Signaling Role for Phospholipase Cγ2 in Platelet Glycoprotein Ibα Calcium Flux and Cytoskeletal Reorganization

IN Volvement of a Pathway Distinct from FcRγ Chain and FcγRIIA*

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Interaction of the platelet GPIb-V-IX complex with surface immobilized von Willebrand factor (vWF) is required for the capture of circulating platelets and their ensuing activation. In previous work, it was found that GPIb/vWF-mediated platelet adhesion triggers Ca2+ release from intracellular stores, leading to cytoskeletal reorganization and filopodia extension. Despite the potential functional importance of GPIb-induced cytoskeletal changes, the signaling mechanisms regulating this process have remained ill-defined. The studies presented here demonstrate an important role for phospholipase C (PLC)-dependent phosphoinositide turnover for GPIb-dependent cytoskeletal remodeling. This is supported by the findings that the vWF-GPIb interaction induced a small increase in inositol 1,4,5-triphosphate (IP3) and that treating platelets with the IP3 receptor antagonist APB-2 or the PLC inhibitor U73122 blocked cytosolic Ca2+ flux and platelet shape change. Normal shape change was observed in Goi−/− mouse platelets, excluding a role for PLCβ isoforms in this process. However, decreased shape change and Ca2+ mobilization were observed in mice lacking PLCγ2, demonstrating that this isotype played an important, albeit incomplete, role in GPIb signaling. The signaling pathways utilized by GPIb involved one or more members of the Src kinase family as platelet shape change and Ca2+ flux were inhibited by the Src kinase inhibitors PP1 and PP2. Strikingly, shape change and Ca2+ release occurred independently of immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors, because these platelet responses were normal in human platelets treated with the anti-FcγRIIA blocking monoclonal antibody IV.3 and in mouse platelets deficient in the FcγRγ chain. Taken together, these studies define an important role for PLCγ2 in GPIb signaling linked to platelet shape change. Moreover, they demonstrate that GPIB-dependent calcium flux and cytoskeletal reorganization involves a signaling pathway distinct from that utilized by ITAM-containing receptors.

Platelets are specialized blood cells that display unique adhesive properties relevant to hemostasis and thrombosis. Platelet adhesion is critically dependent on the synergistic interaction between multiple platelet receptors and adhesive ligands, with the involvement of specific receptor-ligand pairs dependent on the prevailing blood flow conditions (1). Under conditions of high shear stress, von Willebrand factor (vWF) binding to the platelet adhesive receptor, glycoprotein (GP) Ib/V/IX is critical for the initiation of platelet-vessel wall and platelet-platelet interactions and as such is a key adhesive event promoting thrombus growth (2). In addition to its adhesive function, the GPIb-V-IX complex also plays an important role in regulating the organization of the platelet cytoskeleton (3). For example, it has long been recognized from the study of platelets deficient in GPIb-V-IX (Bernard-Soulier syndrome) that this complex is important for maintaining the compact structure of the membrane skeleton, due at least in part, to the physical linkage between GPIb and actin-binding protein (ABP) 280 (ABP-280 or filamin-1) (4). More recently it has been demonstrated that vWF engagement of GPIb can induce cytoskeletal reorganization (5) and that these cytoskeletal changes play a potentially important role in regulating the adhesive function of GPIb (6). The physiological importance of vWF in inducing cytoskeletal reorganization has previously been highlighted from the study of von Willebrand disease pigs (7). Platelets from these animals adhere to sites of vascular injury; however, they exhibit defective cytoskeletal remodeling leading to reduced filopodial extension and poor spreading.

Despite the potential importance of GPIb in initiating platelet activation, the mechanisms by which GPIb transduces signals linked to cytoskeletal remodeling remains incompletely understood. We have previously demonstrated a potentially important role for intracellular Ca2+ mobilization in this process (5); however, to date, the proximal signaling molecules utilized by GPIb-V-IX to induce cytosolic Ca2+ flux have not been identified. The GPIb-V-IX receptor consists of four protein subunits belonging to the leucine-rich repeat superfamily, GPIbα, GPIbβ, GPIX, and GPV (8). The receptor intracellular region does not have catalytic activity, nor does it bind GTP-binding proteins or become phosphorylated by tyrosine kinases. Previous studies have suggested that GPIbα may signal

1 The abbreviations used are: vWF, von Willebrand factor; HvWF, human vWF; GP, glycoprotein; ABP, actin-binding protein; PI, phosphoinositide; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; IP3, inositol 1,4,5-triphosphate; CHO, Chinese hamster ovary; 2-APB, 2-aminoethoxydiphenyl borate; TRITC, tetramethylrhodamine isothiocyanate; mAb, monoclonal antibody; GST, glutathione S-transferase.

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directly as a consequence of its association with the cytoskeletal structural protein, actin-binding protein (ABP-280) (3), and/or signaling molecules such as 14-3-3ζ (9–11), calmodulin (12), Src kinase (13), and phosphoinositide (PI) 3-kinase (14). Alternatively, GPIb may transduce signals indirectly through the physical association with the ITAM-bearing receptors, FcγRII (15, 16), or FcγRIIIA (17, 18). FcγR and FcγRIIA receptor signaling is initiated by Src kinase-dependent tyrosine phosphorylation of the receptors’ ITAM motif, leading to the recruitment of p72syk, PI 3-kinase, and the adaptor proteins (LAT, SLP-76, and vav), which ultimately promote the activation of phospholipase Cγ2 (PLCγ2). The subsequent hydrolysis of phosphatidylinositol 4,5-diphosphate, leading to IP3 generation and intracellular calcium release is a critical event for efficient platelet activation (19, 20).

A consensus model for GPIb signaling has yet to emerge, despite intense investigation from a number of groups. In fact, there remains considerable controversy over a number of basic aspects with regards to GPIb signaling, including the contribution of direct and indirect pathways for platelet activation. One possible reason for the discrepancies in results from different studies reflects the variability in experimental approaches used to examine this process. For example, a wide variety of ligands (venom peptides, soluble human or bovine vWF, recombinant vWF fragments, and immobilized vWF), artificial modulators (ristocetin and botrocin), cell types (human and mouse platelets, GPIb-V-IX-transfected CHO and K562 cell lines), and functional assays (suspension-based aggregation studies, shear-induced platelet aggregation, and static or flow-based adhesion assays) have been utilized to examine GPIb signaling. In many of these studies it is unclear what the direct contribution of the cytoplasmic tails of the GPIb-V-IX complex is for signal generation relative to indirect signaling mediated through the FcγRII chain and FcγRIIIA receptor, soluble agonist release, and integrin αIibβ3 outside-in signaling. Furthermore, it is not always clear that the end-point used to examine GPIb signaling is in fact a direct functional response linked to this receptor.

We have previously established that the vWF-GPIb interaction per se is sufficient to induce cytosolic calcium spikes that are necessary for platelet cytoskeletal remodeling; independent of integrin αIibβ3 (5). In the current study we have utilized platelet shape change and cytosolic calcium transients as bona fide GPIb-dependent responses to examine the proximal signaling events linked to this receptor. Through the use of various pharmacological inhibitors and knock-out mouse models linked to PLC signaling, our studies have demonstrated an important role for Src kinase-dependent activation of the PLCγ2 isofrom in GPIb-dependent calcium flux and cytoskeletal remodeling. In contrast to many other studies, our results do not support an important role for Src kinase-dependent activation of the PLCγ2 chain and FcγRIIIA receptors in GPIb signaling.

EXPERIMENTAL PROCEDURES

Materials—2-Aminoethoxydihydroxyphenyl borate (2-APB), prostaglandin I2, bovine serum albumin, TRITC-phallolidin, fatty acid-free human serum albumin, bovine type I collagen, and serotonin were purchased from Sigma Chemical Co. (St. Louis, MO). U73122 and U73343 were from Calbiochem (San Diego, CA), PP1 and PP2 were from BIOMOL (Plymouth, PA). Ristocetin was obtained from ICN (Costa Mesa, CA), and botrocin was a kind gift from Prof. Michael Berndt (The Baker Institute, Melbourne, Australia). 1-Paraformaldehyde was purchased from Electron Microscopy Sciences (Washington, PA), and glass coverslips were from Polylabo (Strasbourg, France). Anti-αIibβ3, chimeric Fab fragment of monoclonal antibody (mAb) 7E5 (c7E5 Fab from BIOMOL) was from Eli-Lilly (Centocor, Leiden, Netherlands). The anti-FcγRIIA mAb, IV.3, was kindly donated by Dr. Ben Chong (Royal Prince of Wales Hospital, New South Wales, Australia), the anti-phosphotyrosine mAb PY 20 was purchased from BD Biosciences Transduction Laboratories (Lexington, KY), and the anti-PLCγ2 was from Santa Cruz Biotechnology (Santa Cruz, CA). Human vWF (HvWF) was purified from plasma cryoprecipitate according to the method of Montgomery and Zimmerman (21), and human fibrinogen was from Kabi (Stockholm, Sweden). Apyrase was purified from potatoes as previously described (22).

Mouse Strains—C57BL/6 (α-FcγRIIIA−/−) and wild type (α-FcγRIIIA+/+) mouse colonies were established at the animal facilities of the Etablissement Français du Sang-Alsace by breeding the α-FcγRIIIA−/− females (α-FcγRIIIA−/− mice) provided by Prof. Stephan Offeman (Universität Heidelberg, Germany) (23). C57BL/6 FcγR chain-deficient mice (FcγRIIIA−/−) were obtained from Taconic (Germantown, NY). C57BL/6 PLCγ2-deficient mice (PLCγ2−/−) were provided by Prof. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN). (C57BL/6×129Sv)F1 hybrid mice (C57BL/6×129Sv)F1). Cell Line—Chinese hamster ovary (CHO) cells, expressing the GPIb-IX complex, were established as described previously (26).

Platelet Preparation—Blood was drawn from healthy volunteers who had not taken an anti-platelet medication in preceding 2 weeks and collected in an acid citrate dextrose anticoagulant. Platelets were isolated by sequential centrifugation of the blood and washed as previously described (22). Platelets were finally resuspended in Tyrode’s buffer (12 mM NaHCO3, 0.3 mM NaH2PO4, 5 mM Hepes, pH 7.3, 137 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5.5 mM glucose) containing human serum albumin and apyrase (0.02 unit/ml) and kept at 37 °C. Apsionated platelets were obtained by treating cells with 1 μM aspirin for 15 min, prior to final suspension in Tyrode’s buffer. Washed mouse platelets were prepared according to the method described by P. et al. (27). Mouse blood was taken from the abdominal aorta of anesthetized animals and collected in an acid citrate dextrose anticoagulant.

Platelet Aggregation Studies—Aggregation was performed using a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). Platelets (3 × 108 platelets/ml) were stimulated at 37 °C by collagen (1.25 μg/ml) in the presence of human fibrinogen (275 μg/ml), in a final volume of 500 μl. Aggregation was initiated by stirring the platelet mixture at 1100 rpm. When vWF-induced platelet agglutination was examined, aspirinated platelets (3 × 108 platelets/ml) were pre-treated with c7E3 Fab (20 μg/ml) for 10 min or with EDTA (5 mM), then stirred in the presence of human vWF (20 μg/ml) and ristocetin (200 μg/ml). In control studies, the pharmacological PLCγ2 inhibitor, 2-APB, was confirmed by complete inhibition of thrombin (1 unit/ml)-induced platelet aggregation at 50 μM. The ability of IV.3 (5 μg/ml) to block FcγRIIA function was confirmed by inhibition of heat-aggregated immunoglobulins (800 μg/ml)-induced platelet aggregations.

Cell Adhesion and Morphology Analysis—Human platelet adhesion studies were performed as previously described (5). Briefly, platelets (2 × 105/ml) in Tyrode’s buffer were treated with c7E3 Fab (20 μg/ml), then allowed to adhere to a HvWF (10 μg/ml) matrix for 30 min at 37 °C, in the presence of 2 units apyrase. Where indicated, platelets were also preincubated for 10 min with the inhibitors of phospholipase C U73122 (2 μM), 2-APB (20 μM), or ristocetin (200 μg/ml). In control studies, the pharmacological PLCγ2 inhibitor, 2-APB, was added to block aggregation. Platelet shape change was defined by the transformation of platelet morphology (Leica TCSNT) (63× objective). Alternatively, the fixed adherent platelets were stained with TRITC-phallolidin (2 μg/ml) for 30 min and then subjected to fluorescence microscopy. Fluorescent images were obtained at 570 nm, Leica DML microscope (63× objective). The number of adherent cells was scored in five to eight random fields. Platelet shape change was defined by the transformation of platelet morphology from discoid with no filopodial projection to spherical with filopodial projections greater than 0.2 μm in length. Prothrombinase was performed using mouse platelets (3 × 107/ml) or CHO cells (1 × 106/ml). Identical experimental conditions were used except that botrocin (2 μg/ml) was present to support cell adhesion to HvWF, and the integrin-vWF interaction was prevented by the addition of EDTA (5 mM) or c7E3 Fab (20 μg/ml), respectively.

Scanning Electron Microscopy—Adherent platelets were fixed for 45 min in 2.5% glutaraldehyde (0.1 M) and then subjected to fluorescence microscopy (UV illumination at 570 nm, Leica DML microscope) (63× objective). The number of adherent cells was scored in five to eight random fields. Platelet shape change was defined by the transformation of platelet morphology from discoid with no filopodial projection to spherical with filopodial projections greater than 0.2 μm in length. Prothrombinase was performed using mouse platelets (3 × 107/ml) or CHO cells (1 × 106/ml). Identical experimental conditions were used except that botrocin (2 μg/ml) was present to support cell adhesion to HvWF, and the integrin-vWF interaction was prevented by the addition of EDTA (5 mM) or c7E3 Fab (20 μg/ml), respectively.
The vWF-GPIb Interaction Induces IP_3 Formation—We have previously demonstrated that vWF engagement of GPIb induces transient Ca^{2+} spikes that initiate cytoskeletal remodeling (5). In platelets, release of Ca^{2+} from intracellular stores is primarily mediated by inositol 1,4,5-trisphosphate (IP_3), generated through the hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, by one or more phospholipase C (PLC) isoforms. To date, there is limited evidence that the vWF-GPIb interaction can induce PLC activation and IP_3 generation in platelets (16). To examine this possibility, we initially quantified IP_3 levels in platelets aggregated by vWF (10 μg/ml) and ristocetin (1 mg/ml), in the presence of the anti-integrin αIIbβ3 antibody c7E3 Fab. IP_3 levels were determined on platelet aggregates rather than from adhesion assays, because detailed under “Experimental Procedures.” The results demonstrate the relative increase in Ca^{2+} levels in platelets adherent to vWF compared with non-stimulated platelets in suspension (Resting) or following activation with thrombin (1 unit/ml, Thrombin). These results are from one experiment, representative of five.

**Experimental Procedures.** Washed human platelets (2 × 10^9/ml) were treated with c7E3 Fab (20 μg/ml) or from an individual with Glanzmann’s thrombasthenia, were aggregated with HvWf (20 μg/ml) and ristocetin (1 mg/ml) for 10 min. Where indicated, platelets were preincubated with the inhibitors of PLC, U73122 (1 mM) was also present to prevent the integrin αIIbβ3 interaction, 3 mg of platelets were lysed with the inhibitors of Src kinase, PP2 (5 μM); or of Fcγ receptors, 2-APB (20 μM); or of Src kinases, PP2 (5 μM); or of Fe-RIIa, IV.3 mAb (5 μg/ml), prior to adhesion to vWF. When these studies were performed on mouse platelets, adhesion assays were carried out in the presence of botrocetin (1 μg/ml). EDTA (1 mM) was also present to prevent the integrin αIIbβ3-vWF interaction and extracellular Ca^{2+} influx.

**Statistical Analysis—**The statistical significance of differences between means was evaluated using Student’s t test for paired samples, and P values of less than 0.05 were considered to be significant.

**RESULTS**

The vWF-GPIb V-IX Signaling

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**FIG. 1.** The vWF-GPIb interaction induces IP_3 formation. A, washed human platelets (2 × 10^9/ml) treated with the anti-integrin αIIbβ3, mAb (c7E3 Fab, 20 μg/ml) or from an individual with Glanzmann’s thrombasthenia, were aggregated with HvWf (20 μg/ml) and ristocetin (1 mg/ml) for the indicated times. Platelets were then lysed and cellular IP_3 levels determined using a commercial assay kit. Results presented are from one experiment, representative of five. **B** and **C**, the Ca^{2+} flux was defined as a change in fluorescence ratio greater than two standard deviations (2σ = 0.38, n = 390) above the mean fluorescence ratio (the mean fluorescence ratio in resting platelets). Where indicated, platelets were preincubated with the inhibitors of PLC, U73122 (1 μM), or of Src kinase, PP2 (5 μM); or of FcγRIIa, IV.3 mAb (5 μg/ml), prior to adhesion to vWF. When these studies were performed on mouse platelets, adhesion assays were carried out in the presence of botrocetin (1 μg/ml). EDTA (1 mM) was also present to prevent the integrin αIIbβ3-vWF interaction and extracellular Ca^{2+} influx.

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cause we were unable to harvest sufficient quantities of platelet lysates from the latter assays to reliably detect changes in IP₃. Stimulation of platelets in suspension with vWf/ristocetin resulted in a transient increase in IP₃ levels from a resting concentration (1.1 ± 0.1 pmol/10⁹ platelets) up to a peak of 5.2 pmol/10⁹ platelets after 10 s of stimulation. By 30 s, IP₃ levels had returned to basal levels (Fig. 1A). A similar transient increase in IP₃ was also observed in Glanzmann thrombasthenic platelets, confirming the lack of involvement of integrin αIbβ₃ (Fig. 1A). In all experiments, the increase in IP₃ induced by the vWF-GPIb interaction was weak relative to other agonists, with thrombin inducing peak IP₃ levels as high as 90
pmol/10^9 platelets (Fig. 1B and data not shown).

To investigate the relationship between IP_3 generation and calcium mobilization during platelet adhesion on vWF, platelet Ca^{2+} levels were monitored using a quantitative dual-dye ratiometric assay, as detailed under “Experimental Procedures.” As demonstrated in Fig. 1C, the Ca^{2+} concentration in resting
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Fig. 4. The PLC antagonist, U73122, inhibits GPIb-induced filopodial formation in CHO cells. CHO cells expressing GPIb-IX were incubated in the absence (Ctrl) or presence of U73122 (10 μM) or U73343 (10 μM) then allowed to adhere for 30 min to HvWF-coated slides in the presence of 2 μg/ml botrocetin and 5 mM EDTA. After fixation and labeling with TRITC-phalloidin, adherent cells were examined by fluorescence microscopy. Representative phalloidin-stained images are shown (A). The bar graphs demonstrate the total adherent CHO cells in eight random fields and percentage of total adherent cells with filopodial extension (B). Results represent the mean ± S.E. obtained from three independent experiments (****, p < 0.0005).

Platelet adhesion on vWF, in the presence of integrin αIIbβ3 antagonists, results in morphological alterations characterized by sphere forming of the cell body and extension of multiple filopodia (5). To further establish the relationship between IP3 generation, Ca2+ flux, and platelet shape change on vWF, the effects of the IP3 receptor antagonist, 2-aminoethoxydiphenyl borate (APB-2), were examined. As demonstrated in Fig. 2, APB-2 decreased the proportion of platelets undergoing shape change from 85% in control platelets to less than 20% in treated platelets (Fig. 2, A and B). Pretreating platelets with APB-2 (20 μM) completely abolished GPIb-induced Ca2+ spikes (Fig. 2C).

In control studies, we confirmed that vWF-induced cytoskeletal reorganization, adhesion assays were performed on mouse platelets deficient in the α subunit of the Gt heterotrimeric protein (23). Platelets express Gt, but not its structural homologue Gt12, and as a result Gt- deficient platelets do not activate PLCβ (23). Gt- deficient and wild type platelets displayed no difference in their capacity to adhere to a vWF matrix and undergo shape change, indicating that the PLCβ-dependent pathway was not required for vWF-GPIb signaling (Fig. 5, A–C). In control studies, we confirmed that vWF-induced cytoskeletal reorganization in mouse platelets was PLC-dependent, because it was abolished by the PLC inhibitor U73122 (data not shown).

The vWF-GPIb Interaction Induces PLCγ2 Tyrosine Phosphorylation through Src Family Tyrosine Kinases—Previous studies have reported PLCγ2 tyrosine phosphorylation in vWF-aggregated platelets (15, 18), although the functional significance of this event remains unclear. Moreover, it is not clear whether PLCγ2 tyrosine phosphorylation occurs downstream of GPIb or requires vWF binding to integrin αIIbβ3. In platelets aggregated by HvWF (10 μg/ml) and ristocetin (1 mg/ml), in the presence of the anti-integrin αIIbβ3 antibody c7E3 Fab (Fig. 6A), a rapid increase in PLCγ2 tyrosine phosphorylation was observed at 10 s, which slowly decreased over time with kinetics that resemble the production of IP3. In comparison to collagen, phosphorylation induced by vWF was weak (Fig. 6A), consistent with low level phosphoinositide turnover in these platelets. To investigate the ability of GPIb to induce tyrosine phosphorylation

Platelets was low. Following adhesion to vWF, ~60% of adherent platelets elicited transient Ca2+ spikes ranging from 50–200 nM (Fig. 1C). Consistent with the low IP3 levels, GPIb-dependent Ca2+ signals were small relative to those induced by thrombin (up to 2000 nM).

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TRITC-phalloidin, the total number of adherent platelets (ages shown) (Fig. 6). Phosphorylation (Fig. 6).

Platelets were then lysed, immunoprecipitated with an anti-PLCγ2 antibody, and subjected to immunoblotting with either an anti-phosphotyrosine antibody (4G10) or an anti-PLCγ2 antibody. As control, washed platelets in suspension were stimulated in the aggregometer with 1.25 μg/ml Type I collagen in the presence of fibrinogen. B. PLCγ2 phosphorylation was also studied in vWF-adherent platelets in the absence or presence of the Src kinase inhibitor PP2. Washed human platelets (2 × 10⁵/ml) were incubated for 10 min with c7E3 Fab (20 μg/ml). The cells were either lysed in the RIPA buffer while in suspension (Non-adherent) or allowed to adhere to a vWF matrix (10 μg/ml) in the absence (Non-adherent) or presence of PP2 (5 μM) for 20 min at 37°C. The adherent cells were then lysed, and PLCγ2 was immunoprecipitated from the lysates and subjected to immunoblotting with either an anti-phosphotyrosine Ab (PY20) or an anti-PLCγ2 Ab. The results in A and B are from one experiment, representative of three.

Role of PLC in GPIb-V-IX Signaling

The demonstration that PLCγ2 during platelet adhesion on immobilized vWF, platelets were allowed to adhere to a vWF matrix in the presence of the integrin αIIbβ3 antagonist, c7E3 Fab. Platelet adhesion to vWF was associated with the tyrosine phosphorylation of PLCγ2. Pretreating platelets with the Src family kinase inhibitor, PP2, completely eliminated GPIb-induced PLCγ2 phosphorylation (Fig. 6B), confirming an important role for Src kinases in this process.

To investigate the functional significance of Src kinases for GPIb-induced cytoskeletal reorganization, platelet adhesion experiments were performed in the presence of PP1 and PP2. Although PP1 and PP2 had no significant effect on the number of platelets adhering to vWF, they resulted in a >75% reduction in the proportion of platelets undergoing shape change (Fig. 7, A and B), and Ca²⁺ responses (Fig. 7D). Dose-response studies revealed that PP1 and PP2 inhibited shape change induced by vWF and collagen over a concentration range previously demonstrated to be Src kinase-selective (Fig. 7 and data not shown). Taken together, these studies are consistent with a potential role for Src kinase-mediated activation of PLCγ isoforms in GPIb-induced cytoskeletal remodeling.

Cytoskeletal Reorganization Induced by vWF-GPIb Interaction Is Regulated by PLCγ2—PLCγ1 and PLCγ2 isotypes have been detected in platelets, and the latter is clearly implicated in signaling by ITAM-bearing receptors (GPVI and FcγRIIA). Indeed, PLCγ2-deficient platelets have a major defect in collagen signaling (25, 33, 34). To investigate the potential role of PLCγ2 in GPIb signaling, adhesion studies were performed on PLCγ2-deficient platelets. As demonstrated in Fig. 8B, these platelets adhered as efficiently to vWF as PLCγ2-deficient controls, however, PLCγ2-deficient platelets exhibited a significant reduction in their capacity to extend filopodia (Fig. 8, A and C). Studies examining Ca²⁺ flux demonstrated ~35% of adherent wild-type mouse platelets displaying GPIb-induced Ca²⁺ transients similar to those observed for human platelets, with peak Ca²⁺ concentrations ranging from 175 to 500 nM (Fig. 8D). In PLCγ2-deficient mouse, a decreased number of adherent platelets were able to sustain Ca²⁺ oscillations. Interestingly, this defect in platelet shape change and Ca²⁺ mobilization (Fig. 8, D and E) was not as profound as those observed in platelets treated with Src kinase, PLC, or IP₃ receptor antagonists, suggesting the possible involvement of other PLC isoforms in GPIb signaling.

The FcγRIIA Receptor and Fcγ Chain Are Not Required for GPIb-induced Platelet Shape Change—The demonstration that GPIb-induced cytoskeletal reorganization is regulated by Src kinases and PLCγ2, raises the possibility that GPIb utilizes...
ITAM-containing signaling receptors to induce platelet activation. In support of this hypothesis, the two major ITAM-containing receptors in platelets, FcγRIIA receptor and FcγRIIIA chain, have previously been demonstrated to be physically associated with GPIb (15, 17). In addition, recent studies utilizing the FcγRIIA function-blocking mAb antibody, IV.3, have dem-

**Fig. 7.** Src kinases are involved in GPIb-induced cytoskeletal reorganization and intracellular Ca$^{2+}$ mobilization. A–C, washed human platelets (3 × 10$^7$/ml) were treated with 20 μg/ml c7E3 Fab and 5 mM EDTA in the absence (Ctrl) or presence of 10 μM PP1. The platelets were then allowed to adhere to HvWF in the presence of 2 μg/ml botrocetin, fixed, stained with TRITC-phalloidin, and analyzed under fluorescence microscopy. Representative phalloidin-stained platelet images are shown (A). The number of total adherent platelets and cells with filopodial extensions were analyzed in five random fields (×63 objective) and expressed as percentage of total. Results are the mean ± S.E. from three independent experiments (*, p < 0.05) (B). The effects of PP1 on platelet shape change induced by collagen (1.25 μg/ml) were assessed using an aggregometer (C). Results are from one of experiment, representative of three. D, Ca$^{2+}$-dye-loaded platelets (2 × 10$^7$/ml) were incubated with c7E3 Fab (20 μg/ml) in the presence of vehicle (0.25% Me$_2$SO, Control) or PP-2 (5 μM) for 10 min. Platelets were then applied to a vWf matrix (10 μg/ml) in the presence of ristocetin (1 mg/ml) under static conditions. Ca$^{2+}$ flux profiles in three representative platelets (of over 100) are shown. The accompanying bar graph demonstrates the percentage of total adherent platelets undergoing Ca$^{2+}$ oscillations in the absence (Adherent) or presence of PP2 (Adherent + PP2). Note that, in this experiment, <5% of platelets in suspension exhibited transient spontaneous calcium oscillations (Non-Adherent). The results represent the mean ± S.E. from three independent experiments.
onstrated an important role for this receptor in regulating PLCγ2 tyrosine phosphorylation and platelet secretion in vWF-aggregated platelets (18, 35). To examine the involvement of FcγRIIA in GPIb-induced shape change, human platelets were pretreated with IV.3. As demonstrated in Fig. 9, IV.3 had no inhibitory effect on GPIb-induced platelet shape change (A and B) or Ca²⁺ mobilization (C). In control studies, we confirmed that the IV.3 antibody effectively blocks FcγRIIA by its ability to completely abolish platelet aggregation induced by heat-aggregated immunoglobulin (lgG) (data not shown).

A recent study has suggested a potentially important role for the Fcγ chain in promoting vWF-induced PLCγ2 tyrosine phosphorylation and platelet aggregation (16). To examine the necessity of Fcγ chain for GPIb-induced platelet shape change and Ca²⁺ mobilization, studies were performed on platelets derived from Fcγ chain-deficient mice (Fcγ−/−) (24). As demonstrated in Fig. 10, platelets from wild-type (Fcγ+/+) and Fcγ−/− mice changed shape and extended filopodia in an identical manner (Fig. 10A). Analysis of the adherent platelets indicated that the percentage of adherent platelets undergoing Ca²⁺ oscillations was similar in wild-type and Fcγ−/− mouse platelets (Fig. 10B and C). A similar pattern of Ca²⁺ transients was detected in Fcγ+/+ and Fcγ−/− mouse platelets (Fig. 10D).
remodeling following engagement of its adhesive ligand (5). Recent evidence suggests that vWF-induced cytoskeletal reorganization may play a potentially important role in regulating the adhesive function of GPIb, relevant to shear-induced platelet activation (6). Despite the potential functional importance of vWF-induced cytoskeletal changes, the signaling mechanisms regulating this process have remained ill-defined. The studies presented here demonstrate an important role for PLC-dependent phosphoinositide turnover, leading to IP₃ generation and subsequent calcium mobilization, for GPIb-dependent cytoskeletal remodeling. More specifically, through the analysis of PLC-γ2-deficient platelets and pharmacological inhibition of Src kinases, our studies have demonstrated the involvement of PLC-γ2, and potentially a second Src kinase-regulated form of PLC, in GPIb signaling. Finally, our studies do not support a major role for the ITAM-bearing receptors, FcγRIIa or FcγRI chain, in GPIb-dependent cytoskeletal remodeling.

Several lines of experimental evidence support a functionally important role for PLC-mediated phosphoinositide turnover for GPIb-induced cytoskeletal change. First, we have demonstrated that the vWF-GPIb interaction is sufficient to induce a small increase in IP₃, independent of integrin αIbβ₂. Second, that pharmacological blockade of the IP₃ receptor completely blocks platelet shape change. Third, inhibition of PLC with U73122, but not its inactive structural analogue U73343, abolishes GPIb-dependent cytoskeletal changes. Finally, studies on mouse platelets lacking PLC-γ2 demonstrated the involvement of this enzyme in GPIb-dependent cytoskeletal remodeling. These findings, combined with recent reports demonstrating that the vWF-GPIb interaction is sufficient to mobilize calcium from internal stores (5), provide strong evidence that GPIb can induce phosphoinositide turnover in platelets.

The ability of the vWF-GPIb interaction to induce a cytosolic calcium response has remained controversial, with some studies demonstrating transmembrane calcium influx (36, 37), other studies suggesting calcium release from internal stores (38, 39), whereas other studies have failed to detect a cytosolic calcium signal (16, 42). The reason for these discrepancies is likely to reflect technical differences between the studies. From previous studies (5, 38) and the present results it appears that GPIb is a weak activator eliciting weak transient Ca²⁺ response in platelets. Furthermore, in contrast to soluble agonists, vWF does not induce a synchronized calcium response throughout the platelet population, with a low percentage of primary adherent platelets undergoing a calcium response at any one time. The low level, transient IP₃ generation in our studies is consistent with the calcium dynamics observed in primary adherent platelets. These findings may partly explain why other studies have failed to demonstrate IP₃ generation or a detectable Ca²⁺ signal following vWF engagement of GPIb.

Our studies define an important, albeit not absolute requirement for PLC-γ2 for GPIb-dependent cytoskeletal remodeling and Ca²⁺ mobilization. The partial defect in filopodia formation in PLC-γ2−/− platelets was accompanied by a partial decrease in Ca²⁺ signaling suggesting the involvement of another PLC isotype. Goq-deficient mouse platelets have impaired receptor-coupled PLCβ activation and do not aggregate in response to ADP, U46619, or thrombin nor change shape in response to ADP stimulation (23). Although platelets primarily express two PLCβ isoforms (PLCβ2 and PLCβ3), the demonstration that Goq-deficient platelets undergo shape change indistinguishable from normal platelets rules out an important role for these enzymes in GPIb-shape change. Platelets also contain the PLCβ1 and PLCβ1 isoforms, however, their levels of expression are much lower than other PLC isoforms and their roles in platelets and mechanisms of activation remain unclear.

**DISCUSSION**

The GPIb-V-IX receptor complex is unique among adhesion receptors in that it not only regulates the cytoskeletal architecture of resting platelets but can also induce cytoskeletal
Platelets express both members of the PLCγ family: PLCγ1 and PLCγ2; however the latter is expressed at higher levels than PLCγ1 (40). Nonetheless, it remains conceivable that this enzyme may also contribute to GPIb signaling, particularly in light of our observations that inhibition of Src kinases or PLCs has a more profound effect on platelet shape change and Ca2⁺ mobilization.
mobility than that observed in PLCγ2-deficient platelets. The recent observation of a residual activation to collagen in PLCγ2-deficient mice (33, 34) involving the PLCγ1 isotype raises the possibility of a similar involvement of PLCγ1 in GPIb/vWF-triggered activation (34). This possibility will require further investigation.

Our studies do not support an important role for the ITAM-bearing receptors, FcγRIIA and the FcγRI, for GPIb signaling, at least in the context of cytoskeletal remodeling. Evidence for an important role for FcγRIIA in GPIb signaling has been derived from studies demonstrating physical association between the receptors (15, 17) and from functional studies demonstrating that the anti-FcγRIIA-blocking antibody, IV.3, prevents vWF-induced granule release and FcγRIIA tyrosine phosphorylation (18). However, our finding that IV.3 does not inhibit GPIb-dependent cytoskeletal change is not altogether surprising given previous findings that GPIb-δ-V-IX-transfected CHO cells, which do not express Fc receptors (41), undergo cytoskeletal reorganization following adhesion to vWF. Furthermore, in contrast to human platelets, mouse platelets do not naturally express FcγRIIA, yet undergo normal shape change in response to vWF. Given the important role of Src kinases, and in particular the involvement of PLCγ2 in GPIb signaling, we sought evidence for the involvement of FcγRI chain in vWF-induced cytoskeletal changes. It has previously been demonstrated that the FcγRI chain becomes tyrosine-phosphorylated following vWF stimulation of platelets and that it co-immunoprecipitates with GPIb in GST-Syk pull-downs (16). Furthermore, FcγRI-deficient mouse platelets have decreased phosphorylation of Syk, PLCγ2, and linker for activation of T-cell (LAT) and defective platelet aggregate in response to vWF. This has lead to a model in which vWF engagement of GPIb promotes Src-dependent FcγRI chain phosphorylation and assembly of a multicomponent signaling complex involving p72Syk, SLP76, and LAT. However, recent studies (42) have questioned the functional significance of this pathway with respect to GPIb signaling, a finding consistent with our inability to detect differences in the shape change and calcium responses of FcγRI-deficient mouse platelets.

The exact signaling pathway utilized by GPIb to induce PLCγ2 activation remains unclear but undoubtedly involves one or more members of the Src kinase family. The mode of activation of Src kinases by GPIb is not obvious, because the intracellular domains of the GPIb-δ-V-IX complex are devoid of Tyr residues and SH2 domains. A recent study has demonstrated an indirect association between GPIb and Src through a complex involving the p85 subunit of PI 3-kinase (13). Other studies have reported co-precipitation of a non-receptor tyrosine kinase, possibly Src, with GPIb, although the molecular basis for this association has not been defined (43). There is also evidence that GPIb may signal through lipid rafts where GPIb has been found to co-localize with signaling enzymes and adaptor molecules (44), although others have not confirmed these findings (36). GPIb may also signal through the assembly of cytoskeletal signaling complexes, because it has previously been demonstrated that the vWF-GPIb interaction can induce the cytoskeletal association of Src and a range of other signaling enzymes (13). Resolution of this important issue will require more detailed analysis of the key structural domains of the GPIb/δ-V-IX complex involved in signal transduction.

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