Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI.

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Running title: GPVI signals through lipid rafts
Summary

The platelet collagen receptor glycoprotein VI (GPVI) couples to the immune receptor adaptor FcRγ and signals using many of the same intracellular signaling molecules as immune receptors. Studies of immune receptor signaling have revealed a critical role for specialized areas of the cell membrane known as lipid rafts which are enriched in essential signaling molecules. The role of lipid rafts for signaling in non-immune cells such as platelets, however, remains poorly defined. The present study shows that GPVI-FcRγ does not constitutively associate with rafts but is recruited to lipid rafts following receptor stimulation in both GPVI-expressing RBL-2H3 cells and in human platelets. FcRγ is required for GPVI association with lipid rafts as mutant GPVI receptors that do not couple to FcRγ are unable to associate with lipid rafts after receptor clustering. Following GPVI stimulation in platelets virtually all phosphorylated FcRγ is found in lipid rafts but inhibition of FcRγ phosphorylation does not block receptor association with lipid rafts. This work demonstrates that lipid rafts orchestrate GPVI receptor signaling in platelets in a manner analogous to immune cell receptors and supports a model of GPVI signaling in which FcRγ phosphorylation is controlled by ligand-dependent association with lipid rafts.
Introduction

GPVI activates platelets through many of the same downstream kinases, adaptors and effector molecules as Fc, T-cell and B-cell receptors (1,2). Like these immune receptors, GPVI is a multi-subunit receptor in which the ligand binding subunit (GPVI) is non-covalently associated with a signaling subunit (FcRγ) that contains an immunoreceptor tyrosine activation motif (ITAM) (3,4). Cellular signaling by multi-subunit immune receptors is initiated by receptor clustering (5) and platelet activation by GPVI is also believed to result from receptor clustering initiated by interaction with collagen (6) or the GPVI-specific ligand convulxin (CVX) (7). Precisely how clustering of immune receptors initiates signal transduction is not well understood but one proposed mechanism is through receptor association with specialized areas of the cell membrane known as lipid rafts that are enriched in signaling proteins such as Src-family kinases and the transmembrane adaptor LAT (reviewed in (8)).

Lipid rafts, also known as detergent-resistant/insoluble membranes (DRMs/DIMs) or glycolipid-enriched membranes (GEMs), are areas of the cell membrane that are enriched in glycosphingolipids, saturated or near-saturated phospholipids and intercalating cholesterol (9,10). Lipid rafts are too small to be detected with standard microscopy but they are resistant to solubilization at low temperature by non-ionic detergents and have been isolated using density gradients (11,12). Lipid rafts form distinct membrane compartments which exclude most membrane-associated proteins but are enriched for some, including acylated Src family kinases such as Lyn and Fyn and palmitoylated adaptor proteins such as LAT (9,13,14). Lipid rafts are believed to participate in immune receptor signal transduction by sequestering oligomerized
receptors in a micro-environment in which they interact productively with downstream
signaling molecules. The role of lipid rafts as a signaling platform is supported by genetic
studies demonstrating that the raft-associated protein LAT is required for downstream
signaling by the FcεRI and T-cell receptors (15,16). While lipid rafts and raft-associated
proteins have been identified in many cell types, including platelets (9), the role of lipid
rafts for receptor signaling in non-immune cells is largely unexplored.

To assess the role of lipid rafts in GPVI-FcRγ signaling we have taken advantage
of the ability to confer GPVI signaling in the basophilic RBL-2H3 cell line (4) and
studied endogenous GPVI responses in human platelets. RBL-2H3 cells express FcεRI
receptor, which also couples to FcRγ, and activation of FcεRI in these cells results in
transient receptor association with lipid rafts (17-19). As observed for FcεRI, virtually no
GPVI is associated with lipid rafts in RBL-2H3 cells under basal conditions but
activation of GPVI by CVX results in movement of a significant number of receptors to
lipid rafts. Studies using human platelets revealed a similar activation-dependent
movement of GPVI to lipid rafts where Src-family tyrosine kinases were constitutively
present. Following GPVI-FcRγ activation, phosphorylated FcRγ was found exclusively
in lipid rafts, but, surprisingly, inhibition of FcRγ phosphorylation did not block GPVI
association with lipid rafts. In RBL-2H3 cells, clustering of mutant GPVI receptors that
do not couple with FcRγ failed to induce receptor movement to lipid rafts, demonstrating
a critical role for the FcRγ chain in this process. Our results establish a role for lipid rafts
in platelets and support a model of platelet activation by GPVI in which receptor
activation stimulates movement to lipid rafts where FcRγ is phosphorylated to initiate
downstream signaling. Whether other receptors in platelets utilize lipid rafts for signaling
and whether platelet lipid rafts contain unique proteins to facilitate receptor signaling remain to be determined.
Experimental procedures

Antibodies and reagents

All reagents were from Sigma Chemical Co (St Louis, MO) unless stated. The RBL-2H3 cell line that stably expresses human GPVI has been described (4). Convulxin was purified from the venom of the South American rattlesnake, *Crotalus durissus terrificus* by gel filtration (20,21). Anti-phosphotyrosine mouse monoclonal 4G10, rabbit polyclonal anti-LAT and anti-FcRγ were from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine mouse monoclonal PY20, rabbit polyclonal anti-Lyn were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Mouse IgE anti-dinitrophenol (DNP) and a mouse monoclonal antibody recognising the FLAG epitope (bio-M2) was purchased from Sigma as were α- and β-cyclodextrin. The production of a mouse monoclonal anti-human GPVI is described in detail elsewhere (6) and briefly below.

*HY101, a mouse monoclonal antibody recognizing GPVI*

Human GPVI with a transmembrane mutation replacing arginine residue 272 with leucine (R272L) was expressed on the surface of the *Balb/c*-strain of 3T3 fibroblasts. R272L-3T3 were used as an immunogen and injected peritoneally into a *Balb/c* background. Hybridoma cell lines were screened by FACS analysis of RBL-2H3 cells expressing wild-type human GPVI and human platelets, using mouse platelets as an isogenic control.

HY101 was affinity purified using Protein G and covalently modified with one of the following; biotin (Molecular probes Eugene, OR: F-2610), FITC (Molecular Probes F-
6434), or CY3 (APBiotech, Piscawtawy, NJ: PA-33001) according to the accompanying instructions.

**Measurement of cytoplasmic calcium in RBL-2H3 cells**

Adherent cells were detached from culture plates using 5mM EDTA and resuspended in RPMI1640 containing 25mM HEPES and 1mg/ml BSA (RHB medium) at a concentration of 2 x 10⁷ cells/ml. Fura-2/AM (Molecular Probes Inc) was added to 4µg/ml and cells were incubated at 37°C for 30 minutes. Excess FURA-2/AM was removed by washing in RHB buffer. Fluorescence was measured using an Aminco-Bowman Series-2 Luminescence Spectrometer (SLM Instruments Inc, Urbana IL). Fluorescence was measured at 340 and 380 nm for excitation and 510nm for emission. Cells (2 x 10⁶) were stirred continuously during the fluorescence recording. The data was recorded as the relative ratio of fluorescence excited at 340 and 380 nm and the concentration of mobilized calcium using a dissociation constant 224nmol/L for Fura-2:Ca²⁺.

**Receptor movement and lipid raft preparation using non-ionic detergent**

RBL-2H3 (5 x 10⁶) cells were sensitized for one hour on ice in 0.1 M PBS pH 7.4, 3% FCS, with 10 µg per ml ¹²⁵I-labeled mouse anti-DNP IgE. The high affinity Fc receptor for IgE, FcεR1 was clustered using 10 µg per ml DNP₁₇₋₂₅-BSA (Molecular probes Inc) for 3 minutes at 37°C (18,19). 10 µg DNP was added as a control.

¹²⁵I-HY101, was used to label the extracellular domain of GPVI. Antibody binding to the epitope was independent of convulxin binding to the extracellular domain of GPVI. RBL-2H3 cells (5 x 10⁶), into which human GPVI and structural variants of GPVI (as
described in the text), were incubated with 5 µg per ml ¹²⁵I-labeled anti-GPVI HY101 then washed as described above. Human blood was collected into acid-citrate-dextrose buffer (85 mM sodium citrate, 111 mM glucose, 71 mM citric acid containing 1 µg prostacyclin E₁) and platelet-rich plasma (PRP) obtained by centrifugation at 200 x gortex. PRP was incubated with 1 µg ¹²⁵I-labeled anti-GPVI HY101 per 10⁷ human platelets for one hour at room temperature. Platelets were isolated from PRP and free antibody by centrifugation after dilution in a five-fold excess of 150 mM sodium chloride, 10 mM HEPES pH 6.5, 5 mM EDTA and 1 µM prostacyclin. A total of 5 x 10⁷ platelets were used in each gradient assay.

RBL-2H3 GPVI cells and human platelets were allowed to recover for 30 minutes at 37°C. GPVI was clustered using 10 nM CVX for 30 seconds. Untreated cells were used as the negative control for CVX-receptor clustering. Reactions were stopped by lysis in fresh, ice-cold 2x lysis buffer (20 mM Tris pH 8.0, 100 mM sodium chloride, 4 mM sodium vanadate, 60 mM sodium pyrophosphate, 20 mM sodium glycerophosphate, 0.02% v/v sodium azide and a 100-fold dilution of Sigma protease inhibitor cocktail P-8340 and Sigma phosphatase inhibitor cocktail P-2850 and P-5726) with surfactAmps Triton-X100 (Pierce Endogen, Rockford, IL) added immediately before use from 10% w/v stock (final % w/v TX100 concentrations indicated in text). Cell lysate was mixed with an equal volume of 80% w/v sucrose in 25 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA. The final volume was always 2.0 ml at 40% w/v surose. In all experiments, to exclude artifactual possibilities, TX100 was added to the gradient buffers. All gradient solutions and the rotor were suitably pre-cooled. Gradients, from bottom to top were 80% w/v (1.0 ml), 40% (lysate – 2.0 ml), 30% w/v (1.5 ml), 10% w/v (0.50 ml) sucrose (total
5 ml), and were centrifuged at 200,000 x g av using a Beckman SW55 rotor for 18 hours with minimal acceleration and no braking. Fractions (20 fractions at 250 µl each) including the pellet (in fraction 20) were collected sequentially from the top of the gradient and were assayed for movement of receptors into buoyant lipid rafts.

For SDS-PAGE, 250 µl gradient fractions 1 through 20 were combined into ten 500 µl fractions (i through x sequentially from the top of the gradients) and heated to 100°C in 5 x modified Laemmli sample buffer (3.5 M Tris-HCl pH 6.8, 0.5M DTT, 10 % w/v SDS). Alternatively fractions were used for immunoprecipitations (below).

*Immunoprecipitation from cell lysates and gradient fractions.*

For immunoprecipitations from total cell lysates, cells were lysed for one hour at 4°C in ice-cold 2 x lysis buffer (2 % w/v digitonin (Calbiochem), 0.24% v/v TX100 (Pierce), 150 mM NaCl, 0.02% w/v NaN₃, 20 mM triethanolamine pH 7.8 and containing a 1/100 v/v dilution of Sigma mammalian protease phosphatase inhibitor cocktail). Sucrose gradient fractions were diluted in 2 x ice-cold raft lysis buffer (as TX100 raft lysis buffer above and containing 60 mM n-octyl-βD-glucoside to ensure full solubilisation of lipid rafts and associated proteins). Detergent-insoluble cellular debris was pelleted at 10,000 x g av for 15 minutes and the supernatants used for immunoprecipitations. Supernatents were pre-cleared with a mixture of protein G and protein LA beads (50% w/v slurry in lysis buffers. Primary antibodies were added overnight and immunoprecipitated the following day with protein G/LA beads. Beads were pelleted by centrifugation and washed three times in ice-cold washing buffer (50 mM Tris, 150 mM NaCl, pH 8.0, 5 mM CHAPS). Beads were heated to 100°C in an equal volume of 2 x
Laemmli sample buffer (1 M Tris-HCl pH 6.8, 0.2M DTT, 4% w/v SDS, 0.004% bromophenol blue, 20% glycerol) and an aliquot run on 5-20% v/v gradient SDS-PAGE gels for Western blotting-ECL.

**Inhibition of Src-family tyrosine kinases**

Platelets were isolated from platelet-rich plasma by gel filtration through Sepharose 2B (APBiotech) using a modified Tyrodes buffer (137 mM sodium chloride, 20 mM HEPES pH 7.4, 5.6 mM glucose, 1 mg per ml BSA, 1 mM magnesium chloride, 2.7 mM potassium chloride, 3.3 mM sodium dihydrogen phosphate) as the eluent. Gel-filtered platelets (22) were incubated for five minutes at 37°C with indicated concentrations of PP2 kinase inhibitor or the non-specific control for the inhibitor PP3 (both Calbiochem). Resting cells and cells stimulated with 10 nM CVX for 30 seconds were lysed with an equal volume of 2 x Laemmli buffer, resolved by SDS-PAGE and western blots on PVDF probed with mouse monoclonal anti-phosphotyrosine antibodies 4G10 and PY20.

**Cholesterol depletion/repletion from the outer leaflet of the platelet plasma membrane**

Gel filtered platelets were incubated with the indicated concentrations of β-cyclodextrin or the inactive stereoisomer control, α-cyclodextrin (data not shown), for one hour at 30°C. Platelets were washed by centrifugation at 800 x gav at least three times in a five-fold excess of 150 mM sodium chloride, 10 mM HEPES pH 6.5, 5 mM EDTA containing 1 µM prostacyclin. For cholesterol repletion, cholesterol-depleted cells were repleted using cholesterol:β-cyclodextrin as described (23).

**Fluorescent energy resonance transfer**
The efficiency of fluorescence energy transfer (FRET) between FITC- and CY3-labeled mouse monoclonal anti-GPVI HY101 on the surface of RBL-2H3 cells was measured by flow cytometry using a Beckton Dickinson FACStar (Franklin Lakes, NJ) with dual laser excitation (488 nm and 528 nm). The contribution of autofluorescence was determined from unlabeled cells and/or irrelevant FITC and CY3 control. To calculate FRET efficiency for dual-labeled cells, and also to confirm that FRET between FITC-HY101 and CY3-HY101 was due to receptor clustering and was not an artifact of high receptor density, correction factors for spectral overlap were determined from single labeling by substitution of either donor or acceptor fluorochrome with unlabeled HY101. At least 10,000 events were collected from the same cell population every 30 second interval for five minutes after addition of 10 nM CVX.
Results

Activation of GPVI expressed in RBL-2H3 cells results in receptor movement to lipid rafts in a manner identical to that of FcεRI.

To test the role of lipid rafts in signaling by the platelet collagen receptor GPVI we expressed the receptor in RBL-2H3 cells (4), a basophilic cell line which expresses endogenous FcRγ and FcεRI receptor (24). The RBL-2H3 cell line is used as a model cell line to study the earliest membrane-associated events in signaling through the FcεRI receptor (25) Studies by Field et. al., have established that FcεRI receptor signaling in RBL-2H3 cells proceeds through a transient association with lipid rafts which requires precise detergent conditions to capture (18,19). Expression of GPVI in RBL-2H3 cells confers calcium signaling in response to the GPVI-specific agonist convulxin that requires GPVI coupling to endogenous FcRγ (4).

The movement of GPVI receptors during signaling was followed using a radiolabeled anti-GPVI monoclonal antibody, 125I-HY101. HY101 was generated against an undefined epitope on the extracellular domain of human GPVI and receptor binding does not prevent CVX binding to GPVI (Fig 1A) or convulxin-induced calcium responses (Fig 1B). Lipid rafts were isolated as described (see Experimental Procedures) using sucrose gradients and defined as being TX100-insoluble membranes enriched in GM1, a gangioside lipid marker (26), and LAT (27) (Fig 2A). GPVI receptors were detected in lipid rafts at low levels under basal conditions (1.8 ± 0.5%) but receptor association with lipid rafts increased almost 8-fold following receptor activation by CVX (13.5 ± 1.6%, Fig. 2A). Although less quantitative, immunoblot analysis of cell lysate following
sucrose gradient analysis also revealed the movement of GPVI receptors to lipid raft fractions following CVX stimulation (Fig. 2A). As previously reported, activation of endogenous FceRI receptor in these cells results in a similar movement of FceRI receptors to lipid rafts (Figs. 2B and C). FceRI identified in raft fractions was 3.8 ± 0.7% after DNP stimulation (a non-clustering ligand) and 20.6 ± 3.5% after DNP-BSA clustering, a 5.5 fold increase. No GPVI was detected in lipid rafts if GPVI was clustered by CVX following cell lysis in TX100 suggesting that association of activated GPVI with lipid rafts is not merely a biochemical property of clustered receptors (data not shown). Cross-linking of raft GM1 ganglioside by pentavalent cholera toxin B subunit or cross-linking the endogenous FcRγ partner FceRI also had no effect on \(^{125}\text{I}-\text{HY101-GPVI}\) distribution in the sucrose centrifugation gradient (data not shown). Finally, aggregation of GPVI by CVX did not alter the restricted localization of Lyn and LAT in lipid rafts (data not shown). Thus GPVI signaling in RBL-2H3 cells is associated with the movement of a considerable fraction of receptors to lipid rafts, a response that closely mimics FceRI.

**Dependence of GPVI-raft association on raft extraction conditions**

FceRI receptor association with lipid rafts following receptor activation in RBL-2H3 cells is transient and difficult to capture biochemically unless detergent conditions are optimized (18,19). Association of aggregated GPVI with lipid rafts also depended to a great extent on the detergent conditions employed (Fig 3). Optimal recovery of clustered GPVI was observed at 0.025% w/v final TX100 concentration (0.40 mM) in the sucrose gradient (Fig. 3A and Table I). Treatment of lipid rafts isolated using 0.025% w/v TX100
with a two-fold higher TX100 concentration (0.05%) resulted in a drop in GPVI recovery in raft fractions from 19% to 2% despite no detectable loss of the constitutive raft protein LAT (Fig 2B). Cellular lysis and isolation of rafts at physiological temperatures (at which TX100 solubilization of cholesterol-ordered phospholipids is enhanced) or addition of a detergent known to disrupt lipid rafts (60 mM n-octyl-β-D-glucoside (12)) to the cell lysis buffer also led to the exclusive recovery of GPVI in the non-raft-membranes (Fig 3C). The rigorous detergent isolation conditions required to demonstrate association of both GPVI and FcεR1 receptors with lipid rafts is likely to reflect the transient nature of this association.

*GPVI signaling in human platelets proceeds through lipid rafts where FcRγ is exclusively phosphorylated.*

Using the conditions and methods established for following receptor movement in GPVI-expressing RBL-2H3 cells, the association of GPVI receptors with lipid rafts in human platelets was investigated (Fig 4). Lipid rafts isolated from human platelets were enriched in GM1 ganglioside, LAT and the Src-family kinase Lyn (Fig. 4A). As previously observed in RBL-2H3 cells, under resting conditions GPVI receptors were not associated with lipid rafts but following platelet stimulation with CVX a significant number of GPVI receptors were found associated with lipid rafts (Fig. 4A and C). Compared to receptor movement in clonal lines of GPVI-expressing RBL-2H3 cells the movement of GPVI receptors to lipid rafts in human platelets was more variable. Detection of GPVI receptor in lipid rafts under resting conditions was $1.7 \pm 0.9\%$ and rose to $23.7 \pm 12.5\%$ with CVX stimulation, an average fold increase of 12.5 (values represent mean ±
standard deviation of 15 independent experiments performed on platelets from three individuals). The isolation of CVX-clustered GPVI-FcRγ from platelet lipid rafts was sensitive to cholesterol depletion. While the dose response and amount of GPVI-FcRγ recovered from rafts after β-cyclodextrin treatment also showed individual variability, a drop in GPVI recovery from raft fractions to that approaching basal levels was complete using 20 mM β-cyclodextrin and could be reversed by cholesterol repletion (Fig 4B).

Phosphorylation of tyrosine residues on the FcRγ chain is a critical early event in GPVI signaling in platelets (28). To determine the role of lipid rafts in this phosphorylation event FcRγ was immunoprecipitated from each fraction and assayed for phosphotyrosine using immunoblotting. Strikingly, following CVX stimulation of human platelets virtually all phosphorylated FcRγ was detected in lipid rafts despite the presence of only a small percentage of total FcRγ in lipid rafts (Fig. 4A). These results demonstrate that GPVI-FcRγ stimulation in human platelets results in receptor association with lipid rafts and that only those receptors associated with lipid rafts undergo tyrosine phosphorylation and participate in downstream signaling.

FcRγ phosphorylation is not required for GPVI-FcRγ movement to lipid rafts. The finding that FcRγ is exclusively phosphorylated in lipid rafts following receptor stimulation raises the question of whether GPVI movement to lipid rafts is a consequence of FcRγ phosphorylation or vice versa. Since FcRγ is phosphorylated by Src-family tyrosine kinases we addressed this question by determining whether the level of Src-family tyrosine kinases in lipid rafts increases following GPVI activation and by determining whether inhibition of Src family kinases blocks movement of GPVI-FcRγ to
lipid rafts in human platelets (Fig 5). Following CVX stimulation the level of phosphorylated Lyn in lipid rafts was unchanged although the level of phosphorylated LAT greatly increased (Fig 5A). Thus LAT phosphorylation but not Lyn phosphorylation are downstream of GPVI-FcRγ signaling in human platelets. To directly test the requirement of Src-family tyrosine kinase activity for GPVI-FcRγ movement to lipid rafts platelets were stimulated with CVX in the presence of the Src-family kinase inhibitor PP2 or the structurally related non-inhibitor PP3 (29). As previously reported (1) treatment of platelets with PP2 greatly reduces LAT phosphorylation and virtually eliminates FcRγ phosphorylation (Fig. 5B). PP2 treatment did not, however, reduce the movement of GPVI receptors to lipid rafts (Fig. 5C). These results demonstrate that the levels of Src-family kinases in lipid rafts do not change significantly with CVX stimulation of platelets and that movement of GPVI-FcRγ to lipid rafts is independent of FcRγ phosphorylation. Together these findings suggest that FcRγ phosphorylation is likely to be a consequence rather than a cause of receptor movement to lipid rafts.

*GPVI requires associated FcRγ chain for receptor movement to lipid rafts.*

The finding that GPVI-FcRγ movement to lipid rafts following receptor activation is independent of FcRγ phosphorylation suggested that lipid raft association could be entirely independent of FcRγ and mediated by GPVI clustering alone. To define the role of the FcRγ chain for receptor movement to lipid rafts we analyzed the behavior of two GPVI mutants, R272L and R295STOP (R295Δ). We have previously shown that GPVI R272L and GPVI R295Δ bind CVX but do not couple to FcRγ and do not confer signaling responses to CVX when expressed on the surface of RBL-2H3 cells (4). GPVI
R272L has a single amino acid substitution in the receptor’s transmembrane domain while GPVI R295Δ has a wild-type transmembrane domain but lacks most of the intracellular C-tail. In contrast to wild-type GPVI, CVX stimulation of RBL-2H3 cells expressing either GPVI R272L or GPVI R295Δ does not result in the movement of GPVI receptors to lipid rafts (Fig 6). Interestingly, basal association of the mutant receptors with lipid rafts was significantly lower than the wild type receptor (an average of 15-fold lower than wild-type receptor for GPVI R272L and 25-fold lower for R295Δ in 5 experiments).

Clustering of receptors is essential for signaling by FcεRI and is also likely to be required for GPVI signaling. Since both GPVI R272L and GPVI R295Δ-expressing RBL-2H3 cells adhere to CVX-coated surfaces (4) it is likely that CVX clusters GPVI R272L and GPVI R295Δ in a manner similar to that of wild-type GPVI. To test directly for the ability of CVX to cluster these receptors we performed fluorescence energy transfer (FRET) analysis with the anti-GPVI antibody HY101 on RBL-2H3 cells expressing each of these receptors (Fig. 7). Using HY101 covalently labeled with either FITC or CY3 as a donor-acceptor FRET pairing (30), real-time analysis of receptor clustering by CVX was measured on the donor side as quenching and on the acceptor side as fluorescence intensity enhancement. No changes were observed in the presence of only donor or only acceptor only after CVX stimulations (Fig 7). Wild-type GPVI, GPVI R272L and GPVI R295Δ all cluster as shown by reproducible and robust FRET, although clustering of the wild-type receptor by CVX was more efficient than the mutants (Table II). Thus the inability of mutant GPVI receptors to associate with lipid rafts following
CVX stimulation is unlikely to be due to a lack of CVX-mediated clustering and instead reveals a critical role for FcRγ in GPVI receptor association with lipid rafts.
Discussion

Sphingolipids, cholesterol and glycerophospholipids are responsible for the formation of distinct domains within the cell membrane known as lipid rafts (31). The finding that lipid rafts exclude and include specific membrane-associated proteins has led to a model in which rafts participate in receptor signaling through the creation of membrane microdomains that function like signaling scaffolds (8,16). This model has been investigated most thoroughly in immune cells where immune receptors have been shown to associate with lipid rafts during receptor clustering. In this setting rafts are postulated to modulate receptor signaling by mediating receptor-kinase interaction and by contributing critical transmembrane adaptor molecules. Whether lipid rafts serve a similar role for receptor signaling in non-immune cells is not known.

Platelets are highly specialized, non-nucleated cells that bear little functional resemblance to immune cells. Signaling through the immunologic synapse in T cells, a process in which lipid rafts have been shown to actively participate (32), may occur over hours while platelet activation at sites of vessel injury in flowing blood must occur in seconds. It is therefore not obvious that two such different signaling responses would share an initial mechanism of action. Platelets, however, respond to exposed collagen at least in part through the Ig-domain containing receptor GPVI (33,34). GPVI signals through the ITAM of FcRγ (4,35,36), an adaptor also used by Fc receptors such as FcεRI. That GPVI has evolved to function in platelets, however, is clear from an expression pattern that is restricted to mature megakaryocytes and platelets (37). Lipid rafts have been described in platelets but no defined function has been assigned to them in these cells (9). Thus despite the differences in signaling tempo and in vivo function it is
plausible that GPVI signaling in platelets proceeds in a manner analogous to that of immune receptors which utilize lipid rafts.

To address the role of lipid rafts in GPVI signaling we first analyzed GPVI receptor function in GPVI-expressing RBL-2H3 cells, a hematopoietic cell line in which the role of lipid rafts has been well characterized with respect to another FcRγ partner, the FceRI receptor (17,18,38). This approach has two significant advantages. First, the ability to track the movement of stimulated FceRI receptor to lipid rafts in the same cells provides an internal control for receptor association with rafts. Second, the ability to introduce mutant GPVI receptors into RBL-2H3 cells permits structure-function analysis of the mechanism by which GPVI associates with lipid rafts and direct comparison with prior observations in the well-studied FceRI receptor system. GPVI receptors were stimulated with CVX because collagen interacts with platelet surface receptors other than GPVI and because CVX is a more potent agonist on GPVI-expressing RBL-2H3 cells (6). CVX stimulation of GPVI-expressing RBL-2H3 cells resulted in a rapid association of the receptor with lipid rafts that is highly sensitive to detergent conditions and indistinguishable from the responses observed for activated FceRI. Parallel studies in human platelets confirmed that GPVI also associates with lipid rafts in its natural cellular environment. Therefore despite their remarkably different functional roles GPVI and immune receptors appear to share a common mechanism of signal transduction using lipid rafts.

Our studies of CVX-stimulated platelets and GPVI-expressing RBL-2H3 cells strongly support a signaling role for GPVI association with lipid rafts following receptor clustering. First, identical biochemical methods applied to the two very different cell
types demonstrate a similar activation-dependent association with lipid rafts. Second, two distinct GPVI mutants that are unable to couple to FcRγ also do not associate with lipid rafts despite the ability of the mutant receptors to be clustered by CVX. These results demonstrate that clustering of GPVI receptors is not sufficient to detect GPVI association with lipid rafts and reveals an important functional role for FcRγ in mediating association with lipid rafts (discussed below). Finally, following GPVI stimulation in platelets virtually all phosphorylated FcRγ is found associated with lipid rafts where Src-family kinases are concentrated, suggesting that lipid rafts may regulate FcRγ phosphorylation to initiate downstream signaling by GPVI. That lipid raft association is upstream of FcRγ phosphorylation is supported by the inability to inhibit lipid raft association by inhibiting FcRγ phosphorylation. Taken together these data support a model of GPVI signaling much like that postulated for FcεRI signaling (18) in which receptor clustering by ligand results in movement to kinase-rich lipid rafts where FcRγ is phosphorylated and downstream signaling initiated and subsequently coordinated by other raft proteins such as the adaptor LAT.

Comparison of our studies using GPVI to those previously performed with the FcεRI receptor reveals a critical functional and perhaps structural role for the common subunit FcRγ. Mutants of both receptors in which the ligand-binding subunit is uncoupled from FcRγ do not associate with lipid rafts (19), and an uncoupled GPVI receptor with an intact intracellular domain (GPVI R272L) was also deficient. For FcεRI association with lipid rafts neither the FcRβ chain nor the intracellular tail of the FcRγ chain were required (19). These data point to a critical functional role for the transmembrane domains of either FcRγ, its ligand-binding partner or both in mediating ligand-induced receptor
association with lipid rafts. Our finding that GPVI R295Δ, a GPVI receptor mutant with a wild-type transmembrane domain, is deficient in lipid raft association further suggests that the FcRγ transmembrane domain may be what drives oligomerized receptors to lipid rafts. This conclusion is indirectly supported by the fact that GPVI and FcεRI share little homology in their transmembrane domains despite the fact that both couple to the FcRγ chain (4). An alternative explanation for these data is that FcRγ performs a critical structural role in maintaining GPVI and FcεRI in conformations required for lipid raft association. We have no direct evidence for this but the weaker CVX-induced clustering of the FcRγ-uncoupled GPVI mutants observed with FRET is likely to be due to a subtly altered receptor conformation in the absence of FcRγ. It is presently not understood what drives multi-subunit receptor association with lipid rafts in any cell type and the role of the signaling adaptors such as FcRγ merits further attention in this regard.

While we have observed considerable similarities between GPVI and FcεRI signaling through lipid rafts, cell-specific differences in the utilization of lipid rafts are already apparent and are likely to become more so as studies accumulate. The constitutive raft adaptor LAT is required for full FcεRI signaling responses (15) while collagen and CVX signaling in LAT-deficient platelets is preserved at higher concentrations of agonist (39). This is in contrast to loss of the non-raft adaptor protein SLP-76 that completely interrupts signaling by both receptors (40,41). Persistent signaling in LAT-deficient platelets may reflect a difference in the utilization of lipid rafts for signaling in the two cell types or merely reflect the existence of a second adaptor in lipid rafts in platelets. In either case it is clear that the functional roles of lipid rafts for receptor signaling in platelets will differ from those already described for immune cells.
Identification of the proteins found in lipid rafts in platelets and further analysis of receptors which signal through lipid rafts in platelets will provide both a better understanding of platelet biology and of the role of lipid rafts in signal transduction.
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Figure Legends

Figure 1. Binding of HY101 to GPVI does not inhibit subsequent CVX binding and receptor stimulation.

(A) RBL-2H3 cells expressing human GPVI were labeled with CY3-HY101 or IgG-CY3 control and and subsequently exposed to FITC-CVX. Data shown is after 5 minutes FITC-CVX binding. Percentages indicate the number of cells in each quadrant

(B) RBL-2H3 cells expressing human GPVI were super-saturated with HY101 or irrelevant IgG and calcium signaling responses to CVX measured.

Figure 2. GPVI and FcεRI associate with lipid rafts following receptor stimulation in RBL-2H3 cells.

(A) GPVI moves to lipid rafts following CVX stimulation. GPVI-expressing RBL cells pre-labeled with anti-GPVI monoclonal antibody 125I-HY101 were treated with 10 nM CVX for 30 seconds (squares) or left untreated (circles) before lysis in 0.025% w/v TX100. Cell lysate was centrifuged through sucrose gradients as described in ‘Methods’ and fractions taken sequentially from the top (fraction 1) of the gradient. “% receptor” indicates the percentage of 125I-HY101-GPVI in each gradient fraction including the pellet (fraction 20). The position of lipid rafts is identified by dot-blot of the lipid raft marker GM1ganglioside using HRP-conjugated cholera toxin B subunit as a probe. LAT in lipid rafts is shown by immunoblotting. GPVI distribution with and without CVX stimulation was also followed by immunoblotting (lower figures). The experiment shown is representative of seven independent experiments.
(B) FcεRI receptors move to lipid rafts following cross-linking with DNP-BSA. RBL-2H3 cells sensitized with 125I-IgE anti-DNP were lysed with 0.025% w/v TX100 after three minutes stimulation with 10 µg DNP (circles) or 10 µg DNP-BSA (squares). The distribution of 125I-IgE-FcεRI is expressed as a percentage of total 125I in the gradient and is representative of five independent experiments.

(C) The percentage of total GPVI and Fce RI receptors which move to lipid rafts after crosslinking is similar. The percentage of total FcεRI (open bars) and GPVI receptors (filled bars) within lipid raft fractions before and after addition of multivalent ligand is shown (mean ± standard deviation of 7 and 5 experiments respectively).

Figure 3. Sensitivity of the GPVI-raft interaction to isolation conditions.

(A) GPVI-lipid raft association is exquisitely sensitive to Triton X-100 concentration. An equal number of GPVI-expressing RBL-2H3 cells were lysed in TX100 concentrations as indicated after stimulation with 10 nM CVX. The GPVI content in lipid rafts was determined as in Fig 1. The experiment shown is representative of three independent experiments.

(B) Excess TX100 strips clustered GPVI from lipid rafts. 125I-HY101-labeled GPVI-expressing RBL-2H3 cells stimulated with 10 nM CVX were fractionated through sucrose gradients containing 0.025% w/v TX100 (open square). The lipid rafts were pooled, brought to 0.050% w/v TX100 and re-centrifuged through a second gradient containing 0.050% w/v TX100 (closed square). Note that pre-captured GPVI is lost from the low-density lipid rafts but LAT remains associated with raft fractions (analyzed as pools of 2). Data shown are representative of three independent experiments.
Biochemical disruption of rafts destroys GPVI-lipid raft association. Addition of n-octylglucoside detergent (60mM; open circle) to the lysis buffer or TX100 lysis at physiological temperatures (37°C; closed circle) disrupts lipid rafts by enhancing their detergent solubility with concomitant loss of clustered GPVI-FcRγ. Cell lysis in 0.025% w/v TX100 at 4°C is shown as a control (open square).

Figure 4. GPVI associates with lipid rafts in platelets following CVX stimulation.

(A) GPVI on human platelets was labeled and receptor movement followed with (squares) and without (circles) CVX stimulations as described for GPVI-expressing RBL-2H3 cells (Fig. 1). Note the constitutive association of GM1 ganglioside, LAT and the kinase Lyn with platelet lipid rafts. GPVI and associated FcRγ chain movement was also followed using immunoblot analysis of pooled fractions (lower figure). Note that FcRγ chain is phosphorylated only after occupancy of the GPVI receptor by CVX only within sucrose gradient fractions from which lipid rafts can be isolated. FcRγ in unstimulated platelets is not detectably phosphorylated (data not shown).

(B) GPVI-FcRγ is excluded from the lipid rafts following cholesterol depletion (triangles). This exclusion can be reversed by cholesterol repletion (circles). Data shown are from same individual as in Fig 4A and are representative of 6 experiments from 3 individuals.

(C) Quantitation of GPVI association with lipid rafts following receptor stimulation with CVX. Shown are mean ± standard deviation of 15 independent experiments using platelets from 3 individuals.
Figure 5. **GPVI association with lipid rafts following CVX stimulation is independent of FcRγ phosphorylation.**

(A) *Tyrosine phosphorylation of lipid raft proteins.* Anti-phosphotyrosine immunoblotting of cell lysate derived from lipid raft fractions isolated from platelets with and without CVX stimulation is shown. Proteins identified by subsequent blotting of the stripped membrane are labeled. Note the increase in tyrosine phosphorylation of LAT and FcRγ but the lack of change in phospho-Lyn.

(B) *The Src-family kinase inhibitor PP2 inhibits CVX-induced tyrosine phosphorylation of LAT and FcRγ in a dose-dependent manner.* Platelets were incubated with the indicated concentrations of PP2 (*left*) or PP3 (*right*) and then stimulated with 10 nM CVX or vehicle for 30 seconds. SDS-PAGE and immunoblotting of platelet cell lysate for phosphotyrosine is shown. FcRγ chain tyrosine phosphorylation from the same gel is shown below. Note the inhibition of LAT and FcRγ phosphorylation with PP2 but not PP3.

(C) *Inhibition of FcRγ phosphorylation with PP2 does not block GPVI association with lipid rafts.* Movement of GPVI receptors on human platelets was followed without CVX stimulation (*circles*), with CVX stimulation (*open squares*) and with CVX stimulation following PP2 treatment (*filled squares*). Note that GPVI movement to lipid raft fractions following receptor stimulation with CVX is unchanged in the presence of PP2.

Figure 6. **GPVI association with lipid rafts requires FcRγ.**

(A, B) *GPVI mutants unable to couple to FcRγ do not associate with lipid rafts following CVX stimulation.* RBL-2H3 cells expressing wild-type GPVI (WT), GPVI R272L
(R272L) and GPVI R295STOP (R295Δ) were stimulated with CVX and receptor movement followed using ¹²⁵I-HY101 as described. Note the complete absence of mutant receptors in lipid raft fractions. The mean ± standard deviation of GPVI receptor association with lipid rafts in 5 independent experiments is shown in B.

(C) The inability of GPVI mutants to associate with lipid rafts was confirmed using immunoblot analysis of pooled gradient fractions as described in Fig 1.

Figure 7. **CVX clusters wild-type and mutant GPVI receptors.**

FRET between CY3-labeled HY101 and FITC-labeled HY101 after addition of CVX to wild-type and mutant GPVI receptors was used to measure CVX-induced receptor clustering. Changes in FL2 (CY3 emission) after addition of CVX to RBL-2H3 cells expressing wild-type GPVI (WT) and the R272L and R295Δ GPVI mutants are shown at increasing time intervals (colored histograms – closed purple, 0 seconds; green, 30 seconds; pink, 60 seconds; blue, 180 seconds; orange, 300 seconds) on the right. FRET was not seen in single fluorochrome-labeled cells stimulated with CVX (left figure – closed purple, 0 seconds; orange, 300 seconds). Unlabeled RBL-2H3 cells were used to eliminate the contribution of autofluorescence. Single-labeled cells were also used to correct for spectral overlap. The experiment was performed three times with similar results.
Tables

Table I

The influence of TX100 concentration on the recovery of clustered GPVI from lipid rafts in RBL-2H3 cells.

| TX100 % w/v final in sucrose gradient | % GPVI in raft | Fold increase in receptor movement to raft |
|--------------------------------------|--------------|------------------------------------------|
| 0.010                                | 0.74         | 2.89                                     |
| 0.025                                | 1.45         | 11.55                                    |
| 0.050                                | 1.22         | 3.01                                     |
| 0.100                                | 0.85         | 3.42                                     |
| + 10 nM CVX                          | 2.13         | 16.73                                    |
|                                      | 3.67         | 3.67                                     |
|                                      | 2.90         | 2.90                                     |
Table II

**FRET between FITC-HY101 donor and CY3-HY101 acceptor which accompany binding of CVX to GPVI and receptor mutants expressed in RBL-2H3 cells.**

| CY3 emission, % (normalized) | Wild-type | R272L | R295Δ |
|-----------------------------|-----------|-------|-------|
| Time after CVX addition, seconds (*histogram color*) |          |       |       |
| 0 (closed purple)           | 100.00    | 100.00| 100.00|
| 30 (green)                  | 122.33    | 105.98| 106.45|
| 60 (pink)                   | 135.54    | 111.84| 112.19|
| 180 (blue)                  | 151.30    | 112.78| 115.56|
| 300 (orange)                | 176.21    | 119.72| 115.53|
Figure 1. Locke et al.

A

IgG-CY3

0.7% 0.2%

IgG-CY3 + CVX-FITC

0.3% 0.7%

HY101-CY3

33.6% 0.2%

HY101-CY3 + CVX-FITC

14.1% 22.8%

B

Ca^{++} [nM]

200

RBL-GPVI

+ 250 g HY101

+ 250 g IgG

10 nM CVX

Time (min)
Fig 2. Locke et al.,

A

B

C

GM1 ganglioside + CVX

LAT

GPVI

GPVI + CVX

10% w/v sucrose

80%
Fig 3. Locke et al.,

A

B

C

LAT
Fig 4. Locke et al.,

A

% receptor

Fraction

0 10 20 30

GPVI
GPVI + CVX

Lipid raft

SDS-PAGE fractions

i ii iii iv v vi vii viii ix x

GM1 ganglioside

LAT

Lyn

GPVI

GPVI + CVX

FcR chain

FcR chain + CVX

FcR chain phosphorylation (+ CVX only)
Fig 4. Locke et al., (continued)

B

- GPVI
- GPVI + CVX
- GPVI + 20 mM cyclodextrin + CVX
- GPVI/20 mM cyclodextrin + cholesterol + CVX

Fraction

% receptor

10% w/v sucrose

Lipid raft

C

% receptor in rafts

Basal + CVX
Fig 5. Locke et al.,

A

Lipid raft

kDa

92
55
35

CVX - +

FCR ▲

LAT ▲

Lyn ▲

B

Whole platelet lysate

kDa

92
55
35
29
22

CVX - + + + + + - + + + + +

PP2 0 0 1 5 10 20 - - - - -

PP3 - - - - - 0 0 1 5 10 20

C

% receptor

Lipid raft

Fraction 2 4 6 8 10 12 14 16 18 20

GPVI ○
GPVI + CVX □
GPVI + CVX + 20 PP2 ▼
Fig 6. Locke et al.,

A

% receptor in lipid raft

B

% receptor in lipid raft

C

WT + CVX

WT

R272L + CVX

R295 + CVX
Fig 7. Locke et al.,

| WT        | HY101 + HY101-CY3 | FITC-HY101 + HY101-CY3 |
|-----------|--------------------|------------------------|
| R272L     | Cell number        | FL2 channel intensity  |
| R295      | Cell number        | FL2 channel intensity  |

HY101 + HY101-CY3

FITC-HY101 + HY101-CY3
Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI
Darren Locke, Hong Chen, Ying Liu, Changdong Liu and Mark L. Kahn

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