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 \(\gamma\) chain of immunoreceptors (Fc\(\gamma\)Rs) (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 \(C_\gamma\) 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 Fc\(\alpha\)RI 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 Fc\(\alpha\)RI, 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 Fc\(\alpha\)RI (14). KIR family members bind their ligand HLA in the hinge, interdomain region (15), whereas Fc\(\alpha\)RI and Fc\(\gamma\)RI 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).

When using the peptide 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-
tibodies were biotinylated using Dr. G. Smith (University of Missouri, Columbia, MO). Monoclonal an-
were a generous gift from Dr. R. Farndale (Cambridge, UK) (18).

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, Nor-
way) 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 precipi-
tation. Two additional rounds of phage purification 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 centrif-
ugation of the plates, 9O12-binding phages 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 nitrocel-
lulose 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 horseradish peroxidase-diaminobenzidine 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 Con-
vulxin—Experiments were conducted mainly as described (17); microt-

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 125I-labeled convulxin bound to 65,000 sites with a Kd of 0.35 nM on GPVI-transduced cells versus 11,000 sites with a similar Kd on mock-transduced cells. Convulxin induced intracellular calcium mobilization in rGPVI-HEL (not shown). In addition, rGPVI-HEL cells were able to
adhere to immobilized collagen, CRP, and convulxin in a time- and dose-dependent manner (17). rGPVI-HEL expressed a low level of integrin \( \alpha_{\text{v}}\beta_{3} \) 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 \( I_{50} \) was 0.08 nM (Fig. 1A). CRP had a moderate inhibitory effect on the adhesion of rGPVI-HEL to collagen. 2.5 \( \mu \)g/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; maximal inhibition reached 88% with 0.5 nM collagen, and the \( I_{50} \) was 0.08 nM (Fig. 1A). CRP had a moderate inhibitory effect on the adhesion of rGPVI-HEL to collagen. 2.5 \( \mu \)g/ml CRP decreased adhesion by 40%, and 10-fold higher concentrations did not further inhibit the binding to collagen (not shown).

The effect of other mAbs on the binding of rsGPVI-Fc to its ligands was compared with 9O12 using the same concentration of 10 \( \mu \)g/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 \( \mu \)g/ml, and the \( I_{50} \) was 1.6 \( \mu \)g/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 (10% inhibition, Fig. 2). 3F8 dose-dependently inhibited GPVI interaction with convulxin with an \( I_{50} \) of 0.5 \( \mu \)g/ml. Maximal effect was reached with 5 \( \mu \)g/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.

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 \( I_{50} \) of 1.5 \( \mu \)g/ml, and 20 \( \mu \)g/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 \( \mu \)g/ml, and the \( I_{50} \) was 3 \( \mu \)g/ml. In addition, 10 \( \mu \)g/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 \( \mu \)g/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 \( \mu \)g/ml, and the \( I_{50} \) was 1.6 \( \mu \)g/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 (10% inhibition, Fig. 2). 3F8 dose-dependently inhibited GPVI interaction with convulxin with an \( I_{50} \) of 0.5 \( \mu \)g/ml. Maximal effect was reached with 5 \( \mu \)g/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).

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Similar results were obtained when mAbs were tested on the adhesion of rGPVI-HEL to GPVI ligands (not shown). 9O12/20 \( \mu \)g/ml inhibited the adhesion of rGPVI-HEL to collagen by 70% and completely prevented cell adhesion to CRP (17). 20 \( \mu \)g/ml 9E18 reduced the adhesion of GPVI-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. 2B).

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
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 peptides by phage display technology. After three rounds of we screened a combinatorial library of linear pentadecamer ligands, including the physiological collagen. For this purpose, 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—**

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 ($K_d = 0.126$ 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. 5A). 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. 5B). 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).
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.

**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

<table>
<thead>
<tr>
<th>rsGPVI-Fc</th>
<th>Kd ± S.E.</th>
<th>Statistical significance</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>0.079 ± 0.006</td>
<td>NS</td>
</tr>
<tr>
<td>L36A</td>
<td>0.075 ± 0.001</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>V34A</td>
<td>0.125 ± 0.002</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>G30A,V34A,L36A</td>
<td>0.127 ± 0.010</td>
<td>p &lt; 0.001</td>
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**FIG. 5. Binding of rsGPVI-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.

**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.

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 Kd was determined for each interaction. Results are given as mean ± S.E. of three independent experiments. NS, not significant.

**TABLE II**

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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 each other 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 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: CGLXXVC. 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 close vicinity of this epitope.

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REFERENCES