Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily

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Injuries to the vessel wall and subsequent exposure of collagen from the subendothelial matrix result in thrombus formation. In physiological conditions, the platelet plug limits blood loss. However, in pathologic conditions, such as rupture of atherosclerotic plaques, platelet–collagen interactions are associated with cardiovascular and cerebral vascular diseases. Platelet glycoprotein VI (GPVI) plays a crucial role in collagen-induced activation and aggregation of platelets, and people who are deficient in GPVI suffer from bleeding disorders. Based on the fact that GPVI is coupled to the Fc receptor (FcR)-γ chain and thus should share homology with the FcR chains, the genes encoding human and mouse GPVI were identified. They belong to the immunoglobulin (Ig) superfamily and share 64% homology at the protein level. Functional evidence demonstrating the identity of the recombinant protein with GPVI was shown by binding to its natural ligand collagen; binding to convulxin (Cvx), a GPVI-specific ligand from snake venom; binding of anti-GPVI IgG isolated from a patient; and association to the FcR-γ chain. The study also demonstrated that the soluble protein blocks Cvx and collagen-induced platelet aggregation and that GPVI expression is restricted to megakaryocytes and platelets. Finally, human GPVI was mapped to chromosome 19, long arm, region 1, band 3 (19q13), in the same region as multiple members of the Ig superfamily. This work offers the opportunity to explore the involvement of GPVI in thrombotic disease, to develop alternative antithrombotic compounds, and to characterize the mechanism involved in GPVI genetic deficiencies. (Blood. 2000;96:1798-1807)

Introduction

Glycoprotein VI (GPVI) is a 58-kd (62 kd after reduction) platelet membrane glycoprotein that plays a crucial role in the collagen-induced activation and aggregation of platelets.[1,2] Upon injury to the vessel wall and subsequent damage to the endothelial lining, exposure of the subendothelial matrix to the blood flow results in deposition of platelets. Collagen fibers are the most thrombogenic macromolecular components of the extracellular matrix, with collagen types I, III, and VI being the major forms found in blood vessels. Platelet interaction with collagen occurs as a 2-step procedure: (1) the initial adhesion to collagen is followed by (2) an activation step leading to platelet secretion, recruitment of additional platelets, and aggregation.[3,4] In physiologic conditions the resulting platelet plug is the initial hemostatic event limiting blood loss. However, platelet–collagen interactions may also have major pathologic consequences. Exposure of collagen after rupture of atherosclerotic plaques is a major stimulus of thrombus formation associated with myocardial infarction or stroke.

The mechanism of collagen–platelet interactions is complex. It involves direct or indirect binding of collagen to several platelet receptors including the GPIb-IX-V complex, integrin α2β1, GPIV, GPVI, and 65- and 85-kd proteins.[5] These receptors are sequentially enrolled in both adhesion and activation steps. A major indirect mechanism involves the collagen-bound von Willebrand factor, which interacts with its receptor, the GPIb/IX/V complex, resulting in platelet adhesion to the vessel wall at a high shear rate. The platelets are subsequently fully arrested by direct interaction between collagen and integrin α2β1 (GPIa/Iia and very late activation antigen [VLA]–2). Strengthening of platelet attachment is followed by an activation step that involves at least one other platelet collagen receptor. Several lines of evidence indicate that GPVI is a major mediator in this event. Patients deficient in platelet GPVI suffer from mild hemorrhagic diathesis,[6,7] and their platelets fail to aggregate in response to collagen. One patient developed antibodies to GPVI.[8] Anti-GPVI immunoglobulin G (IgG) and $F(ab)'_2$ fragments derived from this patient activate platelets while monovalent Fab fragments block platelet aggregation induced by collagen and IgG. GPVI-deficient platelets show defects in the second phase of adhesion, ie, the recruitment phase mainly attributable to platelet–platelet aggregation. It is therefore believed that GPVI has a minor part in the first step of adhesion and that its key role is in the second step of collagen–platelet interaction, which results in full platelet activation and consequently the formation of platelet aggregates.[4]

Collagen, collagen-related peptides (CRPs), and a snake venom

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protein, convulxin (Cvx), activate platelets similar to anti-GPVI Fab′-2 fragments via GPVI-coupled signaling pathway.[9-11] Protein-tyrosine phosphorylation is a key signaling event in this pathway. GPVI is constitutively associated with the Fc receptor–γ (FcR-γ) chain, which is essential for activation of platelets by collagen.[12] After cross-linking of GPVI by anti-GPVI $F(ab)'_2$ fragments or platelet activation by collagen, CRP, or Cvx, the FcR-γ chain is phosphorylated on tyrosine residues,[13,14] presumably within the immunoreceptor tyrosine-based activation motif (ITAM). Activation through GPVI results in the binding of Syk to the FcR-γ chain, which leads to Syk activation and tyrosine phosphorylation. This in turn leads to activation of phospholipase C gamma-2 (PLC–γ-2).[15] GPVI-deficient platelets do not demonstrate activation of Syk by collagen.[16] Implication of the Src family kinase p59 fyn and p53/56 lyn has also been demonstrated following GPVI stimulation.[15] Recently, it has been shown that platelet stimulation by collagen or the GPVI-specific ligand Cvx involves association between phosphatidylinositol 3–kinase (PI3-kinase), the adapter protein linker for activator of T cells (LAT) and FcR-γ.[17,18] These studies have shown strong similarities between the GPVI signaling pathway in platelets and those used by receptors for immune complexes including the FcR,[19] such as the high- and low-affinity receptors for IgG, IgE, and IgA (Fc-γ/RI, Fc-γ/RIII, Fc-ε/RI, and Fc-α/RI, respectively).[20] These receptors signal via the FcR-γ chain and Syk. Expression of FcRs capable of signaling via the FcR-γ chain in platelets is limited to Fc-ε/RI.[21]

Due to the strong similarity between GPVI and the multimeric Fc receptors, mouse and human megakaryocyte libraries were examined for the presence of an FcR-like complementary DNA (cDNA). A cDNA clone with these characteristics was identified in both murine and human megakaryocyte libraries. Our results demonstrate that these clones encode for GPVI.

Recently, another group also reported the cloning of human GPVI.[22] Commensurate with their results, we show that GPVI belongs to the Ig superfamily. In addition, we have isolated the murine homologue of GPVI and have shown that expression of both human and mouse GPVI is restricted to the megakaryocyte/platelet lineage. We also present considerable functional evidence including its association with the FcR-γ chain. Furthermore, we demonstrate that recombinant GPVI is capable of inhibiting platelet aggregation induced by both collagen and Cvx. Finally, we also show the chromosomal linkage of GPVI to a group of Ig superfamily members.

Materials and methods

Library construction

Human megakaryocytes were obtained from mobilized $CD34^+$ cells. After receiving informed consent, aliquots of leukopheresis units were drawn from patients following mobilization by chemotherapy and granulocyte colony-stimulating factor (G-CSF). After collection, low-density cells were separated over a 1077 Ficoll-Metrizoate gradient (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). Mononuclear cells were separated using a magnetic-activated cell sorting (MACS) system (mini-MACS; Miltenyi Biotec GmbH; Bergisch Gladbach, Germany) in accordance with the manufacturer's recommendations. The cells were passed twice through the column. Purity, which was evaluated by flow cytometry after labeling with a phycoerythrin (PE)–anti-CD34 monoclonal antibody (mAb) (HPCA-2, clone 8G2; Becton Dickinson, Franklin Lakes, NJ), was about 90%. The $CD34^+$ cells were grown for 10 days in Iscove modified Dulbecco medium (IMDM, Gibco BRL, Grand Island, NY) containing the following (all from Sigma Chemical, St Louis, MO): 250 U/mL penicillin, 250 μg/mL streptomycin, and 2 mmol/L glutamine; 1.5% deionized bovine serum albumin (BSA) (Cohn fraction V); 300 mg/mL iron-saturated human transferrin; and a mixture of 20 μg/mL sonicated lipids prepared as previously reported.[23] The medium was supplemented with a combination of pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) (Amgen, Thousand Oaks, CA) and recombinant human stem cell factor (rhSCF) (Amgen). After 10 days of culture, the megakaryocytes were purified by the same technique as the $CD34^+$ cells by using an anti-CD61 mAb (Y2/51; gift from D. Mason, Oxford, England). Purity was more than 90%, as attested by labeling with a PE–anti-CD41a mAb (Pharmingen, San Diego, CA).

Primary mouse megakaryocytes were obtained from day-15 fetal livers, cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco), 10% fetal calf serum (FCS), and PEG-rHuMGDF for 4 days as previously described.[24] cDNA libraries of both human and mouse megakaryocytes were constructed using a previously described method.[25] Random EST sequences of these libraries were searched using Basic Local Alignment Search Tool (BLAST) algorithms to find novel membrane-bound proteins.

Chromosomal mapping

The mouse mapping primers used for polymerase chain reaction (PCR) amplification were forward primer 5′-CTGTAGCTGTTTTCAGACACACC-3′ and reverse primer 5′-CCATCACCTCTTTCTGGTTAC-3′. All PCRs were performed with an annealing temperature of 52°C followed by 35 cycles of extension times at 72°C for 50 seconds, with a final extension of 5 minutes on an MJ Research Peltier PCT-225 Thermal Cycler. The T31 Mouse/Hamster Radiation Hybrid (Research Genetics, Huntsville, AL) was used as a mapping panel for mGPVI.[26] PCRs were performed under the PCR conditions described above and with the mouse primers described above.

For human mapping, the primers were designed from the 3′-UTR (untranslated region) of the sequence: forward primer 5′-ACTCAAAGACTGGCGTGTGTG-3′ and reverse primer 5′-GTTCTGAGAGACGAAAGGAG-3′. The PCR amplification conditions were the same as those used for the mouse mapping. The products were amplified from the G4 Radiation Hybrid Panel (Research Genetics, Huntsville, AL), run on a 2% agarose gel, and individually scored as to the presence or absence of the human band in each of the 93 cell line DNAs in addition to a negative water control. The results were analyzed with the MapManager software program.

Protein preparation

The open-reading frame of the predicted extracellular domain of T268/GPVI was PCR-amplified from the Kozak sequence before the first methionine to asparagine 269, immediately prior to the predicted transmembrane sequence. The PCR fragment was ligated into a pCDM8 host vector containing the genomic sequence of the human IgG1 Fc domain, such that the extracellular part of the hGPVI cDNA was fused at its C-terminus via a 3 alanine linker to the hFc sequence. The sequenced DNA construct was transiently transfected into HEK 293T cells in 150-mm plates using Lipofectamine (Gibco) according to the manufacturer's protocol. After 72 hours of transfection, the serum-free conditioned medium (OptiMEM, Gibco) was harvested, spun, and filtered. The cells were refed with fresh medium and harvested as above for another 72 hours. Using an antihuman IgG Fc polyclonal antibody, analysis of supernatants on Western blot after reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) showed significant amounts of the recombinant human soluble GPVI fusion (rhusGPVI:Fc) protein in the supernatants, with a relative molecular mass of approximately 75-80 kd relative to the Mark 12 molecular weight standards cocktail (Novex, San Diego, CA).

The conditioned media was passed over a 10-mL Prosep-G protein G column (Bioprocessing, Princeton, NJ). The column was then washed with phosphate-buffered saline (PBS) (pH 7.4) and eluted with 200 mmol/L glycine (pH 3.0) at 7 mL/min. Fractions from the 280-nm elution peak containing rhusGPVI:Fc were pooled and dialyzed in 8000 MWCO dialysis tubing against 2 changes of 4 liters PBS (pH 7.4) at 4°C with constant stirring. The buffered exchanged material (0.2 μm) was then sterile filtered (Millipore, Bedford, MA) and frozen at −80°C.

In situ hybridization

In situ hybridization (ISH) was performed with day-12.5 C57BL/B6 mouse embryos and normal 4- to 6-week-old C57BL/6 mouse femurs. The tissues were fixed in 10% formalin, paraffin embedded, and subsequently sectioned at 4 μm onto Superfrost plus slides. The femurs were decalcified in TBD-2 (Shandon, Pittsburgh, PA) prior to paraffin embedding. The sections were deparaffinized in xylene, hydrated through a series of graded ethanol washes, and placed in diethyl pyrocarbonate (DEPC)–treated PBS (pH 7.4) before being processed for ISH. The sections were incubated in 20 μg/mL proteinase K (Sigma) in DEPC-PBS for 15 minutes at 37°C and then immersed in 4% formaldehyde/PBS for 5 minutes.

The sections were treated with 0.2 N HCl for 10 minutes followed by DEPC-PBS. The sections were then rinsed in 0.1 mol/L triethanolamine HCl (TEA, pH 8.0), incubated in 0.25% acetic anhydride–TEA for 10 minutes, rinsed in DEPC-PBS, dehydrated through a series of graded ethanol washes, and air dried. Labeling and hybridization of sulfur 35 (^{35}S)–radio-labeled 2.5×10^7 cpm/mL cRNA antisense and sense RNA probes encoding a 599-bp fragment of the 5′-end of the *GPVI* gene (generated with the PCR primers forward 5′-CAGCCTCACCCACTT-TCTTC-3′, nucleotides [nt] 8-27, and reverse 5′-CCACAAGCACTA-GAGGGTCA-3′, nt 607-588) were performed as previously described.[27] Following hybridization, the sections were dehydrated rapidly through serial ethanol and 0.3 mol/L sodium acetate before being air dried, dipped in a nuclear track emulsion (NTB-2; Eastman Kodak, Rochester, NY), and exposed for 60 days at room temperature. The slides were developed with D-19 (Eastman Kodak), stained with hematoxylin and eosin-Y, and added to cover slips.

Cell lines

The HEL (erythroid/MK), U937 (monoblast), K562 (erythroid), CEM (T cell), HEPG2, and Hela cell lines (American Type Culture Collection [ATCC], Manassas, VA) and the FDC-P1 and 32D cell lines (D. Metcalf, The Walter and Eliza Hall Institute, Melbourne, Australia) were used in this study. The UT7 (erythroid/MK) transduced by c-mpl,[28] TF1 (erythroid), KG1 (myeloblast), HL60 (myeloblast/promyelocyte), MO-7E (MK), Meg-01 (MK), and DAMI (MK) were obtained from the different laboratories that derived them.[29-35]

The HEL, U937 HL60, Meg-01, KG1, and K562 human cell lines were cultured in IMDM with 10% FCS (Stem Cell Technology, Vancouver, British Columbia, Canada). The c-mpl UT7, TF1, and MO-7E are factor-dependent and were grown in the presence of either 2 ng/mL granulocyte macrophage(GM)–CSF or 10 ng/mL PEG-rHuMGDF in IMDM with 10% FCS. CEM and Hela were grown in Roswell Park Memorial Institute medium (RPMI) (Gibco). FDC-P1, 32D, and Ba/F3 murine cell lines were cultured in DMEM with 10% FCS (Stem Cell Technology). The cultures were performed at 37°C in a fully humidified atmosphere of 5% carbon dioxide (CO_2).

Samples

Human megakaryocytes were obtained as described for the human libraries from mobilized or cord blood $CD34^+$. A fetal liver was obtained following abortion at 12-week gestation after obtaining informed consent.

Northern blot/reverse transcriptase–PCR analysis

Human multiple tissue Northern blots (Clontech, Palo Alto, CA) were hybridized to a 1.0-kb human GPVI probe as described by the manufacturer. Total RNA was isolated using RNA PLUS (Bioprobe Systems, France), a modification of the acid-guanidinium thiocyanate-phenylchloroform extraction method of Chomczynski et Sacchi.[36] RNA was reverse transcribed with random hexamers using SUPERSCRIPT reverse transcriptase (RT) (Gibco BRL/Life Technologies, Cergy Pontoise, France).

For human cell lines and tissues, after reverse transcription, each sample was subjected to a specific amplification of GPVI and β_2 microglobulin cDNA. The sequences of the specific primers were: GPVI sense primer, 5′-TTCTGTCTTGGGCTGTGTCTG-3′; GPVI antisense primer, 5′-CCCGCCAGGATTATTAGGATC-3′; β_2 microglobulin sense primer, 5′-CCTGAAGCTGACAGCATTCGG-3′; and β_2 microglobulin antisense primer, 5′-CTCCTAGAGCTACCTGTGGAG-3′. PCR was performed in a 25-μL reaction mixture containing 0.3 U *Thermus aquaticus* (*Taq*) polymerase (ATGC, Noisy-le-Grand, France), 200 μmol/L dNTP (deoxynucleoside 5′-triphosphate), 30 pmol oligonucleotide sense and 30 pmol antisense for GPVI amplification, and 10 pmol oligonucleotide sense and 10 pmol antisense for β_2 microglobulin amplification in ATGC buffer. The reaction mixture was subjected to denaturation for 5 minutes at 95°C and then amplified by 35 cycles as follows: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension at 72°C for 1 minute, with a final 7-minute extension at 72°C in a Thermocycler 2400 (Perkin Elmer, Courtaboeuf, France). We electrophoresed 9 μL PCR products on a 2% agarose gel. The fragments were visualized by illumination after ethidium bromide staining. MassRuler DNA Ladder, Low Range (MBI Fermentas, Amherst, NY) was used as marker.

GPVI-expressing cell lines

Chinese hamster ovary (CHO) cells were transfected using lipofectamine (Gibco) according to manufacturer's instructions. Full-length GPVI cDNA was cloned into an expression vector driven by an SRalpha promoter. Control CHO cells were transfected with the empty vector. The cells were collected 2 days after transfection and lysed in 12 mmol/L Tris (tris[hydroxymethyl] aminomethane), 300 mmol/L sodium chloride (NaCl), and 12 mmol/L EDTA (ethylenediamine tetraacetic acid) containing 2 μmol/L leupeptin, 2 mmol/L PMSF (phenylmethylsulfonyl fluoride), 5 KIU aprotinin, and 0.2% (vol/vol) NP40 (Sigma). After 20 minutes at 4°C under agitation, the samples were centrifuged at 13 000*g* for 15 minutes at 4°C, and the supernatants were frozen at −80°C for analysis.

The human cell lines HEL, U937, and K562 and the murine cell lines FDC-P1, 32D, and Ba/F3 were engineered[37] to express GPVI using the pMSCVpac retrovirus.[38] Briefly, viruses carrying the full-length cDNA encoding human or murine GPVI were constructed using base-perfect PCR-amplified fragments of the cDNAs (Clontech Laboratories, Palo Alto, CA). Viral supernatants were generated into the 293-EBNA cells (Invitrogen, Carlsbad, CA) by transfecting the retroviral construct and 2 pN8-ϵ vectors containing the *gag/pol* genes, which are derived from the murine moloney leukemia virus (MMLV), or the vesicular stomatitis virus envelope glycoprotein G (*VSV-G*) gene (gift from J. Morgenstern, Millennium Pharmaceuticals, Cambridge, MA). Concentrated viral supernatants were prepared by centrifugation at 4°C using an SW28 rotor at 50 000g (25 000 rpm) for 2 hours. The pellets were resuspended in 1.5 mL DMEM at 4°C for 24 hours, shaken at 4°C for 24 hours, and frozen at −80°C. For transduction, the cell lines were incubated with the viral supernatant overnight in 24-well plates, 10×10^5 cells per mL, and selected during 1 week using 4 μg/mL puromycin (Sigma). Human and murine GPVI were transduced in human and murine cell lines, respectively. Expression of the genes was verified using PCR analysis. The control cells were transduced with the empty virus.

Platelet preparation

Blood from healthy volunteers was collected by venipuncture on acid-citrate-dextrose anticoagulant (ACD-A). When needed, the platelets were labeled by incubating the platelet-rich plasma (PRP) with 0.6 μmol/L carbon 14 5-hydroxytryptamine (^{14}C 5-HT) at 37°C for 30 minutes. The platelet pellets were obtained by centrifugation of PRP and were washed 2 times as previously described.[10]

Platelet aggregation and secretion

Aggregation of washed platelets (3×10^8/mL) in reaction buffer was initiated by collagen type I (Bio/Data, Horsham, PA) or Cvx. The experiments were performed with stirring at 37°C in an aggregometer (Chrono-Log, Haverton, PA). Release of ^{14}C 5-HT was measured as described previously.[10]

Cvx preparation

Cvx was purified from the venom of *Crotalus durissus terrificus* mainly as described by Francischetti et al,[39] using a 2-step gel filtration procedure of

sephadex G75 (Pharmacia Biotech, Uppsala, Sweden) followed by sephacryl S100 (Pharmacia Biotech). Cvx was labeled with iodine 125 (^{125}I) using the iodogen procedure (Pierce Chemical, Rockford, IL) and Na-^{125}I (Amersham, Les Ulis, France). Cvx was coupled to fluorescein isothiocyanate (FITC) by mixing Cvx in 50 mmol/L NaHCO3 and 150 mmol/L NaCl (pH 9.5) with a 100-fold molar excess of FITC (Aldrich, St Quentin Fallavier, France) at 4°C overnight. FITC-coupled Cvx was separated from free FITC by chromatography on a sephadex G25 column in 20 mmol/L phosphate and 150 mmol/L NaCl (pH 7.4) in PBS. Bothrojaracin, a specific thrombin inhibitor purified from the venom of *Bothrops jararaca* as previously described,[40] was coupled to FITC using the same procedure.

Flow cytometry

Cells transduced with human or murine GPVI viruses or the control virus were incubated in the presence of 20 nmol/L FITC-Cvx or FITC-bothrojaracin for 60 minutes at room temperature. After dilution in PBS, the cells were analyzed by a fluorescence-activated cell sorter (FACS) flow cytometer (FACSort; Becton Dickinson, Franklin Lakes, NJ).

Protein analysis

Platelets, megakaryocytes, and cell lines were lysed in a buffer composed of 12 mmol/L Tris, 300 mmol/L NaCl, and 12 mmol/L EDTA containing 2 μmol/L leupeptin, 2 mmol/L PMSF, 5 KIU aprotinin, and 0.2% (vol/vol) NP40. After 20 minutes at 4°C under agitation, the samples were centrifuged at 13 000*g* for 15 minutes at 4°C, and the supernatants were frozen at −80°C. The protein concentration was determined using a protein assay (Bio-Rad Laboratories, Ivry-sur-Seine, France). For blotting experiments, the proteins were further solubilized with 2% SDS at 100°C for 5 minutes. The proteins were separated by electrophoresis on acrylamide slab gels (Mini Protean II, Bio-Rad) and transferred on a polyvinylidene fluoride (PVDF) membrane. The membranes were soaked with 5% nonfat dry milk and incubated with either 6×10^3 Bq/mL ^{125}I-Cvx in PBS (pH 7.4) containing 0.1% (vol/vol) Tween 20, or with 9 μg/mL anti-GPVI IgG in PBS (pH 8) containing 0.02% (vol/vol) Tween 20 in the absence or presence of 0.5 μmol/L cold Cvx. Anti-GPVI IgG was obtained as previously described[10] from patient plasma (gift of Prof M. Okuma, Kyoto, Japan).

The antibodies were revealed using peroxidase-coupled protein A and enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden). For immunoprecipitation, cell lysates were precleared by incubation with protein A-sepharose at 4°C for 30 minutes followed by centrifugation. Cleared lysates were incubated overnight at 4°C with 10 μg/mL polyclonal anti–FcR-γ chain antibodies (Upstate Biotechnology, NY) followed by the addition of protein A/G-sepharose (Pharmacia Biotech) at room temperature for 2 hours. Immunoprecipitated proteins were eluted with 2% SDS, subjected to SDS-PAGE, blotted onto PVDF membranes, and then probed using anti-GPVI and anti–FcR-γ chain antibodies as described above.

Cell adhesion

Collagen type I (2 μg) (Chrono-Log), 1.4 μg Cvx, or 2 μg BSA (Sigma) in 100 μL PBS were immobilized on Immulon II plates (Dynatech, St Cloud, France) at 4°C overnight. The plates were then saturated with 2 mg/mL BSA in PBS for 1 hour and washed with PBS. The cells in culture medium were labeled with chromium 51 (^{51}Cr) (CIS Bio International, Gif sur Yvette, France) at 37°C for 1 hour after centrifugation at 150*g* for 10 minutes. The cells were washed with Hank's balanced salt solution containing 2 mg/mL BSA and resuspended in the same buffer. The cells were added to the wells. After 60 minutes at room temperature, the wells were emptied and washed, and the samples were counted for ^{51}Cr.

Results

Cloning of GPVI

Using computer-based searching of EST sequences from a murine megakaryocyte library, we isolated a clone, named T268, that showed striking homology to the inhibitory family of receptors. The human homologue was identified from a human megakaryocyte library by sequence homology. These clones encode proteins of amino acids 313 and 339, respectively, which are predicted to be type I membrane proteins belonging to the Ig superfamily. Alignment of the mouse and human T268 sequences shows 64.4% conservation in amino acids between species (Figure 1A). Alignment of the mouse and human T268 nucleotide sequences shows 67.3 % identity (Figure 1B). The closest database matches for these proteins are the natural killer (NK) inhibitory receptors, although it is clear that there are no inhibitory or activation motifs present in the relatively short cytoplasmic domain. The extracellular domain contains 2 Ig-like C2 domains and a conserved N-linked glycosylation site at asparagine 93. A second potential glycosylation site is also found at asparagine 244 in the mouse protein. It is interesting to note the presence of a charged residue in the transmembrane domain (R273 in hT268 and R270 in mT268), which may act as an interaction site for association with other membrane proteins.

These characteristics of human GPVI are identical to those recently reported by Clemetson et al.[22] The mouse gene for GPVI

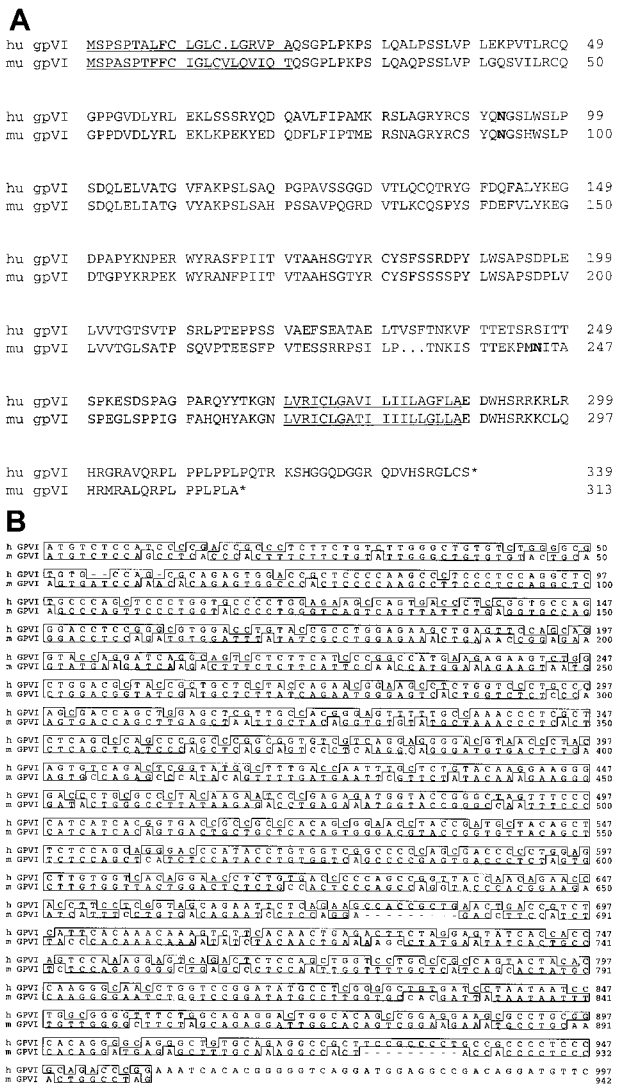

Figure 1. Sequence alignment of human and mouse GPVI. (A) The predicted signal peptides and transmembrane domains are underlined. Potential N-linked glycosylation sites are indicted in bold. The amino acid sequences of human and mouse GPVI share 64.4% identity. (B) The nucleotide sequences of human and mouse GPVI cDNA share 67.3% identity.

was mapped on radiation hybrid panels to chromosome 7, between the flanking markers D7Mit152 and D7Mit178. The human gene was localized to the long arm of human chromosome 19 (19q), 11.1cR distal to genetic marker WI-5264 and 19.3cR proximal to genetic marker WI-5423. This region is equivalent to 19q13.32-q13.33 (region 3, band 2 to band 3). This region contains the melanoma inhibitory activity gene and is part of the leukocyte receptor complex (LRC) that contains various members of the Ig superfamily. The closest mapped genes to GPVI included the FcR-α and the leukocyte-associated Ig-like receptor 1 (LAIR1), a killer cell inhibitory receptor located in 19q13.4. Interestingly, both of these molecules also function via crosslinking with the FcR-γ.

Tissue expression of GPVI

To determine tissue distribution of both mouse and human GPVI, Northern blot, RT-PCR, and ISH were performed. Human tissues were studied using Northern blot or RT-PCR analysis. Northern blots (Figure 2A) revealed no specific message in samples of brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, or lymph node. A 2-kb transcript was observed only in bone marrow and fetal liver. A signal was inconsistently observed with peripheral blood cells, probably due to platelet RNA contamination in some samples. Indeed, transcripts for platelet GPIIb, a platelet specific protein, were also detected in these positive samples (data not shown).

Using RT-PCR analysis (Figure 2B), the GPVI transcript was detected only in platelets, not the other blood cells. In cell lines, a strong PCR signal was observed in the HEL, MEG01, DAMI, TPO-stimulated MO7E, and mpl-transduced UT7 cell lines.[28] A very low signal was also detected in the K562 and KG1 cell lines, 2 cell lines that also express GPIIb at a low level. But there was no expression observed in the HEPG2, CEMT, TF1, U937, HL60, and Hela cells. CD41$^+$ cells (including more than 95% megakaryocytes) isolated from normal cord blood or chemotherapy-induced mobilized peripheral blood displayed a strong RT-PCR signal.

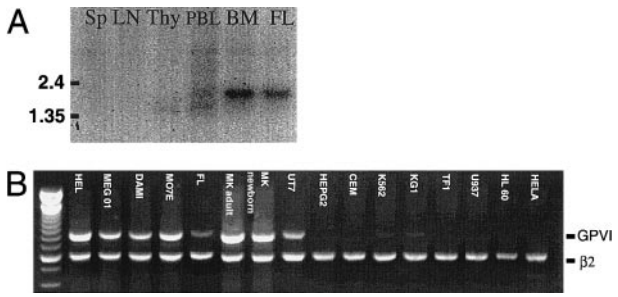

Figure 2. Tissue expression of human GPVI using RT-PCR or Northern blot analysis. (A) Northern blot analysis of human tissues. A 2-kb transcript is observed only in bone marrow and fetal liver. A signal is also observed with peripheral blood leukocytes (PBLs). However, when the same blot was hybridized with a GPIIb probe, a platelet protein absent in PBLs, transcripts were also detected, which suggests that the signal was due to platelet RNA contamination. There was no signal observed in a different PBL sample or in samples of brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, or lymph node (data not shown). Sp indicates spleen; LN, lymph node; Thy, thymus; PBL, peripheral blood leukocytes; BM, bone marrow; and FL, fetal liver. (B) RT-PCR analysis from human samples. We coamplified β_2 microglobulin (β_2) and GPVI transcripts. The high molecular weight fragment (830 base pair [bp]) was generated from the GPVI primers, and the low molecular weight fragment (603 bp) was generated from the β_2 microglobulin primers. The β_2 microglobulin PCR product, used as a loading control, is present in all the samples in similar quantity. In contrast, GPVI is amplified only in megakaryocyte-enriched samples (adult and newborn); in cell lines displaying strong MKC features (HEL, MEG01, DAMI, MO7E, and mpl-UT7); and to a lesser extent, in fetal liver cells. A very low signal was also detected in the K562 and KG1 cell lines, 2 cell lines that also express platelet GPIIb at low levels, but there was no expression detected in the other samples.

Fetal liver cells expressed a moderate level of expression compared to megakaryocyte-enriched samples.

Mouse tissues were studied using Northern blot and ISH analysis. ISH reveals that GPVI was exclusively found in the liver during embryogenesis (Figure 3A,B). The signal pattern was strong and multifocal, suggestive of expression by a scattered cell population. This signal was observed at embryonic day 13.5, 14.5, and 16.5 and decreased in intensity at day 18.5 and in a 1.5-day-old newborn (data not shown). In adults, the expression in liver was no longer observed, but a strong multifocal signal was seen in spleen and bone marrow. There was no signal observed in any other tissues including samples of brain, eye, harderian gland, submandibular gland, bladder, white fat, stomach, brown fat, heart, adrenal gland, colon, small intestine, liver, placenta, thymus, lymph node, lung, spinal cord, pancreas, skeletal muscle, and testes. Photoemulsion processing of the spleen and bone marrow showed that this expression was restricted to megakaryocytes (Figure 3C).

In conclusion, despite screening a large number of human and mouse tissues, GPVI expression was detected only in megakaryocytes and platelets. This result strongly suggests that GPVI is restricted to this hematopoietic lineage.

Identification of recombinant GPVI

Cvx, a very high-affinity specific ligand of GPVI, was used to detect the expression of recombinant GPVI. The CHO cells transfected with the empty vector or the vector containing human T268 cDNA were tested for ^{125}I-Cvx binding in ligand blotting experiments. ^{125}I-Cvx clearly labeled a single band in CHO cells only transfected with the vector containing human T268 cDNA (Figure 4), which strongly suggested that recombinant GPVI is encoded by T268. The migration of the hypothetical recombinant GPVI is slightly faster than that of platelet GPVI (approximately 54 kd vs approximately 58 kd) but very close to that of megakaryocyte GPVI (approximately 55 kd). GPVI production by CHO cells was confirmed by Western blot with human anti-GPVI antibodies (Figure 4B). In platelets, a 58-kd band corresponding to GPVI and a high molecular weight band corresponding to platelet IgG revealed by protein A were observed. Several proteins were nonspecifically labeled in CHO, but a clear 54-kd band was observed exclusively in lysats from T268-transfected CHO. Proof that Cvx and anti-GPVI bind to the same protein is provided by the observation that Cvx inhibits binding of anti-GPVI to recombinant GPVI as well as to platelet GPVI (Figure 4C), indicating that anti-GPVI and Cvx compete for binding to the corresponding protein. Together, these results demonstrate that the protein encoded by T268 is recombinant GPVI. The difference between the molecular mass of platelet GPVI and recombinant GPVI is most probably due to an incomplete or differential processing of the protein in CHO cells compared with platelets or megakaryocytes.

Functional studies

Flow cytometry. To determine whether recombinant GPVI was expressed at the cell surface, different human or murine hematopoietic cell lines were transduced with recombinant retroviruses expressing human or murine GPVI and with the control retrovirus. We observed that the cell lines used for this study express FcR-γ chain, as indicated by immunoblotting studies using a polyclonal anti–FcR-γ antibody (data not shown). The transduced cells were analyzed by flow cytometry using FITC-conjugated Cvx. As a control, we used FITC-conjugated bothrojaracin, another snake venom protein that is structurally very close to Cvx but is a pure

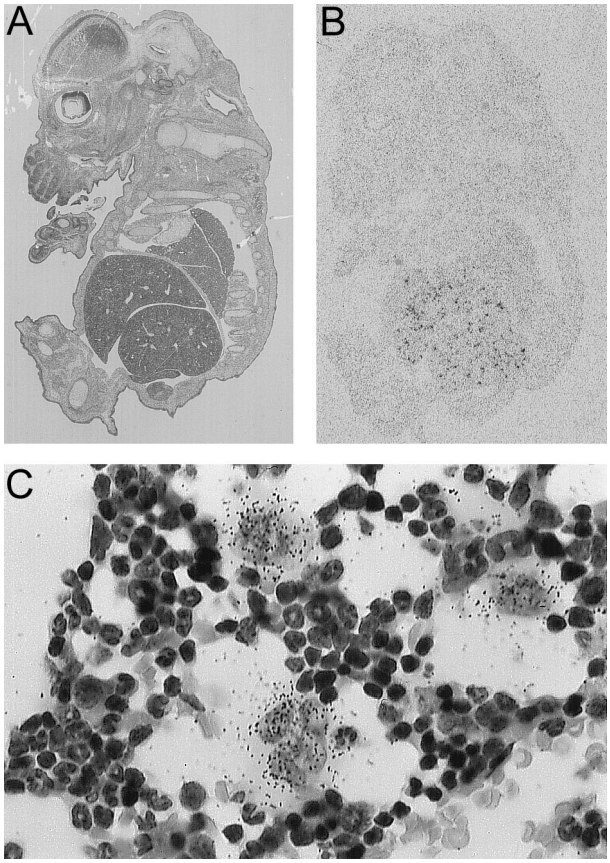

Figure 3. Tissue expression mouse GPVI using ISH analysis. (A) Day-12.5 mouse embryo stained with hematoxylin. (B) ISH of day-12.5 embryo using a GPVI probe. Hybridization is exclusively observed in the liver during embryogenesis. There was no signal seen with the sense probe (data not shown). High magnitude resolution shows that the only positive cell population corresponded to fetal megakaryocytes (data not shown). In adults, expression in the liver was no longer observed. (C) ISH of 6-week-old mouse femur with GPVI probe. Section shows expression restricted to megakaryocytes. Except for the spleen, there was no signal observed in any other adult tissues analyzed.

thrombin inhibitor that does not bind to platelets. Transduction of murine 32D cells with the retrovirus-expressing murine GPVI resulted in a strong Cvx-associated staining compared to cells transduced with the control virus, indicating that these transduced cells express GPVI at their surface (Figure 5). Similar results were obtained with FDC-P1 and Ba/F3 (all murine cell lines) (Figure 5)

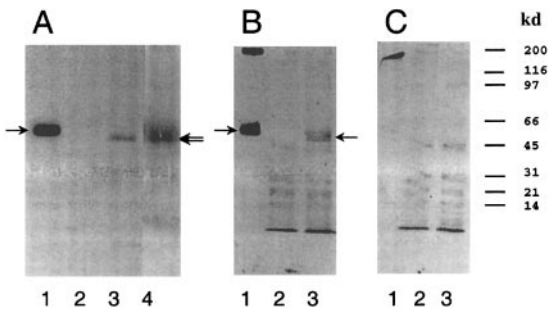

Figure 4. Identification of the protein encoded by human GPVI cDNA. Proteins from platelets (1) or proteins from CHO cells transfected with an empty vector (2), the same vector containing human T268 cDNA (3), or the same vector containing human megakaryocytes (4) were separated by SDS-PAGE and blotted on PVDF membranes. The membranes were incubated (A) with ^{125}I-Cvx, (B) with patient polyclonal anti-GPVI IgG antibody alone,[8] or (C) in the presence of 0.5 μmol/L cold Cvx. ^{125}I-Cvx was revealed by autoradiography. Anti-GPVI IgG was detected with peroxidase-coupled protein A and enhanced chemiluminescence.

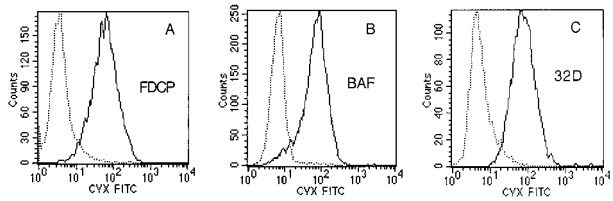

Figure 5. Binding of Cvx to murine hematopoietic cell lines. Hematopoietic cell lines were transduced with a retrovirus expressing murine GPVI (mGPVI). Control cells were transduced with the empty virus. The cells were incubated with FITC-coupled Cvx or FITC-coupled bothrojaracin as a control and analyzed by flow cytometry. (A) FDC-P1, (B) Ba/F3, and (C) 32D. Control cells transduced with the empty virus are indicated by the dotted line. The cells transduced with the retrovirus carrying mGPVI are indicated by the plain line.

and with K562 and U937 (human cell lines, data not shown). Cvx was also found to bind to the wild type HEL cells, but the binding was clearly increased after retroviral transduction indicated an increased expression in cells already constitutively expressing GPVI (data not shown).

Cell adhesion. Because GPVI appeared to be expressed at the cell surface of transduced cells, we tested its capacity to promote cell adhesion in a static system, to either immobilized Cvx or collagen, and then compared this to immobilized BSA. We tested 2 cell lines: U937 and FDC-P1. Neither the cells expressing GPVI nor the control cells bound to immobilized BSA. However, expression of recombinant human or mouse GPVI in U937 or FDCP-1, respectively, clearly promotes the adhesion of these cells to immobilized collagen and to a greater extent to immobilized Cvx (Figure 6).

Association of recombinant GPVI with FcR-γ chain

To analyze whether recombinant GPVI was associated with the FcR-γ chain, we performed immunoprecipitation studies with an anti–FcR-γ polyclonal antibody on GPVI-transduced U937 cell

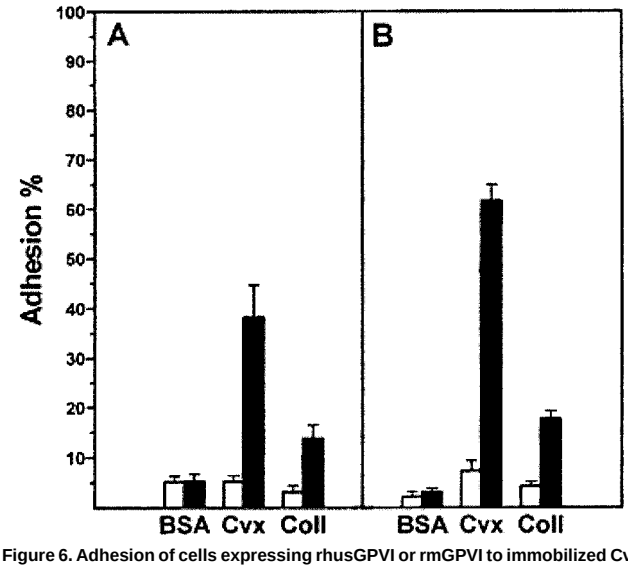

Figure 6. Adhesion of cells expressing rhusGPVI or rmGPVI to immobilized Cvx or collagen. BSA, Cvx, or collagen type I (Coll) were immobilized on microtitration plates. (A) rhuGPVI transduced or control U937 cells or (B) rmGPVI transduced or control FDC-P1 cells were labeled with ^{51}Cr and incubated for 60 minutes in the wells. After aspiration of the nonbound cells and washing, radioactivity associated to the wells was counted to determine the adherent cell number. The results are expressed as the percentage of the cells added to the wells and are the mean plus or minus SEM of 3 values. The control cells are indicated by the open bars, and the GPVI-expressing cells are indicated by the darkened bars.

lysates. Figure 7 shows the precipitated proteins analyzed by immunoblotting with a mixture of anti–FcR-γ and anti-GPVI antibodies. We observed 3 bands in all samples: a high molecular weight band corresponding to IgG, an approximately 50-kd nonidentified band, and a 14-kd doublet corresponding to the FcR-γ chain. In addition, 1 band corresponding to GPVI is present in platelets and also in U937 transduced with the GPVI virus, but it is not present in U937 transduced with the control virus. This indicates that recombinant GPVI is associated with the FcR-γ chain.

Inhibition of collagen and Cvx-induced platelet activation by rhusGPVI:Fc

We produced and purified the rhusGPVI:Fc protein to investigate its ability to compete with membrane-bound platelet GPVI. The RhusGPVI:Fc protein is composed of 2 amino acid fragments (1-269) of GPVI linked by their C-terminus to a human IgG1 Fc domain, has a molecular mass of 75-80 kd, and is divalent. The rhusGPVI:Fc protein did not induce platelet aggregation or granule secretion alone. When 0.25-5 μg/mL rhusGPVI:Fc was incubated with Cvx prior to the addition of platelets, aggregation and dense granule secretion was fully inhibited (Figure 8A). In addition, when 1 μg/mL rhusGPVI:Fc was added to the platelet suspension prior to Cvx, it also inhibited aggregation and secretion, indicating that it could compete with membrane-bound platelet GPVI for Cvx (Figure 8A).

When the agonist was collagen, incubation of rhusGPVI:Fc with collagen prior the addition of platelets inhibited platelet aggregation and secretion (Figure 8B). This inhibition was dose-dependent, with a 10-fold higher concentration of rhusGPVI:Fc (2.5 μg/mL) needed to reach a full inhibitory effect than that required for Cvx. Furthermore, when up to 5 μg rhusGPVI:Fc was added to platelets prior to the collagen, no inhibition was observed (Figure 8B).

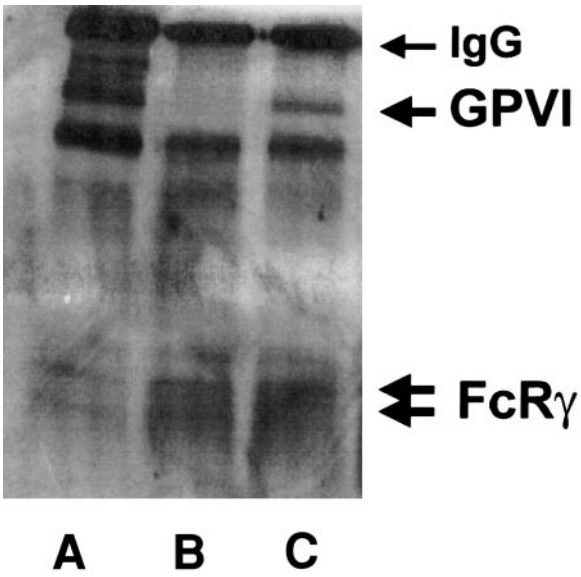

Figure 7. Coexpression of rhusGPVI with FcR-γ chain. Lysates from (A) platelets, (B) control, or (C) GPVI-transduced U937 cells were incubated with a polyclonal anti–FcR-γ antibody and protein A–sepharose. Immunoprecipitated proteins were separated by SDS-PAGE and blotted on a PVDF membrane. The membrane was incubated with a mixture of anti–FcR-γ and anti-GPVI antibodies revealed with peroxydase-coupled protein A and chemiluminescence.

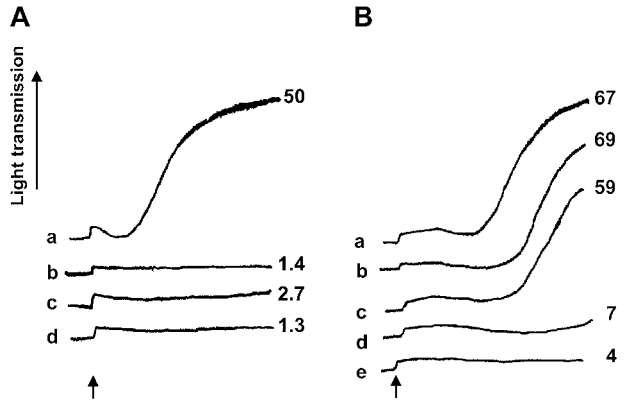

Figure 8. Inhibition Cvx- or collagen-induced platelet activation by rhusGPVI:Fc. (A) Trace a: platelets were activated by 100 pmol/L Cvx; trace b: platelet suspension was incubated with 1 μg GPVI:Fc for 2 minutes before the addition of Cvx; traces c and d: Cvx was preincubated with 0.25 μg and 0.5 μg GPVI:Fc for 2 minutes, respectively, before the addition to platelets. (B) Trace a: platelets were activated by collagen type I; trace b: platelets were preincubated with 5 μg GPVI:Fc for 2 minutes before the addition of collagen; traces c to e: collagen was preincubated with 1 μg, 2.5 μg, and 5 μg GPVI:Fc, respectively, for 2 minutes before the addition to platelets. ^{14}C 5-HT–labeled washed platelets were used. The percentage of ^{14}C 5-HT release measured in each condition is indicated.

Discussion

GPVI has long been recognized as an important receptor for platelet function. Identification of its structure and characterization of its expression are important steps in clarifying its role in thrombosis. Based on the hypothesis that GPVI should share structural similarities with the Fc receptors coupled to the γ-chain, we identified human and mouse GPVI from megakaryocytic cDNA libraries.

As predicted, GPVI belongs to the Ig superfamily of cell surface molecules. It is a type I membrane protein containing 2 extracellular Ig-like domains. The closest relatives of GPVI in the public databases are the growing family of inhibitory receptors, although this relationship is due solely to similarities in the extracellular domain. The sequence of our human clone is identical to that recently reported by Clemetson et al.[22] The homology between mouse and human GPVI is approximately 64%. This homology is spread throughout the molecule, but is slightly higher (78%) in the Ig-like domains. Interestingly, both human and mouse GPVI contain conserved variants of the WSXWS box (residues 97-101 and 192-196). This motif is a signature of class I hematopoietic receptors, but variants are also found in the sequences of all killer-cell inhibitory receptors (KIRs).[41] These motifs have been shown to contribute to tertiary folding. GPVI has a relatively short cytoplasmic tail with no obvious signaling motifs analogous to the ITAMs and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of other signaling receptors. GPVI has a positively charged residue in the transmembrane domain that should allow it to form complexes with the FcR-γ chain, which acts as signaling subunit.[12] Indeed, the Fc-α/RI-α chain, which shows structural homology to GPVI, also contains a positively charged residue in its transmembrane domain that has been shown to be essential for its association with the FcR-γ chain.[20]

Human GPVI was mapped to 19q13.32-q13.33. Multiple members of the Ig superfamily, including KIRs, Ig-like transcripts (ILT-1, -2, and -3), the gp49b family, and FcR-α (CD89), also map to this region. These various receptors differ considerably with respect to function and expression, and it may be hypothesized that

functional differentiation occurred after duplication of a common ancestral gene.

The tissue distribution of GPVI is currently poorly defined. To determine the expression pattern of GPVI, we examined an extensive panel of human and mouse tissues. The only cells where we could detect a significant amount of GPVI RNA were megakaryocytes and platelets. Furthermore, by searching internal and public gene databases, the only cDNA sources that were found were various Millennium megakaryocyte libraries. These results strongly suggest that the expression of GPVI is highly specific to the megakaryocytic lineage. It remains to be determined if GPVI expression is regulated throughout megakaryopoiesis. Presently, there are very few molecules that are specific to the megakaryocyte lineage. GPIIb (integrin α-IIb), which was long considered to be the prototypic megakaryocyte marker, is also expressed on a subset of hematopoietic progenitors.[42-44] Other megakaryocyte proteins, such as GPIb-α, GPI-β, and GPIX (CD42) are also expressed by activated endothelial cells.[45] Only GPV and PF4 appear to be specific to the megakaryocyte/platelet lineage.[46] For this reason the PF4 promoter has been used to target the megakaryocytes in various transgenic models.[47] However, it is not clear that the PF4 promoter allows expression of the transgene in early megakaryocyte progenitors. It would be of interest to further characterize the specificity and stage of expression of GPVI and to analyze its promoter.

Functional characterization of recombinant GPVI was performed using transfected cells that have either no levels of endogenous GPVI (U937 and FDC-P1) or low levels (HEL). In contrast to the work described by Clemetson et al,[22] who used DAMI cells that expressed GPVI messenger RNA, this characterization allowed us to study GPVI functional properties independently of endogenous GPVI. Our extensive analysis showed that the recombinant protein GPVI was based on binding to Cvx, binding to collagen, binding of anti-GPVI IgG isolated from a patient, and association to the FcR-γ chain. We also demonstrate that the extracellular domain of GPVI is active in blocking Cvx- and collagen-induced platelet aggregation.

Cvx, a snake venom protein, has been shown to bind specifically to platelet GPVI.[10,11,17,39] In ligand binding studies we demonstrated that Cvx bound to CHO cells that transiently expressed the cloned genes. The observed molecular weight of GPVI in these cells was lower than that attributed to GPVI in platelets but similar to that observed in primary megakaryocytes. This difference in migration may be due to differences in glycosylation, and it is possible that this is dependent on the stage of cell differentiation. Indeed, GPVI appears to be heavily glycosylated. The predicted molecular weight based on the cDNA sequence is around 38 kd, but when the molecular weight is deduced from SDS-PAGE under nonreducing conditions, the molecular weight is 54 kd in CHO cells and 58 kd in platelets. This is consistent with glycosylation and accounts for approximately one-third of the mass of the protein. Human GPVI has only 1 N-linked glycosylation site, which appears to account for only 3 kd (data not shown). It is likely that the majority of glycosylation is due to O-linked carbohydrates on the large clusters of serine and threonine residues present in the extracellular domain.

Using ligand-binding fluorescence analysis, we showed that Cvx binds to the human recombinant protein in U937 and K562 cells and to the mouse recombinant protein in FDC-P1, 32D, and Ba/F3. It is known that Cvx recognized mouse GPVI from previous studies showing that Cvx is a potent platelet activator of both human and mouse platelets.[17] The expression of recombinant GPVI at the cell surface may have been facilitated by the coexpression of the FcR-γ chain in these cells. It has been previously shown that expression of the FcR-γ chain is required for surface expression of Fc-γ/RIII and Fc-ϵ/RI[48] and for activation of platelets by collagen.[12] However, in the case of Fc-α/RI association to the FcR-γ chain is required for receptor function but not for cell surface expression.[49] The coprecipitation of the FcR-γ chain and recombinant GPVI in transduced U937 cells demonstrates the physical association between these 2 proteins. As with Fc-α/RI,[20] the linkage probably involves charged residues within the transmembrane domain: R273 or R270, respectively, for hGPVI and mGPVI and D11 in the FcR-γ chain. Our preliminary results, obtained in CHO cells, suggest that GPVI can be expressed at the cell surface in the absence of the FcR-γ chain, as was demonstrated for Fc-α/RI (data not shown).

The importance of GPVI in collagen-induced platelet activation was discovered following the characterization of a patient suffering from immune thrombocytopenic purpura.[8] This patient developed self-antibodies that recognized GPVI and induced platelet aggregation. In contrast, Fab fragments do not induce platelet activation and instead inhibit the aggregation induced by collagen. In this paper we show that antibodies from this patient recognize the protein in CHO cells expressing our clone. Interestingly, Cvx inhibited the binding of anti-GPVI antibodies to the recombinant protein, as previously observed on platelets.

GPVI, despite its essential role in collagen-induced platelet aggregation, is described as having a minor role in platelet adhesion to collagen. Other receptors, such as the GPIb-IX-V complex or the integrin α2β1, are major players responsible for platelet adhesion to collagen. However, we have previously shown that immobilized Cvx is able to induce platelet adhesion, which indicates that GPVI may be involved in adhesion in static conditions.[10] In this study, we demonstrated that expression of GPVI in U937 and FDCP-1 cells induces cell adhesion to a collagen- or Cvx-coated surface. The number of cells that bound to immobilized Cvx was significantly higher than those bound to collagen. This probably reflects differences in the density of GPVI binding sites on the 2 surfaces. Cvx is a pure GPVI ligand and, when immobilized, produces a highly reactive surface, while GPVI binding sites should be disseminated on collagen fibers, thereby resulting in a less reactive surface. Nevertheless these results indicate that recombinant GPVI mimics the physiological function of platelet GPVI (ie, binding to collagen).

The difference in reactivity between collagen and Cvx is further emphasized by the differences in the inhibitory effect that the soluble GPVI protein has on collagen- and Cvx-induced platelet activation. Indeed, soluble GPVI inhibits Cvx-induced platelet activation in the absence of preincubation with Cvx, while it requires a preincubation with collagen to inhibit collagen-induced platelet activation. This probably reflects the rapid kinetics of interaction between GPVI and Cvx compared to those between GPVI and collagen. The affinity of soluble GPVI for Cvx is probably very high for 2 reasons: (1) soluble GPVI is expressed in a divalent Fc fusion form, and (2) Cvx is multivalent due to its trimeric α3β3 structure.[50] This suggests that GPVI binding sites on collagen fibers are probably dispersed and poorly accessible. Alternatively, these observations could also suggest that the binding of collagen to its other receptors, including the integrin α2β1, promotes its subsequent interaction with GPVI.

GPVI may play an important role in the development of thrombi because it is the receptor that appears to govern platelet activation at the contact of collagen and thus induces platelet recruitment.

Indeed, patients with GPVI deficiency, whether it was associated or not associated with an anti-GPVI antibody, displayed bleeding disorders.[7,8] The molecular cloning of GPVI provides the opportunity to characterize the mechanism of these deficiencies, not only the precise interaction between GPVI and the integrin $\alpha 2\beta 1$ in collagen-induced platelet activation, but also the role of GPVI in thromboembolic diseases. GPIIb-IIIa (integrin $\alpha IIb\beta 3$) and the ADP receptor are platelet receptors against which efficient antagonists have so far been developed.[51,52] The mode of action of thienopyridines is indirect because they require conversion to unidentified metabolites that are noncompetitive inhibitors of ADP. Their established antithrombotic effect is probably nonspecific and related to the inhibition of platelet recruitment by ADP upon platelet activation by subaggregatory concentrations of agonists.

Even if GPIIb-IIIa were involved in platelet adhesion, its principal role is to bind fibrinogen, thereby allowing platelet aggregation and serving as the final common pathway of platelet thrombus formation regardless of the metabolic pathway initiating platelet activation. In contrast, GPVI is involved in an early step of platelet activation occurring immediately when platelets contact the subendothelial matrix. GPVI may represent an alternative and more specific target for new antithrombotic compounds. The antagonist can be directed against either of the 2 players, ie, collagen GPVI binding sites or GPVI itself. Because our observations suggest that the GPVI binding sites are not easily accessible on collagen fibers, one might speculate that an antagonist directed against GPVI may be more efficient than an antagonist directed against collagen.

Altogether, our results provide evidence that a new member of the Ig superfamily is platelet GPVI, a collagen receptor specific for the megakaryocyte lineage. GPVI characterization allows us to get further insight into its role as collagen receptor, and forthcoming studies will explore its involvement in thrombotic disease, bleeding disorders, and megakaryocyte maturation.

Acknowledgments

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