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Identification of Residues within Human Glycoprotein VI Involved in the Binding to Collagen

EVIDENCE FOR THE EXISTENCE OF DISTINCT BINDING SITES*

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Glycoprotein VI (GPVI) has a crucial role in platelet responses to collagen. Still, little is known about its interaction with its ligands. In binding assays using soluble or cell-expressed human GPVI, we observed that (i) collagen, and the GPVI-specific ligands collagen-related peptides (CRP) and convulxin, competed with one another for the binding to GPVI and (ii) monoclonal antibodies directed against the extracellular part of the human receptor displayed selective inhibitory properties on GPVI interaction with its ligands. Monoclonal antibody 9E18 strongly reduced the binding of GPVI to collagen/CRP, 3F8 inhibited its interaction with convulxin, whereas 9O12 prevented all three interactions. These observations suggest that ligand-binding sites are distinct, exhibiting specific features but at the same time also sharing some common residues participating in the recognition of these ligands. The epitope of 9O12 was mapped by phage display, along with molecular modeling of human GPVI, which allowed the identification of residues within GPVI potentially involved in ligand recognition. Site-directed mutagenesis revealed that valine 34 and leucine 36 are critical for GPVI interaction with collagen and CRP. The loop might thus be part of a collagen/CRP-binding site.

Platelet interaction with a damaged vessel wall initiates thrombus formation. Collagen is the main component of the subendothelial matrix allowing platelet adhesion and activation. Several collagen receptors are involved in this process (1). Among them, glycoprotein VI (GPVI)[1] plays a crucial role in platelet responses to collagen. GPVI belongs to the immunoglobulin superfamily of receptors (2, 3) and is expressed at the platelet surface as a non-covalent complex with its signaling subunit, the common γ chain of immunoreceptors (FcRγ) (3, 4). In addition to fibrous collagen, two specific agonists are used to study GPVI function: the synthetic, triple-helical, collagen-related peptides (CRP) based on a repeated GPO motif (glycine-proline-hydroxyproline) (5) and convulxin, a multimeric, C-type lectin-like toxin from the venom of a rattlesnake (6, 7).

The signaling pathway of GPVI is analogous to that of immune receptors; it involves a cascade of Tyr phosphorylation by Src family kinases and the association of the adaptor protein LAT with phosphatidylinositol 3-kinase. It eventually leads to phospholipase $C\gamma_2$ activation, increase in intracellular calcium concentration, and platelet activation (8). GPVI participates in stable platelet adhesion to subendothelial collagen and is responsible for collagen-induced platelet activation, leading to platelet aggregation and anionic phospholipid exposure, thus providing a procoagulant surface for thrombin formation (9–12).

GPVI shares a high degree of homology with the low affinity IgA receptor FcαRI and killer cell inhibitory receptors (KIRs) (2). The genes encoding these receptors are located on the same chromosome (19q13.4) within the leukocyte receptor cluster (13). The extracellular (EC) domain of GPVI contains two Ig-like loops of the C2 type. The EC1 N-terminal loop is of the IgC2-1 type, as is the EC1 domain of FcαRI, whereas the GPVI EC2 loop is of the IgC2-2 subtype found in KIRs (13). The respective role of EC1, EC2, and the interdomain region varies from one receptor of the leukocyte receptor cluster family to another. The IgA-binding site has been shown to involve structures located in the EC1 domain of FcαRI (14). KIR family members bind their ligand HLA in the hinge, interdomain region (15), whereas FcεRI and FcγRII bind their ligand via their EC2 membrane proximal domain (16).

To locate ligand-binding site(s) on human GPVI, we have produced a panel of monoclonal antibodies (mAbs) directed against the extracellular part of human GPVI. Their ability to prevent GPVI interactions with collagen, CRP, and convulxin was measured in a binding assay using purified proteins and in a cell adhesion assay. The results indicate that the binding sites on GPVI for the diverse ligands seem to be distinct, exhibiting specific features but at the same time also sharing some common residues participating in the recognition of these ligands. Furthermore, we have previously characterized mAb 9O12 as a potent antagonist of GPVI function *in vitro* (17). Using phage display, we have identified its epitope within the GPVI structure. Site-directed mutagenesis has been performed on a soluble form of the receptor. The characterization of the GPVI mutants showed that a loop of the EC1 domain partici-

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[1] The abbreviations used are: GPVI, glycoprotein VI; rGPVI, recombinant human GPVI; rsGPVI, recombinant soluble human GPVI; mAb, monoclonal antibody; CRP, collagen-related peptides; KIR, killer cell inhibitory receptors; EC, extracellular; HRP, horseradish peroxidase; WT, wild type; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FcR, Fc receptor; LIR, leukocyte Ig-like receptors.

pates in the binding to collagen, with, in particular, Val-34 and, to a lesser extent, Leu-36 being critically involved in the interaction.

EXPERIMENTAL PROCEDURES

Materials

Prostaglandin E_1, grade VII apyrase, ortho-phenylenediamine dihydrochloride, para-nitrophenylphosphate disodium salt, horseradish peroxidase (HRP)-coupled streptavidin, and polyoxyethylenesorbitan monolaurate (Tween 20) were from Sigma, bovine serum albumin Cohn fraction V was from Research Organics Inc. (Cleveland, OH), and polyclonal anti-phage M13 antibody-HRP was from Amersham Biosciences. Goat anti-mouse-HRP was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Collagen type I from equine tendon (Horm collagen) came from Nycomed (Munich, Germany). Convulxin was purified from the venom of *Crotalus durrissus terrificus* (Latoxan, France) as described (6). CRP were a generous gift from Dr. R. Farndale (Cambridge, UK) (18).

The pentadecamer phage display peptide library was a kind gift of Dr. G. Smith (University of Missouri, Columbia, MO). Monoclonal antibodies were biotinylated using *N*-hydroxysuccinimide-LC-Biotin (Pierce) according to the manufacturer's instructions.

The human hematopoietic HEL cell line was engineered to express full-length human GPVI (rGPVI-HEL), as reported (3).

Anti-human GPVI Monoclonal Antibodies

The negative isotype-matched control antibody (7I20) and mAbs 9O12, 9E18, and 3F8, directed against the extracellular part of human GPVI, were produced and purified by affinity chromatography on HiTrap protein A-Sepharose, as described (3, 17). As shown previously for 9O12 (17), the newly characterized 9E18 and 3F8 bind to human platelets as measured by flow cytometry and label a 58-kDa band corresponding to GPVI in Western blot analysis of human platelet proteins but do not recognize GPVI under reducing conditions, Also, their IgGs induce aggregation of human platelets with threshold concentration ≤ 5 μg/ml.

Wild Type and Mutant Recombinant Soluble Human GPVI

The cDNA of the recombinant soluble human GPVI (rsGPVI-Fc) was designed and produced as described (3). Residues Gly-30, Val-34, and Leu-36 of rsGPVI-Fc were altered to an alanine using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sense oligonucleotides of the mutagenic pairs were as follows: 5′-ACCTCCGGGCGCGGACCTGTACCGC-3′, V34A; 5′-CCGGGCGTGGACGCGTACCGCCTGGAG-3′, L36A; 5′-CCTCCGGTGCCAGGCACCTCCGGGCGCGGACGCGTACCGCCTGGA-3′, G30A-V34A-L36A. Mutant constructs were confirmed by sequencing. Wild type (WT) and mutant rsGPVI-Fc were produced as already reported (3) and purified by affinity chromatography on HiTrap protein A-Sepharose. Purity was checked by SDS-PAGE.

Isolation of Phages Binding to 9O12 mAb

To identify the epitope of 9O12, a linear pentadecamer phage display library was used, essentially as described elsewhere (19). Biotinylated 9O12 (10 μg) was incubated with phages in low ionic strength buffer, 0.5% Tween 20. Streptavidin-coated magnetic beads (Dynal, Oslo, Norway) were added to the mixture. After five steps of washing in LIB, 0.5% Tween 20, bound phages were eluted with 0.1 M glycine, pH 2. Phages were amplified by infection of *Escherichia coli* K91 Kan cells and partially purified from the supernatant by polyethylene glycol precipitation. Two additional rounds of panning were performed, and after each round, phages were amplified. A dilution of the final eluted phage pool was plated on Luria broth agar plates containing tetracycline. Single colonies were picked from these plates and grown overnight in 2× TY medium containing tetracycline in a 96-well plate. After centrifugation of the plates, 9O12-binding phages contained in the supernatant were detected in ELISA. Phage DNA was prepared by phenol/chloroform extraction for sequencing.

SDS-PAGE and Immunoblotting

Proteins from phages were separated on a 10% acrylamide slab gel, under non-reducing and reducing conditions, and blotted on a nitrocellulose membrane (Amersham Biosciences). After blocking with a 5% milk powder solution, the membrane was incubated with biotinylated 9O12 (2 μg/ml in PBS, 0.4% milk powder) for 2 h. The membrane was washed with PBS containing 0.05% Tween 80 before a 2-h incubation with streptavidin-HRP. The membrane was developed using the ECL detection system (Amersham Biosciences).

ELISA and Binding Assays Using Purified Proteins

Measurement of Phage Binding to 9O12—Purified 9O12 (10 μg/ml in PBS) was coated overnight in a 96-well plate. After blocking with a 2% milk powder solution, dilution series of the individual phage clones selected after the final panning round were added to the wells (all in PBS, 0.2% milk powder). An irrelevant phage that specifically binds to von Willebrand factor (20) was used as a negative control. Phages were incubated at room temperature for 90 min. After washing with PBS, 0.1% Tween 20, a polyclonal anti-M13-HRP-conjugated antibody was added to the wells and incubated for 1 h. Bound phages were detected, after washing, by hydrolysis of ortho-phenylenediamine dihydrochloride. Alternatively, phages were preincubated 30 min with increasing doses of rsGPVI-Fc before addition to the 9O12-containing wells.

Binding of rsGPVI-Fc (WT and mutants) to Collagen, CRP or Convulxin—Experiments were conducted mainly as described (17); microtitration wells were coated overnight with collagen type I, CRP, or convulxin, in PBS, and blocked with 0.2% BSA for 2 h. WT or mutant rsGPVI-Fc (in PBS, 0.1% BSA, 0.1% Tween 20) were added to the wells and incubated for 2 h. Alternatively, rsGPVI-Fc was preincubated for 30 min with 10 μg/ml anti-GPVI mAb before addition to the wells. After washing with PBS, 0.1% BSA, 0.1% Tween 20, bound rsGPVI-Fc was detected using HRP-coupled protein A or HRP-coupled goat anti-mouse antibody, as indicated. To study the binding of convulxin to rsGPVI-Fc, microtitration wells were coated with a non-blocking anti-GPVI mAb (1P10, 5 μg/ml) and saturated with BSA. 0.5 nM rsGPVI-Fc (in PBS, 0.2% BSA, 0.1% Tween 20) was added for 2 h at room temperature. After washing, immobilized rsGPVI-Fc was incubated 45 min with anti-GPVI mAbs or buffer, and then ^{125}I-convulxin was added, and incubation was prolonged for 10 min. Wells were washed with PBS, 0.2% BSA, 0.1% Tween 20 and counted for ^{125}I-convulxin in a γ-counter.

Cell Adhesion Assay and Preparation of Human Platelets

Experiments were mainly performed as described previously (3, 21). Microtitration wells were coated with collagen type I, convulxin, or CRP (20 μg/ml each in PBS). After blocking with BSA, rGPVI-HEL cells (5.10^6/ml) in Tyrode-Hepes buffer were added to the wells and allowed to adhere for 1 h under gentle agitation. For competition studies, cells were preincubated for 30 min with increasing doses of collagen, CRP, or convulxin before adhesion. Adherent cells were quantified by measuring alkaline phosphatase activity using para-nitrophenylphosphate disodium salt. Human washed platelets (3.10^8/ml) were prepared according to a previously described procedure (7).

Homology Modeling of GPVI

A three-dimensional model of the extracellular domain of human GPVI was built by comparative modeling using the 2.1-Å x-ray structure of the first two Ig-like domains of human LIR-1 (Protein Data Bank code 1G0X (22)) as template. The sequences of the extracellular domain of human GPVI and the crystallized ligand-binding domain of LIR-1 were first aligned using the Clustal algorithm (23). The quality of the final alignment was evaluated by identity (48%) and homology score (77%). The resulting alignment was used as input for Modeler v6.0 (salilab.org/modeler) (24). Several three-dimensional structures were generated ($n = 20$) and evaluated using the Modeler objective function. Root mean square deviation was inferior to 0.3 Å. Only the first model was used.

Statistical Analysis

Data are expressed as mean ± S.E. Student's t test or one-factor analysis of variance followed by the Bonferroni test was used to evaluate statistical significance. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Competition between Collagen, CRP, and Convulxin for the Binding to Cell-expressed GPVI—The hematopoietic cell line HEL was engineered to overexpress recombinant human GPVI (rGPVI) (3). rGPVI associated with FcRγ. Equilibrium binding studies indicated that ^{125}I-labeled convulxin bound to 65,000 sites with a K_d of 0.35 nM on GPVI-transduced cells *versus* 11,000 sites with a similar K_d on mock-transduced cells. Convulxin induced intracellular calcium mobilization in rGPVI-HEL (not shown). In addition, rGPVI-HEL cells were able to

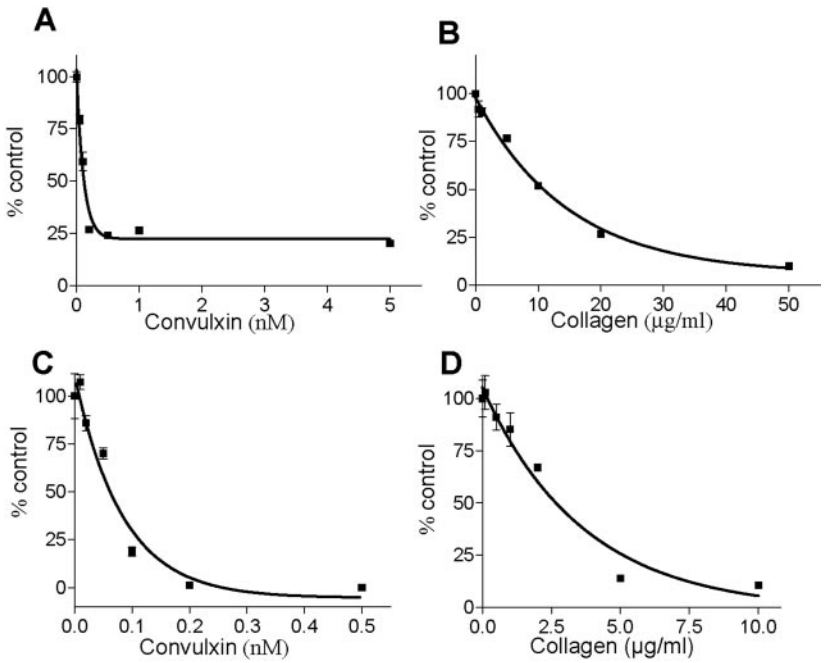

FIG. 1. **Competition between collagen, CRP, and convulxin for binding to cell expressed GPVI.** rGPVI-HEL cells were preincubated for 15 min at room temperature with increasing doses of collagen, convulxin, or CRP. Cells were next allowed to adhere to immobilized ligand (collagen, CRP, or convulxin). Results are presented as follows: adhesion to collagen in the presence of convulxin (*A*), adhesion to convulxin in the presence of collagen (*B*), or adhesion to CRP in the presence of convulxin (*C*) or collagen (*D*). Data are expressed as percentage of control, that is, the binding of rGVPI-HEL in the absence of competing molecules, and is given as mean ± S.E. of $n \geq$ three independent experiments.

adhere to immobilized collagen, CRP, and convulxin in a time- and dose-dependent manner (17). rGPVI-HEL expressed a low level of integrin $\alpha_2\beta_1$ at their surface as indicated by flow cytometry using a fluorescein isothiocyanate-coupled anti-CD49b. We investigated the ability of collagen, CRP, and convulxin to compete with one another for the binding to cell-expressed GPVI by studying the adhesion of rGPVI-HEL to the coated ligand in the presence of the others in soluble form.

Cell adhesion to collagen was reduced by increasing amounts of convulxin; maximal inhibition reached 88% with 0.5 nM convulxin, and the IC_{50} was 0.08 nM (Fig. 1*A*). CRP had a moderate inhibitory effect on the adhesion of rGPVI-HEL to collagen. 2.5 μg/ml CRP decreased adhesion by 40%, and 10-fold higher concentrations did not further inhibit the binding to collagen (not shown).

Adhesion of rGPVI-HEL to convulxin was abolished by increasing amounts of collagen. The IC_{50} was 10 μg/ml, and 95% inhibition was reached with 50 μg/ml collagen (Fig. 1*B*). In contrast, 50 μg/ml CRP only reduced adhesion to convulxin by 20%, but 2.5 μg/ml was sufficient to reach the maximal inhibitory effect (not shown). Adhesion to CRP was fully inhibited by both collagen and convulxin. 95% inhibition was reached with 10 μg/ml collagen (IC_{50} = 2.5 μg/ml) (Fig. 1*D*), whereas the maximal effect was obtained with 0.25 nM convulxin (IC_{50} = 0.06 nM) (Fig. 1*C*). These results demonstrate that collagen and convulxin, and to a lesser extent CRP, compete with each other for the binding to cell-expressed rGPVI.

Identification of Selective Antagonists of GPVI Interaction with Its Ligands—To further characterize ligand-binding sites on human GPVI, we have tested the ability of several mAbs directed against the extracellular domain of the receptor to inhibit the interaction of a recombinant soluble form of GPVI (rsGPVI-Fc) with its ligands collagen, CRP, and convulxin. One of them, 9O12, prevented the interaction of GPVI with both collagen and convulxin (17) (Fig. 2). The binding of rsGPVI-Fc to immobilized collagen was markedly inhibited by 9O12, with an IC_{50} of 1.5 μg/ml, and 20 μg/ml antibody yielded an inhibition of 85%. The binding of convulxin to immobilized rsGPVI-Fc was strongly reduced by the antibody; the maximal inhibition reached 80% for concentrations of 9O12 above 10 μg/ml, and the IC_{50} was 3 μg/ml. In addition, 10 μg/ml 9O12 abolished the binding of rsGPVI-Fc to CRP.

The effect of other mAbs on the binding of rsGPVI-Fc to its ligands was compared with 9O12 using the same concentration of 10 μg/ml (Fig. 2). 9E18 reduced the binding of GPVI to collagen by 45%, reduced the binding of GPVI to CRP by 85%, but had little effect on binding to convulxin (25% inhibition). In addition, inhibition of GPVI interaction with collagen by 9E18 was dose-dependent; the maximal inhibition reached 55% for concentrations of 9E18 above 15 μg/ml, and the IC_{50} was 1.6 μg/ml (not shown). In contrast, 3F8 decreased the binding of rsGPVI-Fc to convulxin by 55% and to CRP by 50%, but not the binding to collagen (l0% inhibition, Fig. 2). 3F8 dose-dependently inhibited GPVI interaction with convulxin with an IC_{50} of 0.5 μg/ml. Maximal effect was reached with 5 μg/ml antibody and did not increase further with a higher concentration of 3F8 (not shown). In the presence of an isotype-matched control antibody, 7I20, the binding of rsGPVI to neither collagen, convulxin, nor CRP was affected (Fig. 2).

Cross-reactivity studies showed that 9O12, 9E18, and 3F8 did not compete for the same binding site on rsGPVI-Fc; although each antibody prevented its own binding to GPVI, the interaction of the soluble receptor with one immobilized mAb was not impaired in the presence of the others (not shown). This indicated that the three mAbs recognize distinct epitopes on GPVI.

Similar results were obtained when mAbs were tested on the adhesion of rGPVI-HEL to GPVI ligands (not shown). 9O12 (20 μg/ml) inhibited the adhesion of rGPVI-HEL to collagen by 70% and completely prevented cell adhesion to CRP (17). 20 μg/ml 9E18 reduced the adhesion of rGPVI-HEL to collagen by 35%. In contrast, the adhesion of rGPVI-HEL to convulxin was poorly affected by either 9O12 or 3F8, inhibition being only 25 and 10%, respectively. However, when cells were incubated with both 9O12 and 3F8, a synergistic inhibitory effect was observed that reached 65%. This might indicate that both monoclonal antibodies bind to independent sites close to or belonging to the convulxin-binding site. All three antibodies abolished the binding of rGPVI-HEL to immobilized CRP by more than 90% (not shown), consistent with the results obtained when using rsGPVI-Fc (Fig. 2*B*).

Epitope Mapping of 9O12 Using Phage Display—Taken together, these results prompted us to focus our efforts on the identification of the epitope of mAb 9O12 since this antibody was the most efficient to prevent GPVI interactions with all its

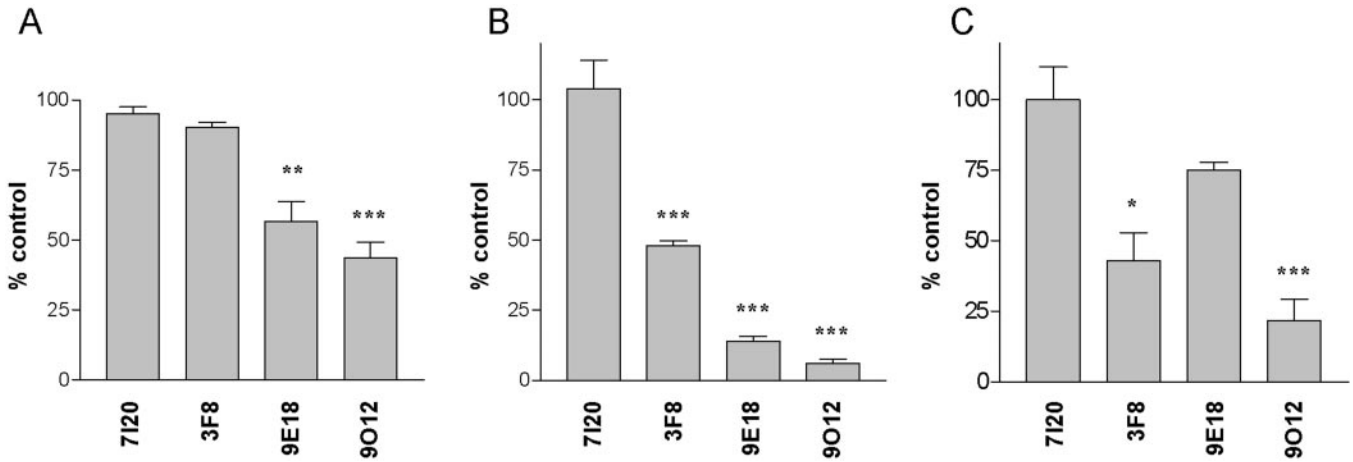

FIG. 2. **Selective inhibition of GPVI interactions with its ligands by anti-GPVI mAbs.** rsGPVI-Fc was preincubated with anti-GPVI mAbs 9O12, 9E18, or 3F8, or with irrelevant isotype-matched mAb 7I20 (10 μg/ml each) for 30 min at room temperature. Immune complexes were then added to collagen- (*A*) or CRP- (*B*) coated wells and incubated 30 min. After washing, bound rsGPVI-Fc was detected using protein A-HRP. *C*, alternatively, coated rsGPVI-Fc was incubated for 45 min with 9O12, 9E18, 3F8, or 7I20 (10 μg/ml each), after which ^{125}I-convulxin was added, and incubated for 10 min. Bound ^{125}I-convulxin was measured by γ counting. Data are given as mean ± S.E. of $n \geq$ four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ligands, including the physiological collagen. For this purpose, we screened a combinatorial library of linear pentadecamer peptides by phage display technology. After three rounds of biopanning, 66 individual clones were obtained. 46 clones were randomly selected and tested for their ability to bind to immobilized 9O12 in ELISA. 12 were positive, as illustrated for three clones on Fig. 3*A*. The binding of 9O12 to the phages was also analyzed by Western blotting. 9O12 IgG recognized the phages under non-reducing conditions, whereas disulfide bridge reduction greatly impaired the binding of 9O12 (Fig. 3*C*).

Importantly, rsGPVI-Fc competed with the selected phages for the binding to 9O12 in ELISA (illustrated on Fig. 3*B* with two clones), suggesting that the peptides displayed by the phages mimic a structure found in GPVI. No binding of the phages to other anti-GPVI mAbs (3F8, 3J24) was observed, demonstrating the specificity of their interaction with 9O12.

Sequencing of these 12 clones resulted in the identification of four sequences (Table I). All sequences share the motif **CGL***XX***VC**. The presence of the two cysteines, together with the fact that the binding of 9O12 to the peptides was impaired upon disulfide bond reduction, strongly suggested that the cysteines were forming a disulfide bridge and that a constrained conformation was required for recognition by 9O12. This was consistent with our previous observation that the epitope of 9O12 within GPVI is conformational (17).

The corresponding cyclic peptides were synthesized for functional analysis. Unfortunately, these were not soluble, and all our attempts to solubilize them remained unsuccessful, even after various modifications. We therefore chose a different approach and used computer modeling of GPVI to identify candidate residues for site-directed mutagenesis.

Epitope Mapping of 9O12 Using Homology Modeling of GPVI Structure—The peptide sequences of the clones were aligned to the sequence of the extracellular domain of human GPVI. The motif **CGL***XX***VC** could not be found as such in the sequence of GPVI, and no obvious similarity was identified. However, we noticed a relative homology with region 30–40 of GPVI, and particularly, the presence of a glycine, a valine, and a leucine in the vicinity of a cysteine in position 28 (Table I).

We used homology modeling to produce a structure of the EC domains of human GPVI. We observed that a loop located at an end of the IgC2-1 domain presented three residues found in the motif: Gly-30, Val-34, and Leu-36. These were in close vicinity, in a constrained environment, and apparently exposed at the surface of the protein, accessible to ligand (Fig. 4). Therefore, we hypothesized that these residues could be involved in collagen recognition.

To test this hypothesis, we produced mutants of rsGPVI-Fc in which Gly-30, Val-34, and Leu-36 were altered to alanine. Three variants were constructed: two consisting of point mutations, V34A and L36A, and one in which the three residues were simultaneously mutated, G30A,V34A,L36A.

Mutation of Val-34 and Leu-36 Inhibits the Binding of GPVI to Collagen and CRP—Mutants V34A, L36A, and G30A,V34A,L36A displayed an apparent molecular weight, determined in SDS-PAGE, identical to that of WT rsGPVI-Fc. They were recognized by a non-inhibitory anti-GPVI mAb (3J24) binding to WT rsGPVI-Fc in Western blot, indicating that proteins were correctly expressed.

We measured the binding properties of 9O12 to immobilized rsGPVI-Fc mutants (2 μg/ml). None of the mutations affected the maximal binding of the antibody. The affinity of 9O12 for L36A was comparable with that measured for WT rsGPVI-Fc (K_d = 0.079 *versus* 0.075 μg/ml) (Table II). However, the affinity of 9O12 for V34A was significantly decreased by a factor of 1.6 (K_d = 0.125 μg/ml), as was the affinity of the antibody for the triple mutant (K_d = 0.127 μg/ml).

The ability of each mutant to bind to immobilized GPVI ligands was then investigated. WT rsGPVI-Fc dose-dependently bound to immobilized collagen with an apparent K_d of 1.2 ± 0.2 μg/ml (Fig. 5*A*). The L36A mutation slightly reduced the capacity of GPVI to bind to collagen; the K_d was of 3.6 ± 0.2 μg/ml. Mutation of Val-34 was far more effective and strongly impaired GPVI interaction with collagen. The apparent K_d increased to 22 ± 2 μg/ml. The triple mutation G30A,V34A,L36A yielded full inhibition of the binding to collagen.

WT rsGPVI-Fc bound to immobilized CRP in a dose-dependent manner, with an apparent K_d of 0.8 ± 0.1 μg/ml comparable with that measured on collagen (Fig. 5*B*). Mutation of Leu-36 did not impair the ability of GPVI to interact with CRP; the resulting affinity was identical to that measured with WT rsGPVI-Fc (K_d = 1 ± 0.2 μg/ml). Mutation of Val-34, on the other hand, markedly reduced the affinity of GPVI toward CRP; the K_d increased to 6.5 ± 1 μg/ml. Again, simultaneous mutation of Gly-30, Val-34, and Leu-36 resulted in full inhibition of the binding to CRP. The K_d increased up to 70 ± 5 μg/ml. None of the mutations reduced the binding of rsGPVI-Fc to immobilized convulxin (data not shown).

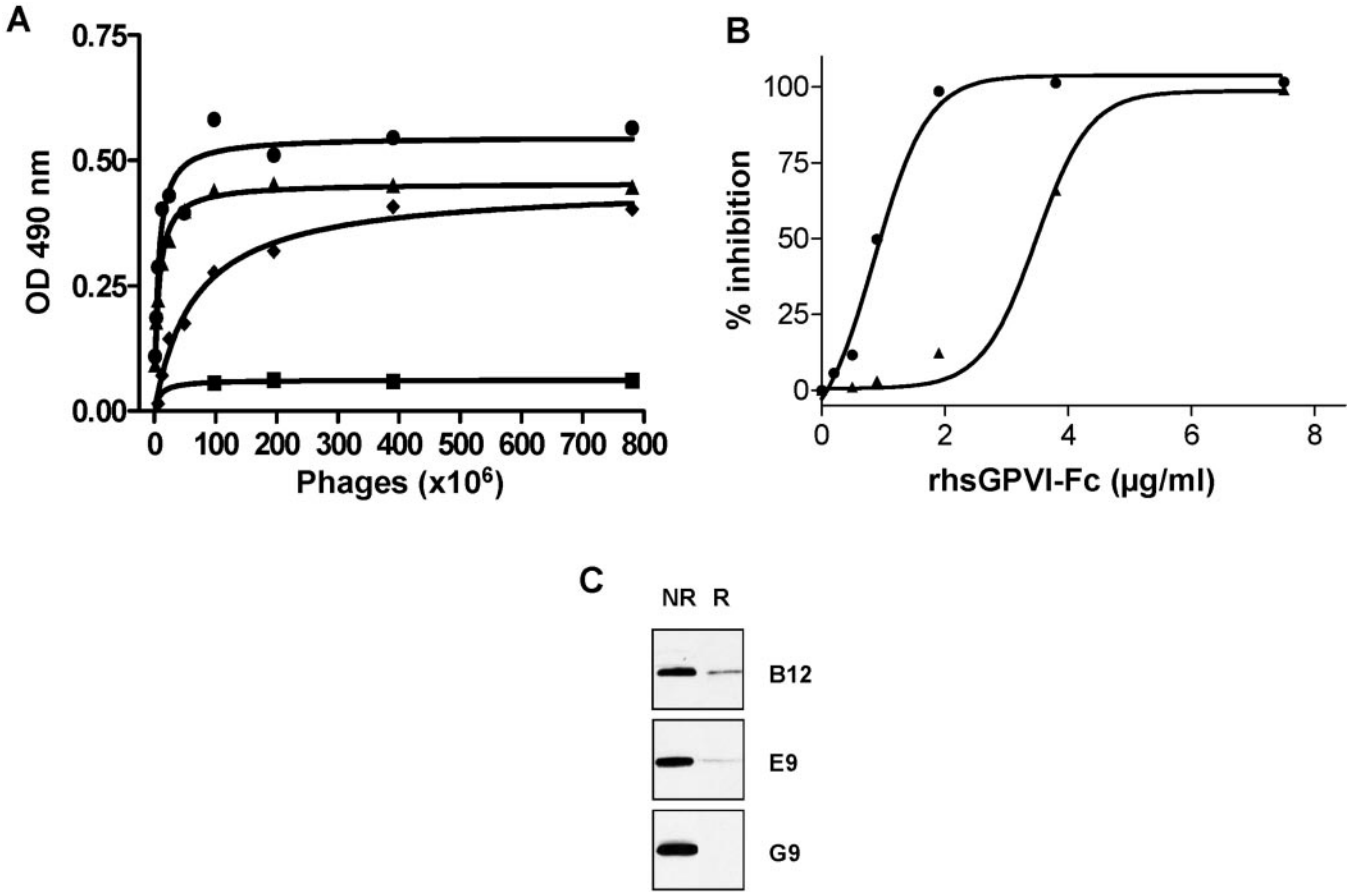

FIG. 3. **Selection and characterization of phage clones binding to 9O12.** *A*, dose-dependent binding of phage clones E9 (●), G9 (▲), B12 (♦), and irrelevant phage (■) to immobilized 9O12. Bound phages were detected using polyclonal anti-M13-HRP antibodies. *B*, inhibition of the binding of phage clone G8 (●) and B12 (▲) to immobilized 9O12 by rsGPVI-Fc. Increasing doses of rsGPVI-Fc were incubated with immobilized 9O12 for 15 min at room temperature. Phages (clones G8 and B12, 1.5×10^9 each) were added to the wells and incubated for 1 h. Bound phages were detected as described above. *C*, proteins from purified phages B12, E9, and G9 ($2{\cdot}10^{10}$) were analyzed in SDS-PAGE using a 10% slab gel under non-reducing (*NR*) and reducing (*R*) conditions. Proteins were transferred to a nitrocellulose membrane, and detection was performed using biotinylated 9O12 (2 µg/ml in PBS, 0.4% milk powder) followed by incubation with streptavidin-HRP.

TABLE I
Peptide sequence of the phage clones selected by anti-GPVI mAb 9O12

Alignment of selected peptide sequences with region 21–40 of the human GPVI EC1 domain. The consensus motif and corresponding residues within GPVI are indicated in bold.

Phage clones	Peptide sequence
F7/B12/E7/E8/B8/C10/F9	S**CGL**GV**VC**GAALVA
G9/E10	GQELLA**CGL**FS**VC**LS
E11/E9	GQRSSVGG**CGL**HL**VC**
G8	KNGVFL**CGL**GL**VC**PD
Human GPVI-(21–40)	EKPVTLR**C**Q**G**PPG**V**D**L**YRLE

We further analyzed the effect of the mutations on GPVI function by testing the ability of the variants to compete with platelet GPVI and to inhibit collagen-induced platelet aggregation (Fig. 6). As already reported elsewhere (3), preincubation of WT rsGPVI-Fc with collagen completely prevented platelet aggregation. Mutant L36A similarly inhibited collagen-induced aggregation. In contrast, the V34A and the triple mutation almost completely reversed the inhibitory effect of rsGPVI-Fc on collagen-induced platelet aggregation. The fact that V34A and G30A,V34A,L36A could hardly prevent platelet activation by collagen correlated well with their respective loss of affinity toward collagen measured in ELISA.

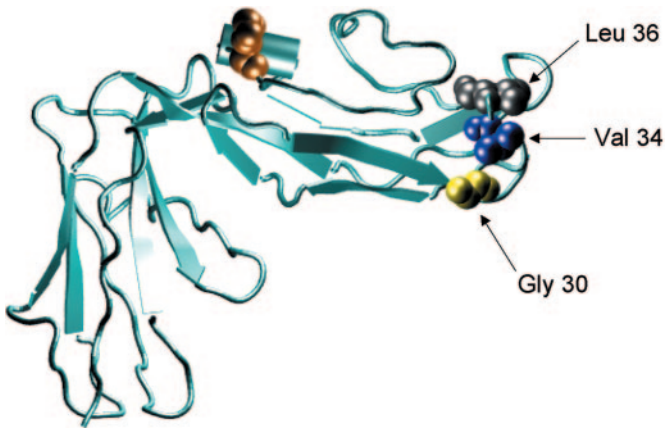

FIG. 4. **Location of 9O12 epitope within human GPVI.** Residues forming the putative epitope of 9O12 in a homology model of human GPVI are shown. The structure of human GPVI is based on the crystallographic data of the human LIR binding site (see "Experimental Procedures"). Hypothesized epitope residues are represented as *space-filling atoms* in the whole molecule, represented as a *ribbon frame*. Lys-59 involved in CRP binding (28) is represented in *brown*.

DISCUSSION

We describe here the characterization of ligand-binding sites in human GPVI using newly developed tools. GPVI is critically involved in collagen-induced platelet activation and thrombus formation following vascular injury (25). Although GPVI has been cloned (2, 3), little is known about the structure of its ligand-binding site. Whereas KIR family members bind their HLA ligands in the interdomain (EC1–EC2) region of the re-

TABLE II
Affinity of mAb 9O12 towards rsGPVI-Fc mutants

The binding of 9O12 to immobilized rsGPVI-Fc (wild type or mutants, 2 μg/ml each) was measured. Detection of bound 9O12 was performed using goat anti-mouse antibody coupled to HRP followed by colorimetric reaction with ortho-phenylenediamine dihydrochloride. Association curves were drawn, and K_d was determined for each interaction. Results are given as mean ± S.E. of three independent experiments. NS, not significant.

rsGPVI-Fc	K_d ± S.E.	Statistical significance
	μg/ml	
Wild type	0.079 ± 0.006	
L36A	0.075 ± 0.001	NS
V34A	0.125 ± 0.002	$p < 0.001$
G30A,V34A,L36A	0.127 ± 0.010	$p < 0.001$

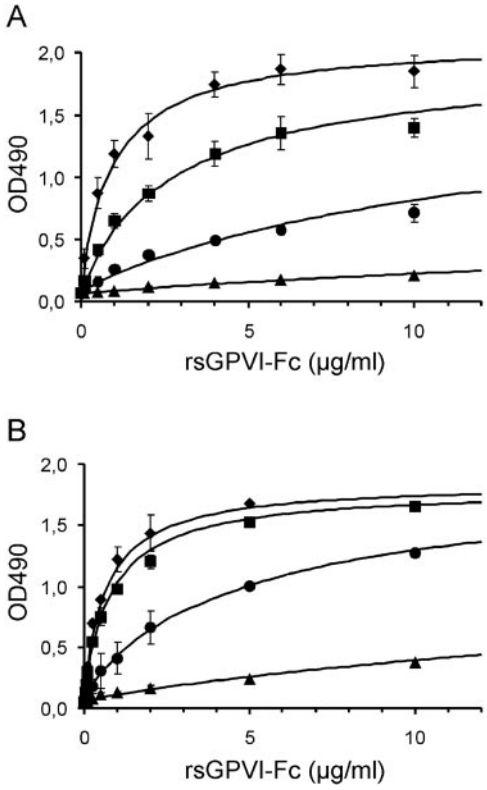

FIG. 5. **Binding of rsGVI-Fc mutants to immobilized collagen and CRP.** Wild type rsGPVI-Fc (♦) and mutant rsGPVI-Fc V34A (●), L36A (■), and G30A,V34A,L36A (▲) were incubated with immobilized collagen (1 μg) (*A*) or CRP (0.5 μg) (*B*) for 2 h. After washing, bound rsGPVI-Fc was detected using protein A-HRP and subsequent colorimetric reaction. Data are given as mean ± S.E. of three independent experiments.

ceptor, the IgA-binding site on FcαRI involves structures in the EC1 domain at a distance from the hinge region (14, 15).

To characterize GPVI interactions with its ligands, we used two complementary approaches. First, we performed a static adhesion assay, using a hematopoietic cell line overexpressing human GPVI (rGPVI-HEL) to determine whether collagen, CRP, and convulxin could compete with one another for the binding to cell-expressed GPVI. Second, we have analyzed a panel of mAbs directed against the extracellular domain of human GPVI and measured their effect on the binding of a recombinant soluble form of human GPVI (rsGPVI-Fc) to its ligands. This led to the identification of three mAbs 9O12, 9E18, and 3F8 with selective inhibitory properties. Together the results obtained indicated that the diverse GPVI ligands interact with binding sites exhibiting specific features but also sharing some residues participating

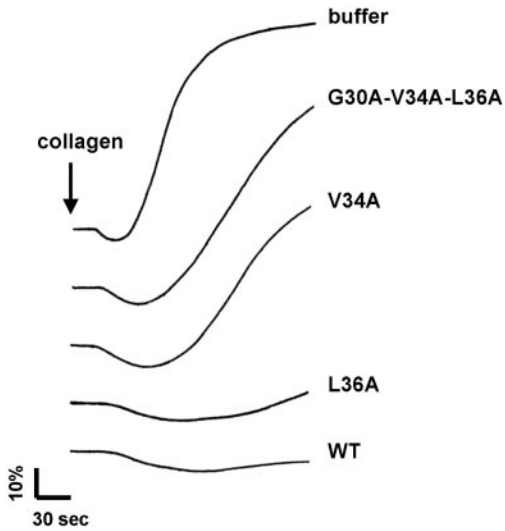

FIG. 6. **Inhibition of collagen-induced platelet aggregation by rsGPVI-Fc mutants.** Human washed platelets (3.10^8/ml) were activated by 0.5 μg/ml collagen. Collagen was preincubated with buffer, with V34A, L36A, G30A,V34A,L36A, or wild type rsGPVI-Fc (10 μg each), for 5 min at 37 °C before addition to platelets. The aggregation curves shown are representative of duplicates from three independent experiments.

in the recognition of these ligands.

This assertion is supported by several observations that follow. (i) Collagen and convulxin compete with each other and with CRP for the binding to GPVI. (ii) mAbs 9O12 and 9E18 both inhibit GPVI interaction with collagen but recognize independent epitopes on GPVI, and the same is observed for 9O12 and 3F8 on GPVI interaction with convulxin. (iii) Only the combination of 9O12 and 3F8 could strongly impair the binding of cell-expressed GPVI to convulxin. Collagen- and convulxin-binding sites thus exhibit distinct features but might also share common residues and be overlapping since 9O12 can prevent GPVI interactions with both ligands. The CRP-binding site is likely to be part of the collagen-binding site. Not only are these multimeric peptides built around the GPO motif derived from the sequence of collagen (18), but collagen and CRP compete with each other for the binding to GPVI. Schulte *et al.* (26) reported that the inhibitory effect of JAQ1, a mAb blocking the CRP-binding site on mouse GPVI, on collagen-induced platelet activation could be overcome by increasing concentrations of collagen. Our results are consistent with these data. However, since in addition convulxin fully prevents rGPVI-HEL adhesion to CRP, this site could be located in the vicinity of the convulxin-binding site or might share common structures with it. Despite the high affinity of the ligand, the interaction of GPVI with CRP is easily disrupted by the binding of anti-GPVI mAbs. This suggests that conformational changes within GPVI may be induced by interactions with antibodies or other ligands and may drastically reduce the accessibility of the CRP-binding site.

9O12 was of particular interest since it inhibits the interaction of GPVI with all its ligands and efficiently blocks all collagen- and CRP-induced platelet responses *in vitro* (17). We thus focused our efforts on the identification of its epitope. Preliminary experiments showed that 9O12 only bound to GPVI in non-reducing conditions, suggesting that its epitope involves a disulfide bridge-maintained structure. By screening a pentadecamer phage display peptide library, we have identified phages that specifically compete with rsGPVI-Fc for the binding to immobilized 9O12. Sequencing of those peptides revealed a consensus motif: CGL*XX*VC. The presence of two cysteines, together with the fact that 9O12 poorly recognized

the peptides under reducing conditions, suggested that the cysteines formed a disulfide bridge and that such constrained structure is required for 9O12 binding. As expected, direct alignment of the peptides with the sequence of human GPVI did not lead to the identification of a linear epitope but allowed the localization of several amino acids found in the consensus motif that were in close vicinity in the GPVI sequence: Gly-30, Val-34, and Leu-36. Homology modeling of the structure of GPVI showed that these residues were exposed at the surface of the receptor and located in a loop within the EC1 domain.

By site-directed mutagenesis, we generated variants of rsGPVI-Fc in which the selected residues were altered to alanine: V34A, L36A, and G30A,V34A,L36A. The characterization of the mutants showed that Val-34 and, to a lesser extent, Leu-36 are critically involved in GPVI interaction with collagen and CRP. By itself, the substitution V34A caused a major inhibition of the binding of GPVI to immobilized collagen and CRP. The triple mutation virtually abolished the binding to both ligands. Furthermore, V34A and G30A,V34A,L36A had a severely impaired ability to prevent collagen-induced platelet activation as compared with wild type rsGPVI-Fc. This loss of inhibitory effect nicely correlated with the decreased affinity of these variants for collagen. Mutation of Val-34 and the triple mutation similarly altered GPVI interaction with both collagen and CRP, confirming that the two ligands bind to common sites. In contrast, 9O12 still bound to the mutants, although its affinity for V34A and the triple mutant is significantly decreased. This suggests that residues other than Val-34 and Leu-36 are part of the 9O12 epitope.

The scFv antibody 10B12, another antagonist of human GPVI function, is thought to map to the CRP-binding site (27). It has recently been shown, using molecular modeling of human GPVI and 10B12, along with site-directed mutagenesis, that lysine 59 in EC1 (Fig. 4, in *brown*) was critically involved in the binding to CRP, with a minor contribution of residues arginine 166 and arginine 117 located in the hinge region and EC2 domain of the receptor (28). In this study, the authors suggested the existence of two CRP-binding sites of different affinity. Whether the motif in the EC1 domain we have identified is the secondary CRP-binding site is plausible.

In conclusion, we have identified a site within GPVI critically involved in recognition of collagen and CRP. This locus comprises Val-34 and Leu-36 and is located in the first IgC2 domain, at a distance from the potential CRP-binding site described by Smethurst *et al.* (28), in the hinge region of the receptor. The collagen-binding site thus appears to be widespread and to cover a larger surface on GPVI.

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