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(54) EPITOPE MAPPING USING NUCLEAR MAGNETIC RESONANCE

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(57) ABSTRACT

This invention relates to methods for mapping or otherwise identifying the amino acid sequence and conformation of a portion of a protein that is involved in ligand binding. This invention finds utility in the process of elucidating the amino acid sequence and conformation of an epitope of, for example, an antigen or an antibody that binds to the antigen.

EPITOPE MAPPING USING NUCLEAR MAGNETIC RESONANCE

FIELD OF INVENTION

[0001] This invention relates to methods for mapping or otherwise identifying the amino acid sequence and conformation of a potion of a protein involved in ligand binding.

BACKGROUND OF THE INVENTION

[0002] When two biological molecules bind to each other, such as when an antibody binds to an antigen or a ligand binds to a receptor, there are discrete interactions between binding domains of the biological molecules themselves. For example, regarding antibody/antigen binding, there are interactions between the amino acids residing primarily within the complementarity determining regions of an antibody and, in the case where the antigen is a protein, the amino acids in the epitope of the antigen.

[0003] Several techniques have been used to determine the identity, conformation, or amino acid make-up of various binding domains. These include the synthesis of peptides on pins or in spots on membrane, the use of synthetic peptides or proteolytic fragments of the antigen in ELISA or competition assays, analysis of the binding of the antibody to antigens in which point mutations have been made, phage display libraries of random peptide or antigen fragments sequences, proteolysis of antigens in the presence or absence of antibody with mass spectral analysis of either binding or non-binding fragments, and X-ray crystallography. Many of these methods are expensive and time consuming, requiring many steps before meaningful results are available. The invention herein provides a new technique for the identification of amino acid sequences involved in molecular interaction between biological molecules using nuclear magnetic resonance (NMR).

SUMMARY OF THE INVENTION

[0004] The invention herein provides new methods of characterizing a binding domain of a labeled, recombinant protein comprising comparing a nuclear magnetic resonance spectrum of a labeled, recombinant protein in the absence of a ligand with a nuclear magnetic resonance spectrum of a labeled, recombinant protein in the presence of a ligand, wherein the binding domain is characterized after the comparing step is undertaken one or more times.

[0005] In one embodiment of the present invention, the recombinant protein can be recombinant protein labeled at multiple sites or a recombinant protein labeled at alternate sites.

[0006] In another embodiment of the present invention, the recombinant protein can be tissue plasminogen activator receptor and the ligand can be tissue plasminogen activator.

[0007] In another embodiment of the present invention, the recombinant protein can be tumor necrosis factor and the ligand can be any antibody that binds to tumor necrosis factor.

[0008] In another embodiment of the present invention, the recombinant protein can be erythropoietin receptor and the ligand can be erythropoietin or an erythropoietin mimetic.

[0009] In another embodiment of the present invention, the recombinant protein or the ligand can be a diagnostic compound or a therapeutic compound. The diagnostic or therapeutic compound can be any of the immunoglobulins, fragments of immunoglobulins, integrins, antigens, growth factors, cell cycle proteins, cytokines, hormones, neurotransmitters, receptors or fusion proteins thereof, blood proteins, antimicrobials, chemokines, or any structural or functional analog thereof.

[0010] In another embodiment of the present invention, the immunoglobulin can be IgG1, IgG2, IgG3, IgG4, IgΛ1, IgΛ2, slgΛ, IgD, IgE, or any structural or functional analog thereof.

[0011] In another embodiment of the present invention, the fragment of immunoglobulin can be $F(ab')_2$, Fab', Fab, Fc, Facb, pFc', Fd, FV, or any structural or functional analog thereof.

[0012] In another embodiment of the present invention, the integrin can be αv, β1, β2, β3, β4, β5, or any structural or functional analog thereof.

[0013] In another embodiment of the present invention, the antigen can be any of the cancer-specific antigens, viral antigens, bacterial antigens, chemical antigens, or any structural or functional analog thereof.

[0014] In another embodiment of the present invention, the growth factor can be any of the insulin-like growth factors, platelet-derived growth factors, fibroblast growth factor 2, epidermal growth factors, tumor growth factors, nerve growth factors, vascular endothelial growth factors, or any structural or functional analog thereof.

[0015] In another embodiment of the present invention, the cell cycle protein can be p13, p27, p34, p53, p60, p80, histone H1, any of the centrosomal proteins, any of the lamins, any of the CDKs, any of the CDK inhibitors, any of the tumor suppressor genes, or any structural or functional analog thereof.

[0016] In another embodiment of the present invention, the cytokine can be IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, any of the interferons, any of the interleukins, any of the lymphokines, any of the cell signal molecules, or any structural or functional analog thereof.

[0017] In another embodiment of the present invention, the hormone can be any of the parathyroid hormones, follicle stimulating hormones, estrogens, progesterones, testosterones, aldosterones, antidiuretic hormones, cortisols, human chorionic gonadotropins, parathormones, growth hormones, secretins, thyroid hormones, or any structural or functional analog thereof.

[0018] In another embodiment of the present invention, the neurotransmitter can be GABA, any of the enkephalins, or any structural or functional analog thereof.

[0019] In another embodiment of the present invention, the receptor or fusion protein thereof can be any of the chemokine receptors, cytokine receptors, cell cycle protein receptors, growth factor receptors, hormone receptors, neurotransmitter receptors, fusion proteins to any one of the foregoing, or any structural or functional analog thereof.

[0020] In another embodiment of the present invention, the blood protein can be any of the TPOs, any of the tPAs, or any structural or functional analog thereof.

[0021] In another embodiment of the present invention, the antimicrobial can be any of the antibiotics, antifungals, antiprotozoals, antivirals, or any structural or functional analog thereof.

[0022] In another embodiment of the present invention, the labeled, recombinant protein can be an isotopically labeled protein. The label can be ^{15}N, ^{13}C, ^{3}H, ^{33}S, ^{17}O, or ^{2}H.

[0023] In another embodiment of the present invention, the comparing step can be accomplished via a computer. The comparing step may also involve data obtained from X-ray crystallography analysis.

[0024] The invention herein also provides new methods of characterizing a binding domain of a ligand comprising comparing a nuclear magnetic resonance spectrum of a labeled, recombinant ligand of a ligand-binding compound in the absence of a ligand-binding compound with a nuclear magnetic resonance spectrum of a labeled, recombinant ligand of a ligand-binding compound in the presence of a ligand-binding compound, wherein the binding domain can be characterized after the comparing step is undertaken one or more times.

[0025] In one embodiment of the present invention, the labeled, recombinant ligand can be a labeled, recombinant ligand labeled at multiple sites or a labeled, recombinant ligand labeled at alternate sites.

[0026] In another embodiment of the present invention, the ligand-binding compound can be tissue plasminogen activator receptor and the labeled, recombinant ligand can be tissue plasminogen activator.

[0027] In another embodiment of the present invention, the ligand-binding compound can be tumor necrosis factor and the labeled, recombinant ligand can be any antibody that binds to tumor necrosis factor.

[0028] In another embodiment of the present invention, the ligand-binding compound can be erythropoietin receptor and the labeled, recombinant ligand can be erythropoietin or an erythropoietin mimetic.

[0029] In another embodiment of the present invention, the ligand-binding compound or the labeled, recombinant ligand can be a diagnostic compound or a therapeutic compound. The diagnostic or therapeutic compound can be any of the immunoglobulins, fragments of immunoglobulins, integrins, antigens, growth factors, cell cycle proteins, cytokines, hormones, neurotransmitters, receptors or fusion proteins thereof, blood proteins, antimicrobials, chemokines, or any structural or functional analog thereof.

[0030] In another embodiment of the present invention, the immunoglobulin can be IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, slgA, IgD, IgE, or any structural or functional analog thereof.

[0031] In another embodiment of the present invention, the fragment of immunoglobulin can be $F(ab')_2$, Fab', Fab, Fc, Facb, pFc', Fd, FV, or any structural or functional analog thereof.

[0032] In another embodiment of the present invention, the integrin can be αv, β1, β2, β3, β4, β5, or any structural or functional analog thereof.

[0033] In another embodiment of the present invention, the antigen can be any of the cancer-specific antigens, viral antigens, bacterial antigens, chemical antigens, or any structural or functional analog thereof.

[0034] In another embodiment of the present invention, the growth factor can be any of the insulin-like growth factors, platelet-derived growth factors, fibroblast growth factor 2, epidermal growth factors, tumor growth factors, nerve growth factors, vascular endothelial growth factors, or any structural or functional analog thereof.

[0035] In another embodiment of the present invention, the cell cycle protein can be p13, p27, p34, p53, p60, p80, histone H1, any of the centrosomal proteins, any of the lamins, any of the CDKs, any of the CDK inhibitors, any of the tumor suppressor genes, or any structural or functional analog thereof.

[0036] In another embodiment of the present invention, the cytokine can be IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, any of the interferons, any of the interleukins, any of the lymphokines, any of the cell signal molecules, or any structural or functional analog thereof.

[0037] In another embodiment of the present invention, the hormone can be any of the parathyroid hormones, follicle stimulating hormones, estrogens, progesterones, testosterones, aldosterones, antidiuretic hormones, cortisols, human chorionic gonadotropins, parathormones, growth hormones, secreting, thyroid hormones, or any structural or functional analog thereof.

[0038] In another embodiment of the present invention, the neurotransmitter can be GABA, any of the enkephalins, or any structural or functional analog thereof.

[0039] In another embodiment of the present invention, the receptor or fusion protein thereof can be any of the chemokine receptors, cytokine receptors, cell cycle protein receptors, growth factor receptors, hormone receptors, neurotransmitter receptors, fusion proteins to any one of the foregoing, or any structural or functional analog thereof.

[0040] In another embodiment of the present invention, the blood protein can be any of the TPOs, any of the tPAs, or any structural or functional analog thereof.

[0041] In another embodiment of the present invention, the antimicrobial can be any of the antibiotics, antifungals, antiprotozoals, antivirals, or any structural or functional analog thereof.

[0042] In another embodiment of the present invention, the ligand-binding compound can be an isotopically labeled protein. The label can be ^{15}N, ^{13}C, ^{3}H, ^{33}S, ^{17}O, or ^{2}H.

[0043] In another embodiment of the present invention, the comparing step can be accomplished via a computer. The comparing step may also involve data obtained from X-ray crystallography analysis.

[0044] Further, the invention herein provides new methods of characterizing a binding domain of a labeled ligand-

binding compound comprising comparing a nuclear magnetic resonance spectrum of a labeled, ligand-binding compound in the absence of a ligand with a nuclear magnetic resonance spectrum of a labeled, ligand-binding compound in the presence of a ligand, wherein the binding domain can be characterized after the comparing step is undertaken one or more times.

[0045] In one embodiment of the present invention, the ligand-binding compound can be a ligand-binding compound labeled at multiple sites or a ligand-binding compound labeled at alternate sites.

[0046] In another embodiment of the present invention, the ligand-binding compound can be tissue plasminogen activator receptor and the ligand can be tissue plasminogen activator.

[0047] In another embodiment of the present invention, the ligand-binding compound can be tumor necrosis factor and the ligand can be any antibody that binds to tumor necrosis factor.

[0048] In another embodiment of the present invention, the ligand-binding compound can be erythropoietin receptor and the ligand can be erythropoietin or an erythropoietin mimetic.

[0049] In another embodiment of the present invention, the ligand-binding compound or the ligand can be a diagnostic compound or a therapeutic compound. The diagnostic or therapeutic compound can be any of the immunoglobulins, fragments of immunoglobulins, integrins, antigens, growth factors, cell cycle proteins, cytokines, hormones, neurotransmitters, receptors or fusion proteins thereof, blood proteins, antimicrobials, chemokines, or any structural or functional analog thereof.

[0050] In another embodiment of the present invention, the immunoglobulin can be IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, slgA, IgD, IgE, or any structural or functional analog thereof.

[0051] In another embodiment of the present invention, the fragment of immunoglobulin can be $F(ab')_2$, Fab', Fab, Fc, Facb, pFc', Fd, FV, or any structural or functional analog thereof.

[0052] In another embodiment of the present invention, the integrin can be αv, β1, β2, β3, β4, β5, or any structural or functional analog thereof.

[0053] In another embodiment of the present invention, the antigen can be any of the cancer-specific antigens, viral antigens, bacterial antigens, chemical antigens, or any structural or functional analog thereof.

[0054] In another embodiment of the present invention, the growth factor can be any of the insulin-like growth factors, platelet-derived growth factors, fibroblast growth factor 2, epidermal growth factors, tumor growth factors, nerve growth factors, vascular endothelial growth factors, or any structural or functional analog thereof.

[0055] In another embodiment of the present invention, the cell cycle protein can be p13, p27, p34, p53, p60, p80, histone H1, any of the centrosomal proteins, any of the lamins, any of the CDKs, any of the CDK inhibitors, any of the tumor suppressor genes, or any structural or functional analog thereof.

[0056] In another embodiment of the present invention, the cytokine can be IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, any of the interferons, any of the interleukins, any of the lymphokines, any of the cell signal molecules, or any structural or functional analog thereof.

[0057] In another embodiment of the present invention, the hormone can be any of the parathyroid hormones, follicle stimulating hormones, estrogens, progesterones, testosterones, aldosterones, antidiuretic hormones, cortisols, human chorionic gonadotropins, parathormones, growth hormones, secreting, thyroid hormones, or any structural or functional analog thereof.

[0058] In another embodiment of the present invention, the neurotransmitter can be GABA, any of the enkephalins, or any structural or functional analog thereof.

[0059] In another embodiment of the present invention, the receptor or fusion protein thereof can be any of the chemokine receptors, cytokine receptors, cell cycle protein receptors, growth factor receptors, hormone receptors, neurotransmitter receptors, fusion proteins to any one of the foregoing, or any structural or functional analog thereof.

[0060] In another embodiment of the present invention, the blood protein can be any of the TPOs, any of the tPAs, or any structural or functional analog thereof.

[0061] In another embodiment of the present invention, the antimicrobial can be any of the antibiotics, antifungals, antiprotozoals, antivirals, or any structural or functional analog thereof.

[0062] In another embodiment of the present invention, the labeled, ligand-binding compound can be an isotopically labeled compound. The label can be ^{15}N, ^{13}C, ^{3}H, ^{33}S, ^{17}O, or ^{2}H.

[0063] In another embodiment of the present invention, the comparing step can be accomplished via a computer. The comparing step may also involve data obtained from X-ray crystallography analysis.

[0064] The invention herein also provides any of the binding domains characterized by any of the methodologies of the present invention.

[0065] The invention herein also provides databases comprising any and/or all of the data collected via any of the methodologies of the present invention. Such databases may or may not be managed by a computer system. Such databases may or may not appear within recordable media.

[0066] Also provided by the present invention are business methods comprising performing for a customer any of the methodologies of the present invention.

[0067] Other objectives, features and advantages of the present invention will become apparent from the following detailed description. The detailed description and the specific examples, although indicating specific embodiments of the invention, are provided by way of illustration only. Accordingly, the present invention also includes those various changes and modifications within the spirit and scope of the invention that can become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

[0068] It is to be understood that this invention is not limited to the particular methodology, protocols, constructs, formulae and reagents described and as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

[0069] It must be noted that as used herein and in the appended claims, the singular forms "a,""and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a protein" is a reference to one or more proteins and includes equivalents thereof known to those skilled in the art, and so forth. Further, reference to a "ligand-binding compound" is a reference to any substance, including but not limited to any recombinant protein, that binds a ligand.

[0070] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

[0071] All publications and patents mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the constructs and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventor is not entitled to antedate such disclosure by virtue of prior invention.

[0072] This invention provides a new procedure for the identification of protein binding domains using recombinant proteins or ligands enriched with isotopically labeled amino acids, in which precise identification of NMR signals is not always required. These binding domains, as the term is used herein, can be the binding regions of any biological molecules, including antibodies, antigens, ligands, and receptors. For convenience, the term 'epitope' is used synonymously with binding domain in many instances throughout the present specification.

[0073] NMR is a technique that identifies specific atoms (generally ^{1}H, ^{13}C, and ^{15}N), and hence identifies amino acid residues, based on the local environment of these atoms within the amino acid molecule. Additionally, carbon and nitrogen NMR spectra are less complex than proton spectra, but natural abundance of the required nuclei can limit sensitivity. With large proteins, there can be considerable overlap in the spectra between atoms in similar environments. Complete assignment of most or all resonances can be done for large proteins, however, given sufficient time and instruments of high enough resolution.

[0074] When a biological molecule binds to a protein, the local environment of some amino acids changes. Those amino acids affected most profoundly are those most likely involved with ligand/protein contact, i.e., the binding domain. Theoretically, it is possible to identify the amino acids of the binding domain by making the NMR assignments for both the protein and the ligand in bound and unbound states, then determining with amino acids have shifted. The complexity of the NMR spectra of an ligand/protein complex makes such an analysis extremely difficult if not impossible, however, with current instruments and methods and is not, therefore, applicable to routine epitope identification. See, e.g., REES ET AL., in PROTEIN & PHARM. ENG'G 35-53 (Craik et al. eds., 1990).

[0075] The present invention provides a simple method for using NMR to determine the amino acids involved in a binding domain. More specifically, using recombinant technology, a ligand, ligand-binding compound, or a recombinant protein, such as an antibody, antigen or receptor, is expressed in a medium in which a single amino acid has been replaced with an isotopically labeled (for example, ^{15}N, ^{13}C, ^{3}H, ^{33}S, ^{17}O, or ^{2}H) counterpart. The ligand, ligand-binding compound, or recombinant protein generated in this manner will have the identical structure and functionality as its unlabeled counterpart.

[0076] The ^{15}N, ^{13}C, ^{3}H, ^{33}S, ^{17}O, or ^{2}H NMR spectra are then run on the ligand, ligand-binding compound, or recombinant protein, separately in the presence and absence of the ligand's binding partner or the recombinant protein's or ligand-binding compound's ligand. The low natural abundance of the resonating nuclei of the unlabeled amino acids provides 5 simplified spectra such that a decoupled spectra will exhibit singlets of ^{15}N spectra and singlets or simple patterns for ^{13}C, ^{3}H, ^{33}S, ^{17}, or ^{2}H spectra, depending on whether the amino acids were uniformly or specifically labeled. In those instances where the labeled amino acid is involved in binding with the target, a shift in resonance is seen.

[0077] As an example, if a protein of 200 amino acids contained ten ^{15}N-labeled alanines, then ten singlets would be seen in the ^{15}N spectra. If, when bound to the ligand, two of these spectra are shifted, this reflects the alteration in the residues' local environment. From this, their location in the binding domain can be inferred. The specific location of the two alanines in the sequence, for example, might not be determined from this single spectrum, but when this process is repeated twenty times with a different amino acid labeled each time, the, composition of the binding domain is identified. Moreover, because the sequence of the protein is known (in a preferred embodiment the protein is produced recombinantly), the location and specific sequence of the binding domain is determined by comparing the known amino acid sequence with the amino acids identified by the NMR studies.

[0078] In another embodiment of the invention, data obtained from the methodologies involving NMR can be considered in light of data obtained from X-ray crystallography analyses. For example, conclusions reached via the NMR-related methodologies of the present invention can be confirmed or partially supported by conclusions reached via X-ray crystallography analyses. Hence, research methodologies comprising NMR-related methodologies and X-ray crystallography analyses are fully within the scope of the present invention. Additionally, in another embodiment of the invention, molecular modeling or algorithms that predict surface-exposed sequences on proteins may provide useful information for epitope identification.

[0079] The methodologies taught herein offer several advantages over current procedures for epitope identification.

[0080] For example, current methods using synthetic peptides (including, but not limited to, pin, spot, or solution and ELISA, or competition assays) or phage or mere fragments of antigen can miss conformational epitopes. Current variations of the spot synthesis (e.g., matrix of peptides) are purportedly better in identifying conformational epitopes, but the number of peptides required for this analysis increases exponentially with the number of amino acids in the protein to the point that approximately two million peptides would be required to characterize a protein of molecular weight 40 kD. See WO 00/72880 A2; Kleinjung et al., 53 BIOPOLYMERS 113-28 (2000); Tugarinov et al., 8 STRUCTURE 385-95 (2000); WINKLER ET AL., in PEPTIDES 1996, 913-14 (Robert Ramage and Roger Epton eds., 1998); Tsang et al., 10 J. MOL. RECOG. 256-62 (1997); Kustanovich & Zvi, 66 EPITOPE MAPPING PROTOCOLS 25-37 (1996); Hubbard et al., 6 PROTEIN SCI. 1945-52 (1997); Zvi et al., 229 EUR. J. BIOCHEM. 178-87 (1995); Cung et al., 31 BIOPOLYMERS 769-76 (1991); Bechtel et al., 11 BULL. MAGN. RESON. 421 (1989); Huang et al., 281 J. MOL. BIOL. 61-67 (1998); Zvi & Anglister, 5 LETTERS IN PEPTIDE SCI. 357-64 (1998). The NMR procedure of the present invention, because it uses the intact protein, will detect conformational epitopes as readily as linear epitopes.

[0081] Another current approach, proteolysis, in combination with mass spectrometry, can identify some conformational epitopes, but the technique destroys the protein and increasingly larger amounts of protein are needed as the molecular weight increases. Bundle et al., 33 BIOCHEM. 5183-92 (1994). In comparison, the NMR procedure described herein is non-destructive. Indeed, if the labeled protein is in short supply it can be recovered after each experiment and reused to map, for example, another ligand.

[0082] The instant invention is also advantageous over point mutations or 'alanine scans' of proteins, which have been used for the identification of both linear and conformational epitope mapping. Cung et al., 11 BULL. MAGN. RESON. 381 (1989). The difficulty of this approach is that each protein requires its own DNA for expression, and for each mutation it must be determined if the target protein has refolded properly upon purification. In contrast, the NMR procedure of the present invention uses the same DNA for all labeled protein, and the labeled and unlabeled proteins are purified and fold identically.

[0083] It has been suggested that X-ray crystallography can be used to identify the binding sites of organic solvents on macromolecules. WO 99/54504. Its drawbacks include the extensive amount of time involved, the amount of protein required, the difficulty of growing a diffraction grade crystal, and the fact that each antibody to the same antigen requires a new protein and a new crystal. Moreover, this approach is not a screening method for rapidly testing many compounds that are chemically diverse, but is limited to mapping the binding sites of only a few organic molecules due to the long time needed to determine the individual crystal structures.

[0084] The nature and source of the compounds involved within the methodologies of the present invention are not limited. For example, the NMR techniques of the present invention can be used to determine the binding domains of an immunoglobulin or fragment thereof which binds an antigen, a cytokine, an integrin, a growth factor, a cell cycle protein, a hormone, a neurotransmitter, a receptor or fusion protein thereof, a blood protein, an antimicrobial, any fragment thereof, and any structural or functional analog thereof. The following is a general discussion of the variety of proteins, peptides and biological molecules that can be used in the in accordance with the teachings herein. These descriptions do not serve to limit the scope of the invention, but rather illustrate the breadth of the invention.

[0085] One embodiment of the methodologies of the present invention involves human or non-human monoclonal antibodies or antibody fragments. Specifically, these antibodies (immunoglobulins) can be isolated, recombinant and/or synthetic human, primate, rodent, mammalian, chimeric, humanized or CDR-grafted, antibodies and anti-idiotype antibodies thereto. Such antibodies or antibody fragments can be produced by enzymatic cleavage, synthetic techniques, or recombinant techniques as known in the art and/or as described herein. Additionally, these antibodies can also be produced in a variety of truncated forms in which various portions of antibodies are joined together chemically by conventional techniques, or prepared as a contiguous protein using genetic engineering techniques. As used presently, an "antibody,""antibody fragment,""antibody variant, ""Fab," and the like, include any protein- or peptide-containing molecule that comprises at least a portion of an immunoglobulin molecule, such as but not limited to at least one CDR of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof, or at least one portion of a receptor or binding protein, which can be identified using the techniques of the present invention. Such antibody optionally further affects a specific ligand, such as but not limited to, where such antibody modulates, decreases, increases, antagonizes, agonizes, mitigates, alleviates, blocks, inhibits, abrogates and/or interferes with at least one target activity or binding, or with receptor activity or binding, in vitro, in situ and/or in vivo.

[0086] Another embodiment of methodologies of the present invention may involve antibodies, or functional equivalents thereof, that are "human," such that they are substantially non-immunogenic in humans. These antibodies can be prepared through any of the methodologies described herein, including the use of transgenic animals, genetically engineered to express human antibody genes. For example, immunized transgenic mice (xenomice) that express either fully human antibodies, or human variable regions have been described. WO 96/34096. In the case of xenomice, the antibodies produced include fully human antibodies and can be obtained from the animal directly (e.g., from serum), or from immortalized B-cells derived from the animal, or from the genes encoding the immunoglobulins with human variable regions can be recovered and expressed to obtain the antibodies directly or modified to obtain analogs of antibodies such as, for example, Fab or single chain Fv molecules. Id. See also, e.g., U.S. patent application Ser. No. 09/920,137; U.S. Provisional Patent Application Nos. 60/223,369; 60/236,826.

[0087] The term "antibody" is further intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including antibody mimetics or comprising portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. The methodologies of the present invention thus encompass the use of antibody fragments capable of binding to a biological molecule (such as an antigen or receptor) or portions thereof, including but not limited to Fab (e.g., by papain digestion), Fab' (e.g., by pepsin digestion and partial reduction) and $F(ab')_2$ (e.g., by pepsin digestion), facb (e.g., by plasmin digestion), pFc' (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and reaggregation), Fv or scFv (e.g., by molecular biology techniques) fragments. See, e.g., CURRENT PROTOCOLS IN IMMUNOLOGY (Colligan et al., eds., John Wiley & Sons, Inc., NY, 1994-2001).

[0088] As with antibodies, other peptide moieties that bind a particular target protein or other biological molecule (target-binding peptides) are utilized within the methodologies disclosed herein. Such target-binding peptides can be isolated from tissues and purified to homogeneity, or isolated from cells which contain the target-binding protein, and purified to homogeneity. Once isolated and purified, such target-binding peptides can be sequenced by well-known methods. From these amino acid sequences, DNA probes can be produced and used to obtain mRNA, from which cDNA can be made and cloned by known methods. Other well-known methods for producing cDNA are known in the art and may effectively be used. In general, any target-binding peptide can be isolated from any, cell or tissue expressing such proteins using a cDNA probe such as the probe described above, isolating mRNA and transcribing the mRNA into cDNA. Thereafter, the protein can be produced by inserting the cDNA into an expression vector, such as a virus, plasmid, cosmid, or other vector, inserting the expression vector into a cell, proliferating the resulting cells, and isolating the expressed target-binding protein from the medium or from cell extract as described above. Alternatively, target-binding peptides can be chemically synthesized using the sequence described above and an amino acid synthesizer, or manual synthesis using chemical conditions well known to form peptide bonds between selected amino acids. Analogues and fragments of target-binding proteins can be produced by chemically modification or by genetic engineering. These fragments and analogues may then be tested for target-binding activity using known methods. See, e.g., U.S. Pat. No. 5,808,029 to Brockhaus et al., issued Sep. 15, 1998.

[0089] Alternatively, target-binding peptides, including antibodies, can be identified using various library screening techniques. For example, peptide library screening takes advantage of the fact that molecules of only "peptide" length (2 to 40 amino acids) can bind to the receptor protein of a given large protein ligand. Such peptides may mimic the bioactivity of the large protein ligand ("peptide agonists") or, through competitive binding, inhibit the bioactivity of the large protein ligand ("peptide antagonists"). Phage display peptide libraries have emerged as a powerful method in identifying such peptide agonists and antagonists. In such libraries, random peptide sequences are displayed by fusion with coat proteins of filamentous phage. Typically, the displayed peptides are affinity-eluted against an immobilized extracellular domain of an antigen or receptor. The retained phages can be enriched by successive rounds of affinity purification and repropagation. The best binding peptides can be sequenced to identify key residues within one or more structurally related families of peptides. The peptide sequences may also suggest which residues can be safely replaced by alanine scanning or by mutagenesis at the DNA level. Mutagenesis libraries can be created and screened to further optimize the sequence of the best binders. See, e.g., WO 0024782, published May 4, 2000, and the references cited therein; U.S. Pat. No. 6,090,382 to Salfeld et al., issued Jul. 18, 2000; WO 93/06213, to Hoogenboom et al., published Apr. 1, 1993.

[0090] Other display library screening methods are known as well. For example, *E. coli* displays employ a peptide library fused to either the carboxyl terminus of the lac-repressor or the peptidoglycan-associated lipoprotein, and expressed in *E. coli*. Ribosome display involves halting the translation of random RNAs prior to ribosome release, resulting in a library of polypeptides with their associated RNAs still attached. RNA-peptide screening employs chemical linkage of peptides to RNA. Additionally, chemically derived peptide libraries have been developed in which peptides are immobilized on stable, non-biological materials, such as polyethylene rods or solvent-permeable resins. Another chemically derived peptide library uses photolithography to scan peptides immobilized on glass slides. These methods of chemical-peptide screening can be advantageous because they allow use of D-amino acids and other unnatural analogues, as well as non-peptide elements. See WO 0024782, published May 4, 2000, and the references cited therein.

[0091] Moreover, structural analysis of binding domains may also be used to suggest peptides that mimic the binding activity of large protein ligands. In such an analysis, the crystal structure may suggest the identity and relative orientation of critical residues of the large protein ligand, from which a peptide can be designed. These analytical methods may also be used to investigate the interaction between, for example, a receptor protein and peptides selected by phage display, which may suggest further modification of the peptides to increase binding affinity. Thus, conceptually, one may discover peptide mimetics of any protein using phage display and the other methods mentioned above. For example, these methods provide for epitope mapping, for identification of critical amino acids in protein-protein interactions, and as leads for the discovery of new therapeutic agents. See WO 00/24782, published May 4, 2000, and the references cited therein.

[0092] The methods of the instant invention may also be used to characterize the binding of synthetic peptides to target receptors or antigens. For example, solution-phase synthesis has been used to create the eptifibatide molecule that binds the platelet receptor glycoprotein IIb/IIIa of human platelets, thus inhibiting platelet aggregation. Eptifibatide, sold commercially as INTEGRILIN® (COR Therapeutics, Belmont, Calif.), is a cyclic heptapeptide containing six amino acids and one mercaptopropionyl (des-amino cysteinyl) residue.

[0093] In one embodiment of the present invention, the methods described herein can be used to determine the binding domain of a growth factor to its receptor or other

binding target. Briefly, growth factors are hormones or cytokine proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Many growth factors are quite versatile, stimulating cellular division in numerous different cell types; while others are specific to a particular cell-type. The following Table 1 presents several factors, but is not intended to be comprehensive or complete, yet introduces some of the more commonly known factors and their principal activities.

TABLE 1

Growth Factors

Factor	Principal Source	Primary Activity	Comments
Platelet Derived Growth Factor (PDGF)	Platelets, endothelial cells, placenta.	Promotes proliferation of connective tissue, glial and smooth muscle cells. PDGF receptor has intrinsic tyrosine kinase activity.	Dimer required for receptor binding. Two different protein chains, A and B, form 3 distinct dimer forms.
Epidermal Growth Factor (EGF)	Submaxillary gland, Brunners gland.	promotes proliferation of mesenchymal, glial and epithelial cells	EGF receptor has tyrosine kinase activity, activated in response to EGF binding.
Fibroblast Growth Factor (FGF)	Wide range of cells; protein is associated with the ECM; nineteen family members. Receptors widely distributed in bone, implicated in several bone-related diseases.	Promotes proliferation of many cells including skeletal and nervous system; inhibits some stem cells; induces mesodermal differentiation. Non-proliferative effects include regulation of pituitary and ovarian cell function.	Four distinct receptors, all with tyrosine kinase activity. FGF implicated in mouse mammary tumors and Kaposi's sarcoma.
NGF		Promotes neurite outgrowth and neural cell survival	Several related proteins first identified as protooncogenes; trkA (trackA), trkB, trkC
Erythropoietin (Epo)	Kidney	Promotes proliferation and differentiation of erythrocytes	Also considered a 'blood protein,' and a colony stimulating factor.
Transforming Growth Factor a (TGF-α)	Common in transformed cells, found in macrophages and keratinocytes	Potent keratinocyte growth factor.	Related to EGF.
Transforming Growth Factor v (TGF-β)	Tumor cells, activated TH_1 cells (T-helper) and natural killer (NK) cells	Anti-inflammatory (suppresses cytokine production and class II MHC expression), proliferative effects on many mesenchymal and epithelial cell types, may inhibit macrophage and lymphocyte proliferation.	Large family of proteins including activin, inhibin and bone morpho-genetic protein. Several classes and subclasses of cell-surface receptors
Insulin-Like Growth Factor-I (IGF-I)	Primarily liver, produced in response to GH and then induces subsequent cellular activities, particularly on bone growth	Promotes proliferation of many cell types, autocrine and paracrine activities in addition to the initially observed endocrine activities on bone.	Related to IGF-II and proinsulin, also called Somatomedin C. IGF-I receptor, like the insulin receptor, has intrinsic tyrosine kinase activity. IGF-I can bind to the insulin receptor.
Insulin-Like Growth Growth Factor-II (IGF-II)	Expressed almost exclusively in embryonic and neonatal tissues.	Promotes proliferation of many cell types primarily of fetal origin. Related to IGF-I and proinsulin.	IGF-II receptor is identical to the mannose-6-phosphate receptor that is responsible for the integration of lysosomal enzymes

[**0094**] Additional growth factors that can be utilized within the methodologies of the present invention include insulin and proinsulin (U.S. Pat. No. 4,431,740); Activin (Vale et al., 321 NATURE 776 (1986); Ling et al., 321 NATURE 779 (1986)); Inhibin (U.S. Pat. Nos. 4,740,587; 4,737,578); and Bone Morphongenic Proteins (BMPs) (U.S. Pat. No. 5,846,931; WOZNEY, CELLULAR & MOLECULAR BIOLOGY OF BONE 131-167 (1993)).

[**0095**] Additional growth factors that can be utilized within the methodologies of the present invention include Activin (Vale et al., 321 NATURE 776 (1986); Ling et al.,

321 NATURE 779 (1986)), Inhibin (U.S. Pat. Nos. 4,737,578; 4,740,587), and Bone Morphongenic Proteins (BMPs) (U.S. Pat. No. 5,846,931; WOZNEY, CELLULAR & MOLECULAR BIOLOGY OF BONE 131-67 (1993)).

[0096] In another embodiment, the methodologies of the present invention can be used to characterize the binding domain of a cytokine or cytokine receptor. Secreted primarily from leukocytes, cytokines stimulate both the humoral and cellular immune responses, as well as the activation of phagocytic cells. Cytokines that are secreted from lymphocytes are termed lymphokines, whereas those secreted by monocytes or macrophages are termed monokines. A large family of cytokines are produced by various cells of the body. Many of the lymphokines are also known as interleukins (ILs), because they are not only secreted by leukocytes, but are also able to affect the cellular responses of leukocytes. More specifically, interleukins are growth factors targeted to cells of hematopoietic origin. The list of identified interleukins grows continuously. See, e.g., U.S. Pat. No. 6,174,995; U.S. Pat. No. 6,143,289; Sallusto et al., 18 ANNU. REV. IMMUNOL. 593 (2000); Kunkel et al., 59 J. LEUKOCYTE BIOL. 81 (1996).

[0097] Additional growth factor/cytokines encompassed in the methodologies of the present invention include pituitary hormones such as CEA, FSH, FSH α, FSH β, Human Chorionic Gonadotrophin (HCG), HCG α, HCG β, uFSH (urofollitropin), GH, LH, LH α, LH β, PRL, TSH, TSH α, TSH β, and CA, parathyroid hormones, follicle stimulating hormones, estrogens, progesterones, testosterones, or structural or functional analog thereof. All of these proteins and peptides are known in the art. Many can be obtained commercially from, e.g., Research Diagnostics, Inc. (Flanders, N.J.).

[0098] The cytokine family also includes tumor necrosis factors, colony stimulating factors, and interferons. See, e.g., Cosman, 7 BLOOD CELL (1996); Gruss et al., 85 BLOOD 3378 (1995); Beutler et al., 7 ANNU. REV. IMMUNOL. 625 (1989); Aggarwal et al., 260 J. BIOL. CHEM. 2345 (1985); Pennica et al., 312 NATURE 724 (1984); R & D Systems, CYTOKINE MINI-REVIEWS, at http://www.rndsystems.com.

[0099] Several cytokines are introduced, briefly, in Table 2 below.

TABLE 2

Cytokines

Cytokine	Principal Source	Primary Activity
Interleukins IL1-α and -β	Primarily macrophages but also neutrophils, endothelial cells, smooth muscle cells, glial cells, astrocytes, B- and T-cells, fibroblasts, and keratinocytes.	Costimulation of APCs and T cells; stimulates IL-2 receptor production and expression of interferon-γ; may induce proliferation in non-lymphoid cells.
IL-2	CD4+ T-helper cells, activated TH_1 cells, NK cells.	Major interleukin responsible for clonal T-cell proliferation. IL-2 also exerts effects on B-cells, macrophages, and natural killer (NK) cells. . IL-2 receptor is not expressed on the surface of resting T-cells, but expressed constitutively on NK cells, that will secrete TNF-α, IFN-g and GM-CSF in response to IL-2, which in turn activate macrophages.
IL-3	Primarily T-cells	Also known as multi-CSF, as it stimulates stem cells to produce all forms of hematopoietic cells.
IL-4	TH_2 and mast cells	B cell proliferation, eosinophil and mast cell growth and function, IgE and class II MHC expression on B cells, inhibition of monokine production
IL-5	TH_2 and mast cells	eosinophil growth and function
IL-6	Macrophages, fibroblasts, endothelial cells and activated T-helper cells. Does not induce cytokine expression.	IL-6 acts in synergy with IL-1 and TNF-α in many immune responses, including T-cell activation; primary inducer of the acute-phase response in liver; enhances the differentiation of B-cells and their consequent production of immunoglobulin; enhances Glucocorticoid synthesis.
IL-7	thymic and marrow stromal cells	T and B lymphopoiesis
IL-8	Monocytes, neutrophils, macrophages, and NK cells.	Chemoattractant (chemokine) for neutrophils, basophils and T-cells; activates neutrophils to degranulate.
IL-9	T cells	hematopoietic and thymopoietic effects
IL-10	activated TH_2 cells, $CD8^+$ T and B cells, macrophages	inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IL-11	stromal cells	synergisitc hematopoietic and thrombopoietic effects

TABLE 2-continued

Cytokines

Cytokine	Principal Source	Primary Activity
IL-12	B cells, macrophages	proliferation of NK cells, INF-γ production, promotes cell-mediated immune functions
IL-13	TH_2 cells	IL-4-like activities
IL-18	macrophages/Kupffer cells, keratinocytes, glucocorticoid-secreting adrenal cortex cells, and osteoblasts	Interferon-gamma-inducing factor with potent pro-inflammatory activity
IL-21	Activated T cells	IL21 has a role in proliferation and maturation of natural killer (NK) cell populations from bone marrow, in the proliferation of mature B-cell populations co-stimulated with anti-CD40, and in the proliferation of T cells co-stimulated with anti-CD3.
IL-23	Activated dendritic cells	A complex of p19 and the p40 subunit of IL-12. IL-23 binds to IL-12R beta 1 but not IL-12R beta 2; activates Stat4 in PHA blast T cells; induces strong proliferation of mouse memory T cells; stimulates IFN-gamma production and proliferation in PHA blast T cells, as well as in CD45RO (memory) T cells.
Tumor Necrosis Factor TNF-α	Primarily activated macrophages.	Once called cachectin; induces the expression of other autocrine growth factors, increases cellular responsiveness to growth factors; induces signaling pathways that lead to proliferation; induces expression of a number of nuclear proto-oncogenes as well as of several interleukins.
(TNF-β)	T-lymphocytes, particularly cytotoxic T-lymphocytes (CTL cells); induced by IL-2 and antigen-T-Cell receptor interactions.	Also called lymphotoxin; kills a number of different cell types, induces terminal differentiation in others; inhibits lipoprotein lipase present on the surface of vascular endothelial cells.
Interferons INF-α and -β	macrophages, neutrophils and some somatic cells	Known as type I interferons; antiviral effect; induction of class I MHC on all somatic cells; activation of NK cells and macrophages.
Interferon INF-γ	Primarily CD8+ T-cells, activated TH_1 and NK cells	Type II interferon; induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, enhances ability of cells to present antigens to T-cells; antiviral effects.
Monocyte Chemoattractant Protein-1 (MCP1)	Peripheral blood monocytes/macrophages	Attracts monocytes to sites of vascular endothelial cell injury, implicated in atherosclerosis.
Colony Stimulating Factors (CSFs)		Stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults.
Granulocyte-CSF (G-CSF)		Specific for proliferative effects on cells of the granulocyte lineage; proliferative effects on both classes of lymphoid cells.
Macrophage-CSF (M-CSF)		Specific for cells of the macrophage lineage.
Granulocyte-MacrophageCSF (GM-CSF)		Proliferative effects on cells of both the macrophage and granulocyte lineages.

[0100] Other cytokines of interest that can be characterized by the invention described herein include adhesion molecules (R & D Systems, ADHESION MOLECULES I (1996), available at http://www.rndsystems.com); angiogenin (U.S. Pat. No. 4,721,672; Moener et al., 226 EUR. J. BIOCHEM. 483 (1994)); annexin V (Cookson et al., 20 GENOMICS 463 (1994); Grundmann et al., 85 PROC. NATL. ACAD. SCI. USA 3708 (1988); U.S. Pat. No. 5,767,247); caspases (U.S. Pat. No. 6,214,858; Thornberry et al., 281 SCIENCE 1312 (1998)); chemokines (U.S. Pat. Nos. 6,174,995; 6,143,289; Sallusto et al., 18 ANNU. REV. IMMUNOL. 593 (2000) Kunkel et al., 59 J. LEUKOCYTE BIOL. 81 (1996)); endothelin (U.S. Pat. Nos. 6,242,485; 5,294,569; 5,231,166); eotaxin (U.S. Pat. No. 6,271,347;

Ponath et al., 97(3) J. CLIN. INVEST. 604-612 (1996)); Flt-3 (U.S. Pat. No. 6,190,655); heregulins (U.S. Pat. Nos. 6,284,535; 6,143,740; 6,136,558; 5,859,206; 5,840,525); Leptin (Leroy et al., 271(5) J. BIOL. CHEM. 2365 (1996); Maffei et al., 92 PNAS 6957 (1995); Zhang et al. (1994) NATURE 372: 425-432); Macrophage Stimulating Protein (MSP) (U.S. Pat. Nos. 6,248,560; 6,030,949; 5,315,000); Neurotrophic Factors (U.S. Pat. Nos. 6,005,081; 5,288,622); Pleiotrophin/Midkine (PTN/MK) (Pedraza et al., 117 J. BIOCHEM. 845 (1995); Tamura et al., 3 ENDOCRINE 21 (1995); U.S. Pat. No. 5,210,026; Kadomatsu et al., 151 BIOCHEM. BIOPHYS. RES. COMMUN. 1312 (1988)); STAT proteins (U.S. Pat. Nos. 6,030,808; 6,030,780; Darnell et al., 277 SCIENCE 1630-1635 (1997)); Tumor Necrosis Factor Family (Cosman, 7 BLOOD CELL (1996); Gruss et al., 85 BLOOD 3378 (1995); Beutler et al., 7 ANNU. REV. IMMUNOL. 625 (1989); Aggarwal et al., 260 J. BIOL. CHEM. 2345 (1985); Pennica et al., 312 NATURE 724 (1984)).

[0101] Also of interest regarding cytokines are proteins or chemical moieties that interact with cytokines, such as Matrix Metalloproteinases (MMPs) (U.S. Pat. No. 6,307,089; NAGASE, MATRIX METALLOPROTEINASES IN ZINC METALLOPROTEASES IN HEALTH AND DISEASE (1996)), and Nitric Oxide Synthases (NOS) (Fukuto, 34 ADV. PHARM 1 (1995); U.S. Pat. No. 5,268,465).

[0102] A further embodiment of the present invention applies the methodologies described herein to the characterization of the binding domains of blood proteins. The term "blood protein" is a generic term for a vast group of proteins generally circulating in blood plasma, and important for regulating coagulation and clot dissolution. See, e.g., Haematologic Technologies, Inc., HTI CATALOG, available at www.haemtech.com. Table 3 introduces, in a non-limiting fashion, some of the blood proteins contemplated by the present invention.

TABLE 3

Blood Proteins

Protein	Principle Activity	Reference
Factor V	In coagulation, this glycoprotein procofactor, is converted to active cofactor, factor Va, via the serine protease α-thrombin, and less efficiently by its serine protease cofactor Xa. The prothrombinase complex rapidly converts zymogen prothrombin to the active serine protease, α-thrombin. Down regulation of prothrombinase complex occurs via inactivation of Va by activated protein C.	Mann et al., 57 ANN. REV. BIOCHEM. 915 (1988); see also Nesheim et al., 254 J. BIOL. CHEM. 508 (1979); Tracy et al., 60 BLOOD 59 (1982); Nesheim et al., 80 METHODS ENZYMOL. 249 (1981); Jenny et al., 84 PROC. NATL. ACAD. SCI. USA 4846 (1987).
Factor VII	Single chain glycoprotein zymogen in its native form. Proteolytic activation yields enzyme factor VIIa, which binds to integral membrane protein tissue factor, forming an enzyme complex that proteolytically converts factor X to Xa. Also known as extrinsic factor Xase complex. Conversion of VII to VIIa catalyzed by a number of proteases including thrombin, factors IXa, Xa, XIa, and XIIa. Rapid activation also occurs when VII combines with tissue factor in the presence of Ca, likely initiated by a small amount of pre-existing VIIa. Not readily inhibited by antithrombin III/heparin alone, but is inhibited when tissue factor added.	See generally, Broze et al., 80 METHODS ENZYMOL. 228 (1981); Bajaj et al., 256 J. BIOL. CHEM. 253 (1981); Williams et al., 264 J. BIOL. CHEM. 7536 (1989); Kisiel et al., 22 THROMBOSIS RES. 375 (1981); Seligsohn et al., 64 J. CLIN. INVEST. 1056 (1979); Lawson et al., 268 J. BIOL. CHEM. 767 (1993).
Factor IX	Zymogen factor IX, a single chain vitamin K-dependent glycoprotein, made in liver. Binds to negatively charged phospholipid surfaces. Activated by factor XIα or the factor VIIa/tissue factor/phospholipid complex. Cleavage at one site yields the intermediate IXα, subsequently converted to fully active form IXaβ by cleavage at another site. Factor IXaβ is the catalytic component of the "intrinsic factor Xase complex" (factor VIIIa/IXa/Ca^{2+}/phospholipid) that proteolytically activates factor X to factor Xa.	Thompson, 67 BLOOD, 565 (1986); Hedner et al., HEMOSTASIS AND THROMBOSIS 39–47 (R. W. Colman, J. Hirsh, V. J. Marder, E. W. Salzman ed., 2nd ed. J. P. Lippincott Co., Philadelphia) 1987; Fujikawa et al., 45 METHODS IN ENZYMOLOGY 74 (1974).
Factor X	Vitamin K-dependent protein zymogen, made in liver, circulates in plasma as a two chain molecule linked by a disulfide bond. Factor Xa (activated X) serves as	See Davie et al., 48 ADV. ENZYMOL 277 (1979); Jackson, 49 ANN. REV. BIOCHEM. 765 (1980); see also Fujikawa et al., 11 BIOCHEM. 4882

TABLE 3-continued

Blood Proteins

Protein	Principle Activity	Reference
	the enzyme component of prothrombinase complex, responsible for rapid conversion of prothrombin to thrombin.	(1972); Discipio et al., 16 BIOCHEM. 698 (1977); Discipio et al., 18 BIOCHEM. 899 (1979); Jackson et al., 7 BIOCHEM. 4506 (1968); McMullen et al., 22 BIOCHEM. 2875 (1983).
Factor XI	Liver-made glycoprotein homodimer circulates, in a non-covalent complex with high molecular weight kininogen, as a zymogen, requiring proteolytic activation to acquire serine protease activity. Conversion of factor XI to factor XIa is catalyzed by factor XIIa. XIa unique among the serine proteases, since it contains two active sites per molecule. Works in the intrinsic coagulation pathway by catalyzing conversion of factor IX to factor IXa. Complex form, factor XIa/HMWK, activates factor XII to factor XIIa and prekallikrein to kallikrein. Major inhibitor of XIa is a_1-antitrypsin and to lesser extent, antithrombin-III. Lack of factor XI procoagulant activity causes bleeding disorder: plasma thromboplastin antecedent deficiency.	Thompson et al., 60 J. CLIN. INVEST. 1376 (1977); Kurachi et al., 16 BIOCHEM. 5831 (1977); Bouma et al., 252 J. BIOL. CHEM. 6432 (1977); Wuepper, 31 FED. PROC. 624 (1972); Saito et al., 50 BLOOD 377 (1977); Fujikawa et al., 25 BIOCHEM. 2417 (1986); Kurachi et al., 19 BIOCHEM. 1330 (1980); Scott et al., 69 J. CLIN. INVEST. 844 (1982).
Factor XII (Hageman Factor)	Glycoprotein zymogen. Reciprocal activation of XII to active serine protease factor XIIa by kallikrein is central to start of intrinsic coagulation pathway. Surface bound α-XIIa activates factor XI to XIa. Secondary cleavage of α-XIIa by kallikrein yields β-XIIa, and catalyzes solution phase activation of kallikrein, factor VII and the classical complement cascade.	Schmaier et al., 18–38, and Davie, 242–267 HEMOSTASIS & THROMBOSIS (Colman et al., eds., J. B. Lippincott Co., Philadelphia, 1987).
Factor XIII	Zymogenic form of glutaminyl-peptide γ-glutamyl transferase factor XIIIa (fibrinoligase, plasma transglutaminase, fibrin stabilizing factor). Made in the liver, found extracellularly in plasma and intracellularly in platelets, megakaryocytes, monocytes, placenta, uterus, liver and prostrate tissues. Circulates as a tetramer of 2 pairs of nonidentical subunits (A_2B_2). Full expression of activity is achieved only after the Ca^{2+}- and fibrin(ogen)-dependent dissociation of B subunit dimer from A_2' dimer. Last of the zymogens to become activated in the coagulation cascade, the only enzyme in this system that is not a serine protease. XIIIa stabilizes the fibrin clot by crosslinking the α and γ-chains of fibrin. Serves in cell proliferation in wound healing, tissue remodeling, atherosclerosis, and tumor growth.	See McDonaugh, 340–357 HEMOSTASIS & THROMBOSIS (Colman et al., eds., J. B. Lippincott Co., Philadelphia, 1987); Folk et al., 113 METHODS ENZYMOL. 364 (1985); Greenberg et al., 69 BLOOD 867 (1987). Other proteins known to be substrates for Factor XIIIa, that can be hemostatically important, include fibronectin (Iwanaga et al., 312 ANN. NY ACAD. SCI. 56 (1978)), a_2-antiplasmin (Sakata et al., 65 J. CLIN. INVEST. 290 (1980)), collagen (Mosher et al., 64 J. CLIN. INVEST. 781 (1979)), factor V (Francis et al., 261 J. BIOL. CHEM. 9787 (1986)), von Willebrand Factor (Mosher et al., 64 J. CLIN. INVEST. 781 (1979)) and thrombospondin (Bale et al., 260 J. BIOL. CHEM. 7502 (1985); Bohn, 20 MOL. CELL BIOCHEM. 67 (1978)).
Fibrinogen	Plasma fibrinogen, a large glycoprotein, disulfide linked dimer made of 3 pairs of non-identical chains (Aa, Bb and g), made in liver. Aa has N-terminal peptide (fibrinopeptide A (FPA), factor XIIIa crosslinking sites, and 2 phosphorylation sites. Bb has fibrinopeptide B (FPB), 1 of 3 N-linked carbohydrate moieties, and an N-terminal pyroglutamic acid. The g chain contains the other N-linked glycos. site, and factor XIIIa cross-linking sites. Two elongated subunits ($(AaBbg)_2$) align in an antiparallel way forming a trinodular arrangement of the 6 chains. Nodes formed by disulfide	FURLAN, Fibrinogen, IN HUMAN PROTEIN DATA, (Haeberli, ed., VCH Publishers, N.Y., 1995); Doolittle, in HAEMOSTASIS & THROMBOSIS, 491–513 (3rd ed., Bloom et al., eds., Churchill Livingstone, 1994); HANTGAN, et al., in HAEMOSTASIS & THROMBOSIS 269–89 (2d ed., Forbes et al., eds., Churchill Livingstone, 1991).

TABLE 3-continued

Blood Proteins

Protein	Principle Activity	Reference
	rings between the 3 parallel chains. Central node (n-disulfide knot, E domain) formed by N-termini of all 6 chains held together by 11 disulfide bonds, contains the 2 IIa-sensitive sites. Release of FPA by cleavage generates Fbn I, exposing a polymerization site on Aa chain. These sites bind to regions on the D domain of Fbn to form proto-fibrils. Subsequent IIa cleavage of FPB from the Bb chain exposes additional polymerization sites, promoting lateral growth of Fbn network. Each of the 2 domains between the central node and the C-terminal nodes (domains D and E) has parallel a-helical regions of the Aa, Bb and g chains having protease-(plasmin-) sensitive sites. Another major plasmin sensitive site is in hydrophilic preturbance of a-chain from C-terminal node. Controlled plasmin degradation converts Fbg into fragments D and E.	
Fibronectin	High molecular weight, adhesive, glycoprotein found in plasma and extracellular matrix in slightly different forms. Two peptide chains interconnected by 2 disulfide bonds, has 3 different types of repeating homologous sequence units. Mediates cell attachment by interacting with cell surface receptors and extracellular matrix components. Contains an Arg-Gly-Asp-Ser (RGDS) cell attachment-promoting sequence, recognized by specific cell receptors, such as those on platelets. Fibrin-fibronectin complexes stabilized by factor XIIIa-catalyzed covalent cross-linking of fibronectin to the fibrin a chain.	Skorstengaard et al., 161 Eur. J. BIOCHEM. 441 (1986); Kornblihtt et al., 4 EMBO J. 1755 (1985); Odermatt et al., 82 PNAS 6571 (1985); Hynes, R. O., ANN. REV. CELL BIOL., 1, 67 (1985); Mosher 35 ANN. REV. MED. 561 (1984); Rouslahti et al., 44 Cell 517 (1986); Hynes 48 CELL 549 (1987); Mosher 250 BIOL. CHEM. 6614 (1975).
β_2-Glycoprotein I	Also called β_2I and Apolipoprotein H. Highly glycosylated single chain protein made in liver. Five repeating mutually homologous domains consisting of approximately 60 amino acids disulfide bonded to form Short Consensus Repeats (SCR) or Sushi domains. Associated with lipoproteins, binds anionic surfaces like anionic vesicles, platelets, DNA, mitochondria, and heparin. Binding can inhibit contact activation pathway in blood coagulation. Binding to activated platelets inhibits platelet associated prothrombinase and adenylate cyclase activities. Complexes between b_2I and cardiolipin have been implicated in the anti-phospholipid related immune disorders LAC and SLE.	See, e.g., Lozier et al., 81 PNAS 2640–44 (1984); Kato & Enjyoi 30 BIOCHEM. 11687–94 (1997); Wurm, 16 INT'L J. BIOCHEM. 511–15 (1984); Bendixen et al., 31 BIOCHEM. 3611–17 (1992); Steinkasserer et al., 277 BIOCHEM. J. 387–91 (1991); Nimpf et al., 884 BIOCHEM. BIOPHYS. ACTA 142–49 (1986); Kroll et.al. 434 BIOCHEM. BIOPHYS. Acta 490–501 (1986); Polz et al., 11 INT'L J. BIOCHEM. 265–73 (1976); McNeil et al., 87 PNAS 4120–24 (1990); Galli et a;. I LANCET 1544–47 (1990); Matsuuna et al., II LANCET 177–78 (1990); Pengo et al., 73 THROMBOSIS & HAEMOSTASIS 29–34 (1995).
Osteonectin	Acidic, noncollagenous glycoprotein (Mr = 29,000) originally isolated from fetal and adult bovine bone matrix. May regulate bone metabolism by binding hydroxyapatite to collagen. Identical to human placental SPARC. An alpha granule component of human platelets secreted during activation. A small portion of secreted osteonectin expressed on the platelet cell surface in an activation-dependent manner	Villarreal et al., 28 BIOCHEM. 6483 (1989); Tracy et al., 29 INT'L J. BIOCHEM. 653 (1988); Romberg et al., 25 BIOCHEM. 1176 (1986); Sage & Bornstein 266 J. BIOL. CHEM. 14831 (1991); Kelm & Mann 4 J. BONE MIN. RES. 5245 (1989); Kelm et al., 80 BLOOD 3112 (1992).
Plasminogen	Single chain glycoprotein zymogen with 24 disulfide bridges, no free sulfhydryls, and 5 regions of internal sequence homology, "kringles", each five triple-	See Robbins, 45 METHODS IN ENZYMOLOGY 257 (1976); COLLEN, 243–258 BLOOD COAG. (Zwaal et al., eds., New York, Elsevier, 1986); see

TABLE 3-continued

Blood Proteins

Protein	Principle Activity	Reference
	looped, three disulfide bridged, and homologous to kringle domains in t-PA, u-PA and prothrombin. Interaction of plasminogen with fibrin and α2-antiplasmin is mediated by lysine binding sites. Conversion of plasminogen to plasmin occurs by variety of mechanisms, including urinary type and tissue type plasminogen activators, streptokinase, staphylokinase, kallikrein, factors IXa and XIIa, but all result in hydrolysis at Arg560–Val561, yielding two chains that remain covalently associated by a disulfide bond.	also Castellino et al., 80 METHODS IN ENZYMOLOGY 365 (1981); Wohl et al., 27 THROMB. RES. 523 (1982); Barlow et al., 23 BIOCHEM. 2384 (1984); SOTTRUP-JENSEN ET AL., 3 PROGRESS IN CHEM. FIBRINOLYSIS & THROMBOLYSIS 197–228 (Davidson et al., eds., Raven Press, New York 1975).
tissue Plasminogen Activator	t-PA, a serine endopeptidase synthesized by endothelial cells, is the major physiologic activator of plasminogen in clots, catalyzing conversion of plasminogen to plasmin by hydrolising a specific arginine-alanine bond. Requires fibrin for this activity, unlike the kidney-produced version, urokinase-PA.	See Plasminogen.
Plasmin	See Plasminogen. Plasmin, a serine protease, cleaves fibrin, and activates and/or degrades compounds of coagulation, kinin generation, and complement systems. Inhibited by a number of plasma protease inhibitors in vitro. Regulation of plasmin in vivo occurs mainly through interaction with a_2-antiplasmin, and to a lesser extent, a_2-macroglobulin.	See Plasminogen.
Platelet Factor-4	Low molecular weight, heparin-binding protein secreted from agonist-activated platelets as a homotetramer in complex with a high molecular weight, proteoglycan, carrier protein. Lysine-rich, COOH-terminal region interacts with cell surface expressed heparin-like glycosaminoglycans on endothelial cells, PF-4 neutralizes anticoagulant activity of heparin exerts procoagulant effect, and stimulates release of histamine from basophils. Chemotactic activity toward neutrophils and monocytes. Binding sites on the platelet surface have been identified and can be important for platelet aggregation.	Rucinski et al., 53 BLOOD 47 (1979); Kaplan et al., 53 BLOOD 604 (1979); George 76 BLOOD 859 (1990); Busch et al., 19 THROMB. RES. 129 (1980); Rao et al., 61 BLOOD 1208 (1983); Brindley, et al., 72 J. CLIN. INVEST. 1218 (1983); Deuel et al., 74 PNAS 2256 (1981); Osterman et al., 107 BIOCHEM. BIOPHYS. RES. COMMUN. 130 (1982); Capitanio et al., 839 BIOCHEM. BIOPHYS. ACTA 161 (1985).
Protein C	Vitamin K-dependent zymogen, protein C, made in liver as a single chain polypeptide then converted to a disulfide linked heterodimer. Cleaving the heavy chain of human protein C converts the zymogen into the serine protease, activated protein C. Cleavage catalyzed by a complex of α-thrombin and thrombomodulin. Unlike other vitamin K dependent coagulation factors, activated protein C is an anticoagulant that catalyzes the proteolytic inactivation of factors Va and VIIIa, and contributes to the fibrinolytic response by complex formation with plasminogen activator inhibitors.	See Esmon, 10 PROGRESS IN THROMB. & HEMOSTS. 25 (1984); Stenflo, 10 SEMIN. IN THROMB. & HEMOSTAS. 109 (1984); Griffen et al., 60 BLOOD 261 (1982); Kisiel et al., 80 METHODS ENZYMOL. 320 (1981); Discipio et al., 18 BIOCHEM. 899 (1979).
Protein S	Single chain vitamin K-dependent protein functions in coagulation and complement cascades. Does not possess the catalytic triad. Complexes to C4b binding protein (C4BP) and to negatively charged phospholipids, concentrating C4BP at cell surfaces	Walker, 10 SEMIN. THROMB. HEMOSTAS. 131 (1984); Dahlback et al., 10 SEMIN. THROMB. HEMOSTAS., 139 (1984); Walker 261 J. BIOL. CHEM. 10941 (1986).

TABLE 3-continued

Blood Proteins

Protein	Principle Activity	Reference
	following injury. Unbound S serves as anticoagulant cofactor protein with activated Protein C. A single cleavage by thrombin abolishes protein S cofactor activity by removing gla domain.	
Protein Z	Vitamin K-dependent, single-chain protein made in the liver. Direct requirement for the binding of thrombin to endothelial phospholipids. Domain structure similar to that of other vitamin K-dependant zymogens like factors VII, IX, X, and protein C. N-terminal region contains carboxyglutamic acid domain enabling phospholipid membrane binding. C-terminal region lacks "typical" serine protease activation site. Cofactor for inhibition of coagulation factor Xa by serpin called protein Z-dependant protease inhibitor. Patients diagnosed with protein Z deficiency have abnormal bleeding diathesis during and after surgical events.	Sejima et al., 171 BIOCHEM. BIOPHYSICS RES. COMM. 661 (1990); Hogg et al., 266 J. BIOL. CHEM. 10953 (1991); Hogg et al., 17 BIOCHEM. BIOPHYSICS RES. COMM. 801 (1991); Han et al., 38 BIOCHEM. 11073 (1999); Kemkes-Matthes et al., 79 THROMB. RES. 49 (1995).
Prothrombin	Vitamin K-dependent, single-chain protein made in the liver. Binds to negatively charged phospholipid membranes. Contains two "kringle" structures. Mature protein circulates in plasma as a zymogen and, during coagulation, is proteolytically activated to the potent serine protease α-thrombin.	Mann et al., 45 METHODS IN ENZYMOLOGY 156 (1976); Magnusson et al., PROTEASES IN BIOLOGICAL CONTROL 123–149 (Reich et al., eds. Cold Spring Harbor Labs., New York 1975); Discipio et al., 18 BIOCHEM. 899 (1979).
α-Thrombin	See Prothrombin. During coagulation, thrombin cleaves fibrinogen to form fibrin, the terminal proteolytic step in coagulation, forming the fibrin clot. Thrombin also responsible for feedback activation of procofactors V and VIII. Activates factor XIII and platelets, functions as vasoconstrictor protein. Procoagulant activity arrested by heparin cofactor II or the antithrombin III/heparin complex, or complex formation with thrombomodulin. Formation of thrombin/thrombomodulin complex results in inability of thrombin to cleave fibrinogen and activate factors V and VIII, but increases the efficiency of thrombin for activation of the anticoagulant, protein C.	45 METHODS ENZYMOL. 156 (1976).
β-Thromboglobulin	Low molecular weight, heparin-binding, platelet-derived tetramer protein, consisting of four identical peptide chains. Lower affinity for heparin than PF-4. Chemotactic activity for human fibroblasts, other functions unknown.	See, e.g., George 76 BLOOD 859 (1990); Holt & Niewiarowski 632 BIOCHIM. BIOPHYS. ACTA 284 (1980); Niewiarowski et al., 55 BLOOD 453 (1980); Varma et al., 701 BIOCHIM. BIOPHYS. ACTA 7 (1982); Senior et al., 96 J. CELL. BIOL. 382 (1983).
Thrombopoietin	Human TPO (Thrombopoietin, Mpl-ligand, MGDF) stimulates the proliferation and maturation of megakaryocytes and promotes increased circulating levels of platelets in vivo. Binds to c-Mpl receptor.	Horikawa et al., 90(10) BLOOD 4031–38 (1997); de Sauvage et al., 369 NATURE 533–58 (1995).
Thrombospondin	High-molecular weight, heparin-binding glycoprotein constituent of platelets, consisting of three, identical, disulfide-linked polypeptide chains. Binds to surface of resting and activated platelets, may effect platelet adherence and aggregation. An integral component of basement membrane in different tissues. Interacts with a variety of extracellular macromolecules including heparin, collagen, fibrinogen and fibronectin,	Dawes et al., 29 THROMB. RES. 569 (1983); Switalska et al., 106 J. LAB. CLIN MED. 690 (1985); Lawler et al. 260 J. BIOL. CHEM. 3762 (1985); Wolff et al., 261 J. BIOL. CHEM. 6840 (1986); Asch et al., 79 J. CLIN. CHEM. 1054 (1987); Jaffe et al., 295 NATURE 246 (1982); Wright et al., 33 J. HISTOCHEM. CYTOCHEM. 295 (1985); Dixit et al., 259 J. BIOL. CHEM. 10100 (1984); Mumby et al., 98 J. CELL. BIOL. 646

TABLE 3-continued

Blood Proteins

Protein	Principle Activity	Reference
	plasminogen, plasminogen activator, and osteonectin. May modulate cell-matrix interactions.	(1984); Lahav et al, 145 EUR. J. BIOCHEM. 151 (1984); Silverstein et al, 260 J. BIOL. CHEM. 10346 (1985); Clezardin et al. 175 EUR. J. BIOCHEM. 275 (1988); Sage & Bornstein (1991).
Von Willebrand Factor	Multimeric plasma glycoprotein made of identical subunits held together by disulfide bonds. During normal hemostasis, larger multimers of vWF cause platelet plug formation by forming a bridge between platelet glycoprotein IB and exposed collagen in the subendothelium. Also binds and transports factor VIII (antihemophilic factor) in plasma.	Hoyer 58 BLOOD 1 (1981); Ruggeri & Zimmerman 65 J. CLIN. INVEST. 1318 (1980); Hoyer & Shainoff 55 BLOOD 1056 (1980); Meyer et al., 95 J. LAB. CLIN. INVEST. 590 (1980); Santoro 21 THROMB. RES. 689 (1981); Santoro, & Cowan 2 COLLAGEN RELAT. RES. 31 (1982); Morton et al., 32 THROMB. RES. 545 (1983); Tuddenham et al., 52 BRIT. J. HAEMATOL. 259 (1982).

[0103] Additional blood proteins contemplated herein include the following human serum proteins, which may also be placed in another category of protein (such as hormone or antigen): Actin, Actinin, Amyloid Serum P, Apolipoprotein E, B2-Microglobulin, C-Reactive Protein (CRP), Cholesterylester transfer protein (CETP), Complement C3B, Ceruplasmin, Creatine Kinase, Cystatin, Cytokeratin 8, Cytokeratin 14, Cytokeratin 18, Cytokeratin 19, Cytokeratin 20, Desmin, Desmocollin 3, FAS (CD95), Fatty Acid Binding Protein, Ferritin, Filamin, Glial Filament Acidic Protein, Glycogen Phosphorylase Isoenzyme BB (GPBB), Haptoglobulin, Human Myoglobin, Myelin Basic Protein, Neurofilament, Placental Lactogen, Human SHBG, Human Thyroid Peroxidase, Receptor Associated Protein, Human Cardiac Troponin C, Human Cardiac Troponin I, Human Cardiac Troponin T, Human Skeletal Troponin I, Human Skeletal Troponin T, Vimentin, Vinculin, Transferrin Receptor, Prealbumin, Albumin, Alpha-1-Acid Glycoprotein, Alpha-1-Antichymotrypsin, Alpha-1-Antitrypsin, Alpha-Fetoprotein, Alpha-1-Microglobulin, Beta-2-microglobulin, C-Reactive Protein, Haptoglobulin, Myoglobulin, Prealbumin, PSA, Prostatic Acid Phosphatase, Retinol Binding Protein, Thyroglobulin, Thyroid Microsomal Antigen, Thyroxine Binding Globulin, Transferrin, Troponin I, Troponin T, Prostatic Acid Phosphatase, Retinol Binding Globulin (RBP). All of these proteins, and sources thereof, are known in the art. Many of these proteins are available commercially from, for example, Research Diagnostics, Inc. (Flanders, N.J.).

[0104] Another embodiment applies the methodologies of the present invention to the characterization of the binding domain of a neurotransmitter or the receptor of a neurotransmitter. Neurotransmitters are chemicals, some of them proteinaceous, made by neurons and used by them to transmit signals to the other neurons or non-neuronal cells (e.g., skeletal muscle, myocardium, pineal glandular cells) that they innervate. Neurotransmitters produce their effects by being released into synapses when their neuron of origin fires (i.e., becomes depolarized) and then attaching to receptors in the membrane of the post-synaptic cells. This causes changes in the fluxes of particular ions across that membrane, making cells more likely to become depolarized, if the neurotransmitter happens to be excitatory, or less likely if it is inhibitory. Neurotransmitters can also produce their effects by modulating the production of other signal-transducing molecules ("second messengers") in the post-synaptic cells. See generally COOPER, BLOOM & ROTH, THE BIOCHEM. BASIS OF NEUROPHARMACOLOGY (7th Ed. Oxford Univ. Press, NYC, 1996); http://web.indstate.edu/thcme/mwking/nerves. Neurotransmitters contemplated in the present invention include, but are not limited to, Acetylcholine, Serotonin, γ-aminobutyrate (GABA), Glutamate, Aspartate, Glycine, Histamine, Epinephrine, Norepinephrine, Dopamine, Adenosine, ATP, Nitric oxide, and any of the peptide neurotransmitters such as those derived from pre-opiomelanocortin (POMC), as well as antagonists and agonists thereof.

[0105] The binding domains of numerous other proteins or peptides can be characterized by the methods of the present invention. Table 4 presents a non-limiting list and description of some pharmacologically active peptides which can be incorporated into the methods contemplated by the present invention.

TABLE 4

Pharmacologically active peptides

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
EPO receptor (intrapeptide disulfide-bonded)	EPO mimetic	Wrighton et al., 273 SCIENCE 458–63 (1996); U.S. Pat. No. 5,773,569, issued Jun. 30, 1998.

TABLE 4-continued

Pharmacologically active peptides

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
EPO receptor (C-terminally cross-linked dimer)	EPO mimetic	Livnah et al., 273 SCIENCE 464–71 (1996); Wrighton et al., 15 NATURE BIOTECHNOLOGY 1261–5 (1997); Int'l Patent Application WO 96/40772, published Dec. 19, 1996.
EPO receptor (linear)	EPO mimetic	Naranda et al., 96 PNAS 7569–74 (1999).
c-Mpl (linear)	TPO-mimetic	Cwirla et al., 276 SCIENCE 1696–9 (1997); U.S. Pat. No. 5,869,451, issued Feb. 9,1999; U.S. Pat. No. 5,932,946, issued Aug. 3,1999.
c-Mpl (C-terminally cross-linked dimer)	TPO-mimetic	Cwirla et al., 276 SCIENCE 1696–9 (1997).
(disulfide-linked dimer)	stimulation of hematopoesis ("G-CSF-mimetic")	Paukovits et al., 364 HOPPE-SEYLERS Z. PHYSIOL. CHEM. 30311 (1984); Laerumgal., 16 Exp. HEMAT. 274–80 (1988).
(alkylene-linked dimer)	G-CSF-mimetic	Batnagar et al., 39 J. MED. CHEM. 38149 (1996); Cuthbertson et al., 40 J. MED. CHEM. 2876–82 (1997); King et al., 19 Exp. HEMATOL. 481 (1991); King et al., 86(Suppl. 1) BLOOD 309 (1995).
IL-1 receptor (linear)	inflammatory and autoimmune diseases ("IL-1 antagonist" or "IL-1 ra-mimetic")	U.S. Pat. No. 5,608,035; U.S. Pat. No. 5,786,331; U.S Pat. No. 5,880,096; Yanofsky et al., 93 PNAS 7381–6 (1996); Akeson et al., 271 J. BIOL. CHEM. 30517–23 (1996); Wiekzorek et al., 49 POL. J. PHARMACOL. 107–17 (1997); Yanofsky, 93 PNAS 7381–7386 (1996).
Facteur thyrnique (linear)	stimulation of lymphocytes (FTS-mimetic)	Inagaki-Ohara et al., 171 CELLULAR IMMUNOL. 30–40 (1996); Yoshida, 6 J. IMMUNOPHARMACOL 141–6 (1984).
CTLA4 MAb (intrapeptide di-sulfide bonded)	CTLA4-mimetic	Fukumoto et al., 16 NATURE BIOTECH. 267–70 (1998).
TNF-α receptor (exo-cyclic)	TNF-α antagonist	Takasaki et al., 15 NATURE BIOTECH. 1266–70 (1997); WO 98/53842, published Dec. 3, 1998.
TNF-α receptor (linear)	TNF-α antagonist	Chirinos-Rojas, J. IMM., 5621–26.
C3b (intrapeptide di-sulfide bonded)	inhibition of complement activation; autoimmune diseases (C3b antagonist)	Sahu et al., 157 IMMUNOL. 884–91 (1996); Morikis et al., 7 PROTEIN SCI. 6 19–27 (1998).
vinculin (linear)	cell adhesion processes, cell growth, differentiation wound healing, tumor metastasis ("vinculin binding")	Adey et al., 324 BIOCHEM. J. 523–8 (1997).
C4 binding protein (C413P) (linear)	anti-thrombotic	Linse et al. 272 BIOL. CHEM. 14658–65 (1997).
urokinase receptor (linear)	processes associated with urokinase interaction with its receptor (e.g. angiogenesis, tumor cell invasion and metastasis; (URK antagonist	Goodson et al., 91 PNAS 7129–33 (1994); International patent application WO 97/35969, published Oct. 2, 1997.
Mdm2, Hdm2 (linear)	Inhibition of inactivation of p53 mediated by Mdm2 or hdm2; anti-tumor ("Mdm/hdm antagonist")	Picksley et al., 9 ONCOGENE 2523–9 (1994); Bottger et al. 269 J. MOL. BIOL. 744–56 (1997); Bottger et al., 13 ONCOGENE 13: 2141–7 (1996).
$p21^{WAFI}$ (linear)	anti-tumor by mimicking the activity of $p21^{WAFI}$	Ball et al., 7 CURR. BIOL. 71–80 (1997).
farnesyl transferase (linear)	anti-cancer by preventing activation of ras oncogene	Gibbs et al., 77 CELL 175–178 (1994).
Ras effector domain (linear)	anti-cancer by inhibiting biological function of the ras oncogene	Moodie et at., 10 TRENDS GENEL 44–48 (1994); Rodriguez et al., 370 NATURE 527–532 (1994).
SH2/SH3 domains (linear)	anti-cancer by inhibiting tumor growth with activated tyrosine kinases	Pawson et al, 3 CURR. BIOL. 434–432 (1993); Yu et al., 76 CELL 933–945 (1994).

TABLE 4-continued

Pharmacologically active peptides

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
$p16^{INK4}$ (linear)	anti-cancer by mimicking activity of p16; e.g., inhibiting cyclin D-Cdk complex ("p, 16-mimetic")	Fahraeus et al., 6 CURR. BIOL. 84–91 (1996).
Src, Lyn (linear)	inhibition of Mast cell activation, IgE-related conditions, type I hypersensitivity ("Mast cell antagonist").	Stauffer et al., 36 BIOCHEM. 9388–94 (1997).
Mast cell protease (linear)	treatment of inflammatory disorders mediated by release of tryptase-6 ("Mast cell protease inhibitors")	International patent application WO 98/33812, published Aug. 6, 1998.
SH3 domains (linear)	treatment of SH3-mediated disease states ("SH3 antagonist")	Rickles et al., 13 EMBO J. 5598–5604 (1994); Sparks et al., 269 J. BIOL. CHEM. 238536 (1994); Sparks et al., 93 PNAS 1540–44 (1996).
HBV core antigen (HBcAg) (linear)	treatment of HBV viral antigen (HBcAg) infections ("anti-HBV")	Dyson & Muray, PNAS 2194–98 (1995).
selectins (linear)	neutrophil adhesion inflammatory diseases ("selectin antagonist")	Martens et al., 270 J. BIOL. CHEM. 21129–36 (1995); European Pat. App. EP 0 714 912, published Jun. 5, 1996.
calmodulin (linear, cyclized)	calmodulin antagonist	Pierce et al, 1 MOLEC. DIVEMILY 25965 (1995); Dedman et al., 267 J. BIOL. CHEM. 23025–30 (1993); Adey & Kay, 169 GENE 133–34 (1996).
integrins (linear, cyclized)	tumor-homing; treatment for conditions related to integrin-mediated cellular events, including platelet aggregation, thrombosis, wound healing, osteoporosis, tissue repair, angiogenesis (e.g., for treatment of cancer) and tumor invasion ("integrin-binding")	International patent applications WO 95/14714, published Jun. 1, 1995; WO 97/08203, published Mar. 6,1997; WO 98/10795, published Mar. 19,1998; WO 99/24462, published May 20, 1999; Kraft et al., 274 J. BIOL. CHEM. 1979–85 (1999)
fibronectin and extracellular matrix components of T-cells and macrophages (cyclic, linear)	treatment of inflammatory and autoimmune conditions	International patent application WO 98/09985, published Mar. 12, 1998.
somatostatin and cortistatin (linear)	treatment or prevention of hormone-producing tumors, acromegaly, giantism, dementia, gastric ulcer, tumor growth, inhibition of hormone secretion, modulation of sleep or neural activity	European patent application EP 0 911 393, published Apr. 28, 1999.
bacterial lipopoly-saccharide (linear)	antibiotic; septic shock; disorders modulatable by CAP37	U.S. Pat. No. 5,877,151, issued Mar. 2, 1999.
parclaxin, mellitin (linear or cyclic)	antipathogenic	International patent application WO 97/31019, published 28 Aug. 1997.
VIP (linear, cyclic)	impotence, neuro-degenerative disorders	International patent application WO 97/40070, published Oct. 30, 1997.
CTLs (linear)	cancer	European patent application EP 0 770 624, published May 2, 1997.
THF-gamma2 (linear)		Burnstein, 27 BIOCHEM. 4066–71 (1988).
Amylin (linear)		Cooper, 84 PNAS 8628–32 (1987).
Adreno-medullin (linear)		Kitamura, 192 BBRC 553–60 (1993).

TABLE 4-continued

Pharmacologically active peptides

Binding partner/ Protein of interest (form of peptide)	Pharmacological activity	Reference
VEGF (cyclic, linear)	anti-angiogenic; cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis ("VEGF antagonist")	Fairbrother, 37 BIOCHEM. 17754–64 (1998).
MMP (cyclic)	inflammation and autoimmune disorders; tumor growth ("MMP inhibitor")	Koivunen, 17 NATURE BIOTECH. 768–74 (1999).
HGH fragment (linear)		U.S. Pat. No. 5,869,452, issued Feb. 9, 1999.
Echistatin	inhibition of platelet aggregation	Gan, 263 J. BIOL. 19827–32 (1988).
SLE autoantibody (linear)	SLE	International patent application WO 96/30057, published Oct. 3, 1996.
GD1 alpha	suppression of tumor metastasis	Ishikawa et al., 1 FEBS LETT. 20–4 (1998).
anti-phospholipid β-2 glycoprotein-1 (β2GPI) antibodies	endothelial cell activation, anti-phospholipid syndrome (APS), thromboembolic phenomena, thrombocytopenia, and recurrent fetal loss	Blank Mal., 96 PNAS 5164–8 (1999).
T-Cell Receptor β chain (linear)	diabetes	International patent application WO 96/101214, published Apr. 18, 1996.

[0106] Regarding a particular embodiment of the present invention, there are two pivotal cytokines in the pathogenesis of rheumatoid arthritis, IL-1 and TNF-α. They act synergistically to induce each other, other cytokines, and COX-2. Research suggests that IL-1 is a primary mediator of bone and cartilage destruction in rheumatoid arthritis patients, whereas TNF-α appears to be the primary mediator of inflammation.

[0107] Thus, an embodiment of the present invention applies the methodologies described herein to characterizing the binding domain of an anti-TNF-α antibody. Anti-TNF-α antibodies have shown great promise as therapeutics. For example, Infliximab, provided commercially as REMICADE® by Centocor, Inc. (Malvern, Pa.) has been used for the treatment of several chronic autoimmune diseases such as Crohn's disease and rheumatoid arthritis. Treacy, 19(4) HUM. EXP. TOXICOL. 226-28 (2000); see also Chantry, 2(1) CURR. OPIN. ANTI-INFLAMMATORY IMMUNOMODULATORY INVEST. DRUGS 31-34 (2000); Rankin et al., 34(4) BRIT. J. RHEUMATOLOGY 334-42 (1995). Alternatively, murine-derived anti-TNF-α antibodies can be utilized as ligands in the methodologies of the present invention. Saravolatz et al., 169(1) J. INFECT. DIS. 214-17 (1994).

[0108] Alternatively, a type of binding domain that can be characterized by the methodologies of the present invention can be a binding domain for or of a thrombolytic. For example, the thrombolytic can be tPA, or a functional variation thereof. RETAVASE®, produced by Centocor, Inc. (Malvern, Pa.), is a variant tPA with a prolonged half-life. Interestingly, in mice, the combination of Retavase and the IIb/IIIa receptor antagonist 7E3F(ab')$_2$ markedly augmented the dissolution of pulmonary embolism. See U.S. Provisional Patent Application Serial No. 60/304409.

[0109] Another type of binding domain that can be characterized by the methodologies of the present invention can be all or part of a TNF receptor, or protein or peptide that interacts with such a receptor. For example, Etanercept is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fe portion of human IgG1. The Fc component of etanercept contains the C_H2 domain, the C_H3 domain and hinge region, but not the C_H1 domain of IgG1. Etanercept is produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system. It consists of 934 amino acids and has an apparent molecular weight of approximately 150 kilodaltons. Etanercept can be obtained as ENBREL™, manufactured by Immunex Corp. (Seattle, Wash.). See also Hughes et al., (6) BIODRUGS 379-93 (2001).

[0110] Another example of a human TNF receptor exists as well, identified as p55. Kalinkovich et al., J. INFERON & CYTOKINE RES. 15749-57 (1995). This receptor has also been explored for use in therapy. See, e.g., Qian et al., 118 ARCH. OPHTHALMOL. 1666-71 (2000). A previous formulation of the soluble p55 TNF receptor had been coupled to polyethylene glycol [r-metHuTNFbp PEGylated dimer (TNFbp)], and demonstrated clinical efficacy but was not suitable for a chronic indication due to the development antibodies upon multiple dosing, which resulted in increased clearance of the drug. A second generation molecule was designed to remove the antigenic epitopes of TNFbp, and can be useful in treating patients with rheumatoid arthritis. Davis et al., Presented at the Ann. European Cong. Rheumatology, Nice, France (Jun. 21-24, 2000).

[0111] Another embodiment of the present invention applies the methodologies described herein to the characterization of an IL-1 receptor antagonist (IL-1Ra), a natu-

rally occurring cytokine antagonist that demonstrates anti-inflammatory properties by balancing the destructive effects of IL-1α and IL-1β, in rheumatoid arthritis but does not induce any intracellular response. Hence, in a preferred embodiment of the invention, the methodologies comprise IL-1Ra, or any structural or functional analog thereof. Two structural variants of IL-1Ra exist: a 17-kDa form that is secreted from monocytes, macrophages, neutrophils, and other cells (sIL-1Ra) and an 18-kDa form that remains in the cytoplasm of keratinocytes and other epithelial cells, monocytes, and fibroblasts (icIL-1Ra). An additional 16-kDa intracellular isoform of IL-1Ra exists in neutrophils, monocytes, and hepatic cells. Both of the major isoforms of IL-1Ra are transcribed from the same gene through the use of alternative first exons. The production of IL-1Ra is stimulated by many substances including adherent IgG, other cytokines, and bacterial or viral components. The tissue distribution of IL-1Ra in mice indicates that sIL-1Ra is found predominantly in peripheral blood cells, lungs, spleen, and liver, while icIL-1Ra is found in large amounts in skin. Studies in transgenic and knockout mice indicate that IL-1Ra is important in host defense against endotoxin-induced injury. IL-1Ra is produced by hepatic cells with the characteristics of an acute phase protein. Endogenous IL-1Ra is produced in human autoimmune and chronic inflammatory diseases. The use of neutralizing anti-IL-1Ra antibodies has demonstrated that endogenous IL-1Ra is an important natural antiinflammatory protein in arthritis, colitis, and granulomatous pulmonary disease. Patients with rheumatoid arthritis treated with IL-1Ra for six months exhibited improvements in clinical parameters and in radiographic evidence of joint damage. Arend et al., 16 ANN. REV. IMMUNOL. 27-55 (1998).

[0112] Yet another example of an IL-1Ra that can be characterized by the methods of the present invention is a recombinant human version called interleukin-1 17.3 Kd met-IL1ra, or Anakinra, produced by Amgen, (San Francisco, Calif.) under the name KINERET™. Anakinra has also shown promise in clinical studies involving patients with rheumatoid arthritis. Presented at the 65th Ann. Sci. Meeting of Am. College Rheumatology (Nov. 12, 2001).

[0113] Another method of the present invention incorporates a moiety that targets cyclooxigenase-2 (COX-2). COX-2 selective inhibitors-such as valdecoxib, etoricoxib, celecoxib and rofecoxib are less toxic to the gastrointestinal (GI) tract than conventional nonsteroidal anti-inflammatory drugs (NSAIDs), while possessing equivalent analgesic efficacy for conditions such as osteoarthritis (OA), rheumatoid arthritis (RA), dental pain and menstrual pain.

[0114] The methodologies of the present invention may also be applied to characterize a selective p38 Mitogen-Activated Protein Kinase (p38 MAP kinase) inhibitor. For example, the compound SB 242235 is a potent and selective p38 MAP kinase inhibitor. The compound is active in the adjuvant arthritic rat, where it inhibits inflammation and has significant joint-protective effects as measured by changes in bone mineral density, magnetic resonance imaging, micro-computed tomography, and histology. These studies indicate that cytokine-suppressing, low molecular weight p38 inhibitors can be orally active, disease-modifying agents in the treatment of rheumatoid arthritis. Badger et al, *Disease-Modifying Activity of SB* 242235, *A Selective Inhibitor of p*38 *Mitogen-Activated Protein Kinase,* in *Rat Adjuvant-Induced Arthritis,* Proceedings of the 1999 AACR, NCI, EORTC Int'l Conference, Am. Assoc. for Cancer Res.

[0115] The methodologies of the present invention may also be applied to characterize the binding domain associated with interleukin 12 (IL-12), a heterodimeric cytokine consisting of glycosylated polypeptide chains of 35 and 40 kD which are disulfide bonded. The cytokine is synthesized and secreted by antigen presenting cells, including dendritic cells, monocytes, macrophages, B cells, Langerhans cells and keratinocytes, as well as natural killer (NK) cells. IL-12 mediates a variety of biological processes and has been referred to as NK cell stimulatory factor (NKSF), T-cell stimulating factor, cytotoxic T-lymphocyte maturation factor and EBV-transformed B-cell line factor. Curfs et al., 10 CLIN. MICRO. REV. 742-80 (1997). Interleukin-12 can bind to the IL-12 receptor expressed on the plasma membrane of cells (e.g., T-cells, NK cells), thereby altering (e.g., initiating, preventing) biological processes. For example, the binding of IL-12 to the IL-12 receptor can stimulate the proliferation of pre-activated T-cells and NK cells, enhance the cytolytic activity of cytotoxic T-cells (CTL), NK cells and LAK (lymphokine activated killer) cells, induce production of gamma interferon (IFN GAMMA) by T-cells and NK cells and induce differentiation of naive Th0 cells into Th1 cells that produce IFN GAMMA and IL-2. Trinchieri, 13 ANN. REV. IMMUNOLOGY 251-76 (1995). In particular, IL-12 is vital for the generation of cytolytic cells (e.g., NK, CTL) and for mounting a cellular immune response (e.g., a Th1 cell mediated immune response). Thus, IL-12 is critically important in the generation and regulation of both protective immunity (e.g., eradication of infections) and pathological immune responses (e.g., autoimmunity). Hendrzak et al., 72 LAB. INVESTIGATION 619-37 (1995). Accordingly, an immune response (e.g., protective or pathogenic) can be enhanced, suppressed or prevented by manipulation of the biological activity of IL-12 in vivo, for example, by means of an antibody.

[0116] The methodologies of the present invention may also be applied to characterize the binding domain of an integrin or integrin-binding receptor. Integrins have been implicated in the angiogenic process, by which tumor cells form new blood vessels that provide tumors with nutrients and oxygen, carry away waste products, and to act as conduits for the metastasis of tumor cells to distant sites. Gastl et al., 54 ONCOL. 177-84 (1997). Integrins are heterodimeric transmembrane proteins that play critical roles in cell adhesion to the extracellular matrix (ECM) which, in turn, mediates cell survival, proliferation and migration through intracellular signaling. During angiogenesis, a number of integrins that are expressed on the surface of activated endothelial cells regulate critical adhesive interactions with a variety of ECM proteins to regulate distinct biological events such as cell migration, proliferation and differentiation. Specifically, the closely related but distinct integrins αVβ3 and αVβ5 have been shown to mediate independent pathways in the angiogenic process. An antibody generated against αVβ3 blocked basic fibroblast growth factor (bFGF) induced angiogenesis, whereas an antibody specific to αVβ5 inhibited vascular endothelial growth factor-induced (VEGF-induced) angiogenesis. Eliceiri et al., 103 J. CLIN. INVEST. 1227-30 (1999); Friedlander et al., 270 SCIENCE 1500-02 (1995). Such integrins may include, but are not limited to α1, α2, α3, α4, α5, α6, α7, α8, α9, αD, αL, αM, αV, αX, αIIb, αIELb, β1, β2, β3,

β4, β5, β6, β7, β8, α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α4β7, α6β4, αDβ2, αLβ2, αMβ2, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, αXβ2, αIIbβ3, αIELbβ7, and any structural or functional analog thereof.

[0117] In another preferred embodiment of the invention, the methodologies of the present invention can be used to characterize the binding domain of a biomolecule that serves as a glycoprotein IIb/IIIa receptor antagonist. The IIb/IIIa receptor antagonist can be, but is not limited to, an antibody, a fragment of an antibody, a peptide, or an organic molecule. See, e.g., U.S. Pat. Nos. 5,976,532, 5,877,006, 5,770,198; Coller, 78 THROM HAEMOST. 730-35 (1997); JORDAN ET AL., in ADHESION RECEPTORS AS THERAPEUTIC TARGETS 281-305 (Horton, ed. CRC Press, New York, 1996); Jordan et al., in NEW THERAPEUTIC AGENTS IN THROMBOSIS & THROMBOLYSIS (Sasahara & Loscalzo, eds. Marcel Kekker, Inc. New York, 1997).

[0118] As noted previously, the techniques of the present invention can be used to characterize the binding domains of receptors, or fragments thereof, and activated receptors, i.e., peptides associated with their corresponding receptors, or fragments thereof. These complexes may mimic activated receptors and thus affect a particular biological activity. Alternatively, the receptor can be genetically re-engineered to adopt the activated conformation. For example, the thrombin-bound conformation of fibrinopeptide A exhibits a strand-turn-strand motif, with a -turn centered at residues Glu-11 and Gly-12. Molecular modeling analysis indicates that the published fibrinopeptide conformation cannot bind reasonably to thrombin, but that reorientation of two residues by alignment with bovine pancreatic trypsin inhibitor provides a good fit within the deep thrombin cleft and satisfies all of the experimental nuclear Overhauser effect data. Based on this analysis, a researchers were able to successfully design and synthesize hybrid peptide mimetic substrates and inhibitors that mimic the proposed β-turn structure. The results indicate that the turn conformation is an important aspect of thrombin specificity, and that the turn mimetic design successfully mimics the thrombin-bound conformation of fibrinopeptide. Nakanishi et al., 89(5) PNAS 1705-09 (1992).

[0119] Another example of activated-receptor moieties that can be characterized by the present invention concerns the peptido mimetics of the erythropoietin (Epo) receptor. By way of background, the binding of Epo to the Epo receptor (EpoR) is crucial for production of mature, red blood cells. The Epo-bound, actvated EpoR is a dimer. See, e.g., Constantinescu et al., 98 PNAS 4379-84 (2001). In its natural state, the first EpoR in the dimer binds Epo with a high affinity whereas the second EpoR molecule binds to the complex with a low affinity. Bivalent anti-EpoR antibodies have been reported to activate EopR, probably by dimerization of the EpoR. Additionally, small synthetic peptides, that do not have any sequence homology with the Epo molecule, are also able to mimic the biologic effects of Epo but with a lower affinity. Their mechanism of action is probably also based on the capacity to produce dimerization of the EpoR. Hence, an embodiment of the present invention provides for a methodology involving an activated EpoR mimetic. Indeed, a particularly useful application of the present invention allows for the construction of peptido mimetics based on the information obtained from the NMR characterization described herein.

[0120] The methodologies contemplated by the present invention may also be used to identify the binding domains associated with antimicrobial agents or portions thereof, which include antibacterial agents, antiviral agents, antifungal agents, antimycobacterial agents, and antiparasitic agents. Such binding domains can be part of the antimicrobial agents or the proteins or receptors to which such agents bind. In one embodiment of the present invention, either an agent or a protein/receptor can be isotopically labeled. Antibacterials include, but are not limited to, Beta-lactams (such as Penicillins and Cephalosporins), Aminoglycosides (such as Gentamicin), Macrolides (such as Erythromycin), Fluoroquinolones, Metronidazole, Sulfonamides, Tetracyclines, Trimethroprim, and Vancomycin. Antifungal agents include, but are not limited to Amphotericin, Fluconazole, Flucytosine, Itraconazole, and Ketoconazole. Antiparasitic agents include, but are not limited to, Ivermectin, Mebendazole, Mefloquine, Pentamidine, Praziquantel, Pyrimethamine, and Quinine. Antiviral agents include, but are not limited to, Acyclovir, Amantadine, Didanosine, Famciclovir, Foscarnet, Ganciclovir, Rimatandine, Stavudine, Zalcitabine, and Zidovudine. Antimycobacterial agents include, but are not limited to, Isoniazid, Rifampin, Streptomycin, Dapsone. SANFORD ET AL., GUIDE TO ANTIMICROBIAL THERAPY (25th ed., Antimicrobial Therapy, Inc., Dallas, Tex. 1995).

[0121] In another embodiment of the invention, the NMR methodologies may identify the binding domain of a cell cycle protein. Cell cycle proteins are known in the art, and include cyclins, such as G_1 cyclins, S-phase cyclins, M-phase cyclins, cyclin A, cyclin D and cyclin E; the cyclin-dependent kinases (CDKs), such as G_1 CDKs, S-phase CDKs and M-phase CDKs, CDK2, CDK4 and CDK 6; and the tumor suppressor genes such as Rb and p53. Cell cycle proteins also include those proteins involved in apoptosis, such as Bcl-2 and caspase proteins; proteins associated with Cdc42 signaling, p70 S6 kinase and PAK regulation; and integrins, discussed elsewhere. Also included in the cell cycle proteins of the present invention are anaphase-promoting complex (APC) and other proteolytic enzymes. The APC triggers the events leading to destruction of the cohesins and thus allowing sister chromatids to separate, and degrades the mitotic (M-phase) cyclins. Other relevant cell cycle proteins include S-phase promoting factor, M-phase promoting factor that activates APC. Kimball, *Kimball's Biology Pages,* at http://www.ultranet.com/~jkimball/BiologyPages. Cell cycle proteins also include p13, p27, p34, p60, p80, histone H1, centrosomal proteins, lamins, and CDK inhibitors.

[0122] As noted previously, the NMR methodologies of the present invention can be useful to identify the epitope of a particular antigen. Antigens, in a broad sense, may include any molecule to which an antibody, or functional fragment thereof, binds. Such antigens can be pathogen derived, and be associated with either MHC class I or MHC class II reactions. These antigens can be proteinaceous. These antigens may also be non-proteinaceous, for example in instances where the antigens comprise non-identical, repeating units such as, for example, carbohydrate units. Carbohydrate antigens are present on cell surfaces of all types of cells, including normal human blood cells and foreign, bacterial cell walls or viral membranes. Nucleic acids may also be antigenic when associated with proteins, and are hence included within the scope of antigens encompassed in

the present invention. See SEARS, IMMUNOLOGY (W. H. Freeman & Co. and Sumanas, Inc., 1997), available online at http://www.whfreeman.com/immunology.

[0123] For example, antigens can be derived from a pathogen, such as a virus, bacterium, mycoplasm, fungus, parasite, or from another foreign substance, such as a toxin. Such bacterial antigens may include or be derived from *Bacillus anthracis, Bacillus tetani, Bordetella pertusis;* Brucella spp., *Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Coxiella burnetii, Francisella tularensis, Mycobacterium leprae, Mycobacterium tuberculosis, Salmonella typhimurium, Streptocccus pneumoniae, Escherichia coli, Haemophilus influenzae,* Shigella spp., *Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningiditis, Treponema pallidum, Yersinia pestis, Vibrio cholerae.* Often, the oligosaccharide structures of the outer cell walls of these microbes afford superior protective immunity, but must be conjugated to an appropriate carrier for that effect.

[0124] Viruses and viral antigens that can be involved with the methodologies of the current invention include, but are not limited to, HBeAg, Hepatitis B Core, Hepatitis B Surface Antigen, Cytomegalovirus B, HIV-1 gag, HIV-1 nef, HIV-1 env, HIV-1 gp41-1, HIV-1 p24, HIV-1 MN gp120, HIV-2 env, HIV-2 gp 36, HCV Core, HCV NS4, HCV NS3, HCV p22 nucleocapsid, HPV L1 capsid, HSV-1 gD, HSV-1 gG, HSV-2 gG, HSV-II, Influenza A (HIN1), Influenza A (H3N2), Influenza B, Parainfluenza Virus Type 1, Epstein Barr virus capsid antigen, Epstein Barr virus, Poxviridae Variola major, Poxviridae Variola minor, Rotavirus, Rubella virus, Respiratory Syncytial Virus, Surface Antigens of the *Syphilis spirochete,* Mumps Virus Antigen, *Varicella zoster* Virus Antigen and Filoviridae.

[0125] Other parasitic pathogens such as *Chlamydia trachomatis, Plasmodium falciparum,* and *Toxoplasma gondii* may also provide antigens for the methodologies contemplated by the present invention. Numerous bacterial and viral, and other microbe-generated antigens are available from commercial suppliers such as Research Diagnostics, Inc. (Flanders, N.J.).

[0126] Toxins, toxoids, or antigenic portions of either, utilized within the methodologies of the present invention include those produced by bacteria, such as diphteria toxin, tetanus toxin, botulin toxin and enterotoxin B; those produced by plants, such as Ricin toxin from the castor bean *Ricinus cummunis.* Mycotoxins, produced by fungi, that may serve in the present invention include diacetoxyscirpenol (DAS), Nivalenol, 4-Deoxynivalenol (DON), and T-2 Toxin. Other toxins and toxoids produced by or derived from other plants, snakes, fish, frogs, spiders, scorpions, blue-green algae, snails may also be incorporated into the methodologies of the present invention.

[0127] Additionally, epitopes identified by the methodologies of the present invention can be those derived from antigens that serve as markers for particular cell types, or as targets for an agent interacting with that cell type. Examples include Human Leukocyte Antigens (HLA markers), MHC Class I and Class II, the numerous CD markers useful for identifying T-cells and the physiological states thereof. Alternatively, antigens involved with the methodologies of the present invention can be those antibodies that serve as "markers" for a particular disease or condition, or as targets of a therapeutic agent. Examples include Prostate Specific Antigen, Pregnancy specific beta 1 glycoprotein (SP1), Thyroid Microsomal Antigen, and Urine Protein 1. Antigens may include those defined as "self" implicated in autoimmune diseases. Haptens, low molecular weight compounds such as drugs or antibiotics that are too small to cause an immune response unless they are coupled with much larger entities, can be involved in the methodologies contemplated by the present invention. See ROITT ET AL., IMMUNOLOGY (5th ed., 1998); BENJAMINI ET AL., IMMUNOLOGY, A SHORT COURSE (3rd ed., 1996).

[0128] Also within the scope of the present invention are databases comprising any portion or all of the data obtained by the methodologies of the present invention. A particular embodiment of the involves methods of doing business by providing a customer with any portion or all of the data obtained by the methodologies of the present invention. Another embodiment of the present invention involves any computer system and any form of recordable medium comprising any portion or all of the data obtained by the methodologies of the present invention. The term "recordable medium" includes, but is not limited to, any book, floppy disk, CD-ROM, ZIP disk, and any functional equivalent thereof.

EXAMPLE

[0129] Without further elaboration, it is believed that one skilled in the art, using the preceding description, can utilize the present invention to the fullest extent. The following example is illustrative only, and not limiting of the remainder of the disclosure in any way whatsoever.

[0130] Human Papillomavirus (HPV) E2

[0131] The papillomaviruses are a family of small DNA viruses that cause genital warts and have been associated with cervical carcinomas. The E2 protein of HPV regulates viral transcription and is required for viral replication. Thus, molecules that block the binding of E2 to DNA can be useful therapeutic agents against HPV. The protein rather than the DNA should be chosen as a target, because it is expected that agents with greater selectivity would be found that bind to the protein rather than the DNA.

[0132] An ^{15}N-labeled human papillomavirus E2 protein can be produced via recombinant technologies known to persons of ordinary skill in the art and overexpressed in bacteria using the T7 expression system. ^{15}N-Alanine-labeled protein can be isolated from bacteria grown on a minimal medium. The protein can be purified from the bacterial cell lysate using an S-sepharose FastFlow column pre-equilibrated with buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH=8.3).

[0133] The protein can be eluted with a linear gradient of 100-500 mM NaCl in buffer, pooled, and applied to a Mono-S column at a pH=7.0. The protein can be eluted with a salt gradient (100-500 mM), concentrated to 0.3 mM, and exchanged into a TRIS (50 mM, pH=7.0 buffered H_2O/D_2O (9/1) solution containing sodium azide (0.5%).

[0134] Characterization of the E2 Binding Domain using NMR Correlation Spectral Analysis

[0135] The E2 protein can be prepared in accordance with the procedures outlined above. The protein solutions used in

the screening assay may contain ^{15}N-Alanine-labeled E2 (0.3 mM), acetohydroxamic acid (500 mM), $CaCl_2$ (20 mM), and sodium azide (0.5%) in a H_2O/D_2O (9/1) TRIS buffered solution (50 mM, pH=7.0), and/or any other components known to persons of ordinary skill in the art.

[0136] Two-dimensional ^{15}N NMR spectra can be generated at 29° C. on a Bruker AMX500 NMR spectrometer equipped with a triple resonance probe and Bruker sample changer. A delay of 1 second between scans and 8 scans per free induction decay (fid) can be employed in the data collection. All NMR spectra can be processed and analyzed on Silicon Graphics computers using software.

[0137] Spectra can be acquired for the ^{15}N-Alanine-labeled E2 molecule in the absence of any ligand as described above. Similar experiments are run with recombinant E2 in which different amino acids are labeled.

[0138] The various labeled E2 proteins may then be exposed any member of a database of known ligands to E2. Stock solutions of the compounds can be made at 100 mM and 1M. One must note, however, that the pH of the 1M stock solution should be adjusted with acetic acid and ethanolamine so that no pH change will be observed upon a 1/10 dilution with a 100 mM phosphate buffered solution (pH=7.0). It is important to adjust the pH, because small changes in pH can alter the chemical shifts of the biomolecules and complicate the interpretation of the NMR data.

[0139] Additional spectra can be acquired for the ^{15}N-labeled E2 target molecule in the presence of any one of-the known ligands as described above.

[0140] Comparisons of the spectra acquired for the ^{15}N-labeled E2 protein in the absence of any ligand and the spectra acquired for the ^{15}N-labeled E2 protein in the presence of any ligand may then be undertaken. In particular, comparisons between and among spectra should focus upon varying shifts in resonance suggesting what portions of the labeled E2 are involved in binding with a ligand. One may also compare the spectral data with the known sequence of the E2 protein. Via such comparisons, one may characterize the binding domain of E2.

What is claimed is:

1. A method for characterizing a binding domain of a labeled, recombinant protein, comprising comparing at least one nuclear magnetic resonance spectrum of a labeled, recombinant protein in the absence of a ligand with a nuclear magnetic resonance spectrum of a labeled, recombinant protein in the presence of a ligand.

2. A method according to claim 1, wherein the recombinant protein is a recombinant protein labeled at multiple sites or a recombinant protein labeled at alternate sites.

3. A method according to claim 1, wherein the recombinant protein is a tissue plasminogen activator receptor and the ligand is tissue plasminogen activator.

4. A method according to claim 1, wherein the recombinant protein is tumor necrosis factor and the ligand is any antibody that binds to tumor necrosis factor.

5. A method according to claim 1, wherein the recombinant protein is erythropoietin receptor and the ligand is erythropoietin or an erythropoietin mimetic.

6. A method according to claim 1, wherein the recombinant protein or the ligand is a diagnostic compound or a therapeutic compound.

7. A method according to claim 6, wherein the diagnostic or therapeutic compound is selected from at least one immunoglobulin, at least one fragment of an immunoglobulin, at least one integrin, at least one antigen, at least one growth factor, at least one cell cycle protein, at least one cytokine, at least one hormone, at last one neurotransmitter, or at least one receptor or at least one fusion proteins thereof, at least one blood protein, at least one antimicrobial, at least one chemokine, or any structural or functional analog thereof.

8. A method according to claim 7, wherein the immunoglobulin is at least one of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, slgA, IgD, IgE, or any structural or functional analog thereof.

9. A method according to claim 7, wherein the fragment of immunoglobulin is selected from at least one of $F(ab')_2$, Fab', Fab, Fc, Facb, pFc', Fd, Fv, or any structural or functional analog thereof.

10. A method according to claim 7, wherein the integrin is selected from at least one of αv, β1, β2, β3, β4, β5, or any structural or functional analog thereof.

11. A method according to claim 7, wherein the antigen is selected from at least one of a cancer-specific antigen, a viral antigen, a bacterial antigen, a chemical antigen, or any structural or functional analog thereof.

12. A method according to claim 7, wherein the growth factor is selected from at least one of an insulin-like growth factor, a platelet-derived growth factor, a fibroblast growth factor 2, an epidermal growth factor, a tumor growth factor, a nerve growth factors, a vascular endothelial growth factor, or any structural or functional analog thereof.

13. A method according to claim 7, wherein the cell cycle protein is at least one selected from a p13, a p27, a p34, a p53, a p60, a p80, a histone H1, a centrosomal protein, a lamin, a CDK, a CDK inhibitor, a umor suppressor gene, or any structural or functional analog thereof.

14. A method according to claim 7, wherein the cytokine is at least one selected from an IL-1, an IL-2, an IL-3, an IL-4, an IL-5, an IL-6, an IL-7, an IL-8, an IL-9, an IL-10, an IL-11, an IL-12, an IL-13, an IL-14, an IL-15, an IL-16, an IL-17, an IL-18, an IL-19, an IL-20, an IL-21, an IL-22, an IL-23, an interferon, an interleukin, a lymphokine, a cell signal molecule, or any structural or functional analog thereof.

15. A method according to claim 7, wherein the hormone is selected from at least one a parathyroid hormone, a follicle stimulating hormone, an estrogen, a progesterone, a testosterone, an aldosterone, an antidiuretic hormone, a cortisols, a human chorionic gonadotropin, a parathormone, a growth hormone, a secretin, a thyroid hormone, or any structural or functional analog thereof.

16. A method according to claim 7, wherein the neurotransmitter is selected from at least one of a GABA, an enkephalins, or any structural or functional analog thereof.

17. A method according to claim 7, wherein the receptor or fusion protein thereof is selected from at least one of a chemokine receptor, a cytokine receptor, a cell cycle protein receptors, a growth factor receptor, a hormone receptor, a neurotransmitter receptor, a fusion protein to any one of the foregoing, or any structural or functional analog thereof.

18. A method according to claim 7, wherein the blood protein is at least one selected from a TPO, a tPA, or any structural or functional analog thereof.

19. A method according to claim 7, wherein the antimicrobial is at least one of an antibiotic, an antifungal, an antiprotozoal, an antiviral, or any structural or functional analog thereof.

20. A method according to claim 1, wherein the labeled, recombinant protein is an isotopically labeled protein.

21. A method according to claim 20,wherein the label is selected from at least one of ^{15}N, ^{13}C, ^{3}II, ^{33}S, ^{17}O, or ^{2}II.

22. A method according to claim 1, wherein the comparing step is accomplished via a computer.

23. A method according to claim 22, wherein the the comparing step optionally further comprises includes the use of data obtained from X-ray crystallography analysis.

24. Recordable media, comprising data corresponding to at least one binding domain characterization obtained by a method according to claim 1.

25. A binding domain of a protein corresponding to at least a portion of the data according to claim 24.

26. A computer system, comprising recordable media according to claim 24.

27. A database comprising data corresponding to at least one binding domain characterization obtained by a method according to claim 1.

28. A business method, comprising the step of performing for a customer at least one method according to claim 1.

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摘要(译)

本发明涉及用于作图或以其他方式鉴定参与配体结合的蛋白质的一部分的氨基酸序列和构象的方法。本发明可用于阐明例如抗原或与抗原结合的抗体的表位的氨基酸序列和构象的过程。

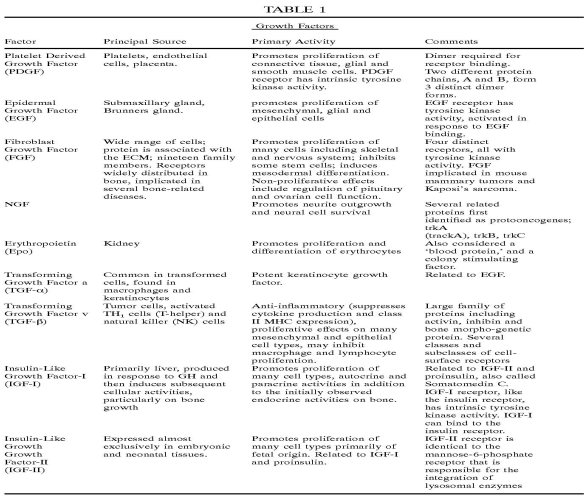

TABLE 1

Growth Factors

Factor	Principal Source	Primary Activity	Comments
Platelet Derived Growth Factor (PDGF)	Platelets, endothelial cells, placenta.	Promotes proliferation of connective tissue, glial and smooth muscle cells. PDGF receptor has intrinsic tyrosine kinase activity.	Dimer required for receptor binding. Two different protein chains, A and B, form 3 distinct dimer forms.
Epidermal Growth Factor (EGF)	Submaxillary gland, Brunners gland.	promotes proliferation of mesenchymal, glial and epithelial cells	EGF receptor has tyrosine kinase activity, activated in response to EGF binding.
Fibroblast Growth Factor (FGF)	Wide range of cells; protein is associated with the ECM, nineteen family members. Receptors widely distributed in bone, implicated in several bone-related diseases.	Promotes proliferation of many cells including skeletal and nervous system; inhibits some stem cells; induces mesodermal differentiation. Non-proliferative effects include regulation of pituitary and ovarian cell function.	Four distinct receptors, all with tyrosine kinase activity. FGF implicated in mouse mammary tumors and Kaposi's sarcoma.
NGF		Promotes neurite outgrowth and neural cell survival	Several related proteins first identified as protooncogenes; trkA (trackA), trkB, trkC
Erythropoietin (Epo)	Kidney	Promotes proliferation and differentiation of erythrocytes	Also considered a "blood protein," and a colony stimulating factor.
Transforming Growth Factor a (TGF-α)	Common in transformed cells, found in macrophages and keratinocytes	Potent keratinocyte growth factor.	Related to EGF.
Transforming Growth Factor v (TGF-β)	Tumor cells, activated TH_1 cells (T-helper) and natural killer (NK) cells	Anti-inflammatory (suppresses cytokine production and class II MHC expression), proliferative effects on many mesenchymal and epithelial cell types, may inhibit macrophage and lymphocyte proliferation.	Large family of proteins including activin, inhibin and bone morpho-genetic protein. Several classes and subclasses of cell-surface receptors
Insulin-Like Growth Factor-I (IGF-I)	Primarily liver, produced in response to GH and then induces subsequent cellular activities, particularly on bone growth	Promotes proliferation of many cell types, autocrine and pamcrine activities in addition to the initially observed endocrine activities on bone.	Related to IGF-II and proinsulin, also called Somatomedin C. IGF-I receptor, like the insulin receptor, has intrinsic tyrosine kinase activity. IGF-I can bind to the insulin receptor.
Insulin-Like Growth Growth Factor-II (IGF-II)	Expressed almost exclusively in embryonic and neonatal tissues.	Promotes proliferation of many cell types primarily of fetal origin. Related to IGF-I and proinsulin.	IGF-II receptor is identical to the mannose-6-phosphate receptor that is responsible for the integration of lysosomal enzymes