Physical Association of the Small GTPase Rho with a 68-kDa Phosphatidylinositol 4-Phosphate 5-Kinase in Swiss 3T3 Cells

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Our previous work showed that post-translationally modified Rho in its GTP-bound state stimulated phosphatidylinositol 4-phosphate 5-kinase (PIP5K) activity in mouse fibroblast lysates. To investigate whether Rho physically interacts with PIP5K, we incubated immobilized Rho-GST with Swiss 3T3 cell lysates and tested for retained PIP5K activity. Rho-GST, but not Ras-GST or GST alone, bound significant PIP5K activity. The binding of PIP5K was independent of whether Rho was in a GTP- or GDP-bound state. An antibody against a 68-kDa human erythrocyte type I PIP5K recognized a single 68-kDa protein eluted from Rho-GST column. The Rho-associated PIP5K responded to phosphatidic acid differentially from the erythrocyte type I PIP5K, suggesting that it could be a distinct isoform not reported previously. Rho co-immunoprecipitated with the 68-kDa PIP5K from Swiss 3T3 lysates, demonstrating that endogenous Rho also interacts with PIP5K. ADP-ribosylation of Rho with C3 exoenzyme enhanced PIP5K binding by approximately eightfold, consistent with the ADP-ribosylated Rho functioning as a dominant negative inhibitor. These results demonstrate that Rho physically interacts with a 68-kDa PIP5K, although whether the association is direct or indirect is unknown.

INTRODUCTION

The small GTPase Rho plays a key role in the regulation of actin filament polymerization and cell growth (Hall, 1994; Olson *et al.*, 1995). Like other GTPases, Rho proteins cycle between a GDP-bound inactive state and a GTP-bound active state. In the GTP-bound state, Rho activates downstream effector(s), leading to the assembly of actin stress fibers and focal adhesions. These effects can be blocked by ADP-ribosylation of endogenous Rho protein within the putative effector-binding region (amino acids 32–42) with C3 exoenzyme from *Clostridium botulinum* (Paterson *et al.*, 1990; Ridley and Hall, 1992). Rho proteins (193 amino acids)

are post-translationally modified at the C-terminus by geranylgeranylation of cysteine-190, proteolytic removal of the C-terminal three residues, and methylation of cysteine-190. These modifications are necessary for Rho to translocate to membranes during cell activation, and to interact with its regulatory proteins such as GDP-dissociation inhibitor and stimulator proteins (reviewed in Narumiya and Morii, 1993).

Like Rho, phosphatidylinositol 4,5-bisphosphate (4,5-PIP₂), the catalytic product of phosphatidylinositol 4-phosphate 5-kinase (PIP5K), is implicated in the regulation of both actin polymerization and cell growth (reviewed in Lee and Rhee, 1995). 4,5-PIP₂ regulates the interaction of profilin, α -actinin, and a number of actin-capping proteins with actin, potentially leading to assembly/disassembly of actin fila-

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ments (Hartwig *et al.*, 1995). 4,5-PIP₂ is also critical for cellular signaling, as it can serve as a membrane anchor site for signaling proteins containing pleckstrin homology domains, and as a substrate for both phospholipase-C γ and phosphatidylinositol 3-kinase. Action of these enzymes generates second messengers such as inositol triphosphate, diacylglycerol, and 3,4,5-PIP₃. In addition, both 4,5-PIP₂ and Rho have been reported to activate phospholipase D (Liscovitch *et al.*, 1994; Malcolm *et al.*, 1994; Singer *et al.*, 1995), which catalyzes the hydrolysis of phosphatidylcholine to produce phosphatidic acid and choline. Thus, PIP5K is positioned in the pathway such that it could conceivably mediate several effects of Rho on cell function and structure.

PIP5K activity and 4,5-PIP₂ levels can be enhanced by integrin-mediated cell attachment to the extracellular matrix protein fibronectin (McNamee *et al.*, 1993). In this system, levels of 4,5-PIP₂ correlated with the ability of cells to hydrolyze 4,5-PIP₂, and activate downstream events in response to growth factors such as thrombin or platelet-derived growth factor (PDGF) (Schwartz and Lechene, 1992; McNamee *et al.*, 1993). Recently, we found that Rho stimulated PIP5K activity in extracts from mouse fibroblasts (Chong *et al.*, 1994). This activation was GTP-dependent and was blocked by pre-treatment of Rho with C3 exoenzyme. We also found that when adherent cells that normally have high levels of 4,5-PIP₂ were microinjected with C3 exoenzyme to inhibit Rho, their response to thrombin or PDGF was markedly inhibited. Conversely, injection of an activated variant of Rho allowed nonadherent cells that normally have low levels of 4,5-PIP₂ to respond well to PDGF and thrombin (Chong *et al.*, 1994). These results show that a PIP5K is a downstream element in a Rho-dependent signaling pathway, and suggest that Rho mediates the activation of PIP5K by integrins. Whether Rho directly activates a PIP5K, or whether the activation is mediated by one or more intermediate steps is, however, unknown.

The present study was undertaken to determine whether PIP5K can physically interact with Rho, and therefore whether it might be a direct downstream effector of Rho. Our results show that Rho can associate with a PIP5K. We also provide some initial characterization of the Rho-associated kinase.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP was purchased from Amersham (Arlington Heights, IL). [adenylate-³²P]NAD and [³⁵S]GTP γ S were obtained from NEN (Boston, MA). Phosphatidylinositol 4-phosphate and GTP γ S were obtained from Boehringer Mannheim (Indianapolis, IN). Phosphocellulose P-11 and Silica Gel-60 thin layer chromatography plates were obtained from Whatman (Fairfield, NJ). Other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. Recombinant RhoA-GST and H-Ras-GST expressing bacteria were

kindly provided by Dr. A. Hall (University College, London, UK) and Dr. L. Feig (Tufts University, Boston, MA), respectively. Bacteria expressing C3 exoenzyme-GST fusion protein was a gift from Dr. S. Dillon (Tufts University). Affinity-purified rabbit anti-human erythrocyte type I and type II PIP5K antibodies were described (Jenkins *et al.*, 1994).

Purification of GST-Fusion Proteins from *Escherichia coli*

GST, Rho-GST, Ras-GST, and C3 exoenzyme were purified as described (Smith and Johnson, 1988) with minimal modifications. Briefly, bacteria were sonicated on ice in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μ M GDP. The supernatant from the sonication was incubated with glutathione-agarose beads in the presence of 1% Triton X-100 at 4°C for > 3 h. After washing with the above buffer containing 1 M NaCl, the beads were aliquoted and frozen in liquid nitrogen. The Rho-GST, Ras-GST, and GST beads had no detectable PIP5K activities. Alternatively, the proteins were cleaved off the GST beads by thrombin, and the thrombin was removed by incubation with benzamidine-agarose beads. The active small GTPases were measured by [³⁵S]GTP γ S binding assay (Knaus *et al.*, 1992). The amount of active small GTPases on the beads was estimated by comparing the [³⁵S]GTP γ S binding activities in the *E. coli* lysates before and after incubation with glutathione beads. The amount of protein on the beads was also estimated by SDS-PAGE in comparison with bovine serum albumin (BSA) standards. Approximately 90% of the small GTPases on the beads could bind GTP.

ADP-Ribosylation of Rho

ADP-ribosylation reaction was carried out as described (Tominaga *et al.*, 1993) with modifications. Briefly, Rho samples were incubated with 10 μ M NAD at 30°C for 60 min in the presence of 100 mM Tris, pH 8.0, 5 mM MgCl₂, 10 mM thymidine, 0.2 mM GTP, 1 mM dithiothreitol, and 10 μ g/ml C3 exoenzyme. For radiolabeling, [³²P]NAD was added to the reaction at 10 μ Ci/ml. For ribosylation of milligram quantities of Rho-GST, NAD, GTP, and thymidine were present in at least 10-fold excess. A second ribosylation of this material with [³²P]NAD showed that >90% of the GST-Rho was modified.

Preparation of Cell Lysate

Swiss 3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum in 10-cm tissue culture plastic dishes. Confluent cells were washed three times with cold PBS and lysed in 0.5–1 ml Buffer A (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 10 μ g/ml each of aprotinin and leupeptin, and 1 mM PMSF). The lysates were clarified by centrifugation at 13,000 $\times$ g for 30 min at 4°C, and filtered (0.2- μ m pore size) before affinity chromatography.

Isolation of PIP5K from Cell Lysates Using Rho-GST Agarose Beads

Immobilized Rho-GST (4 μ l beads, 16 μ g protein) was loaded with GDP or GTP γ S by incubation in 200 μ l 100 mM Tris, pH 8.0, 50 mM NaCl, 0.5 mM MgCl₂, 2 mM EDTA, 1 mM dithiothreitol, and 0.1 mM GDP or GTP γ S at 30°C for 10 min. The reaction was stopped by adding 400 μ l cold buffer containing 20 mM MgCl₂ and 100 mM Tris, pH 8.0. The beads were washed twice with Buffer A, blocked with 3% BSA in Buffer A at 4°C for 1–2 h, and incubated with 100 μ l cell lysate (approximately 200 μ g protein) at 4°C for 30 min with constant rotation. After washing five times by low speed centrifugation in Buffer B (50 mM Tris, pH 7.4, 0.1% Triton X-100, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol), PIP5K activity was determined either directly on the beads or after elution

in Buffer C (50 mM Tris, pH 7.4, 5 mM MgCl₂, 0.1 mM EGTA, 1 mM dithiothreitol, 0.02% Triton X-100, and 250–1000 mM NaCl). Alternatively, PIP5K was isolated in larger quantities by affinity chromatography on Rho-GST columns.

PIP5K Assay

Assays were carried out essentially as described (Chong *et al.*, 1994) except the final reaction was in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 1 mM dithiothreitol, 0.02% Triton X-100, 50 μ M 4-PIP, and 25 μ M [γ -³²P]ATP (5 Ci/mmol). Radioactive PIP₂ bands were scraped off the thin layer chromatography plates and quantitated by scintillation counting.

Partial Purification of PIP5K by Phosphocellulose Chromatography

Phosphocellulose was acid-base cycled and pre-equilibrated with Buffer A. Filtered Swiss 3T3 cell lysates were mixed with phosphocellulose and rotated for 30 min at 4°C. After washing with at least 20 column volumes of Buffer B, the kinases were eluted with Buffer C containing 650 mM NaCl. PIP5K containing fractions were pooled, dialyzed into Buffer C containing 150 mM NaCl, and stored frozen at –70°C.

Extraction of PIP5K from Mouse Erythrocyte Membranes

Mouse erythrocytes were washed (2500 $\times$ g) three times in cold PBS containing 10 mM glucose and lysed in 6 volumes of ice cold lysis buffer (5 mM NaH₂PO₄, pH 7.2, 1 mM EDTA, 2 mM dithiothreitol, 1 mM PMSF, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μ g/ml each of aprotinin and leupeptin). The membranes were washed five times with lysis buffer by centrifuging at 100,000 $\times$ g for 60 min and extracted with buffer containing 1 M NaCl as described (Jenkins *et al.*, 1994).

Western Blot Analyses

Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes (Hybond-C, Amersham), and blocked with 5% nonfat milk (Carnation) at 4°C overnight. The membranes were probed with rabbit anti-human erythrocyte type I or type II PIP5K (175 ng/ml), followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (BioSource international, Camarillo, CA). Blots were developed using ECL reagents and Hyperfilm (Amersham).

Immunodepletion

Antibodies against human erythrocyte type I PIP5K were incubated with protein A agarose beads at 4°C overnight, washed three times with Buffer A containing 2% BSA, and blocked with the same buffer at 4°C for 30 min. PIP5K partially purified from ADPR-Rho column was reconstituted into the above buffer and incubated with the beads coupled with antibodies at 4°C for 4 h. The supernatant was sampled for PIP5K activity assay as described above.

Co-immunoprecipitation

Swiss 3T3 cell lysate (400 μ l) prepared in Buffer A containing 10 mM NaCl and 10% glycerol was incubated at 4°C for 4 h with protein A agarose coupled with rabbit anti-human type I or type II PIP5K antibodies or nonimmune rabbit IgG. The beads were washed three times with 0.8 ml each of 50 mM Tris, pH 7.4, containing 0.1% Triton X-100, 10 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, and 10% glycerol, and divided. PIP5K activity was assayed in one portion. The other portion was used for ³²P-ADP-ribosylation (in the presence of 0.05%

SDS), and then separated by SDS-PAGE and analyzed by autoradiography to assay the presence of Rho.

Modulation of PIP5K Activity by Phosphatidic Acid

PIP 5-kinases were immunoprecipitated from mouse erythrocyte membrane extracts or Swiss 3T3 cell lysate using 250 ng anti-human type I PIP5K antibody, and washed three times with 50 mM Tris, pH 7.4, 0.1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, and 1 mM dithiothreitol, and once with 50 mM Tris, pH 7.4, 1% Triton X-100, 10 mM MgCl₂, and 1 mM EGTA. PIP5K assays were performed as described (Jenkins *et al.*, 1994).

RESULTS

Interaction of Rho-GST with a PIP5K in Swiss 3T3 Cell Lysate

To test whether there is a physical association between Rho and PIP5K, a Rho-GST fusion protein expressed in *E. coli* was immobilized on glutathione-agarose beads, loaded with GDP or GTP γ S, and incubated with lysate from Swiss 3T3 cells. After washing, the bound PIP kinase activity was assayed with [γ -³²P]ATP and phosphatidylinositol 4-phosphate as substrate. Although GST and Ras-GST beads retained minimal PIP5K activity, Rho-GST bound significant levels (Figure 1A). To confirm that the lipid phosphorylation was due to PIP5K, the spots containing PIP₂ were scraped off the thin layer chromatography plates, eluted, and deacylated. High performance liquid chromatography analysis showed that the PIP₂ was essentially 100% 4,5-PIP₂ with no detectable 3,4-PIP₂ (Figure 1B), indicating that the PIP kinase associated with Rho was a PIP5K.

The association of PIP5K with Rho was independent of whether the bound nucleotide was GTP γ S or GDP. Considering that there are guanine nucleotide-exchange factors and significant amounts of GTP in cell lysates, it is possible that the preloaded nucleotides were exchanged during the incubation, making the binding independent of the initially bound nucleotide. We therefore partially purified PIP5K by chromatography on a phosphocellulose column. Although we could not ensure the elimination of exchange activity toward Rho, the partially purified PIP5K was free of nucleotides and endogenous Rho (as detected by ³²P-ADP-ribosylation). The purified PIP5K was then supplemented with either GDP or GTP γ S (10 μ M), and incubated with Rho-GST preloaded with GDP or GTP γ S, respectively. Under these conditions, GDP-Rho and GTP-Rho still bound to PIP5K equally well.

Enhanced Association of PIP5K with ADP-ribosylated Rho

Stimulation of PIP5K in the cell lysate by GTP-bound Rho can be blocked by ADP-ribosylation of Rho with C3 exoenzyme (Chong *et al.*, 1994). To investigate the

Figure 1. Association of PIP5K with Rho. (A) Immobilized Rho-GST, Ras-GST, and GST were loaded with GDP or GTP γ S, incubated with Swiss 3T3 cell lysate, and the bound PIP5K activity was assayed as described in MATERIALS AND METHODS. Values represent the fold increase relative to the PIP5K activity on GST-beads (means $\pm$ SE, $n = 3$). (B) Product of the Rho-associated PIP kinase co-migrated with authentic 4,5-PIP $_2$. The product of a kinase reaction using [32 P]ATP, 4-PIP, and PIP kinase eluted from the GST-Rho column was mixed with a [3 H]4,5-PIP $_2$ standard and analyzed by high performance liquid chromatography (Kachintorn *et al.*, 1993). Fractions were counted for 32 P (left axis) and 3 H (right axis). 3,4-PIP $_2$ eluted 2–3 min earlier than 4,5-PIP $_2$. (C) Binding of PIP5K in cell lysate to ADPR-Rho-GST was compared with unmodified Rho-GST. Values are means $\pm$ SE from 11 experiments.

effect of ADP-ribosylation of Rho on its binding to PIP5K, Rho-GST-beads were ADP-ribosylated before incubation with cell lysates. ADP-ribosylated Rho (ADPR-Rho) showed an approximately eightfold higher retention of PIP5K activity (Figure 1C), whereas Rho that had been mock ribosylated (in the absence of C3) showed similar binding activity to untreated Rho. Additional experiments revealed that complete elution of the bound PIP5K from ADPR-Rho beads required a higher concentration of NaCl (1 M) compared with unmodified Rho (250 mM), suggesting that ADPR-Rho has a higher affinity for PIP5K. ADPR-Rho bound up to 15% of the total PIP5K activity in cell lysates.

Rho-associated PIP5K Is a 68-kDa Protein

Several PIP 5-kinases of distinct molecular masses have been identified. A 110-kDa PIP5K was purified from rat brain (Cochet and Chambaz, 1986) and bovine brain membranes (Moritz *et al.*, 1990), and two types (a 68-kDa enzyme termed type I and a 53-kDa enzyme termed type II) were purified from human erythrocytes (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994) (The type I PIP5K was defined by its earlier elution from phosphocellulose column than the type II kinase). Antibodies against human erythrocyte type I and type II PIP5K do not cross-react, indicating that the two kinases are distinct (Bazenet *et al.*, 1990; Jenkins *et al.*, 1994). These antibodies recognized two type I (68 kDa and 90 kDa) and one type II (53 kDa) PIP5K, respectively, in bovine brain cytosol (Jenkins *et al.*, 1994). The ability of these antibodies to recognize PIP5K eluted from Rho beads was tested by immunoblotting. Both Swiss 3T3 cell lysate and mouse erythrocyte membrane extracts contained a 68-kDa protein that reacted with the anti-type I antibody, and a 53-kDa protein that reacted with the anti-type II antibody (Figure 2A, lanes 1 and 3). The Swiss 3T3 PIP5K eluted from the Rho column showed only a 68-kDa band, which was recognized by the anti-type I antibody (Figure 2A, left panel, lane 2) but not the anti-type II antibody (Figure 2A, right panel, lane 2). In addition, ADPR-Rho bound significantly more of the 68-kDa

Figure 2. Western immunoblot analyses using antibodies against human erythrocyte type I and type II PIP5K. (A) Swiss 3T3 cell lysate (lanes 1), Swiss 3T3 PIP5K eluted from ADPR-Rho beads (lanes 2), and mouse erythrocyte membrane extract (lanes 3) were blotted with anti-type I (left panel) and anti-type II (right panel) PIP5K antibodies. (B) Swiss 3T3 cell lysate (lane 1), mouse erythrocyte membrane extract (lane 2), Swiss 3T3 PIP5K eluted from ADPR-Rho beads (lane 3), unmodified Rho beads (lane 4), or GST beads (lane 5) were blotted with anti-type I antibody as described in MATERIALS AND METHODS.

Figure 3. Immuno-depletion of PIP5K activity eluted from Rho-GST. PIP5K purified from Rho-GST column was incubated with protein A beads coupled with increasing amount of antibodies against type I erythrocyte PIP5K (closed circles) or pre-immune rabbit IgG (open circles). Shown here is the PIP5K activity in the supernatants. cpm refers to total counts incorporated into 4,5-PIP₂. The data are representative of two experiments.

protein compared with untreated Rho (Figure 2B), consistent with its higher retention of PIP5K activity (Figure 1B).

To confirm that the 68-kDa protein recognized by the type I antibody fully accounts for the PIP5K activity, proteins eluted from Rho-GST column were incubated with anti-type I antibody bound to protein A beads, and the PIP5K activity remaining in the supernatant was tested. The antibody depleted essentially 100% of the PIP5K activity eluted from Rho-GST (Figure 3). Taken together, these results indicate that the Rho-associated PIP5K is a 68-kDa protein immunologically related to the 68-kDa erythrocyte type I PIP5K.

Interaction between Endogenous Rho and PIP5K

To determine whether the interaction observed in vitro with recombinant Rho protein occurs in 3T3 cells, the antibodies described above were used to immunoprecipitate PIP5K from Swiss 3T3 cell lysate. Anti-type I antibody precipitated a significant amount of PIP5K activity, whereas anti-type II antibody or nonimmune IgG did not (Figure 4A). Subsequent ³²P-ADP-ribosylation showed that only the type I immunoprecipitates contained Rho protein (Figure 4B), demonstrating that an association can be observed between endogenous Rho and a 68-kDa PIP5K.

Figure 4. Co-immunoprecipitation of Rho and PIP5K. (A) PIP5K assay of the cell lysate (1 μl, lane L) and the immunoprecipitates using antibodies against human type I (lane I) and type II (lane II) PIP5K and nonimmune rabbit IgG (lane G). (B) Autoradiogram after ADP-ribosylation and SDS-PAGE of the cell lysate (12.5 μl) and the immunoprecipitates. The data are representative of two experiments.

Enzymatic Characterization of Rho-associated PIP5K

The PIP5K activity eluted from Rho columns was further characterized. Consistent with most of the PIP 5-kinases reported so far, the activity of the Rho-associated PIP5K was magnesium dependent, with optimal activity at 5–10 mM MgCl₂ and little or no activity without magnesium or at higher concentrations (Figure 5A). Absence of magnesium also inhibited binding of PIP5K to Rho. Concentrations of NaCl above 150 mM also inhibit the kinase activity (Figure 5B). The inhibitory effects of magnesium and NaCl were reversible.

Enzymatic activity of some isoforms of PIP 5-kinases can be modulated by phospholipids such as phosphatidylserine (Ling *et al.*, 1989) and phosphatidic acid (Moritz *et al.*, 1992; Jenkins *et al.*, 1994). Rho-associated PIP5K was not stimulated by phosphatidylserine, similar to erythrocyte type I PIP5K. A key characteristic of erythrocyte type I PIP5K is its stimulation by phosphatidic acid (Jenkins *et al.*, 1994). We therefore compared the effect of phosphatidic acid on the Rho-associated PIP5K and type I PIP5K from mouse erythrocytes. Type I PIP5K immunoprecipitated from mouse erythrocyte membrane extracts showed an approximately twofold stimulation by phosphatidic

Figure 5. Enzymatic characterization of Rho-associated PIP5K activity. (A) Effect of magnesium on PIP5K activity. Assays were carried out as described in MATERIALS AND METHODS with various concentrations of magnesium chloride. Values represent PIP5K activities in cpm (means $\pm$ range, $n = 2$). (B) Effect of NaCl on PIP5K activity. Assays were performed in buffers containing 5 mM MgCl₂ and various concentrations of NaCl. Values represent PIP5K activities in cpm (means $\pm$ range, $n = 2$). (C) Effect of phosphatidic acid on PIP5K activity. Values represent PIP5K activity (total cpm incorporated into 4,5-PIP₂) assayed in the presence of phosphatidic acid relative to that in the absence of phosphatidic acid (means $\pm$ SE, $n = 3$).

acid, as expected. PIP5K in immunoprecipitates from Swiss 3T3 cells did not respond to phosphatidic acid, and the PIP5K eluted from Rho beads was inhibited by phosphatidic acid (Figure 5C). These results suggest that the Rho-associated PIP5K, although immunologically related to erythrocyte type I PIP5K, is biochemically distinct.

DISCUSSION

We have previously shown that post-translationally modified Rho in its GTP-bound state stimulates PIP5K activity in cell lysates (Chong *et al.*, 1994), suggesting that a PIP5K is a downstream element in a Rho-de-

pendent signaling pathway. Those experiments did not, however, address the question of whether PIP5K is a direct effector of Rho, or whether several intermediate steps lie in between. We therefore used a Rho-GST fusion protein expressed in *E. coli* to test whether there was a physical interaction between Rho and PIP5K. We detected an *in vitro* association between the recombinant Rho protein and a 68-kDa PIP5K in Swiss 3T3 cells. We also demonstrated by co-immunoprecipitation that endogenous Rho associates with a 68-kDa PIP5K in 3T3 cell lysates. These experiments clearly demonstrate that Rho physically interacts with a PIP5K. They do not, however, rule out the possibility that other proteins might also co-associate or contribute to the interaction.

Because only GTP-Rho stimulated PIP5K activity (Chong *et al.*, 1994), one would have predicted that only GTP-Rho could bind PIP5K, as is usually the case for the effectors of small GTPases (Moodie *et al.*, 1993; Manser *et al.*, 1994; Rodriguez-Viciano *et al.*, 1994). In this study, however, the association between Rho-GST and PIP5K was independent of whether Rho bound GDP or GTP. In a recent independent study, Tolias *et al.* (1995) also showed that Rac (a member of Rho family GTPases), in both GTP- and GDP-bound states, associated with a PIP5K (though its properties are distinct from those of the Rho-associated kinase). Tolias *et al.* failed to observe binding of PIP 5-kinase to Rho in their system; the reason for this discrepancy is unknown and efforts are underway to sort out the difference. In their experiments, the same Rac-GST beads retained phosphatidylinositol 3-kinase activity in a GTP-dependent fashion. It is possible that the GTP-independence is a consequence of using GST fusion proteins expressed in bacteria and that post-translationally modified Rho will bind in a GTP-dependent manner. It seems more likely to us, however, that binding and activation of PIP5K by Rho are separate events. In other words, the PIP5K-binding site may be in a region that does not change conformation after Rho exchanges GDP for GTP. This notion implies that activation of PIP5K may require another Rho effector. Consistent with this hypothesis, Rho is found to have a second effector-binding domain in addition to the "classical" one at residues 32–42, and both sites are required for Rho effects on actin reorganization (Self *et al.*, 1993). Work is underway to test this hypothesis by mapping the sites required for binding and activation of PIP5K.

We also found in this study that ADP-ribosylated Rho binds to PIP5K approximately eightfold better than untreated Rho. It has been established that C3 exoenzyme specifically ADP-ribosylates and functionally inactivates Rho, leading to disruption of actin stress fibers and focal adhesions (Paterson *et al.*, 1990; Ridley and Hall, 1992). Pretreatment of Rho with C3 exoenzyme can abolish its stimulation of PIP5K activ-

ity in cell lysates (Chong *et al.*, 1994). How ADP-ribosylation inactivates Rho is not clear. ADP-ribosylation does not affect the interaction of Rho with its upstream regulators such as RhoGAP (29 kDa) (Pateron *et al.*, 1990; Morii *et al.*, 1991), GDP dissociation inhibitor, or stimulator proteins (Narumiya and Morii, 1993), nor does ADP-ribosylation alter its intrinsic or GAP-stimulated GTPase activities, or its intrinsic GDP/GTP exchange rate (Kikuchi *et al.*, 1988; Quilliam *et al.*, 1989; Pateron *et al.*, 1990). It is therefore likely that ADP-ribosylation affects the interaction of Rho with its effector(s). It was found that microinjection of ADPR-Rho, at a level where unmodified Rho protein had no detectable effect, caused cell rounding and actin filament reorganization similar to injecting C3 exoenzyme. Furthermore, co-injection of a ribosylation-resistant Rho variant with C3 exoenzyme did not overcome the effect of C3 exoenzyme (Pateron *et al.*, 1990; Ridley and Hall, 1992). These results suggest that ADPR-Rho binds with higher affinity to and prevents the activation of its downstream effector (Morii *et al.*, 1992). This scheme is therefore consistent with our data that ADPR-Rho associates with PIP5K more tightly than untreated Rho (Figures 1C and 2B), but cannot activate PIP5K (Chong *et al.*, 1994). It is also possible, however, that other Rho effectors exist, and ADP-ribosylation may block the physical interaction of Rho with some of these effectors (Pateron *et al.*, 1990).

Multiple PIP 5-kinases of different molecular masses and distinct biochemical features have been identified in several tissues and species (Cochet and Chambaz, 1986; Ling *et al.*, 1989; Bazenet *et al.*, 1990; Jenkins *et al.*, 1994; Boronenkov and Anderson, 1995). Our data suggests that Rho selectively associates with one of the isoforms of PIP5K, although differences due to post-translational modification or regulatory subunits cannot be excluded. Recently, Toliás *et al.* (1995) reported that Rac is associated with a PIP5K whose activity is stimulated by phosphatidic acid. It is therefore likely that Rho and Rac bind to different PIP 5-kinases that can be differentially regulated. Studies of how interactions between Rho family small GTPases and PIP 5-kinases regulate actin filament organization and focal contact formation will be greatly facilitated after cDNA cloning of the relevant PIP 5-kinases.

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