Expression of the Platelet Receptor GPVI Confers Signaling via the Fc Receptor γ-Chain in Response to the Snake Venom Convulxin but Not to Collagen*

Yun-Min Zheng‡§, Changdong Liu‡§, Hong Chen‡, Darren Locke‡, James C. Ryan¶, and Mark L. Kahn‡¶

From the ‡Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the ¶Department of Medicine, University of California at San Francisco and the Veterans Administration Medical Center, San Francisco, California 94121

Platelet activation is essential for both normal hemostasis and arterial thrombosis that occurs in the setting of vascular diseases such as stroke and myocardial infarction. One of the earliest steps in arterial thrombosis is the adhesion of circulating platelets to areas of injured vessel wall and the activation of adherent platelets, which recruits additional platelets to form a hemostatic plug. Activation of platelets at sites of vascular injury occurs in response to exposed subendothelial matrix proteins, the most important of which is collagen. Exposed collagen initiates two essential platelet functions: the adhesion of circulating platelets to the site of injury and the activation of platelet signaling, which stimulates thrombus growth. Platelet adhesion to collagen has been shown to occur both indirectly, via interaction of platelet GPIb with von Willebrand’s factor bound to exposed collagen (1), and directly, via collagen interaction with the platelet integrin α2β1 (2). In contrast, although the activation of platelets by collagen has been observed for over 30 years (3), the receptors and signaling pathways that mediate platelet activation by collagen are only beginning to be fully understood. Indirect evidence suggests that both α2β1 and GPIb can initiate signaling when bound to collagen (1, 4, 5). However, this signaling does not appear to be sufficient to account for the magnitude of the platelet response to collagen.

GPVI is a recently cloned 62-kDa surface protein (6, 7) first identified by iodination of platelet surface glycoproteins (8). GPVI was proposed as a signaling receptor for collagen following the description of individuals with bleeding disorders whose platelets could not be activated by collagen and lacked GPVI despite having normal levels of the platelet integrin α2β1 (9, 10). Significant evidence suggests that GPVI signaling is sufficient to activate platelets and that GPVI may mediate collagen signaling in platelets. Platelets are activated by cross-linked anti-GPVI antibodies (11) and by convulxin (CVX), a multimeric snake venom protein isolated from a South American rattlesnake which is capable of desensitizing platelets specifically to collagen and which binds specifically to GPVI (12, 13). In addition, collagen signaling and GPVI signaling in platelets both employ the immunoreceptor signaling pathway (14) and require Fc Rγ (15). These data have led to a model of collagen activation of platelets in which adhesive roles are played by the integrin α2β1 as well as GPIb and signaling roles are played by GPVI-Fc Rγ, α2β1, as well as perhaps GPIb (14). This model has not been adequately tested, however, because of the absence of systems in which the contribution of each receptor can be studied in isolation.

The recent cloning of human and mouse GPVI (6, 7) reveals that GPVI is a type I transmembrane protein whose deduced amino acid sequence identifies it as an Ig domain-containing receptor homologous to the Fc and killer Ig-like receptors, some...
of which are known to signal via Fc γRy (16). Consistent with its putative role as an Fc Ry partner, GPVI has a charged arginine residue in its transmembrane domain that may mediate interaction with the Fc Ry transmembrane domain in a manner analogous to that of the known Fc Ry partners FcεRI and PIR-A (16–18). Direct functional evidence demonstrating that GPVI is a collagen receptor and that GPVI signaling is mediated by Fc Ry, however, are lacking. Transient expression of GPVI has been demonstrated to confer a slight calcium signal to collagen in the DAMI megakaryocytic cell line (6), but these cells express endogenous GPVI (data not shown and Ref. 6), αβγ alters (19), and perhaps other collagen receptors; it is therefore not clear if that response is mediated directly by GPVI or if GPVI expression is sufficient to enhance signaling by αβγ or other unidentified collagen receptors.

To address the functional role of GPVI, we have stably expressed the receptor in RBL-2H3 cells, a rat basophilic leukemia cell line that expresses Fc Ry and reproduces the platelet collagen responses of intracellular calcium mobilization and degranulation but does not express endogenous GPVI or αβγ. Our studies reveal that GPVI cross-linking by the GPVI-specific ligand convulxin initiates intracellular signaling but that GPVI alone is incapable of mediating a signaling response to collagen. A small signaling response is elicited by collagen-related peptides (CRPs), however, and static adhesion studies support interaction with convulxin and CRP but not collagen. Finally, site-directed mutagenesis of the GPVI transmembrane domain and intracellular C-tail demonstrates that both the GPVI transmembrane arginine and the receptor C-tail are necessary for Fc Ry interaction and intracellular signaling. These results provide insight into GPVI signal transduction and suggest that GPVI-signaling in response to collagen requires coreceptors for both ligand binding and intracellular signal transduction.

**EXPERIMENTAL PROCEDURES**

Materials—Type I collagen derived from equine tendons was obtained from Chronolog (Havertown, PA) and used for all studies shown. Structural studies were performed with type I collagen purified from calf skin (Sigma). Convulxin was obtained from Sigma and purified from the venom of the Crotalus durissus rattlesnake using gel filtration as previously described (13). CRPs were synthesized as previously described using cross-linked cysteine residues (21). All other reagents were obtained from Sigma.

**Cloning and Epitope Tagging of GPVI**—A GPVI cDNA was generated by PCR from human platelet cDNA using primers based on published 5′- and 3′-untranslated sequences (sense strand primer: 5′-TCAGCAA-CAGGGTCTAGAACC-3′; antisense strand primer: 5′-TTGGATAC-GACCGTGCTCGGGG-3′). Three distinct amplified 1.1-kilobase pair products were sequenced to obtain a consensus sequence that exhibited several differences from the published cDNA (6) but agreed with the published cDNA sequence deposited directly in GenBank™ (accession number AB035073). All GPVI receptor amino acids reported here correspond to the protein predicted by the open reading frame of this cDNA starting at nucleotide number 13. FLAG-tagged GPVI was generated by replacing the endogenous signal peptide with that of interleukin-1 and placing the FLAG epitope (DYKDDDK) in frame with GPVI at amino acid number 21, the predicted site of signal peptide cleavage (SignalP V1.1). HA-tagged GPVI was generated in an identical manner. Wild-type and epitope-tagged GPVI were expressed using the mammalian expression vector pCDNA3.0 (Invitrogen).

**Site-directed Mutagenesis of GPVI**—Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene). The oligonucleotide used for the R272L mutation was 5′-GCAACCTGGTACTGATGCTCGCGGTCTGGCTGTTG-3′. The oligonucleotide used for the R295STOP mutation was 5′-GGCAGAGGACTGACAGCTAGAGAAGGCGCTGC-3′. All mutants were made as epitope-tagged receptors as described above.

**Platelet Aggregation Studies**—Blood was collected into citrate buffer and platelet-rich plasma obtained as previously described (22). All studies were performed using platelet-rich plasma at a platelet density of 2 × 10^10 platelets/ml.
uously described (26). Briefly, 20 μl of 0.5% Triton X-100 in PBS were added to each well to lyse the bound cells. 80 μl of 1 mM substrate (p-nitrophenol N-acetyl-β-D-glucosaminide; Sigma catalog no. N9376) in 0.05 M citrate buffer, pH 4.5, were subsequently added. After 1 h of incubation at 37 °C, 100 μl of 0.1 M sodium carbonate, 0.1 M sodium bicarbonate were added per well. The A<sub>405</sub> was measured with an Emax precision microplate reader (Molecular Devices, Inc., Sunnyvale, CA).

RESULTS

GPVI-expressing RBL-2H3 Cells Signal in Response to Convulxin and CRP but Not to Collagen—RBL-2H3 cells stably expressing wild-type GPVI (GPVI-RBL) and FLAG-tagged GPVI (FLAG GPVI-RBL) were identified using adhesion to convulxin and flow cytometry to detect surface FLAG epitope, respectively. The ability of the putative GPVI ligands collagen, CRP, and convulxin to initiate calcium signaling in RBL, GPVI-RBL, and FLAG GPVI-RBL cells was tested in parallel with aggregation studies of human platelets performed using the same reagents on the same day (Fig. 1, Table I, and data not shown). GPVI-RBL and FLAG GPVI-RBL, but not untransfected RBL cells, responded to convulxin with a threshold concentration only 1.6-fold greater than that found necessary to aggregate human platelets (0.3 nM). These results demonstrate functional GPVI signaling and close concordance between the two assays for GPVI dose response, suggesting similar GPVI receptor density in the two cell types. In contrast, collagen elicited no response even at concentrations 500 times greater than that necessary to aggregate human platelets (100 μg/ml). Similar results were obtained using three distinct GPVI-RBL clones and three distinct FLAG-GPVI RBL clones. The data shown are representative of experiments performed in RPMI with Chronolog Type I collagen, which elicits the most robust platelet

![Fig. 1: GPVI signaling in RBL-2H3 cells](image)

**Table I**

Comparison of signaling by putative GPVI ligands in GPVI-expressing RBL cells and in platelets

| Agonist | Platelet activation | GPVI-RBL signaling | Platelet/GPVI RBL signaling ratio |
|---------|---------------------|---------------------|---------------------------------|
| Convulxin | 0.3 nM | 0.5 nM | 1:1.6 |
| CRP | 0.1 μg/ml | 50 μg/ml | 1:500 |
| Collagen | 0.2 μg/ml | >100 μg/ml | >1:500 |
responses in our hands (data not shown). In addition, no signal responses were observed using a second source of type I collagen, type III collagen, or BSA, and adhesion of wild-type and GPVI-expressing RBL cells was measured at 405 nm using a colorimetric substrate of the endogenous RBL enzyme hexosaminidase. a, adhesion of wild type RBL cells (open bars) and GPVI-expressing RBL cells (black bars) in the PBS with 0.9 mM calcium and 0.4 mM magnesium. b, adhesion of wild type RBL cells (open bars) and GPVI-expressing RBL cells (black bars) in the presence of EDTA (5 mM). The results shown are the mean and S.D. of quadruplicate samples from a single experiment. Each experiment is representative of 3–5 similar experiments performed with distinct GPVI-expressing clones. Note the strong adhesion of GPVI-expressing cells to CVX, intermediate adhesion to CRP, and lack of detectable adhesion to collagen.

**Fig. 2. Adhesion of GPVI-expressing RBL cells to putative GPVI ligands.** Microtiter plates were coated with type I collagen, CRP, CVX, fibronectin, or BSA, and adhesion of wild-type and GPVI-expressing RBL cells was measured at 405 nm using a colorimetric substrate of the endogenous RBL enzyme hexosaminidase. a, adhesion of wild type RBL cells (open bars) and GPVI-expressing RBL cells (black bars) in the PBS with 0.9 mM calcium and 0.4 mM magnesium. b, adhesion of wild type RBL cells (open bars) and GPVI-expressing RBL cells (black bars) in the presence of EDTA (5 mM). The results shown are the mean and S.D. of quadruplicate samples from a single experiment. Each experiment is representative of 3–5 similar experiments performed with distinct GPVI-expressing clones. Note the strong adhesion of GPVI-expressing cells to CVX, intermediate adhesion to CRP, and lack of detectable adhesion to collagen.

**Loss of the GPVI Transmembrane Domain Arginine and C-tail Results in Loss of Coupling to Fc Rγ**—To determine why the R272L and R295STOP mutants of GPVI no longer supported signaling by CRP, we compared the ability of wild-type and mutant GPVI receptors to interact with Fc Rγ by a functional assay in HEK-293T cells stably expressing FLAG-Fc Rγ and by direct biochemical means using coprecipitation. As for HEK-293T cells engineered to express low levels of the homologous immunoreceptor signaling adaptor DAP-12 (25), FLAG-Fc Rγ is not expressed on the cell surface of these cells in the absence of a coexpressed Fc Rγ partner (Fig. 4A). The ability of an expressed receptor to rescue surface expression of FLAG-Fc Rγ therefore measures functional association with Fc Rγ. Wild-type GPVI expression rescued 10 times more surface FLAG-Fc Rγ than mock-transfected cells (Fig. 4A). In contrast, GPVI R272L expression failed to rescue any FLAG-Fc Rγ, consistent with a complete loss of association with Fc Rγ (Fig. 4A). GPVI R295STOP expression also failed to rescue surface FLAG-Fc Rγ, indicating a lack of Fc Rγ interaction (Fig. 4A). Surface staining for the HA epitope confirmed that wild-type and mutant GPVI receptors were expressed at equivalent levels (Fig. 4B) and demonstrates that, as for the related Fc Rγ partner PI(18), GPVI expression in HEK-293T cells does not require Fc Rγ interaction. Loss of Fc Rγ interaction in GPVIR272L and GPVIR295STOP was confirmed biochemically using convulxin to precipitate GPVI and subsequently assaying for associated Fc Rγ by immunoblotting (Fig. 5). Convulxin precipitation of wild-type GPVI, but neither mutant receptor resulted in coprecipitation of Fc Rγ (Fig. 5). Interestingly, immunoblotting of GPVI-R295STOP protein with anti-FLAG antibody reveals the presence of mature protein at the predicted molecular mass of ~57 kDa (5 kDa smaller than the wild-type and R272L receptors) and the presence of a large

**The GPVI Transmembrane Arginine and Intracellular C-tail Are Both Necessary for GPVI Signaling**—The signaling roles of the GPVI transmembrane (TM) domain arginine (R272) and intracellular C-tail were tested by generating RBL cell lines expressing FLAG-tagged receptors in which the TM arginine is replaced by leucine (R272L-RBL) and the C-tail is truncated shortly following the TM domain (R295STOP-RBL). Both mutant GPVI receptors were expressed on the surface of RBL cells at levels equal to or greater than clones expressing wild-type GPVI (Fig. 3B). Consistent with the results of analogous mutations in related receptors, R272L-RBL did not signal in response to convulxin, confirming a necessary role for the GPVI TM domain arginine for signal transduction. Surprisingly, unlike similar C-tail truncation mutants of the Fc Rγ RI and Fc Rγ RIII (see Fig. 6), RBL cells expressing the GPVI C-tail truncation mutant R295STOP also failed to signal to convulxin, demonstrating an unexpected necessary role for the GPVI C-tail (Fig. 3C).
amount of protein at 36–38 kDa, the predicted size for unglycosylated, incompletely processed protein. It is possible that this lower molecular mass species represents protein that cannot reach the cell surface because it cannot couple to FcRγ partners and is therefore targeted for degradation. The fact that the abundantly expressed R272L mutant escapes this fate supports the role of the transmembrane arginine in targeting unpartnered receptors for degradation. These results show that the GPVI transmembrane arginine is necessary but not sufficient for functional association with the receptor’s signaling coreceptor FcRγ and that loss of signaling following truncation of the GPVI C-tail is due to loss of FcRγ interaction rather than loss of an unidentified, distinct signaling function.

**DISCUSSION**

Recent studies of human platelets that are unresponsive to collagen (10), mouse knockouts (27), and platelet signaling (reviewed in Ref. 14) have generated the hypothesis that the platelet surface protein GPVI mediates collagen signaling and does so through its interactions with the immunoreceptor signaling adaptor FcRγ. We have expressed GPVI in RBL-2H3 cells and studied GPVI signaling in a heterologous system to directly and formally address this hypothesis. RBL-2H3 cells express endogenous FcRγ and are a model cell line for studying FcεRI receptor signaling (28). Like platelets, the signaling end points achieved by FcεRI receptor cross-linking and FcRγ signaling in RBL-2H3 cells include mobilization of intracellular calcium and degranulation. Unlike the megakaryocytic cell lines DAMI, HEL, and MEG-01, however, RBL-2H3 cells express neither endogenous GPVI nor the integrin receptor for collagen α2β1 (data not shown). Thus, RBL-2H3 cells express the appropriate signaling machinery to study GPVI signaling without the ambiguity of endogenous collagen receptor expression.

Expression of wild-type and FLAG-GPVI conferred robust calcium signaling to the snake venom protein convulxin at a threshold concentration equivalent to that necessary to activate human platelets but no detectable response to collagen at a concentration more than 500 times greater than that necessary to activate human platelets (Fig. 1). Unlike collagen, convulxin has been demonstrated to directly bind GPVI (13, 29) and was used by Clemetson et al. (6) to purify the GPVI protein from platelets. Thus, RBL-2H3 cells express the appropriate signaling machinery to study GPVI signaling without the ambiguity of endogenous collagen receptor expression.

**FIG. 3. Expression and function of GPVI transmembrane and C-tail mutants in RBL-2H3 cells.** FLAG-tagged wild-type (WT), R272L, and R295STOP GPVI receptors were stably expressed in RBL cells and tested for their signaling responses to the GPVI ligand CVX using the calcium-sensitive dye Fura-2. **A.** schematic diagrams of the wild-type and mutant receptors expressed are shown to highlight the sites of mutation and the proposed point of interaction with the signaling adaptor FcRγ. **EC**, receptor extracellular domain; **TM**, receptor transmembrane domain; **IC**, receptor intracellular domain; **R**, the GPVI transmembrane domain arginine; **L**, the leucine substituted for R272 in the R272L mutant; **D**, the FcRγ transmembrane domain aspartate; **Y**, FcRγ ITAM tyrosine residues. **B.** receptor surface expression measured with anti-FLAG antibody in control RBL cells (thin lines) and GPVI-expressing RBL cells (thick lines). **C.** calcium signaling of GPVI-expressing RBL cells in response to CVX (10 nM) and thrombin (10 nM). These results are representative of identical experiments performed with 3–5 distinct clones for each receptor type.
convulxin and weaker binding to CRP but no detectable binding to collagen. Thus, the adhesion to immobilized proteins conferred by GPVI expression parallels the signaling responses observed to soluble agonists. These results are in contrast to those recently reported by Jandrot-Perrus et al. (7), who detected a small amount of collagen binding in a monocytic cell line (U937) stably expressing human or mouse GPVI. This discrepancy could reflect a difference in GPVI receptor density between the stable cell lines used or differences in methodology. In our hands, however, even clones expressing very high levels of GPVI such as the R272L clones (whose extracellular domains are wild type) confer adhesion to both CRP and convulxin but not collagen (data not shown and Fig. 3).

Is GPVI a *bona fide* collagen receptor, and, if so, why is GPVI expression insufficient to confer collagen signaling? Inadequate receptor density on RBL-2H3 cells is not a likely explanation, since the dose-response to CVX is similar in platelets and in GPVI-expressing RBL cells (Table I). One potential explanation for these results is that GPVI does mediate collagen signaling but that another coreceptor is required. This coreceptor might facilitate direct GPVI-collagen binding, or GPVI might mediate collagen signaling indirectly by linking a ligand-binding coreceptor to the signaling adaptor Fc Rγ. Of the reported platelet collagen receptors, including the integrin α2β1 (2), glycoprotein IV (30), and p65 (20), the most likely candidate is the α2β1 integrin, whose high affinity for collagen may bring collagen to the platelet surface in an apparent concentration and/or configuration necessary for GPVI binding and signal transduction, although the precise role of α2β1 remains controversial (31). Alternatively, GPVI may not be involved in collagen signaling, and an as yet unrecognized Fc Rγ partner may be the true collagen receptor.

Several lines of evidence support the model that collagen is a GPVI ligand but that GPVI absolutely requires a coreceptor such as α2β1 for productive collagen interaction. CRPs, which structurally closely resemble collagen but are more potent activators of platelets (21), initiate a small amount of intracellular signaling in GPVI-expressing but not wild-type RBL-2H3...
GPVI Signals via Fc Rγ to Convulxin but Not to Collagen

Fig. 5. Co precipitation of Fc Rγ with wild-type and mutant GPVI receptors expressed in RBL cells. The interaction of Fc Rγ with wild-type and mutant GPVI receptors in RBL cells was determined biochemically by precipitation of GPVI receptors with convulxin-coated beads. CVX-precipitated protein was probed for GPVI using anti-Fc Rγ antibody (upper panel) and for Fc Rγ using anti-Fc Rγ antibody (lower panel). RBL, untransfected RBL cells; RBL-GPVI, RBL cells stably expressing wild-type GPVI; RBL-GPVR272L, RBL cells stably expressing GPVR272L; RBL-GPVR295STOP, RBL cells stably expressing GPVR295STOP; +, precipitation with convulxin-coated beads; −, control precipitations with BSA-coated beads. The GPVI-expressing RBL cell lines used were the same as those analyzed for surface expression and signaling in Fig. 3.

Amino acid alignment of the transmembrane and intracellular domains of GPVI and other Fc Rγ receptor partners. Alignment of the deduced amino acid sequences of human GPVI (hGPVI), mouse GPVI (mGPVI), Fcα, PIRα, Fcγ RI, and Fcγ RIII receptors was performed using the ClustalW program (Macvector). Amino acid identities shared among the GPVI, Fcγ RI, and Fcγ RIII receptors are boxed. In boldface type, * the site of C-tail truncation for GPVI R295STOP; **, the site of C-tail truncation for GPVI R272L; boxed, portion of GPVI C-tail containing a significant number of basic amino acids; proline, portion of GPVI C-tail containing a cluster of proline residues; S/T, portion of human GPVI C-tail with a significant number of serine and threonine residues.
C-tail facilitates Fc Rγ interaction and whether GPVI-related receptors also require their C-tails for Fc Rγ coupling remains uncertain and awaits further mutational analysis.

These studies provide the first functional analysis of GPVI as a signaling receptor and raise several important questions regarding the role of GPVI in vivo. The inability of GPVI to respond directly to collagen may suggest the evolution of a receptor adapted to operate in a highly specialized cellular environment in cooperation with other collagen receptors such as the integrin α_IIbβ_3 (a hypothesis supported by the megakaryocytic-specific pattern of expression of the receptor’s mRNA (data not shown, and see Ref. 27), but the possibility that GPVI does not mediate collagen signaling cannot yet be definitively excluded. Our results extend the proposed model of platelet collagen signaling to one requiring no fewer than four receptor subunits and establish a heterologous system in which to further dissect this signaling pathway. Identification of the receptors involved in collagen activation of platelets and the molecular basis for this response may provide novel targets for anti-platelet therapies, which act at a critical point in thrombogenesis, the activation of newly adherent platelets at sites of atherosclerotic rupture.

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