Activation of Type I Phosphatidylinositol 4-Phosphate 5-Kinase Isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42*

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Type I phosphatidylinositol 4-phosphate 5-kinase (PI5K) catalyzes the formation of the phospholipid, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which is implicated in many cellular processes. The Rho GTPases, RhoA and Rac1, have been shown previously to activate PI5K and to bind PI5K. Three type I PI5K isoforms (Iα, Iβ, and Iγ) have been identified; however, it is unclear whether these isoforms are differentially or even sequentially regulated by Rho GTPases. Here we show that RhoA and Rac1, as well as Cdc42, but not the Ras-like GTPases, RaIA and Rap1A, markedly stimulate PI(4,5)P2 synthesis by all three PI5K isoforms expressed in human embryonic kidney 293 cells, both in vitro and in vivo. RhoA-stimulated PI(4,5)P2 synthesis by the PI5K isoforms was mediated by the RhoA effector, Rho-kinase. Stimulation of PI5K isoforms by Rac1 and Cdc42 was apparently independent of and additive with RhoA- and Rho-kinase, as shown by studies with C3 transferase and Rho-kinase mutants. RhoA, and to a lesser extent Rac1, but not Cdc42, interacted in a nucleotide-independent form with all three PI5K isoforms. Binding of PI5K isoforms to GDP-bound, but not GDP-bound, RhoA could be displaced by Rho-kinase, suggesting a direct and constitutive PI5K-Rho GTPase binding, which, however, does not trigger PI5K activation. In summary, our findings indicate that synthesis of PI(4,5)P2 by the three PI5K isoforms is controlled by RhoA, acting via Rho-kinase, as well as Rac1 and Cdc42, implicating that regulation of PI(4,5)P2 synthesis has a central position in signaling by these three Rho GTPases.

Synthesis and turnover of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), is appreciated to be involved in various, apparently very different, cellular processes. As substrate for phospholipase C and phosphoinositide 3-kinase, PI(4,5)P2 embodies a crucial component in cellular signaling (1, 2). In addition, PI(4,5)P2 is directly involved in diverse fundamental cellular processes, including control of actin polymerization (3), focal adhesion assembly (4, 5), cytoskeleton-plasma membrane linkage (6), modulation of ATP-sensitive potassium channels (7), and membrane trafficking (8), comprising regulated exocytosis (9, 10), as well as clathrin-mediated endocytosis (11, 12). This astonishing variety of functions demands the cellular level of PI(4,5)P2, and, hence, the lipid kinases involved in its synthesis, to be detailed and distinctly regulated.

A critical role in PI(4,5)P2 synthesis takes type I phosphatidylinositol 4-phosphate 5-kinase (PI5K), which phosphorylates phosphatidylinositol 4-phosphate (PI(4)P) at the D-5 position of the inositol ring, committing the final step in PI(4,5)P2 synthesis in vivo (13, 14). In line with this function, overexpression of type I PI5K isoforms was shown to modulate actin cytoskeletal dynamics and to induce stress fibers (15, 16), membrane ruffles (17), microvilli (18), and motile actin comets (19). Type I PI5Ks are now clearly distinguished from type II PI kinases, which actually phosphorylate phosphoinositides at the D-4 position (20, 21). Theoretically, type II isoforms might contribute to PI(4,5)P2 synthesis by phosphorylating PI(4)P, but the importance of this alternative pathway in vivo is not yet clarified. Three isoforms of type I PI5K have been identified, designated Iα, Iβ, and Iγ, with molecular masses of 68 kDa (Iα and Iγ) and 90 kDa (Iγ), respectively (22–24). The differential regulation and/or subcellular localization of the PI5K isoforms obviously could represent a mechanism for cells to coordinate their PI(4,5)P2 production. Indeed, PI5K-Iα was found to specifically localize to membrane ruffles (25), PI5K-Iγ targeted to focal adhesions (26) and nerve terminals (27), whereas the PI5K pool involved in endocytosis may primarily be produced by PI5K-Iβ (28).

Small GTPases of the Rho family are key regulators of cytoskeletal organization (29) and have been implicated in the regulation of PI(4,5)P2 synthesis (30). Thus, inactivation of Rho GTPases by Clostridium difficile toxin B reduced cellular PI(4,5)P2 levels, resulting in inhibition of receptor-mediated inositol phosphate formation by phospholipase C and PI(4,5)P2-sensitive phospholipase D (PLD) (31, 32). RhoA (33) and Rac1 (34) have been shown to stimulate PI(4,5)P2 synthesis, and PI5K isoforms appear to function downstream of RhoA and Rac1 in actin organization (15, 35). We have shown recently that the RhoA effector, Rho-kinase, which mediates signals from RhoA to the actin cytoskeleton (36), is involved in RhoA-dependent regulation of PI5K activity (37). In agreement, in intact CV1 cells (16) and platelets (38), PI(4,5)P2 formation is reduced by the Rho-kinase inhibitor, Y27632, and in N1E-115 neuroblastoma cells, PI5K functions as downstream effector of RhoA- and Rho-kinase in neurite remodeling (39, 40). Rho GTPases were also found to physically associate with type I PI5K. Whereas Ren et al. (41) showed that RhoA bound a 68-kDa PI5K isoform in the plasma membrane, we find essentially no PI5K-Iα bound to RhoA in our subcellular fractionation studies, and we show that the RhoA effectors, Rho-kinase and ROCK, associate with a specific PI5K-Iβ isoform.
Swiss 3T3 cell lysates, Tobias et al. (42) found that PIP5K activity from rat liver cytosol associated with Rac1 but not with RhoA or Cdc42. Both studies do agree in the observation that the binding of PIP5K is independent of the nucleotide-binding state (GDP or GTP) of the Rho GTases.

In the present study, we have examined the interaction of type I PIP5K isoforms with Rho GTases. We find that, upon expression in HEK-293 cells, all three type I PIP5K isoforms are positively regulated by RhoA and Rac1, but also by Cdc42, resulting in enhanced cellular PIP2 levels. The stimulation of PIP5K isoforms by RhoA is entirely mediated by Rho kinase, whereas the effects of Rac1 and Cdc42 are largely independent of the RhoA/Rho-kinase relay. In addition, we show that all three type I PIP5K isoforms physically associate with RhoA and Rac1, but not with Cdc42.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNAs encoding murine PIP5K-I, PIP5K-Iβ, PIP5K-1γ, and the kinase-deficient PIP5K-Iα A138, as well as the isoform-specific antisera, have been described before (22, 24). The cDNAs encoding wild-type Rho-kinase, the catalytic domain of Rho-kinase (Rho-kinase-CAT; amino acids 6–553), dominant-negative Rho-kinase (DBP/PH (TT); amino acids 941–1088 with mutations T1036 and T1037), and the Rho-binding domain of Rho-kinase (RB; amino acids 941–1075) were kindly provided by Drs. Mutsuki Amano and Kozo Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan) (36, 43, 44). The cDNAs encoding wild-type and inactive Rho GTases and C3 transferase were generously provided by Dr. Alan Hall (University College London, London, United Kingdom). RalA and Rap1A constructs were kindly provided by Drs. Jean de Gunzburg and Jacques Camonis (INSERM U528, Paris, France). Myosin light chain (MLC) was from Sigma. All other materials were from previously described sources (37, 45).

**Cell Culture and Transfection—HEK-293 cells were routinely passaged in Dulbecco’s modified Eagle’s medium/F-12 medium with 10% fetal calf serum (46). For transient expression of proteins, subconfluent monolayers on 145-mm culture dishes were transfected with cDNAs encoding PIP5K-Iα, PIP5K-Iα A138, PIP5K-Iβ (all in pCMV), PIP5K-1γ (in pCDNA3), RhoA and RhoA N19 (in pRK5), Rac1, Rac1 N17, Cdc42, and Cdc42 N17 (all in pHEXI, Rho-kinase, Rho-kinase-CAT, RB/PH (TT), and C3 transferase (all in pHEF), and Rap1A, Rap1A A26, Rap1A, and Rap1A N17 (all in pPK5) by the calcium phosphate method (47). Cells were, unless otherwise stated, transfected with 30 or 50 μg of DNA of the indicated PIP5K isoforms for functional and binding studies, respectively. Assays were performed 48 h after transfection. Cells were washed twice in phosphate-buffered saline and lysed in P-40, 25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin.

**PIP5K Assay—Cell lysates were diluted 1:10 with kinase buffer (25 mM Tris/HCl, pH 7.4, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol), and aliquots were tested for PIP5K activity as described previously (37). Reaction mixtures (50 μl) contained 50 μg of protein, 70 μM PIP2, 35 μM phosphatidylserine, 0.1% Nonidet P-40, and 20 μM [γ-32P]ATP (1 μCi/assay). Reactions were allowed for 5 min at 25 °C, stopped by adding 0.3 ml of methanol, 1 nM HCl (1:1, v/v) and extracted with 0.25 ml of chloroform. The organic phase was chromatographed on a Silica Gel 60 column, the samples were assayed for IP3 by the displacement of [3H]IP3 from bovine adrenal cortical IP3-binding protein (48). Values were converted into original PIP2 masses and expressed as pmol per mg protein.

**Preparation and Nucleotide Loading of GST Fusion Proteins—Glutathione S-transferase (GST) fusion proteins of RhoA, Rac1, Cdc42, and RB were expressed in Escherichia coli and purified with glutathione-Sepharose 4B beads as described (49). Fusion proteins were eluted with a buffer containing 50 mM Tris/HCl, pH 7.5, 2 mM EGTA, 1 mM dithiothreitol, and 10 mM glutathione. Alternatively, native proteins were obtained by cleaving the fusion constructs with thrombin (10 U) in 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl2, and 1 mM dithiothreitol. The beads were recovered by centrifugation and used as the respective control GST beads. Thrombin was inactivated by treatment with p-aminobenzenzamide beads. Protein concentrations were determined with the Bradford method, and the preparations were analyzed for their homogeneity by SDS-PAGE and Coomassie staining. Immobilized GTases were nucleotide-depleted by incubation in Buffer A (25 mM Tris/HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol) for 30 min at 25 °C. The beads were resuspended in Buffer B (Buffer A with 5 mM MgCl2 substituted for 5 mM EDTA), loaded with 100 μM GDP or 100 μM GTP-S for 30 min at 25 °C, and washed in Buffer B (50).

**Binding of PIP5K to Rho/GST GTPase Fusion Proteins—**Cell lysates were clarified by centrifugation for 20 min at 40,000 x g. Aliquots of the supernatants (typically 500 μg of protein) were incubated with GST or GST fusion proteins bound to Sepharose beads (20 μg of protein) for 1 h at 4 °C with gentle rocking. The beads were centrifuged at 5,000 x g and rapidly washed three times with Buffer B and once with kinase buffer. The beads were assayed for PIP5K activity or incubated in Laemmli buffer for 10 min at 95 °C, followed by separation of the released proteins by SDS-PAGE (10%). The proteins were transferred onto nitrocellulose filters, which were blocked with 5% non-fat milk for 1 h. The blots were probed with isoform-specific anti-peptide antisera (1,500 dilution) against PIP5K-Iα, PIP5K-Iβ, and PIP5K-1γ (22, 24) and developed by chemiluminescence.

**Immunoprecipitation—**Clariﬁed cell lysates (1.0 mg of protein) were incubated with antibodies (1 μg) to RhoA, Rac1, or Cdc42 or with non-immune IgG (Santa Cruz Biotechnology, Inc.), coupled to protein A-Agarose (20 μl) for 2 h at 4 °C, and the precipitates were washed four times with lysis buffer. The precipitated proteins were washed with kinase buffer and assayed for PIP5K activity or analyzed by immunoblotting (SDS-PAGE on 15% acrylamide gels) with the respective anti-GTase antibodies.

**Phosphorylation of MLC—**Beads were incubated in 50 μl of protein kinase buffer (20 mM Tris/HCl, pH 7.5, 5 mM MgCl2, 1 mM CaCl2) with 50 μl [γ-32P]ATP (5 μCi/assay) and 2 μl of MLC for 10 min at 30 °C. Reactions were terminated by the addition of concentrated Laemmli buffer and heating for 10 min at 95 °C. Released proteins were separated by SDS-PAGE (15%), the gels were dried, and phosphoproteins were visualized by autoradiography.

**RESULTS**

**Stimulation of Type I PIP5K Isoforms by RhoA and Rho-kinase—**Previously, we have shown that RhoA and its effector, Rho-kinase, enhance the synthesis of PIP2 by stimulating the activity of PIP5K (37). We now separately examined the individual type I PIP5K isoforms for their responsiveness to RhoA and Rho-kinase. For this, PIP5K-Iα, PIP5K-Iβ, and PIP5K-1γ were transiently expressed in HEK-293 cells, and PIP5K activity was determined in whole cell lysates, using PIP4 and [γ-32P]ATP as substrates. Transfection of all three PIP5K isoforms manifested in a dose-dependent, robust, and specific increase in [γ-32P]PIP2 synthesis (Fig. 1). Synthesis of [γ-32P]PIP from cellular or exogenous (not shown) PI was only marginally affected by expression of the PIP5K isoforms. The identity of the produced PIP2 was confirmed to be exclusively phosphatidylinositol 4,5-bisphosphate by high performance liquid chromatography after deacylation (not shown). Cells transfected with equal amounts of cDNA encoding the catalytically inactive PIP5K-Iα A138 did not exhibit increased lipid kinase activity (Fig. 1). The strong increases in PIP2 synthesis confirmed the biological activity of the constructs and enabled us to study the regulation of the individual isoforms in a cellular context. To examine whether the isoforms are regulated by Rho, cells were transfected with the PIP5K isoforms, together with RhoA or C3 transferase. Overexpression of RhoA resulted in a marked, up to 8-fold increase in the kinase activity of all three isoforms (see below), whereas inhibition of endogenous Rho by expression of C3 transferase reduced the synthesis of [γ-32P]PIP2 by 20–30%.
by Rho-kinase was seen in cells transfected with PIP5K-I/H9251. Transfection of cells with PIP5K-I left panels. The panels show the essential sections of typical autoradiograms, with the migration positions of PIP and PIP 2 indicated.

forms were stimulated by Rho-kinase, but [32P]PIP2 synthesis of PIP5K isoforms with Rho-kinase-CAT. All three isoforms were stimulated by Rho-kinase, but [32P]PIP2 synthesis was elevated the strongest in cells expressing PIP5K-I-B (−40-fold), as compared with PIP5K-Iα (−6-fold) or PIP5K-Iγ (−8-fold) (Fig. 2B, left panel). No potentiation of [32P]PIP2 synthesis by Rho-kinase was seen in cells transfected with PIP5K-Iα A138. Very similar effects were obtained when wild-type Rho-kinase was co-transfected with PIP5K isoforms instead of Rho-kinase-CAT (not shown). The increase in PIP5K activity by Rho-kinase was reflected in elevated in vivo PIP2 levels in the transfected cells. Expression of Rho-kinase-CAT caused an increase in cellular PIP2 by 40–90% in cells overexpressing PIP5K-Iα, PIP5K-Iβ, and PIP5K-Iγ, but again, had no effect in cells expressing PIP5K-Iα A138 (Fig. 2B, right panel).

Stimulation of PIP5K Isoforms by Rac1 and Cdc42—We then investigated the regulation of PIP5K isoforms by Rac1 and Cdc42. Similar to RhoA, overexpression of Rac1 and Cdc42 enhanced in vitro PIP5K activity (Fig. 3A). Stimulation of endogenous PIP5K by the Rho GTPases amounted to 50–80% (Fig. 3A, upper panel). The effect was strongly potentiated in cells co-transfected with PIP5K-Iα, PIP5K-Iβ, and PIP5K-Iγ, indicating that all three isoforms respond to the three Rho GTPases (Fig. 3A, lower panels). Stimulation of PIP5K activity was also seen in cells transfected with constitutively active mutants of the Rho GTPases (not shown), whereas expression of the inactive mutants, RhoA N19, Rac1 N17, and Cdc42 N17, had no effect (Fig. 3B, upper panel). In contrast to the Rho GTPases, overexpression of the Ras-related GTPases, Rap1A and Rap1A, did not increase the synthesis of [32P]PIP2 (Fig. 3B, lower panel). We next examined whether, similar to RhoA- and Rac1- and Cdc42, affect the cellular level of PIP2. Transfection of cells with PIP5K-Iα and increasing amounts of either Rac1 or Cdc42 elevated the cellular PIP2 mass by up to 40% (Fig. 3C). In contrast, expression of the inactive mutants, RhoA N19, Rac1 N17, and Cdc42 N17, reduced cellular PIP2 levels by 30–40%, whereas expression of inactive Rap1A A26 and Rap1A N17 had no effect (Fig. 3D). Thus, synthesis of PIP2 by the three PIP5K isoforms is specifically regulated by the Rho GTPases, RhoA, Rac1, and Cdc42, but not by the Ras-related GTPases, Rap1A and Rap1A.

Control of PIP2 Synthesis by Rac1 and Cdc42 Is Independent of RhoA- and Rho-kinase—Rho GTPases have been shown to be interconnected in cellular pathways, and RhoA has been suggested to act upstream (51), as well as downstream (52), of Rac1. Thus, the effects of Rac1 and Cdc42 on PIP5K might have been achieved by cellular activation of Rho and its effector, Rho-kinase. To investigate this possibility, cells were co-transfected with PIP5K-Iβ, the Rho GTPases, and either C3 transferase or dominant-negative Rho-kinase. The stimulatory effect of RhoA on PIP2 synthesis was completely abolished by co-expression of C3 transferase, confirming the fidelity of the RhoA effect on PIP5K activity, as well as the effectiveness of the C3 transferase (Fig. 4A). In contrast, the stimulation of PIP2 synthesis by Rac1 and Cdc42 was not or only partially reduced by C3 transferase, suggesting that activation of Rho does not represent a major mechanism by which Rac1 and Cdc42 stimulate PIP5K. Virtually the same conclusion can be drawn for the involvement of Rho-kinase. Expression of the dominant-negative Rho-kinase construct, RB/PH (TT) (44), potently and completely suppressed stimulation of PIP2 synthesis by RhoA (Fig. 4B). The kinetics by which RB/PH (TT) affected PIP5K stimulation by Rac1 and Cdc42 were clearly different. Whereas transfection of 20 μg RB/PH (TT) DNA completely blocked the effect of RhoA, this amount had no effect on the Rac1- or Cdc42-induced PIP5K stimulation. At the highest amount of RB/PH (TT) DNA, stimulation by Rac1 and Cdc42 was reduced by ~50%. Conversely, stimulation of PIP5K by wild-type Rho-kinase was reduced by co-expression of RhoA N19, whereas dominant-negative Rac1 N17 and Cdc42 N17 did not interfere (Fig. 4C). Stimulation by the constitutively active Rho-kinase-CAT was not affected by any of the inactive Rho-family members (Fig. 4C). PIP5K stimulation induced by Rho-kinase-CAT was also not further increased by co-expression of RhoA. In contrast, co-expression of Rac1 and Cdc42 further enhanced PIP5K activity in an additive (Rac1) or even synergistic (Cdc42) manner (Fig. 4D). Taken together, these data indicate that the effects of both Rac1 and Cdc42 on PIP2 synthesis are largely independent of RhoA- and Rho-kinase.

Binding of Type I PIP5K Isoforms to Rho GTPases—As RhoA activated all three PIP5K isoforms, and an association between RhoA and a 68-kDa type I PIP5K has been reported before (41), we examined which of the three PIP5K isoforms does actually bind to RhoA. For this, lysates of HEK-293 cells transfected (data not shown). To screen the responsiveness of the PIP5K isoforms to Rho-kinase, cells were co-transfected with the catalytic domain of Rho-kinase (Rho-kinase-CAT) (36), and the lysates were assayed for PIP5K activity. Stimulation of endogenous PIP5K activity by Rho-kinase (−1.4-fold) was strongly potentiated in cells overexpressing the PIP5K isoforms (Fig. 2A). Phosphorylation of PI was not affected by the co-expression of PIP5K isoforms with Rho-kinase-CAT. All three isoforms were stimulated by Rho-kinase, but [32P]PIP2 synthesis was increased 6-fold, as compared with PIP5K-Iα (−6-fold) or PIP5K-Iγ (−8-fold). No potentiation of [32P]PIP2 synthesis by Rho-kinase was seen in cells transfected with PIP5K-Iα A138. Very similar effects were obtained when wild-type Rho-kinase was co-transfected with PIP5K isoforms instead of Rho-kinase-CAT (not shown). The increase in PIP5K activity by Rho-kinase was reflected in elevated in vivo PIP2 levels in the transfected cells. Expression of Rho-kinase-CAT caused an increase in cellular PIP2 by 40–90% in cells overexpressing PIP5K-Iα, PIP5K-Iβ, and PIP5K-Iγ, but again, had no effect in cells expressing PIP5K-Iα A138 (Fig. 2B, right panel).

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with PIP5K-Iα, PIP5K-Iβ, or PIP5K-Iγ were incubated with RhoA-GST immobilized on glutathione-Sepharose beads. After washing, the beads were assayed for PIP5K activity. RhoA-GST incubated with lysates of untransfected cells clearly retained PIP5K activity as detected by the synthesis of \( [32P] \)PIP2 (Fig. 5A, upper panel). After incubation with lysates of cells transfected with PIP5K-Iα, PIP5K-Iβ, or PIP5K-Iγ, the kinase activity associated with RhoA-GST was greatly enhanced, indicating that all three isoforms bound to RhoA. The kinase activity found on GST beads was negligible throughout (Fig. 5A, upper panel). No phosphorylation of PI4P by any bead preparation was observed in the absence of cell lysates (data not shown). In accordance with the initial observation (41), the association of the PIP5K isoforms was observed in the absence of cell lysates (data not shown). In accordance with the initial observation (41), the association of the PIP5K isoforms was observed in the absence of cell lysates (data not shown). In accordance with the initial observation (41), the association of the PIP5K isoforms was observed in the absence of cell lysates (data not shown).
stimulation by Rho-kinase-CAT. Taken together, these data indicate that RhoA specifically and directly binds all three PIP5K isoforms and that this interaction is independent of the nucleotide-binding state of RhoA.

We next examined the possible interaction of the PIP5K isoforms with Rac1 and Cdc42. As shown in Fig. 7A, in addition to RhoA, all three PIP5K isoforms also bound to Rac1, but not to Cdc42. Binding of PIP5K to Rac1 was found to be less effective as binding to RhoA and amounted to ~60% of the kinase activity associated with RhoA (57 ± 12% for PIP5K-Iα, 64 ± 23% for PIP5K-Iβ, and 56 ± 11% for PIP5K-Iγ; mean ± S.E. from three independent experiments). Similar to RhoA, binding of PIP5K isoforms to Rac1 was found to be independent of the nucleotide (GDP or GTP\(\gamma\)S) occupation of Rac1 (not shown). PIP5K activity retained by Cdc42-GST did not significantly differ from the kinase activity bound to GST alone.

To investigate the physiological relevance of the binding of PIP5K to recombinant RhoA and Rac1, we immunoprecipitated endogenous RhoA, Rac1, and Cdc42 from HEK-293 cells and assayed the precipitates for associated PIP5K. PIP5K activity was found to co-precipitate with RhoA and, to a lesser extent, with Rac1 (Fig. 7B). No kinase activity was observed after immunoprecipitation with anti-Cdc42 antibody or a non-immune serum. To exclude that the absence of PIP5K in Cdc42 immunoprecipitates was because of the low(er) expression level of the GTPase, immunoprecipitations were repeated with cells overexpressing RhoA, Rac1, and Cdc42. Again, PIP5K activity was found exclusively in RhoA and Rac1 precipitates; activity associated with Cdc42 was equal to the activity precipitated from the same lysate with a non-immune serum (Fig. 7C, left panel). The amount of the precipitated GTPases was found to be similar by immunoblotting of the precipitates with the respective antibodies (Fig. 7C, right panel). Thus, co-precipitation of PIP5K with cellular RhoA and Rac1 closely mimicked
Fig. 4. RhoA- and Rho-kinase-independent stimulation of PIP5K by Rac1 and Cdc42. HEK-293 cells were transfected with 20 µg of PIP5K-β DNA, together with 50 µg of RhoA (black bars), Rac1 (dark gray bars), or Cdc42 DNA (light gray bars), without (-) or with (+) 50 µg of C3 transferase DNA (A), with 50 µg of RhoA (closed circles), Rac1 (open circles), or Cdc42 DNA (closed boxes), together with the indicated amounts of RB/PH (TT) DNA (B), with 50 µg of Rho-kinase or 30 µg of Rho-kinase-CAT DNA, together with 50 µg of the indicated inactive GTPases (C), or with 50 µg of the GTPases (light gray bars), 30 µg of Rho-kinase-CAT (dark gray bars), and the combinations (black bars) (D). In all transfections, DNA amounts were adjusted for with empty vector. After 48 h, cell lysates were assayed for PIP5K activity. Kinase activities are expressed as -fold increase over control (PIP5K-β alone) (A and D) or as a percent of the mean of PIP5K stimulation by the respective GTPases (B) and the Rho-kinase mutants (C). Data are mean ± S.E. of three experiments (A, B, and D) or mean ± S.E. of three experiments (C).

**DISCUSSION**

We have shown previously (37) that RhoA and its effector, Rho-kinase, affect phosphoinositide metabolism by stimulating the catalytic activity of PIP5K. Here we show that besides RhoA, both Rac1 and Cdc42 markedly stimulate PIP5K activity and elevate the cellular level of PIP2. Several data indicate that the stimulatory effects of Rac1 and Cdc42 on PIP2 synthesis are specific and independent of RhoA and Rho-kinase. First, in contrast to Rac1 and Cdc42, wild-type and inactive mutants of RalA and Rap1A did not affect PIP2 synthesis, showing that expression of monomeric GTPases per se did not influence PIP2 metabolism. Second, stimulation of PIP5K activity by Rac1 and Cdc42 was not or only partly sensitive to inhibition of RhoA by C3 transferase. Third, in contrast to RhoA, regulation of PIP5K activity by Rac1 and Cdc42 was largely independent of Rho-kinase. Furthermore, stimulation of PIP2 synthesis by wild-type Rho-kinase was inhibited by inactive RhoA, but not by inactive Rac1 or Cdc42 mutants. Finally, stimulation of PIP2 synthesis by constitutively active Rho-kinase could be further increased by co-expression of Rac1 and Cdc42, but not RhoA. In COS-7 cells, RhoA has been localized downstream of Rac1 in receptor-mediated translocation of PIP5K-β to the plasma membrane, suggesting that Rac1 and RhoA sequentially operate in a single signaling pathway (54). In platelets, however, Rac1 and RhoA/Rho-kinase seem to mediate receptor-dependent formation of PIP2 by heterotrimeric Gq and Gi1/2/3 proteins, respectively, in two independent pathways (38). Thus, by directly monitoring PIP5K activity, we conclude that Rac1 and Cdc42 control PIP2 synthesis largely independent from the RhoA/Rho-kinase pathway.

The identification of the three type I PIP5K isoforms raised the expectation of a differential regulation by cellular signal transduction components. Until now, the regulatory properties of PIP5K-α, PIP5K-β, and PIP5K-γ appeared to be remarkably similar. All three isoforms are stimulated by phosphatidic acid (24) and are reported to be negatively regulated by PI-dependent autophosphorylation (55) and protein kinase A (56). We now demonstrate that all three type I PIP5K isoforms are equally regulated by Rho family GTPases and by Rho-kinase. Some quantitative differences were seen, e.g. high sensitivity of PIP5K-β for Rho-kinase, but the significance of this observation is not yet clear. Alternatively, the subcellular partitioning of particular signaling units, i.e. GTPases and PIP5K isoforms, may be decisive to yet coordinate the demand of PIP2 for different cellular processes. Indeed, Rac1 was shown to associate with PIP5K-α and PIP5K-β, but only PIP5K-α seemed to induce actin filament uncapping downstream of Rac1 in permeabilized platelets (35) and to localize in Rac1-induced membrane ruffles (25).

Reports about the association of PIP5K with Rho GTPases have been controversial. Binding of recombinant and cellular RhoA to PIP5K has initially been demonstrated by Ren et al. (41). On the other hand, Tolias et al. (42) reported that PIP5K activity from rat liver cytosol exclusively associated with recombinant Rac1, but not RhoA or Cdc42, and that PIP5K activity was present in Rac immunoprecipitates. We now show that RhoA, and to a lesser extent Rac1, but not Cdc42, associate with all three type I PIP5K isoforms, both in vitro and in vivo. In agreement with our observation, only the C-terminal peptides of RhoA and Rac1 were found recently (57) to precipitate PIP5K activity from HL60 cell lysates. Our data support the finding that PIP5K associates with both GDP- and GTP-bound RhoA and Rac1 (41, 42). Besides PIP5K, a diacylglycerol kinase that synthesizes phosphatidic acid has also been shown to associate with GDP-bound Rac1 (58). Most other effectors of Rho GTPases exclusively bind to the active, GTP-loaded.
Fig. 5. **Association of RhoA with type I PIP5K isoforms.** A, GST (upper panel) or RhoA-GST (lower panel) bound to glutathione-Sepharose beads was loaded with 100 μM GDP or 100 μM GTPγS and subsequently incubated with lysates (500 μg of protein) of HEK-293 cells transfected with empty vector (Con), PIP5K-ια, PIP5K-ιβ, or PIP5K-ιγ DNA. The beads were washed and assayed for PIP5K activity. The panels show autoradiograms of the PIP₂ position. B, lysates (50 μg) of HEK-293 cells transfected with PIP5K-ια, PIP5K-ιβ, or PIP5K-ιγ DNA were separated by SDS-PAGE and transferred to nitrocellulose filters. The blots were incubated with anti-PIP5K isoform-specific antisera. The panels show non-adjacent lanes from the same blot. C, GST or RhoA-GST bound to glutathione-Sepharose beads was incubated with lysates (500 μg) of HEK-293 cells transfected with the indicated PIP5K isoforms. The beads were washed, subjected to SDS-PAGE, and immunoblotted with the respective isoform-specific antibodies. The positions of PIP5K-ια and PIP5K-ιβ (68 kDa), and PIP5K-ιγ (90 kDa) are indicated.
GTPases. As these effectors are released from the inactivated, GDP-bound GTPase, they unlikely will function as scaffolding proteins, suggesting that PIP5K directly binds to the Rho proteins. Although the interaction of PIP5K with RhoA and Rac1 is apparently specific and direct and likely exists in intact cells, binding of the PIP5K isoforms to the Rho GTPases appears not to be a trigger or prerequisite for kinase activation. First, Cdc42, which activated the PIP5K isoforms as effectively as RhoA and Rac1, did not bind the kinases, either in vitro or in vivo. Second, GDP-bound RhoA and Rac1 bound the PIP5K isoforms equally as well as GTPγS-bound RhoA and Rac1, whereas the GDP-bound inactive mutants of these Rho GTPases did not lead to PIP5K activation and even decreased cellular PIP2 levels. Finally, as demonstrated for PIP5K-RhoA binding, upon activation of the GTPase it binds further effectors, such as Rho-kinase, which then displace the bound PIP5K. A similar scenario can be envisaged for the interaction of PIP5K with Rac1. Thus, the constitutive PIP5K-RhoA/Rac1 binding apparently serves another cellular function, e.g. recruitment of the PIP2-forming kinases by the Rho GTPases to

FIG. 6. Competition of PIP5K with Rho-kinase for binding to GTPγS-RhoA. A, immobilized RhoA-GST was loaded with GTPγS and incubated with lysates of HEK-293 cells transfected with vector (Con), PIP5K-Iα, PIP5K-Iβ, or PIP5K-Iγ, in the absence (−) or presence (+) of 2 μM purified recombinant RB. The beads were assayed for bound PIP5K activity. B, RhoA-GST loaded with GTPγS was incubated with lysates of cells expressing PIP5K-Iα in the presence of the indicated concentrations of RB. Data are means ± range of duplicate determinations and representative of two experiments. C, RhoA-GST loaded with GDP or GTPγS was incubated with lysates of cells expressing PIP5K-Iα in the absence (−) or presence (+) of 2 μM RB (upper panel) or 2 μM recombinant RhoA (lower panel). D, immobilized GTPγS-loaded RhoA-GST was incubated with lysates of cells transfected with vector (Con), PIP5K-Iα, Rho-kinase (left panel), or Rho-kinase-CAT (right panel), alone or together with PIP5K-Iα (Both). The beads were assayed for MLC phosphorylation or PIP2 generation. The autoradiograms in A, C, and D show [32P]PIP2 spots after chromatography and [32P]MLC bands after SDS-PAGE and are representative of at least two independent experiments with duplicate determinations. The lower panels in D show autoradiograms with different exposure times.
Activation of PIP5K by Rho GTPases

The stimulation of PIP5K by RhoA was specifically, potently, and fully blocked by the dominant-negative Rho-kinase construct RB/PH (TT), suggesting that the effect of RhoA on PIP2 metabolism is completely mediated by Rho-kinase. Stimulation of PIP5K by Rho-kinase is unlikely to be the result of changes in cellular architecture, as PIP5K activation was maintained outside the cellular context. Furthermore, acute administration of recombinant Rho-kinase outside the cellular context. Furthermore, acute administration of recombinant Rho-kinase in vitro likewise stimulated PIP5K activity (37). The exact mechanisms by which Rho-kinase, as well as Rac1 and Cdc42, control PIP5K activity are presently unclear. We did not find evidence for direct phosphorylation of PIP5K by Rho-kinase (37). Furthermore, there was no specific interaction of activated Rac1 with PIP5K, and Cdc42 did not bind PIP5K at all, suggesting participation of additional factors in PIP5K stimulation by the GTPases. The stimulation of PIP5K by RhoA, Rac1, and Cdc42 stresses the central position of PIP2 in Rho GTPase signaling. A further pivotal enzyme in phospholipid metabolism, PLD, that produces phosphatidic acid, has also been shown to be regulated by RhoA- and Rho-kinase, as well as by Rac1 and Cdc42 (59, 60). This similarity in regulation of PIP5K and PLD by Rho GTPases, and the reciprocal stimulation of these enzymes by their respective products, i.e. PIP2 by phosphatidic acid and PLD by PIP2, points to concerted and complex mechanisms in cellular actions. PIP2 is an intrinsic membrane recognition signal essentially involved in membrane trafficking processes such as endocytosis, exocytosis, and vesicle motility (61). This function obviously demands a localized, highly dynamic PIP2 turnover, apparently under control of Rho GTPases. Thus, Rac1 can activate PIP5K to produce PIP2, but also inhibits endocytosis by direct interaction with the inositol 5-phosphatase, synaptojanin 2 (62). Cdc42 and PIP2 have been shown to coordinately regulate actin nucleation by the WASP-ARP2/3 complex by binding to regulatory domains on WASP (63, 64). Our data now suggest that Cdc42 may additionally support vesicle transport by stimulation of PIP5K activity and, thus, local PIP2 synthesis.

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Activation of Type I Phosphatidylinositol 4-Phosphate 5-Kinase Isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42
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