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D3 Phosphoinositides and Outside-in integrin Signaling by Glycoprotein IIb-IIIa Mediate Platelet Actin Assembly and Filopodial Extension Induced by Phorbol 12-Myristate 13-Acetate*

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Phorbol 12-myristate 13-acetate (PMA) uncaps a small number of the fast-growing (barbed) ends of actin filaments, thereby eliciting slow actin assembly and extension of filopodia in human blood platelets. These reactions, which also occur in response to immunologic perturbation of the integrin glycoprotein (GP) IIb-IIIa, are sensitive to the phosphoinositide 3-kinase inhibitor wortmannin. Platelets deficient in GPIIb-IIIa integrins or with GPIIb-IIIa function inhibited by calcium chelation or the peptide RGDS have diminished PMA responsiveness. The effects of PMA contrast with thrombin receptor stimulation by 5 μM thrombin receptor-activating peptide (TRAP), which causes rapid and massive wortmannin-insensitive actin assembly and lamellar and filopodial extension. However, we show here that wortmannin can inhibit filopod formation if the thrombin receptor is ligated using suboptimal doses (<1 μM) of TRAP. Phosphatidylinositol 3,4-bisphosphate inhibits actin filament severing and capping by human gelsolin *in vitro*. The findings implicate D3 polyphosphoinositides and integrin signaling in PMA-mediated platelet stimulation and implicate D3 containing phosphoinositides generated in response to protein kinase C activation and GPIIb-IIIa signaling as late-acting intermediates leading to filopodial actin assembly.

Extracellular perturbations cause cells to change cell shape and translocate by remodeling diverse intracellular actin-based structures. The relative functional simplicity of these changes in the anucleate human blood platelet makes it a useful subject for working out the pathways leading to this remodeling. Of particular utility is the fact that actin remodeling events in the platelet predominantly take place sequentially in time rather than the actin rearrangements that occur simultaneously in space during cell locomotion.

The resting platelet is a rigid disc stabilized by an actin filament gel that links to a submembrane skeleton and to transmembrane proteins (1, 2). Over 98% of the actin filaments in resting platelets have their fast growing ("barbed" as defined by myosin head fragment binding) ends stabilized by specific barbed-end capping proteins. In response to thrombin, a calcium transient in the platelet activates gelsolin, which then severs actin filaments in the periphery of the actin filament gel and caps the newly formed barbed ends of the severed filaments (3). Subsequently, phosphoinositides accumulate and inactivate gelsolin as well as another abundant barbed end binding factor, capping protein. Phosphoinositide-mediated uncapping of at least 25% of the filament barbed ends eventuates in massive actin assembly as micromolar quantities of monomeric subunits, previously prevented from spontaneous nucleation by sequestering proteins, add onto the uncapped filament barbed ends (4–6).

The actin assembly following thrombin-induced platelet activation mediates the appearance of two types of surface protrusions, ruffling lamellae that spread circumferentially and threadlike filopodial protrusions. The filopodia are particularly associated with GPIIb[1]-IIIa-mediated platelet aggregation and clot retraction (7, 8). Lamellae contain an orthogonal actin filament network cross-linked by ABP-280 (platelet filamin) that forms only in response to the calcium- and gelsolin-mediated severing of the filamentous (F)-actin in the resting platelet. In contrast, filopodial actin assembly proceeds whether or not intracellular calcium rises and also takes place in platelets lacking gelsolin (3, 9). In part, the calcium dependence of lamellar actin assembly is anatomical. Without efficient severing of long filaments in the resting platelet, no template arises off of which to nucleate an orthogonal network. However, results of studies with cultured cells have also shown that lamellar and filopodial actin structures arise from activation of distinct signaling pathways. Activation of the GTPase *Rac* induces lamellae, whereas a different GTPase, Cdc42Hs, leads to extension of filopodia (10, 11). In this report we provide evidence that different phosphoinositides, formed in response to activation of these signaling pathways, mediate formation of the distinct surface structures. We have examined the platelet responses to the diacylglycerol analog, phorbol 12-myristate 13-acetate

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[1] The abbreviations used are: GP, glycoprotein; PMA, phorbol 12-myristate 13-acetate; DAG, 1,2-dioctanoyl-*sn* -glycerol; PtdIns, phosphatidylinositol; PtdIns-3-P, phosphatidylinositol 3-phosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-3,4-P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PI-3-K, phosphoinositide 3-kinase; TRAP, thrombin receptor-activating peptide; TRAF, thrombin receptor-activating fragment peptide; FITC, fluorescein isothiocyanate; LIBS, ligand-induced binding site; TRITC, tetramethylrhodamine B isothiocyanate.

(PMA) and conclude that it induces filopodial extension by generation of a D3 phosphoinositide, PtdIns-3,4-P_2, presumably following a synthetic pathway set in motion by protein kinase C (PKC). In keeping with the association between platelet filopodia and GPIIb-IIIa, we also show that PMA-activated filopodial actin assembly requires signaling through this integrin.

EXPERIMENTAL PROCEDURES

Materials—Human thrombin, PMA, prostaglandin E_1, wortmannin, bovine serum albumin, fibrinogen, phallacidin, FITC-phalloidin, iodomethacin, PtdIns-4,5-P_2 from bovine brain, and other general chemical reagents were purchased from Sigma. PtdIns-4,5-P_2 has primarily stearate and arachidonate as the SN1 and 2 acyl chains. Synthetic dipalmitoyl PtdIns-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3 were obtained from Matreya, Inc. (Pleasant Gap, PA). RGDS, TRAP (SFLLRNPNQKYEPF), and TRAF (SFLRRN) (thrombin receptor-activating fragment peptide) peptides were purchased from BACHEM, King of Prussia, PA. [^{32}P]Orthophosphoric acid was obtained from DuPont NEN. Fura2 AM was purchased from Molecular Probes, Eugene, OR. Ligand-induced binding site (LIBS) antibody was generously provided by Dr. Mark H. Ginsberg, Scripps Research Institute, La Jolla, CA.

Isolation and Activation of Platelets—Human blood from either healthy volunteers or a volunteer patient with well characterized Glanzmann's thrombasthenia, drawn into 0.1 volume of a citrate-based anticoagulant (Aster-Jandl), was centrifuged at 110 × *g* for 15 min. The platelet-rich plasma was gel-filtered through a Sepharose 2B column, equilibrated, and eluted with platelet buffer (145 mM NaCl, 10 mM HEPES, 10 mM glucose, 0.5 mM Na_2HPO_4, 5 mM KCl, 2 mM $MgCl_2$, and 0.3% bovine serum albumin, pH 7.4). Platelet concentrations were determined using a Coulter counter (Coulter Corp., Miami, FL), and gel-purified platelets were typically eluted from the Sepharose 2B column at concentrations of ~ 2–3 × 10^8 cells/ml. Gel-purified platelets were incubated for at least 30 min at 37 °C before exposure to PMA or TRAP to ensure a resting state. Platelets were preincubated with 10 μM indomethacin, 100 nM wortmannin, or 300 μM RGDS at 37 °C for 5, 15, or 5 min, respectively, before agonist treatment. Platelets were activated with PMA or TRAP without stirring. Phospholipid labeling experiments were performed in stirred cells in the presence of 500 μg/ml of fibrinogen. In these [^{32}P]orthophosphoric acid-labeling experiments, platelets were purified from plasma by two centrifugations at 800 × *g* for 10 min in the presence of 1 μM prostaglandin E_1.

Phospholipid Labeling and Extraction—Platelets isolated by centrifugation (~10^9/ml) were incubated for 1 h at 37 °C with 2 mCi/ml of [^{32}P]orthophosphoric acid. Free ^{32}P was separated from platelets by gel filtration over a Sepharose 2B column in the presence of 1 μM prostaglandin E_1. Following activation of platelets with thrombin or PMA with stirring in the presence of 500 μg/ml fibrinogen, lipids were extracted in chloroform:methanol:HCl, deacylated, and analyzed by high pressure liquid chromatography as previously described (12, 13).

Measurement of F-actin Content and Barbed End Nuclei—The F-actin content of resting and activated cells was determined by quantitation of FITC-phalloidin binding to formaldehyde fixed platelets after permeabilization with Triton X-100 (14, 15). Platelets were fixed with an equal volume of 3.6% formaldehyde at various times after activation at 37 °C for a minimum of 30 min. The fixed cells were permeabilized with 0.1 volume of 1% Triton X-100 containing 10 μM FITC-phalloidin at 25 °C for a minimum of 60 min. Labeled platelets were gated by forward and side scattering, and the mean fluorescence of 10,000 cells quantitated in a flow cytometer using the image II software (FACScan, Becton Dickinson, Mansfield, PA). The results are expressed as percentage of F-actin using the well established value of 40% as the amount of F-actin in resting cells (16). The number of exposed barbed filament ends was determined by measuring the rate and extent of pyrene-labeled actin polymerization in detergent lysates as described previously (3). Platelets stimulated with PMA or TRAF in the presence or absence of wortmannin were permeabilized with 0.1 volume of PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$) containing 0.75% Triton X-100, 1 μM phallacidin, and protease inhibitors (3). To 100 μl of this detergent lysate, 185 μl of 100 mM KCl, 0.2 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM ATP, 10 mM Tris-HCl, and 0.5 mM dithiothreitol, pH 7.0, were added. The polymerization rate assay was started by the addition of pyrene-labeled rabbit skeletal muscle actin to a final concentration of 1 μM and the fluorescence quantitated with a Perkin-Elmer spectrophotometer at excitation and emission wavelengths of 366 and 386 nm, respectively. The number of barbed ends required to support the actin assembly rate was determined as described previously (3).

Morphological Studies—The morphology of TRAP, PMA and LIBS3-treated cells was evaluated by light and electron microscopy. Platelet suspensions (2 × 10^8/ml) were either PMA and TRAP activated or first preincubated with 4 μM LIBS3 Fab for 5 min then exposed to 30 μg/ml of fibrinogen at 37 °C. Cells were fixed by the addition of 1 volume of platelet buffer containing 2% glutaraldehyde for 15 min. For light microscopy, cells were adhered to the surface of 12-mm poly-L-lysine-coated coverslips by centrifugation. For electron microscopy, the fixed cells were adhered to the surface of poly-L-lysine coated glass coverslips, washed into distilled water, rapidly frozen, freeze-dried at −90 °C, and rotary coated with 1.8 nm of platinum and 5 nm of carbon without rotation in a Cressington CFE-50 apparatus (Cressington Instrument Co., Watford, UK). The replicas were separated from the coverslips using 25% hydrofluoric acid, and protein was removed using 100% household bleach and picked up using 200 mesh copper grids coated with Formvar and carbon. The replicas were examined and photographed in a JEOL-1200 EX electron microscope.

Evaluation of Platelet Morphology—Platelets were fixed in suspension with an equal volume of 3.7% paraformaldehyde in phosphate-buffered saline, pH 7.4. Fixed cells were attached to glass coverslips by centrifugation, the coverslips were mounted on glass slides, and the cells were photographed using phase-contrast optics in a Olympus microscope. Cells were enlarged to 500 times, and their morphology was evaluated. Cells were scored into the following categories: discoid (rest) or activated having filopodia (one or more); contractile, small amorphic cells with dense cell centers and no apparent filopodia (17, 18); or lamellar, cells having large pseudopodia.

Assay for Gelsolin-mediated Filament Severing—The ability of micelles of polyphosphoinositides to inhibit the actin filament-severing ability of human gelsolin was determined in a pyrene-labeled F-actin depolymerization assay as previously reported (19). Gelsolin's severing activity was measured by its affect on accelerating the fluorescence decrease associated with depolymerization of pyrene-labeled F-actin diluted below its critical monomer concentration. To evaluate the inhibition of gelsolin by phosphoinositides, 50 nM gelsolin was incubated for 5–10 s with various amounts of phosphoinositides in F-actin buffer (2 mM Tris, 0.2 mM $CaCl_2$, 0.2 mM dithiothreitol, 0.5 mM ATP, 2 mM $MgCl_2$, 150 mM KCl, pH 7.4). 30% pyrene-labeled F-actin was then added from a 12 μM stock solution to a dilution of 300 nM. Severing activity was taken to be proportional to the initial slope of the fluorescence decrease and expressed relative to this rate in the absence of phosphoinositides. Other details of the method and data analysis have been presented previously (20). PtdIns-4,5-P_2 micelles, known to inactivate gelsolin, were compared to the D3 phospholipids. All phospholipid solutions were made in 10 mM Tris-HCl, pH 7.4, from lyophilized solids or from chloroform solutions dried in a nitrogen stream and sonicated at maximal power for three 20-s bursts as described previously (21).

RESULTS

Phorbol Myristate Acetate Induces Actin Assembly in Platelets—As shown in Fig. 1, platelets exposed to 30 nM and 100 nM PMA assemble an equivalent amount of F-actin with similar kinetics. Compared with thrombin receptor perturbation, a maximally effective (100 nM) PMA concentration is slower and of much smaller magnitude. Platelets treated with ≥5 μM TRAP increase their F-actin content 2-fold within 30–60 s (Fig. 2) (5). Treatment of platelets with 10 μM indomethacin and/or 1 unit/ml apyrase had no effect on the ability of PMA to induce actin assembly. Therefore, the release of thromboxane or ADP in response to PMA is not responsible for actin assembly (Fig. 1*B*).

Wortmannin Markedly Inhibits PMA-induced Actin Assembly but Affects TRAP-induced Actin Assembly Only at Low Doses of TRAP—Wortmannin markedly reduced the PMA-stimulated F-actin increase (Fig. 3*A*) but, as previously reported, had little effect on actin assembly induced by ≥5 μM TRAP (Fig. 2) (13). When 0.5–1 μM doses of TRAP were used, however, inhibition of actin assembly by wortmannin could be observed (Fig. 2*B*). LY294002, a different and structurally unrelated inhibitor of PI-3-K, inhibits the PMA-induced actin assembly response in platelets to the same extent as 100 nM wortmannin (data not shown).

PMA Induces Wortmannin-inhibitable Actin Filament

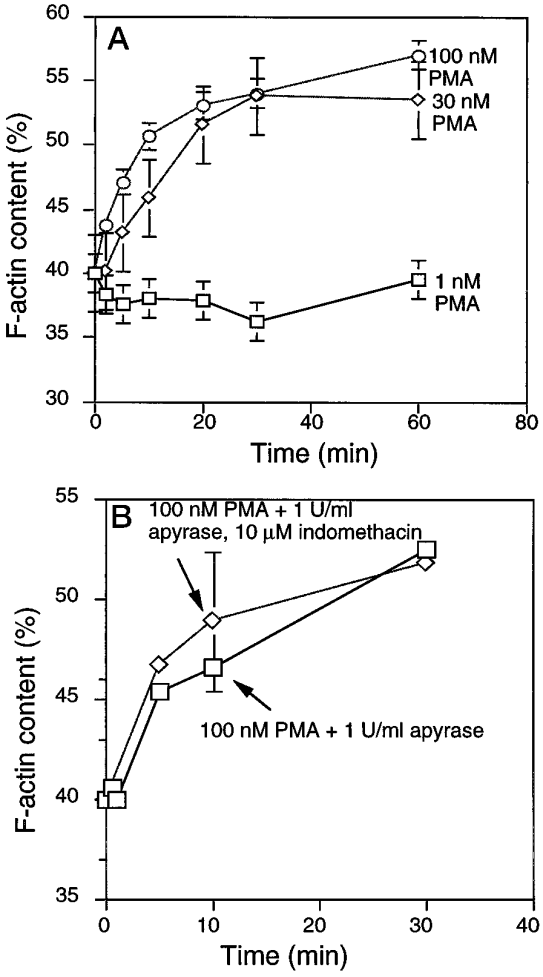

FIG. 1. **Actin assembly induced in human platelets by PMA.** Gel-filtered human platelets were treated with the designated concentrations of PMA. F-actin content was quantitated after the indicated times by TRITC-phalloidin binding as described under "Experimental Procedures." *A*, assembly in the presence of 0.1 mM $CaCl_2$ in the bathing medium. *B*, effect of 1 unit/ml apyrase and 10 μM indomethacin on PMA-induced actin assembly. Cells were preincubated with apyrase and indomethacin for 5 min prior to exposure to PMA. The data are from three separate experiments (mean ± S.D.).

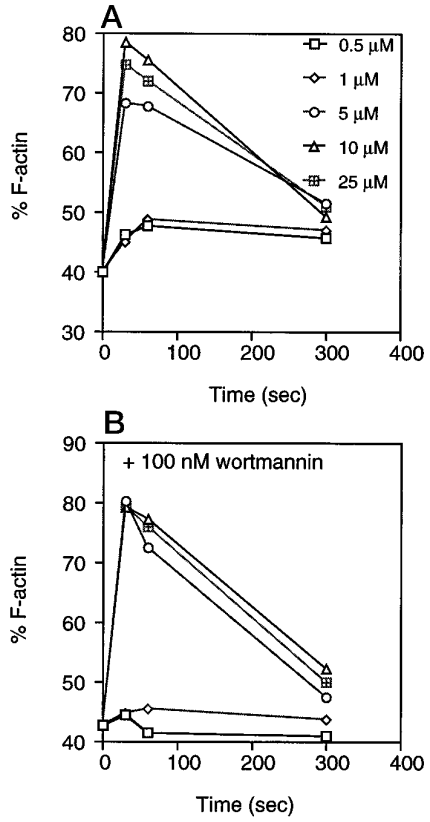

FIG. 2. **Wortmannin inhibits actin assembly only after suboptimal ligation of the thrombin receptor.** Resting platelets were incubated without (*A*) or with 100 nM wortmannin (*B*) for 15 min, then activated using TRAP. F-actin content was measured using FITC-phalloidin binding. Concentrations of TRAP used are indicated in *panel A*.

Barbed End Exposure—PMA stimulates platelets to expose a small number of barbed filament ends (Fig. 3*B*). PMA for 10 min produced 60 ± 18 more barbed filament ends over the resting level, an increase of 67%. This effect is small and slow in contrast to platelets activated with TRAF, which increases the number of free barbed ends in cells by 250% within 1 min. Treatment of the cells with 100 nM wortmannin greatly reduced barbed filament end exposure in response to PMA (Fig. 3*B*).

PMA Induces Primarily PtdIns-3,4-P_2 and PtdIns-4-P Production in Platelets—PtdIns-3,4-P_2 levels increase 2 min after PMA addition and remain elevated thereafter for the 10-min duration of the assay (Fig. 4*A*). PtdIns-3-P and PtdIns-3,4,5-P_3 levels do not detectably differ for 10 min after addition of PMA. Fig. 4*C* shows the concomitant changes in PtdIns-4-P and PtdIns-4,5-P_2 following the addition of PMA. PtdIns-4-P synthesis increased 2.9- and 2.6-fold at 2 and 3 min, respectively, while the PtdIns-4,5-P_2 levels showed a more moderate increase, *i.e.* a maximal increase of 1.5-fold at 3 min. Preincubation with 100 nM wortmannin abrogated any increase in D3 phosphoinositides in PMA-stimulated platelets (Fig. 4*B*) but had only a modest effect on PtdIns-4-P and PtdIns-4,5-P_2 production (Fig. 4*C*), consistent with our previous findings on the effect of wortmannin on phosphoinositide production in platelets activated through the thrombin receptor (13).

Functional GPIIb-IIIa Participation in Actin Assembly and PtdIns-3,4-P_2 Accumulation Induced by PMA—Three experimental approaches established a requirement for intact function of the GPIIb-IIIa complex in the PMA response of platelets. First, as shown in Fig. 5*A*, irreversible inactivation of GPIIb-IIIa by calcium chelation of platelets in 1 mM EGTA for 45 min (22, 23) inhibited the PMA-induced actin assembly response by ~75%. Second, the tetrapeptide RGDS diminished by 56–82% the actin filament assembly response elicited by 100 nM PMA in platelets (Fig. 5*B*). Fig. 6 shows that addition of RGDS to platelets reduced not only the actin assembly response in PMA treated cells, but also PtdIns-3,4-P_2 production elicited by PMA. In addition, RGDS diminished the cellular levels of PtdIns-3,4-P_2 resulting from PMA treatment by 83% at 5 min (Fig. 6*A*). RGDS had no effect on PtdIns-4-P production (Fig. 6*B*), although it had a small effect on the amount of PtdIns-4,5-P_2 induced by exposing platelets to 100 nM PMA at a 5-min time point. Third, platelets from a patient with Glanzmann's thrombasthenia with ~2–3% the normal level of GPIIb-IIIa on their surface[2] were 50% less responsive in actin assembly at 2 and 10 min after PMA exposure compared to platelets isolated from normal human donors (Fig. 5*C*). However, the actin assembly response of these GPIIb-IIIa-deficient platelets to TRAF was identical to that of normal platelets (data not shown).

PMA Activation of Platelets Results in Filopodial Extension—As shown in Fig. 7, *b* and *c*, PMA-treated platelets become spherical and extend filopodia from their surfaces (Table

[2] A. Michelson, personal communication.

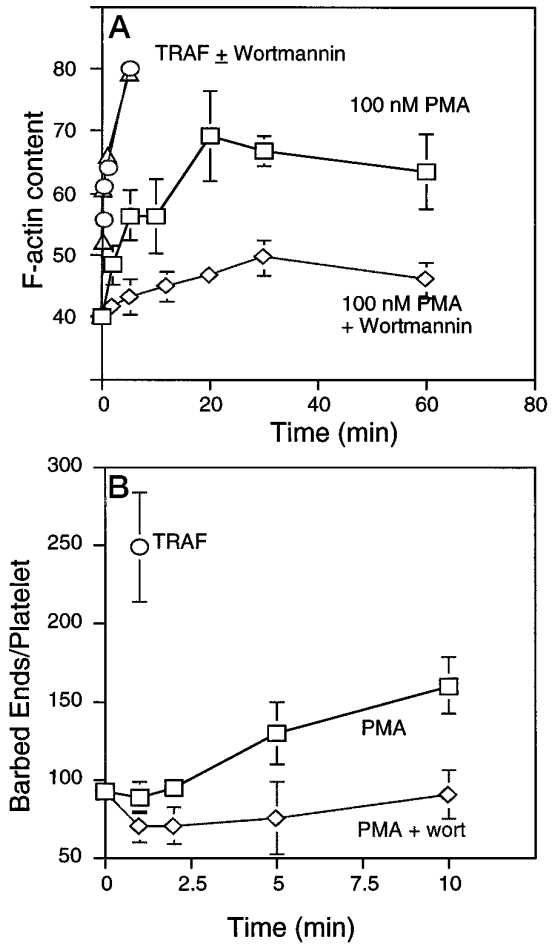

FIG. 3. **Wortmannin inhibits actin assembly and exposure of barbed filament ends induced by PMA but not by TRAF.** *A*, gel-filtered platelets were incubated with 100 nM Wortmannin for 15 min prior to the addition of 100 nM PMA or 25 μM TRAF. Data are expressed as mean ± S.D., $n = 6$. *B*, the number of exposed barbed filament ends in platelet lysates was determined in cells treated with 100 nM PMA as described previously (5). The data are expressed as mean ± S.D., $n = 3$.

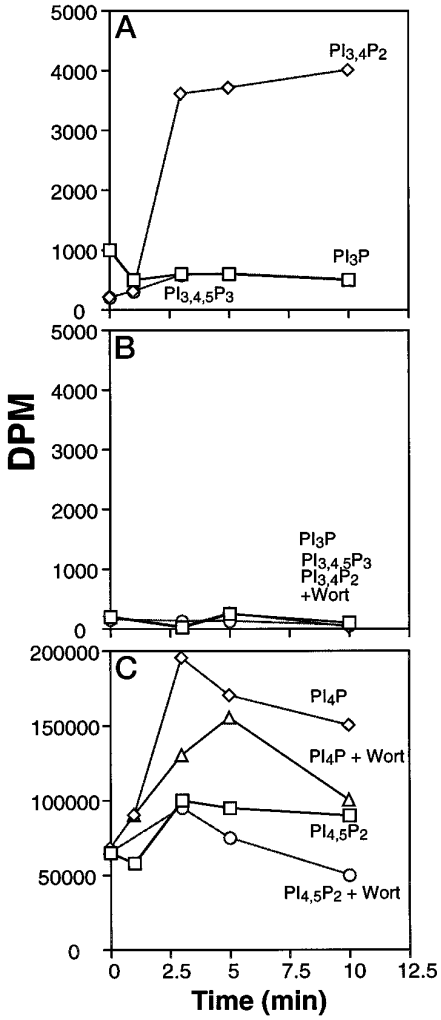

FIG. 4. **PMA treatment of platelets causes the production of primarily PtdIns-3,4-P_2 and PtdIns-4-P and the production of PtdIns-3,4-P_2 is highly sensitive to wortmannin.** *A*, temporal change in D3 phosphoinositides produced in stirred platelets treated with 100 nM PMA in the presence of fibrinogen. PtdIns-3,4-P_2 is the major species induced in response to PMA being stimulated by 70-fold after 3 min. *B*, wortmannin inhibits the production of PtdIns-3,4-P_2 as well as the other D3 lipids in response to PMA. Resting platelets were incubated with wortmannin for 15 min prior to PMA stimulation. *C*, temporal change in PtdIns-4-P and PtdIns-4,5-P_2 following PMA treatment of platelets. Although the cellular contents of PtdIns-4-P and PtdIns-4,5-P_2 are increased by PMA, PtdIns-4-P production is stimulated to a greater extent (3-fold *versus* 1.4-fold). The formation of PtdIns-4-P or PtdIns-4,5-P_2 were not significantly affected by wortmannin. The results are representative of three separate experiments.

I). Fig. 7*b* is a representative view of a platelet exposed to PMA for 5 min and shows that the filopods first appear at the margins of the resting discoid form prior to conversion into a spherical shape. Fig. 7*c* shows that filopods on platelets exposed to PMA for 20 min, are larger, and that the cell body has become smaller and spherical. Fig. 7*d* shows filopod extension to be dependent on actin filament assembly, since inclusion of 2 μM cytochalasin B inhibits their formation. Cytochalasin B-treated cells maintain, for the most part, their discoid resting shapes (compare Fig. 7, *d* with *a*). As shown in Table I, wortmannin inhibited the extension of filopodia from PMA-treated platelets but did not inhibit the formation of the contracted form.

Activation of GPIIb-IIIa with LIBS Fab also Leads to Cytochalasin B-sensitive Filopodial Extension—We have previously reported that direct activation of GPIIb-IIIa molecules with antibodies leads to the net production of PtdIns-3,4-P2 and PtdIns-4-P in platelets (13). Fig. 7*f* shows that ligation of GPIIb-IIIa on the surface of resting platelets with this same antibody (LIBS3 Fab) in the presence of fibrinogen causes discoid resting cells to be converted into their active forms after 5 min. Cells treated with the antibody develop filopodial extensions. Antibody and ligand induced shape change was sensitive to cytochalasin B or the RGDS peptide showing actin assembly as well as receptor cross-linking and post-occupancy outside-in signaling events to be required.

Wortmannin Inhibits the Extension of Filopods by Low Concentrations of TRAP—Although inhibition of PI-3-K activity does not detectably inhibit the massive lamellar actin assembly accompanying thrombin receptor perturbation of platelets with ≥5 μM TRAP, 100 μM wortmannin prevents actin assembly at suboptimal doses of TRAP (Fig. 2). Evaluation of platelets activated by low doses of TRAP showed primarily the extension of filopodia (Fig. 8*A*). Wortmannin inhibited this filopodial extension (Fig. 8*B*).

D3 Polyphosphoinositides Inhibit the Interaction of Gelsolin with Actin—Since D3 phosphoinositide production is critical for filament uncapping and actin assembly in PMA-treated platelets, we determined if D3 phosphoinositides could inhibit barbed-end capping proteins. The ability of micelles of PtdIns-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3 to inhibit the severing

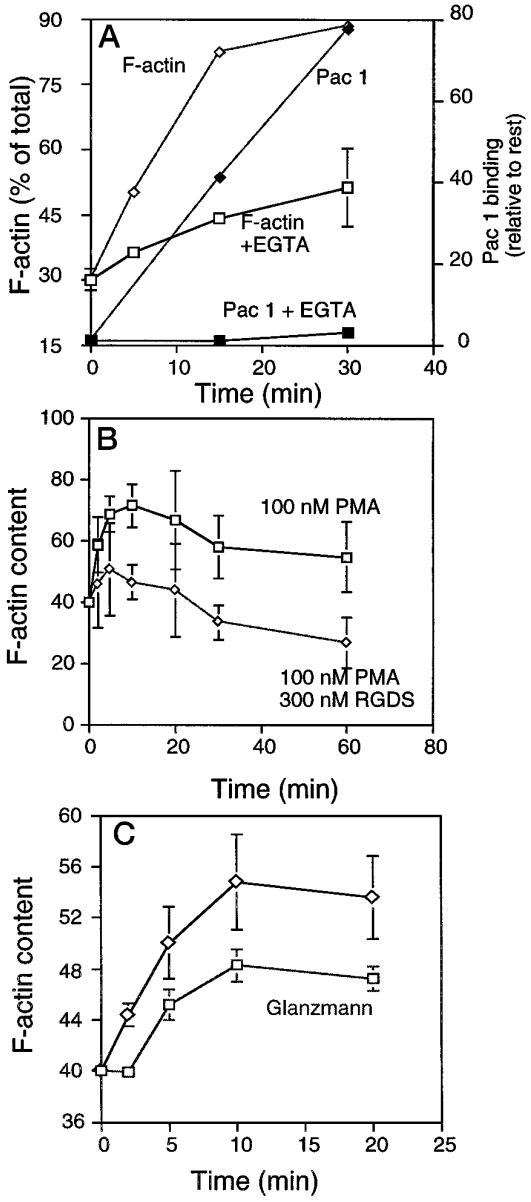

FIG. 5. **Outside-in integrin signaling is required for PMA-induced actin assembly.** *A*, relationship of GPIIb-IIIa activity state to F-actin content in platelets activated with 100 nM PMA. Activation of GPIIb-IIIa was quantified using FITC-labeled Pac-1 IgM binding. GPIIb-IIIa was irreversibly dissociated by preincubating the platelets in a buffer containing 1 mM EGTA for 30 min before the addition of PMA. The Pac-1 antibody was added at time 0. Platelets were fixed at 0, 15, and 30 min after PMA using 1 volume of 4% paraformaldehyde. *B*, F-actin assembly induced by PMA is sensitive to the RGDS peptide. Addition of 300 nM of the GPIIb-IIIa binding and cross-linking inhibiting tetrapeptide RGDS markedly diminishes the actin assembly response in PMA treated cells. Data are expressed as mean ± S.D., $n = 3$. *C*, the F-actin increase in normal and thrombasthenic platelets from a Glanzmann's patient. Thrombasthenic platelets have a diminished actin assembly response to 100 nM PMA relative to platelets from normal volunteers. Thrombasthenic platelets assemble actin normally in response to TRAF. Data are expressed as mean ± S.E., $n = 12$ control, $n = 6$ Glanzmann's patient platelets.

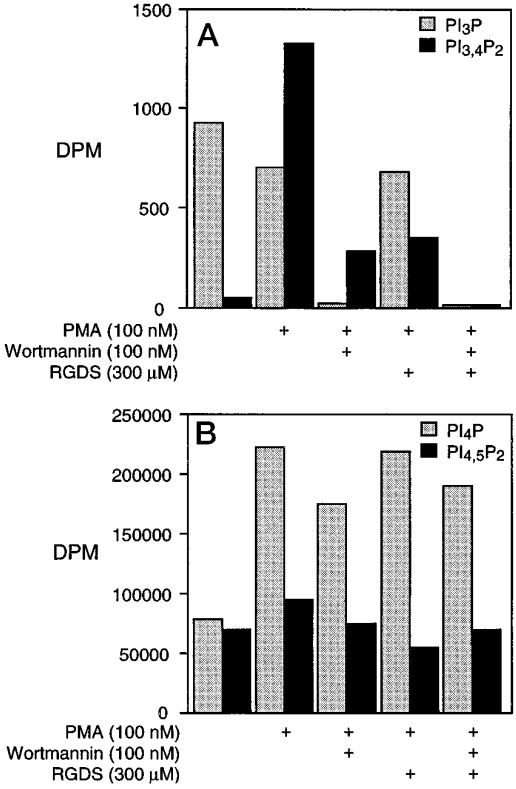

FIG. 6. **D3 phosphoinositide production is sensitive to the RGDS peptide.** Effect of the RGDS peptide on phosphoinositide production 5 min. after stimulation by 100 nM PMA. Platelets were preincubated with 300 μM RGDS for 5 min. PtdIns-3,4-P_2 and PtdIns-4-P production are decreased by 83 and 120%, respectively, by the presence of the RGDS peptide. 100 nM wortmannin inhibited only the production of the D3 phosphoinositides.

activity of human plasma gelsolin was compared to that of micelles of PtdIns-4,5-P_2. The inhibitory dose response for actin filament severing in Fig. 9 shows that PtdIns-3,4-P_2 was highly effective in inhibiting gelsolin and that this phospholipid inactivated gelsolin at very low phospholipid concentrations. The rank order of lipid potency in inhibiting gelsolin severing activity was PtdIns-3,4-P_2 ≫ PtdIns-3,4,5-P_3 > PtdIns-3-P or PtdIns-4,5-P_2. However, although all phospholipid solutions were prepared under similar conditions, the details of their packing into micelles and bilayer vesicles are not well characterized and certain to differ. In addition to differences in head group structure, the difference in acyl chains in the D3 phospholipids (palmitate) and PtdIns-4,5-P_2 (stearate and arachidonate) may also influence the lipid packing. Nevertheless, these results show that phosphoinositides containing a D3 phosphate strongly influence the function of gelsolin and that D3 lipids may be as or more potent on a molar basis than PtdIns-4-P or PtdIns-4,5-P_2.

DISCUSSION

Exposure of diverse cell types to tumor-promoting phorbol esters such as PMA cause morphological changes associated with net actin assembly (24–28). The actin assembly induced by PMA is slower and more sustained that the actin polymerization response to chemoattractants and growth factors, and PMA, unlike these other stimuli, does not induce cell translocation (26, 28, 29). In platelets, PMA generates an irreversible shape change (18, 30), centripetal contractions (31, 32) and cell-cell aggregation (30). We have now defined a signal transduction pathway by which PMA mediates a slow actin assembly that extends filopods from the surface of blood platelets. The general principle underlying this pathway, that it ends with phosphoinositide-mediated uncapping of actin filament barbed ends, is the same as previously defined for thrombin-mediated actin assembly (5).

Thrombin and PMA, however, utilize distinct phosphoinositides to uncap markedly different quantities of actin filaments in platelets. Platelets activated by thrombin rapidly produce PtdIns-4-P and PtdIns-4,5-P_2 to uncap 5–10 times more barbed filament ends than uncap with PMA. The very

modest number of actin filaments uncapped, which is mirrored by the relatively minor quantity of polymerizing actin (explaining why some researchers possibly starting with partially activated platelets failed to observe it), makes for too small a signal to detect in a permeabilization approach that we used to document thrombin-mediated actin filament uncapping (5). However, compensating for this lack was the finding that PI-3-K inhibitors block the PMA-initiated signaling pathway, pointing to an involvement of D3 phosphoinositides in the PMA response. The actin uncapping and assembly that results from thrombin is insensitive to inhibitors of PI-3-K, indicating that PtdIns-4-P and PtdIns-4,5-P_2 are sufficient for this reaction.

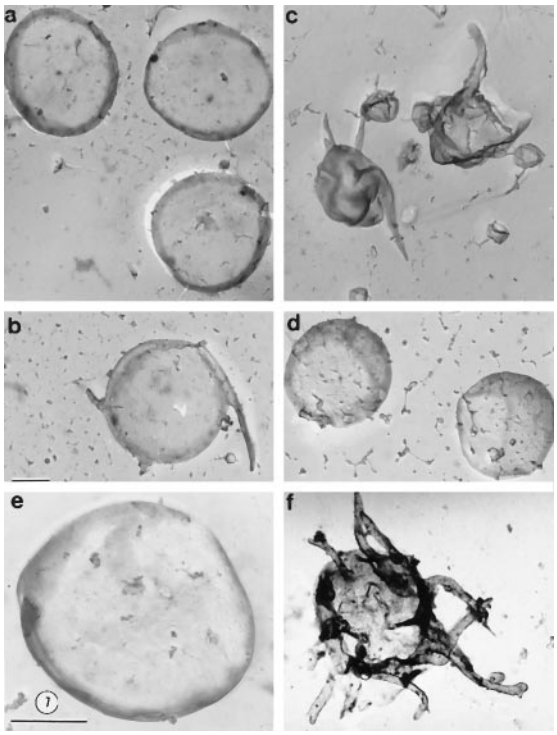

FIG. 7. **Morphology of resting, PMA- and LIBS-treated platelets.** Resting platelets in the presence of 30 μg/ml of fibrinogen at 37 °C for 5 min were fixed with 3.6% formaldehyde before application to glass coverslips fixed after 5 (*a*) or 20 (*b*) min of exposure to 100 nM PMA (*c*), or 20 min after PMA treatment in the presence of 2 μM cytochalasin B (*d*). Fixed cells were adhered to polylysine-coated coverslips by centrifugation at 100 × *g* for 10 min. and then prepared for the electron microscope by liquid-helium freezing, freeze-drying, and metal coating with platinum and carbon. Resting cells are discoid in shape. Cells treated with PMA convert into active forms having predominately filopodia. Filopodial protrusion in response to PMA is inhibited by cytochalasin B. All micrographs have the same final magnification. *e* and *f*, resting platelets were treated in suspension with (*f*) or without 4 μM LIBS3 Fab antibody fragments (*e*) before fixation and application to glass coverslips as described above. Platelets exposed to LIBS3 Fab and fibrinogen for 5–10 min activate predominately by extending filopodia. The *bar* is 1 μm.

The magnitude of the thrombin-induced actin assembly mediated by PtdIns-4-P and PtdIns-4,5-P_2, however, could mask a small contribution operating through D3 phosphoinositides, an effect that is detectable in isolation following stimulation of platelets with PMA. The results presented here support this idea and indicate that D3 phosphoinositide-mediated filopodial actin assembly is also a component of the thrombin response.

We confirmed that PMA induces the synthesis of both PtdIns-3,4-P_2 (Fig. 5) (33–35) and PtdIns-4-P (Fig. 5). At a concentration of 100 nM, wortmannin inhibits primarily PtdIns-3,4-P_2 synthesis and decreased actin assembly induced by PMA by ~70%. The D3 phosphoinositides uncap actin filaments in permeabilized platelets, and PtdIns-3,4-P_2 is as effective as PtdIns-4,5-P_2 in promoting the exposure of barbed filament ends (5). Here we have shown that D3 phosphoinositides also inhibit the activity of actin capping proteins *in vitro*. PtdIns-3,4-P_2, like PtdIns-4,5-P_2, binds to gelsolin inhibiting the severing activity of this protein (Fig. 8). PMA also stimulates PtdIns-4-P production, and this phosphoinositide is likely to account for the wortmannin insensitive component of PMA-induced platelet actin assembly.

PMA activates PKC isoforms, leading to the inference that the phosphorylation of critical substrates by this enzyme results in cellular actin restructuring. The first leg of this PKC-associated pathway requires PI-3-K-dependent signaling from PKC to the major platelet integrin, GPIIb-IIIa, leading to its activation (Fig. 10, *Step 1*). The two known physiological activators for platelet PKCs are diacylglycerol (DAG) and the D3 phosphoinositides, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3. The PMA effect is direct, whereas in response to thrombin, DAG must first accumulate as a result of degradation of PtdIns-4,5-P_2 and phosphatidylcholine to activate PKCs. PKCs heavily phosphorylate the protein pleckstrin, and this phosphorylation correlates with the synthesis of PtdIns-3,4-P_2 in platelets (36, 37). Wortmannin inhibits the PMA-induced pleckstrin phosphorylation, and the addition of exogenous PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 to permeabilized cells leads to an increase in pleckstrin phosphorylation (36, 37). Moreover, pleckstrin phosphorylation correlates with activation of GPIIb-IIIa to bind fibrinogen (38), although some have proposed that direct phosphorylation of GPIIb-IIIa by PKC mediates its activation (39). The major products of PI-3-K, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, are essential for sustaining GPIIb-IIIa following its activation by thrombin (13).

In the second step, outside-inside signaling by the activated integrin GPIIb-IIIa is an obligatory step for signaling from activated PKC to actin (Fig. 10, *Step 2*). In support of this model, inactivation of GPIIb-IIIa by chelation destroys the platelet's ability to respond to PMA with actin assembly. Inhibition of PMA-mediated responsiveness by the tetrapeptide RGDS further implicates a requirement for fibrinogen mediated GPIIb-IIIa cross-linking on the platelet surface in the signaling response. Cell-cell cross-linking is not required since

TABLE I
Morphology of platelets activated with 100 nM PMA

Human platelets were activated in suspension for 15 min at 37 °C with PMA. Some of the cells were preincubated with 25 or 100 nM wortmannin for 15 min before the PMA addition. Resting cells or PMA-activated cells were fixed with 1 volume of 3.7% formaldehyde overnight, then attached by centrifugation at 280 × *g* for 5 min to 12-mm round coverslips coated with 1 mg/ml poly-L-lysine. Slides were photographed in an Olympus inverted microscope, printed to a final enlargement of 500 times, and the morphology of ≥500 cells was characterized for each condition.

Condition	Disc	Filopodial	Contractile	Lamellipodial
		%		
Rest	84.9 ± 4.1	8.5 ± 3.0	6.4 ± 2.3	
Rest + 100 nM wortmannin	86.0 ± 3.0	6.0 ± 5.0	7.9 ± 2.4	
100 nM PMA		75.4 ± 7.1	18.4 ± 7.3	4.1 ± 0.7
100 nM PMA + 100 nM wortmannin	4.6 ± 0.9	7.2 ± 0.9	87.1 ± 1.5	
100 nM PMA + 25 nM wortmannin	4.5 ± 2.8	20.6 ± 1.8	74.0 ± 3.2	

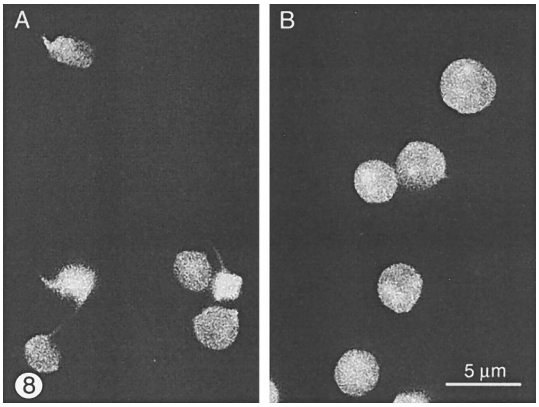

FIG. 8. **Morphology of platelets activated with 0.5 μM TRAP in the absence (*A*) and presence of 100 nM wortmannin (*B*).** Platelets activated in suspension using TRAP were fixed after 60 s by the addition of 1 volume of 4% paraformaldehyde. The cells have been visualized using TRITC-phalloidin after permeabilization with Triton X-100. The magnification is × 300.

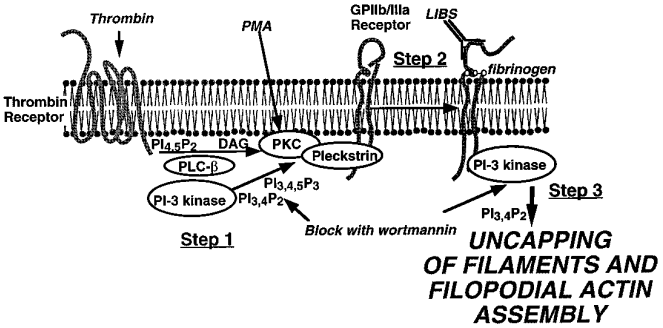

FIG. 10. **Hypothetical pathway for the induction of actin filament assembly from PMA.** PMA activates PKC which phosphorylates pleckstrin and GPIIb-IIIa. Phosphopleckstrin is required for GPIIb-IIIa activation (*Step 2*). Activation of PKC is also accomplished after activation of the thrombin receptor. Activated phospholipase C-β and PI-3-K (*Step 1*) produce DAG and PtdIns-3,4,5-P_3, respectively. Activated GPIIb-IIIa further signals to PI-3-K generating PtdIns-3,4-P_2 (*Step 3*). PtdIns-3,4-P_2 helps maintain the GPIIb-IIIa receptor in its active state (via PKC) as well as mediating actin filament uncapping and actin assembly.

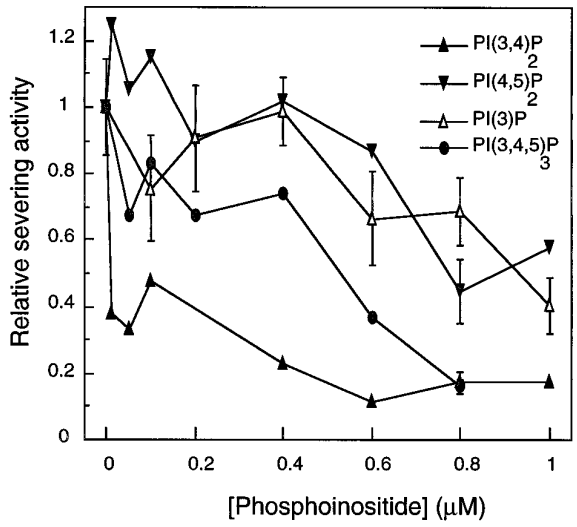

FIG. 9. **Differential inhibition of gelsolin by phosphoinositides.** The severing activity of gelsolin was measured by its effect on accelerating actin depolymerization when added to pyrene-labeled F-actin after a 10 s incubation with various concentrations of PtdIns-3,4,5-P_3, PtdIns-3,4-P_2, PtdIns-4,5-P_2, and PtdIns-3,4,5-P_3. Error bars denote standard deviations of three independent measurements.

activation of actin assembly occurs in the absence of stirring. Also, thrombasthenic platelets mount only a weak actin assembly response to PMA when compared to normal cells. The kinetics and extent of actin assembly induced by PMA is reduced by 50% in these GPIIb-IIIa lacking cells, an inhibition of fibrinogen-mediated GPIIb-IIIa cross-linking using excess extracellular RGDS peptide markedly inhibits the actin assembly response induced by PMA in platelets. RGDS treatment also reduced PtdIns-3,4-P_2 production to levels achievable with wortmannin, but did not greatly affect the production of the PtdIns-4-P or PtdIns-4,5-P_2. These findings suggest that D3 phosphoinositides are generated in post-occupancy signaling events elicited by PMA after fibrinogen binds to active GPIIb-IIIa receptors (Fig. 10, *Step 3*). Another piece of evidence linking GPIIb-IIIa to the PMA response is that direct ligation and activation of surface GPIIb-IIIa molecules using the LIBS3-antibody causes platelets to change from discoid shapes into spiky cells having numerous filopodia (Fig. 7). Actin assembly is required for this protrusivity, because cytochalasin B completely prevents it as it does the PMA response. LIBS3-induced activation of GPIIb-IIIa also results in the production of PtdIns-3,4-P_2 (13). Both RGDS and wortmannin inhibit LIBS-3 mediated D3-phosphoinositide synthesis.

Both thrombin- and PMA-stimulated platelets extrude filopodia. However, we have shown that these structures are the exclusive protrusions extended by PMA- or LIBS3-reacted platelets, whereas thrombin-treated cells also generate ruffling lamellae. The simplest interpretation is that the D3-mediated actin filament barbed end uncapping unique to PMA- or LIBS3-treated platelets is also operating in response to perturbation of the thrombin receptor as a result of DAG production and recruitment of activated PI-3-K. The sensitivity of thrombin receptor-induced filopodial extension to wortmannin confirms the importance of GPIIb-IIIa in the thrombin-mediated filopodial response. It also follows that GPIIb-IIIa signaling is intimately associated with filopodial extension. Dense collections of GPIIb-IIIa molecules have recently been visualized by electron microscopy at the tips and along the sides of platelet filopodia (8). GPIIb-IIIa receptors might signal to PI-3-K through CDC42 which is known to translocate to the activated platelet cytoskeleton in a GPIIb-IIIa-dependent fashion (40). The activated forms of the CDC42, Rho and Rac GTPases bind PI-3-K both *in vitro* and *in vivo* (41, 42). Activation enhances the movement of platelet cytoplasmic GTPase activities into the actin cytoskeleton (40, 43). PI-3-K, at the end of the signal transduction pathway leading to actin filament barbed end uncapping and filopodial growth, is likely to reside on the cytoplasmic tails of GPIIb-IIIa via interactions between it, the cytoplasmic integrin tails, and tensin and $pp125^{FAK}$ molecules (44).

PI-3-K signals both upstream and downstream of GPIIb-IIIa. Upstream signaling could be mediated by PKC isoyzmes activated by the D3 phosphoinositides. Downstream events modulated by PI-3-K could be the priming of the CDC42 and/or Rac GTPases, or the direct interaction of D3 phosphoinositides produced by PI-3-K with actin filament capping proteins. As an example of upstream signaling, PI-3-K primes the Rac GTPase in growth factor activated fibroblasts in a process that eventually leads to membrane ruffling and lamellar assembly. Wortmannin inhibits the ruffling response (45), but this inhibition can be overcome by microinjection of constitutively active Rac, suggesting PI-3-K is upstream from Rac (46). In platelets PI-3-K inhibition has little effect on the massive actin assembly that forms lamellae, although Rac is critical for this lamellar protrusion driven through ligation of the thrombin receptor (16). Platelets appear to be poised to rapidly produce copious

PtdIns-4,5-P_2 to initiate the massive assembly of actin that generates lamellae. PtdIns-3,4-P_2, in contrast, initiates actin assembly only for filopodial growth. The D3 phosphoinositides formed by activated fibroblasts may therefore have different spatial constraints when compared to those in platelets. Candidates to control the spatial distribution of phosphoinositides and phosphoinositide kinases therefore include integrin adhesion receptors.

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D3 Phosphoinositides and Outside-in integrin Signaling by Glycoprotein IIb-IIIa Mediate Platelet Actin Assembly and Filopodial Extension Induced by Phorbol 12-Myristate 13-Acetate

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