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A Selective Role for Phosphatidylinositol 3,4,5-Trisphosphate in the G_i-dependent Activation of Platelet Rap1B*

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The small GTP-binding protein Rap1B is activated in human platelets upon stimulation of a G_i-dependent signaling pathway. In this work, we found that inhibition of platelet adenylyl cyclase by dideoxyadenosine or SQ22536 did not cause activation of Rap1B and did not restore Rap1B activation in platelets stimulated by cross-linking of Fcγ receptor IIA (FcγRIIA) in the presence of ADP scavengers. Moreover, elevation of the intracellular cAMP concentration did not impair the G_i-dependent activation of Rap1B. Two unrelated inhibitors of phosphatidylinositol 3-kinase (PI3K), wortmannin and LY294002, totally prevented Rap1B activation in platelets stimulated by cross-linking of FcγRIIA, by stimulation of the $P2Y_{12}$ receptor for ADP, or by epinephrine. However, in platelets from PI3Kγ-deficient mice, both ADP and epinephrine were still able to normally stimulate Rap1B activation through a PI3K-dependent mechanism, suggesting the involvement of a different isoform of the enzyme. Moreover, the lack of PI3Kγ did not prevent the ability of epinephrine to potentiate platelet aggregation through a G_i-dependent pathway. The inhibitory effect of wortmannin on Rap1B activation was overcome by addition of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), but not PtdIns(3,4)P_2, although both lipids were found to support phosphorylation of Akt. Moreover, PtdIns(3,4,5)P_3 was able to relieve the inhibitory effect of apyrase on FcγRIIA-mediated platelet aggregation. We conclude that stimulation of a G_i-dependent signaling pathway causes activation of the small GTPase Rap1B through the action of the PI3K product PtdIns(3,4,5)P_3, but not PtdIns(3,4)P_2, and that this process may contribute to potentiation of platelet aggregation.

Rap1B is a small GTP-binding protein highly expressed in human platelets (1). In resting cells, it is mainly located at the membrane, but it translocates to the cytosol upon phosphorylation by protein kinase A (2). In activated platelets, Rap1B rapidly interacts with the reorganized actin-based cytoskeleton (3). As other GTPases, Rap1B is activated by binding of GTP. Platelet stimulation by different agonists, such as thrombin, collagen, and ADP, induces the rapid binding of GTP to Rap1B (4, 5). An increase in the intracellular Ca^{2+} concentration in stimulated platelets has been shown to be sufficient to promote Rap1B activation, and specific Ca^{2+}/calmodulin-sensitive guanine nucleotide exchange factors for Rap1B have been identified (5, 6). We (7) and others (8) have recently described a new pathway for Rap1B activation that is initiated by stimulation of membrane G_i-coupled receptors and that is independent of intracellular Ca^{2+} increases. In fact, the sole binding of ADP to the $P2Y_{12}$ receptor, as well as the interaction of epinephrine with the α_{2A}-adrenergic receptor, is sufficient to trigger Rap1B activation. Moreover, we have found that agonists that activate platelets through stimulation of G_q-coupled receptors, such as the thromboxane A_2 analog U46619, or through stimulation of a tyrosine kinase-based pathway, such as in the case of cross-linking of FcγRIIA,[1] totally rely on binding of secreted ADP to the G_i-coupled $P2Y_{12}$ receptor to activate Rap1B (7). Finally, activation of Rap1B induced by ADP or epinephrine is prevented in $G\alpha_{i2}$- and $G\alpha_z$-deficient mice, respectively (8).

During the last few years, activation of a G_i-dependent signaling pathway has been recognized to represent a crucial event absolutely required to elicit full platelet activation. For instance, platelet responsiveness to the thromboxane A_2 analog U46619, to protease-activated receptor-1-activating peptide, or to cross-linking of FcγRIIA is strongly compromised when secretion is prevented by protein kinase C inhibitors or when extracellular ADP is neutralized by specific scavengers, such as apyrase or creatine phosphate/creatine phosphokinase (9–12). It has also been clearly shown that, although ADP can bind to two different G-protein-coupled receptors on the platelet surface (the $P2Y_1$ receptor coupled to G_q and the $P2Y_{12}$ receptor coupled to G_i), only the latter one is responsible for potentiation of platelet activation induced by other agonists (10–12).

The exact mechanism for the G_i-mediated potentiation of platelet activation is still unclear. The α-subunits of the G_i family of heterotrimeric G-proteins are known to inhibit adenylyl cyclase, but several findings indicate that reduction of basal cAMP levels does not contribute to ADP-mediated potentiation of platelet activation (13, 14). By contrast, several studies using specific cell-permeable inhibitors have suggested a crucial role

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[1] The abbreviations used are: FcγRIIA, Fcγ receptor IIA; PI3K, phosphatidylinositol 3-kinase; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; mAb, monoclonal antibody; RBD, Rap-binding domain; GST, glutathione *S*-transferase; RIPA, radioimmune precipitation assay.

for phosphatidylinositol 3-kinase (PI3K) in this event (11, 12, 14, 15). The recent finding that stimulation of a G_i-coupled receptor is sufficient to trigger activation of Rap1B also suggests that this small GTPase could be involved in potentiation of platelet aggregation.

In this work, we have investigated the mechanism of Rap1B activation downstream of stimulation of G_i-coupled receptors in an attempt to reveal a correlation between this small GTPase and the potentiation of agonist-induced platelet activation. We have found that inhibition of adenylyl cyclase is not sufficient to stimulate GTP binding to Rap1B. By contrast, it is shown here that PI3K plays an important role in the G_i-mediated activation of Rap1B. We also provide evidence suggesting that activation of Rap1B downstream of PI3K is associated with the G_i-mediated potentiation of platelet activation.

EXPERIMENTAL PROCEDURES

Materials—Epinephrine, ADP, thrombin, sheep anti-mouse $F(ab')_2$ fragments, apyrase, creatine phosphate, creatine phosphokinase, and adenosine 3′-phosphate 5′-phosphosulfate (PAPS) were from Sigma. Dideoxyadenosine, SQ22536, wortmannin, and LY294002 were from Alexis. AR-C69931MX was a generous gift from AstraZeneca (Charnwood, UK). Di-C_{16}-PtdIns(3,4,5)P_3 and Di-C_{16}-PtdIns(3,4)P_2 were from Matreya, Inc. Monoclonal antibody (mAb) IV.3 against FcγRIIA was obtained from Medarex. Sepharose CL-2B, GSH-Sepharose 2B, and the enhanced chemiluminescence substrate were from Amersham Biosciences. The rabbit polyclonal antiserum against Rap1B was described previously (16). Polyclonal and monoclonal (5G3) antibodies against Akt and phospho-Akt Thr^{308} were from New England Biolabs, Inc. The cDNA for the Rap-binding domain (RBD) of the Ral guanine nucleotide dissociation stimulator was kindly provided by Dr. Johannes L. Bos (Department of Physiological Chemistry, University of Utrecht, The Netherlands). Peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad.

Human Platelet Isolation and Stimulation—Human platelets were isolated by gel filtration on Sepharose CL-2B and eluted with HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.9 mM KCl, and 12 mM $NaHCO_3$, pH 7.4) as previously described (17). Platelet concentration was adjusted to 0.35×10^9 platelets/ml. Platelet samples (0.5 ml) were incubated at 37 °C in an aggregometer under constant stirring and typically stimulated with 10 μM ADP and 1 μM epinephrine or by cross-linking of FcγRIIA through addition of 2 μg/ml mAb IV.3 for 2 min, followed by 30 μg/ml sheep anti-mouse $F(ab')_2$ fragments. Platelet stimulation was typically performed for 1 min. Where indicated, 1 unit/ml apyrase, 5 mM creatine phosphate, 40 units/ml creatine phosphokinase, 500 μM PAPS, or 100 nM AR-C69931MX was added to the platelet samples 2 min before stimulation. Preincubation with wortmannin or LY294002 was performed for 15 min at 37 °C. Inhibition of adenylyl cyclase was achieved by incubation of platelets with 100 μM dideoxyadenosine or 300 μM SQ22536 for 30 min. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were dissolved in Me_2SO and added to the platelet suspension at a final concentration of 30 μM. Measurement of platelet aggregation was performed under the same conditions indicated above, and aggregation was monitored continuously over 10 min.

Rap1B Activation Assay—Activation of Rap1B was evaluated using GST-RBD immobilized on GSH-Sepharose, which is known to bind specifically and selectively the GTP-bound form of Rap1B from a platelet lysate. Platelet stimulation was stopped by addition of an equal volume of ice-cold modified 2× RIPA buffer (100 mM Tris-HCl, pH 7.4, 400 mM NaCl, 5 mM $MgCl_2$, 2% Nonidet P-40, 20% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 0.2 μM aprotinin, and 0.2 mM Na_3VO_4). Cell lysis was performed on ice for 10 min, and the insoluble material was eliminated by centrifugation at 13,000 rpm for 10 min at 4 °C. Recombinant purified GST-RBD was coupled to GSH-Sepharose by incubating 200 μg of the protein with 100 μl of GSH-Sepharose (75% slurry) for 2 h at room temperature under constant tumbling and then added to the cleared platelet lysates (20 μg of GST-RBD/sample). Precipitation of GTP-bound Rap1B was performed by incubation at 4 °C for 45 min. The precipitates were collected by brief centrifugation, washed three times with modified 1× RIPA buffer, and finally resuspended in 25 μl of SDS sample buffer (25 mM Tris, 192 mM glycine, pH 8.3, 4% SDS, 1% dithiothreitol, 20% glycerol, and 0.02% bromphenol blue). Precipitated Rap1B was separated by SDS-PAGE on 10–20% acrylamide gradient gels and transferred to nitrocellulose. The presence of active Rap1B in precipitates with GST-RBD was evaluated by staining the nitrocellulose filters with a specific polyclonal antiserum directed against Rap1B, used at a final dilution of 1:1000. Reactive proteins were detected by enhanced chemiluminescence reaction. Data in all figures are representative of at least three separate experiments.

Measurement of Akt Phosphorylation—Platelet samples (0.2 ml, 10^9 platelets/ml) were incubated at 37 °C and stimulated as indicated in the figure legends for 1 min. Platelets were lysed in 2% SDS in HEPES buffer, and protein concentration was determined. Aliquots containing 80 μg of total platelet lysates were heated at 96 °C for 5 min in SDS sample buffer, separated on 12% acrylamide gel, and transferred to nitrocellulose. Blots were probed with anti-phospho-Akt Thr^{308} antibody and then reprobed with anti-Akt antibody. Analysis of Akt phosphorylation in platelets stimulated by cross-linking of FcγRIIA was performed with immunoprecipitated Akt because preliminary experiments revealed a cross-reactivity of the anti-phospho-Akt Thr^{308} antibody with the heavy chains of mAb IV.3, used to activate FcγRIIA, that compromised the interpretation of the results (data not shown). Platelet samples were lysed in ice-cold RIPA buffer and precleared with protein A-Sepharose. The precleared lysates that were also devoid of mAb IV.3 were used to immunoprecipitate Akt with mAb 5G3. Immunoprecipitates were then analyzed by immunoblotting with anti-phospho-Akt Thr^{308} antibody and reprobed with anti-Akt antibody.

Studies with Mouse Platelets—PI3Kγ-deficient mice were generated as previously described (18). Blood was collected from anesthetized mice from the inferior vena cava into syringes containing heparin solution (5 units/ml). Blood was centrifuged at $90 \times g$ for 10 min, and the platelet-rich plasma was collected. Aggregation studies were directly performed with the platelet-rich plasma upon adjustment of the platelet count to 2×10^8 platelets/ml with autologous platelet-poor plasma. For analysis of Rap1B activation, washed platelets were prepared by centrifuging the platelet-rich plasma at $150 \times g$ for 10 min. The platelet pellet was washed once with 0.038% trisodium citrate, 0.6% glucose, and 0.72 NaCl, pH 7.0, containing 25 ng/ml prostaglandin E_1 and finally resuspended in HEPES buffer at a final concentration of 3×10^8 platelets/ml. Stimulation of platelet samples (0.4 ml), lysis, and pull-down assay for Rap1B activation were performed as described above.

RESULTS

G_i-dependent Activation of Rap1B Does Not Require Inhibition of Adenylyl Cyclase—We have previously demonstrated that stimulation of a G_i-dependent pathway by epinephrine or by binding of ADP to the $P2Y_{12}$ receptor is sufficient to trigger activation of the small GTPase Rap1B and that Rap1B activation promoted by cross-linking of FcγRIIA is completely dependent on the stimulation of the G_i-coupled $P2Y_{12}$ receptor by secreted ADP (7). To investigate the signaling pathway linking G_i to Rap1B, we first analyzed the possible role of $G\alpha_i$-mediated inhibition of adenylyl cyclase. Gel-filtered platelets were treated with two different membrane-permeable inhibitors of adenylyl cyclase (SQ22536 and dideoxyadenosine), and activation of Rap1B was evaluated upon precipitation of the GTP-bound form of the protein with GST-RBD. Although SQ22536 and dideoxyadenosine were used at concentrations reported to maximally inhibit forskolin-stimulated adenylyl cyclase (13), neither compound by itself caused detectable activation of Rap1B (Fig. 1*A*). These results are in agreement with those recently reported by Woulfe *et al.* (8). However, we considered the possibility that, although not sufficient by itself to induce Rap1B activation, inhibition of adenylyl cyclase could contribute to this process in association with co-stimulation of other signaling pathways. In platelets stimulated by cross-linking of FcγRIIA, activation of Rap1B is suppressed by the ADP scavenger creatine phosphate/creatine phosphokinase, but is restored by the simultaneous addition of epinephrine (7). By contrast, in the presence of creatine phosphate/creatine phosphokinase, neither SQ22536 nor dideoxyadenosine was able to restore Rap1B activation upon cross-linking of FcγRIIA (Fig. 1*A*). This indicates that the contribution of the G_i pathway to Rap1B activation does not require the inhibition of adenylyl cyclase by the G-protein α-subunit.

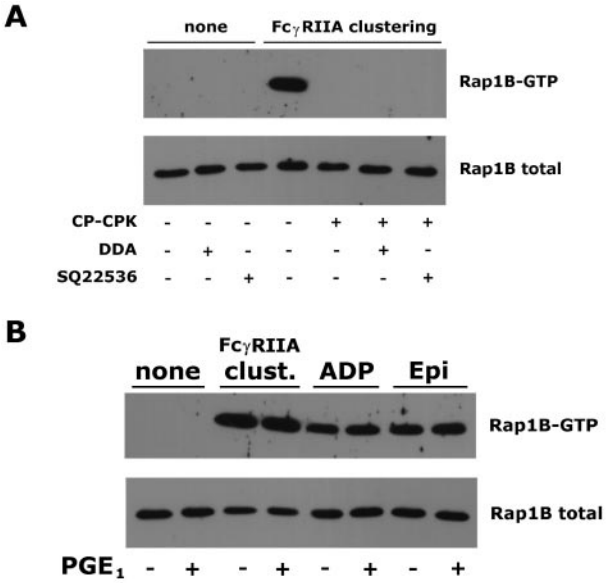

FIG. 1. **G_i-dependent activation of Rap1B is not regulated by cAMP.** *A*, gel-filtered platelets were preincubated at 37 °C with 100 μM dideoxyadenosine (*DDA*), 300 μM SQ22536, or 5 mM creatine phosphate plus 40 units/ml creatine phosphokinase (*CP-CPK*) as indicated and then treated with buffer (*none*) or stimulated by clustering of FcγRIIA with 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse $F(ab')_2$ fragments for 1 min. After cell lysis, active GTP-bound Rap1B was precipitated using GST-RBD and identified by immunoblotting with a specific polyclonal antiserum. Aliquots (20 μl) of each cell lysate were withdrawn before addition of GST-RBD and immunoblotted with anti-Rap1B antiserum to evaluate the level of the protein in the different samples. *B*, gel-filtered platelets were preincubated with or without 10 μM prostaglandin E_1 (*PGE_1*) for 30 min and then treated with buffer (*none*) or stimulated by clustering (*clust.*) of FcγRIIA with 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse $F(ab')_2$ fragments, with 10 μM ADP, or with 1 μM epinephrine (*Epi*). After 1 min, platelets were lysed, and GTP-bound Rap1B was isolated with GST-RBD and visualized by immunoblotting. Total Rap1B in cell lysates was monitored by immunoblotting of 20-μl aliquots.

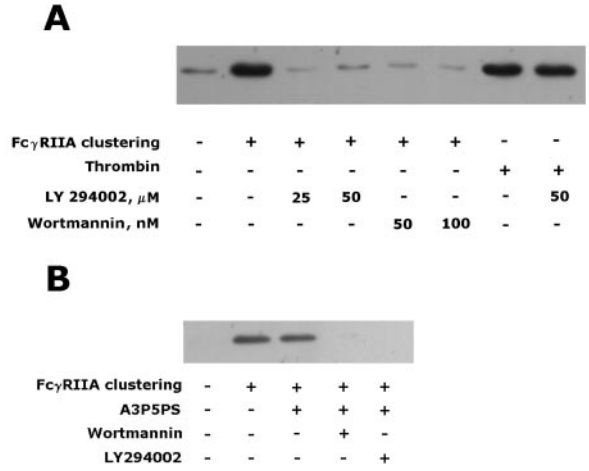

FIG. 2. **PI3K inhibitors prevent Rap1B activation induced by clustering of FcγRIIA.** *A*, platelet samples were preincubated at 37 °C with the indicated concentrations of LY294002 or wortmannin or with an equivalent volume of Me_2SO for 15 min and then stimulated by clustering of FcγRIIA with 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse $F(ab')_2$ fragments or with 1 unit/ml thrombin. After 1 min, GTP-bound Rap1B was precipitated from lysed platelets using GST-RBD and immunoblotted with a specific polyclonal antiserum. *B*, platelets preincubated with Me_2SO, 25 μM LY294002, or 50 nM wortmannin were stimulated by clustering of FcγRIIA for 1 min in the absence or presence of 500 μM PAPS (*A3P5PS*) as indicated. The immunoblot shows active Rap1B isolated by precipitation with GST-RBD.

We also considered that the very low intracellular levels of cAMP in resting platelets may represent a permissive condition to allow agonist-induced Rap1B activation. To verify this possibility, human platelets were incubated with prostaglandin E_1 to stimulate adenylyl cyclase and to increase cAMP levels and then treated with agonists that activate Rap1B through stimulation of G_i. Fig. 1*B* shows that, even when intracellular cAMP levels were increased by prostaglandin E_1, activation of Rap1B induced by clustering of FcγRIIA, epinephrine, or ADP occurred normally. Taken together, these results indicate that the G_i-mediated activation of Rap1B is completely independent of the modulation of intracellular cAMP levels.

G_i-mediated Activation of Rap1B Is Regulated by PI3K—It is known that activation of G_i is necessary to support full platelet secretion and aggregation as well as Rap1B activation induced by U46619 or by cross-linking of FcγRIIA (7, 10, 12). Moreover, it has been shown that PI3K plays a crucial role in the G_i-mediated potentiation of platelet activation and is required for irreversible aggregation (11, 12, 14, 15). Therefore, we investigated the role of PI3K in FcγRIIA-mediated activation of Rap1B using two structurally unrelated inhibitors of the enzyme, wortmannin and LY294002. Fig. 2*A* shows how activation of Rap1B induced by clustering of FcγRIIA was totally suppressed by both compounds. By contrast, Rap1B activation induced by thrombin was not significantly affected by LY294002 (Fig. 2*A*) or by wortmannin (data not shown). We have previously shown that FcγRIIA-induced activation of Rap1B requires the binding of secreted ADP to the $P2Y_{12}$ receptor (7). To verify whether, upon FcγRIIA recruitment, PI3K signals to Rap1B downstream of the G_i-coupled receptor for ADP, human platelets were stimulated by cross-linking of FcγRIIA in the presence of PAPS, a selective antagonist of the $P2Y_1$ receptor for ADP. Under these conditions, secreted ADP can bind exclusively to the G_i-coupled $P2Y_{12}$ receptor. As shown in Fig. 2*B*, binding of secreted ADP to the $P2Y_{12}$ receptor was sufficient to allow Rap1B activation in response to FcγRIIA cross-linking. Moreover, under these conditions, inhibition of PI3K by wortmannin or LY294002 still completely suppressed activation of Rap1B (Fig. 2*B*).

To further demonstrate that PI3K lies downstream of the G_i-coupled $P2Y_{12}$ receptor, we analyzed Rap1B activation in response to exogenous ADP. Fig. 3*A* shows that, when exogenous ADP was allowed to bind exclusively to the $P2Y_{12}$ receptor, *i.e.* in the presence of PAPS, activation of Rap1B was prevented by the PI3K inhibitors wortmannin and LY294002. Finally, we analyzed the role of PI3K in Rap1B activation induced by epinephrine, which binds exclusively to the G_i-coupled α_{2A}-adrenergic receptor on the platelet surface. Fig. 3*B* shows that both wortmannin and LY294002 almost completely suppressed epinephrine-induced activation of Rap1B. These results confirm and extend previously reported data (8) and indicate that PI3K is a key element in the G_i-dependent pathway for Rap1B activation.

PI3Kγ Is Not Involved in Either Rap1B Activation or Potentiation of Platelet Aggregation Mediated by G_i—It is known that human platelets express different isoforms of PI3K (23). At least two members of the class I PI3K family, *viz.* PI3Kβ and PI3Kγ, are activated by G-protein βγ-dimers (24–26) and therefore may represent the isoforms linking G_i to Rap1B. To investigate the possible role of PI3Kγ, we compared agonist-induced activation of Rap1B in platelets from PI3Kγ-deficient and wild-type mice. Because mouse platelets do not express FcγRIIA, these studies have been performed using ADP and epinephrine as Rap1B activators. As shown in Fig. 4, activation of Rap1B induced by ADP or epinephrine in PI3Kγ-deficient platelets was almost identical to that observed in platelets from wild-type mice. This finding argues against a role for PI3Kγ in coupling activation of G_i to Rap1B. However, because several

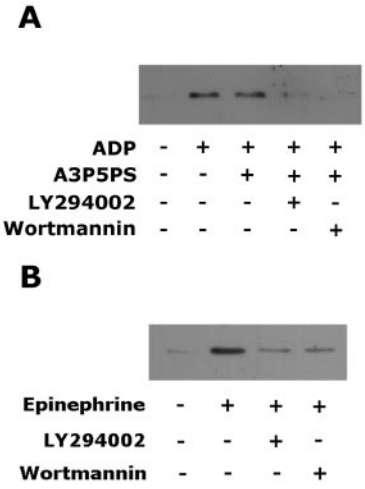

FIG. 3. **Rap1B activation by ADP and epinephrine is prevented by PI3K inhibitors.** PI3K was inhibited by incubation of platelets with 25 μM LY294002 or 50 nM wortmannin for 15 min. Control samples were preincubated with an equal volume of Me_2SO. Platelets were then stimulated with 10 μM ADP in the absence or presence of 500 μM PAPS (*A3P5PS*) (*A*) or with 1 μM epinephrine (*B*) for 1 min. Accumulation of GTP-bound Rap1B was evaluated as described under "Experimental Procedures."

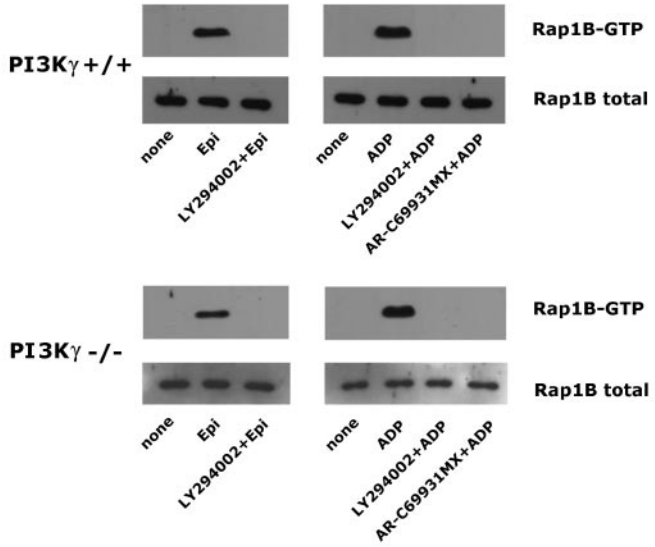

FIG. 4. **Activation of Rap1B in PI3Kγ-deficient mice.** Platelets were isolated from wild-type ($PI3K^{+/+}$) or PI3Kγ-deficient ($PI3K^{-/-}$) mice and stimulated with buffer (*none*), 10 μM epinephrine (*Epi*), or 10 μM ADP for 1 min. Preincubation with 25 μM LY294002 was performed for 15 min before stimulation, whereas preincubation with 100 nM AR-C69931MX was for 2 min before addition of ADP. Active Rap1B was precipitated from platelet lysates with GST-RBD and revealed by immunoblotting with a specific polyclonal antiserum. The amount of total Rap1B in cell lysates was also monitored by immunoblotting.

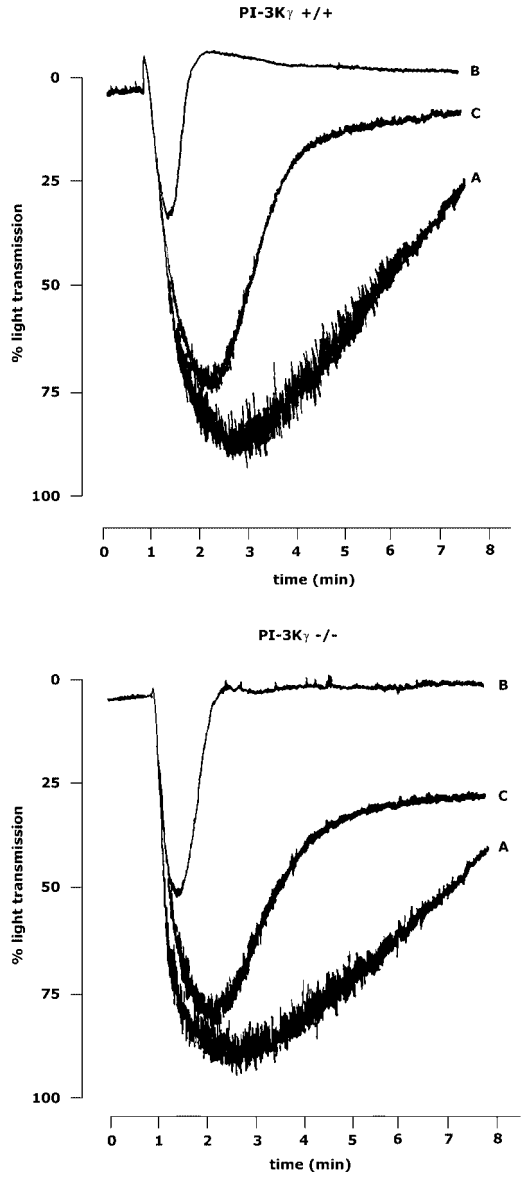

FIG. 5. **Epinephrine restores ADP-induced platelet aggregation blocked by a $P2Y_{12}$ receptor antagonist in wild-type and $PI3K\gamma^{-/-}$ mice.** Platelets from $PI3K\gamma^{-/-}$ mice (*lower panel*) or wild-type littermates (*upper panel*) were placed in an aggregometer and preincubated with buffer (*trace A*) or with 100 nM AR-C69931MX for 2 min (*traces B* and *C*). Samples were then stimulated with 10 μM ADP (*traces A* and *B*) or with 10 μM ADP and 10 μM epinephrine (*trace C*). Representative traces of platelet aggregation are reported.

intracellular messengers, including calcium, diacylglycerol, and tyrosine kinases, can mediate Rap1 activation (6), we considered that, in the absence of PI3Kγ, other signaling pathways may become predominant and result in an almost equally efficient activation of Rap1B. By using a selective antagonist of the $P2Y_{12}$ receptor, we found that, in both wild-type and $PI3K\gamma^{-/-}$ mice, ADP-induced activation of Rap1B was equally dependent on stimulation of the G_i-coupled receptor (Fig. 4). Moreover, in both control and PI3Kγ-deficient platelets, ADP- or epinephrine-induced activation of Rap1B was still prevented by the PI3K inhibitors LY294002 (Fig. 4) and wortmannin (data not shown). These results clearly indicate that a PI3K isoform different from PI3Kγ is involved in the G_i-mediated activation of Rap1B.

Both PI3K and Rap1B have been hypothesized to be involved in the G_i-dependent potentiation of platelet aggregation. Because activation of Rap1B downstream of G_i occurs normally in $PI3K\gamma^{-/-}$ mice, these platelets represent a good model to test the specific contribution of PI3Kγ *versus* Rap1B to epinephrine-induced potentiation of platelet aggregation. In mouse platelets, ADP caused a reversible platelet aggregation that required the concomitant stimulation of the G_q-coupled $P2Y_1$ and G_i-coupled $P2Y_{12}$ receptors. A small (but significant) reduction of ADP-induced platelet aggregation in $PI3K\gamma^{-/-}$ mice was reported in an earlier study (32). This effect was more evident when low doses of ADP were used to stimulate washed platelets. In the present work, such inhibition was negligible because we used high doses of ADP to stimulate platelets in

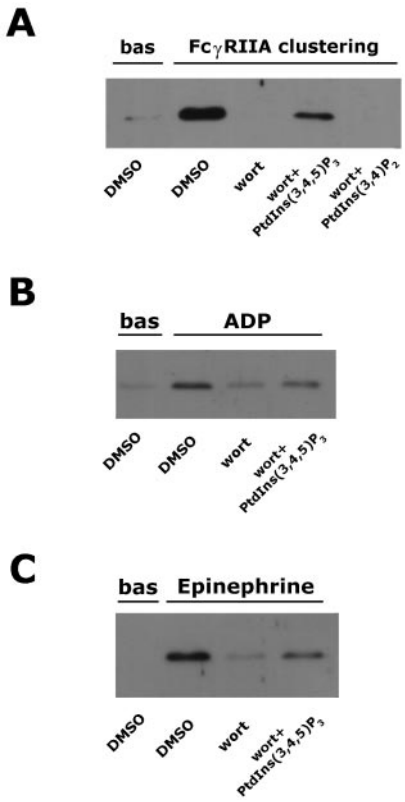

FIG. 6. **Wortmannin-induced inhibition of Rap1B activation is reversed by PtdIns(3,4,5)P_3.** Platelets were preincubated with 50 nM wortmannin (*wort*) or an appropriated volume of Me_2SO (*DMSO*) for 15 min and then treated with buffer (*bas*) or stimulated with 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse F(ab′)$_2$ fragments to induce clustering of FcγRIIA (*A*), with 10 μM ADP (*B*), or with 1 μM epinephrine (*C*) for 1 min. Where indicated, PtdIns(3,4,5)P_3 (30 μM) or PtdIns(3,4)P_2 (30 μM) was added together with the agonists. Active Rap1B was precipitated and revealed by immunoblotting with a specific polyclonal antiserum.

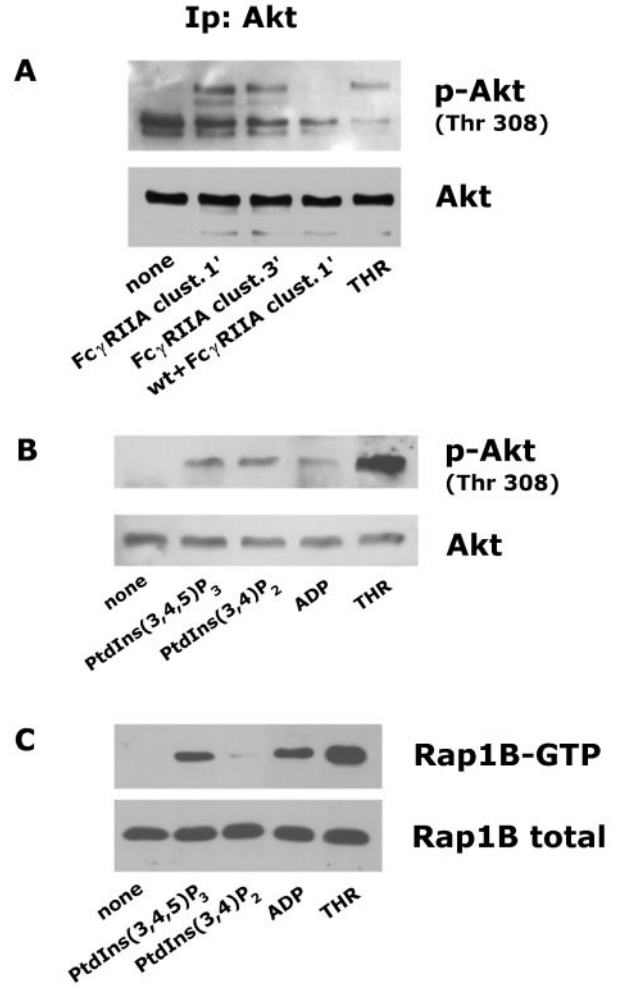

FIG. 7. **Phosphorylation of Akt by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.** *A*, gel-filtered platelets were stimulated by clustering (*clust.*) of FcγRIIA with 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse F(ab′)$_2$ fragments for 1 (*1′*) or 3 (*3′*) min or with 1 unit/ml thrombin (*THR*) for 1 min. Preincubation with 50 nM wortmannin (*wt*) was for 15 min before stimulation. Platelets were lysed in RIPA buffer, and Akt was immunoprecipitated (*Ip*) from the precleared lysates with mAb 5G3 and then analyzed by immunoblotting with anti-phospho-Akt Thr^{308} antibody (*p-Akt (Thr 308)*). Blots were stripped and reprobed with anti-Akt antibody (*Akt*). *B*, gel-filtered platelets were treated with 30 μM PtdIns(3,4,5)P_3, 30 μM PtdIns(3,4,5)P_3, 10 μM ADP, or 1 unit/ml thrombin at 37 °C for 1 min. Samples were lysed in 2% SDS, and the levels of Akt phosphorylation (*p-Akt*) at Thr^{308} were measured by immunoblotting with phospho-specific antibodies. Blots were then stripped and reprobed with anti-Akt antibody. *C*, similar samples were lysed in RIPA buffer and processed for measurement of Rap1B activation.

platelet-rich plasma. However, Fig. 5 shows that selective blockade of the $P2Y_{12}$ receptor strongly inhibited platelet aggregation induced by high doses of ADP in both wild-type and PI3K$\gamma^{-/-}$ mice. However, in the presence of the $P2Y_{12}$ receptor antagonist, addition of epinephrine, which activates a G_i-dependent signaling pathway leading to stimulation of Rap1B activation, strongly, although not completely, restored ADP-induced platelet aggregation in both wild-type and PI3K$\gamma^{-/-}$ platelets. This indicates that PI3Kγ is involved neither in Rap1B activation nor in the G_i-dependent potentiation of platelet aggregation.

PI3K-dependent Activation of Rap1B Is Mediated by the Lipid Product PtdIns(3,4,5)P_3, but Not by PtdIns(3,4)P_2—Activation of PI3K leads to the accumulation of the lipid products PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, which, in turn, promote stimulation of Akt. To investigate the role of 3-phosphorylated phosphoinositides in Rap1B activation, we analyzed their ability to relieve the inhibition of Rap1B activation in wortmannin-treated platelets. Preliminary experiments with permeabilized platelets revealed that saponin treatment caused an almost total loss of platelet responsiveness to the analyzed agonists (data not shown). However, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 have been shown to trigger biological responses even when added to whole cells (19–21). Moreover, when dissolved in Me_2SO and then added to intact platelets, both lipids have been found to be rapidly incorporated into the cell membrane (22). Fig. 6*A* shows that, in platelets stimulated by cross-linking of FcγRIIA, inhibition of Rap1B activation by wortmannin was partially reversed by addition of PtdIns(3,4,5)P_3. By contrast, the related lipid PtdIns(3,4)P_2 was unable to relieve the wortmannin-induced inhibition of Rap1B activation. Similarly, even when activation of Rap1B was triggered by direct stimulation of G_i-coupled receptors by ADP or epinephrine, PtdIns(3,4,5)P_3 was able to partially counteract the inhibitory effect of wortmannin (Fig. 6, *B* and *C*). These results indicate that the lipid product PtdIns(3,4,5)P_3, but not PtdIns(3,4)P_2, is directly involved in the PI3K-dependent activation of Rap1B downstream of stimulation of G_i.

We next investigated the ability of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 to promote Akt phosphorylation using phospho-specific antibodies against phospho-Akt Thr^{308}. As shown in Fig. 7*A*, platelet activation by cross-linking of FcγRIIA induced the phosphorylation of Akt at Thr^{308}, which was prevented by preincubation with wortmannin. When analyzed using the

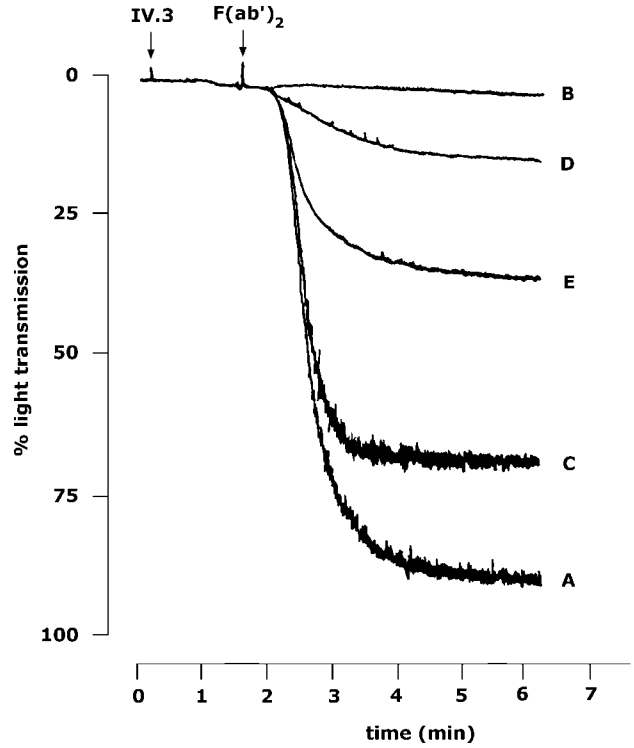

FIG. 8. **Inhibition of FcγRIIA-mediated platelet aggregation by apyrase is reversed by PtdIns(3,4,5)P_3.** Gel-filtered platelets were placed in an aggregometer, and clustering of FcγRIIA was induced by addition of 2 μg/ml mAb IV.3 and 30 μg/ml sheep anti-mouse F(ab′)$_2$ fragments as indicated by the *arrows*. Aggregation was monitored continuously. The representative traces shown refer to samples treated as follows. *trace A*, no treatment; *trace B*, 1 unit/ml apyrase; *trace C*, 1 unit/ml apyrase and 10 μM epinephrine; *trace D*, 1 unit/ml apyrase and 30 μM PtdIns(3,4,5)P_3; *trace E*, 1 unit/ml apyrase and 60 μM PtdIns(3,4,5)P_3. Apyrase was added together with mAb IV.3, whereas epinephrine or PtdIns(3,4,5)P_3 was added together with sheep anti-mouse F(ab′)$_2$ fragments.

same experimental approach, phosphorylation of Akt induced by thrombin was found to be comparable to that induced by stimulation of FcγRIIA (Fig. 7*A*). Fig. 7*B* shows that addition of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 to intact platelets caused phosphorylation of Akt to a similar extent, as evaluated by immunoblotting of whole platelet lysates. The level of Akt phosphorylation induced by the 3-phosphorylated phosphoinositides was lower than that observed in platelets stimulated with thrombin, but comparable to that induced by ADP. Parallel analysis of Rap1B activation showed that addition of PtdIns(3,4,5)P_3 to platelets was sufficient to stimulate GTP binding to Rap1B (Fig. 7*C*). By contrast, PtdIns(3,4)P_2, although able to promote Akt phosphorylation, did not stimulate activation of Rap1B. These results indicate that exogenous PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 can mimic the effects of endogenous lipids and that phosphorylation of Akt does not seem to be required for Rap1B activation.

In the presence of ADP scavengers, platelet aggregation induced by cross-linking of FcγRIIA is inhibited, but can be restored by the simultaneous addition of epinephrine (12). Under the same conditions, also activation of both PI3K and Rap1B is prevented and can be restored by epinephrine (7, 12). Therefore, we wondered whether direct activation of Rap1B by PtdIns(3,4,5)P_3 could by-pass the need for PI3K activation to induce platelet aggregation. Fig. 8 shows that FcγRIIA-mediated platelet aggregation was totally prevented when platelets where stimulated in the presence of apyrase, but could be almost completely restored by epinephrine. Addition of PtdIns(3,4,5)P_3 at a concentration shown to restore Rap1B activation also resulted in a limited (but significant) recovery of platelet aggregation. At higher concentration of PtdIns(3,4,5)P_3, the recovery of platelet aggregation was even more evident, although clearly less marked than that induced by epinephrine. Taken together, these results indicate that, when ADP secreted upon cross-linking of FcγRIIA is neutralized, exogenous PtdIns(3,4,5)P_3 causes both activation of Rap1B and restoration of platelet aggregation.

DISCUSSION

During the last few years, a number of studies using scavenger systems and receptor antagonists demonstrated the essential role played by secreted ADP in potentiating platelet activation in response to many different extracellular agonists (9–12). Although the biological effects of ADP on human platelets require concomitant activation of two different G-protein-coupled receptors, P2Y$_1$ (coupled to G_q) and P2Y$_{12}$ (coupled to G_i), potentiation of platelet activation induced by other agonists is mediated mainly by activation of a G_i-dependent pathway through the P2Y$_{12}$ receptor (10, 12). In an attempt to identify the intracellular messengers promoting G_i-dependent potentiation of platelet activation, we (7) and others (8) have recently found that the small GTPase Rap1B is indeed activated upon the sole binding of ADP to the P2Y$_{12}$ receptor or upon activation of the G_i-coupled α_{2A}-adrenergic receptor by epinephrine. Moreover, in platelets from mice that lack $G\alpha_{i2}$ or $G\alpha_z$, activation of Rap1B by ADP or epinephrine is abolished (8). Rap1 is emerging as a modulator of cell adhesion and integrin function (6), and a very recent study by Bertoni *et al.* (27) demonstrates that active Rap1B regulates the affinity state of integrin $\alpha_{IIb}\beta_3$ in mouse megakaryocytes. Therefore, Rap1B may represent a link between activation of a G_i pathway and stabilization of integrin-mediated platelet aggregation.

In this work, we investigated the biochemical mechanism of Rap1B activation downstream of stimulation of a G_i signaling pathway. By using inhibitors and activators of adenylyl cyclase, we demonstrated that the reduction of the intracellular levels of cAMP promoted by the G_i α-subunits is not required to induce activation of Rap1B. These results confirm and extend those recently reported by Woulfe *et al.* (8) and strengthen the idea that $G\alpha_i$-mediated inhibition of adenylyl cyclase does not directly contribute to the G_i-promoted potentiation of platelet activation (13, 14).

In addition to the activated α-subunits, stimulation of a G_i-coupled receptor generates free βγ-dimers that are able to stimulate different intracellular effectors, including selective isoforms of the lipid-metabolizing enzyme PI3K, such as PI3Kβ and PI3Kγ. In this work, we have actually demonstrated that activation of PI3K is an essential step in the G_i-mediated signaling pathway leading to Rap1B activation. In fact, the PI3K inhibitors wortmannin and LY294002 have been found to totally suppress Rap1B activation upon clustering of FcγRIIA by acting downstream of P2Y$_{12}$ receptor stimulation by secreted ADP. Moreover, as recently described (8), we also found that PI3K inhibitors prevented activation of Rap1B induced by direct binding of ADP to the P2Y$_{12}$ receptor or of epinephrine to the α_{2A}-adrenergic receptor. Therefore, PI3K appears to link G_i-coupled receptors to the small GTPase Rap1B.

How activation of G_i results in the stimulation of PI3K and how PI3K promotes activation of Rap1B are still unclear. Free βγ-dimers generated upon stimulation of a G_i-coupled receptor may activate some class I PI3K isoforms, *viz.* PI3Kβ and PI3Kγ (24–26). PI3Kγ is highly expressed in platelets, and it has been reported to play an essential role in ADP-induced platelet activation downstream of the G_i-coupled P2Y$_{12}$ receptor (32).

Therefore, we hypothesized that this isozyme could link G_i to activation of Rap1B. However, studies with platelets from PI3Kγ knockout mice clearly showed that the lack of expression of this isoform does not compromise the ability of epinephrine or ADP to induce activation of Rap1B. A variable decrease in agonist-induced Rap1B activation in PI3K$\gamma^{-/-}$ mice has been recently reported (8). Although some small differences were occasionally observed in our experiments (see, for instance, Rap1B activation induced by epinephrine in the representative experiment reported in Fig. 4), the analysis of several determinations led us to conclude that these occasional differences were not significant and that the contribution of PI3Kγ to G_i-mediated Rap1B activation, if any, is negligible.

Our results also demonstrate that the G_i-mediated pathway for platelet aggregation, which can be monitored by the ability of epinephrine to replace ADP, is normally functional in PI3K$\gamma^{-/-}$ mice. This is consistent with a role for activated Rap1B, but not for PI3Kγ, in the signaling pathway from G_i to platelet aggregation. We also have shown that, in PI3K$\gamma^{-/-}$ mice, G_i-mediated activation of Rap1B is still prevented by PI3K inhibitors. This clearly indicates that a different isoform of the enzyme is mainly involved. Interestingly, platelets express high levels of PI3Kβ (32), which is also activated by $\beta\gamma$-dimers (25, 26). Moreover, a recent report demonstrates that PI3Kβ, but not PI3Kγ, represents a link between $\beta\gamma$-dimers released upon stimulation of the G_i-coupled receptor for lysophosphatidic acid and activation of the small GTPase Ras (33). Therefore, it is most likely that PI3Kβ represents the main PI3K isoform involved in the regulation of Rap1B.

In this work, we have further strengthened the functional correlation between PI3K and Rap1B activation by demonstrating that exogenous PtdIns(3,4,5)P_3, but not PtdIns(3,4)P_2, can restore wortmannin-inhibited activation of Rap1B in platelets stimulated with ADP or epinephrine or by cross-linking of FcγRIIA. This indicates that the action of PI3K on Rap1B activation is actually mediated, at least in part, by the main lipid product of this enzyme. It is interesting to note that, in stimulated platelets, accumulation of PtdIns(3,4,5)P_3 is rapid and transient, whereas synthesis of PtdIns(3,4)P_2 occurs mainly as a consequence of integrin $\alpha_{IIb}\beta_3$-mediated platelet aggregation (28, 29). Therefore, the ability of PtdIns(3,4,5)P_3, but not PtdIns(3,4)P_2, to support Rap1B activation is consistent with a role for this GTPase in an early phase of platelet stimulation that precedes integrin $\alpha_{IIb}\beta_3$ activation and cell aggregation. Evaluation of Akt activation by exogenous PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 revealed that both lipids were able to stimulate phosphorylation of Akt at Thr^{308}. A previous report suggested that, in contrast to PtdIns(3,4)P_2, addition of PtdIns(3,4,5)P_3 to intact platelets does not activate Akt (20). Although we cannot provide a definitive explanation for this discrepancy, our results are clearly in line with the current concept of PtdIns(3,4,5)P_3 being a major regulator of Akt (24). It is interesting to note that, although both 3-phosphorylated phosphoinositides were able to activate Akt, only PtdIns(3,4,5)P_3 caused activation of Rap1B. The finding that PtdIns(3,4)P_2 induces Akt phosphorylation, but not Rap1B activation, suggests a dissociation between the two events. This is also supported by the finding that ADP induced normal, PI3K-dependent activation of Rap1B in PI3K$\gamma^{-/-}$ mouse platelets (Fig. 4) under conditions in which phosphorylation of Akt has been reported to be totally suppressed (32).

The data in Fig. 6 clearly show that the restoration of Rap1B activation by PtdIns(3,4,5)P_3 never returns the amount of active Rap1B to that observed in control platelets stimulated in the absence of wortmannin. This indicates either that generation of PtdIns(3,4,5)P_3 is not the only mechanism by which PI3K participates in Rap1B activation or that endogenously generated lipids act more efficiently. In this regard, it should be noted that stimulators of G_i, such as ADP and epinephrine, are really weak stimulators of PI3K (12, 30). For instance, epinephrine induces the accumulation of <20% of the amount of PtdIns(3,4,5)P_3 measured in thrombin-stimulated platelets (12). Despite this, the finding that PI3K inhibitors totally suppressed Rap1B activation clearly indicates that such a small amount of 3-phosphoinositides plays a crucial role. Thus, the endogenously generated PtdIns(3,4,5)P_3 seems to have a very high efficiency in stimulating Rap1B activation. Although the reason for this effect is not known, it may be hypothesized that a specific and optimal localization of endogenously generated PtdIns(3,4,5)P_3 may improve its effect on Rap1B. In this regard, it is interesting to note that a preferential distribution of the PI3K-generated lipids in platelet membrane rafts has been recently reported (31). In this work, we have also provided evidence that PtdIns(3,4,5)P_3-mediated activation of Rap1B is an important step in the G_i-dependent pathway for platelet aggregation. In fact, exogenous PtdIns(3,4,5)P_3, in addition to partially restore Rap1B activation, can overcome the inhibition of FcγRIIA-mediated platelet aggregation promoted by ADP scavengers. In this context, the effect of PtdIns(3,4,5)P_3 resembles that of epinephrine, which activates a truly G_i-dependent pathway. Our data show that PtdIns(3,4,5)P_3 is much less efficient than epinephrine in promoting restoration of FcγRIIA-mediated platelet aggregation in apyrase-treated platelets. However, this fits well with the reduced ability of this lipid to restore Rap1B activation.

In conclusion, we have shown that stimulation of a G_i signaling cascade leads to activation of the small GTPase Rap1B through the action of the PI3K lipid product PtdIns(3,4,5)P_3, rather than through inhibition of adenylyl cyclase. These results also suggest that Rap1B represents a downstream effector for PI3K in the G_i-dependent signaling pathway for potentiation of platelet aggregation.

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A Selective Role for Phosphatidylinositol 3,4,5-Trisphosphate in the G_i-dependent Activation of Platelet Rap1B

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