Delineation of the Region in the Glycoprotein VI Tail Required for Association with the Fc Receptor γ-Chain*

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The glycoprotein VI (GPVI)–Fc receptor γ-chain (FcRγ-chain) complex is the major activation receptor for collagen on platelets. GPVI cross-linking mediates activation through tyrosine phosphorylation of an ITAM (immunoreceptor tyrosine-based activation motif) in the FcRγ-chain by Src family kinases. It has been previously shown that a transmembrane arginine and the cytoplasmic domain of GPVI are required for association with the FcRγ-chain in immortalized cell lines. In this study, we have delineated the regions in the GPVI tail that promote binding to FcRγ-chain and mediate functional responses to the snake venom convulxin by reconstitution of mutant forms of GPVI in RBL-2H3 cells. Sequential truncation of the cytoplasmic tail of GPVI revealed a major role for the basic region and a minor role for the juxtamembrane six amino acids in the association with FcRγ-chain and functional responses to convulxin. Analysis of selective deletions in the GPVI tail supported this conclusion. In addition, we show that the proline-rich domain is required for optimal Ca²⁺ release, whereas it is dispensable for FcRγ-chain association.

The response of platelets to vessel wall injury is a primary event in arterial thrombosis (1). The adhesion and activation of platelets by subendothelial collagen fibers initiates aggregate formation at sites of vessel damage. Glycoprotein VI (GPVI) is the major activator collagen receptor in platelets and megakaryocytes. GPVI forms a complex with the Fc receptor (FcR)γ-chain (2, 3) and this association is essential for the generation of intracellular signals (3). The ITAM (immunoreceptor tyrosine-based activation motif) of FcRγ-chain is phosphorylated by two Src kinases, Lyn and Fyn (4, 5), leading to recruitment and activation of Syk (6), which initiates a signaling cascade that culminates in activation of phosphatidylinositol 3-kinase, phospholipase Cγ2, and small G proteins, leading to platelet activation (7).

GPVI is a member of the immunoglobulin (Ig) superfamily, showing close homology to Fce receptor, GPVI along with FcRγ-chain is sufficient to reconstitute responses to the GPVI-specific snake venom toxin, convulxin (11–13). Collagen has also been shown to mediate activation of cells expressing a high level of GPVI and the FcRγ-chain (14). GPVI has a charged arginine residue in its transmembrane domain that is necessary for association with the FcRγ-chain (12, 13).

The human GPVI cytoplasmic domain is 50 amino acids long and can be divided into several distinct regions: juxtamembrane, basic, proline-rich, and C-terminal (Fig. 1A) (13). We have shown previously that the proline-rich sequence mediates association with Fyn and Lyn via their Src homology 3 domains (15) and is required for tyrosine phosphorylation of the ITAM in transfected COS-7 cells. The basic region contains a sequence that has been shown to mediate association with calmodulin (16).

In the present study we have investigated the amino acids sequences in the cytoplasmic domain of GPVI required for association with the FcRγ-chain and activation of functional responses. Using RBL-2H3 cells stimulated with GPVI-agonist snake venom convulxin, we demonstrate that 6 amino acids of the juxtamembrane domain and the basic region of GPVI cooperate to mediate binding to the FcRγ-chain and subsequent generation of Ca²⁺ release. In addition, we show that the proline-rich region is not required for association with the FcRγ-chain but is necessary for optimal signaling.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Convulxin and anti-convulxin antibody were generous gifts from Drs. Mireille Leduc and Cassian Bon (Institut Pasteur, Paris, France). FITC-conjugated anti-rabbit IgG (Fab')2 fragments and anti-FLAG M2 monoclonal antibody were purchased from Sigma. Anti-FcRγ-chain polyclonal antibody was purchased from Upstate Biotechnology, Inc. (TCS Biological Ltd., Botesph, Claydon, UK). Dulbecco’s modified Eagle’s medium and Trypsin-EDTA were from Invitrogen. Other reagents were from previously described sources (12, 17).

Cell Culture—COS-7 cells and RBL-2H3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum under 5% CO₂ at 37 °C in a humidified incubator. Cells were kept at exponential phase of growth. Adherent cells were detached by incubating with trypsin-EDTA for 5 min at 37 °C before washing.

Constructs—The cDNA for human GPVI was subcloned into HindIII/XbaI sites of pRC plasmid (FLAG-tagged). The construct encoded a fusion protein with the N-terminal region corresponding to human GPVI and FLAG sequence at the C terminus. The cDNA for human FcRγ-chain was subcloned into pMIG (Invitrogen, San Diego, CA). All sequences were verified by sequencing.

Site-directed Mutagenesis of GPVI—Site-directed mutagenesis of GPVI was performed using the QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotide sequence used for mutagenesis was 5′-cgtcgccctccccctttgagactaacaag-3′; for Δ309GPVI, 5′-ggtccagggcgctttcatgactaacaag-3′; for Δ289GPVI, 5′-gaagcagccgctttgagactaacaag-3′.

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GPVI-FcR γ-Chain Complex

| Transmembrane | juxta | basic | proline | C-terminal tail |
|---------------|-------|-------|---------|----------------|
| KGNLRVIGAVIIILAGFLAEDWRRKLRHGRAVQQPPLPPPLPPPLPPQTRKSGQGDGRQDVQSRGLCS | 267-339 | h-GPVI |
| KGNLRVIGAVIIILAGFLAEDWRRKLRHGRAVQQPPLPPPLPPPLPPQTRKSGQGDGRQDVQSRGLCS | 265-313 | m-GPVI |
| TONGRAGVLAIIIILAGFLAEDWRRKLRHGRAVQQPPLPPPLPPPLPPPLPPQ | 129-191 | h-FccR |
| MELSGMCVQSGSLAQATRHPPLAQ | 644-690 | m-PRIA |
| PQLCYLDAFLPLYGIVLTLVCRKLQVRKADASRESDKDAYSQGLNTRQETYTLKHEFKPQ | 22-86 | r-FcγRγ-chain |

Fig. 1. Amino acid alignment of the transmembrane and intracellular domains of GPVI and related receptors. A, alignment of the deduced amino acid sequences of human GPVI (h-GPVI), mouse GPVI (m-GPVI), human FcγR-I (h-FcγR-I), mouse PIR-A receptors (m-PIR-A), and rat FcγR chain (r-FcγR chain). Amino acids that are shared among the receptors are shaded. B, sequences of wild type, C-terminal truncated, deleted, and point-mutated GPVI receptors used in the study. Point mutations are shown with a gray background.

tacaag-3' for ΔbasicGPVI, 5'-gagagccgtgacaagcagctgagagcagc-3' for ΔjuxtaGPVI, 5'-gctgggtgctgcaagcagctgagagcagc-3' for E289A/D290A GPVI, 5'-gctgggtgctgcaagcagctgagagcagc-3' for W291A/H292A/S293A, 5'-gctgggtgctgcaagcagctgagagcagc-3' for W291A, 5'-gctgggtgctgcaagcagctgagagcagc-3' for H292AGPVI, 5'-gctgggtgctgcaagcagctgagagcagc-3' and for S293A GPVI, 5'-gctgggtgctgcaagcagctgagagcagc-3'. The constructs Δ286GPVI and R272AGPVI have been described previously (12, 15). All mutated sequences were verified by sequencing.

**Transient and Stable Transfections**—For stable transfections, 1 × 10⁷ RBL-2H3 cells were washed once with serum-free medium and once with Cytomix buffer (8.9 mg/ml KCl, 1.8 mg/ml CaCl₂, 1.7 mg/ml KH₂PO₄, 0.1 mg/ml KH₄PO₄, 6 mg/ml HEPES, 8.5 mg/ml EGTA, 5 mm MgCl₂, pH 7.6) supplemented on the day of the experiment with 0.37 mg/ml aprotinin, and 1 mg/ml leupeptin, 1 mg/ml bovine serum albumin. The constructs Δ286GPVI and R272AGPVI were transfected into COS-7 cells, the extract was incubated with 10 μg/ml convulxin for 2 h. 0.4 μg/ml anti-convulxin antibody was added, and the sample was left overnight. On the following day, 30 μl of protein A-Sepharose (v/v) was added and incubated for 1 h before washing the samples three times with TBS-T-Tris-buffered saline/Tween 20 before final resuspension in Laemmli sample buffer without 2-mercaptoethanol (non-reducing condition).

**Immunoblotting and Ligand Blotting**—Proteins were separated by SDS-PAGE on 10–15% gels, electrotransferred, and blotted as described previously (17). For GPVI detection, membranes were incubated with 0.1 mg/ml convulxin for 1 h at room temperature and incubated with anti-convulxin antibody as described previously (18). Cells were stimulated with low Ca²⁺-RBL-2H3 cells were resuspended in RPMI 1640 (Invitrogen) with HEPES 25 mg/ml and 1 mg/ml bovine serum albumin. Cells were loaded with the Ca²⁺ reporter dye Fura-2 by incubation with 3 μM Fura-2-AM for 1 h at 37 °C. Cells were stimulated with low Ca²⁺ in a fluorometer with an excitation wavelength of 530 nm and emission wavelengths at 330 and 380 nm. The ratio of Fura-2 emissions was measured and analyzed using FWinlab software. Ratios were plotted using a FACScalibur (BD Biosciences). Data were recorded and analyzed using CellQuest software.

**RESULTS**

**Effect of Truncation of Cytoplasmic Amino Acids of GPVI on Functional Responses in RBL-2H3 Cells**—We demonstrated previously that transmembrane arginine 272 and the cytoplasmic tail of GPVI are necessary for association with the Fcγ γ-chain and activation of the tyrosine kinase Syk (12). To identify the region within the cytoplasmic tail of GPVI mediating association with Fcγγ-chain and subsequent functional responses, we generated a series of sequential deletion mutants.
as illustrated in Fig. 1B. A FLAG sequence was added at the C-terminal end of all GPVI constructs to facilitate detection of transfected proteins. Δ316GPVI lacks the last 22 C-terminal amino acids, which are absent in murine GPVI (9). Δ309GPVI lacks additional 7 amino acids covering the proline-rich region, which promotes binding to the Src homology 3 domains of the GPVI-FcR γ-Chain Complex.
Src family kinases, Fyn and Lyn (15). Δ294GPVI lacks additional 15 amino acids covering the basic region, which has been shown to mediate association with calmodulin. Δ294GPVI possesses 6 cytosolic amino acids and has been termed the juxtamembrane domain (amino acids 289–294). The same 6 amino acids are conserved in the juxtamembrane region of two other FcR γ-chain-associated receptors, Fcε receptor and PIR-A (Fig. 1B) (19, 20). Δ288GPVI is truncated at the interface of the transmembrane and cytoplasmic domains.

As many of the proteins in the GPVI signaling cascade are restricted to hematopoietic cells, we chose to express GPVI in RBL-2H3 cells, a rat basophilic leukemia cell line that expresses the FcRγ-chain but not GPVI (13). Cells were stably transfected with GPVI and its various mutants. All clones expressed a level of GPVI at the membrane surface similar to that in cells transfected with the wild type receptor, as measured by flow cytometry using the GPVI snake venom agonist convulxin as shown in Fig. 2A. The significance of small differences in expression levels between clones was addressed through the use of more than one clone for each construct. Convulxin stimulated a rapid and robust increase in intracellular Ca\(^{2+}\) in RBL-2H3 cells transfected with wild type and Δ316GPVI, which lacks the last 22 amino acids; there was no apparent difference in the pattern of response in the two cell lines (Fig. 2B).

In Δ309GPVI-expressing cells, which also lack the proline-rich region, a maximally effective concentration of convulxin (10 μg/ml) stimulated a peak level of intracellular Ca\(^{2+}\) similar to that in cells transfected with wild type receptor, although the time to the plateau was significantly delayed (Fig. 2B). Accordingly, in Δ309GPVI-expressing cells, the dose-response curve for Ca\(^{2+}\) elevation to convulxin is shifted by more than 1 order to the right of that in cells expressing wild type GPVI (Fig. 2C). This demonstrates that the proline-rich sequence in the GPVI cytosolic tail is required for optimal signaling by convulxin in transfected RBL cells. This is consistent with our previous observation of a role for this region of GPVI in mediating activation through the recruitment of the Src kinases Fyn and Lyn to GPVI-FcRγ-chain complex (15).

In Δ294GPVI-expressing cells, convulxin (10 μg/ml) stimulated a slower increase in intracellular Ca\(^{2+}\), which reached a plateau that was less than 50% of that obtained in cells trans-

![Graph](image-url)
separated by SDS-PAGE under nonreducing conditions. Membranes and GPVI was precipitated with anti-FLAG M2 antibody. Samples were the cytoplasmic tail of GPVI in mediating activation.

not shown), suggesting an important role for the basic region of polyvinylidene difluoride membranes, and blotted with anti-FcR vulxin. Samples were separated by 12.5% SDS-PAGE, transferred to WT (GPVI or other mutant receptors were transiently transfected with FcR/H9253). This demonstrates a critical role for the juxtamembrane domain in response to convulxin in RBL-2H3 cells.

Experiments were designed to investigate whether the inhibition of the Ca2+ signal brought about by the truncation of GPVI could be explained by an alteration in the association with the FcRγ-chain. This question was investigated in the stably transfected RBL-2H3 mutants as well as in COS-7 cells transiently transfected with the same mutants.

A similar level of association between GPVI and FcRγ-chain was observed in RBL cells transfected with wild type, Δ316GPVI, and Δ309GPVI as estimated by co-precipitation (Fig. 3A). These results show that the C-terminal domain and proline-rich region are dispensable for the association with the FcR γ-chain. The likely explanation, therefore, for the reduction in response observed in the cells expressing Δ309GPVI is the loss of association with Fyn and Lyn as described previously (15).

In contrast, the FcR γ-chain was barely detectable in GPVI precipitates from Δ294GPVI-expressing cells, suggesting an involvement of the basic region in the association with FcRγ-chain (Fig. 3A). Significantly, we also identified an additional contribution of the juxtamembrane sequence in the association with the FcR γ-chain, because the co-precipitation with FcRγ-chain was totally lost in Δ288GPVI-expressing cells (Fig. 3A). The strong decrease in association and absence of association obtained with the Δ294 and Δ288 constructs, respectively, corresponds with the increases in Ca2+ induced by convulxin in the transfected lines.

Complementary experiments were performed in COS-7 cells co-transfected with FcR γ-chain and cytosolic mutants of GPVI. We initially confirmed that wild type GPVI co-precipitates with FcR γ-chain, whereas Δ288GPVI, which lacks the cytosolic sequence, does not (12). Interestingly, all of the other GPVI mutants including Δ294GPVI, which possesses only the 6 juxtamembrane amino acids, associate with transfected FcR γ-chain in COS-7 cells (Fig. 3B). This result indicates that when both GPVI and FcR γ-chain are overexpressed, the 6 juxtamembrane amino acids of GPVI are sufficient to mediate association with FcR γ-chain or that there is a difference in the association of GPVI with human and rat FcR γ-chain.

**Effect of Deletion of Functional Domains in GPVI Cytosolic Tail on Ca2+ Mobilization**—To evaluate further the ability of the juxtamembrane and the basic regions of GPVI to induce functional responses through FcR γ-chain, we created additional mutants of GPVI that lacked the functional sequences in the cytosolic tail. ΔjuxtaGPVI is lacking the 6 amino acids of the juxtamembrane domain and ΔbasicGPVI is lacking the first 5 amino acids of the basic region, disrupts the calmodulin binding site (16) (Fig. 1B). These GPVI mutants were stably transfected into RBL-2H3 cells, and clones were selected that expressed similar levels of GPVI on their cell surface (Fig. 4A). ΔbasicGPVI-transfected cells exhibited a robust increase in Ca2+ similar to cells expressing wild type GPVI (Fig. 4B) demonstrating that this region is not necessary for the generation of intracellular signals. In comparison, in cells transfected with ΔjuxtaGPVI, no increase in Ca2+ was detected in response to convulxin. In addition, ΔjuxtaGPVI presents a very marginal association with FcR γ-chain suggesting that the absence of the increase in Ca2+ in response to convulxin was because of lack of association with FcR γ-chain (Fig. 4C). These results demonstrate that the 6 juxtamembrane amino acids of GPVI are necessary for association with the FcR γ-chain and the generation of intracellular signals by convulxin.

**Negative and Positive Charges of Juxtamembrane Domain of GPVI Are Not Necessary for Ca2+ Release**—The proximal amino acids of the GPVI-juxtamembrane domain are conserved in PIR-A and Fco receptor (19); the mutation of a negatively charged amino acid in this sequence from PIR-A (Glu-643 of mPIR-A) decreases its association with FcR γ-chain (20). To evaluate the involvement of the conserved negative charges in the juxtamembrane domains of GPVI, Ghu-289 and Asp-290 of GPVI were mutated to alanine (E289A/D290A GPVI) and stably transfected in RBL-2H3 cells. We also created mutants of the conserved juxtamembrane amino acids Trp-291, His-292, Ser-293, independently and together (W291A, H292A, S293A and W291A/H292A/S293A).
These mutants were stably transfected in RBL-2H3 cells and clones were selected that expressed levels similar to those measured by flow cytometry (Fig. 5A). All mutant GPVI-expressing RBL-2H3 cells responded to the snake venom convulxin in a manner similar to cells expressing wild type GPVI, demonstrating that these amino acids are not required for the generation of intracellular signals (Fig. 5B). In contrast, and in agreement with previous studies (12), cells expressing a point mutation of the transmembrane arginine (R272A GPVI) did not exhibit a functional response to convulxin (Fig. 5B). The lack of signaling by convulxin in R272L GPVI-expressing cells was in agreement with the inability of R272L GPVI to co-precipitate with FcR γ-chain (Fig. 5C).

**DISCUSSION**

The collagen receptor GPVI forms a complex with the FcR γ-chain in platelets. The molecular basis of GPVI-FcR γ-chain interaction involves a conserved arginine residue in the transmembrane domain (Arg-272) and the cytoplasmic domain (12, 13). Using sequential truncations of the cytoplasmic domain of GPVI, we have demonstrated the involvement of the basic and juxtamembrane regions in the association with FcRγ-chain.

**Fig. 4. Ca²⁺ mobilization in response to convulxin in RBL-2H3 cells expressing deleted GPVI.** A, RBL-2H3 cells nontransfected (shaded) or transfected with wild type (WT) GPVI or mutants of GPVI (unshaded) were incubated with convulxin, anti-convulxin, and FITC-labeled anti-rabbit IgG. Fluorescence (FL 1-H) was analyzed by flow cytometry. B, RBL-2H3 cells stably expressed wild type GPVI or mutant GPVI receptors were stimulated with 10 μg/ml convulxin. Intracellular Ca²⁺ mobilization was measured by the ratio of Fura-2 emissions in a fluorimeter. Curves presented are representative of results (n = 3–6) from at least two independent clones expressing stably GPVI. C, cells were lysed and GPVI precipitated with anti-FLAG antibody. Samples were separated by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti-FcRγ chain (top). GPVI expression was detected by convulxin ligand blotting (bottom). Results are representative of three experiments. IP, immunoprecipitate.
Truncation of the amino acids that lie C-terminal to the basic region did not alter the association with FcRγ-chain, demonstrating that the last 22 amino acids, which are absent in murine GPVI, and the proline-rich regions are not required for this interaction. In contrast, truncation of the basic region (construct Δ294) led to a dramatic decrease in the association of GPVI to FcRγ-chain in RBL-2H3 cells, whereas the additional loss of the juxtamembrane domain (construct Δ288) abolished the interaction. This indicates that both the basic and juxtamembrane regions participate in the interaction with the FcRγ-chain. Importantly, and consistent with these results, the release of intracellular Ca²⁺ in response to convulxin is strongly decreased by truncation above the basic region and is abolished by deletion above the juxtamembrane domain. These observations demonstrate that the basic and juxtamembrane domains are necessary for optimal GPVI-FcRγ-chain interaction and efficient Ca²⁺ release. Interestingly, in transiently transfected COS-7 cells, the interaction between GPVI and FcRγ-chain...
γ-chain was not altered by truncation of the basic region, suggesting that overexpression of FeR γ-chain is sufficient to restore the interaction of the Δ294 construct with GPVI.

Like GPVI, a conserved arginine in the transmembrane domain of the immunoglobulin receptors PIR-A and Fco is crucial for their association to the FeR γ-chain (19, 20). The proximal amino acids of GPVI juxtamembrane domain are also conserved in PIR-A and Fco receptor (19). Although to our knowledge the deletion of this region has not been undertaken for these receptors, mutation of a negatively charged amino acid in this sequence from PIR-A (Glu-643 of mPIR-A) has been shown to decrease its association with FeR γ-chain and to inhibit Ca\(^{2+}\) mobilization (20). However, mutation of the two negative charges to alanine residues in juxtamembrane region of GPVI (Glu-289 and Asp-290) did not affect Ca\(^{2+}\) mobilization in response to convulxin. These results suggest that despite having the same juxtamembrane sequences, GPVI and PIR-A seem to interact differently with the FeR γ-chain. In addition, mutation of the subsequent three amino acids (Trp-291, His-292, and Ser-293), independently or together, did not affect the release of intracellular Ca\(^{2+}\) by convulxin. Therefore, although deletion of the juxtamembrane region abolished the functional response, site-directed mutations in this sequence had no effect. The latter observation demonstrates that the individual amino acids in the juxtamembrane region are not required for the interaction with the FeR γ-chain, which is mediated primarily through the basic domain in combination with a minor contribution from the complete juxtamembrane sequence. The loss of association that was seen on deletion of the juxtamembrane region is most likely explained by the misalignment of the GPVI tail and FeR γ-chain. Interestingly, deletion of the first 5 amino acids of the basic region did not alter the release of Ca\(^{2+}\), suggesting that the remaining amino acids of the basic domain are able to mediate the interaction with FeR γ-chain.

We have shown previously that the proline-rich region of the GPVI cytoplasmic tail is involved in the recruitment of the Src family kinases, Fyn and Lyn, to the GPVI-FeR γ-chain complex (15). With truncated GPVI, in which the proline-rich region was absent (Δ309GPVI), we determined a normal level of association with the FeR γ-chain in RBL-2H3 cells. In comparison, the Ca\(^{2+}\) increase was slower in cells transfected with Δ309GPVI relative to cells transfected with wild type receptor, and the dose-response curve to convulxin was shifted to the right. This demonstrates that the proline-rich sequence of GPVI contributes to functional responses in RBL cells independent of influencing the interaction with FeR γ-chain. This reinforces the idea that the proline-rich domain of GPVI plays a role in the activation of cellular signaling via the recruitment of Src kinases (15).
Recently, an association between calmodulin and GPVI has been reported in platelets. Calmodulin is associated with GPVI in unstimulated cells but undergoes partial dissociation upon activation. The basic region of the cytoplasmic domain of GPVI is conserved in other proteins that bind calmodulin and contains the calmodulin binding sequence (16). Deletion of the first 5 amino acids in this region in GPVI (Δbasic), which disrupts the predicted helical wheel of the putative calmodulin binding site, does not affect Ca²⁺ mobilization in response to convulxin in RBL-2H3 cells. This suggests that the calmodulin binding site is dispensable, at least with respect to the generation of Ca²⁺ signals, provided that the rest of the C-terminal amino acid sequence is present.

In this study, we have highlighted the crucial role of the juxtamembrane region and the basic region of GPVI in forming a complex with FcRγ-chain and inducing functional responses in RBL-2H3 cells. Our results demonstrate that the GPVI-FcRγ-chain complex is supported primarily by the positively charged transmembrane arginine and by the basic region of GPVI, with a more minor contribution from the proximal juxtamembrane region.

Addendum—An important role for the basic region in signaling by GPVI was published following submission of this paper (21).

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