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IN FOCUS

Glycoprotein (GP) VI dimer as a major collagen-binding site of native platelets: direct evidence obtained with dimeric GPVI-specific Fabs

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See also Herr AB. Direct evidence of a native GPVI dimer at the platelet surface. This issue, pp 1344–46.

Summary. *Background:* The platelet collagen receptor glycoprotein (GP) VI is suggested to exist as a dimer on the platelet surface, but no direct proof of the functional importance of dimer formation has been provided. *Objectives:* To obtain direct evidence for GPVI dimers on the platelet membrane and their functional importance, Fab antibodies were developed that bind to GPVI dimer (GPVI-Fc_2) but not to GPVI monomer (GPVIex) through a phage display method. *Results:* Ssix Fabs were found: B–F, only reactive with GPVI-Fc_2, and A, mainly reactive with GPVI-Fc_2, with some reactivity towards GPVIex; each Fab (Fab-dHLX-MH) forms a bivalent dimer (b-Fab) by dimerizing the dHLX domains from two Fab molecules. Fab F was subcloned to a monovalent format by deleting its dHLX domain. All b-Fabs induced platelet aggregation, but the monomeric form of Fab F (m-Fab-F) specifically inhibited collagen-induced aggregation. All b-Fabs and m-Fab-F inhibited GPVI-Fc_2 binding to fibrous collagen. Immunoblotting showed that b-Fab-F and m-Fab-F bound weakly to GPVI-Fc_2. Adding the anti-GPVI monoclonal antibody 204-11 increased the B_{max} of m-Fab-F binding to GPVI-Fc_2, suggesting that 204-11 binds to GPVI-Fc_2 molecules not already in the appropriate conformation to recognize the Fab, converting them to a conformation reactive to the Fab. *Conclusions:* GPVI forms a specific structure by dimerization that is necessary for the binding of this receptor to collagen fibrils. The binding of m-Fab-F to platelets directly demonstrates that GPVI is present as a functionally relevant dimer on the platelet surface.

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Introduction

The platelet-specific collagen receptor glycoprotein (GP) VI binds to collagen fibers, inducing strong platelet activation that leads to platelet aggregate formation under physiologic conditions. GPVI is complexed with the Fc receptor γ-chain (FcRγ) in the platelet membrane, but, owing to the small size of the FcRγ extracellular domain, the GPVI extracellular domain, particularly the two immunoglobulin-like moieties, is almost totally responsible for collagen binding. FcRγ transmits the signal-transducing reactions after the initial ligand binding, mainly through the immunoreceptor tyrosine-based activation motif domain of its cytoplasmic domain. FcRγ is also involved in GPVI expression in platelets [1,2], so FcRγ-deficient mouse platelets do not express GPVI [3].

GPVI forms a complex with FcRγ via a salt bridge between Arg252 of the transmembrane domain of GPVI and an Asp in the transmembrane domain of FcRγ [1,4]. As FcRγ is a homodimer crosslinked by a disulfide bond, two GPVIs may bind to one FcRγ [5], although recent reports have suggested that there is one-to-one complex formation between GPVI and FcRγ [6,7]. High-density expression of GPVI seems to be necessary for the expression of collagen-binding activity [4,8], suggesting that GPVI dimer formation is important for this receptor's binding activity. Thus, most of the reported data, although indirect, have suggested that dimer formation of the GPVI extracellular domain is critical for its binding to collagen fibrils. The recombinant GPVI extracellular domain by itself has no strong affinity for collagen fibrils, but when the same extracellular domain is fused to the Fc domain of IgG, so that it becomes a dimeric form by disulfide bonding at the Fc domains, the resultant dimer shows high affinity for collagen fibrils and inhibits collagen-induced platelet aggregation and platelet adhesion to immobilized collagen [9,10]. Finally, the crystal structure of the extracellular immunoglobulin domain

recently elucidated by Horii *et al.* [11] clearly indicates that the GPVI extracellular domain forms a back-to-back dimer; the docking algorithm identified two parallel binding sites for collagen on the GPVI dimer surface, separated by 55 Å, which is sufficient space to fit three triple helical collagen molecules between the two collagen-binding sites [11]. This model could explain why GPVI binds preferably to polymerized collagen fibrils rather than collagen monomer. Receptors with two binding sites would be expected to have higher affinity than those with one. However, the difference in affinity (K_D) of the GPVI dimer and its monomer for collagen is markedly larger than the difference in affinity of these two species for collagen-related peptide (CRP) [9], suggesting that dimer formation of GPVI results in a unique conformation specific for collagen binding.

In the present study, a screen was performed for recombinant immunoglobulin Fab fragments that specifically bind to recombinant GPVI dimer, but do not bind to GPVI monomer. The obtained recombinant Fabs bind only to GPVI dimer and inhibit the binding of the dimer to collagen, thereby inhibiting collagen-induced platelet aggregation. These results suggest that GPVI forms a dimer-specific conformation that is involved in collagen binding.

Materials and methods

Materials

Recombinant GPVI extracellular domain (GPVIex: GPVI monomer) and the same domain fused with the Fc domain of human IgG (GPVI-Fc_2: GPVI dimer) were prepared as described previously [9]. GPVI-Fc_2 was labeled with $Na^{125}I$ by using Iodobeads (Pierce, Rockford, IL, USA) at room temperature, according to the manufacturer's instructions. The specific radioactivity of GPVI was $3–10 \times 10^5$ c.p.m. (μg protein)$^{-1}$. Bovine type III collagen was from Koken Co., Ltd (Tokyo, Japan), and fibrous collagen was prepared as described previously [12]. Human IgG was from Sigma (St Louis, MO, USA).

Fab preparation

Recombinant Fabs that specifically react with GPVI dimer, but not the monomer, were obtained with the phage display method by MorphoSys AG (Planegg, Germany). Each recombinant Fab was generated from the HuCAL GOLD human antibody library (MorphoSys AG) [13,14] by selecting a Fab-expressing phage that specifically binds to GPVI-Fc_2. Prior to selection, the phage library was blocked with GPVIex and human IgG to deplete antibodies against GPVIex and the Fc domain of IgG. Each Fab has a small homodimerization domain (termed dHLX), a Myc tag, and a His-6 tag at the C-terminus of the heavy chain. Each obtained Fab (Fab-dHLX-MH) forms a bivalent dimer by dimerizing dHLX domains from two Fab molecules. One of the obtained Fab-dHLX-MH clones was subcloned to a monovalent Fab-MH format by deleting its dHLX domain. The enriched Fab genes were inserted into *Escherichia coli* vectors that lead to functional periplasmic expression of the corresponding Fabs. Fab proteins were purified by affinity chromatography on an Ni^{2+}–nitril-oacetic acid–agarose column.

Platelet preparation and aggregation assays

All studies involving human subjects were conducted according to the Declaration of Helsinki, following protocols approved by the ethical committee of Kurume University. Blood was drawn from the cubital vein of healthy volunteers into a 0.1 volume of 3.8% sodium citrate, and washed platelets were then prepared [9]. Platelet aggregation was monitored by a blood aggregometer (Chrono-Log Corp, Haverton, PA, USA) with stirring at 37 °C, using washed platelets in modified HEPES–Tyrode buffer (buffer A: 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 5.5 mM glucose, 5 mM HEPES, pH 7.4). The aggregation-inducing activity of bivalent Fabs (b-Fabs) and the inhibitory activity of monovalent Fab (m-Fab) against aggregation induced by collagen (Chrono-Log Corp) were analyzed.

Enzyme-linked immunosorbent assay (ELISA) analysis of the binding of m-Fab to GPVI

m-Fab-F binding to GPVI-Fc_2 and GPVIex was measured by ELISA. GPVI-Fc_2 (10 μg mL^{-1}) and GPVIex (10 μg mL^{-1}) were immobilized to microtiter-plate wells (Nunc-immuno module; Nunc A/S, Roskilde, Denmark) by reacting each with the wells overnight at 4 °C. The GPVI-bound wells were blocked with 2% bovine serum albumin (BSA) in buffer A, and washed with buffer A; different concentrations of m-Fab-F were then added to the wells and incubated for 1 h at room temperature. The wells were washed three times with buffer A, reacted with peroxidase-conjugated anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h, and washed four times with 0.005% Tween-20/buffer A; bound m–Fab-F was then visualized with 1-Step Turb TMB-ELISA (Pierce). For comparison, the anti-GPVI monoclonal antibody 204-11 [15] was also reacted with GPVI-Fc_2 and visualized similarly but by using peroxidase-conjugated anti-mouse IgG antibody (Biosource International, Camarillo, CA, USA). To determine whether 204-11 affects m-Fab-F binding to GPVI-Fc_2, m-Fab-F was mixed with 204-11 (1 μg mL^{-1}) and then reacted with immobilized GPVI-Fc_2. In these experiments, Fab binding to BSA-blocked wells was taken to be non-specific binding and subtracted from the binding to the immobilized GPVI-Fc_2 or immobilized GPVIex surface. Experiments were each performed in triplicate.

Effects of Fabs on GPVI binding to fibrous collagen

Binding of radiolabeled GPVI-Fc_2 to insoluble fibrous collagen was measured as previously described [12,15]. Various amounts of b-Fab or m-Fab were added to radiolabeled GPVI-Fc_2

(10 μg mL^{-1}) and reacted with fibrous type III collagen (0.2 mg mL^{-1}). After 45 min at room temperature, a 50-μL aliquot of each reaction mixture was transferred to the top of a tube containing 300 μL of buffer A containing 5% sucrose and then centrifuged at 10 000 *g* for 10 min at 25 °C. Collagen-bound GPVI is precipitated with the collagen fibers, so after freezing of the tubes, the tip of each was cut off and counted in a gamma counter. Quadruplicate assays were performed for each condition; data were expressed as the amount of GPVI-Fc_2 protein.

Immunoblotting analysis

Washed platelets in buffer A were lysed with an equal volume of 2% sodium dodecylsulfate (SDS)/8 M urea solution, and Laemmli's SDS sample buffer without reducing reagent was added; the mixture was heated at 100 °C for 3 min, and then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE). GPVI-Fc_2 and GPVIex were also analyzed by SDS-PAGE with the same procedure. After electrophoresis, the separated protein bands were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany), reacted with Fab or 204-11, and visualized by the chemiluminescence technique using peroxidase-conjugated anti-human IgG F(ab′)$_2$ or peroxidase-conjugated anti-mouse IgG antibody.

Flow cytometry analysis

Washed platelets (5×10^8 mL^{-1}) were reacted with control human IgG F(ab′)$_2$ (50 μg mL^{-1}; Jackson ImmunoResearch Laboratories) or m-Fab-F (50 μg mL^{-1}) in the presence or absence of 204-11 (2 μg mL^{-1}) for 25 min, and then with fluorescein isothiocyanate (FITC)-conjugated anti-His tag antibody (Kamiya Biomedical Co., Seattle, WA, USA) or FITC-conjugated anti-human F(ab′)$_2$ antibody (Jackson ImmunoResearch Laboratories) for 25 min. The platelet suspension was mixed with an equal volume of 1% paraformaldehyde solution for 30–60 min, and then diluted with IsoFlow solution (Beckman Coulter, Miami, FL, USA) for flow cytometry (Epics XL, Beckman Coulter).

Data analyses

Calculation of K_D and B_{max} values of Fab binding and analysis of statistical significance was performed with GRAPHPAD PRISM (GraphPad Software, La Jolla, CA, USA).

Results

Screening for recombinant Fab specifically recognizing GPVI dimer

Screening for recombinant Fabs that bind to GPVI-Fc_2 and not to GPVIex gave six strains of Fab. These Fabs have the helix–turn–helix structure needed to form the dimeric b-Fab. Figure 1 (first six lanes from the left) shows the GPVI-Fc_2-binding and GPVIex-binding properties of these b-Fabs. Although b-Fab-A showed some binding to GPVIex, the other b-Fabs bound only to GPVI-Fc_2, which suggests that these b-Fabs recognize a structure specific to GPVI-Fc_2. None of the b-Fabs had affinity for human IgG, indicating that they do not interact with the Fc domain of GPVI-Fc_2. The b-Fabs also showed no non-specific interaction with BSA and other proteins (ubiquitin and CD33). m-Fab (Fig. 1, first three lanes from the right) was made from one of the b-Fab strains, b-Fab-F, by deleting the helix–turn–helix domain. The obtained m-Fab-F also specifically bound to GPVI-Fc_2 (5 and 50 μg mL^{-1}), but with weaker affinity than b-Fab-F.

Effects of the Fabs on platelet aggregation

All of the b-Fabs induced platelet aggregation (Fig. 2A). In particular, b-Fab-B and b-Fab-F, which showed higher affinity for GPVI-Fc_2 (Fig. 1), strongly induced platelet aggregation as compared with b-Fab-C, b-Fab-D, and b-Fab-E, which each had weaker binding affinity and weaker aggregation-inducing activity. Platelet aggregation induced by b-Fab-F (10 μg mL^{-1}) was similar to that induced by 1.25 μg mL^{-1} collagen (Fig. 2B). Although b-Fab-F activated platelets, m-Fab-F did not induce aggregation (bottom aggregation curve) and inhibited collagen-induced platelet aggregation (Fig. 2C, blue aggregation curves), with the concentration-dependency shown in Fig. 2D. These results indicate that the b-Fabs induce platelet aggregation and that m-Fab-F inhibits platelet aggregation induced by collagen, which suggests that all of these Fabs, b-Fabs and m-Fab, recognize the structure of native GPVI in platelets. It is interesting that b-Fab-F could induce full aggregation at 10 μg mL^{-1} but a six-fold higher concentration of m-Fab-F (60 μg mL^{-1}) was needed for strong inhibition of aggregation; such a difference in the effective concentrations of Fab could be explained by the enhanced affinity of b-Fab-F conferred by its bivalency. m-Fab-F inhibited CRP-induced and convulxin-induced platelet aggregation at concentrations similar to that needed for inhibition of collagen-induced aggregation, but did not affect thrombin-induced aggregation (data not shown).

Immunoblotting using b-Fabs and m-Fabs

Binding of b-Fabs and m-Fabs to GPVI was also analyzed by immunoblotting. Platelet lysate, GPVI-Fc_2, and GPVIex were separated by non-reducing SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with b-Fabs and m-Fabs, and Fab binding was visualized by peroxidase-conjugated antibody against human IgG F(ab′)$_2$. Both b-Fab-F and m-Fab-F bound very weakly to GPVI-Fc_2, but they did not bind to GPVIex and GPVI from platelet lysate (Fig. 3). As these Fabs did not show any stained GPVI bands after SDS-PAGE under the reducing condition (data not shown), these results suggest that some GPVI-Fc_2 with intact structure remained after immunoblotting under the present

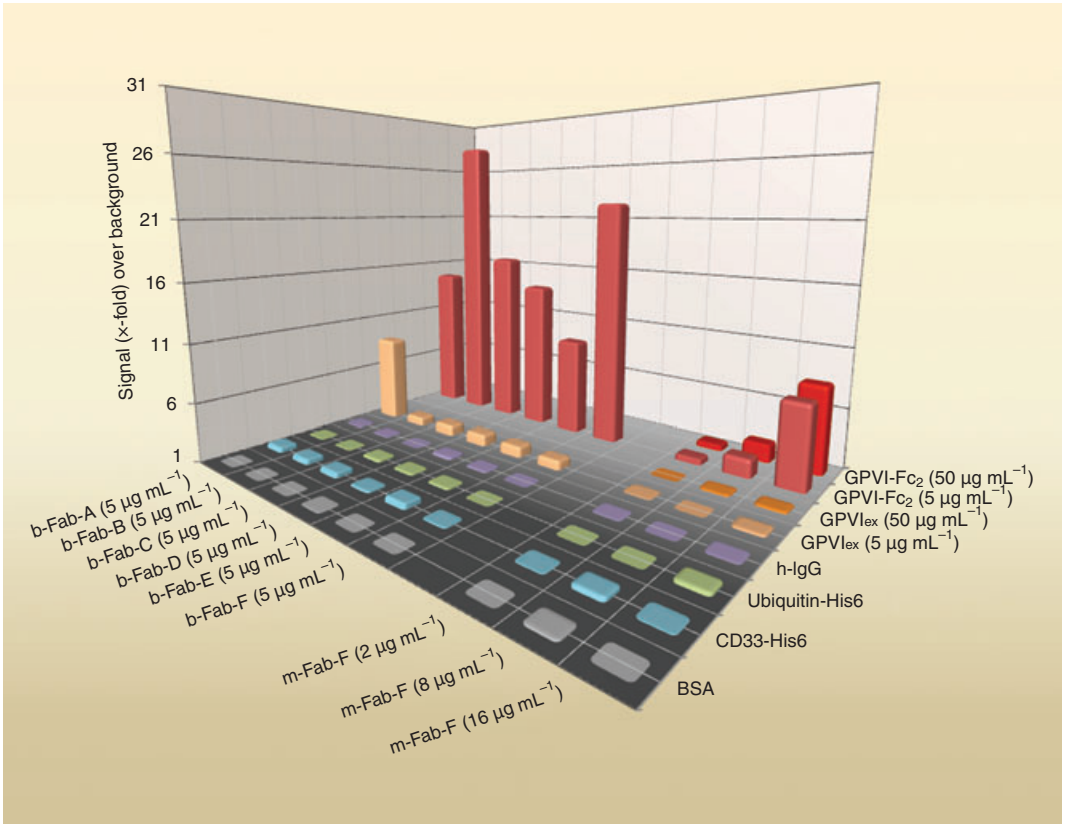

Fig. 1. Binding properties of screened Fabs that specifically react with glycoprotein VI (GPVI) dimer (GPVI-Fc_2). Enzyme-linked immunosorbent assay was used to determine the interaction of the selected bivalent Fabs (b-Fabs) (first six sets of data from the left side) and monovalent Fab-F (m-Fab-F) (first three sets of data from the right side) with GPVI-Fc_2 (GPVI dimer), GPVIex (GPVI monomer), and other proteins. Each b-Fab (5 μg mL^{-1}) was reacted with wells coated with one of the following proteins: GPVI-Fc_2, GPVIex, h-IgG, ubiquitin-His6, CD33-His6, and bovine serum albumin (BSA); extents of binding of the b-Fabs and m-Fab-F were then measured. Although b-Fab-A reacted with both GPVI-Fc_2 and GPVIex (to a small extent), the other b-Fabs and m-Fab-F were specific for GPVI-Fc_2 and did not interact with GPVIex or the other proteins.

non-reducing conditions. Monoclonal antibody against GPVI (204-11) reacted with all GPVI forms. The strong protein band (about 250 kDa) could be non-specific staining, as peroxidase-conjugated secondary antibody by itself stained this band. Other forms of b-Fab also showed a similar staining pattern; that is, they only bound to GPVI-Fc_2 (data not shown). Although the Fabs recognized GPVI on the platelet membrane, they did not bind to GPVI after solubilization by SDS, indicating that they recognized the native three-dimensional structure of GPVI or a dimer-specific structure of GPVI. These results also confirm that the b-Fabs and m-Fab-F recognized the GPVI-Fc_2-specific conformation.

Inhibition of GPVI binding to collagen fibers by b-Fabs and m-Fabs

The effects of b-Fabs and m-Fabs on GPVI-Fc_2 binding to collagen fibers were analyzed with the method [12] used for measuring GPVI-Fc_2 binding to insoluble collagen fibers. Radiolabeled GPVI-Fc_2 (10 μg mL^{-1}) binding to collagen fibers was inhibited by the b-Fabs and m-Fab-F (Fig. 4A). b-Fab-B and b-Fab-F, having higher affinity (Fig. 1), showed greater inhibitory activity, and b-Fab-E, with the lowest affinity, showed very weak inhibitory activity. Monovalent m-Fab-F had much weaker inhibitory activity than its bivalent form, b-Fab-F (Fig. 4B). These results indicate that these Fabs bound to a dimer-specific structure of GPVI and inhibited GPVI binding to collagen fibers, suggesting that the epitope of these Fabs could be closely related to the collagen-binding site of GPVI.

Analysis of Fab binding to GPVI

The ELISA assay results (Fig. 5A) showed that m-Fab-F bound to immobilized GPVI-Fc_2, but did not interact with immobilized GPVIex, confirming the results of Fig. 1. m-Fab-F bound to GPVI-Fc_2 with a K_D of 20.4 ± 14.1 μg mL^{-1} (4.08 ± 2.83 × 10^{-7} M) (n = 13). Comparison with the K_D of the monoclonal anti-GPVI antibody 204-11 [K_D = 0.163 ± 0.063 μg mL^{-1} (1.09 ± 0.420 × 10^{-9} M) (n = 3)] indicates that the m-Fab antibody bound with about 400 times weaker affinity than 204-11 (Fig. 5B).

m-Fab-F and 204-11 do not bind to a common site on GPVI-Fc_2, because adding the Fab at a concentration as high as 200 μg mL^{-1} (Fig. 6A) did not significantly interfere with

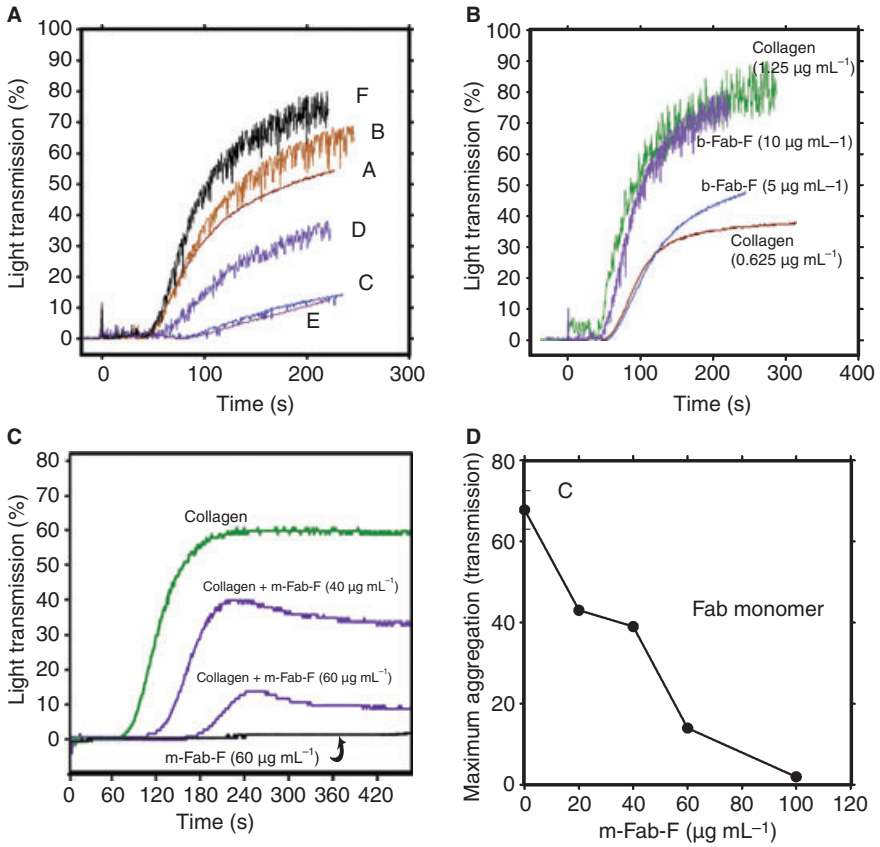

Fig. 2. Effects of bivalent Fabs (b-Fabs) and monovalent Fab-F (m-Fab-F) on platelet aggregation. (A) One of the b-Fabs (10 μg mL^{-1}) was added to the stirred washed platelet sample (2.5×10^8 platelets mL^{-1}) in the aggregometer, and aggregation was then monitored as light transmission increase. Aggregation curves A–F: b-Fab-A to b-Fab-F, respectively. All b-Fabs induced aggregation. (B) The aggregation-inducing activity of b-Fab-F was compared with that of collagen. (C) Effects of m-Fab-F on collagen-induced platelet aggregation. m-Fab-F alone at concentrations as high as 100 μg mL^{-1} (bottom curve shows data for the 60 μg mL^{-1} concentration) did not aggregate platelets, but it concentration-dependently inhibited collagen-induced platelet aggregation (blue curves). (D) Inhibitory activity of m-Fab-F against collagen (1.67 μg mL^{-1})-induced aggregation. Maximum aggregation was plotted against m-Fab-F concentration. m-Fab-F inhibited collagen-induced platelet aggregation but at concentrations higher than those needed for the aggregation-inducing activity of b-Fab-F.

the binding of 204-11 (Fig. 6A). However, adding 204-11 to the binding reaction of Fab and GPVI-Fc_2 increased the binding of Fab to GPVI-Fc_2. This suggests that the binding of 204-11 to GPVI-Fc_2 induces a conformational change of GPVI-Fc_2 that may allow it to bind more efficiently to m-Fab-F. This effect of 204-11 is GPVI-Fc_2 -specific, because 204-11 had no effect on Fab binding to GPVIex (Fig. 6B). Analyses of the binding of m-Fab-F to GPVI-Fc_2 in the absence or presence of 204-11 (Fig. 6C) indicated that the difference in K_D values [26.8 ± 30.7 ($5.36 \pm 6.13 \times 10^{-7}$ M) and 11.8 ± 5.75 μg mL^{-1} ($2.36 \pm 1.15 \times 10^{-7}$ M) for Fab only and Fab in the presence of 204-11 (1 μg mL^{-1}), respectively] was not significant ($P = 0.214$, $n = 6$), but the B_{max} values (0.223 ± 0.128 and 0.342 ± 0.150 OD at 450 nm in the absence or presence of 204-11, respectively) were significantly different ($P = 0.011$, $n = 6$). This increase in the number of binding sites in the presence of 204-11 suggests that some of the GPVI-Fc_2 molecules are not fully reactive towards the Fab and the binding of 204-11 to GPVI-Fc_2 to these could induce a change to a conformation reactive or more reactive to Fab.

Analysis of Fab binding to platelets

Two different procedures were tried, because it was difficult to detect m-Fab-F binding to platelets, owing to the weak association between m-Fab-F and GPVI. In the binding assay using ^{125}I-labeled m-Fab-F, we could not detect any specific binding to platelets, probably because the bound Fab dissociated from platelets during sucrose-gradient centrifugation to remove free ligand (data not shown). Next, flow cytometry was performed using Fab-treated platelets that were reacted with fluorescence-labeled anti-His-tag antibody and then fixed with paraformaldehyde. This allowed detection of the specific binding of m-Fab-F to platelets (Fig. 7), and, as in Fig. 6, the binding was increased in the presence of 204-11. With the use of fluorescence-labeled anti-human IgG $F(ab')_2$, a similar result was obtained, except that there was a smaller difference in the fluorescence between the control [human $F(ab')_2$] and m-Fab-F (data not shown). These results indicate that GPVI is present as a dimer complex on the platelet surface.

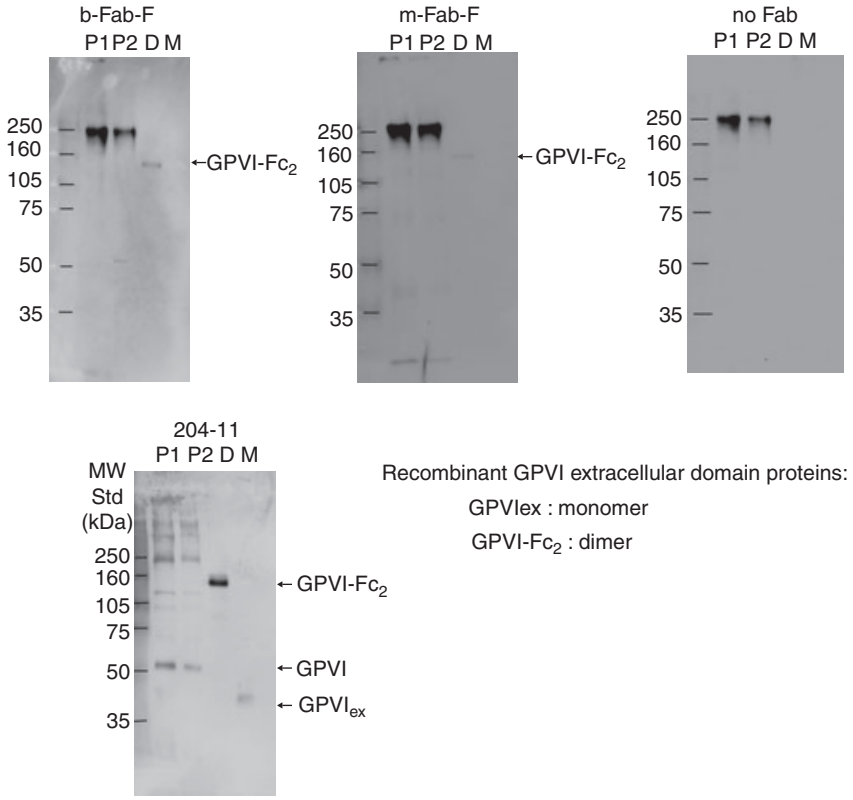

Fig. 3. Immunoblotting analyses using monovalent Fab-F (m-Fab-F) and bivalent Fab-F (b-Fab-F). Platelet lysates (P1, 10 μg; P2, 5 μg; as proteins), glycoprotein VI (GPVI) dimer (GPVI-Fc_2) (D, 0.25 μg) and GPVI monomer (GPVIex) (M, 0.25 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and then electrophoretically transferred to a nitrocellulose membrane. The membranes were reacted with anti-GPVI antibody 204-11(10 μg mL^{-1}), b-Fab-F (10 μg mL^{-1}), or m-Fab-F (10 μg mL^{-1}); reacted with secondary antibody; and then visualized by the chemiluminescence method. Non-specific staining was analyzed by staining with only the secondary antibody (no Fab). Both Fabs reacted very weakly with GPVI-Fc_2 but did not react at all with GPVI of platelet lysate.

Discussion

Recent developments in antibody technology have made it possible to obtain conformation-specific antibodies by screening for antibodies that react specifically with a specific conformation of a protein. With the phage display method, the Fab-expressing phages from the antibody phage library were screened for their ability to specifically bind to GPVI-Fc_2 (GPVI dimer) while showing no affinity for GPVIex (GPVI monomer). Six clones were obtained that specifically bind to GPVI-Fc_2, with one of them, b-Fab-A, showing less specificity for GPVI-Fc_2 because it also weakly interacted with GPVIex (Fig. 1). These Fabs are bivalent forms (Fab-dHLX-MH) that are dimerized through their dHLX domains. The monovalent form of Fab-F was made; this lacks the dHLX domain and so does not dimerize. All of the b-Fabs induced platelet aggregation (Fig. 2). In contrast to b-Fab-F, m-Fab-F inhibited collagen-induced platelet aggregation (Fig. 2C). Sugiyama *et al*. [16] reported a similar characteristic for an antibody from a patient with autoimmune thrombocytopenia: the bivalent IgG fraction induced platelet aggregation but its monovalent form, Fab, inhibited aggregation. The crosslinking of GPVI dimers on the platelet surface by bivalent immunoglobulin or b-Fab would induce platelet aggregation.

All of the b-Fabs inhibited GPVI-Fc_2 binding to collagen fibers, and m-Fab-F also inhibited GPVI binding to collagen fibrils, as b-Fab-F did (Fig. 4). However, the m-Fab-F concentrations required to effectively inhibit collagen-induced platelet aggregation and those needed to inhibit GPVI-Fc_2 binding to fibrous collagen were much higher than the respective concentrations of b-Fab-F. An explanation for this difference is that the bivalency of b-Fab-F could give it higher affinity for GPVI than m-Fab-F. We can try to apply a similar mechanism to explain the affinity differences in collagen binding between monomeric and dimeric GPVI. Although GPVIex showed only insignificant binding to collagen, GPVI-Fc_2 showed a very high affinity for collagen fibers, but, in this case, the difference in affinities was too large to be explained by the effect of bivalency alone [9]. Thus, it would be completely reasonable to hypothesize that the dimerization of GPVI could induce a special structure that significantly contributes to the binding of GPVI to collagen fibers. This hypothesis was checked by using the GPVI-Fc_2-specific antibodies developed in this study. The Fabs specifically recognize the GPVI-dimer conformation, and all of them inhibited the interaction between GPVI and collagen. Although not quantitatively related, the clones with strong affinity for GPVI-Fc_2, b-Fab-B and b-Fab-F, showed higher platelet aggregating activity and greater

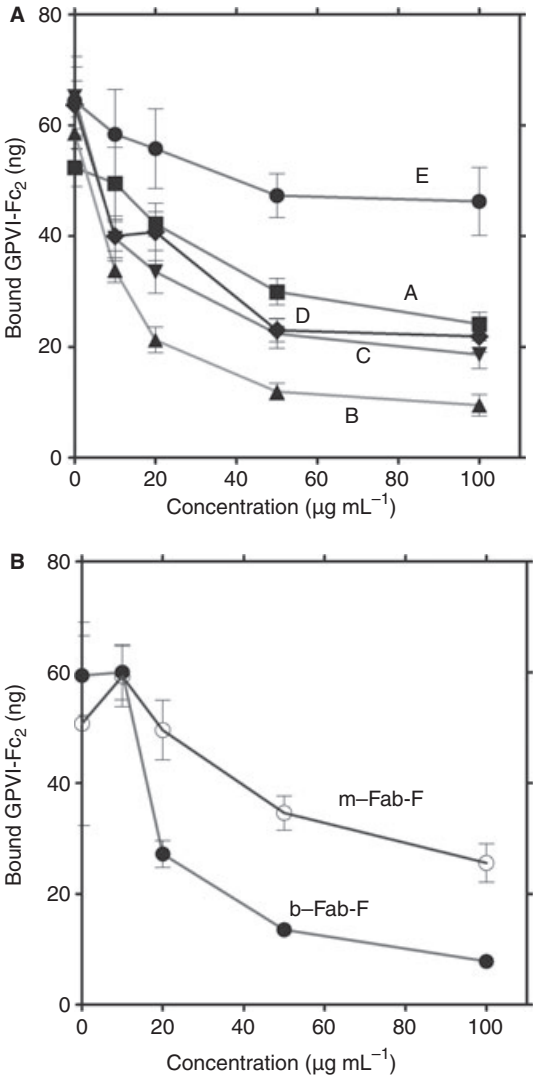

Fig. 4. Effects of the Fabs on glycoprotein VI dimer (GPVI-Fc_2) binding to collagen fibrils. Radiolabeled GPVI-Fc_2 was reacted with collagen fibrils in the presence or absence of Fab, and GPVI binding to collagen fibrils was measured (see Materials and methods). (A) Effects of bivalent Fabs (b-Fabs). Curves (A)–(E) indicate the presence of b-Fab-A to b-Fab–E, respectively. (B) Comparison of the effects of b-Fab-F and monovalent Fab-F (m-Fab-F).

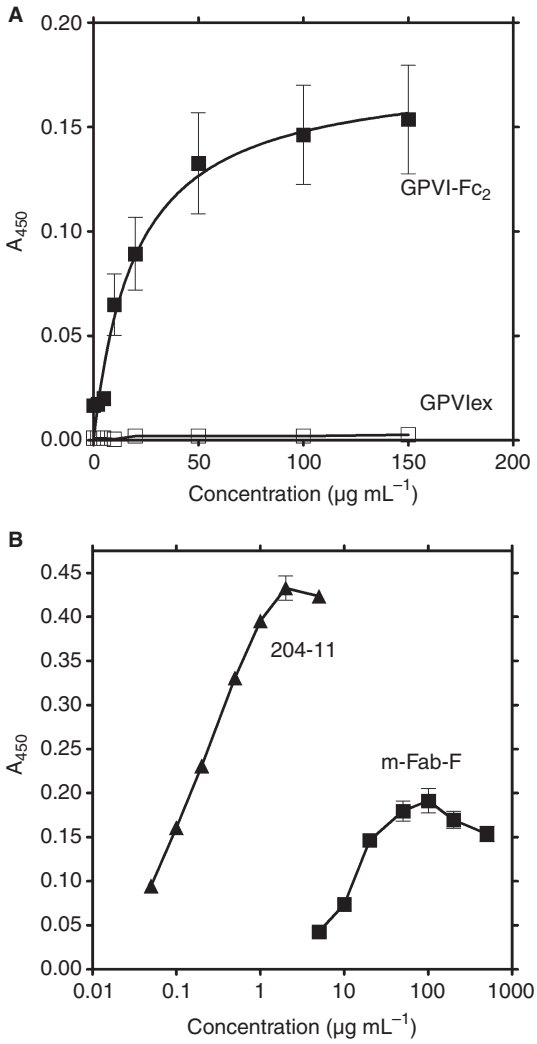

Fig. 5. Analysis of the interaction between monovalent Fab-F (m-Fab-F) and glycoprotein VI (GPVI). (A) The extents of binding of m-Fab-F to GPVI dimer (GPVI-Fc_2)-coated or GPVI monomer (GPVIex) coated wells were analyzed by enzyme-linked immunosorbent assay using horseradish peroxidase-conjugated anti-human IgG $F(ab')_2$ antibody. m-Fab-F bound specifically to GPVI-Fc_2. (B) Comparison of the extents of binding of monoclonal antibody 204-11 and m-Fab-F to GPVI-Fc_2.

inhibitory activity against GPVI binding to collagen; and b-Fab-E, with the weakest affinity for GPVI-Fc_2, had the weakest aggregatory and inhibitory activities. As each of these Fabs exhibits platelet-aggregating activity and inhibitory activity against GPVI binding to collagen that are qualitatively commensurate with its affinity for dimeric GPVI (GPVI-Fc_2), this means that all of these GPVI-Fc_2-specific Fabs recognize a similar conformational structure of GPVI dimer. Thus, the data obtained in this study indicate that this specific dimeric GPVI structure strongly contributes to the interaction between GPVI and collagen.

By analyzing the crystals of the GPVI extracellular domain (GPVI monomer), Horii *et al.* [11] indicated that GPVI exists in a dimeric structure that forms the binding site for collagen. This suggests that molecules of the GPVI extracellular domain would interact with each other and form a dimer in a conformation appropriate for collagen binding when present at sufficiently high concentrations. By extension, this could also be true for the dimer GPVI-Fc_2, in which each component GPVI extracellular domain would be constrained to be in close proximity to the other by the disulfide-linked Fc_2 domains, thereby forming the specific binding site for collagen in GPVI-Fc_2. Thus, the reported crystal structure of GPVI would correspond to the dimer conformation discussed in this article, but it cannot be predicted what part of the reported structure corresponds to the dimer-specific conformation.

Analysis of m-Fab-F binding to GPVI-Fc_2 provided a rather interesting result. The binding of Fab to GPVI-Fc_2 was stimulated when a low concentration of the anti-GPVI

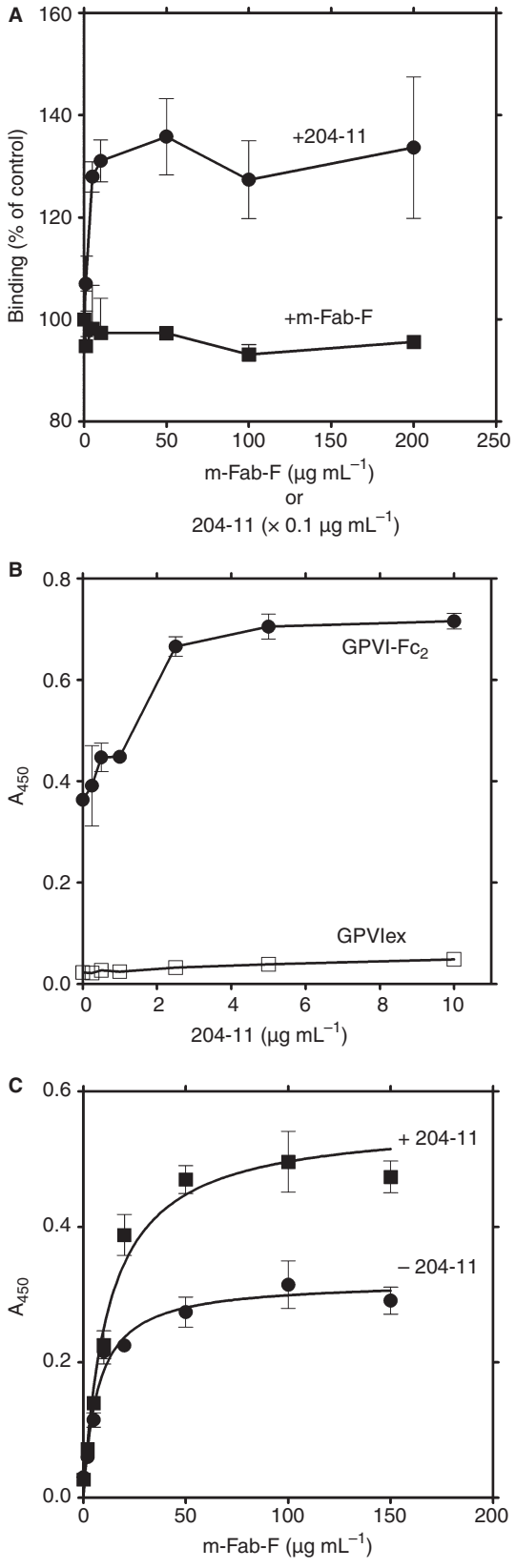

Fig. 6. Effect of monoclonal antibody 204-11 on the interaction between monovalent Fab-F (m-Fab-F) and glycoprotein VI dimer (GPVI-Fc_2). (A) The effect of 204-11 on the binding of m-Fab-F to GPVI-Fc_2 (●) and the effect of m-Fab-F on 204-11 binding (■) were analyzed by enzyme-linked immunosorbent assay. m-Fab-F had no effect on 204-11 binding, but 204-11 increased the binding of m-Fab-F to GPVI-Fc_2 by about 30%. (B) 204-11 increased the binding of m-Fab-F to GPVI-Fc_2 by about 30%, but showed no effect on m-Fab-F binding to GPVI monomer (GPVIex). (C) Analysis of binding of m-Fab-F to GPVI-Fc_2 in the presence (■) or absence of 204-11 (●). Monoclonal antibody 204-11 (1 μg mL^{-1}) increased the number of binding sites for GPVI-Fc_2, consistent with there being a conformational change of GPVI-Fc_2.

monoclonal antibody 204-11 was present. The effective 204-11 concentration range for this effect corresponded to the range for its binding to GPVI (data not shown), suggesting that 204-11 first binds to immobilized GPVI-Fc_2, resulting in increased binding of GPVI dimer to Fab. This is dimer-specific, because no stimulating effect was observed for Fab binding to immobilized GPVIex. The increased Fab binding is apparently not due to a change in affinity, the presence of 204-11 not significantly changing the K_D value, but is due instead to an increase in B_{max}. These results suggest that 204-11 converts a proportion of the GPVI-Fc_2 molecules (i.e., those not already in the appropriate conformation) to a conformation that is reactive with Fab.

Although there have been many reports suggesting that GPVI may exist in a dimeric form on the platelet surface [6,9,11,17], there has been no direct evidence for this until now. The results presented here, showing that all of the b-Fabs induce platelet aggregation, m-Fab-F inhibits collagen-induced platelet aggregation, and m-Fab-F specifically binds to plate-

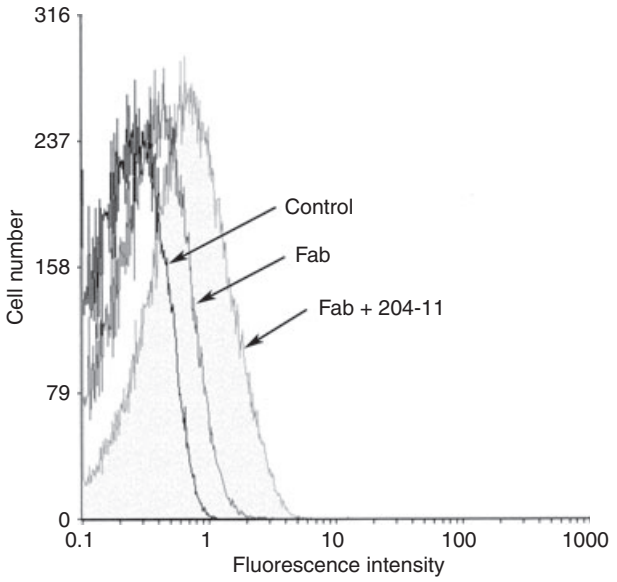

Fig. 7. Flow cytometric analysis of monovalent Fab-F (m-Fab-F) binding to platelets. m-Fab-F (50 μg mL^{-1}) in the presence or absence of monoclonal antibody 204-11 (2 μg mL^{-1}) was incubated with platelet suspension, and the binding of m-Fab-F was analyzed by flow cytometry using fluorescein isothiocyanate-conjugated anti-His-tag antibody. Human IgG $F(ab')_2$ was used as the control. m-Fab-F bound to platelets, and the binding was increased in the presence of 204-11 (shaded curve). This directly indicates that GPVI is present as a dimer on the platelet surface.

lets, as assessed by flow cytometry, all indicated the presence of GPVI dimers on the platelet surface. The present article is the first report directly demonstrating the presence of GPVI dimers on the surface of native platelets. However, the number of GPVI dimers on each platelet could not be quantitatively measured because of the low affinity of the GPVI dimer-specific Fab.

In conclusion, we have developed Fabs that specifically react with the GPVI dimer and demonstrated that this dimer-specific structure is strongly related to the binding of collagen. The binding of these GPVI dimer-specific Fabs to platelets provides direct evidence that GPVI exists as functionally relevant dimers on the platelet surface.

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Disclosure of Conflict of Interests

The author states that he has no conflict of interest.

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