We have cloned the platelet collagen receptor glycoprotein (GP) VI from a human bone marrow cDNA library using rapid amplification of cDNA ends with platelet mRNA to complete the 5’ end sequence. GPVI was isolated from platelets using affinity chromatography on the snake C-type lectin, convulxin, as a critical step. Internal peptide sequences were obtained, and degenerate primers were designed to amplify a fragment of the GPVI cDNA, which was then used as a probe to screen the library. Purified GPVI, as well as Fab fragments of polyclonal antibodies made against the receptor, inhibited collagen-induced platelet aggregation. The GPVI receptor cDNA has an open reading frame of 1017 base pairs coding for a protein of 339 amino acids including a putative 23-amino acid signal sequence and a 19-amino acid transmembrane domain between residues 247 and 265. GPVI belongs to the immunoglobulin superfamily, and its sequence is closely related to FcαR and to the natural killer receptors. Its extracellular chain has two Ig-C2-like domains formed by disulfide bridges. An arginine residue is found in position 3 of the transmembrane portion, which should permit association with Fcγ and its immunoreceptor tyrosine-based activation motif via a salt bridge. With 51 amino acids, the cytoplasmic tail is relatively long and shows little homology to the C-terminal part of the other family members. The ability of the cloned GPVI cDNA to code for a functional platelet collagen receptor was demonstrated in the megakaryocytic cell line Dami. Dami cells transfected with GPVI cDNA mobilized intracellular Ca2+ in response to collagen, unlike the nontransfected or mock transfected Dami cells, which do not respond to collagen.

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 hemostatic 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 including CD36 (1) and a p65 collagen type I specific receptor (2), but the major ones are now considered to be the integrin αβ1 and the nonintegrin glycoprotein (GP)1 IV. Although α2β1 is well characterized and both subunits were cloned and sequenced several years ago (3, 4), the structure of GPVI has remained elusive; however, several features have been identified. It was determined about 20 years ago that GPVI is a major platelet glycoprotein with a molecular mass in the 60–65-kDa range and an acidic pl (5). Its role as a putative collagen receptor was established following the identification of a patient in Japan with a mild bleeding disorder whose platelets had a specific defect in response to collagen and lacked this receptor (6). This patient had also developed autoantibodies to the deficient receptor, and these were used to characterize the molecule further (7). More recently it was established that GPVI is associated noncovalently with the common Fcγ subunit, which acts as the signaling part of the complex (8, 9). 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 (10). The GPVI-Fcγ complex was shown to signal to the platelet interior by an immune receptor-like mechanism, involving activation of p72SYK and leading by a cascade of kinase-phosphatase-adaptor protein interactions to activation of PLCγ2 and hence to release of granule contents and to platelet aggregation (11). A further step in characterization 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 (12, 13). Convulxin was shown to bind specifically to GPVI, providing a tool for purification of this receptor in conjunction with established approaches. Using this method we have now purified amounts of GPVI adequate for preliminary characterization and for peptide sequencing. The sequences were used to design primers for PCR to identify a positive sequence in a cDNA library. This DNA sequence was then used as a probe to isolate an almost complete cDNA sequence from the library, and the missing 5′-sequence was obtained using a RACE method on platelet RNA.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose, peroxidase-conjugated goat anti-rabbit antibodies, bovine serum albumin, C. durissus terrificus venom,

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wheat germ agglutinin, N-hydroxysuccinimidyl chloroformate-activated cross-linked 4% beaded agarose, Triton X-114, puromycin, and fura 2-AM were from Sigma. Octyl-N-methylglucamide and nonanoyl-N-methylglucamide were from Oxyl Chemie (Böbingen, Germany). Convulxin was isolated from C. durissus terrificus venom as described previously (14) in the presence of protease inhibitors. The final human bone marrow library (CLONTECH, Palo Alto, CA) was amplified into the expression vector SRα (provided by Dr. F. Arenzana-Seisdedos, Pasteur Institute, Paris, France) (17) as a BamHI fragment. For the murine 300-19 cells, 20 μg of linearized vector was transfected by electroporation, and transfectants were grown in the presence of 1.5 μg/ml puromycin and screened by flow cytometry with fluorescein isothiocyanate-labeled convulxin. The cells were tested with agonists for elevation of intracellular Ca2+, using fura-2 fluorescence.

Transfection of Dami Cells with cDNA Coding for GPVI—The SRα/puroGPVI construct described above was used for transfection into Dami cells. In addition, the GPVI cDNA was introduced in the pcDNA3 vector (Invitrogen, Groningen, The Netherlands). Transfection was performed with Effectene reagent (Qiagen AG, Basel, Switzerland). Dami cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal calf serum (Biological Industries, Kibbutz Beitz Haeme, Israel) and 1% penicillin/streptomycin. 1 day before transfection, the cells in suspension were centrifuged and resuspended at 10^6 cells/ml in a new bottle. 1 h before transfection, the cells were seeded at 1 × 10^5 cells/ml in 5.4 ml of RPMI medium, without serum in Petri dishes. pcDNA3/GPVI DNA (2 μg), SRα/puroGPVI DNA (2 μg), or empty vectors, transfection reagents, and RPMI without serum were mixed and added to the cells following the supplier's instructions. After 36 h, the cells were harvested and tested for their response to agonists.

Measurement of Cytoplasmatic Ca2+ [Ca2+]i, in Transfected and Mock Transfected Dami Cells after Addition of Agonists—Nontransfected and transfected Dami cells were harvested 36 h after transfection and washed with 20 μM Hepes, pH 7.4, 4.8 μM KCl, 136 mM NaCl, 5 mM glucose, and 4 mM CaCl2, and 105 cells were resuspended in 400 μl of the same buffer and incubated with 2.5 μM fura-2AM for 30 min at 37 °C. Agonist was added to 2.5 × 105 cells, and fura-2 fluorescence was measured.

RESULTS
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 (14). Because GPVI represents a very small fraction of the platelet membrane glycoprotein pool, the specificity of the snake C-type lectin convulxin was used for isolation of this receptor. Affinity chromatography on convulxin coupled to agarose yielded as major components 65- and 60-kDa proteins corresponding respectively to intact GPVI and its proteolytic fragment (Fig. 1, A and B). Minor amounts of uncharacterized material of higher molecular mass, which co-eluted with GPVI, could not be removed by extensive washing of the column. Therefore, preparative gel electrophoresis on 8.5% polyacrylamide gel was used as a final step of purification. Fractions containing the 65-kDa band of GPVI were pooled and gave a single band on reanalysis (Fig. 1C). Purified GPVI was tested for its ability to block platelet aggregation by collagen. The GPVI was dissolved in 10 mM Tris/HCl containing 0.1% octyl-N-methylglucamide, which is a very mild detergent and did not affect either the ability of collagen to activate platelets (data not shown) or platelets to be activated...
by agonists. A slight inhibitory effect was observed when aliquots (0.5 μg) of GPVI solution were added to the platelet suspension before adding collagen (1 μg) (Fig. 2). However, when GPVI was preincubated with collagen before adding the mixture to the platelet suspension, aggregation was inhibited in a dose-dependent manner. These platelets still aggregated when fresh collagen was added (not shown), indicating that the GPVI had bound to the first collagen. The purified GPVI was used to prepare in rabbits polyclonal antibodies that recognized GPVI had bound to the first collagen. The purified GPVI was used to prepare in rabbits polyclonal antibodies that recognized GPVI. Detection was by chemiluminescence using a peroxidase-linked second antibody.

Two sequences of 7 amino acids showing the least degeneracy in the genetic code were selected for the synthesis of DNA primers to amplify part of the GPVI cDNA by PCR. Because the positions 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'-TYATHCCNGCNATGAARMG-3' primer coding for the sequence PAMKRSL with the antisense 5'-TTRTANARNGCAAYTGRTC-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 DQFALYK. The amplified DNA was digested with the restriction enzymes SauI or EcoRI and belonged to the IgG superfamily. The fourth one had a 4.6-kb insert by SauI digestion and gave two fragments of 2300 and 1300 bp, respectively when treated by EcoRI. Its DNA encoded protein sequence including the sequences of the 10 peptides derived from amino acid sequencing of GPVI but stopped short of the N terminus. No starting methionine or leader sequence could be found, but more than 2000 bp of previously sequenced (on data base) nonreading 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 348 bp including 58 bp new on the sequence of the fourth clone at bp 1987 corresponding to 14 amino acids including the first methionine were found before falling back on
the established GPVI sequence. The SalI DNA insert was isolated from the fourth clone. A 1700-bp fragment was generated by SstI and ligated with the DNA amplified by RACE. Thus, a cDNA containing a total of 2001 bp together with the complete 3′-untranslated region and with an open reading frame of 1017 bp coding for a protein including leader sequence with 339 amino acids could be sequenced. All the amino acid sequences from the peptides were present and are shaded in the translated cDNA sequence (Figs. 3 and 4).

In a first approach, the cloned GPVI collagen receptor was expressed in the murine pre-B cell line 300-19. Stable transfectants, which expressed GPVI on their surface, were obtained. However, neither elevation of cytoplasmic Ca\(^{2+}\) nor the activation of a kinase cascade could be detected when the cells were treated with collagen or convulxin. Then Dami cells were chosen because they synthesized a very small amount of mRNA for GPVI, which was detectable using reverse transcription PCR with GPVI-specific primers. Flow cytometry using the rabbit polyclonal anti-GPVI antibodies or fluorescein isothiocyanate-labeled convulxin revealed traces of the glycoprotein on the cell surface. Furthermore, a weak increase of \([\text{Ca}^{2+}]\) was observed when the cells were treated with convulxin but was never detected with collagen as agonist. Transfection was performed with both vectors pcDNA3/GPVIDNA and SR\textalpha\textgamma puro/GPVIDNA. As expected, in relation to the strength of the promoter, the cells transfected with SR\textalpha\textgamma puro gave a stronger signal than those transfected with pcDNA3. The \([\text{Ca}^{2+}]\) elevation induced by thrombin was used as a standard reference between transfected, mock transfected and nontransfected cells. As shown in Fig. 5 the transfected Dami cells responded to collagen (Fig. 5A), whereas the mock transfected cells, like the nontransfected cells did not (Fig. 5B). The \([\text{Ca}^{2+}]\) elevation elicited by convulxin (0.1 μg/ml)
in the transfected cells was more than twice that in the non-transfected ones and comparable with the signal induced by 1 unit of thrombin. Addition of saturating amounts of anti-GPVI IgG to the transfected cells before collagen totally inhibited the increase of cytoplasmic Ca^{2+} by this agonist. The results shown here are typical of those with cells from three transfection experiments with this amount of DNA.

**DISCUSSION**

A method was developed for the isolation of the platelet GPVI collagen receptor from platelets by affinity chromatography and preparative gel electrophoresis. The isolated protein was used to produce polyclonal antibodies and was also tested for its effects on collagen-induced platelet aggregation. When GPVI was added to a platelet suspension followed by collagen it had only a minor effect on the rate of aggregation. However, when GPVI was incubated together with collagen and the mixture was then added to platelets, there was a dose-dependent reduction in the platelet response compared with that induced by collagen incubated with buffer. This demonstrates that the soluble GPVI binds to and blocks the GPVI-binding sites on collagen preventing the collagen from interacting with GPVI on platelets and thereby activating them. The IgG from polyclonal antibodies to GPVI as well as F(ab')2 fragments strongly activated platelets, as previously reported for human alloantibodies to GPVI (7). Fab fragments of these antibodies were capable of inhibiting collagen-induced platelet aggregation when they were preincubated with the platelets, again demonstrating that GPVI collagen interactions are necessary for platelet aggregation by collagen (Fig. 2). 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 cDNA contains an open reading frame of 1017 bp encoding 339 amino acids and an untranslated 3’ region. Hydrophobicity analysis of the amino acid sequence revealed the presence of two putative hydrophobic domains, a putative 23-amino acid signal sequence and a 19-amino acid transmembrane domain between residues 247 and 265 of the mature protein. The sequence and its amino acid translation are shown in Fig. 3. A comparison with the amino acid sequence of the most similar molecules found in a search of SwissProt is shown in Fig. 4. GPVI clearly belongs to the immunoglobulin superfamily, and the extracellular domain contains two Ig C2-domain loops formed by two disulfide bridges. It is a transmembrane class one molecule with the N terminus at the exterior and traverses the membrane once. The most closely related molecules in the SwissProt data base are the Fc receptor, a mouse mast cell receptor, and both inhibitory and activatory members of the natural killer (NK) receptor class. GPVI clearly belongs to the activatory subclass not only through its function but also because unlike the inhibitory class it has no immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic domain. Nor does it contain any tyrosine residues that could be phosphorylated. There are some threonine and serine residues in this C-terminal region, but they do not match any kinase consensus sequences. Like the Fc receptor and some of the NK activatory class of receptors, GPVI contains a charged amino acid in the transmembrane domain. An arginine residue in position 3 of the transmembrane domain is postulated to form a salt bridge to an aspartic acid residue in the transmembrane domain of the FcY subunit, which, expressing immunoreceptor tyrosine-based activation motifs, initiates signal transduction upon phosphorylation. The cytoplasmic tail comprises 51 amino acids, showing only minor similarities (in the region just below the membrane) to the cytoplasmic domains of other members of this family. This suggests that this region in GPVI may associate with different types of cytoplasmic molecules than the other family members. GPVI contains only a single putative N-glycosylation site at Asn^9. However, there is a short sequence just above the membrane, before the β sheets of the Ig domain finish, that contains a mucin-like region rich in threonine and serine residues. These could provide O-glycosylation sites such as are found in GPIbα and GPV. The main function of this O-glycosylation in the other receptors seems to be to extend the receptor structures out from the platelet surface to facilitate the interactions with their bulky ligands and to protect against proteolysis. Because GPVI was earlier established to be 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.

GPVI is therefore the third platelet receptor with no direct immune function belonging to the immunoglobulin superfamily together with ICAM-2 (CD102) and PECAM (CD31). To provide the evidence that the cloned GPVI is really the collagen receptor expressed on platelets, the cDNA was transfected into cell lines. Surface expression might be expected to modify the collagen binding properties of the transfected cell. However, to be functional and transduce a signal after binding collagen not only does GPVI have to be present but also the necessary signal transducing molecules, including Fcy, which is needed to link GPVI to several pathways (8). As a first trial we used a mouse pre-B cell line that has been widely described in expression and Ca^{2+} mobilization studies and that should contain many of the signaling molecules required. Although this cell line when transfected with GPVI cDNA expressed this receptor as detected by anti-GPVI antibodies, no Ca^{2+} signal was measured after activation with collagen or convulxin. As an alternative we therefore selected the Dami cell line, which is a well-differentiated megakaryocyte-like line that responds to several platelet agonists. In particular Dami has been shown to express α2β1 but does not show a Ca^{2+} transient in response to collagen, suggesting that collagen receptors or signaling molecules are deficient (18). Several authors found that Dami adheres to collagen, indicating that the problem might lie with GPVI (18, 19). However, we found that Dami responded weakly to convulxin. In addition, traces of GPVI mRNA were detected by reverse transcription PCR. When transfected with GPVI cDNA, the Dami line showed a marked increase in response to convulxin and now gave a clear response to collagen, indicating that the signaling machinery was present and capable of transducing signals from the transfected GPVI. Presumably the traces of GPVI present in the untransfected cells were not enough for a detectable response from collagen, which is a weaker agonist for GPVI than convulxin. Although the GPVI-Fcy complex resembles immune cell receptors and their signaling pathways have much in common, not all the molecules involved have been identified. Therefore it is possible that nonmegakaryocytic cells lack critical components. Signaling by the transfected Dami cells in response to collagen was completely blocked by antibodies to GPVI, again demonstrating that, as in platelets (7, 20), blocking one receptor is sufficient to prevent collagen-induced activation.

The structure of NK receptors of the two domain type has been established by x-ray crystallographic studies (21), and the two Ig-like domains were shown to form an angle with the receptor site for the peptide-carrying HLA antigens lying on the outside of the elbow. A comparison of the structure of the HLA peptide-binding site with that of collagen immediately suggests that these receptors have a common origin because the multiple α-helical structures of the HLA-binding site and the peptide it contains strongly resemble the triple helical structure of collagen. The sites recognized by the NK receptor
The Platelet Collagen Receptor GPVI

include the parts of the HLA structure binding the peptide. The NK receptors are postulated to work by a dimerization mechanism with two receptors recognizing two separate HLA sites on the cell with which the NK cell interacts. Possibly this dimerization is part of the activation or deactivation mechanism, depending on the class of receptor. In the case of GPVI, cross-linking of two or more molecules by collagen may also be the critical mechanism. All of the signaling subunits of these types of receptors whether Fcγ, Δ or the activating NK receptor subunit DAP12 are dimers, suggesting that they may be associated with two ligand-binding subunits, although the stoichiometry is not known. Thus, there may also be the possibility for two GPVI molecules to associate with one Fcγ, because each monomer of the Fcγ dimer has a recognition sequence. 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-Fcγ complexes that are clustered together.

Recent research has emphasized that polymorphisms in platelet collagen receptor α2β1 that cause differences in the level of expression of this receptor may have an important role in susceptibility to cardiovascular problems (22, 23). It was earlier shown that platelets lacking GPVI do not adhere to collagen, although they express α2β1 normally (6). While much remains to be learned about the mechanisms involved, this suggests that synergy between these receptors is critical for the platelet response to this agonist. Thus, not only α2β1 but also GPVI should be considered an attractive target for controlling platelet reactivity as an alternative strategy to GPIIb-IIIα inhibitors. The cloning and sequencing of GPVI should provide the basis for a detailed analysis of its role in platelet response to various types of collagen as well as the molecular mechanism of its interaction with collagen. In addition, the purification of GPVI and the possibility of preparing the recombinant molecule should activate the search for pharmacologically effective inhibitors as an approach to the prevention and treatment of cardiovascular disorders.

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