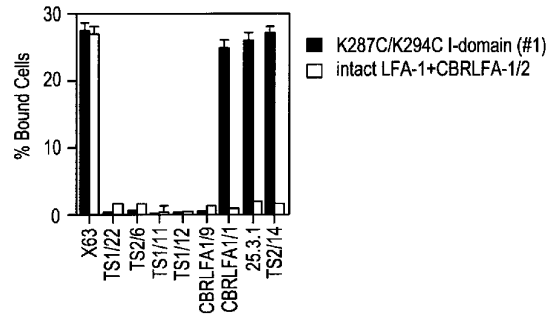


FIG. 9A

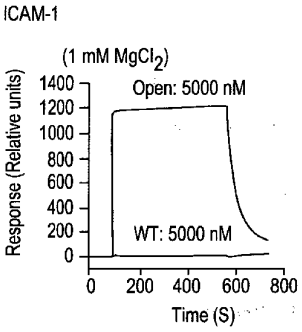


FIG. 9B

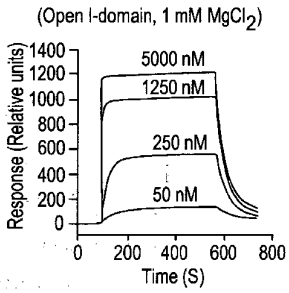


FIG. 9C

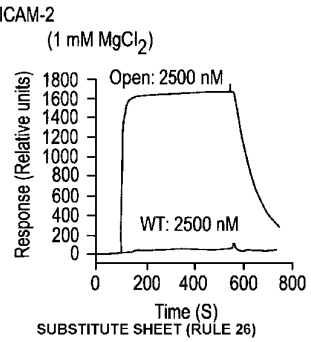


FIG. 9D

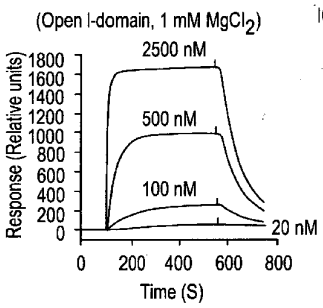


FIG. 9E

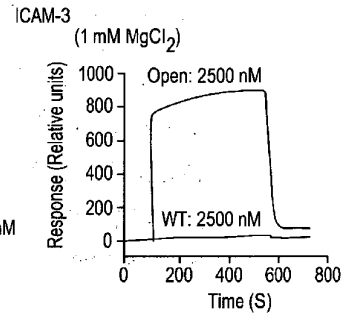


FIG. 9F

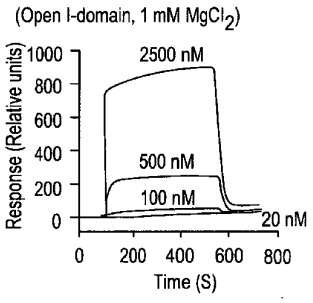


FIG. 10A

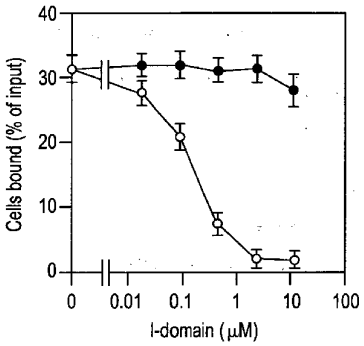


FIG. 10B

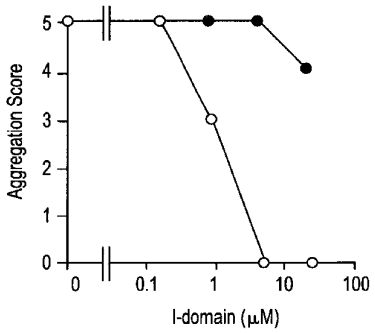


FIG. 11A

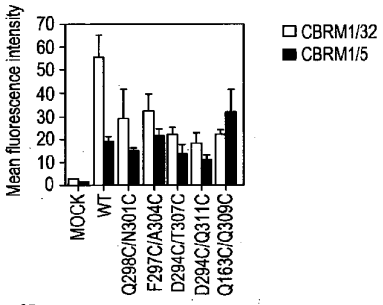


FIG. 11B

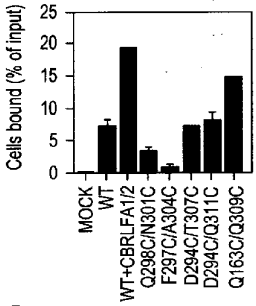


FIG. 11C

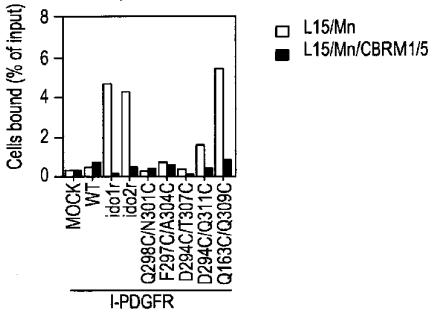


FIG. 12A

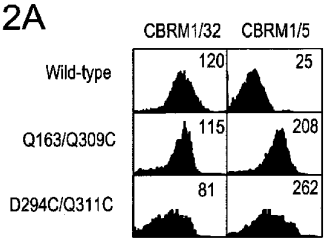


FIG. 12B

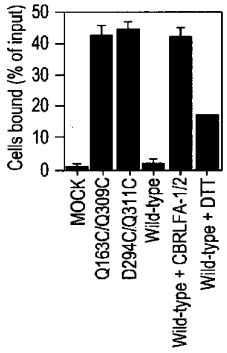
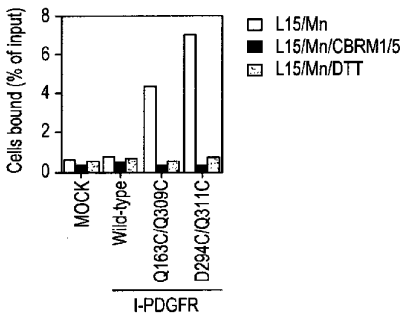


FIG. 12C



【国際調査報告】

INTERNATIONAL SEARCH REPORT

Int[illegible] ial Application No
PC[illegible]US 01/27227

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 G06F17/50 A01K67/027 A61K38/17
A61K31/7088 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N G01N G06F A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PETRUZZELLI LILLI ET AL: "Activation of lymphocyte function-associated molecule-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) mimicked by an antibody directed against CD18." JOURNAL OF IMMUNOLOGY, vol. 155, no. 2, 1995, pages 854-866, XP002209615 ISSN: 0022-1767 the whole document -/--	25-30

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2002	27/08/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Smalt, R

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INTERNATIONAL SEARCH REPORT

Inte ... ial Application No
PC1/US 01/27227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAZES B ET AL: "MODEL BUILDING OF DISULFIDE BONDS IN PROTEINS WITH KNOWN THREE-DIMENSIONAL STRUCTURE" PROTEIN ENGINEERING, vol. 2, no. 2, 1988, pages 119-126, XP001074212 ISSN: 0269-2139 cited in the application	38-43
Y	the whole document	1-35, 44-72
Y	SHIMAOKA M ET AL: "COMPUTATIONAL DESIGN OF AN INTEGRIN I DOMAIN STABILIZED IN THE OPEN HIGH AFFINITY CONFORMATION" NATURE STRUCTURAL BIOLOGY, NEW YORK, NY, US, vol. 8, no. 7, August 2000 (2000-08), pages 674-678, XP008006358 ISSN: 1072-8368 the whole document	1-7, 12-28, 32-35, 44-55, 57,58, 61,62,72
Y	QU AIDONG ET AL: "Crystal structure of the I-domain from the CD11a/CD18 (LFA-1, alpha-L-beta-2) integrin." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 22, 1995, pages 10277-10281, XP002209616 1995 ISSN: 0027-8424 cited in the application the whole document	1-11, 16-35, 44-72
A	SALI A ET AL: "COMPARATIVE PROTEIN MODELLING BY SATISFACTION OF SPATIAL RESTRAINTS" JOURNAL OF MOLECULAR BIOLOGY, LONDON, GB, vol. 234, 1993, pages 779-815, XP002948615 ISSN: 0022-2836 cited in the application the whole document	
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INTERNATIONAL SEARCH REPORT

Int[illegible]al Application No
PC[illegible]/US 01/27227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	LEE JIE-OH ET AL: "Two conformations of the integrin A-domain (I-domain): A pathway for activation?" STRUCTURE (LONDON), vol. 3, no. 12, 1995, pages 1333-1340, XP008006249 ISSN: 0969-2126 cited in the application the whole document ---	
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A	LI RUI ET AL: "Two functional states of the CD11b A-domain: Correlations with key features of two Mn2+-complexed crystal structures." JOURNAL OF CELL BIOLOGY, vol. 143, no. 6, 14 December 1998 (1998-12-14), pages 1523-1534, XP002209418 ISSN: 0021-9525 the whole document ---	
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A	HUTH J R ET AL: "NMR AND MUTAGENESIS EVIDENCE FOR AND I DOMAIN ALLOSTERIC SITE THAT REGULATES LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN 1 LIGAND BINDING" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 10, 9 May 2000 (2000-05-09), pages 5231-5236, XP000938664 ISSN: 0027-8424 the whole document --- -/--	

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 01/27227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	LU CHAFEN ET AL: "An isolated, surface-expressed I domain of the integrin alphaLbeta2 is sufficient for strong adhesive function when locked in the open conformation with a disulfide bond." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 5, 27 February 2001 (2001-02-27), pages 2387-2392, XP002209618 February 27, 2001 ISSN: 0027-8424 the whole document	1-11, 16-23, 25-30, 32-35, 38-45
P,X	LU CHAFEN ET AL: "Locking in alternate conformations of the integrin alphaLbeta2 I domain with disulfide bonds reveals functional relationships among integrin domains." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 5, 27 February 2001 (2001-02-27), pages 2393-2398, XP002209619 February 27, 2001 ISSN: 0027-8424 the whole document	1-11, 16-23, 25-30, 32-35, 38-43
P,X	SHIMAOKA MOTOMU ET AL: "Reversibly locking a protein fold in an active conformation with a disulfide bond: Integrin alphaL I domains with high affinity and antagonist activity in vivo." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 11, 22 May 2001 (2001-05-22), pages 6009-6014, XP002209620 May 22, 2001 ISSN: 0027-8424 the whole document	1-11, 16-23, 25-30, 32-35, 38-43
P,X	ORCHEKOWSKI RANDAL P ET AL: "alphaMbeta2 (CD11b/CD18, Mac-1) integrin activation by a unique monoclonal antibody to alphaM I domain that is divalent cation-sensitive." JOURNAL OF LEUKOCYTE BIOLOGY, vol. 68, no. 5, November 2000 (2000-11), pages 641-649, XP008006357 ISSN: 0741-5400 the whole document	25,26,28

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/27227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	SHIMAOKA M ET AL: "CONFORMATIONAL REGULATION OF INTEGRIN STRUCTURE AND FUNCTION" ANNUAL REVIEW OF BIOPHYSICS AND BIOMOLECULAR STRUCTURE, ANNUAL REVIEWS INC., PALO ALTO, CA, US, vol. 31, no. 31, July 2002 (2002-07), pages 485-516,C01-C08, XP008006352 ISSN: 1056-8700 page 494, paragraph 4 -page 495, paragraph 2 page 497, paragraph 2 -page 500, paragraph 2	

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INTERNATIONAL SEARCH REPORT

ational application No.
PCT/US 01/27227

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 46-64 and 68-70 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

ormation on patent family members

Int nal Application No
PC I/US 01/27227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9517412 A	29-06-1995	US 5437958 A	01-08-1995
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		AU 1860395 A	10-07-1995
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		US 5817515 A	06-10-1998
		US 5837478 A	17-11-1998

Form PCT/ISA/210 (patent family annex) (July 1992)

フロントページの続き

(51)Int.Cl.[7]	FI		テーマコード（参考）
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Ａ６１Ｋ 45/00	Ａ６１Ｋ 45/00		
Ａ６１Ｋ 48/00	Ａ６１Ｋ 48/00		
Ａ６１Ｐ 1/04	Ａ６１Ｐ 1/04		
Ａ６１Ｐ 9/00	Ａ６１Ｐ 9/00		
Ａ６１Ｐ 9/10	Ａ６１Ｐ 9/10		
Ａ６１Ｐ 11/00	Ａ６１Ｐ 11/00		
Ａ６１Ｐ 11/06	Ａ６１Ｐ 11/06		
Ａ６１Ｐ 13/12	Ａ６１Ｐ 13/12		
Ａ６１Ｐ 17/00	Ａ６１Ｐ 17/00		
Ａ６１Ｐ 17/02	Ａ６１Ｐ 17/02		
Ａ６１Ｐ 17/06	Ａ６１Ｐ 17/06		
Ａ６１Ｐ 19/02	Ａ６１Ｐ 19/02		
Ａ６１Ｐ 25/04	Ａ６１Ｐ 25/04		
Ａ６１Ｐ 29/00	Ａ６１Ｐ 29/00		
Ａ６１Ｐ 37/02	Ａ６１Ｐ 29/00	１０１	
Ｃ０７Ｋ 14/705	Ａ６１Ｐ 37/02		
Ｃ０７Ｋ 16/28	Ｃ０７Ｋ 14/705		
Ｇ０１Ｎ 33/15	Ｃ０７Ｋ 16/28		
Ｇ０１Ｎ 33/50	Ｇ０１Ｎ 33/15	Ｚ	
Ｇ０１Ｎ 33/53	Ｇ０１Ｎ 33/50	Ｚ	
Ｇ０１Ｎ 33/566	Ｇ０１Ｎ 33/53	Ｄ	
	Ｇ０１Ｎ 33/566		
	Ａ６１Ｋ 37/02		

(81)指定国 AP(GH,GM,KE,LS,MW,MZ,SD,SL,SZ,TZ,UG,ZW),EA(AM,AZ,BY,KG,KZ,MD,RU,TJ,TM),EP(AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE,TR),OA(BF,BJ,CF,CG,CI,CM,GA,GN,GQ,GW,ML,MR,NE,SN,TD,TG),AE,AG,AL,AM,AT,AU,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CO,CR,CU,CZ,DE,DK,DM,DZ,EC,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KP,KR,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,PH,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,US,UZ,VN,YU,ZA,ZW

（特許庁注：以下のものは登録商標）
Windows

(72)発明者 シモアカ, モトム
アメリカ合衆国 マサチューセッツ州 ０２４４６ ブルックライン ロングウッド アベニュー ７３
(72)発明者 リュー, チャーフェン
アメリカ合衆国 マサチューセッツ州 ０２１６７ ニュートン ウッドマン ロード ３６

Ｆターム(参考) 2G045 BB14 BB20 BB50 BB51 CB01 DA13 FA11 FA37 FB03 FB07 FB12 GC15
4B024 AA01 BA63 CA04 DA03 DA06 EA04 GA11
4C084 AA02 AA06 AA07 AA13 AA19 BA01 BA02 BA08 BA22 BA23 CA53 MA01 MA02 NA14 ZB081 ZB111
4C085 AA03 AA13 AA14 BB11 DD61 EE01 EE05
4C087 AA01 AA02 BC83 MA02 NA13 NA14 ZB08 ZB11
4H045 AA10 AA20 AA30 BA50 CA40 DA50 DA75 EA22 EA31 FA74

专利名称(译)	用所需构象稳定的修饰多肽和产生所述多肽的方法		
公开(公告)号	JP2004527217A	公开(公告)日	2004-09-09
申请号	JP2002522490	申请日	2001-08-31
[标]申请(专利权)人(译)	该中心血液研究油墨		
申请(专利权)人(译)	该中心血液研究油墨		
[标]发明人	スプリンガーティモシーエイ シモアカモトム リューチャーフェン		
发明人	スプリンガー, ティモシー, エイ. シモアカ, モトム リュー, チャーフェン		
IPC分类号	A01K67/027 A61K35/76 A61K38/00 A61K39/00 A61K39/395 A61K45/00 A61K48/00 A61P1/04 A61P9/00 A61P9/10 A61P11/00 A61P11/06 A61P13/12 A61P17/00 A61P17/02 A61P17/06 A61P19/02 A61P25/04 A61P29/00 A61P37/02 C07K14/705 C07K16/28 C12N15/09 C12N15/12 G01N33/15 G01N33/50 G01N33/53 G01N33/566		
CPC分类号	A61K38/00 A61P1/04 A61P11/00 A61P11/06 A61P13/12 A61P17/00 A61P17/02 A61P17/06 A61P19/02 A61P25/04 A61P29/00 C07K14/70546 Y10S930/26		
FI分类号	C12N15/00.ZNA.A A01K67/027 A61K35/76 A61K39/00.H A61K39/395.D A61K39/395.N A61K45/00 A61K48/00 A61P1/04 A61P9/00 A61P9/10 A61P11/00 A61P11/06 A61P13/12 A61P17/00 A61P17/02 A61P17/06 A61P19/02 A61P25/04 A61P29/00 A61P29/00.101 A61P37/02 C07K14/705 C07K16/28 G01N33/15.Z G01N33/50.Z G01N33/53.D G01N33/566 A61K37/02		
F-TERM分类号	2G045/BB14 2G045/BB20 2G045/BB50 2G045/BB51 2G045/CB01 2G045/DA13 2G045/FA11 2G045/FA37 2G045/FB03 2G045/FB07 2G045/FB12 2G045/GC15 4B024/AA01 4B024/BA63 4B024/CA04 4B024/DA03 4B024/DA06 4B024/EA04 4B024/GA11 4C084/AA02 4C084/AA06 4C084/AA07 4C084/AA13 4C084/AA19 4C084/BA01 4C084/BA02 4C084/BA08 4C084/BA22 4C084/BA23 4C084/CA53 4C084/MA01 4C084/MA02 4C084/NA14 4C084/ZB081 4C084/ZB111 4C085/AA03 4C085/AA13 4C085/AA14 4C085/BB11 4C085/DD61 4C085/EE01 4C085/EE05 4C087/AA01 4C087/AA02 4C087/BC83 4C087/MA02 4C087/NA13 4C087/NA14 4C087/ZB08 4C087/ZB11 4H045/AA10 4H045/AA20 4H045/AA30 4H045/BA50 4H045/CA40 4H045/DA50 4H045/DA75 4H045/EA22 4H045/EA31 4H045/FA74		
优先权	60/229700 2000-09-01 US		
其他公开文献	JP5113314B2		
外部链接	Espacenet		

摘要(译)

本发明提供了通过向多肽中引入至少一个二硫键来稳定所需构象的蛋白质的方法。使用计算机设计，指定在哪个位置引入半胱氨酸残基，二硫化物仅由一种蛋白质构象形成，因此该蛋白质是否可以以某种构象固定。因此，选择对所需蛋白质构象和小分子治疗特异的抗体。本发明还提供了用所需构象稳定的修饰的整联蛋白I-结构域多肽。此外，本发明还提供了利用本发明的修饰的整联蛋白I结构域的筛选试验和治疗方法。

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