Fc Rγ-independent Signaling by the Platelet Collagen Receptor Glycoprotein VI*

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The platelet collagen receptor glycoprotein VI (GPVI) is structurally homologous to multisubunit immune receptors and signals through the immune receptor adapter Fc Rγ. Multisubunit receptors are composed of specialized subunits thought to be dedicated exclusively to ligand binding or signal transduction. However, recent studies of the intracellular region of GPVI, a ligand-binding subunit, have suggested the existence of protein-protein interactions that could regulate receptor signaling. In the present study we have investigated the signaling role of the GPVI intracellular domain by stably expressing GPVI mutants in RBL-2H3 cells, a model system that accurately reproduces the GPVI signaling events observed in platelets. Studies of mutant GPVI receptor protein-protein interaction and calcium signaling reveal the existence of discrete domains within the receptor’s intracellular tail that mediate interaction with Fc Rγ, calmodulin, and Src family tyrosine kinases. These receptor interactions are modular and mediated by non-overlapping regions of the receptor transmembrane and intracellular domains. GPVI signaling requires all three of these domains as receptor mutants able to couple to only two interacting proteins exhibited severe signaling defects despite normal surface expression. Our results demonstrate that the ligand-binding subunit of the GPVI-Fc Rγ receptor participates directly in receptor signaling by interacting with downstream signaling molecules other than Fc Rγ through an adaptor-like mechanism.

Activation of platelets in response to exposed collagen is a critical step in response to vascular injury and the formation of intravascular thrombi associated with stroke and myocardial infarction. A necessary step in the activation of platelets by collagen is signaling by the glycoprotein VI (GPVI) receptor (1–3). The GPVI receptor is homologous to immune receptors and associates non-covalently with Fc Rγ (3, 4), a transmembrane protein that mediates signaling by several immune receptors through an immunoreceptor tyrosine activation motif (ITAM) (5). ITAM signaling is initiated by tyrosine phosphorylation mediated by Src family tyrosine kinases (6), a process associated with receptor movement to specialized regions of the cell membrane known as lipid rafts (7, 8). In platelets and other hematopoietic cells ITAM activation ultimately results in phospholipase Cγ activation (9) and intracellular calcium release through a series of signaling proteins including the non-receptor tyrosine kinase SYK (3, 10) and the adaptors SLP-76 (11) and LAT (12, 13).

We and others (14, 15) have shown that mutation of a single transmembrane arginine in GPVI (GPVI R272L) is sufficient to uncouple the receptor from the Fc Rγ chain. GPVI R272L is unable to activate the release of intracellular calcium in RBL-2H3 cells (14), a hematopoietic cell model in which heterologous expression of GPVI confers collagen-dependent calcium signaling (16). Unexpectedly, and unlike previously studied Fc Rγ partners (17, 18), truncation of the GPVI intracellular domain also abrogated GPVI signaling despite preservation of the critical transmembrane arginine (14, 15). These results suggest that the GPVI intracellular domain might play an important role in GPVI signaling, an idea recently confirmed by the identification of an interaction between the Src family kinase Lyn and the GPVI intracellular domain (19). Biochemical studies have also shown that the GPVI intracellular domain interacts with calmodulin (20). The functional importance of these interactions for signal transduction by GPVI, however, is unknown.

To further investigate the role of the GPVI intracellular domain during signal transduction we have stably expressed a series of receptor truncation mutants and amino acid substitution mutants in RBL-2H3 cells to analyze receptor signaling and protein-protein interactions. These studies identify two critical functional domains within the GPVI intracellular tail, a highly basic region that mediates interaction with calmodulin and a proline-rich region that mediates interaction with Src family kinases. Interruption of either one of these domains significantly impairs GPVI signaling despite normal association with Fc Rγ. In addition, the function of these domains appeared autonomous, i.e. loss of calmodulin binding, Lyn association or Fc Rγ coupling had little effect on GPVI association with the other two interacting proteins. Our results reveal an important independent role for the GPVI intracellular tail in the regulation of receptor signaling and suggest that the ligand-binding subunit of this receptor functions as an adaptor to bind downstream signaling proteins. The extent to which the intracellular domains of similar multisubunit receptors also function to modulate receptor signaling in an adaptor-like fashion remains to be investigated.

EXPERIMENTAL PROCEDURES

Materials—All reagents were from Sigma unless stated. Convulxin was purified from the venom of the South American rattlesnake (Crotalus durissus terrificus) by gel filtration as described (14). Mouse monoclonal anti-calmodulin and anti-FcRγ antibody were from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-Lyn polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GPVI monoclonal antibody HY101 was made as described (16) and affinity-purified from hybridoma supernatants by HiTrap Sepharose-
**RESULTS**

_Calmodulin Affinity Chromatography_—A crude membrane pellet was obtained from RBL 2H3 GPVI cells by Dounce homogenization (~30 times on ice) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, containing protease inhibitors. The homogenate was centrifuged at 1500 \( \times g \), for 10 min at 4 °C and the supernatant stored on ice. The pellet was resuspended in half the original volume of homogenization buffer supplemented with 5 mM magnesium chloride, pH 8.0 and disrupted by Dounce homogenization. The homogenate was centrifuged at 1500 \( \times g \), for 10 min at 4 °C. The pooled supernatants were centrifuged at 109,000 \( \times g \), for 1 h at 4 °C. The supernatant was discarded and the pellet resuspended to a protein concentration of ~10 mg/ml in 250 mM sucrose, 50 mM potassium chloride, 0.1 mM calcium chloride, 20 mM MOPS-Tris, pH 7.2.

Affinity chromatography of cell membrane lysates on calmodulin-Sepharose (Ambersham Biosciences) was performed using a method described by Klaerke et al. (23). CHAPS was added to the crude membrane preparation from a 0.5% stock to a detergent protein ratio of 1:1 (w/w). After 60 min the unsolubilized protein was removed by centrifugation. Sepharose-calmodulin beads, which had been equilibrated with 50 mM HEPES pH 7.4, 1 mM magnesium chloride, 1 mM dithiothreitol, 10 mM CHAPS, were added to the supernatant to 30% (v/v). Lysates were supplemented with 1 mM calcium chloride and incubated at 30 °C for 120 min. The beads were loaded into a disposable 0.7 ml estradiol-Sepharose 4B column (Pharmacia) and the flow through was collected and stored at -80 °C.

**Fluorescence Spectroscopy**—Emission spectra were obtained at 25 °C using a Varian Cary Eclipse Fluorescence spectrophotometer with well-plate attachment (Varian, Walnut Creek, CA). The intensity of the emission spectra was measured and subsequently corrected for the light-scattering contribution. The excitation wavelength was set at 280 nm and the emission spectra were recorded from 300 to 400 nm. The excitation and emission slits were set at 5 nm in a 1-cm cuvette, and the samples were equilibrated for 10 min in 10 mM HEPES pH 7.4, 0.1 mM nonyl N-methylglucamide.

**RESULTS**

_Calmodulin Associates with GPVI in a Specific and High Affinity Manner_—A theoretical calmodulin-binding region in GPVI was identified by screening for a basic amphipathic sequence between the transmembrane and cytoplasmic receptor.

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domains. A 14 amino acid sequence between amino acids Trp-292 and Val-306 shares homology to classical calmodulin-binding motifs and to the recently identified calmodulin-binding wheel projection of the membrane-proximal region of the GPVI intracellular tail (Trp-292–Val-306).

To more precisely define the interaction between GPVI and calmodulin we measured the intrinsic tryptophan fluorescence of GPVI before and after addition of calmodulin. Because calmodulin has no tryptophan residues, this technique provides information about the environment surrounding tryptophan residues of calmodulin-binding proteins. Affinity chromatography of GPVI-expressing RBL-2H3 cell lysates on CVX-Sepharose yielded a major protein component identified by Western blotting as GPVI, migrating at 66 kDa under reducing conditions (Fig. 2a). Minor protein components were removed by extensive salt washing and were further eliminated by non-denaturing preparative PAGE (data not shown). Purified GPVI had an emission peak at 352 nm after excitation of tryptophan at 295 nm. Calmodulin titrations were performed in the presence of calcium, magnesium, and/or EDTA. In the presence of calcium, addition of bovine calmodulin to purified GPVI resulted in a decrease on the emission maxima (a “blue shift”) to 344 nm that did not occur in the presence of EDTA or when magnesium was present in place of calcium (Fig. 2b and data not shown). Titration experiments (Fig. 2a, c and d) revealed approximately molar saturation between the two proteins and a high affinity interaction between GPVI and calmodulin (K_D of 35 nM). Thus calmodulin interacts with GPVI in living cells in a high affinity and specific manner.

**GPVI-Calmodulin Interaction Is Mediated Exclusively by the Basic Amino Acids in the GPVI Intracellular Tail**—The basic amino acids within the GPVI intracellular domain are predicted to form the calmodulin-binding site if arranged in the shape of an α-helix (Fig. 1). To directly test the role of these amino acids for calmodulin binding and for signal transduction by GPVI we generated GPVI mutants in which: (i) the four amino-terminal basic residues were mutated to alanines (GPVI RK1), (ii) the four carboxyl-terminal basic residues were mutated to alanines (GPVI RK2), and (iii) all eight basic amino acids were mutated to alanines (GPVI RK1/2). Significantly, all mutations of this domain were made in the context of the full-length receptor rather than in cognate peptides or through receptor truncation. Mutant receptors were expressed stably at roughly equivalent levels in RBL-2H3 cells and co-immunoprecipitation studies used to identify the residues critical for interaction with calmodulin in live cells. Mutation of either the amino-terminal or carboxyl-terminal basic amino acids alone (GPVI RK1 or GPVI RK2) did not disrupt calmodulin binding, but mutation of all eight basic amino acids (GPVI RK1/2) abrogated the interaction of these two proteins (Fig. 3b). GPVI truncation mutants lacking residues carboxyl to these basic amino acids (A303STOP and T318STOP) or the transmembrane domain critical for Fc R_y coupling (R272L) bound calmodulin normally, but a truncation mutant lacking the basic domain (R295STOP) did not. These results demonstrate that the basic amino acids between Trp-292 and Val-306 in the GPVI intracellular domain mediate calmodulin interaction and that calmodulin interaction is independent of either Fc R_y or Lyn association (discussed further below).

**Calmodulin Is Released by GPVI during Receptor Stimulation**—To determine the role played by calmodulin during GPVI signaling we analyzed calmodulin-GPVI interaction before and after receptor stimulation with CVX, a high affinity GPVI ligand that activates strong calcium signaling in GPVI-expressing but not wild-type RBL-2H3 cells (14). In resting cells, calmodulin could be co-immunoprecipitated with GPVI (Fig. 3, b and d). Following stimulation of GPVI with CVX, however, calmodulin was released from GPVI within 30–60 s (Fig. 3, b and c). As seen in Fig. 3b, calmodulin could be easily co-immunoprecipitated with GPVI from resting cell lysate when using HY101, a non-clustering anti-GPVI antibody (16). When GPVI was precipitated using the clustering ligand CVX, however, very little calmodulin was co-precipitated. These results suggest that calmodulin associates with GPVI in resting cells and calmodulin-GPVI interaction is regulated by receptor-ligand interaction in a manner that may be independent of downstream GPVI signaling.

**GPVI Receptors Unable to Bind Calmodulin Exhibit a Signaling Defect Despite Preserved Interactions with Fc R_y and Lyn**—Although the interaction of calmodulin with several platelet receptors has recently been identified, the role of calmodulin during signal transduction by platelet receptors remains unknown. To detect a role for calmodulin in GPVI signal
transduction we examined the calcium signals stimulated by the high affinity GPVI ligand CVX in cells expressing wild-type GPVI (hGPVI). GPVI lacking one-half the basic residues of the calmodulin-binding domain (GPVI RK1 and GPVI RK2) and GPVI lacking all basic residues in the calmodulin-binding domain and unable to bind calmodulin (GPVI RK1/2). As previously observed, expression of GPVI in RBL-2H3 cells conferred robust calcium signaling in response to CVX (Fig. 4 and Ref. 14). GPVI receptors lacking only half the basic residues of the calmodulin-binding domain but still able to bind calmodulin demonstrated wild-type calcium responses to CVX with the exception of a lag in the time required to initiate calcium signaling for the GPVI RK2 mutant receptor (Fig. 4). GPVI receptors unable to bind calmodulin, however, demonstrated severely reduced calcium responses to CVX despite a receptor surface expression equivalent to that of wild-type GPVI (Fig. 4). These results suggest that the GPVI receptor domain required for interaction of calmodulin with GPVI is also required for normal receptor signaling.

**Loss of Signaling by GPVI Receptors Unable to Bind Calmodulin Is Not Caused by Loss of Association with Fc Rγ or Lyn—** Previous studies have revealed an unexpected role for the intracellular GPVI C-tail in mediating association of GPVI with Fc Rγ (14, 15), raising the possibility that the loss of signaling observed in GPVI RK1/2-expressing RBL-2H3 cells might be caused by loss of Fc Rγ association rather than loss of a specific function mediated by the calmodulin-binding domain of GPVI. To test this possibility we performed co-immunoprecipitation assays to compare the ability of wild-type GPVI (WT GPVI) and the GPVI RK mutants to associate with Fc Rγ in RBL-2H3 cells. As expected, Fc Rγ co-precipitated with wild-type GPVI but not with the GPVI R272L mutant in which a transmembrane arginine critical for Fc Rγ association is mutated (Fig. 5). Significantly, Fc Rγ was also co-precipitated with all the RK mutants, including the GPVI receptor lacking all of the basic amino acids (GPVI RK1/2) required for calmodulin interaction. Similarly co-immunoprecipitation studies also demonstrated preserved interaction with the Src family kinase Lyn (Fig. 5). These results suggest that the loss of GPVI signaling associated with loss of the calmodulin-binding domain is not merely the result of loss of Fc Rγ or Lyn association and that interaction with calmodulin or an unidentified protein may be required for normal GPVI signal transduction.

GPVI Association with the Src Family Kinase Lyn Is Independent of GPVI Association with Calmodulin and Fc Rγ—Recent studies have demonstrated that the proline-rich region within the GPVI C-tail interacts with the SH3 domain of the Src family tyrosine kinase Lyn (19), but the importance of the interaction for signal transduction by GPVI is not established. To further dissect the domains of the GPVI C-tail and test their functional importance for receptor signaling we stably expressed three GPVI truncation mutants, GPVI T318STOP, GPVI A303STOP, and GPVI R295STOP, in RBL-2H3 cells. As shown in Fig. 3a, GPVI T318STOP deletes the region of human

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**FIG. 2.** Calmodulin binds GPVI with high affinity. a, isolation of GPVI from GPVI-expressing RBL-2H3 cells by affinity chromatography on CVX-Sepharose. Lane 1, material bound to a CVX-Sepharose column from GPVI-expressing RBL-2H3 cell lysates. Lanes 2 and 3, material remaining after washing with high and low salt buffers respectively. Lane 4, material eluted from the column using high pH buffer containing detergent. The identity of the purified 66-kDa protein was established as GPVI was confirmed by Western blotting using anti-GPVI MAb HY101 (not shown). b, intrinsic tryptophan fluorescence of GPVI before and after addition of calmodulin. Measurements were taken at an excitation wavelength of 295 nm and scanning emission with 10 μM GPVI (closed triangle) or 10 μM GPVI + 10 μM calmodulin (open circle), both with the addition of Ca²⁺ to saturation. c, calmodulin titration of GPVI. GPVI (10 μM) was titrated with calmodulin and the change in emission calculated as described under “Experimental Procedures”). Each data point was determined by two individual measurements and was plotted as the fraction of the maximum emission shift. Excitation and emission parameters were as described in b, d, determination of K_d and the Hill coefficient of slope for the interaction of purified GPVI with calmodulin. Data were transformed from fluorescence titration experiments and values calculated by linear regression (SigmaPlot 2002). Titration data are representative of Fig. 2c.
GPVI without homology to mouse GPVI, and GPVI A303STOP deletes the proline-rich domain predicted to interact with Lyn as well as the non-homologous region. GPVI R295STOP (Fig. 3a and Ref. 14) deletes virtually all of the intracellular C-tail of GPVI, including the calmodulin-binding domain, the proline-rich domain, and the non-homologous domain. To determine whether the functions of these predicted domains were truly discrete or if there was significant overlap of the GPVI domains required to mediate interaction with calmodulin and Lyn we performed co-immunoprecipitation experiments with each receptor in RBL-2H3 cells. As expected, wild-type GPVI but not GPVI R272L co-precipitated with Fc Rγ as well as Lyn and calmodulin (Fig. 5). Surprisingly, all the truncation mutants also associated with Fc Rγ, although the association of GPVI R295STOP with Fc Rγ was generally weak and could not always be detected (Fig. 5, Ref. 14, and data not shown). Thus Fc Rγ association is primarily mediated by the GPVI transmembrane domain with secondary modulation by the GPVI intracellular tail.

In contrast to its association with Fc Rγ, the association of GPVI with Lyn correlated completely with the presence of the proline-rich domain in the GPVI intracellular C-tail. Wild-type GPVI associated with Lyn but GPVI truncation mutants lacking the proline-rich domain (GPVI R295STOP and GPVI A303STOP) did not (Fig. 5). A GPVI truncation mutant retaining the proline-rich domain (GPVI T318STOP) however, did maintain an interaction with Lyn (Fig. 5). Surprisingly, the domains of GPVI required for interaction with Lyn were completely distinct from those required for interaction with Fc Rγ or calmodulin. Thus the transmembrane mutant GPVI R272L interacted normally with Lyn and calmodulin despite complete loss of Fc Rγ association (Fig. 5 and Ref. 14). GPVI RK1/2 was unable to interact with calmodulin but associated normally with both Lyn and Fc Rγ (Fig. 5). Finally, GPVI A303STOP associated normally with both Fc Rγ and calmodulin despite a loss of Lyn interaction. Together, these results demonstrate the presence of discrete functional domains within the GPVI C-tail that mediate non-overlapping protein-protein interactions.

**Productive Interaction with Calmodulin, Lyn, and Fc Rγ Are All Required for Normal GPVI Signal Transduction**—The biochemical studies described above suggested that the functional importance of GPVI interaction with calmodulin, Lyn, and Fc Rγ could be distinguished using GPVI mutant-expressing RBL-2H3 cells. As previously reported, loss of Fc Rγ association resulted in a complete loss of GPVI signal transduction despite preserved interaction with calmodulin and Lyn (GPVI R272L, Fig. 6 and Ref. 14). As predicted by its normal biochemical profile, loss of the non-homologous region of the human GPVI receptor had no discernable effect on CXV-induced calcium signals (GPVI T318STOP, Fig. 6). Loss of either the calmodulin-binding domain (GPVI RK1/2) or the Lyn-binding domain resulted in significant but not absolute loss of CVX-induced calcium signaling (Fig. 6), while mutants retaining normal calmodulin and Lyn binding (GPVI RK1 and GPVI RK2) demonstrated normal signaling responses to CVX. Interestingly, despite its ability to associate weakly with Fc Rγ, the GPVI R295STOP mutant unable to associate with either calmodulin or Lyn demonstrated a complete loss of function in this assay (Fig. 6).
DISCUSSION

The predicted amino acid sequence of the gene encoding the platelet collagen receptor GPVI establishes the receptor as a member of the family of multisubunit receptors that signal through the ITAM domains of non-covalently associated co-receptors (21). Subsequent studies have indeed demonstrated that GPVI associates with a signaling co-receptor, Fc Rγ, and that Fc Rγ association is required both for the receptor’s surface expression in platelets (4) and for its ability to mobilize intracellular calcium (14). Fc Rγ-dependent GPVI signaling proceeds through tyrosine phosphorylation of two Fc Rγ intracellular tyrosines (the Fc Rγ ITAM), which bind and activate the non-receptor tyrosine kinase SYK to activate downstream effectors such as phospholipase Cγ (13). Like many of the immune receptor ligand-binding subunits (17, 25), however, GPVI has a sizable intracellular domain whose role in receptor signaling, if any, is not established.

Recent biochemical studies have suggested that the intracellular domain of GPVI binds proteins other than Fc Rγ, which may participate in downstream GPVI signaling events, strengthening the possibility that GPVI signaling is regulated in an Fc Rγ-independent fashion by the receptor C-tail. Two proteins in particular, calmodulin and the Sre family tyrosine kinase Lyn, have been found to interact directly with peptides corresponding to the GPVI intracellular C-tail and to co-immunoprecipitate with GPVI in platelet lysates (20). In the present study we have extended these observations to address the functional importance of these interactions during GPVI signaling by addressing the following questions. (i) What is the stoichiometry and affinity of GPVI-calmodulin interaction? (ii) Does GPVI interact with Fc Rγ, calmodulin, and Lyn through discrete protein domains or does the receptor interact with these proteins in an interdependent fashion that requires for-
mation of a complex in which some or all must be present? (iii) What is the importance of GPVI interaction with each of these proteins for signal transduction by this receptor? We have approached these questions through analysis of wild-type and mutant GPVI receptors expressed in RBL-2H3 cells, a cellular system previously demonstrated to confer GPVI-dependent collagen signals in a manner that accurately reflects platelet responses (16).

Studies of GPVI-calmodulin interaction demonstrate that these proteins constitutively associate at a 1:1 ratio through a high affinity interaction. Our results using purified GPVI receptor agree closely with previous predictions of GPVI-calmodulin interaction based on the use of GPVI C-tail peptides (20). Our results also confirm reports that GPVI association with calmodulin is disrupted during receptor activation in a time course that is consistent with that required to generate calcium signals. Thus calmodulin interaction with GPVI is tied to receptor inactivity and calmodulin is either regulated by receptor-mediated calcium signals or vice versa.

The juxtamembranous region of GPVI rich in basic amino acids has been suggested as a calmodulin-binding site based on its homology to a calmodulin-binding site in GPIbα and on the ability of cognate peptides to interact with calmodulin (20). We have tested that interaction directly in the context of the full-length receptor through the generation and expression in RBL-2H3 cells of mutant GPVI receptors in which some or all of the charged amino acids in this domain were replaced by alanines. Co-immunoprecipitation studies demonstrate clearly that this region is indeed required for calmodulin interaction but that either the amino-terminal or carboxyl-terminal basic residues of this domain are sufficient to mediate the GPVI-calmodulin interaction despite the fact that GPVI and calmodulin associate in a 1:1 ratio.

The release of calmodulin from GPVI during receptor activation suggests that calmodulin might regulate GPVI signaling and that a GPVI mutant unable to interact with calmodulin might therefore exhibit altered signaling. Indeed, a GPVI mutant lacking all the basic residues in this domain and unable to bind calmodulin exhibited a significant loss of function. Unexpectedly, this loss of function was not due to a failure to interact with FcγR or Lyn, suggesting that this region serves an independent role in regulating GPVI signal transduction. We cannot exclude the possibility that the loss of signaling exhibited by GPVI RK1/2 is due to an effect unrelated to calmodulin binding such as loss of interaction with an unidentified protein. However, evidence that GPVI RK1/2 associated normally with FcγR and Lyn and that calmodulin binding by GPVI RK1 and GPVI RK2 correlated closely with calcium signaling suggests that calmodulin binding may regulate GPVI signaling directly.

The ability to dissociate GPVI interaction with calmodulin from that with FcγR and Lyn suggested that the GPVI intracellular C-tail might regulate GPVI signaling through an adapter-like function, i.e. by mediating interaction with signaling effectors through discrete and non-overlapping domains. To further test this model we generated a series of truncation mutants (GPVI T318STOP, GPVI A303STOP, and GPVI R295STOP) and tested them for interaction with the known GPVI-interacting proteins FcγR and Lyn. The carboxyl-terminal region of human GPVI (deleted in GPVI T318STOP) has no homologous counterpart in the mouse GPVI receptor, and loss of this region did not disrupt association with any of these GPVI partners or have any discernable effect on GPVI-mediated calcium signaling in RBL-2H3 cells. In contrast, loss of the proline-rich domain (deleted in GPVI A303STOP) disrupted GPVI interaction with Lyn. Importantly, despite normal association with FcγR and calmodulin,
GPVI signaling was clearly impaired in the absence of the proline-rich domain, suggesting that GPVI-mediated recruitment of Src family tyrosine kinases to Fc Rγ may be an important step for ITAM phosphorylation and the initiation of GPVI-Fc Rγ signaling. Previous studies have shown that GPVI associates with lipid rafts in an activation-dependent manner and that phosphorylation of Fc Rγ occurs exclusively in lipid-raft associated receptors (7), suggesting that ligand-induced movement of receptors to lipid rafts regulates the association of GPVI-Fc Rγ with active Lyn kinase. The finding that the Lyn-binding domain of GPVI is required for normal receptor signaling suggests either that receptor association with Src family binding domain of GPVI is required for normal receptor signaling or that association with the Fc Rγ adaptor is necessary for normal signaling. The transmembrane domain is critical for association of GPVI intracellular domain with lipid rafts in an activation-dependent manner of receptors to lipid rafts regulates the association of GPVI-Fc Rγ with active Lyn kinase. The finding that the Lyn-binding domain of GPVI is required for normal receptor signaling suggests either that receptor association with Src family binding domain of GPVI is required for normal receptor signaling or that association with the Fc Rγ adaptor is necessary for normal signaling. The transmembrane domain is critical for association of GPVI intracellular domain with lipid rafts in an activation-dependent manner.

Taken together, our studies of GPVI protein-protein interaction and signaling in RBL-2H3 cells demonstrate an important role for the GPVI intracellular domain during receptor signaling that is independent of Fc Rγ association. In many ways the GPVI intracellular domain appears to function as a tethered adaptor. The transmembrane domain is critical for association with Fc Rγ, a necessary signal-transducing co-receptor. The juxtamembraneous basic domain mediates interaction with calmodulin and the proline-rich domain binds Src family tyrosine kinases and perhaps other SH3 domain-containing proteins. As for many adaptor proteins, these interactions appear discrete and non-overlapping. Surprisingly, loss of any of these domain functions significantly impairs GPVI receptor signaling, providing a more complex view of signal transduction by this ligand-binding receptor subunit than mere coupling to Fc Rγ. Previous mutational analysis of two other Fc Rγ-associated ligand-binding subunits, FcyRIII and FcyRI, have also suggested functional roles for their cytoplasmic domains (17, 26). Whether these observations reflect modulation of receptor signaling through discrete protein-protein interactions in a manner similar to GPVI is a question with important implications for signaling in many circulating cells.

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