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(54) **Antibodies against recombinant platelet collagen receptor glycoprotein VI**

Antikörper gegen Rekombinantes Plättchencollagen-Rezeptor-Glycoprotein VI

Anticorps dirigés contre la glycoprotéine VI recombinante de récepteur de collagène de plaquette

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(73) Proprietor: **Sanofi-Aventis Deutschland GmbH**
65926 Frankfurt am Main (DE)

(72) Inventor: **Clementson, Kenneth J.**
3065 Bern (CH)

(74) Representative: **Zwicker, Jörk**
ZSP Patentanwälte
Partnerschaftsgesellschaft
Radtkoferstrasse 2
81373 München (DE)

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Description

[0001] The invention relates to Glycoprotein VI (GPVI), its isolation, purification, and methods for recombinant production. Especially, the invention relates to the use of GPVI, preferably recombinant GPVI, in the treatment of disorders and pathological events correlated directly or indirectly to blood coagulation disorders such as thrombotic and cardiovascular diseases. The extracellular recombinant protein can also be used for establishing screening assays to find potential inhibitors of the membrane bound GPVI in order to inhibit interaction of thrombocytes and collagen.

[0002] Glycoprotein VI is a 62/65 kDa (non-reduced/reduced respectively) platelet membrane glycoprotein which forms a complex together with the Fc γ common subunit. The GPVI subunit contains the collagen binding site and the Fc γ subunit is responsible for signalling. The complex forms one of the major collagen receptors on the platelet surface critical for platelet activation in response to collagen. The recognition sequence on collagen consists of (GlyProHyp) $_n$ sequences. Patients are known from Japan who have a genetic deficiency of GPVI. They have mild bleeding problems and their platelets respond only weakly to collagen, presumably via other receptors. A great deal has been learned about the signalling cascades originating at GPVI which strongly resemble those from immune receptors including T-cell receptors, B-cell receptors and natural killer cell receptors. These cascades involve src family tyrosine kinases such as Fyn and Lyn as well as p72^{SYK} and many other tyrosine kinases and phosphatases and adaptor proteins such as LAT. A main target of these cascades is activation of phospholipase C γ 2 which splits phospholipids to give the second messengers diacylglycerol and IP $_3$. GPVI is thought to be involved in activation of the platelet integrin α 2B1 which has a major role in platelet adhesion to damaged vessel wall. Mice with the Fc γ subunit "knocked-out" have platelets which still show responses to collagen implying that the resting state of α 2B1 may also be regulated by the GPVI/Fc γ complex.

[0003] The platelet collagen receptor GPVI is closely related to the natural killer activatory receptors of the p58KAR family.

[0004] The adhesion and activation of resting, circulating platelets at a site of vascular injury is the first step in a process leading to the formation of a thrombus which is converted into a haemostatic plug. Collagen is one of the major components of the vessel wall responsible for platelet activation. Many types of collagen exist and seven of these are found in the subendothelial layers. Several different receptors for collagen have been identified on platelets but the major ones are now considered to be the integrin α 2B1 and the non-integrin GPVI. Although α 2B1 is well characterised and both subunits were cloned and sequenced several years ago, the structure of GPVI has remained elusive although several features have been identified. It was determined about twenty years ago that GPVI is a major platelet glycoprotein with a molecular mass in the 60-65 kDa range and an acid pI. Its role as putative collagen receptor was established following the identification of a patient in Japan with a mild bleeding disorder whose platelets showed a specific defect of response to collagen and lacked this receptor. This patient had also developed autoantibodies to the deficient receptor and these were used to characterise the molecule further. More recently it was established that GPVI is associated non-covalently with the common Fc γ subunit which acts as the signalling part of the complex. It was also demonstrated that the recognition sequence on collagen for GPVI is a repeated Gly-Pro-Hyp triplet within the Collagen triple helical structure and that synthetic peptides based on this structure could be used as specific GPVI directed agonists. The GPVI/Fc γ complex was shown to signal to the platelet interior by an immune receptor-like mechanism, involving activation of p72^{SYK} and leading by a cascade of kinase/phosphatase/adaptor protein interactions to activation of PLC γ 2 and hence to release of granules and platelet aggregation. A further step in characterisation of this molecule was the demonstration that the snake C-type lectin, convulxin, from the Tropical Rattlesnake, *Crotalus durissus terrificus* was able to activate platelets by clustering GPVI through a multimeric interaction. Convulxin was shown to bind specifically to GPVI providing a method for purification of this receptor in conjunction with established approaches.

[0005] Sugiyama et al. (Blood 69 (1987) 1712-1720) describe a novel platelet aggregating factor. This factor has been found in a patient with autoimmune thrombocytopenia. The factor is the patient's IgG or the Fab or F(ab) $_2$ fragments thereof. The F(ab) $_2$ fragments induced irreversible aggregation in normal platelet-rich plasma. Moreover, the Fab fragments specifically inhibited aggregation induced by collagen and by the patient's IgG. The major component of an immunoprecipitate made with the patient's IgG from radiolabelled membrane proteins of normal platelet extract had a molecular weight of 62 kDa.

[0006] Sugiyama et al. (International Journal of Hematology 58 (1993) 99-104) examined the functional role of P62, the antigen recognized by the Fab fragment of IgG isolated from the patient with autoimmune thrombocytopenia. It is disclosed that the patient's platelet showed normal adherence to collagen and that the Fab fragment inhibited normal platelet adhesion to collagen in a dose dependent manner.

[0007] Ichinohe et al. (The Journal of Biological Chemistry 270 (1995) 28029-28036) examined the role of the GPVI protein using the F(ab) $_2$ fragments of the patient's IgG with autoimmune thrombocytopenia. The GPVI engagement is coupled to cAMP-insensitive activation of c-Src and Syk accompanied by tyrosine phosphorylation of numerous substrates including phospholipase C- γ 2 in a manner similar to collagen stimulation.

[0008] Gibbins et al. (FEBS Letters 413 (1997) 255-259) disclose that GPVI is the collagen receptor in platelets which couples collagen-stimulation of platelets to tyrosine phosphorylation of the Fc receptor γ -chain. The authors use anti-

GPVI F(ab)₂ fragments of the IgG of the patient with autoimmune thrombocytopenia.

[0009] Jandrot-Perrus et al. (The Journal of Biological Chemistry 272 (1997) 27035-27041) disclose the adhesion and activation of human platelets induced by convulxin. Adhesion and activation involves GPVI and integrin alpha2beta1. Platelet exocytosis and aggregation induced by convulxin were inhibited by the Fab fragments of the IgG of the patient with autoimmune thrombocytopenia.

[0010] Ezumi et al. (The Journal of Experimental Medicine 188 (1998) 267-276) investigated the involvement of the Src family in the proximal signals through the GPVI-FcR γ complex using convulxin and anti-GPVI F(ab)₂ fragments of the IgG of the patient with autoimmune thrombocytopenia. The results indicated that the Src family plays a critical role in platelet activation via the collagen receptor GPVI- FcR γ complex.

[0011] Heemskerk et al. (Thrombosis and Haemostasis, Schattauer GmbH 81 (1999) 782-792) disclose the function of GPVI and integrin alpha2beta1 in the procoagulant response of single collagen-adherent platelets. The authors use GPVI IgG and the corresponding Fab fragments of the IgG of the patient with autoimmune thrombocytopenia.

[0012] Ishibashi et al. (International Journal of Hematology 62 (1995) 107-115) identified the intercellular adhesion molecule 2 (ICAM-2) using the antibody of the patient with autoimmune thrombocytopenia in an improved immunoprecipitation technique. The ICAM-2 molecule has a molecular weight of about 62 kDa.

[0013] EP 0 896 002 relates to chimeric proteins consisting of an integrin and an immunoglobulin, their heterodimer complexes, a production process thereof and their applications as drugs and reagents. Furthermore, EP 0 896 002 relates to medicinal application of the integrin-immunoglobulin chimeric protein as a platelet substitute. The integrins are molecules belonging to the integrin superfamily. The alpha chains include 15 alpha chains, inter alia, alpha1. The beta chains include eight beta chains, inter alia beta1. It was found that an integrin alpha2beta1-immunoglobulin chimeric protein heterodimer complex has extracellular matrix binding capability. Therefore, it can be used as a therapeutic or preventive agent against the congenital and acquired bleeding tendency due to platelet abnormality, and also widely as a platelet transfusion substitute. Moreover, the integrin alpha2beta1-immunoglobulin chimeric protein heterodimer complex can be a therapeutic or preventive agent for conditions of diseases where vascular endothelial cell disorder is a problem.

[0014] Thus, it is clear from the prior art that GPVI seems to be a very interesting compound in many therapeutical fields above all concerning with applications which are related, widely spoken, directly or indirectly, to blood coagulation events which depend on collagen - platelet interaction. It was, therefore, a goal of the present invention is to provide GPVI in a recombinant form and to show its efficiency as direct therapeutical target or as tool for screening of short compounds, especially chemically synthesized or synthesizable compounds having the capability to inhibit or block the natural platelet-collagen interaction.

[0015] It was another goal of the present invention to provide anti-GPVI antibodies.

[0016] In view of this, the present invention relates to a polyclonal anti-GPVI antiserum, anti-GPVI Fab obtainable from said anti-GPVI antiserum or anti-GPVI F(ab')₂ obtainable from said anti-GPVI antiserum, wherein GPVI has the sequence of SEQ ID NO: 3.

[0017] The present invention also relates to a polyclonal anti-GPVI antiserum, anti-GPVI Fab obtainable from said anti-GPVI antiserum or anti-GPVI F(ab')₂ obtainable from said anti-GPVI antiserum as disclosed above, wherein GPVI is obtainable from platelets using wheat-germ agglutinin and convulxin and applying gel electrophoresis on polyacrylamide of about 8.5 %.

[0018] The present invention furthermore relates to a polyclonal anti-GPVI antiserum, anti-GPVI Fab obtainable from said anti-GPVI antiserum or F(ab')₂ obtainable from said anti-GPVI antiserum as disclosed above, wherein the polyclonal anti-GPVI antiserum is generated in rabbits.

[0019] Moreover, the present invention relates to an anti-GPVI Fab fragment of humanized mouse monoclonal antibodies against GPVI, wherein GPVI has the sequence of SEQ ID NO: 3.

[0020] The present invention also relates to a monoclonal or polyclonal antibodies recognising the N-terminal region of GPVI, wherein GPVI has the sequence of SEQ ID NO: 3.

[0021] The invention discloses portions or fragments of the GPVI protein which have maintained their biological activity which is the binding to collagen.

[0022] The invention was successful in purifying adequate amounts of GPVI for preliminary characterisation and for peptide sequencing. The sequences were used to design primers for PCR to identify a positive sequence in a DNA library. This DNA sequence was then used as a probe to isolate an almost complete cDNA sequence from the library and missing 5'-sequence was obtained using a RACE method from a platelet cDNA library.

[0023] The invention was also successful in showing the use of recombinant GPVI as therapeutically applicable compound which is capable, when administered in a patient with e.g. damaged blood vessels, to bind collagen, thus preventing thrombocytes bearing membrane- bound GPVI from binding to said collagen. The recombinant soluble extracellular domain of GPVI contains the collagen binding site and can prevent platelet activation by collagen. It is therefore applicable to treatment of disease conditions involving increased platelet activation with collagen such as atherosclerotic plaque rupture in diseases such as unstable angina or during surgical treatment such as Percutaneous Transluminal

Coronary Angioplasty (PTCA) where arteries are reopened by inflation of a balloon catheter causing considerable damage to the vessel wall and much platelet activation and often resulting in reclosure of the vessel later. The advantage of recombinant GPVI fragments compared to present treatment methods is that they act at an earlier stage by preventing or reducing platelet activation rather than by suppressing events after platelet activation, such as aggregation by GPIIb-IIIa antagonists. Thus, smaller amounts of platelet granule contents are released including growth factors and chemokines which are involved not only in wound repair but in the remodelling of the vessel wall by smooth muscle migration and in attraction of phagocytic cells such as monocytes known to contribute to atherosclerosis. Fab fragment of humanised mouse monoclonal antibodies against GPVI could be used with similar effect to block GPVI on the platelet surface with similar applications as above.

[0024] Recombinant GPVI as disclosed herein can also be used in a binding assay to collagen to screen for small Molecules (in combinatory libraries for example) capable of inhibiting this interaction and which can be used to develop therapeutic compounds which are inhibitors of the collagen-platelet interaction. By suitable derivatisation these compounds are made orally available. Again the main objective is to prepare compounds reducing GPVI-collagen interactions and hence platelet activation in situations where platelets come into contact with collagen. The screening technology as such used in this invention is well established in the prior art. By such screening assays the invention enables to find and develop new targets which can inhibit the natural membrane-bound GPVI on the thrombocyte surface as collagen antagonist. Such targets which may be short chemical molecules may be then basis for further inventions.

[0025] Another major application of GPVI and reagents that recognize specific domains of GPVI is as markers of platelet age and functionality. Young platelets are generally thought to be more active and functional than older ones. Young platelets bind to and are activated by the snake venom C-type lectin convulxin, which is specific for GPVI, and as they age both the binding and degree of activation decrease. This can be due to either proteolytic or conformational changes in GPVI or its association with Fcγ due to platelet activation or damage in the circulation. This can be a useful parameter to measure the age and function profile of platelets in patients as well as in normal persons during medical controls. The platelet age profile changes in many diseases affecting the bone marrow or the immune system and could be an important diagnostic criterion if better methods for its determination were available. For example, patients with diseases

[0026] involving increased platelet turnover will show more young platelets whereas patients on chemotherapy or radiation treatment will show a steadily aging population. Thus, such an age profile can be used for a precise monitoring of treatment. In a normal healthy population very little is known about the age profile distribution and its role as a predictor of changes in health. At present thiazole orange is used to detect young reticulated platelets containing mRNA. This mRNA soon decays, restricting the method to only the youngest platelets. Reagents which could be used in such an assay would include GPVI-specific snake venom proteins such as convulxin, or monoclonal or polyclonal antibodies recognising the N-terminal region of GPVI or monoclonal antibodies recognising new sites or conformations exposed by proteolysis of the N-terminal domain or specific conformations present either in the intact molecule and not in the one or vice versa. These reagents would be labelled with a fluorescent marker, or together with a fluorescent labelled second antibody or affinity reagent and used in flow cytometry to measure the platelet binding profile. At a later stage alternative, less labour intensive measuring techniques based on automated measuring of platelet profiles could be adopted. Using cell sorting methods with flow cytometry or magnetic beads it should be possible to isolate young and old platelets to examine the factors involved in removal of old platelets from the circulation.

[0027] The present invention discloses a DNA coding for Glycoprotein VI or biological active fragments thereof, especially the sequence of Fig. 2.

[0028] The present invention further discloses a DNA coding for Glycoprotein VI comprising the amino acid sequence of Fig. 1.

[0029] The present invention discloses a pharmaceutical composition comprising recombinant GPVI together with a pharmaceutically acceptable diluent, carrier or excipient therefore, and its use for the manufacture of a medicament in the therapeutic field of thrombotic and cardiovascular events and disorders related to thrombocyte-collagen interactions

[0030] The present invention discloses the use of recombinant GPVI in a screening tool for detecting specific inhibitors of platelet-collagen interactions. Possible medical indications and applications, respectively, are, for example, unstable angina pectoris, PTCA, use of stents in this field, operations on coronary vessels, general operations on blood vessels, operations which may damage thicker blood vessels such as hip joint operations. Moreover, all indications are included which relate to thromboembolic events caused by disorders of the interaction between the vessel wall and the coagulation system with a high risk of formation of thrombi and blocking of vessels.

[0031] As indicated above, the GPVI protein and fragments thereof are suitable as pharmaceutically effective compounds in pharmaceutical compositions and combinations.

[0032] The pharmaceutical formulations optionally may comprise additional active ingredients like anti-coagulants such as hirudin or heparin or thrombolytic agents such as plasminogen activator or hementin.

[0033] The novel protein, and its biological active fragments, respectively, may form pharmaceutically acceptable salts with any non-toxic, organic or inorganic acid. Inorganic acids are, for example, hydrochloric, hydrobromic, sulphuric or

phosphoric acid and acid metal salts such as sodium monohydrogen orthophosphate and potassium hydrogen sulfate. Examples for organic acids are the mono, di and tri carboxylic acids such as acetic, glycolic, lactic, pyruvic, malonic, succinic, glutaric, fumaric, malic, tartaric, citric, ascorbic, maleic, hydroxymaleic, benzoic, hydroxybenzoic, phenylacetic, cinnamic, salicylic and sulfonic acids such as methane sulfonic acid. Salts of the carboxy terminal amino acid moiety include the non-toxic carboxylic acid salts formed with any suitable inorganic or organic bases. These salts include, for example, alkali metals such as sodium and potassium, alkaline earth metals such as calcium and magnesium, light metals of Group IIIA including aluminium, and organic primary, secondary and tertiary amines such as trialkylamines, including triethylamine, procaine, dibenzylamine, 1-ethenamine, N,N'-dibenzylethylone-diamine, dihydroabietylamine and N-alkylpiperidine. As used herein, the term "pharmaceutically acceptable Carrier" means an inert, non toxic solid or liquid filler, diluent or encapsulating material, not reacting adversely with the active compound or with the patient. Suitable, preferably liquid carriers are well known in the art such as sterile water, saline, aqueous dextrose, sugar solutions, ethanol, glycols and oils, including those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil and mineral oil.

[0034] The formulations may be administered as unit doses containing conventional non-toxic pharmaceutically acceptable carriers, diluents, adjuvants and vehicles which are typical for parenteral administration.

[0035] The term "parenteral" includes herein subcutaneous, intravenous, intra-articular and intratracheal injection and infusion techniques. Also other administrations such as oral administration and topical application are suitable. Parenteral compositions and combinations are most preferably administered intravenously either in a bolus form or as a constant fusion according to known procedures. Tablets and capsules for oral administration contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablets may be coated according to methods well known in the art.

[0036] Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives like suspending agents, emulsifying agents, non-aqueous vehicles and preservatives.

[0037] Topical applications may be in the form of aqueous or oily suspensions, solutions, emulsions, jellies or preferably emulsion ointments.

[0038] Unit doses may contain daily required amounts of the protein according to the invention, or sub-multiples thereof to make up the desired dose. The optimum therapeutically acceptable dosage and dose rate for a given patient (mammals, including humans) depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health, Sex, diet, time and route of administration, rate of clearance the object of the treatment, i. e., therapy or prophylaxis and the nature of the thrombotic disease to be treated, antiplatelet or anticoagulant activity.

[0039] Therefore, in compositions and combinations useful as anticoagulants in a treated patient (in vivo) a pharmaceutical effective daily dose of the peptides of this invention is between about 0.01 and 100 mg/kg body weight, preferably between 0.1 and 10 mg/kg body weight. According to the application form one single dose may contain between 0.5 and 10 mg of the thrombin inhibitor. To achieve an anticoagulant effect in extracorporeal blood a pharmaceutically effective amount of the inventive peptides is between 0.2 and 150 mg/l, preferably between 1 mg and 20 mg/l extracorporeal blood.

Detailed description of the invention

[0040] Two sequences of 7 amino acids showing the least degeneracy in the genetic code were chosen for the synthesis of DNA primers in order to amplify part of the of GPVI cDNA by PCR. As the location of both peptides in the protein were totally unknown, for each of them, two degenerate primers, one sense and one antisense were prepared. These primers were used to amplify a human bone-marrow library. The combination of the sense 5' TYA THC CNG CNA TGA ARMG 3' primer coding for the sequence PAMKRSL with the antisense 5' TTR TAN ARN GCR AAY TGR TC 3' one corresponding to DQFALYK amplified a DNA fragment of 221 bp. In addition to the selected peptides, the amplified DNA coded for the LysC/AspN peptide DQLELVATGVFAKPSLSAQPGPAVSS, clearly linking the sequence to the cDNA for GPVI.

[0041] Screening 600.000 pfu from a bone marrow library with this 221 bp DNA fragment produced 4 positive pfu. Three had inserts of 1350 bp whether cut by the restriction enzymes Sal I or EcoR I and belonged to the IgG superfamily. The fourth one had an 4.6 kb insert by Sal I digestion and gave two fragments of 2300 bp and 1300 bp respectively when treated by EcoRI. Its DNA enclosed the sequence for the 10 peptides derived from amino acid sequencing of GPVI but stopped short of the amino terminal. No starting methionine or leader sequence could be found but more than 2000 bp of previously sequenced non-reading frame DNA terminating in an Alu sequence were present. The 5' end RACE experiment was completed on platelet poly A RNA with primers located in a part of the GPVI sequence which had been corroborated by that of the peptides. A Fragment of 318 bp including 70 bp new and x bp on the sequence of the fourth pfu at bp 1987 corresponding to 14 amino acids including the first methionine were found before falling back on the established GPVI sequence. Thus, a cDNA containing a total of 1249 bp and coding for a protein with an Open reading frame including leader sequence with 339 amino acids could be sequenced.

[0042] A cDNA coding for platelet GPVI was cloned and sequenced from a human bone marrow cDNA library using RACE with platelet mRNA to supply missing 5' sequence. The Open reading frame of 1017 bp encodes 339 amino acids and a untranslated 3' region. Hydrophobicity analysis of the amino acid sequence revealed the presence of two putative transmembrane domains, a putative 23 amino acid signal sequence, and a 19 amino acid domain between residues 247 and 265 of the mature protein. The sequence and its amino acid translation are shown in Fig. 2 and Fig. 1. A comparison with the amino acid sequence of the most similar molecules found in a search of GenBank reveals clearly that it belongs to the immunoglobulin superfamily and the extracellular domain contains two Ig C2-domain loops formed by two disulphide bridges. It is a membrane crossing protein class one molecule with the N-terminus at the exterior and traverses the membrane once. The most closely related molecules belong to the natural killer receptor class which contains both inhibitory and activatory types. GPVI clearly belongs to the activatory subclass not only through its function but also because unlike the inhibitory class it does not contain ITIM sequences in its cytoplasmic domain. Neither does it contain any tyrosine residues which might be involved in phosphorylation. There are some threonine and serine residues in this domain but they do not match any criteria for kinase Consensus sequences. Like the activatory class of NK receptors, GPVI contains an arginine residue as the third amino acid of the membrane crossing domain which is involved in the complex formation with the Fc γ subunit. The cytoplasmic domain contains 51 amino acids, showing only a minor similarity (in the region just below the membrane) to the cytoplasmic domains of other members of this family. This suggests that this domain in GPVI may associate with different types of cytoplasmic molecule than the other family members. GPVI contains only a single putative N-glycosylation site at Asn69. The domain just above the membrane before the beta sheets of the Ig domain start, however, is rich in threonine and serine residues which could provide O-glycosylation sites such as are found in GPIIb α and GPV. The main function of this O-glycosylation seems to be to present the receptor structures well-extended from the platelet surface to facilitate the interactions with their bulky ligands. Since GPVI was earlier established as a sialoglycoprotein, the difference in molecular mass between the theoretical amino acid mass (37 kDa) and the mass determined by gel electrophoresis (65 kDa reduced) must be due to this glycosylation.

[0043] The structure of natural killer receptors of the two domain type has been established by X-ray crystallographic studies and the two Ig-domains were shown to form an acute angle with the receptor site for the peptide-carrying HLA antigens lying on the outside of the elbow. A direct comparison of the structure of the HLA peptide binding site with that of collagen immediately suggests why these receptors have a common origin because the multiple alpha-helical structures of the HLA binding site and the peptide it contains strongly resemble the triple helical structure of collagen. Further GPVI contains multiple PGX sequences which could mimic an additional collagen strand in interacting with a collagen fibre. The natural killer receptors are postulated to work by a dimerisation mechanism with two receptors recognising two separate HLA sites on the cell which the natural killer cell is investigating. Possibly this dimerisation is part of the activation or deactivation mechanism, depending on the class of receptor. In the case of GPVI there may as well be the possibility for two GPVI molecules to associate with one Fc γ , since each monomer of the Fc γ dimer has a recognition sequence. However, the stoichiometry is not yet known, and based upon the structure of collagens, collagen-like peptides that act via GPVI and convulxin, it seems likely that the strength of the signal is related to the number of GPVI/Fc γ complexes that are clustered together. Other platelet receptors belonging to this Ig family include ICAM-2 (CD) and PECAM (CD31).

[0044] All microorganisms, cell lines, expression systems, expression hosts, plasmids, promoters, resistance markers, replication origins, restriction sites or other fragments or parts of vectors which are mentioned in the description not directly in connection with the claimed invention are commercially or otherwise generally available. Provided that no other hints are given, they are used only as examples and are not essential with respect to the invention, and can be replaced by other suitable tools and biological materials, respectively.

[0045] The techniques which are essential according to the invention are described in detail below and above. Other techniques which are not described in detail correspond to known standard methods which are well known to a person skilled in the art, or are described more in detail in the cited references and patent applications and in the standard literature (e.g. Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor; Harlow, Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor).

EXAMPLES

[0046] Example 1: Materials-Protein A-Sepharose, peroxidase-conjugated goat antimouse and anti-rabbit antibodies, bovine Serum albumin, *Crotalus durissus terrificus* venom, wheat germ agglutinin (WGA), N-hydroxysuccinimidyl chloroformate-activated cross-linked 4% beaded agarose and Triton X-1 14 were from Sigma Chemical Co. (St Louis, MO), Octyl-N-methyl-glucamide (ONMG) and nonanoyl-N-methyl-glucamide (NNMG) were from Oxy Chemie (Bobingen, Germany).

[0047] Example 2: GPVI isolation from platelets - Membrane glycoproteins were isolated from platelets as previously described. Briefly, platelets (from 40 buffy coats) were washed and lysed in 2% Triton X-1 14 in the presence of protease inhibitors. The Triton X-114 and aqueous phases were separated and the detergent phase was loaded on a column of wheat-germ agglutinin coupled to Sepharose 4B. The platelet glycoproteins were eluted with 10 mM Tris HCl, pH 7.4,

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30 mM NaCl, 0.2% octyl-N-methylglucamide (ONMG) and 2% N-acetylglucosamine. After dialysis and concentration, the solution of glycoproteins was loaded on a column of convulxin bound to N-hydroxylsuccinamidyl-p-nitrophenyl chloroformate activated cross-linked 4% beaded agarose (1 mg/ml). The column was washed with 4 volumes of 10 mM Tris HCl, pH7.4, 30 mM NaCl, 0.2% nonanoyl-N-methylglucamide (NNMG), and then with 4 volumes of 10 mM Tris HCl, pH7.4, 30 mM NaCl and 2% NNMG. GPVI was eluted with 0.08% SDS in 10 mM Tris/HCl, pH 7.4. The solution was concentrated and loaded on a preparative gel of 8.5 % polyacrylamide using the Model 491 Prep Cell (BioRad, CA). The preparative electrophoresis was performed under non-reduced conditions following the manufacturer instructions. GPVI eluted as a single band at 65 kDa. The fractions were pooled, concentrated on Centricon-30 (Amicon, Beverly, MA) and resuspended in 10 mM Tris/HCl, pH7.4 and 0.1 % ONMG.

[0048] *Example 3: Amino acid analysis of GPVI* - GPVI was digested with the endoproteases LysC and AspN (Boehringer Mannheim, Germany). The 10 peptides generated were separated by reverse-phase HPLC and sequenced on an Applied Biosystem model 477A pulsed-liquid-phase protein sequencer with a model 120A on-line phenylthiohydantoin amino acid analyser.

[0049] *Example 4: Amplification of a 221 bp fragment coding for part of GPVI from a λ gt 77 cDNA library* -

[0050] A sample (10^{10} pfu) (plaque forming units) from a human bone marrow library (Clonetech, Palo Alto, CA) was amplified using 2 combinations of 4 degenerate primers. The final primer concentrations were 2 μ M, the dNTP concentration was 200 μ M and 2 U/100 μ l reaction AmpliTaq Gold (Perkin Elmer, Rotkreuz, Switzerland) were used. The PCR conditions were 5 cycles at 37°C followed by

30 cycles at 44°C. The sense 19mer 5' TYATHCCNGCNATGAARMG 3' and the antisense 20mer 5' TTRTAN-ARNGCRAAYTGRTC 3' amplified a 221 bp fragment which was subcloned in Bluescript KS⁺ (Stratagene, La Jolla, CA) and sequenced using the T7 Sequenase kit (Amersham, Switzerland).

[0051] *Example 5: Screening the λ gt11 cDNA library with - the 221 bp GPVI Probe-* The 221 bp fragment was cut from the plasmid, cleaned and labelled with α^{32} P-ATP (20MBq/50 μ l, Hartmann Analytik, Braunschweig, Germany) using the High Prime Labelling kit (Boehringer Mannheim, Switzerland). The human bone marrow library was screened following the manufacturer instructions. Positive phages were grown, their DNA isolated and subcloned in BlueScript using either EcoRI or Sal I sites and sequenced. Sequencing was performed using the ABS system of RACE- Platelet poly A RNA was prepared as previously described (). Reverse transcription (30 μ l) was performed using 5 μ g of poly A RNA with the primer 5'TGAATGAGACGGTCAGTTCAGC 3' (20 μ M), dNTP (1mM), RNAsin (40 U), 1X AMV buffer and 20 U AMV reverse transcriptase for 20 min at 45°C followed by 20 min at 52°C. The reaction mixture was treated with 2 μ l 6N NaOH at 65°C for 30 min, neutralised with 2 μ l 6N acetic acid, and concentrated in a Centricon 30 (Amicon). An anchor was ligated to the first strand DNA following the protocol of Aptes and Siebert () in BioTechniques 15: 890-893 (1993). Nested PCR was performed using a primer complementary to the anchor and the primer 5' TTGTACAGAGCAAATTGGTC 3' (35 cycles, 55°C) and followed by the primer 5' GACCAGAGGCTCCGTTCTG 3' (30 cycles at 53°C). The highest band (350 bp) was separated by agarose electrophoresis from the lower ones, subcloned into BlueScript, and sequenced.

[0052] *Example 6: Preparation of anti-GPVI Fab and F(ab')₂*-Polyclonal antisera against human GPVI was generated in rabbits. IgG from rabbit anti-GPVI antiserum was purified as described. Digestion of IgG with immobilized papain (Pierce) to generate Fab fragments was performed according to the standard protocol of the supplier. Fab fragments were separated from undigested IgG and Fc fragments using an immobilized Protein A (Sigma) column. The flowthrough was transferred to dialysis tube, concentrated using solid polyethyleneglycol 20 000, intensively dialysed against 20 mM Hepes, 140 mM NaCl, 4 mM KCl, pH 7.4 and stored at 4°C until used. F(ab')₂ fragments were prepared by pepsin digestion of IgG, 1 :50 enzyme to substrate ratio (w/w), in 0.5 M acetate buffer, pH 4.0, at 37°C for 18 hr. The pH was corrected to 7.4 with diluted NaOH and the sample was dialysed against 20 mM phosphate, pH 7.4. F(ab')₂ fragments were separated from undigested IgG and Fc fragments using Protein A chromatography. The flow-through was transferred to dialysis tube, concentrated using solid polyethyleneglycol 20 000, intensively dialysed against 20 mM Hepes, 140 mM NaCl, 4 mM KCl, pH 7.4 and stored in aliquots at -20°C. Washed platelets were lysed in Triton X-114 and phase separation was performed on the soluble material before isolating the membrane glycoproteins associated with the Triton X-114 phase by affinity chromatography on wheat-germ agglutinin-Sepharose 4B as described previously. As GPVI represents a very small fraction of the platelet membrane glycoprotein pool, we used the specificity of the snake C-type lectin convulxin for isolation of this receptor. Affinity chromatography on convulxin coupled to Sepharose 4B yielded a 65 kDa protein as major product. However, uncharacterized material of both higher and lower Mr CO-eluted with GPVI and could not be removed by extensive washing of the column. Preparative gel electrophoresis on 8.5 % polyacrylamide was added as a final step of purification. Fractions containing GPVI were pooled and gave a single band on reanalysis. Purified GPVI was tested for its ability to block platelet aggregation by collagen. A slight inhibitory effect was observed when aliquots of GPVI solution were added to the platelet suspension. However, by preincubating GPVI with collagen before adding the mixture to the platelet suspension, aggregation could be inhibited in a dose-dependant manner. These platelets still aggregated when fresh collagen was added. Under non-reducing conditions, the isolated protein has a Mr of 62 kDa with a shift toward a slightly higher Mr (65 kDa) under reducing conditions. As the amino

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terminus of GPVI was found to be blocked, the protein was digested with the enzymes LysC and LysC/AspN which produced 4 and 6 peptides, respectively, from which sequence was obtained. The peptides were separated by reverse phase HPLC on a C4 column and sequenced using the Edman method. The amino acid sequences of these peptides are underlined in the translated cDNA sequence (Fig. 1).

5

Description of Seq IDs

[0053]

- 10 Seq ID Nr. 1 Nucleotide sequence of open reading frame of GPVI gene as depicted in fig. 2.
Seq ID Nr. 2 Amino acid sequence of presequence of GPVI protein as depicted by amino acids 1 to 23 of fig. 1.
Seq ID Nr. 3 Amino acid sequence of mature GPVI protein as depicted by amino acids 24 to 316 of fig. 1.

SEQUENCE LISTING

15

[0054]

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<120> Recombinant platelet collagen receptor glycoprotein VI and its pharmaceutical use

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<223> mature GPVI protein

45

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50

55

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 caaacctcg ctctcagccc agccccggccc ggcggtgtcg tcaggagggg acgtaacct 420
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 5 Leu Glu Lys Pro Val Thr Leu Arg Cys Gln Gly Pro Pro Gly Val Asp
 10 Leu Tyr Arg Leu Glu Lys Leu Ser Ser Ser Arg Tyr Gln Asp Gln Ala
 15 Val Leu Phe Ile Pro Ala Met Lys Arg Ser Leu Ala Gly Arg Tyr Arg
 20 Glu Leu Val Ala Thr Gly Val Phe Ala Lys Pro Ser Leu Ser Ala Gln
 25 Pro Gly Pro Ala Val Ser Ser Gly Gly Asp Val Thr Leu Gln Cys Gln
 30 Ala Pro Tyr Lys Asn Pro Glu Arg Trp Tyr Arg Ala Ser Phe Pro Ile
 35 Ile Thr Val Thr Ala Ala His Ser Gly Thr Tyr Arg Cys Tyr Ser Phe
 40
 45
 50
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Ser Ser Arg Asp Pro Tyr Leu Trp Ser Ala Pro Ser Asp Pro Leu Glu
 165 170 175
 5 Leu Val Val Thr Gly Thr Ser Val Thr Pro Ser Arg Leu Pro Thr Glu
 180 185 190
 Pro Pro Ser Ser Val Ala Glu Phe Ser Glu Ala Thr Ala Glu Leu Thr
 10 195 200 205
 Val Ser Phe Thr Asn Lys Val Phe Thr Thr Glu Thr Ser Arg Ser Ile
 210 215 220
 15 Thr Thr Ser Pro Lys Glu Ser Asp Ser Pro Ala Gly Pro Ala Arg Gln
 225 230 235 240
 Tyr Tyr Thr Lys Gly Asn Leu Val Arg Ile Cys Leu Gly Ala Val Ile
 20 245 250 255
 Leu Ile Ile Leu Ala Gly Phe Leu Ala Glu Asp Trp His Ser Arg Arg
 260 265 270
 25 Lys Arg Leu Arg His Arg Gly Arg Ala Val Gln Arg Pro Leu Pro Pro
 275 280 285
 Leu Pro Pro Leu Pro Gln Thr Arg Lys Ser His Gly Gly Gln Asp Gly
 290 295 300
 30 Gly Arg Gln Asp Val His Ser Arg Gly Leu Cys Ser
 305 310 315

35 **Claims**

1. Polyclonal anti-GPVI antiserum, anti-GPVI Fab obtainable from said anti-GPVI antiserum or anti-GPVI F(ab)₂ obtainable from said anti-GPVI antiserum, wherein GPVI has the sequence of SEQ ID NO: 3.
- 40 2. Polyclonal anti-GPVI antiserum, anti-GPVI Fab obtainable from said anti-GPVI antiserum or anti-GPVI F(ab)₂ obtainable from said anti-GPVI antiserum according to claim 1, wherein GPVI is obtainable from platelets using wheat-germ agglutinin and convulxin and applying gel electrophoresis on polyacrylamide of about 8.5 %.
- 45 3. Polyclonal anti-GPVI antiserum, anti-GPVI Fab obtainable from said anti-GPVI antiserum or F(ab)₂ obtainable from said anti-GPVI antiserum according to claim 1 or 2, wherein the polyclonal anti-GPVI antiserum is generated in rabbits.
4. Anti-GPVI Fab fragment of humanized mouse monoclonal antibodies against GPVI, wherein GPVI has the sequence of SEQ ID NO: 3.
- 50 5. Monoclonal or polyclonal antibodies recognising the N-terminal region of GPVI, wherein GPVI has the sequence of SEQ ID NO: 3.

55 **Patentansprüche**

1. Polyklonales Anti-GPVI-Antiserum, Anti-GPCTI-Fab, das von dem Anti-GPVI-Antiserum erhältlich ist, oder Anti-GPVI F(ab)₂, das von dem Anti-GPVI-Antiserum erhältlich ist, wobei GPVI die Sequenz gemäß SEQ ID NO: 3 aufweist.

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- 5
2. Polyklonales Anti-GPVI-Antiserum, Anti-GPVI-Fab, das von dem Anti-GPVI-Antiserum erhältlich ist, oder Anti-GPVI F(ab')₂, das von dem Anti-GPVI-Antiserum erhältlich ist nach Anspruch 1, wobei GPVI von Plättchen unter Verwendung von Weizenkeimagglutinin und Convulxin und Durchführen einer Gelelektrophorese auf ungefähr 8,5%igem Polyacrylamid erhältlich ist.
- 10
3. Polyklonale Anti-GPVI-Antiserum, Anti-GPVI-Fab, das von dem Anti-GPVI-Antiserum erhältlich ist, oder Anti-GPVI F(ab')₂, das von dem Anti-GPVI-Antiserum erhältlich ist nach Anspruch 1 oder 2, wobei das polyklonale Anti-GPVI-Antiserum in Kaninchen erzeugt wird.
- 15
4. Anti-GPVI-Fab-Fragment von humanisierten monoklonalen Mausantikörpern gegen GPVI, wobei GPVI die Sequenz gemäß SEQ ID NO: 3 aufweist.
5. Monoklonale oder polyklonale Antikörper, die die N-terminale Region von GPVI erkennen, wobei GPVI die Sequenz von SEQ ID NO: 3 aufweist.

Revendications

- 20
1. Antisérum anti-GPVI polyclonal, Fab anti-GPVI pouvant être obtenu à partir dudit antisérum anti-GPVI ou F(ab')₂ anti-GPVI pouvant être obtenu à partir dudit antisérum anti-GPVI, GPVI ayant la séquence de SEQ ID NO: 3.
- 25
2. Antisérum anti-GPVI polyclonal, Fab anti-GPVI pouvant être obtenu à partir dudit antisérum anti-GPVI ou F(ab')₂ anti-GPVI pouvant être obtenu à partir dudit antisérum anti-GPVI selon la revendication 1, GPVI pouvant être obtenu à partir de plaquettes par utilisation d'agglutinine de germe de blé et de convulxine et application d'électrophorèse sur gel sur polyacrylamide d'environ 8,5 %.
- 30
3. Antisérum anti-GPVI polyclonal, Fab anti-GPVI pouvant être obtenu à partir dudit antisérum anti-GPVI ou F(ab')₂ anti-GPVI pouvant être obtenu à partir dudit antisérum anti-GPVI selon la revendication 1 ou 2, l'antisérum anti-GPVI polyclonal étant généré chez des lapins.
- 35
4. Fragment Fab anti-GPVI d'anticorps monoclonaux de souris humanisés contre GPVI, GPVI ayant la séquence de SEQ ID NO: 3.
5. Anticorps monoclonaux ou polyclonaux reconnaissant la région N-terminale de GPVI, GPVI ayant la séquence de SEQ ID NO: 3.
- 40
- 45
- 50
- 55

Figure 2

GPVI definitive nucleotide sequence covering Open reading frame of 1017 bp plus 3' and 5' regions total 1249 bp

GAGCTCAGGA CAGGGCTGAG GAACCATGTC TCCATCCCCG ACCGCCCTCT (50)
TCTGTCTTGG GCTGTGTCTG GGGCGTGTGC CAGCGCAGAG TGGACCGCTC (100)
CCCAAGCCCT CCCTCCAGGC TCTGCCCAGC TCCCTGGTGC CCCTGGAGAA
GCCAGTGACC CTCCGGTGCC AGGGACCTCC GGGCGTGGAC CTGTACCGCC (200)
TGGAGAAGCT GAGTTCAGC AGGTACCAGG ATCAGGCAGT CCTCTTCATC
CCGGCCATGA AGAGAAGTCT GGCTGGACGC TACCGCTGCT CCTACCAGAA (300)
CGGAAGCCTC TGGTCCCTGC CCAGCGACCA GCTGGAGCTC GTTGCCACGG
GAGTTTTTGC CAAACCCTCG CTCTCAGCCC AGCCCGGCC GCGGGTGTCTG (400)
TCAGGAGGGG ACGTAACCCT ACAGTGTCAG ACTCGGTATG GCTTTGACCA
ATTTGCTCTG TACAAGGAAG GGGACCCTGC GCCCTACAAG AATCCCGAGA (500)
GATGGTACCG GGCTAGTTTC CCCATCATCA CGGTGACCGC CGCCCACAGC
GGAACCTACC GATGCTACAG CTTCTCCAGC AGGGACCCAT ACCTGTGGTC (600)
GGCCCCCAGC GACCCCCTGG AGCTTGTGGT CACAGGAACC TCTGTGACCC
CCAGCCGGTT ACCAACAGAA CCACCTTCCT CGGTAGCAGA ATTCTCAGAA (700)

GCCACCGCTG AACTGACCGT CTCATTCACA AACAAAGTCT TCACAACTGA
 GACTTCTAGG AGTATCACCA CCAGTCCAAA GGAGTCAGAC TCTCCAGCTG (800)
 GTCCTGCCCG CCAGTACTAC ACCAAGGGCA ACCTGGTCCG GATATGCCTC
 GGGGCTGTGA TCCTAATAAT CCTGGCGGGG TTTCTGGCAG AGGACTGGCA (900)
 CAGCCGGAGG AAGCGCCTGC GGCACAGGGG CAGGGCTGTG CAGAGGCCGC
 TTCCGCCCTT GCCGCCCTC CCGCAGACCC GGAAATCACA CGGGGGTCAG (1000)
 GATGGAGGCC GACAGGATGT TCACAGCCGC GGGTTATGTT CATGACCGCT
 GAACCCAGG CACGGTCGTA TCCAAGGGAG GGATCATGGC ATGGGAGGCG (1100)
 ACTCAAAGAC TGGCGTGTGT GGAGCGTGGA AGCAGGAGGG CAGAGGCTAC
 AGCTGTGGAA ACGAGGCCAT GCTGCCTCCT CCTGGTGTTT CATCAGGGAG (1200)
 CCGTTCGGCC AGTGTCTGTC TGTCTGTCTG CCTCTCTGTC TGAGGGCAC (1249)

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	抗重组血小板胶原受体糖蛋白VI的抗体		
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申请号	EP2011001216	申请日	2000-04-25
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当前申请(专利权)人(译)	SANOFI-AVENTIS DEUTSCHLAND GMBH		
[标]发明人	CLEMENTSON KENNETH J		
发明人	CLEMENTSON, KENNETH J.		
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其他公开文献	EP2341141A3 EP2341141A2		
外部链接	Espacenet		

摘要(译)	gagctcagga cagggtgag gaaccatgtc tccatccccg accgccctct tctgtcttgg 60
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本发明涉及糖蛋白VI (GPVI)，其分离，纯化和重组生产方法。特别地，本发明涉及GPVI，优选重组GPVI在治疗直接或间接与凝血障碍如血栓形成和心血管疾病相关的病症和病理事件中的用途。细胞外重组蛋白也可用于建立筛选试验。寻找膜结合GPVI的潜在抑制剂，以分别抑制血小板和血小板与胶原蛋白的结合。