Role for phosphoinositide 3-kinase in FcγRIIA-induced platelet shape change

Kurt L. Barkalow, Hervé Falet, Joseph E. Italiano, Jr., Andrew van Vugt, Christopher L. Carpenter, Alan D. Schreiber, and John H. Hartwig

Division of Hematology, Brigham and Women’s Hospital, Department of Medicine, Harvard Medical School, Boston 02115; and Beth Israel Deaconess Medical Center, Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115; and Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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BLOOD PLATELETS are uniformly structured subcellular particles that activate in response to soluble and solid-phase ligands at sites of vascular injury to form plugs that stop hemorrhage. To form effective plugs, platelets change from their circulating discoid shapes into active forms capable of interacting with one another. Shape change occurs in two distinct morphological steps (18). Platelets first convert from compact disks to irregular spheres. They then flatten on the damaged vascular structure by extending actin-rich lamellae and filopodia. The long actin filaments in filopodia originate from the center of the platelet and radiate outward, coalescing into a bundle of filaments. Orthogonal arrays of actin filaments fill extended lamellae. Many receptors are positioned on the platelet surface that lead to actin assembly and platelet shape change when ligated. Shape change, the signaling cascade that initiates actin assembly, and proteins that regulate actin assembly reactions have been extensively in platelets activated via the thrombin receptor protease-activated receptor (PAR)-1 or the collagen receptor glycoprotein (GP)VI (4, 12, 18, 19) or after chilling (22, 39). Signaling from PAR-1 begins with the activation of pertussis toxin-sensitive trimeric G proteins (6, 7), which couples to phospholipase C-β to cleave phosphatidylinositol 4,5-biphosphate (PI_{4,5}P_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (25). Subsequently, IP_3 gives rise to elevated intracellular calcium and diacylglycerol activates protein kinase C (5). Elevated cytosolic calcium also activates gelsolin, which binds to actin filaments to sever them and to transiently associate with their barbed ends (26). PAR-1 also couples to the small GTPases Rac and Cdc42 (19). Synthesis of polyphosphoinositides (pPIs) follows, and these new pPIs mediate barbed end exposure by dissociating actin filament barbed end capping proteins (4, 19). Barbed ends, exposed when gelsolin family members dissociate from actin filaments, are subsequently amplified by Arp2/3 complex (14), which is also activated by pathways involving pPIs (22, 32). This process is intimately connected to PAR-1-induced actin filament assembly because mice null for gelsolin have only 25–50% of the wild-type actin filament barbed end exposure/nucleation activity after stimulation with thrombin (14, 40).

Early studies indicate that immunoglobulins can activate platelets (21). Immune thrombocytopenic purpura and heparin-induced thrombocytopenia have been linked to ligation of the immunoglobulin receptor FcγRIIA (CD32A) on platelets, suggesting that FcγRIIA can bind immune complexes in blood and that this leads to clearance of platelets from the blood by macrophage-mediated phagocytosis (28). Platelet acti-
vation via FcγRIIA initiates all the well-defined responses believed to be important for platelet function (16, 33). However, the normal hemostatic function of FcγRIIA has not been determined.

In this study, we investigated the mechanisms of shape change in FcγRIIA-stimulated platelets. Like ligation of PAR-1 (8, 15, 24, 31), the cross-linking of FcγRIIA leads to actin assembly. However, the cytoplasmic signaling cascades set in motion by these two stimuli differ. Platelet shape change initiated by FcγRIIA is particularly dynamic, with readily observed filopodia, membrane ruffling, and extension of lamellae resulting in platelet spreading. The underlying structures that support these movements are actin rich, and actin assembly proceeds in a reproducible and robust response with exposure of actin filament barbed ends following FcγRIIA cross-linking. In addition, proteins that modulate barbed end exposure, such as Arp2/3 complex (14), associate with actin in FcγRIIA-activated platelets. Importantly, the data place phosphoinositide 3-kinase (PI3-kinase) as an essential element in actin assembly reactions triggered by FcγRIIA. In particular, PI3-kinase activity is required for the association of the actin filament barbed end amplifier Arp2/3 complex with the platelet actin cytoskeleton.

MATERIALS AND METHODS

Platelet preparation and activation. Platelet isolation from blood included differential centrifugation and chromatography as previously described (4). Briefly, freshly drawn anticoagulated blood was centrifuged at 110 g for 15 min to obtain platelet-rich plasma (PRP). Platelets were separated from the PRP by chromatography on a Sepharose 2B column equilibrated and run in (mM) 145 NaCl, 10 HEPES, 10 glucose, 0.2 Na2HPO4, and 5 KCl with 0.3% BSA, pH 7.4 (platelet buffer). Column-purified platelets were rested for 30 min in a 37°C water bath before use. Approval was obtained from the Institutional Review Board of Brigham and Women’s Hospital, and informed consent was approved according to the Declaration of Helsinki.

Immunofluorescent staining. Adherent platelets were fixed for 10 min with 1% glutaraldehyde in PHEM buffer (in mM: 60 PIPES, 25 HEPES, 10 EGTA, and 2 MgCl2, pH 6.9) containing 1 μM phallacidin, protease inhibitors (10 μg/ml each of leupeptin, aprotinin, and benzamidine), and 0.75% Triton X-100 for 2 min. Cytoskeletons were processed for electron microscopy as previously reported (18). They were fixed for 10 min with 1% glutaraldehyde in PHEM buffer containing 0.1 μM phallacidin, washed in deionized, filtered water and rapidly frozen, freeze dried, and coated with 1.4-nm tungsten-tantalum with rotation and 2.5-nm carbon without rotation. Cytoskeletons were examined and photographed in a JEOL 1200-EX electron microscope at an accelerating voltage of 100 kV.

Actin assembly assay. Platelets were activated with 25 μM thrombin receptor PAR-1 activating peptide (TRAP) or with 3 μg/ml IV.3 (the concentration that gave the maximum response in all quantitative experiments) for 3 min, followed by 30 μg/ml goat anti-mouse F(ab)2 (GAM), and then fixed at the desired time point by the addition of an equal volume of 6.8% formalin. The samples were extracted and labeled in PHEM buffer with 0.1% Triton X-100, and the actin filaments were labeled with 1 μM FITC-phalloidin for 30 min. FACS analysis with a BD Biosciences FACSCalibur (San Jose, CA) used forward and side scattering to identify platelets and measure the mean fluorescence of 10,000 platelets to quantitate filamentous actin content.

Immunofluorescent staining of actin filament barbed end exposure in platelets. The average number of barbed ends exposed per cell was obtained from a nucleation assay with pyrene-labeled rabbit skeletal muscle actin as previously described (18, 19). Ninety microliters of platelets at 2 × 10^9/ml was activated with TRAP or IV.3-GAM and extracted at various time points as described in Actin assembly assay. Platelet extracts were diluted to 300 μl with buffer B (0.1 M KCl, 0.2 mM MgCl2, 0.1 mM EGTA, 0.5 mM ATP, 10 mM Tris, and 0.5 mM β-mercaptoethanol pH 7.4). The assay was started by the addition of 1 μM monomeric pyrene-labeled actin, and fluorescence followed in a Perkin-Elmer LS-50B fluorimeter with an excitation wavelength of 366 nm and an emission wavelength of 386 nm. Actin filament barbed ends were calculated as described previously (18, 19).

When actin nucleation was tested in the presence of recombinant PI3-kinase subunits p85 and p110, resting platelets were permeabilized with 0.25–0.4% octylglucoside (OG) for 30 s and then treated for 2 min with various concentrations of the kinase. Kinase-treated and control platelets were then diluted in buffer B, and the polymerization of 1 μM pyrene-actin was followed as above. Gelsolin and CapZ release from OG-permeabilized platelets was analyzed for sensitivity to added pp1 micelles. OG-permeabilized platelets were washed twice to remove soluble proteins by two successive rounds of centrifugation at 10,000 g for 1 min after resuspension in PHEM buffer with 0.1 μM phallacidin. These platelet cytoskeletal “ghosts” were treated with pp1, in the form of sonicated micelles, and the F-actin-associated pellet and soluble fractions were separated by centrifugation for 15 min at 15,000 g to generate a pellet and soluble fraction. Gelsolin and CapZ were detected in immunoblots of all fractions.

GTP-Rac and -Cdc42 trapping assay. Platelets activated through FcγRIIA in suspension were lysed with 0.1% Triton X-100 in PHEM buffer containing 1 μM phallacidin and protease inhibitors. Triton releases all of the Rac and Cdc42 into the soluble phase (2), allowing platelet lysates to be clarified by centrifugation at 100,000 g for 1 h. GTP-Rac and -Cdc42, in the soluble fraction, were selectively adsorbed to glutathione S-transferase (GST)-GTPase-binding domain (GST) domain of human p21-activated kinase 1 (PAK-1) (amino acids 67–150) adsorbed to glutathione Sepharose beads in an overnight incubation at 4°C. Unbound material was removed with five successive washes in PBS containing 0.02% Tween 20. Bound Rac and Cdc42 were released from the glutathione Sepharose beads with SDS-PAGE sample
buffer, displayed by 12% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and detected by antibodies directed against Rac and Cdc42 (2, 3). As controls, Rac and Cdc42 in the detergent-soluble lysate from resting platelets were loaded with guanosine 5'-O-(2-thiodiphosphate) (GDP[S]) or guanosine 5'-O-(3-thiotriphosphate) (GTP[S]) and incubated with affinity GBD-domain beads. GDP[S]-Rac and -Cdc42 did not bind to PAK-1, GTP[S]-Rac and -Cdc42 bound to the GST-PAK-1 beads (2).

**Immunoblot analysis of platelet cytoskeletal proteins.** Resting and activated platelets were lysed with a final Triton X-100 concentration of 0.1% in PHEM buffer. The lysates were centrifuged at 100,000 g for 30 min at 4°C, the resultant pellets and supernatants were separated, and SDS-PAGE loading buffer containing 5% β-mercaptoethanol was added. The samples were boiled for 5 min. Proteins were displayed by SDS-PAGE and transferred onto Immobilon-P membrane (Millipore). Membranes were blocked with 5% dry milk in 100 mM NaCl-20 mM Tris·HCl, pH 7.4, and then probed with specific antibodies and appropriate peroxidase-tagged secondary antibodies. Detection was performed with an enhanced chemiluminescence system (Pierce).

**Intracellular calcium measurement.** Isolated platelets were preincubated in the dark with 2 μM indo 1-AM (Molecular Probes, Eugene, OR) for 45 min at room temperature. Indo 1-labeled platelets were collected by centrifugation at 800 g and resuspended in platelet buffer containing 1 mM CaCl₂. Platelets were activated as described in *Actin assembly assay*, and fluorescence was recorded (excitation 331 nm, emission 410 nm). The intracellular calcium concentration ([Ca²⁺]) was calculated as described previously (17): [Ca²⁺] = K_d × (F - F_min)/(F_max - F) where K_d = 250 nM, F is fluorescence, F_min is fluorescence after extraction with detergent in the absence of free calcium, and F_max is fluorescence after extraction with detergent in the presence of millimolar free calcium.

**RESULTS**

**FcyRIIA cross-linking leads to platelet shape change and actin assembly.** FcyRIIA cross-linking is sufficient to support platelet spreading (Fig. 1A). Spreading on IV.3-coated coverslips begins with adhesion of discoid platelets and conversion into irregular spheres, followed by filopodial growth from the periphery. Lamellae next fill between filopodia, and platelets flatten. Surfaces coated with BSA (no anti-FcyRIIA antibody) do not support platelet adhesion or shape change, and inhibition of actin assembly with cytochalasin B prevents the extension of both filopodia and lamellae in IV.3-activated platelets (data not shown).

Cross-linking of FcyRIIA on platelets in solution with the IV.3 antibody also initiates shape change and actin assembly, which peaks ~2 min after addition of the cross-linking secondary antibody (Fig. 1B). Actin assembly in response to FcyRIIA cross-linking is dose dependent and saturates when the amount of IV.3 antibody is 3 μg/ml (data not shown). Actin assembly induced through FcyRIIA shows a lag period compared with that seen when platelets are activated through PAR-1, although activation through FcyRIIA and PAR-1 both lead to similar numbers of exposed actin filament barbed ends (Fig. 1C). As expected, there is temporal coupling between actin filament barbed end exposure and actin assembly such that the kinetics of barbed end exposure parallel those of actin filament assembly.

Whereas light microscopy underscores the dynamic nature of platelet filopodial extension, membrane ruffling, and spreading when platelets adhere to IV.3-coated surfaces, high-resolution electron microscopy reveals the ultrastructure of the underlying actin cytoskeleton in these processes (Fig. 2A). Filamentous actin fills ruffles, lamellae, and filopodia. Actin filaments in filopodia and lamellae organize into bundles and networks comparable to those seen in platelets activated by contact with glass (18). Filopodia are cored by long actin filaments, which originate from the cell center, coalesce into bundles at the base of filopodia, and extend to their tips. Lamellae have a dense three-

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**Fig. 1.** Human platelets change shape and assemble actin when activated via FcyRIIA. A: differential interference contrast (DIC) micrograph showing the morphology of spread platelets after activation on a surface coated with the anti-FcyRIIA IgG IV.3. Because the attachment of platelets to the surface is random, this field contains both fully spread platelets and platelets that have just begun to spread. This image was photographed 20 min after the first platelets attached. Bar, 5 μm. B: F-actin content in resting and activated platelets was determined by flow cytometry with FITC-phalloidin. Data are means ± SD; n = 7. C: barbed end content was determined fluorometrically from the rate of pyrene-actin assembly in the presence and absence of permeabilized platelets. Data are means ± SD; n = 3. TRAP, thrombin receptor protease-activated receptor (PAR)-1-activating peptide.

*AJP-Cell Physiol* • Vol 285 • October 2003 • www.ajpcell.org
dimensional network of shorter actin filaments. Ruffles, rare in glass-activated platelets, have densely packed actin filament arrays that appear to form on top of the less dense actin arrays of lamellae.

Role of PI3-kinase in platelet shape change and actin assembly induced by FcγRIIA cross-linking. The p85 subunit of PI3-kinase associates with FcγRIIA in immunoprecipitates, and platelets activated by IV.3 synthesize phospholipids phosphorylated in the D-3 position of the inositol ring (9, 16). Consistent with these observations, Fig. 3A shows that the platelet mass of phosphatidylinositol 3,4-bisphosphate (PI3,4P2) and phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P3) increases after FcγRIIA activation. Inhibition of PI3-kinase by 25 nM wortmannin blocked D-3 phosphorylation induced by FcγRIIA cross-linking.

We examined the requirement for D-3 phosphoinositide synthesis in the actin assembly reaction by inhibiting PI3-kinase activity (Fig. 3B). Actin filament assembly induced by IV.3, detectable by FACS analysis with FITC-labeled phalloidin, was completely inhibited by pretreatment of platelets with wortmannin concentrations as low as 10 nM, specific for PI3-kinase. Wortmannin, in a dose-dependent manner, also markedly reduced but did not completely inhibit actin filament barbed end exposure; 20% of the normal barbed end nucleation sites remained exposed.
mained wortmannin insensitive. The PI3-kinase inhibitor LY-294002 also blocked actin assembly at concentrations consistent with the selective inhibition of PI3-kinase (data not shown).

Platelet spreading on IV.3 coated surfaces is highly sensitive to wortmannin (Fig. 2B). Wortmannin-treated platelets, however, do round up and grow sparse filopodia after stimulation of FcγRIIA. Electron micrographs of cytoskeletons from platelets treated with 25 nM wortmannin and activated on IV.3-coated surfaces lack well-developed actin cortices but have many filopodia containing long actin filaments arranged in bundles. Because filopodial growth in the presence of wortmannin is inhibited by cytochalasin B (data not shown), some residual PI3-kinase-insensitive actin filament assembly remained that is necessary to elaborate the filopodia. The dose response for inhibition of barbed end exposure by wortmannin paralleled that found for its inhibition of actin assembly (Fig. 3B).

Inhibition of PI3-kinase also blunts other platelet activation responses, such as secretion and activation of the fibrinogen receptor, the integrin αIIbβ3 (9, 16, 33). We therefore examined platelets lacking αIIbβ3 from a patient with Glanzmann thrombasthenia and found that αIIbβ3 is not required for spreading on IV.3-coated surfaces or for actin assembly in response to FcγRIIA cross-linking (data not shown). Similarly, other reports have suggested that release of ADP by platelets activated via FcγRIIA is a costimulus. However, using apyrase to scavenge ADP did not affect the actin filament barbed end exposure reaction in response to FcγRIIA cross-linking (data not shown). Similarly, inhibition of thromboxane production during FcγRIIA-induced spreading does not contribute to the shape change because indomethacin had no effect on spreading (data not shown).

FcγRIIA cross-linking leads to activation of small GTPases Rac and Cdc42. One potential downstream target of FcγRIIA signaling to actin is the small GTPase Rac. Because Triton X-100 treatment releases all of platelet Rac independently of its guanine nucleotide-bound state (2), we trapped and segregated GTP-Rac in soluble lysates with the GST-PAK-1 construct that binds GTP-Rac but not GDP-Rac in Triton-soluble platelet extracts (Fig. 4A). These and other earlier experiments (2) reveal that the majority of Rac in resting platelets is GDP bound. Activation of platelets through FcγRIIA increased the amount of GTP-Rac in platelets to 20 ± 10% of the total at 3 min, the time of maximal actin assembly and barbed end exposure. Treatment of platelets with 30 μM LY-294002 completely prevented Rac charging, indicating that activation of Rac by FcγRIIA cross-linking is dependent on PI3-kinase.

GTP charging of Cdc42 was evaluated with the same approach (Fig. 4B). The amount of active Cdc42 in

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Fig. 4.** Effect of phosphoinositide 3-kinase (PI3-kinase) inhibition on Rac, Cdc42, Arp2/3 complex, and CapZ in FcγRIIA-activated platelets. GTP loading of Rac (A) and Cdc42 (B) was detected by trapping GTP-Rac and GTP-Cdc42 with the glutathione S-transferase (GST)-PAK-1 construct and quantitated by immunoblotting. Unbound Rac and Cdc42 contain bound GDP. Treatment of platelets for 10 min with 30 μM LY-294002 (LY) completely inhibits the formation of GTP-Rac in FcγRIIA-activated platelets. PI3-kinase inhibition by 25 nM wortmannin (Wort) had little effect on GTP-Cdc42 charging. C and D: Arp2/3 complex (C) and CapZ (D) content in cytoskeletons of FcγRIIA-activated platelets incubated with or without 30 μM LY-294002 or 25 nM wortmannin. Data are means ± SD; n = 4. Data shown for Cdc42 are means of 2 independent experiments.
resting platelets was small, representing <5% of the total, as reported previously (2, 34, 36). Cross-linking of FcγRIIA increased the amount of GTP-Cdc42 over the time course of the experiment. The extent of Cdc42 converted to the GTP state, however, was small, representing only 10% of the total after 1 min. Treatment of platelets with 25 nM wortmannin had little effect on the amount of GTP-Cdc42 in resting and FcγRIIA-activated platelets.

**Incorporation of Arp2/3 complex and CapZ in platelet actin cytoskeleton.** Proteins that regulate actin filament exposure in platelets were examined for their association with F-actin in platelet cytoskeletons activated through FcγRIIA with or without PI3-kinase inhibitors (Fig. 4, C and D). The actin filament barbed end amplifier Arp2/3 complex (Fig. 4C) and the capping protein CapZ (Fig. 4D) both associated with the cytoskeleton of FcγRIIA-activated platelets with kinetics that slightly lagged behind the formation of actin filament barbed ends. Twenty-five percent of the total platelet Arp2/3 complex associated with the resting platelet cytoskeleton. FcγRIIA activation of platelets increased the amount of Arp2/3 complex in the cytoskeleton to a maximum of 65% of the total in 2–3 min. In similar fashion, ~35–40% of the actin filament barbed end capping protein CapZ associated with the Triton-insoluble cytoskeletal actin in resting platelets. Platelet activation by FcγRIIA increased the amount of CapZ in the detergent-insoluble cytoskeleton to 60–65%. PI 3-kinase inhibition by wortmannin and/or LY-294002 diminished the association of Arp2/3 complex and CapZ with the active cytoskeleton by 80% and 40%, respectively.

**Immunofluorescence localization of Arp2/3 complex in platelets.** In resting platelets spread on anti-FcγRIIA IgG showed it to move to the platelet cortex (Fig. 5A), as described previously (13, 14). A portion of Arp2/3 complex also concentrated in foci in the center of these platelets. Analysis of Arp2/3 complex distribution in surface-activated platelets pretreated with LY-294002 revealed that Arp2/3 complex failed to move to the cell periphery and instead incorporated only into distinct foci within the cell center (Fig. 5C). Resting platelets showed diffuse labeling for the Arp2/3 complex throughout the platelet cytoskeleton (14). F-actin staining with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin confirmed that platelets adhered less well to IV.3-coated surfaces after exposure to LY-294002 (Fig. 5, B and D). The platelets that did adhere rounded up and extended some filopodia but were unable to protrude lamellae or make ruffles.

**Role of calcium mobilization in platelet actin assembly induced by FcγRIIA cross-linking.** We examined whether a calcium transient is required for FcγRIIA-mediated actin assembly (Fig. 6). Figure 6A compares the free calcium increases in FcγRIIA- and PAR-1-activated platelets. IV.3 induced a sustained rise in free calcium after a 1-min lag, from a resting value of ~170 nM to a maximum of 500 nM after 3 min. Calcium remained elevated for the time of the assay (5 min). TRAP, on the other hand, mobilized calcium

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*AJP-Cell Physiol • VOL 285 • OCTOBER 2003 • www.ajpcell.org*
within seconds and was maximal by 15 s, after which the calcium transient decayed. Mobilization of calcium by FcγRIIA activation requires the activity of PI3-kinase because it is inhibited by wortmannin (Fig. 6A) or LY-294002 (data not shown).

Chelation of intracellular calcium with EGTA-AM markedly reduced the ability of activated platelets to expose actin filament barbed ends in both FcγRIIA- and PAR-1-stimulated platelets (Fig. 6B). The inability to generate actin filament barbed ends translated into reduced actin assembly in platelets preloaded with EGTA-AM and activated through FcγRIIA (Fig. 6C). Furthermore, platelets preloaded with EGTA-AM, at sufficient levels to buffer intracellular calcium (20), did not spread on IV.3-coated surfaces, although filopod-like processes were extended, and movement of Arp2/3 complex to the cell cortex was impaired in platelets treated with EGTA-AM (data not shown). Arp2/3 complex once again was clustered in intracellular foci, as was observed when PI3-kinase was inhibited.

**Products of PI3-kinase mediate actin assembly in OG-permeabilized platelets.** We further measured the ability of ppIIs phosphorylated in positions D-3 and D-4 to release the F-actin capping proteins CapZ and gelsolin from OG-permeabilized platelets (Fig. 7A). Ten percent of platelet CapZ and gelsolin is found in the OG-insoluble fraction (4). PI3,4,5P3 and PI3,4P2 release both CapZ and gelsolin from these preparations. Control experiments using phosphatidylserine showed no release of these proteins (4). These experiments demonstrate that gelsolin and CapZ are two putative downstream effectors of D-3-containing ppIIs that can lead to barbed end exposure in permeabilized platelets. Addition of >5 nM PI3-kinase to permeabilized platelets induced a maximal response of ~500 barbed ends per platelet. This implies that PI3-kinase and the synthesis of D-3 phosphorylated ppIIs can lead to actin filament barbed end exposure in permeabilized platelets.

**Actin filament barbed end exposure was also measured in OG-permeabilized platelets after addition of the p85-p110 PI3-kinase complex.** Figure 7B shows the PI3-kinase dose dependence of barbed end exposure in permeabilized platelets. Addition of >5 nM PI3-kinase to permeabilized platelets induced a maximal response of ~500 barbed ends per platelet. This implies that PI3-kinase and the synthesis of D-3 phosphorylated ppIIs can lead to actin filament barbed end exposure in permeabilized platelets.

**Products of PI3-kinase mediate actin assembly in OG-permeabilized platelets.** We further measured the ability of ppIIs phosphorylated in positions D-3 and D-4 to release the F-actin capping proteins CapZ and gelsolin from OG-permeabilized platelets (Fig. 7A). Ten percent of platelet CapZ and gelsolin is found in the OG-insoluble fraction (4). PI3,4,5P3 and PI3,4P2 release both CapZ and gelsolin from these preparations. Control experiments using phosphatidylserine showed no release of these proteins (4). These experiments demonstrate that gelsolin and CapZ are two putative downstream effectors of D-3-containing ppIIs that can lead to barbed end exposure in platelets.

**Actin filament barbed end exposure was also measured in OG-permeabilized platelets after addition of the p85-p110 PI3-kinase complex.** Figure 7B shows the PI3-kinase dose dependence of barbed end exposure in permeabilized platelets. Addition of >5 nM PI3-kinase to permeabilized platelets induced a maximal response of ~500 barbed ends per platelet. This implies that PI3-kinase and the synthesis of D-3 phosphorylated ppIIs can lead to actin filament barbed end exposure in permeabilized platelets.

**Fig. 6. A rise in free cytosolic calcium is required for actin assembly in FcγRIIA-activated platelets.** A: cross-linking of FcγRIIA with IV.3 induces a sustained calcium transient. After addition of the cross-linking antibody (arrowhead), there is an ~1-min lag before calcium begins to rise to its sustained maximum value of ~500 nM. The kinetics of calcium release when PAR-1 is activated differ markedly from those of FcγRIIA. TRAP induces an immediate and rapid release of cytoplasmic calcium that peaks between 15 and 20 s. Calcium is then resequestered after TRAP activation. Platelets pretreated for 5 min with 25 nM wortmannin mount weak calcium responses to FcγRIIA activation. [Ca2+]i, intracellular Ca2+ content. B: actin polymerization is blunted in platelets preloaded with EGTA-AM. C: EGTA-AM loading inhibits barbed end exposure to both FcγRIIA and PAR-1. Data are means ± SD; n = 3 experiment.

**Fig. 7. PI3-kinase and ppiI micelles expose barbed ends by releasing capping proteins.** A: release of gelsolin and CapZ from octylglucoside (OG)-permeabilized platelets with buffer or with increasing concentrations of PI3,4P2 or PI3,4,5P3 as indicated. B: purified PI3-kinase, added to permeabilized platelets, increases the number of exposed barbed ends in a dose-dependent manner.
DISCUSSION

With a single immunoglobulin G Fc receptor, human platelets provide a simplified model system for examining cellular responses triggered by FcγRIIA. Platelet activation on surfaces coated with anti-FcγRIIA induces aggressive spreading over the substratum driven by a robust actin assembly reaction and actin filament barbed end exposure. PI3-kinase rapidly and transiently associates with FcγRIIA in platelets on clustering of FcγRIIA (9). Consistent with previous results (9, 16, 33), we find both Pl3,4,5P2 and Pl3,4,5P3 to be synthesized in platelets activated through FcγRIIA and posits PI3-kinase at a critical control point for a broad spectrum of activation-related events in platelets stimulated through FcγRIIA. The tyrosine kinase Syk is also recruited to FcγRIIA (10) and is required for FcγRIIA-dependent phagocytosis (11). We have observed that inhibitors of Syk kinase activity prevent FcγRIIA-induced actin assembly (data not shown). PI3-kinase may associate with FcγRIIA either directly via its SH2 domain or indirectly by using Syk as an adapter. In the latter case, Syk would bind first to the immunoreceptor tyrosine-based activation motif (ITAM) of FcγRIIA and then PI3-kinase would dock on to the phosphorylated tyrosine residues on Syk (9, 23).

Platelet actin assembly induced by FcγRIIA requires the enzymatic activity of PI3-kinase. Platelets adhere less well to surfaces coated with anti-FcγRIIA antibody after exposure to LY-294002, and the platelets that do adhere round up and extend some filopodia but are unable to protrude lamellae or make ruffles. Some residual actin assembly and filopodia growth remain in wortmannin-treated and FcγRIIA-activated platelets that are inhibited by cytochalasin B. These findings suggest either that the small amount of actin assembly required to grow filopodia is not detectable in our assays or that actin disassembly is coupled to filopodial actin assembly under these conditions. PI3-kinase-independent activation of Cdc42 occurs after cross-linking of FcγRIIA with the IV.3 antibody. Further experimentation is required to determine the precise role of Cdc42 in filopodia formation in platelets. Interestingly, platelets lacking the Cdc42 effector WASp normally assemble actin and change shape (13).

Our findings couple Rac signaling to actin in a PI3-kinase-dependent and -independent fashion. GTP-Rac can activate PIP5-kinase Iα to synthesize the ppI P2,3 to the cytoplasmic face of the plasma membrane, which can bind and dissociate capping proteins to uncap actin filament barbed ends (19, 35) and stimulate activation of Arp2/3 complex (32). Rac may activate Arp2/3 complex via cortactin (37), as cortactin translocation to the cell cortex and to actin-enriched ruffles is Rac dependent (36, 38). Arp2/3 complex fails to migrate to the cell cortex and, instead, aggregates into foci in the cell interior when PI3-kinase is inhibited. It is therefore possible that Arp2/3 complex activation is initiated in a Rac-dependent pathway (29) with Rac activation initiated by PI3-kinase (30, 34).

Unusual localization and activation of Arp2/3 complex have previously been linked to low barbed end production when the actin filament severing and nucleating protein gelsolin is either impaired or absent (14). However, signaling to Arp2/3 complex appears to be impaired here because Arp2/3 complex association with actin is normal in gelsolin-deficient platelets (14). It has been shown that PI3-kinase is not necessary for actin assembly and Arp2/3 complex recruitment during FcγRIIA-mediated phagocytosis (1, 27). The data suggest that FcγRIIA signaling cascades to actin during platelet spreading or phagocytosis by macrophages may differ. CapZ, a heterodimeric actin filament barbed end capping protein, also associates with the cytoskeleton in FcγRIIA-activated platelets as it does after PAR-1 ligation (4). CapZ binding to filament barbed ends both stabilizes newly formed filaments and stops filament elongation.

Platelet shape change and actin assembly in response to activation via FcγRIIA is calcium dependent. Loading platelets with EGTA-AM, at a concentration that buffers intracellular calcium rise (20), dramatically reduces the exposure of actin filament barbed ends and prevents platelet spreading. The requirement for calcium ions in actin assembly and shape change supports previous reports in which calcium-dependent actin filament severing by gelsolin is required for maximal barbed end exposure and actin assembly during platelet activation (4, 18, 40). Atypical broad filopodia elaborated when EGTA-loaded platelets are activated through FcγRIIA also resemble those previously described in glass-activated and EGTA-loaded platelets (18). In addition, because calcium levels do not increase when PI3-kinase is inhibited in FcγRIIA-activated platelets, gelsolin-dependent uncapping of actin filaments to generate free barbed ends does not occur and Arp2/3 complex amplification of actin filament barbed ends is therefore minimized.

In conclusion, we have shown that activation of FcγRIIA results in platelet spreading in a manner comparable to other mechanisms of platelet activation. However, the signaling pathway to actin in FcγRIIA-activated platelets differs from that seen in PAR-1-stimulated platelets. We have shown that activation of PI3-kinase is essential for normal shape change and actin assembly driven by FcγRIIA. In particular, Arp2/3 complex requires PI3-kinase activity for binding to actin, where, in concert with gelsolin, it amplifies actin filament barbed ends (14). These findings establish a framework in which we can further understand platelet activation and signaling between Fcγ receptors and actin and clarify physiological roles for FcγRIIA on human platelets.

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DISCLOSURES

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