The von Willebrand Factor-Glycoprotein Ib/V/IX Interaction Induces Actin Polymerization and Cytoskeletal Reorganization in Rolling Platelets and Glycoprotein Ib/V/IX-transfected Cells*

(Received for publication, August 19, 1999, and in revised form, September 30, 1999)

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Platelet adhesion to sites of vascular injury is initiated by the binding of the platelet glycoprotein (GP) Ib-V-IX complex to matrix-bound von Willebrand factor (vWF). This receptor-ligand interaction is characterized by a rapid on-off rate that enables efficient platelet tethering and rolling under conditions of rapid blood flow. We demonstrate here that platelets adhering to immobilized vWF under flow conditions undergo rapid morphological conversion from flat discs to spiny spheres during surface translocation. Studies of Glanzmann thrombasthenic platelets (lacking integrin αIibβ3) and Chinese hamster ovary (CHO) cells transfected with GPIb/IX (CHO-Ib/IX) confirmed that vWF binding to GPIb/IX was sufficient to induce actin polymerization and cytoskeletal reorganization independent of integrin αIibβ3. vWF-induced cytoskeletal reorganization occurred independently of several well characterized signaling processes linked to platelet activation, including calcium influx, prostaglandin metabolism, protein tyrosine phosphorylation, activation of protein kinase C or phosphatidylinositol 3-kinase but was critically dependent on the mobilization of intracellular calcium. Studies of Oregon Green 488, 1,2-bis(o-amino-5-fluorophenoxy)ethane-N,N,N,N-tetraacetic acid tetraacetoxymethyl ester-loaded platelets and CHO-Ib/IX cells demonstrated that these cells mobilize intracellular calcium in a shear-dependent manner during surface translocation on vWF. Taken together, these studies suggest that the vWF-GPIb interaction stimulates actin polymerization and cytoskeletal reorganization in rolling platelets via a shear-sensitive signaling pathway linked to intracellular calcium mobilization.

The ability of platelets to adhere to subendothelial matrix proteins and to other activated platelets at sites of vascular injury is essential for the arrest of bleeding and for subsequent vascular repair. The first step in the hemostatic process involves the binding of the platelet adhesion receptor, GPIIb/IIIa, to the vascular adhesive protein, vWF.1 Under conditions of rapid blood flow this receptor-ligand interaction is indispensable for tethering platelets to the injured vessel wall as a prerequisite step for integrin-mediated cell arrest (1, 2). This multi-step adhesion mechanism is remarkably similar to that utilized by leukocytes to adhere to post-capillary venules in vivo. Rolling of leukocytes is mediated by one or more selectin family members, whereas irreversible cell adhesion requires activation of β3 integrins (3, 4).

vWF is a unique adhesive ligand in that it has the ability to support both the initial transient phase of platelet adhesion as well as integrin αIibβ3-mediated cell arrest. The A1 domain of vWF contains the binding site for GPIIbα, whereas the C1 domain peptide sequence Arg-Gly-Asp-Ser (RGD) binds integrin αIibβ3 (platelet GPIIb/IIIa) (5). Bond formation between vWF and GPIIb is rapid, reversible, and inherently resistant to detachment by high shear forces. This latter property of the vWF-GPIIb interaction is essential for sustaining platelet tethering under high shear, presumably as a consequence of the multivalency of the vWF-GPIIb interaction (6) as well as anchorage of the receptor complex to the membrane skeleton (7). There is growing evidence that GPIIb/IIIa not only mediates platelet adhesion but also transduces signals required for platelet activation (8). One of these activation events involves the conversion of integrin αIibβ3 from a low affinity to a high affinity receptor (9, 10). The interaction between activated integrin αIibβ3 and vWF is characterized by a slow dissociation rate that supports irreversible platelet adhesion and stable platelet aggregation (1, 9, 10). These adhesive interactions are physiologically important as evidenced by the severe bleeding disorders experienced by individuals with congenital deficiencies of vWF, GPIIb/IIIa, or integrin αIibβ3.

In addition to supporting irreversible platelet adhesion, vWF binding to platelets induces dramatic cytoskeletal reorganization transforming resting disc-shaped platelets to fully spread forms (11, 12). The physiological importance of vWF in promoting cytoskeletal reorganization has been highlighted by in vivo

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* This work was supported by grants from the National Health and Medical Research Council of Australia and from the National Heart Foundation of Australia. 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.

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1 The abbreviations used are: vWF, von Willebrand factor; BvWF, bovine vWF; DIC, differential interference contrast; F-actin, filamentous actin; GP, glycoprotein; HvWF, human vWF; IP, phosphoinositide; PKA, protein kinase A; PKC, protein kinase C; PBS, phosphate-buffered saline; RGD, Arg-Gly-Asp-Ser; P3, inositol (1,4,5)-trisphosphate; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PGE, prostaglandin E; BAPTA-AM, 1,2-bis(o-amino-5-fluorophenoxy)ethane-N,N,N,N-tetraacetic acid tetraacetoxymethyl ester; CHO, Chinese hamster ovary; DIOC6, 3,3′-dihexyloxacarbocyanine iodide.
experiments on pigs with von Willebrand disease (vWD) (13). In these studies, platelet adhesion to injured coronary arteries of normal pigs was associated with platelet filopodial extension and cell spreading. In contrast, platelets from vWD pigs extended few filopodia and failed to spread following adhesion to the subendothelium, indicating that cytoskeletal reorganization of adherent platelets in vivo is primarily a vWF-dependent process. Despite the potential importance of vWF-induced cytoskeletal reorganization, the molecular basis for this phenomenon has been poorly defined. Studies of human platelets congenitally deficient in integrin αIIbβ3 (Glanzmann thrombasthenia) have demonstrated an important role for this receptor in inducing platelet spreading at sites of vessel wall injury (14, 15). The role of the vWF-GPIb/V/IX interaction in inducing cytoskeletal reorganization is less clear. A previous study by Cunningham et al. (16) has demonstrated that the vWF-GPIb/IX interaction can induce pseudopodial extension and cell spreading in GPIb/IX-transfected CHO cells, although a follow-up study by the same group has suggested that these cytoskeletal changes require activation of endogenous CHO cell integrins (17). The ability of the vWF-GPIb interaction to induce cytoskeletal reorganization and shape change is a potentially important issue. Other rolling receptors, such as the selectins, do not induce cytoskeletal reorganization during the rolling process (3). This is not surprising given that the spherical shape of resting leukocytes is ideally suited for a rolling-type adhesion and that morphological change in these cells reduces their ability to adhere under flow (3). Circulating platelets, however, exhibit a flat discoid morphology, well suited for efficient cell transport through the microvasculature but less ideal for cell rolling.

In this study we have examined the possibility that the vWF-GPIb/IX interaction not only mediates platelet rolling but also transduces signals leading to cytoskeletal reorganization during the rolling process. We demonstrate that platelets tethering to a vWF matrix under flow undergo dramatic cytoskeletal reorganization, leading to platelet spreading and filopodial extension. These morphological changes were also observed in Glanzmann thrombasthenic platelets and GPIb/IX-transfected cells demonstrating that these cytoskeletal changes occur independently of integrin αIIbβ3. The ability of the vWF-GPIb interaction to induce these cytoskeletal changes did not require calcium influx, activation of PKC, PI 3-kinase, protein tyrosine phosphorylation, or prostaglandin metabolism but was critically dependent on the mobilization of calcium from intracellular stores.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies—**DNA, DNase I, FITC-conjugated phalloidin, Arg-Gly-Asp-Ser (RGDS) peptide, Wortmannin, and calphostin D were purchased from Sigma. Latrunculin B, methoxetate, and EGTA-AM were obtained from Calbiochem. Oregon Green 488 BAPTAM-AM was from Molecular Probes Inc. (Eugene, OR). G418 and polyethylene chain reaction reagents were obtained from Roche Molecular Biochemicals. Gm and Zeocin was from Invitrogen (San Diego, CA). Pfu polymerase was from Stratagene (GmbH, Germany). Genistein, tyrophostin, erbstatin, calphostin, and bisindolylmaleimide were from Biomol Research Laboratories (Plymouth Meeting, PA). Ly294002 was from LC laboratories (Coats Mesa, CA). Human (HvWf) and bovine vWf (BvWf) were purified from plasma cryoprecipitate according to the method of Montgomery and Fox et al. (21). Washed platelets (3 × 10^8/ml) and transfected K562 or CHO cells (3 × 10^6/ml) were aggregated with either HvWf (20 μg/ml), Ptc calphostin (1 μM) or bisindolylmaleimide (200 μM), or protein tyrosine kinases (genistein (100 μM), tyrophostin (200 μM), or erbstatin (25 μg/ml)). The prostaglandin pathway was inhibited by pretreating platelets with aspirin (1 μM) for 90 min at 37 °C or by donor ingestion of 300 mg of aspirin per day for 3 consecutive days before blood collection. In each case the pharmacological activity of the inhibitors was confirmed as follows. Wortmannin and Ly294002 caused dose-dependent inhibition of thrombin-stimulated production of phosphatidylinositol (3,4)-bisphosphate; genistein, tyrphostin, or erbstatin completely inhibited vWF-induced protein tyrosine phosphorylation; calphostin or bisindolylmaleimide abolished PAC-1 binding to washed platelets adherent to a vWF matrix; aspirin abolished arachidonic acid-induced platelet aggregation; RGDS and cT3 Fab blocked ADP-induced platelet aggregation. Resuspension of platelets in EDTA-containing buffers abolished calpain activation in thrombin-stimulated platelets. Analysis of Platelet Adhesion Under Flow—Platelet adhesion under flow was performed using a modified method of Cranmer et al. (7). Microslides (flat, rectangular microcapillary tubes) were coated with HvWf (100 μg/ml) in phosphate-buffered saline (PBS) for 2 h at room temperature or overnight at 4 °C. For flow studies using citrated whole blood, the microcapillary tubes were left unblocked; however, for washed platelet studies the vWF-coated tubes were blocked with either 25% heat-inactivated human serum (containing 50 μg/ml phenylmethlysulfonyl fluoride) or 1% bovine serum albumin for 60 min. In general, the results were similar with either blocking method although there was less nonspecific adhesion on the serum-blocked vWF matrices. For whole blood studies, platelets were fluorescently labeled by incubating circulating blood with DioC6 (1 μM) for 10 min. The labeled whole blood was perfused through the vWF-coated microcapillary tubes at a shear rate of 150 or 750 s⁻¹, and the non-adherent blood cells were removed by perfusing Tyrode's buffer. cT3 Fab (20 μg/ml) was present throughout the experiments to prevent irreversible platelet adhesion. For wash studies, platelet flow studies, cells were washed twice in platelet washing buffer, pH 6.5, containing the platelet activation inhibitors, PGE1 (200 ng/ml), and theophylline (10 μM). Platelets were finally resuspended in the same buffer (3 × 10^9/ml) and perfused over a vWF matrix at a shear rate of 150 s⁻¹. In the majority of experiments with washed platelets, flow was ceased for 5 min to maximize platelet adhesion onto the matrix. Flow was reintiated at a shear rate of 750 s⁻¹ and platelets perfused with platelet washing buffer (containing platelet activating inhibitors) or Tyrode's buffer. In some studies, platelets were preincubated with cytoskeletal (10 μM) or latrunculin B (500 ng/ml) for 10 min, or with inhibitors of tyrosine kinases, PKC, PI 3-kinase, the prostaglandin pathway, or with EGTA-AM or BAPTA-AM for 30 min, as described under “Platelet Aggregation Studies.” Rolling platelets were visualized using fluorescence or differential interference contrast (DIC) microscopy (100× objective) (Leica DMIRB) and videotaped for subsequent image analysis.

**DNase I Inhibition Assays—**Actin polymerization in vWF-aggregated platelets, transfected K562, and CHO cells was analyzed using a quantitative DNase I inhibition assay as described by Blikstad et al. (20) and Fox et al. (21). Washed platelets (3 × 10^9/ml) and transfected K562 or CHO cells (3 × 10^6/ml) were aggregated with either HvWf (20 μg/ml), unless otherwise specified) in the presence of ristocetin (1 mg/ml) or BvWf (20 μg/ml) alone with stirring for the indicated time points. Cells were then lysed in 1% Triton X-100-containing buffer (20 mM Tris, pH 7.4, 1 mM phenylmethlysulfonyl fluoride, 2 mM EDTA, 1 mM benzamidine, and 0.1 μM phallacidin) and immediately subjected to DNase I inhibition assay. An alternative method (22), involving sedimentation of cytoskeletal actin by ultracentrifugation (100,000 g for 3 h) of whole platelet lysates, yielded similar results to the DNase I inhibition assay.

**Expression of the GPIb-IX Complex on the Surface of CHO and K562 Cells—**Full-length cDNAs for GPIbα, Ibβ and IX cloned into the pDI vector were generous donations from Dr. J. Lopez (Houston, TX). Gpvi was cloned into the pZeoSV vector. K562 cells were cultured in RPMI 36242 vWF-Induced Cytoskeletal Reorganization.
media supplemented with 10% fetal calf serum. CHO cells expressing GPIbβ and IX (CHOIII, obtained from Dr. J. Lopez, Houston) were cultured in Dulbecco's modified Eagle's/F-12 media supplemented with 10% fetal calf serum, 400 μg/ml G418, and 100 μM methotrexate. K562 cells were transfected with the cDNAs for GPIbα, GPIbβ, GPIX, and GPV, whereas CHOIII cells were transfected with the CDNA for full-length wild type GPIbα cloned into pDX, using FuGene TP6 Transfection Reagent (Roche Molecular Biochemicals). Briefly, FuGene TP6 (3 μl) and all plasmid (0.9 μg) were mixed with pZeoSV (0.1 μg) and incubated with K562 or CHOIII cells for 3–7 h. Selection was initiated 3 days after transfection with 200 μg/ml Zeocon or G418. The individual Zeocon or G418-resistant CHO cell clones were isolated with glass cloning rings, and K562 cells were isolated by limiting dilution 2 weeks following selection. CHO cell clones expressing GPIbαIX (CHOIII) and K562αβIX cells were cultured for a further 2 weeks and subjected to immunofluorescence and fluorescence-activated cell sorter analysis to examine protein expression on the cell surface.

K562 and CHO Cell Adhesion Studies—CHO and K562 cells (1 × 10⁶/ml) in Tyrode's buffer were applied to a HvWf (10 μg/ml) matrix in the presence of botrocetin (1 μg/ml) and allowed to adhere for 60 min at 37 °C, unless otherwise specified. In some studies, the cells were pre-treated for 10 min with anti-GPIbα mAb (AK2), RGDS peptide (1 mM), EDTA (2 mM), cytochalasin D (1 μM), or 30 min with 80 μM EGTA-AM prior to application to vWF matrices. Where indicated, cells were also fixed with 3.7% formaldehyde for 10 min prior to application to vWF matrices. Cells were then washed to allow adherence to poly-L-lysine (100 μg/ml)-coated coverslips. Non-adherent cells were removed by three gentle washes with PBS. Adherent cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 30 min, and stained with FITC-conjugated phalloidin for 30 min. The cells were then washed three times with PBS and subjected to confocal fluorescence microscopy (Leica TCSNT, Germany) (16 and 100× objective). The images were reconstructed using VoxBlast software. The presence of membrane projections on a given cell was arbitrarily defined as membrane extensions (>2 μm in length) covering the entire surface of the cell. When CHO cell adhesion studies were performed under flow conditions, CHOIII cells (1 × 10⁶/ml) were resuspended in Ca²⁺-free Tyrode's buffer containing 1 mM EDTA. Cells were perfused through a BvWf (100 μg/ml)-coated microcapillary tube at a shear rate of 150 or 1500 s⁻¹, and flow was allowed for 10 min by perfusing Tyrode's buffer (containing 1 mM EDTA). The adherent cells were then fixed, permeabilized, stained with FITC-conjugated phalloidin and subjected to fluorescence microscopy.

Analysis of Intracellular Calcium Mobilization in Platelets and GPIb/IX-transfected CHO Cells—Washed platelets (1 × 10⁹/ml) or CHOIII cells (1 × 10⁶/ml) were resuspended in Ca²⁺-free Tyrode's buffer containing EGTA (1 mM). The cells were then incubated with the Ca²⁺-indicator dye, Oregon Green 488 BAPTA-AM-1 (1 μM), for 45 min at 37 °C. The platelets were then pelleted by centrifugation (2000 × g for 5 min) and either resuspended in calcium-free Tyrode's buffer or washed autologous red blood cells to a final concentration of 1 × 10¹¹/ml. Oregon Green 488 BAPTA-AM-1-loaded CHOIII cells were resuspended in calcium-free Tyrode's buffer. In all experiments, EGTA (1 mM) was included in the cell suspensions to chelate trace quantities of extracellular calcium. Platelets were maintained at 37 °C for 30 min in the presence of Fab c7E3 (20 μg/ml), prior to perfusion through BvWf (10 μg/ml)-coated microcapillary tube at a shear rate of 600, 1800, or 3000 s⁻¹. In the indicated studies PGE₁ (100 ng/ml), and theophylline (10 mM) for 60 min. Non-adherent cells were aspirated, and adherent cells were allowed to spread for the indicated times following perfusion. Cells were then fixed, permeabilized, stained with FITC-conjugated phalloidin, and imaged using confocal fluorescence microscopy. B, washed normal platelets (1 × 10⁹/ml) were pretreated with control buffer or c7E3 Fab (20 μg/ml) for 10 min and then allowed to adhere for 60 min to either human HvWf (100 μg/ml) or bovine HvWf (10 μg/ml). Images were obtained using confocal fluorescence microscopy (100× objective). The cells presented are representative of >90% of the total population of platelets from four independent experiments, and the results for Glanzmann thrombasthenic platelets from one experiment.

RESULTS

Distinct Roles for GPIb/IX and Integrin αⅡbβ₃ in Mediating vWF-induced Cytoskeletal Reorganization in Human Platelets—A striking feature of the platelet adhesion process is the dramatic change in cell morphology induced by vWF binding to GPIb/IX in normal platelets or in integrin αⅡbβ₃. Two actin-based structures are required for platelet spreading, filopodial bundles, and lamellipodial networks. The filopodial bundles are formed from long actin filaments radiating from the center of the platelet, whereas lamellipodial networks are formed from a circumferential zone of orthogonally arrayed short actin filaments (23). The mechanism(s) by which vWF induces platelet spreading remains poorly understood, although previous studies with Glanzmann thrombasthenic platelets (congenitally deficient in integrin αⅡbβ₃) indicate that integrin αⅡbβ₃ plays an important role in this process (14, 15). In our initial studies we compared the ability of normal and Glanzmann thrombasthenic platelets to extend filopodia and lamellipodia following adhesion onto a purified vWF matrix. As demonstrated in Fig. 1A, normal platelets adherent to a vWF matrix initially adopted a spherical morphology and extended multiple filopodial projections (Fig. 1A, 10°). This was followed by the extension of broad lamellipodial sheets between adjacent filopodia, converting the platelet from a dendritic to a fully spread form (20° and 60°). In contrast, Glanzmann thrombasthenic platelets adopted a spherical morphology and extended filopodia; however, lamellipodial formation was completely absent (Fig. 1A). These results suggest that the vWF-GPIb/IX interaction may be sufficient to induce platelet shape change and filopodial extension, whereas the binding of vWF to integrin αⅡbβ₃ is essential for the formation of lamellipodial networks. Additional evidence in support of this hypothesis was derived from studies of normal platelets pretreated with the anti-αⅡbβ₃ chimeric Fab fragment of 7E3 (c7E3 Fab). Similar to Glanzmann thrombasthenic platelets, blocking ligand binding to integrin αⅡbβ₃ abolished lamellipodial formation but had no effect on the ability of platelets to change shape and extend filopodia (Fig. 1B). A technical limitation of using HvWf for these studies was the inability of this protein to support stable adhesion in the presence of inhibitors of integrin αⅡbβ₃. It is possible that repeated washing may preferentially removed discoid cells from the matrix leaving non-representative dendritic platelets. We therefore performed adhesion assays with purified BvWf, which supports stable platelet adhesion in the absence of integrin αⅡbβ₃. As with HvWf, all platelets pretreated with c7E3 Fab became spherical and extended filopodia after adhering to BvWf but were incapable of extending lamellipodia (Fig. 1B).
Cytoskeletal Reorganization and Filopodial Extension in Rolling Platelets—The ability of the vWF-GP Ib/VIIa interaction to induce cytoskeletal reorganization in static adhesion assays raised the interesting possibility that similar morphological changes may occur during the process of platelet translocation on a vWF matrix. To adequately visualize the morphology of platelets under flow conditions, we performed all adhesion assays in flat rectangular microcapillary tubes, as described under “Experimental Procedures.” This assay system enabled adequate resolution to distinguish between resting discoid cells and dendritic forms. We initially performed flow studies on anticoagulated whole blood using DiOC<sub>6</sub>-labeled platelets. DiOC<sub>6</sub> stains membrane lipid and enables visualization of membrane projections during the process of platelet activation (24). Consistent with previous reports (1), perfusion of whole blood over a vWF matrix at 150 s<sup>-1</sup> in the presence of c7E3 Fab (20 μg/ml) revealed that all cells tethering to the matrix underwent shape change during surface translocation. Identical results were obtained using whole blood from an individual with Glanzmann thrombasthenia, confirming that these morphological changes occur independently of integrin α<sub>IIb</sub>β<sub>3</sub> (data not shown).

Further evidence confirming that the vWF-GP Ib interaction can induce cytoskeletal reorganization during the process of platelet translocation was obtained from studies using washed platelets. In these experiments washed platelets were perfused through vWF-coated microcapillary tubes in the presence of the platelet activation inhibitors, PGE<sub>1</sub> and theophylline, to avoid platelet activation prior to cell contact with the vWF matrix. As detailed under “Experimental Procedures” considerable care was taken to ensure that platelets were not activated during the washing procedure. This included maintaining the platelet suspension at pH 6.5 and including platelet activation inhibitors from the moment of blood collection, throughout all washing steps and during platelet perfusion experiments. We confirmed that platelets were maintained in a resting state under these conditions by their lack of surface expression of P-selectin and by the inability of the activation-dependent antibody, PAC-1, to recognize integrin α<sub>IIb</sub>β<sub>3</sub> on the cell surface (data not shown). Analysis of the morphology of c7E3 Fab-treated washed platelets (in the presence of PGE<sub>1</sub> and theophylline) by DIC microscopy demonstrated that these cells exhibited a resting discoid morphology (Fig. 2B, Fixed) and rolled in a stop-start, side-to-side “flipping” manner over the vWF matrix (Fig. 2B, –PGE<sub>1</sub>/Theo). Removing PGE<sub>1</sub> and theophylline from the rolling platelets by perfusing the microcapillary tubes with Tyrode’s buffer resulted in the platelets adopting a spherical morphology and extending filopodia, in an identical manner to that observed in whole blood. The extent of filopodial formation reflected the time of platelet contact with the vWF matrix. Translocating platelets at the inlet of the microcapillary tube (Fig. 2B, –PGE<sub>1</sub>/Theo-Inlet), which had only a brief contact with the vWF matrix, exhibited a small number of short filopodia, whereas cells that had translocated to the outlet of the microcapillary tube (Fig. 2B, –PGE<sub>1</sub>/Theo-Outlet) extended large prominent filopodia. The filopodia were dynamic structures that extended and retracted from the cell surface and, along with the cell body, participated in the adhesion process by forming transient adhesion contacts with the matrix. The extension of filopodia from the surface of rolling platelets was dependent on actin polymerization as it was completely inhibited by cytochalasin D (Fig. 2C, +CD) or latrunculin B (data not shown). Cytochalasin D prevents actin filament elongation in platelets by binding to the fast-growing (barbed) ends of actin filaments (21, 25), whereas latrunculin B prevents actin polymerization by binding monomeric actin (26). As with the platelet activation inhibitors, removing cytochalasin D from the rolling platelets subsequently resulted in filopodial extension in all cells (Fig. 2C, –CD). These studies, combined with our experiments in whole blood, provide strong evidence that the vWF-GP Ib/VIIa interaction can induce actin polymerization and cytoskeletal reorganization during the process of platelet translocation.

vWF-induced Actin Polymerization in Aggregated Platelets—In addition to mediating platelet adhesion to the injured vessel wall, vWF also plays an important role in mediating platelet-platelet adhesion contacts (aggregation) under conditions of high shear stress (27–30). We have previously established that a number of signaling processes occurring in adherent platelets also occur in vWF-aggregated platelets (12).

We therefore investigated the ability of vWF to induce actin polym-
vWF-Induced Cytoskeletal Reorganization

FIG. 3. vWF-induced actin polymerization in aggregated platelets. A, washed platelets (3 x 10^9/ml) were aggregated with HvWF (20 µg/ml) in the presence of ristocetin (1 mg/ml) for the indicated time points while stirring. In all experiments, the F-actin content in whole cell lysates was determined using the DNase I inhibition assay, as described under “Experimental Procedures.” Results represent the mean ± S.E. from seven experiments. B, platelets were stirred in the presence of buffer (Resting), ristocetin (1 mg/ml) alone (Ristocetin), HvWF (20 µg/ml) and ristocetin (1 mg/ml)(HvWF + Ristocetin), or BvWF (BvWF) alone (20 µg/ml) for 10 min. C, platelets were aggregated with the indicated concentrations of BvWF for 10 min. Results in B and C represent the mean ± S.E. from four independent experiments performed in duplicate. Statistical analysis was performed using the Student’s t test comparing resting versus vWF-aggregated platelets (**p < 0.001).

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FIG. 4. The vWF-GPIb/V/IX interaction induces actin polymerization independently of α5β3. A, effect of EDTA on BvWF-induced platelet aggregation and F-actin accumulation. Washed platelets (3 x 10^9/ml) were aggregated with the indicated concentration of BvWF for 5 min in the presence (+) or absence (−) of 2 mM EDTA. The rate of aggregation was determined by changes in light transmission in the washed platelet suspension using a platelet aggregometer. F-actin content in whole cell lysates was determined as described in Fig. 3. Results are from one experiment, representative of three experiments performed in duplicate. B, effect of EDTA, c7E3 Fab, and RGDS on HvWF-induced actin polymerization. Platelets were either unstimulated (Resting) or stimulated with HvWF (20 µg/ml) and ristocetin (1 mg/ml), in the presence of control buffer (Control), EDTA (2 mM), c7E3 Fab (20 µg/ml), or RGDS (1 mM). Note: in these experiments, using high concentrations of vWF in the presence of inhibitors of α5β3 did not reduce the rate of platelet aggregation. Results are the mean ± S.E. from four individual experiments performed in duplicate. **p < 0.0001. C, vWF-induced actin polymerization in Glanzmann thrombasthenic platelets. Washed normal or Glanzmann thrombasthenic platelets were aggregated with HvWF (20 µg/ml) in the presence of ristocetin (1 mg/ml) for 10 min. Results are derived from one experiment.

A strong correlation between the rate and extent of platelet aggregation and the degree of actin polymerization (Fig. 4A). Studies on vWF-aggregated Glanzmann thrombasthenic platelets confirmed the ability of the vWF-GPIb/V/IX interaction to induce actin polymerization independently of integrin α5β3 (Fig. 4C).

vWF-Induced Cytoskeletal Reorganization in GPIb/V/IX-transfected K562 Cells—To investigate further the relationship between vWF binding to GPIb and subsequent actin polymerization and cytoskeletal reorganization, we performed studies on GPIb/V/IX-transfected K562 cells. K562 cells are derived from a human leukemic cell line and express many of the signaling components present in platelets but do not express detectable levels of GP Ib/V/IX or β3 integrin on the cell surface (data not shown). These cells therefore represent a potentially useful cell model to express the GPIb-V-IX complex and examine adhesion and signaling processes linked to this receptor. In initial studies we compared the ability of non-transfected (K562) or transfected K562 cells (K562αβVIX) to adhere to immobilized HvWF in the presence of botrocetin. As demonstrated in Fig. 5A (upper panel), only transfected cells formed stationary adhesion contacts with the vWF matrix. This adhesion was dependent on GPIb as it was effectively blocked by...
pretreating K562αVIX cells with the anti-GPib antibody, AK2. Examination of the morphology of adherent K562αVIX cells demonstrated that ~80% of these cells extended numerous membrane projections (Fig. 5A, lower panel, and 5B). This contrasted with K562αβVIX cells that exhibited a featureless round morphology (Fig. 5A, lower panel) prior to adhesion to the vWf matrix, suggesting that the vWf-GPib interaction induced these morphological changes. The extension of membrane projections required actin polymerization as they were completely abolished by pretreating the cells with cytochalasin D (Fig. 5A, lower panel). Furthermore, EDTA or RGDS had no effect on the ability of K562αVIX cells to adhere to the vWf matrix or extend membrane projections (Fig. 5, A and B), excluding a role for integrins in this process.

Cytoskeletal Reorganization in GPIb/IX-transfected CHO Cells—Previous studies examining vWf-induced cytoskeletal reorganization in GPIb/IX-transfected CHO cells have suggested an important role for endogenous CHO cell integrins in this process (16, 17). To investigate possible differences between CHO and K562 cells, we performed CHO cell adhesion studies on HvWf using cells transfected with GPIbα, Ibβ, and IX (CHOαβIX). As demonstrated in Fig. 6A, CHOαβIX cells adhered to HvWf in the presence of botrocetin. In control studies we demonstrated that this adhesion required the vWf-GPib interaction as it was blocked by AK2 and was not observed with CHO cells expressing GPIbβ and GPIX alone (CHOβIX). Morphological analysis of adherent CHOαβIX cells revealed that >80% of the cells extended membrane projections similar to those observed with transfected K562 cells (Fig. 6A, lower panel, 6B). Furthermore, approximately a third of the cells exhibited stress fibers and spread on the vWf matrix. As with K562αβVIX, these cytoskeletal changes appeared to be dependent on the vWf-GPib interaction as cells fixed in suspension prior to exposure to a vWf-matrix exhibited a round morphology (Fig. 6A, lower panel). The addition of RGDS or EDTA to the adhesion assays had no effect on the level of adhesion or the extension of membrane projections; however, stress fibers and spreading were completely abolished (Fig. 6, A and B). Furthermore, all cytoskeletal changes were prevented by pretreating CHOαβIX cells with cytochalasin D (Fig. 6A, lower panel).

These results support our findings in GPIb/IX-transfected K562 cells suggesting that the vWf-GPib/IX interaction is sufficient to induce cytoskeletal reorganization independently of integrins; however, subsequent spreading and stress fiber formation appear to be an integrin-dependent phenomenon. They also establish that cytoskeletal reorganization is not dependent on the presence of GPIV. To confirm that the vWf-GPib interaction was able to induce cytoskeletal reorganization in the absence of an artificial modulator, CHO-Ib/IX cells were perfused through BvWf-coated microcapillary tubes at venous (150 s⁻¹) or arterial (1500 s⁻¹) shear rates. As demonstrated in Fig. 6C, CHOαβIX cells exhibited a smooth round appearance prior to the performance of flow experiments. Exposure of these cells to low shear resulted in the extension of numerous small filopodial projections over the entire cell surface. At high shear, filopodia were much more prominent and specifically localized to the upstream edge of the cell. The reason for this polarity is unclear but may reflect the shear differential experienced by the leading and trailing edge of the cell. It should also be noted that in the static adhesion assays filopodia developed slowly, within 5–10 min of adhesion and were maximal by 60 min. In
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Fig. 7. vWF-induced actin polymerization in GPIb/IX-transfected CHO and GPIb/IX-transfected K562 cells. A, CHOoβIX cells (3 \times 10^6/ml) were transfected with BvWf (20 μg/ml) to induce cell aggregation. Cells were then lysed, and the level of F-actin in the whole cell lysates was determined using the DNase I inhibition assay. Results are from one experiment representative of four performed in duplicate. B, CHOoβIX, CHOβIX, and K562oβVIX cells (3 \times 10^6/ml) were transfected with the anti-GPIbα mAb (AK2) (5 μg/ml), EDTA (2 mM), or latrunculin B (LB) (500 ng/ml) for 10 min prior to the addition of BvWf. Results represent the mean ± S.E. from four independent experiments performed in duplicate. ***, p < 0.0001.

contrast, cells exposed to shear extended filopodia within 1–2 min with maximal extension by 5 min.

vWF-induced Increase in Filamentous Actin in GPIb/IX-transfected CHO and K562 Cells—To investigate the extent of actin polymerization induced by vWF binding to GPIb/IX in CHOoβIX and K562oβVIX cells, DNase I inhibition assays were performed on whole cell lysates from aggregated cells. BvWf-induced aggregation of CHOoβIX and K562oβVIX resulted in an overall increase in the cellular content of F-actin from ~30 to 45% (Fig. 7). In general the rate and extent of aggregation as well as the increase in F-actin in transfected cells were less that than observed in platelets (Figs 3 and 4), probably reflecting lower receptor density on these cells. The increase in F-actin was dependent on the vWF-GPIbα interaction in that it was abolished by pretreating the cells with AK2 and was not observed in CHOβIX cells (Fig. 7B). Similar to platelets, the vWF-induced increase in F-actin was not inhibited by EDTA but was completely abolished by latrunculin B (Fig. 7B).

Signaling Processes Regulating vWF-induced Actin Polymerization—It is now well established that vWF binding to platelets induces multiple signaling events, including calcium influx (28, 29), protein tyrosine phosphorylation (12, 19, 34, 35), prostaglandin metabolism (8), activation of PKC (31), and PI 3-kinase (19). To investigate the potential importance of these signaling events in vWF-induced actin polymerization and cytoskeletal reorganization, we pretreated washed platelets with a range of well characterized signal transduction inhibitors. These included pharmacological inhibitors of tyrosine kinases (genistein, tyrphostin or erbstatin), PI 3-kinase (wortmannin or LY294002), PKC (calphostin C or bisindolylmaleimide), prostaglandin metabolism (aspirin), and a chelator of extracellular calcium (EDTA). The effects of these inhibitors on vWF-induced actin polymerization and cytoskeletal reorganization are summarized in Fig. 8. None of these inhibitors prevented platelet shape change and filopodial extension in rolling platelets (Fig. 8D) or inhibited platelet aggregation and actin polymerization in response to 20 μg/ml HvWf (Fig. 8A). In control studies, each inhibitor was demonstrated to be pharmacologically active and capable of inhibiting other functional platelet responses (see “Experimental Procedures”). Consistent with our earlier studies on rolling platelets (Fig. 2), the signaling processes responsible for vWF-induced actin polymerization appeared to be sensitive to the inhibitory effects of PKA, as pretreating platelets with PGE_1 and theophylline led to an ~70% reduction in actin polymerization (Fig. 8A) without affecting vWF-induced aggregation. Taken together, these studies suggest the existence of alternative signaling pathways linking the vWF-GPIbα interaction to cytoskeletal reorganization.

Calcium Mobilization in Rolling Platelets—In further studies we investigated the possibility that the vWF-GPIbα interaction may induce mobilization of calcium from intracellular stores, independent of calcium influx. Cytosolic calcium plays a key role in activating the actin filament severing mechanism as an essential step for platelet sphering, normal filopodial formation, and lamellipodial development (23). To investigate changes in the cytosolic concentration of calcium during platelet rolling, studies were performed on washed platelets loaded with the calcium indicator dye, Oregon Green 488 BAPTA-AM-1. These cells were perfused over a vWF matrix at low (150 s^{-1}), intermediate (600 s^{-1}), or high (1500 and 3000 s^{-1}) shear rates, and in all experiments the platelets were resuspended in EGTA-containing buffers to examine specifically the contribution of intracellular calcium mobilization to changes in cytosolic calcium. As demonstrated in Fig. 8A, real-time confocal imaging of platelets exposed to low shear (150 s^{-1}) demonstrated that 2–3% of cells exhibited a high level of fluorescence intensity during surface translocation. In contrast, exposing these cells to thrombin (1 unit/ml) induced high fluorescence emission in >80% of the cells examined, consistent with the ability of this agonist to induce calcium mobilization from intracellular stores. This thrombin-induced increase in cytosolic calcium was prevented by pretreating platelets with PGE_1 (data not shown), a finding consistent with the ability of PKA to
antagonize agonist-induced mobilization of intracellular calcium. Exposing platelets to increasing shear in the absence of thrombin resulted in individual cells exhibiting intermittent calcium pulsing during the rolling process (Fig. 9, B and C). As demonstrated in Fig. 9B, the percentage of cells pulsing at any one time increased as a function of wall shear rate with 3–5% pulsing at 600 s⁻¹, 10–13% at 1800 s⁻¹, and 18–24% at 3000 s⁻¹ (n = 4). As with thrombin-stimulated platelets, the shear-induced increase in cytosolic calcium was abolished by pre-treating platelets with PGE₁ (Fig. 9B). To confirm that the vWF-GPIb interaction was sufficient to induce mobilization of intracellular calcium independently of integrin ability, studies were performed on Oregon Green 488 BAPTA-AM-1-loaded CHOαβIX cells resuspended in EGTA-containing buffers. These cells exhibited a low basal level of fluorescence when exposed to serum-blocked micropipette tubes (Fig. 9D). Following adhesion to vWf under static conditions, a small percentage of cells exhibited high fluorescence emission (Fig. 9D). As with platelets, the percentage of cells fluorescing at any one time increased as a function of wall shear rate with 5–8% of cells exhibiting high fluorescence intensity at 150 s⁻¹ and 18–23% at 1500 s⁻¹.

Comparative imaging of unloaded platelets by DIC and Oregon Green 488 BAPTA-AM-1-loaded platelets by fluorescence microscopy highlighted the close relationship between shear-induced calcium pulsing and cytoskeletal reorganization. For example, time-lapse imaging of platelet shape change and filopodial extension under static conditions demonstrated that these morphological changes occur slowly over a 5–10-min time period, consistent with the weak calcium response under these experimental conditions. In contrast, the rate of platelet shape change increased as a function of shear with the majority of platelets undergoing morphological changes within the first 15–30 s of tethering at high shear rates.

A Critical Role for Cytoplasmic Calcium in Mediating vWf-induced Shape Change—To investigate the functional importance of intracellular calcium mobilization in inducing cytoskeletal changes in platelets adherent to vWf, washed platelets were incubated with the membrane-permeable calcium chelators, EGTA-AM or BAPTA-AM, and examined for morphological changes under static or flow conditions. As demonstrated in Fig. 10, chelating cytosolic calcium prevented the normal disc-to-sphere transformation in platelets adherent under static conditions and flow. Many of the rolling platelets lost their smooth surface appearance and developed irregular margins (Fig. 10, upper and middle panel). Moreover, there was a marked reduction in the number and size of filopodia extending from the cell surface. In general, the filopodia were short and had a thickened bulbous appearance, consistent with the role of calcium in inducing actin filament severing during normal filopodial development. EGTA-AM loading of CHO-Ib/IX cells resulted in a similar defect in filopodial formation with the majority of membrane extensions having a short irregular appearance (Fig. 10, bottom panel).

1.7-s intervals over 6.8 s. D, CHOαβIX (1 × 10⁹/ml) cells were loaded with Oregon Green 488 BAPTA-AM-1 and applied to serum-coated micropipette tubes under static conditions or, alternatively, perfused through BvWf (20 μg/ml)-coated micropipette tubes at 150 s⁻¹ or 1500 s⁻¹ in the presence of EGTA (1 mM), as described under “Experimental Procedures.” The fluorescent images (top panel) demonstrate a shear-dependent increase in cytosolic calcium. DIC images of the same cells are demonstrated in the bottom panels. The images in this figure are from one experiment, representative of four independent experiments performed in duplicate.
incubated with EGTA-AM (80 mM EGTA and c7E3 Fab (20 μg/ml) for 30 min in the presence of 1 mM EDTA and 108/ml) were then fixed, permeabilized, stained with FITC-phalloidin, and recorded real-time on video tape. CHO-Ib/IX cells (1 X 10^9/ml) were preincubated with EGTA-AM (80 μM) for 30 min in the presence of EDTA (1 mM). The cells were adhered to a HvWF (10 μg/ml)-coated coverslip for 60 min at 37 °C, in the presence of botrocetin (1 μg/ml). The adherent cells were then fixed, permeabilized, stained with FITC-phalloidin, and subjected to fluorescence microscopy. Images presented are from one experiment, representative of five experiments performed in duplicate.

**FIG. 10.** vWF-induced calcium mobilization is required for cytoskeletal reorganization in rolling platelets and GPIb/IX-transfected cells. Washed platelets (3 X 10^9/ml) were incubated with Me₂SO (Control) or EGTA-AM (50 μM) for 30 min in the presence of 1 mM EDTA and c7E3 Fab (20 μg/ml). The cells were then allowed to adhere to HvWF (100 μg/ml)-coated coverslips for 60 min (Static). Alternatively, the washed platelets were perfused through HvWF (100 μg/ml)-coated capillary tubes at a shear rate of 1500 s⁻¹ (Flow). The cell images were obtained using DIC microscopy (63× objective) and recorded real-time on video tape. CHO-Ib/IX cells (1 X 10^9/ml) were preincubated with EGTA-AM (80 μM) for 30 min in the presence of EDTA (1 mM). The cells were adhered to a HvWF (10 μg/ml)-coated coverslip for 60 min at 37 °C, in the presence of botrocetin (1 μg/ml). The adherent cells were then fixed, permeabilized, stained with FITC-phalloidin, and subjected to fluorescence microscopy. Images presented are from one experiment, representative of five experiments performed in duplicate.

**DISCUSSION**

The results presented here demonstrate that platelets tethering to a vWF matrix under physiological flow conditions undergo rapid cytoskeletal reorganization during the rolling process. Studies using Glanzmann thrombasthenic platelets and GPIb/IX-transfected cells have demonstrated that the vWF-GPIb interaction is sufficient to induce these cytoskeletal changes independent of integrin αIbβ3. Cytoskeletal reorganization in rolling platelets is a shear-dependent event that does not involve several well characterized signaling processes previously linked to vWF-induced platelet activation, including calcium influx, protein tyrosine phosphorylation, prostaglandin metabolism, and activation of PKC and PI 3-kinase. In contrast, our studies suggest that the vWF-GPIb interaction activates a shear-sensitive signaling process linked to intracellular calcium mobilization. This signaling pathway is sensitive to the inhibitory effects of PKA and is functionally linked to cytoskeletal reorganization in rolling platelets.

Our studies with Glanzmann thrombasthenic platelets have confirmed previous reports suggesting a critical role for integrin αIbβ3 in inducing cytoskeletal reorganization as a necessary event for platelet spreading. Despite the presence of at least four other integrins on the platelet surface, including receptors for collagen (α2β1), laminin (α5β1), vitronectin (α5β1), and fibronectin (α5β1), integrin αIbβ3 appears to play an indispensable role in the normal spreading process even when platelets are exposed to a heterogeneous mix of adhesive substrates in the subendothelium (14, 15). A previous report has demonstrated that GPIb/IX-transfected CHO cells extend filopodia and spread on a human vWF matrix in the absence of integrin αIbβ3 (16). However a follow-up study by the same group suggested that vWF binding to GPIb/IX was not sufficient to induce these cytoskeletal changes independently of endogenous CHO cell integrins (17). Whereas our studies have also suggested an important role for integrins in mediating platelet and CHO-Ib/X cell spreading on a vWF matrix, several lines of evidence indicate that binding of vWF to GPIb was sufficient to induce actin polymerization and cytoskeletal reorganization independently of other major platelet adhesion receptors. First, Glanzmann thrombasthenic platelets and normal platelets treated with inhibitors of integrin αIbβ3 were able to change shape and extend filopodia under static and flow conditions. Second, vWF-induced aggregation of Glanzmann thrombasthenic platelets or normal platelets pretreated with EDTA, RGDS, or c7E3 Fab was associated with a marked increase in the level of F-actin. Third, vWF was able to induce actin polymerization in cells expressing GPIbα, Ibβ, and IX but not in cells expressing GPIbβ and GPIX alone. Fourth, adhesion of GPIb/IX-transfected cells to a vWF matrix resulted in actin filament reorganization and the extension of numerous membrane projections, in the presence of inhibitors of endogenous integrins.

Our studies define a key role for intracellular calcium in mediating cytoskeletal changes in rolling platelets. Chelating intracellular calcium prevented the normal disc-to-sphere transition in rolling platelets and resulted in a substantial reduction in the number and size of filopodia extending from the surface of platelets and GPIb/IX-transfected CHO cells. These observations are in keeping with previous studies demonstrating an indispensable role for cytosolic calcium in activating the actin filament severing mechanism as a necessary event for platelet spreading and normal filopodial extension (23). The ability of EGTA-AM-loaded platelets to extend short filopodia is consistent with previous findings demonstrating that cytosolic calcium is not essential for actin filament growth but is required for severing elongated filaments (23). Instead of terminating at the filopodial tips these elongated filaments make U-turns at the filopodial ends and run back into the cell body resulting in filopodia adopting a short bulbous appearance similar to those observed in our study.

An important issue for future investigation will be to determine the mechanism by which the vWF-GPIb interaction induces increases in cytosolic calcium in rolling platelets. Previous studies examining platelet activation in a cone-plate viscometer have demonstrated that shear-induced binding of soluble vWF to the GPIb-V-IX complex leads to an increase in cytosolic calcium (28, 29). This increase is not dependent on ligand binding to integrin αIbβ3, but is completely abolished by chelating extracellular calcium, suggesting the existence of a membrane calcium channel functionally linked to the GPIb-V-IX complex. Our studies in rolling platelets and CHO-Ib/IX cells demonstrate for the first time that the vWF-GPIb interaction can induce increases in cytosolic calcium and cytoskeletal reorganization independently of calcium influx. The simplest and most likely explanation for our findings is that the vWF-GPIb interaction induces PI turnover in platelets, leading to the generation of inositol (1,4,5)-trisphosphate (IP3) and release of calcium from IP3-sensitive stores. PI turnover would also explain the ability of platelets to extend short filopodia in EGTA-AM-loaded platelets as the synthesis of endogenous d-4 phosphinositides has previously been linked to actin filament uncapping in human platelets (36). These observations would also explain why inhibitors of 0-3 phosphoinositide synthesis (wortmannin and LY294002) had no effect on vWF-induced actin polymerization and filopodial extension. To date, there are no reports of a direct link between the GPIb-V-IX complex and enzymes involved in PI metabolism. In fact, previous studies demonstrating PI turnover in vWF-aggregated platelets have suggested that this signaling event occurs indirectly, via the generation of thromboxane A2 (8). Studies are currently
underway to examine the relationship between platelet rolling and PI turnover and to elucidate the mechanism by which vWF binding to the GPIb/V/IX complex induces calcium mobilization.

An important unresolved issue is whether the GPIb-V-IX complex transduces signals directly, through signaling proteins such as 14-3-3ζ, or involves an indirect pathway dependent on other surface receptors, such as FcγRIIA. In addition to a key role for the FcγRIIA receptor in collagen-induced platelet activation (37), there is recent evidence that this receptor may also transduce signals initiated by the vWF-GPIb interaction (38, 39). Although our studies cannot exclude an important role for FcγRIIA in vWF-induced calcium mobilization and cytoskeletal regulation in human platelets, it is clearly not essential for these changes in CHO cells as these cells lack Fc receptors (40).

Our studies demonstrating calcium mobilization in translocating platelets differs from a recent report (40) suggesting that all changes in cytosolic calcium in platelets interacting with a vWF matrix under flow are dependent on vWF engagement of integrin αIIbβ3. The reason for this discrepancy from our results is not immediately apparent but may reflect methodological differences between the two studies. For example, Kuwahara et al. (41) loaded washed platelets with high concentrations of the fluorescent dyes, Fura red AM and Calcium Green-1 AM, which may cause calcium buffering and potentially reduce the sensitivity of calcium detection. In addition, the confocal imaging system used in our study has superior sensitivity for detecting changes in cytosolic calcium compared with epifluorescence videomicroscopy. It is unlikely that the calcium pulsing observed in our platelet experiments was due to incomplete integrin αIIbβ3 blockage by cTE3 as these cells failed to form stationary adhesion contacts under flow, and furthermore, calcium pulsing was observed during surface translocation. Studies of GPIb/IX-transfected CHO cells also exclude an essential role for integrin αIIbβ3 in this process.

The ability of the vWF-GPIb/IX interaction to induce actin polymerization and filopodial extension in rolling platelets raises a number of important issues regarding the role of cytoskeletal reorganization in regulating the dynamics of the platelet-vessel wall interaction. One possibility is that the conversion of platelets from discoid to spherical forms is important for improving the efficiency of the rolling process. For example, flat discoid platelets would experience high transient torque during rotational movement, thus potentially undermining the ability of these cells to maintain contact with the matrix under conditions of high shear stress. Experimental evidence demonstrating that cell shape plays an important role in regulating cell-matrix interactions under flow is based on the observation that PMA-induced conversion of spherical neutrophils to bipolar elongated forms has a profound inhibitory effect on adhesion to a P-selectin matrix under flow but only a modest effect under static conditions (3).

In addition to platelet spherical, filopodial extension may also play an important role in regulating the dynamics of the platelet-vWF interaction. Based on our observations of rolling platelets by DIC microscopy, the filopodia themselves also appeared to be involved in the rolling process. Cells appeared to form transient adhesion contacts with the vWF matrix through both their filopodial projections and cell body. These observations raise the interesting possibility that at least a subset of GPIb-IX complexes become localized to filopodia during the rolling process. However, GPIb redistribution to filopodia in activated platelets remains a controversial issue. Whereas some studies have demonstrated GPIb on the surface of filopodia (42), other studies have not supported these findings (43). This is a potentially important issue as the ability of GPIb to transiently immobilize filopodial extensions may increase the probability of integrin αIIbβ3, which has been reported to be enriched on the surface of filopodia (44), forming adhesion contacts with the matrix and stabilizing platelet adhesion. The extension of filopodia may also enhance accessibility of integrin αIIbβ3 to immobilized vWF. Platelets are covered by a dense glyocalyx due to the high concentration of glycosylated GPIb on the cell surface. The vWF-binding site on GPIb extends 60 nm above the surface membrane, significantly higher than the vWF-binding site on integrin αIIbβ3 which at most is likely to be 20–30 nm above the level of the plasma membrane (45). It is possible that the dense glyocalyx may sterically hinder access of integrin αIIbβ3 to immobilized vWF. Thus the relative density of GPIb/IX and integrin αIIbβ3 on the surface of filopodia may be an important determinant regulating platelet rolling and irreversible adhesion.

In conclusion, our findings suggest a novel function for the GPIb-IX complex. In addition to its function as a rolling receptor and in transducing signals involved in integrin αIIbβ3 activation, we have demonstrated an important role for this receptor in regulating cytoskeletal reorganization during the rolling process and potentially during platelet aggregation. Although it is well known that GPIb/IX plays a key role in maintaining the normal cytoskeletal architecture of resting discoid platelets, it has not previously been established that ligand binding to this receptor per se can induce cytoskeletal reorganization, especially under physiological flow conditions. To our knowledge this is the first example of a cell surface receptor that not only maintains the normal cytoskeletal architecture of the resting cell but is also able to induce rapid morphological alterations following engagement of an adhesive substrate.

Acknowledgments—We thank Marie-Jeanne Baas and Martine Morales for their technical assistance in cell transfection experiments. We also thank Dr. John Hartwig for helpful suggestions and Dr. Jose Lopez for generously donating the GPIb/IX receptor constructs and for supplying the CHO-GPIb/IX cell line. We also thank Prof. Michael Berndt for the generous supply of monoclonal antibodies.

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