Lipid Rafts Facilitate the Interaction of PECAM-1 with the Glycoprotein VI-FcR γ-Chain Complex in Human Platelets*

Received for publication, August 18, 2006, and in revised form, October 10, 2006 Published, JBC Papers in Press, October 26, 2006, DOI 10.1074/jbc.M607930200

Fiona A. Lee†, Marjolijn vanier‡, Ingrid A. M. Relou§, Loraine Foley¶, Jan-Willem N. Akkerman†, Harry F. G. Heijnen‡, and Richard W. Farndale†1

From the †Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom and the ‡Laboratory for Thrombosis and Haemostasis, Department of Haematology, and the §Cell Microscopy Centre and the Institute of Biomembranes, University Medical Centre, Utrecht, 3584 CX, Utrecht, the Netherlands

Glycoprotein (GP) VI, the main signaling receptor for collagen on platelets, is expressed in complex with the FcR γ-chain. The latter contains an immunoreceptor tyrosine-based activation motif, which becomes phosphorylated, initiating signaling cascades leading to the rapid activation and aggregation of platelets. Previous studies have shown that signaling by immunoreceptor tyrosine-based activation motif-containing receptors is counteracted by signals from receptors with immunoreceptor tyrosine-based inhibitory motifs. Here we show, by immunoprecipitation, that the GPVI-FcR γ-chain complex associates with the immunoreceptor tyrosine-based inhibitory motif-containing receptor, PECAM-1. In platelets stimulated with collagen-related peptide (CRP-XL), tyrosine phosphorylation of PECAM-1 precedes that of the FcR γ-chain, implying direct regulation of the former. The GPVI-FcR γ-chain complex and PECAM-1 were present in both lipid raft and soluble fractions in human platelets; this distribution was unaltered by activation with CRP-XL. Their association occurred in lipid rafts and was lost after lipid raft depletion using methyl-β-cyclodextrin on platelets, is expressed in complex with the FcR γ-chain. The latter contains an immunoreceptor tyrosine-based activation motif, which becomes phosphorylated, initiating signaling cascades leading to the rapid activation and aggregation of platelets. Previous studies have shown that signaling by immunoreceptor tyrosine-based activation motif-containing receptors is counteracted by signals from receptors with immunoreceptor tyrosine-based inhibitory motifs. Here we show, by immunoprecipitation, that the GPVI-FcR γ-chain complex associates with the immunoreceptor tyrosine-based inhibitory motif-containing receptor, PECAM-1. In platelets stimulated with collagen-related peptide (CRP-XL), tyrosine phosphorylation of PECAM-1 precedes that of the FcR γ-chain, implying direct regulation of the former. The GPVI-FcR γ-chain complex and PECAM-1 were present in both lipid raft and soluble fractions in human platelets; this distribution was unaltered by activation with CRP-XL. Their association occurred in lipid rafts and was lost after lipid raft depletion using methyl-β-cyclodextrin. We propose that lipid raft clustering facilitates the interaction of PECAM-1 with the GPVI-FcR γ-chain complex, leading to the down-regulation of the latter.

The discovery of specialized microdomains in the plasma membrane known as lipid rafts or detergent-resistant membranes has opened a new field of research, providing insight into the role of the plasma membrane in signal transduction (1). Lipid rafts are enriched in cholesterol and sphingolipids, imparting enhanced stability and resistance at 4 °C to Triton X-100 (2). Lipid rafts provide a platform where signaling complexes can assemble, and thereby regulate the resultant signaling process (3). The first study of lipid rafts in platelets by Dorahy et al. (4) established CD36 as constitutively and exclusively expressed in the lipid raft. Glycosylphosphatidylinositol (GPI)3-linked or palmitoylated proteins, such as the Src family kinases and the linker for activation of T cells (LAT), are enriched in lipid rafts. Recently, the main signaling receptor for collagen, glycoprotein (GP) VI, along with the FcR γ-chain, was also located in lipid rafts (5–7).

Platelets adhere to collagen exposed in damaged vasculature, resulting in their activation and aggregation (8). GPVI, a 62-kDa protein, is a member of the Ig superfamily (9) found only on platelets and late megakaryocytes (10). Its interaction with the FcR γ-chain via a salt bridge is crucial for both function and expression (11, 12). Stimulation of GPVI with collagen leads to the phosphorylation of the FcR γ-chain on its immunoreceptor tyrosine-based activation motif (ITAM) by the Src family kinases, Fyn and Lyn. This allows the recruitment and activation of syk by its tandem SH2 domains, resulting in a series of phosphorylations involving the proteins SLP-76 and LAT and the assembly of a signaling complex that culminates in the activation of PLCγ2. This pathway causes the rise in intracellular calcium that is essential for platelet activation and aggregation.

Recent studies have shown that ITAM-dependent signaling is regulated by counteracting pathways involving receptors that possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) (13). PECAM-1, one of the few ITIM-containing proteins in human platelets, may be a key receptor in regulating signals produced by the ITAM signaling pathways. Jackson et al. (14) have shown that PECAM-1 recruits the tyrosine phosphatase, SHP-2, during platelet aggregation and have described the physical and functional interplay between PECAM-1 and the ITAM-containing FcyRIIa on platelets (15). PECAM-1 has been shown to exert negative regulation on the GPib-IX-V complex, providing further evidence of its inhibitory role (16). This suggested to us that PECAM-1 might co-localize with other ITAM receptors, such as the GPVI-FcR γ-chain, and might modulate their signals, either directly or otherwise. In support of this concept, Jones et al. (17) and Patil et al. (18) showed that PECAM-1 deficiency results in exaggerated platelet adhesion and activation by collagen. In addition, Wu and Lian (19) and Cicmil et al. (20) have shown that PECAM-1 ligation by a specific antibody impaired the aggregation response to collagen, con-

* This work was supported by grants from the Dutch Heart Foundation, (to M. v. L.) and from Heart Research UK and the Medical Research Council UK (to L.F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of a British Heart Foundation studentship.

2 To whom correspondence should be addressed: Dept. of Biochemistry, University of Cambridge, Downing Site, Cambridge CB2 1QW, United Kingdom. Tel.: 44-1223-766111; Fax: 44-1223-333345; E-mail: rwf10@cam.ac.uk.

3 The abbreviations used are: GPI, glycosylphosphatidylinositol; GP, glycoprotein; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; CRP-XL, collagen-related peptide; LAT, linker for activation of T cells; GPO, glycine-proline-hydroxyproline; HRP, horseradish peroxidase; MβCD, methyl-β-cyclodextrin; PLC, phospholipase C.
vulxin, and thrombin and have described aggregation-independent phosphorylation of PECAM-1 (21), implying a more specific role for PECAM-1 in regulating primary activation processes.

The use of collagen to study the ITAM and ITIM pathways poses a problem because several collagen receptors are expressed on the platelet surface (22). Using the GPVI-selective agonist, collagen-related peptide (CRP-XL), which binds GPVI via its glycine-proline-hydroxyproline (GPO) content, we were able to exclude any secondary signals that may arise from other receptors when using native collagen fibers. The synthetic peptide is a repeat of GPO triplets, which is cross-linked to form a quaternary structure that is a highly potent platelet agonist (23, 24).

Co-localization of receptors on the plasma membrane may be mediated by the clustering of lipid rafts, which allows for efficient regulation of signaling. Here we show by immunoprecipitation that there is a physical association of PECAM-1 with the GPVI-FcR γ-chain complex and that this is facilitated by lipid rafts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine tendon type I collagen fibers were a gift from Ethicon Inc. (Somerville, NJ) and were dialyzed against 0.01 M acetic acid and stored at 1 mg/ml for use. CRP-XL (monomer sequence, H-GPC-(GPO)$_{10}$-GPCG-NH)$_{2}$), was synthesized and cross-linked as described by Morton et al. (24), again dialyzed against 0.01 M acetic acid. MβCD and apyrase (grade VI) were obtained from Sigma (Dorset, UK). Cholera toxin-B subunit, HRP-linked, was from Calbiochem-Novabiochem (Nottingham, UK). Protein G-coated magnetic beads were from Dynal Biotech (Wirral, UK). The following antibodies were used: mouse anti-phosphotyrosine (4G10) was from TCS (Nottingham, UK). Protein G-coated magnetic beads were from Dynal Biotech (Wirral, UK). The following antibodies were used: mouse anti-phosphotyrosine (4G10) was from TCS Biologicals; rabbit anti-GPVI was kindly provided by Dr. P. Smethurst (National Blood Service, Cambridge, UK); rabbit anti-γ subunit, T cell receptor, Fc receptor, were obtained from Upstate Biotechnology and used for immunoprecipitation of FcRγ-chain; mouse anti-T cell receptor, Fc receptor γ subunit, from United States Biological were used for immunodetection; mouse anti-PECAM-1 (1.3) and mouse PECAM-1 F(ab’)$_2$ fragments were kindly provided by Prof. P. Newman to J.-W.N.A., and rabbit and mouse anti-LAT were purchased from Upstate Biotechnology; rabbit and mouse anti-PLCγ2 and rabbit and goat anti-PECAM-1 were obtained from Autogen Bioclear. Materials obtained from Perbio Science Ltd. (Cheshire, UK) include ECL reagents and the PAGE-PREP kit. Protease inhibitor tablets containing chymotrypsin (1.5 μg/ml), thermolysin (0.8 μg/ml), papain (1 μg/ml), Pronase (1.5 μg/ml), pancreatic extract (1.5 μg/ml), and trypsin (0.002 μg/ml) were from Roche Applied Science (Welwyn Garden City, UK).

**Platelet Isolation for Aggregation**—Turbidimetric aggregation was monitored over 5 min at 37 °C, using washed platelets stimulated with CRP-XL or thrombin, at 1100 rpm in a Series 490 aggregometer (Chrono-Log, Haverton, PA). Blood was obtained from the antecubital vein of informed volunteers, in accordance with the Helsinki protocol, into 0.105 M citrate Vacutainers®. Platelet-rich plasma was prepared from after two spins for 1 min at 1200 × g, 10% (v/v) of acid citrate dextrose buffer (39 mM citric acid, 75 mM tri-sodium citrate, 135 mM d-glucose, pH 4.5) and prostaglandin E$_1$ (280 nM final concentration) was added, and the platelets were pelleted for 12 min at 700 × g and then resuspended in 6 ml of buffer (5.5 mM d-glucose, 128 mM NaCl, 4.26 mM Na$_2$HPO$_4$, 7.46 mM NaH$_2$PO$_4$, 4.77 mM tri-sodium citrate, 2.35 mM citric acid, 0.35% bovine serum albumin, pH 6.5). Prostaglandin E$_1$ was added as above, and the platelets were spun for 6 min at 700 × g. Platelets were resuspended to 2 × 10$^8$ platelets/ml in buffer.

**Platelet Isolation and Stimulation for Signaling Experiments**—Platelets from pooled concentrate obtained from the National Blood Service, Cambridge, UK were prepared by centrifugation (250 × g for 15 min) to remove contaminating red blood cells. Apyrase (0.25 units/ml) was added to the platelet suspension, which was then centrifuged (700 × g) for 15 min. The platelet pellet was washed with HEPES-buffered saline (145 mM NaCl, 10 mM HEPES, 5 mM KCl, 10 mM glucose, 1 mM MgSO$_4$, 0.5 mM EGTA, pH 7.4) and spun (700 × g) for 10 min. Washed platelets were counted using a Beckman Coulter counter and diluted to 1 × 10$^9$ cells/ml. A maximal dose of CRP-XL (10 μg/ml) was used to stimulate platelets for 90 s unless otherwise indicated in the figure legends. Ligand was diluted in acetic acid (0.01 M), which was used as a control as required. For PECAM-1 activation, platelets were stimulated with 10 μg/ml PECAM-1 F(ab’)$_2$ fragments for 90 s or preincubated for 10 min before CRP-XL stimulation.

**Lipid Raft Isolation**—Platelets (450 μl) prepared as described above were preincubated at 37 °C with MβCD (10 mM) for 30 min, where indicated, and then stimulated with CRP-XL (10 μg/ml) for 90 s. The reaction was terminated by the addition of 2 × lipid raft lysis buffer (20 mM Tris, 100 mM NaCl, 60 mM sodium pyrophosphate, 20 mM sodium glycerocephosphate, 0.02% w/v sodium azide, 0.025% Triton X-100, 2 mM sodium vanadate, protease inhibitor tablet, pH 8.0). Lysates were vortexed and incubated on ice for 30 min. Samples were mixed with equal volumes of 80% w/v sucrose to give 40% w/v final concentration. This was transferred to an ultracentrifuge tube (14 × 95 mm) where 5 ml of 36% w/v sucrose was layered on top followed by another 5-ml layer of 5% w/v sucrose. Each sucrose solution also contained 0.025% w/v Triton X-100. Tubes were ultracentrifuged in an Optima LE-80K at 200,000 × g for 18 h at 4 °C. Sequential 1-ml fractions were collected from the top of each sample.

**Cholera Toxin-B Assay**—Washed platelets (450 μl) were incubated with cholera toxin-B HRP (1930 milliunits in 3 μl) for 1 h at 37 °C before stimulation. After lipid raft isolation, 75 μl of each fraction was dispensed to a 96-well plate and mixed with 50 μl of the colorimetric substrate 3,3′,5,5′-tetramethylbenzidine at room temperature until a change in color could be seen. The reaction was quenched by the addition of 125 μl of 2.5 M H$_2$SO$_4$, and absorbance was measured at 450 nm. Standard curves with activity ranges of 0–100 microunits were produced in parallel with experimental samples to test the linearity and sensitivity of the assay. Control curves were produced to ensure that sucrose from the fractions did not interfere with the assay. Protein content of the fractions (20 μl) was also measured, using the MicroBCA assay kit (Perbio, Rockford II).

**Immunoprecipitation Studies**—Lipid raft fractions or platelets stimulated with CRP-XL (10 μg/ml) were combined with
Interaction of PECAM-1 and GPVI-FcRγ in Lipid Rafts

2× lysis buffer (2% Nonidet P-40, 300 mM NaCl, 50 mM Tris, 2 mM Na3VO4, protease tablet, pH 7.3), vortexed, and incubated on ice for 30 min. Lysates were precleared with 20 μl of protein G Dynal beads for 1 h. The antibody-bead complex was added to the lysates for 3 h, after which the beads were washed in 1× lysis buffer and resuspended in SDS-sample buffer. Proteins were separated using 8 or 15% (SDS-PAGE) and transferred to a nitrocellulose membrane in a Hoefer semi-dry blotter for 2 h at 0.8 mA/cm². Uniform transfer was confirmed by staining with Ponceau S (0.1% w/v Ponceau S, 5% v/v acetic acid). Membranes were blocked in 5% w/v bovine serum albumin in Tris-buffered saline-Tween (20 mM Tris, 140 mM NaCl, 0.1% v/v Tween 20, pH 7.6) for 1 h to prevent nonspecific binding of antibodies. All primary antibodies were diluted in 5% w/v bovine serum albumin in Tris-buffered saline-Tween and incubated overnight at 4 °C, after which the membranes were incubated with the appropriate secondary antibody. Antibodies were visualized by ECL using commercial reagents. Bands were quantified by video densitometry using a Leica Q550C image analyzer (Leica Imaging Systems, Cambridge, UK).

Confocal Microscopy—Washed platelets from whole blood were allowed to sediment and adhere under gravity to CRP-XL-coated coverslips (10 μg/ml) for 30 min and fixed with 2% paraformaldehyde. Samples were quenched with 0.1M ammonium chloride before labeling with rabbit anti-GPVI, mouse anti-PECAM-1, mouse anti-LAT, or rabbit anti-LAT as indicated. Fluorescein isothiocyanate- and Alexa Fluor-conjugated secondary antibodies were used to detect the proteins using a Leica DMIRB fluorescence microscope, with a ×63 PlanApo objective (Leica, Voorburg, the Netherlands), interfaced with a Leica TCS4D confocal laser microscope (Leica Lasertechnik, Heidelberg, Germany).

RESULTS

Cholera Toxin-B Subunit Detects Lipid Rafts—The ability of the cholera toxin-B subunit to bind the lipid raft marker, GM1 (ganglioside 1), was exploited to identify lipid raft fractions in the sucrose gradient reproducibly and accurately in a 96-well colorimetric assay. Lysates subjected to sucrose fractionation separate into two populations: lipid raft protein and soluble protein (Fig. 1A). The profile of cholera toxin-B HRP activity had two peaks (Fig. 1B). Lipid raft fractions that are enriched in cholesterol and sphingolipid were found at the interface between the 5 and 36% sucrose, and soluble protein and cytoskeleton, including the cholera toxin-B that either had not bound to GM1 or may have been internalized upon binding to GM1, was found in the denser sucrose fractions. This second peak may also contain rafts tightly associated with the cytoskeleton (25). The distributions for stimulated and non-stimulated platelets were similar.

GPVIdependent Tyrosine Phosphorylation and Aggregation Is Impaired by Methyl-β-cyclodextrin Treatment—Western blots of platelets stimulated with CRP-XL after incubation with MβCD or under control conditions revealed that MβCD treatment decreased the level of tyrosine phosphorylation induced by 10 μg/ml CRP-XL (Fig. 2A). Phosphorylation in the 120-kDa region, which contains proteins such as PECAM-1 and PLCγ2, was reduced by MβCD treatment. Whole gel lanes were analyzed by densitometry (Fig. 2A, right panel). The characteristic two waves of phosphorylation seen with a high level of CRP-XL (10 μg/ml) were apparent regardless of treatment with MβCD, but phosphorylation levels were lower (<60%) than in control platelets.

CRP-XL at low concentrations (0.002 and 0.02 μg/ml) was unable to induce aggregation (Fig. 2B, right panel). Using CRP-XL at 0.2 μg/ml, 5 and 10 mM MβCD treatments inhibited aggregation by 29.5 ± 8 and 37 ± 10.2%, respectively. At 20 mM MβCD, the level of aggregation was decreased by 71.7 ± 7.3% of control activity. Higher concentrations of CRP-XL were able to overcome the effects of MβCD, but platelet aggregation did not reach control levels. The pA50 of CRP-XL in control platelets was 1.09 ± 0.07 μg/ml, and this was decreased to 0.95 ± 0.12, 0.99 ± 0.07, and 0.61 ± 0.31 μg/ml for 5, 10, and 20 mM MβCD-treated platelets, respectively.

Bodin et al. (26) have reported that thrombin-induced platelet aggregation is not lipid raft-dependent. However, in our hands, aggregation induced by 0.1 units/ml thrombin was decreased by 19 ± 7.8 and 35.7 ± 16.8% with 5 and 10 mM MβCD treatment, respectively (Fig. 2C, left panel). With 20 mM

![FIGURE 1. Isolation and detection of lipid rafts using cholera toxin-B subunit.](image-url)
FIGURE 2. The effect MβCD on tyrosine phosphorylation and aggregation. A, washed platelets from concentrate were preincubated with or without MβCD, stimulated with CRP-XL (10 μg/ml) for 90 s, lysed with SDS sample buffer, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with mouse anti-tyrosine phosphorylation (4G10). Whole lanes were analyzed using densitometry, and data shown are the mean integrated optical density, expressed relative to basal, ± S.E., n = 3. Statistical analysis was carried out using a two-way analysis of variance (***, p < 0.001). Aggregation traces are shown for control and MβCD-treated platelets stimulated with 0.2 μg/ml CRP-XL (B) and 0.1 units/ml thrombin (C). The percentage of transmittance was measured at 5 min, and concentration-dependent curves were plotted in the presence of the indicated level of MβCD. Means ± S.E. are shown for three separate experiments.
Interaction of PECAM-1 and GPVI-FcRγ in Lipid Rafts

MβCD treatment, the level of aggregation was reduced by 52 ± 0.06% when compared with control platelets. Although this reduced aggregation is smaller than that seen in CRP-XL-stimulated platelets, MβCD treatment clearly inhibits thrombin-induced aggregation. The pA50 of thrombin in control platelets peaked at 90 s and remained constant up to 300 s (Fig. 3A, panel i). In contrast, the tyrosine phosphorylation of the FcRγ-chain, detected indirectly in syk SH2 domain pull downs, was assessed. Platelets stimulated with 10 μg/ml CRP-XL or 10 μg/ml PECAM-1 Fab(1), fragments (1.3) were immunoprecipitated for PLCγ2 for 3 h before washing with lysis buffer. Beads were resuspended in SDS-PAGE sample buffer, and protein was subjected to SDS-PAGE, transferred to nitrocellulose, and probed for tyrosine phosphorylation and total protein. WCL, whole cell lysate. WB, Western blot. A, panel i, bands were analyzed using densitometry and plotted against time. Data shown are mean integrated optical density ± S.E., n = 4. B, panel i, platelets stimulated with either 10 μg/ml CRP-XL or 10 μg/ml PECAM-1 Fab(1) fragments (1.3) were immunoprecipitated for PLCγ2 for 3 h at 4 °C. Immunoprecipitates were separated on SDS-PAGE and probed with 4G10 and for total protein. B, panel ii, the same immunoprecipitation was performed with platelets preincubated with PECAM-1 Fab(1)2 fragments for 10 min before stimulation with CRP-XL (10 μg/ml). In each panel, the suffix P is used to denote phosphorylated forms of the target proteins.

Maximal Tyrosine Phosphorylation of PECAM-1 Precedes That of FcRγ-Chain—To determine the temporal relationship between activation of PECAM-1 and the FcRγ-chain, tyrosine phosphorylation of each protein was examined in immunoprecipitates from washed platelets stimulated with CRP-XL for the indicated times (Fig. 3A, panel i). PECAM-1 phosphorylation peaked at 90 s and remained constant up to 300 s (Fig. 3A, panel ii). In contrast, the tyrosine phosphorylation of the FcRγ-chain increased for up to 180 s, after which it declined. These data are consistent with those obtained by Tsuji et al. (12), who stimulated platelets with an anti-GPVI F(ab)2, although the massive dose of collagen used by these authors elicited more rapid phosphorylation of the FcRγ-chain, detected indirectly in syk SH2 domain pull downs. The present results confirm that GPVI activation can elicit PECAM-1 phosphorylation (17), which occurs in advance of that of the FcRγ-chain and is sustained throughout the course of FcRγ-chain activation.

The tyrosine phosphorylation of PLCγ2, a protein downstream of the ITAM-containing receptor, was assessed. Platelets stimulated with 10 μg/ml CRP-XL or PECAM-1 F(ab)2, for 90 s were immunoprecipitated with anti-PLCγ2 and then probed with 4G10. PLCγ2 tyrosine phosphorylation was only apparent in CRP-XL-stimulated cells and not in those that were stimulated with PECAM-1 F(ab)2 (Fig. 3B, panel i). Preincubation of platelets with PECAM-1 F(ab)2, for 10 min before stimulation with 10 μg/ml CRP-XL, however, did decrease the phosphorylation of PLCγ2 by 46 ± 9% (n = 3), suggesting a down-regulation of an ITAM-dependent pathway by an ITIM receptor (Fig. 3B, panel ii).

Co-immunoprecipitation Studies in Whole Cell Lysates Show That PECAM-1, GPVI, and FcRγ-Chain Are Associated—Co-immunoprecipitation studies were performed in platelet lysates, either from resting cells or after stimulation, for 90 s with 10 μg/ml CRP-XL. Whole cell lysates and immunoprecipitates of GPVI, the FcRγ-chain, or PECAM-1 were separated by SDS-PAGE, and then associated proteins were immunodetected. The majority of GPVI was recovered in the immunoprecipitate fraction since GPVI could not be detected either in bead-only controls or in the bead supernatants (results not shown). PECAM-1 was detected in immunoprecipitates of GPVI from both resting and stimulated cells (Fig. 4A) but was detected much more strongly after CRP-XL treatment. The FcRγ-chain immunoprecipitates similarly contained detectable PECAM-1 (Fig. 4B). When the immunoprecipitatin and detecting antibodies were reversed, GPVI was found with PECAM-1 under both resting and stimulated conditions (Fig. 4C), but the FcRγ-chain was undetectable in PECAM-1 immunoprecipitates. GPVI immunoprecipitates were probed for the FcRγ-chain and viro versa as positive controls (Fig. 4D). Isotype-matched nonspecific antibodies did not pull down PECAM-1, the FcRγ-chain, or GPVI (Fig. 4E).

The GPVI-FcRγ-Chain Complex, PECAM-1, and LAT Are Present in Lipid Rafts—The presence of GPVI, the FcRγ-chain, and PECAM-1 was examined in lipid rafts, using LAT and β3-integrin as positive and negative controls. Lipid rafts were isolated from CRP-XL-stimulated lysates and concentrated with PAGE-PREP. GPVI, the FcRγ-chain, PECAM-1, and LAT were present in both lipid raft and soluble fractions (Fig. 5); however, GPVI and the FcRγ-chain were enriched in the former (60 ± 5 and 75 ± 8% of the total, respectively), whereas only 26 ± 5% of total PECAM-1 was located in lipid rafts, measured densitometrically. LAT was largely found in lipid rafts (>80%). The multiple bands seen for the FcRγ-chain are presumed to correspond to the different phosphorylation states of the receptor (27). In the soluble fractions, two forms of the FcRγ-chain were observed: the lower band (i) representing the nonphosphorylated protein and the upper band (ii) being a phos-
The activated FcR γ-chain (iii and iv) appeared in the lipid raft fractions, however. This suggests either that the activated FcR γ-chain is preferentially recruited to lipid rafts or that the lipid raft environment promotes the phosphorylation of the FcR γ-chain. The salt bridge linking GPVI and the FcR γ-chain dictates that their distribution in the gradient is similar. PECAM-1 was present in lipid rafts but, unlike GPVI and the FcR γ-chain, was not enriched in raft fractions. The presence of LAT confirmed the identity of rafts, whereas the β3 integrin subunit appeared only in the soluble fractions.

**Co-immunoprecipitation of PECAM-1, GPVI, and FcR γ-Chain**

All three receptors were separately immunoprecipitated from lipid rafts isolated from resting and CRP-XL-stimulated platelets. Western blotting confirmed the findings described above, that PECAM-1, GPVI, and the FcR γ-chain are found in lipid raft as well as soluble fractions. Immunoblotting for LAT confirmed the identity of lipid raft fractions (Fig. 6D), but LAT became relatively more abundant in non-raft fractions after MβCD treatment. Immunoprecipitates of both the FcR γ-chain and GPVI were found to contain PECAM-1 (Fig. 6, A and B). In resting platelets, a small amount of PECAM-1 was pulled down with either GPVI or the FcR γ-chain. CRP-XL stimulation enhanced this interaction; the amount of PECAM-1 bound to the GPVI-FcR γ-chain complex increased. However, no association between PECAM-1 and GPVI/FcRγ was detected in material from platelets treated with MβCD. The multiple bands of phosphorylated FcR γ-chain were only present in lipid rafts of CRP-XL-stimulated platelets, and MβCD treatment prevented this phosphorylation, again suggesting that the raft environment favors phosphorylation and/or recruitment of phosphorylated forms of the FcR γ-chain. MβCD treatment itself seemed activatory as a small population of phosphorylated FcR γ-chain existed in lipid rafts of resting cells. Immunoprecipitation of PECAM-1 from resting and stimulated platelets confirmed its association with GPVI (Fig. 6C). Again, this interaction was only seen in lipid raft fractions and was not detected after MβCD treatment. As with the whole cell lysates (Fig. 4), the FcR γ-chain did not co-immunoprecipitate with PECAM-1 from lipid rafts. These results demonstrate that lipid rafts are essential for the association of PECAM-1 with the GPVI-FcR γ-chain complex as disruption of lipid rafts with MβCD results in the loss of interaction.

The stoichiometry of the PECAM-1-GPVI interaction was estimated by densitometry of Western blots obtained from whole platelet lysates, data used to calibrate the amount of each receptor in immunoprecipitates, assuming 2,000 copies of GPVI and 10,000 copies of PECAM-1 per platelet. This allowed us to estimate that one molecule of GPVI associates with two molecules of PECAM-1 in GPVI immunoprecipitates, a ratio not altered greatly after stimulation with CRP-XL. The equivalent calculation from PECAM-1 immunoprecipitates suggests that one copy of GPVI could be recovered from 35 copies of immunoprecipitated PECAM-1, but given the 5-fold excess expression of PECAM-1 when compared with GPVI, these data suggest a 1:7 ratio. Since GPVI, but not PECAM-1, is enriched in lipid rafts, these data should be further modified by the rela-
Interaction of PECAM-1 and GPVI-FcRγ in Lipid Rafts

---

**DISCUSSION**

The main signaling receptor for collagen on platelets is GPVI (22), which relays signals through the ITAM motif of the FcR γ-chain, a key step in the platelet activation pathway and a mechanism that is well understood. Much less is known about the ITIM-containing inhibitory receptors that can modulate these signals, and recent evidence suggests a role for ITIMs in mediating platelet activation (28). The studies described here were designed to investigate the interaction of the ITAM-containing GPVI/FcRγ complex with the ITIM-containing PECAM-1 in platelet lipid rafts.

Although many studies have implied a functional interplay between ITIM and ITAM receptors, few have shown a physical association between them. Jones et al. (17) have shown an enhancement of platelet aggregation and secretion responses with collagen and CRP-XL stimulation in PECAM-1-deficient mice, implying an inhibitory role for PECAM-1. These mice also showed larger thrombi when perfused over a collagen matrix. Coinciding with these results, Cicmil et al. (20) demonstrated that cross-linking PECAM-1 inhibits aggregation and secretion in the response of the platelet to collagen and to the GPVI-selective agonist, convulxin. Tyrosine phosphorylation was also inhibited by the activation of PECAM-1. The first evidence of a physical association between an ITIM- and ITAM-containing receptor in platelets was obtained for PECAM-1 and FcγRIIA (15). The two receptors were shown to be in close proximity to each other by FRET experiments, results that were confirmed by co-immunoprecipitation. However, the mechanism of their association was not investigated.

The importance of lipid rafts in CRP-induced pathways was demonstrated by using MβCD. Treatment with the lipid raft disruptor attenuated levels of tyrosine phosphorylation and impaired aggregation in response to CRP-XL. The selectivity of MβCD was assessed, using aggregation of platelets stimulated with thrombin. The lower aggregation caused by both CRP and thrombin after MβCD treatment suggests that the aggregation process itself is dependent on intact lipid rafts, being sensitive to MβCD, but it is important to note that higher concentrations of either ligand were able to compensate for the effects of MβCD. Treatment with the reagent does not grossly perturb platelet function, therefore, and our data provide no evidence for specificity. Ezumi et al. (6) have shown that phorbol 12-myristate 13-acetate, which activates protein kinase C, was able to induce normal levels of aggregation in MβCD-treated platelets, whereas other groups have also shown impairment by MβCD of receptor-mediated aggregation, induced by ADP and thrombin receptor activating peptide (TRAP) (29, 30).

The use of MβCD alone as an indicator of raft involvement would be open to criticism of the competence of the resulting platelets as well as the specificity of the reagent. To complement these data, therefore, we isolated raft fractions and investigated the presence of GPVI, the FcR γ-chain, and PECAM-1. We show that there is a physical interaction of PECAM-1 with both GPVI and the FcR γ-chain (Fig. 4) and that this occurs only in lipid rafts (Fig. 6). Co-localization of PECAM-1 with GPVI was observed using confocal microscopy (Fig. 7), supporting immunoprecipitation data. The nature of the interaction is still unknown, and whether or not PECAM-1 binds to GPVI, the FcR γ-chain, or both receptors at the same time is difficult to determine since GPVI and the FcR γ-chain are linked via a salt bridge. The association of PECAM-1 with the GPVI-FcR γ chain complex may be indirect since it is markedly enhanced.
upon platelet activation. Thus it may be mediated via an adapter that can bind to both the phosphorylated PECAM-1 and the suitable domains of the GPVI-FcR γ complex (the ITAM of FcR γ or the Pro-rich domain of GpVI), similar to Grb2 that contains both SH2 and SH3 domains. However, direct association cannot be excluded. The clustering of lipid rafts may bring the receptors into close proximity so that activated PECAM-1 can recruit phosphatases such as SHP-1, SHP-2, and SHIP that dephosphorylate proteins and lipids to terminate signals from the ITAM of the FcR γ-chain. There is evidence that lipid rafts are concentrated at the filopodia of platelets (30, 31), which would suggest that the cytoskeleton plays a major role in the integrity of lipid rafts and vice versa (32, 33). Lipid raft clustering may also therefore occur during platelet spreading when the cytoskeleton is most dynamic.

We have confirmed previous findings that PECAM-1 is activated by stimulation with CRP (17), but it is interesting that its phosphorylation precedes and is sustained throughout the process of FcR γ-chain phosphorylation and dephosphorylation (Fig. 3A), suggesting that the activation of PECAM-1 is not dependent upon the FcR γ-chain and that PECAM-1 either is separately regulated or lies upstream of the FcR γ-chain, perhaps exerting an activatory function on the pathway as a whole. A plausible mediator of such effects might be SHP-1, a protein that binds to activated PECAM-1 (34) and has been shown to activate GPVI (35). The Src family kinases are responsible for the activation of both ITAM-containing (36, 37) and ITIM-containing receptors (21) and, being both activated and inhibited by kinase and phosphatase activity, may provide the key link between the pathways. The present study indicates a site of signal divergence, with GPVI clustering leading to activation of kinases that phosphorylate PECAM-1 and potentially other substrates, independently of the classical ITAM-mediated signals. Detailed kinetic analysis may further elucidate these processes.

We were unable to co-immunoprecipitate the FcR γ-chain with PECAM-1 in resting platelets or under any other conditions using PECAM-1 antibodies for immunoprecipitation. The apparent anomaly, depending on which protein is the bait in the immunoprecipitation, may be useful in identifying the site of interaction between PECAM-1 and the FcR γ-chain. A possible reason that PECAM-1 antibodies did not also pull down the FcR γ-chain, although the reverse pull downs were effective, is that the interaction site of the two receptors is at the epitope of the immunoprecipitating goat anti-PECAM-1, which is directed at the carboxyl terminus of PECAM-1. This may contain the site

FIGURE 6. Co-immunoprecipitation of PECAM-1 with the GPVI-FcR γ-chain complex in lipid rafts (LR). Washed platelets were stimulated with 10 μg/ml CRP-XL and lysed in 0.025% Triton X-100 buffer. Samples were mixed with equal volumes of 80% sucrose and transferred to an ultracentrifuge tube where 36 and 5% sucrose was layered on top. Tubes were centrifuged for 16 h at 200,000 × g. 1-ml fractions were taken from the top of the tube and mixed with an equal volume of 2% Nonidet P-40 lysis buffer. Protein G Dynal beads preincubated with Upstate Biotechnology rabbit anti-FcR γ-chain (A), rabbit anti-GPVI (B), and goat anti PECAM-1 (C) were added to the precleared lysates for 3 h before washing with lysis buffer and resuspended in SDS-PAGE sample buffer. Protein was subjected to SDS-PAGE, transferred to nitrocellulose, probed for immunoprecipitated protein, and then stripped and reprobed for co-immunoprecipitating protein (D). Immunoblotting for LAT was performed to confirm lipid raft fractions. Blots are representative of five separate experiments. Sol, soluble fraction; IP, immunoprecipitate; WB, Western blot.
at which it associates with the FcRγ-chain. It is interesting to note that the FcRγ-chain is also associated with GPlb-V-IX in platelets (38, 39), suggesting that lipid rafts may facilitate the interaction of other platelet receptors and so provide an environment that favors platelet signaling. This interpretation is supported by our data that show that raft depletion impairs both platelet aggregation and protein tyrosine phosphorylation. From these results, we demonstrate that lipid rafts provide a platform for signaling complexes to assemble, which is critical for the activation of the platelet.

REFERENCES

1. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–39
2. Schroeder, R. J., Ahmed, S. N., Zhu, Y., London, E., and Brown, D. A. (1998) J. Biol. Chem. 273, 1150–1157
3. Zajchowski, L. D., and Robbins, S. M. (2002) Eur. J. Biochem. 269, 737–752
4. Prowse, P. E., Lincz, L. F., Meldrum, C. J., and Burns, G. F. (1996) Biochem. J. 319, 67–72
5. Wenerow, P., Oberfell, A., Wilde, J. I., Bobe, R., Asazuma, N., Brdicka, T., Leo, A., Schraven, B., Horejsi, V., Shattil, S. J., and Watson, S. P. (2002) Biochem. J. 364, 755–765
6. Ezumi, Y., Kodama, K., Uchiiyama, T., and Takayama, H. (2002) Blood 99, 3250–3255
7. Locke, D., Chen, H., Liu, Y., Liu, C., and Kahn, M. L. (2002) J. Biol. Chem. 277, 18801–18809
8. Farndale, R. W., Sixma, J. J., Barnes, M. J., and de Groot, P. G. (2004) J. Thromb. Haemostasis 2, 561–573
9. Clemetson, J. M., Polgar, J., Magnenet, E., Wells, T. N., and Clemetson, K. J. (1999) J. Biol. Chem. 274, 29019–29024
10. Nieswandt, B., and Watson, S. P. (2003) Blood 102, 449–461
11. Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M., and Watson, S. P. (1997) FEBS Lett. 413, 255–259
12. Tsuji, M., Ezumi, Y., Ariai, M., and Takayama, H. (1997) J. Biol. Chem. 272, 23528–23531
13. Watson, S. P., Asazuma, N., Atkinson, B., Berlange, O., Best, D., Bobe, R., Jarvis, G., Marshall, S., Snell, D., Stafford, M., Tulusne, D., Wilde, J., Wenerow, P., and Frampston, J. (2001) Thromb. Haemostasis 86, 276–288
14. Jackson, D. E., Ward, C. M., Wang, R., and Newman, P. J. (1997) J. Biol. Chem. 272, 6986–6993
15. Thai, Le M., Ashman, L. K., Harbour, S. N., Hogarth, P. M., and Jackson, D. E. (2003) Blood 102, 3637–3645
16. Rathore, V., Stapleton, M. A., Hillery, C. A., Montgomery, R. R., Nichols, T. C., Merrick, E. P., Newman, D. K., and Newman, P. J. (2003) Blood 102, 3658–3664
17. Jones, K. I., Hughan, S. C., Dopheide, S. M., Farndale, R. W., Jackson, S. P., and Jackson, D. E. (2001) Blood 98, 1456–1463
18. Patil, S., Newman, D. K., and Newman, P. J. (2001) Blood 97, 1727–1732
19. Wu, X. W., and Lian, E. C. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 3154–3158
20. Clemetson, K. J., and Clemetson, J. M. (2001) Thromb. Haemostasis 86, 189–197
21. Asselin, J., Gibbins, J. M., Achison, M., Lee, Y. H., Morton, L. F., Farndale, R. W., Barnes, M. J., and Watson, S. P. (1997) Blood 89, 1235–1242
22. Morton, I. F., Hargreaves, P. G., Farndale, R. W., Young, R. D., and Barnes, M. J. (1995) Biochem. J. 306, 337–344
23. Bodin, S., Soulet, C., Tronchere, H., Sie, P., Gachet, C., Plantavid, M., and Payrastre, B. (2005) J. Cell Sci. 25, 25
24. Bodin, S., Soulet, C., Tronchere, H., Sie, P., Gachet, C., Plantavid, M., and Payrastre, B. (2005) J. Cell Sci. 118, 759–769
25. Gibbins, J., Asselin, J., Farndale, R., Barnes, M., Law, C. L., and Watson, S. P. (1996) J. Biol. Chem. 271, 18095–18099
26. Barrow, A. D., Astoul, E., Floto, A., Brooke, G., Relou, I. A., Jennings, N. S., and Jackson, D. E. (1998) Blood 92, 1971–1978
27. Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M., and Watson, S. P. (2000) Blood 96, 1727–1732
28. Farndale, R. W., Barnes, M. J., and Watson, S. P. (1997) J. Biol. Chem. 272, 18095–18099
29. Quinton, T. M., Kim, S., Jin, J., and Kunapuli, S. P. (2005) J. Thromb. Haemostasis 3, 1036–1041
30. Heijnen, H. F., Van Lier, M., Waijenborg, S., Ohno-Iwashita, Y., Waheed, A. A., Inomata, M., Gorter, G., Mobius, W., Akkermans, J. W., and Slot, J. W. (2003) Thromb. Haemostasis 90, 1161–1173
31. Nishi, M., Lee, F., Farndale, R. W., Gorter, G., Verhoef, S., Ohno-Iwashita, Y., Akkermans, J. W., and Heijnen, H. F. (2005) J. Thromb. Haemostasis 3, 2514–2525
32. Villalba, M., Bi, K., Rodriguez, F., Tanaka, Y., Schoenberger, S., and Altman, A. (2001) J. Cell Biol. 155, 331–338
33. Valensin, S., Paccini, S. R., Ullivieri, C., Mercati, D., Pacini, S., Patrussi, L., Hirst, T., Lupetti, P., and Baldari, C. T. (2002) Eur. J. Immunol. 32, 435–446
34. Hua, C. T., Gamble, J. R., Vadas, M. A., and Jackson, D. E. (1998) J. Biol. Chem. 273, 28332–28340
35. Pasquet, J. M., Quek, L., Pasquet, S., Poole, A., Matthews, J. R., Lowell, C., and Watson, S. P. (2000) J. Biol. Chem. 275, 28526–28531
36. Quek, L. S., Pasquet, J. M., Hers, L., Cornall, R., Knight, G., Barnes, M., Hibbs, M. L., Dunn, A. R., Lowell, C. A., and Watson, S. P. (2000) Blood 96, 4246–4253
37. Ezumi, Y., Shindo, K., Tsuji, M., and Takayama, H. (1998) J. Exp. Med. 188, 267–276
38. Wu, Y., Suzuki-Inoue, K., Satohe, K., Asazuma, N., Yatomi, Y., Berndt, M. C., and Ozaki, Y. (2001) Blood 97, 3836–3845
39. Falati, S., Edmead, C. E., and Poole, A. W. (1999) Blood 94, 1648–1656