Phosphorylation of P-Rex1 by the Cyclic AMP-dependent Protein Kinase Inhibits the Phosphatidylinositol (3,4,5)-Trisphosphate and Gβγ-mediated Regulation of Its Activity*

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Linnia H. Mayeenuddin 1 and James C. Garrison
From the Department of Pharmacology, University of Virginia Health System, Charlottesville, Virginia 22908

Rac activation is a key step in chemotaxis of hematopoietic cells, which is both positively and negatively regulated by receptors coupled to heterotrimeric G proteins. P-Rex1, a Rac-specific guanine nucleotide exchange factor, is dually activated by phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and the Gβγ subunits of heterotrimeric G proteins. This study explored the regulation of P-Rex1 by phosphorylation with the cAMP-dependent protein kinase (protein kinase A) in vitro and by Gα- and Gβγ-coupled receptors in HEK293T cells. P-Rex1 isolated from Sf9 and HEK293T cells migrates as two distinct bands that are partially phosphorylated. Phosphorylation of P-Rex1 with protein kinase A (PKA) inhibits the PIP3- and Gβγ-stimulated P-Rex1 guanine nucleotide exchange activity on Rac. The guanine nucleotide exchange factor activity of three different forms of P-Rex1 (native Sf9, de-phosphorylated, and phosphorylated) was examined in the presence of PIP3 and varying concentrations of Gβγ. Gβγγγ was 47-fold less potent in activating the phosphorylated form of P-Rex1 compared with the de-phosphorylated form. HEK293T cells expressing P-Rex1 were labeled with 32P and stimulated with lysophosphatidic acid (LPA) to release Gβγγγ to activate PKA. Treatment with isoprostane or ScAMPs, a potent activator of PKA, increased the incorporation of 32P into P-Rex1. LPA increased the amount of GTP-bound Rac in the cells and isoprostane reduced basal levels of GTP-bound Rac and blunted the effect of LPA. Treatment of the cells with ScAMPs also reduced the levels of GTP-bound Rac. These results outline a novel mechanism for Gγ-linked receptors to regulate the function of P-Rex1 and inhibit its function in cells.

P-Rex1 is a Rac-specific guanine nucleotide exchange factor (Rac-GEF) that is dually modulated by heterotrimeric G protein βγ subunits and PIP3 (1), a phospholipid messenger produced in cells via the activation of phosphatidylinositol 3-kinase (PI3K) and the Gβγ subunits of heterotrimeric G proteins. Although there is an inositol polyphosphate 4-phosphatase (InsP4, 4-phosphatase) domain contained within P-Rex1 and P-Rex2a, neither of these proteins demonstrate InsP4 4-phosphatase activity (1, 3) and this domain is not needed for the protein to act as a Rac-GEF (5).

Like the P-Rex family of GEFs, all GEFs are multimodular proteins containing at least the DH/PH tandem domain and various other functional domains, such as SH2, SH3, Ser/Thr, or tyrosine kinase, Ras-GEF, Rho-GAP, Ran-GEF, PDZ and/or additional PH domains (6–8). Their structural complexity allows for a number of distinct modes of regulation although no universal mode of GEF regulation exists. Thus far, at least five different modes of regulation have been identified for the known proteins exhibiting GEF activity: (a) regulation by localization; (b) regulation by intramolecular interactions; (c) regulation by phosphoinositol kinases; (d) activation by α and βγ subunits of GTP-binding proteins; and (e) activation by protein kinases (6, 7).

There have only been a limited number of studies published on the activity and regulation of the three P-Rex family members. These proteins display differential tissue localization, with P-Rex1 being highly expressed in the brain and peripheral blood leukocytes, such as neutrophils (1). In contrast, P-Rex2a is abundant in skeletal muscle, heart, placenta, kidney, small intestine, and lung (3), and P-Rex2b seems most abundant in cardiac tissues (4). Interestingly, neither P-Rex2a nor P-Rex2b are present in peripheral blood leukocytes (3, 4). Furthermore, P-Rex1 is mainly a cytosolic protein that can be partially membrane associated in non-stimulated cells and is not substantially recruited to the membrane upon cell stimulation (1), suggesting that the PH domain in this protein may act as an inhibitory domain for GEF activity (1).

The regulation of P-Rex proteins via PIP3 produced by the activation of phosphoinositol kinases, specifically by the p110y isoform of phosphatidylinositol 3-kinase, has been reported (1, 4). Welch et al. (1) have demonstrated that when co-expressed in Sf9 cells, phosphatidylinositol 3-kinase, P-Rex1, and Gβγγγ work synergistically to produce Rac activation. Similar results have been observed for P-Rex2 (3, 4). A recent study by Hill et al. (5) has explored the regions of P-Rex1 that are important for its regulation by PIP3 and Gβγ subunits (5). Using targeted deletions, they found that P-Rex1 mutants lacking the PH domain (∆PH) could not be stimulated by PIP3, demonstrating that the PH domain is required for PIP3-dependent activation of P-Rex1 (5). Moreover, the ∆PH mutant was 10 times more active than the wild-type P-Rex1, suggesting that the PH domain plays an inhibitory role in the wild-type P-Rex1 molecule (5). Similarly, the P-Rex1 mutant containing only the isolated DH/PH tandem of P-Rex1 was highly active compared with wild-type P-Rex1. These findings suggest that the PH domain along with the other domains in wild-type P-Rex1 are responsible for the intramolecular regulation of basal P-Rex1 activity (5).

In hematopoietic cells, where P-Rex1 is highly expressed, Gγ-coupled receptors, such as the fMet-Leu-Phe receptor, and Gγ-coupled recep-
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tors, such as the adenosine A2a receptor, have been shown to stimulate and inhibit chemotaxis, respectively (9–16). Because Rac activation is a key step in hematopoietic cell chemotaxis and P-Rex1 plays a major role in Rac activation, the regulation of P-Rex1 by multiple G protein α subunits (17) and βγ dimers (1, 17) has been studied. This work shows that P-Rex1 is not modulated by G protein α subunits but that it can be selectively activated by certain Gβγ subunits, suggesting the possibility that different receptors can release distinct Gβγ dimers (17).

Several Rac-GEFs (Vav1–3, Ect2, and Tiam1) have been shown to be stimulated by phosphorylation (18–23), but the regulation of P-Rex1 by protein kinases has not been studied. However, as activation of receptors such as the adenosine A2a receptor are known to raise the level of cyclic AMP and inhibit chemotaxis (13, 14), we investigated the possibility that phosphorylation of P-Rex1 by the cyclic AMP-dependent protein kinase (PKA) might regulate the GEF activity of P-Rex1 in cells. We found that phosphorylation of P-Rex1 by PKA caused a marked inhibition of P-Rex1, suggesting that PKA stimulation different from the stimulatory effects reported for the phosphorylation of other Rac-GEFs (18–23).

EXPERIMENTAL PROCEDURES

Materials—The reagents used for Sf9 cell culture and purification of βγ dimers have been described (24, 25). Isoproterenol, 4-[3-(cyclopro-tyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram), amyllose resin, maltose, and Triton X-100 were purchased from Sigma. Lysophosphatidic acid (LPA18:1) was purchased from Avanti Polar Lipids. S. c.-CAMPS was obtained from Biomol. The Rac Activation Assay kit and the Rac monoclonal antibody were obtained from Upstate Biotechnol-ogy. PKA was obtained from Promega. PKA inhibitor peptide 5–24 was from Calbiochem. [35S]GTPγS was from PerkinElmer Life Sciences, 32P and [γ-32P]ATP were from ICN. The sources of all other reagents have been published (2, 17).

Recombinant Protein Constructs—The protocols for constructing the G protein βγ subunits and γγ subunits used in this study have been published (17, 26, 27). The cDNA encoding EE-tagged P-Rex1 and the recombinant baculovirus expressing EE-tagged P-Rex1 were kindly pro-vided by Dr. Leonard R. Stephens, Cambridge University, United King-dom (1). The bacterial expression plasmids encoding GST-Rac1, PAK1 p21-binding domain (PBD)-GST, and maltose-binding protein-α-phos-phatase (MBP-α-PPase) (28) were obtained from Dr. Ian Macara, Dr. Martin Schwartz, and Dr. Todd Stukenberg at the University of Vir-ginia, respectively. Bacterial expression plasmids for three other Rac-GEFs, wild-type Vav2 (29), oncogenic Vav2 (29), and N-terminally trunk-ted C-1199 Tiam1 (30, 31) constructs were all provided by Dr. K. S. Ravichandran at the University of Virginia.

Purification of Recombinant G βγ Dimers and EE-tagged P-Rex1 from Sf9 Cells—The protocols for the culture and infection of Sf9 cells with recombinant baculoviruses have been published (2, 17). The methods used to purify the G protein βγ subunits and Rac1 dimer, to prepare the EE-antibody column, and to purify the EE-tagged P-Rex1 protein doublet from Sf9 cells have been described (17).

Purification of Rac1 and PAK1 PBD-GST Fusion Proteins from Bacteria—The method used to purify GST-Rac1 has been described (17). The human PAK1 p21-activated kinase 1 protein contains a PBD that binds activated Cdc42 or Rac with high affinity (32). The recombi-nant PAK1 PBD-binding domain with an N-terminal GST tag was puri-fied using a GST resin (Amersham Biosciences). Two liters of DH5α bacterial culture expressing the GST-tagged PAK1 PBD construct were harvested and lysed using a French Press in 30 ml of lysis buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT) and protease inhibitors (aprotinin, leupeptin, and pepstatin (at 2 μg/ml), benzami-dine at 20 μg/ml, and Pefabloc SC Plus at 100 μg/ml). One percent Triton X-100 was added to the cell lysate and the mixture incubated on ice for 30 min. The cell lysate was then centrifuged at 10,000 × g for 30 min at 4°C. The supernatant (30 ml) was collected and incubated with 1 ml of GST resin for 2 h at 4°C with constant agitation. After the incuba-tion, the GST-Sepharose beads were pelleted at 2000 rpm for 2 min. The beads were washed five times and pelleted at 2000 rpm for 2–3 min after each wash. The resin was washed twice with 15 ml of buffer B (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT and protease inhibitors) and 1% Triton X-100. The beads were subsequently washed three more times with 15 ml of buffer B. The beads were then aliquoted and stored in buffer B containing 50% (v/v) glycerol at −80°C.

Purification of λ-PPase—The recombinant MBP-tagged λ-PPase was purified using an amyllose resin. One liter of BL21 bacterial cell culture expressing λ-PPase was harvested and lysed using a French press in 30 ml of MBP column buffer containing 20 mM Tris, pH 7.4, 200 mM NaCl, 1 mM EDTA, 40 units/μl DNase I, and protease inhibitors. The cell lysate was centrifuged at 10,000 × g for 30 min at 4°C. The supernatant (30 ml) was collected and incubated with ~3 ml of amyllose resin for 2 h at 4°C with constant rocking. After the incubation, the resin was centrifuged at 600 × g for 2–3 min and the superna-tant was removed. The resin was poured into a column and washed with 40 ml of MBP buffer and allowed to drain by gravity flow. The protein was eluted off the column in 2-ml fractions using MBP buffer containing 10% maltose. Column fractions (4 ml) containing λ-PPase were pooled and dialyzed against two changes of 2 liters of storage buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 0.1 mM MnCl2, 0.1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, and 0.01% Brij 35. The purified protein was then dialyzed again overnight against 1 liter of storage buffer containing 50% glycerol. The dialyzed protein was resolved on a 8% SDS-PAGE to determine the purity and quantity of the λ-PPase by visualizing with Coomassie Blue. The MBP-tagged λ-PPase prepared using this protocol was highly pure and migrated as a single major band at ~56 kDa. Approximately, 1 ml of 3 mg/ml of λ-PPase was obtained from 1 liter of BL21 bacterial culture.

Preparation of γ-PPase-treated and PKA-treated P-Rex1—We used two different protocols to de-phosphorylate and phosphorylate P-Rex1. Initial experiments used the purified P-Rex1 protein doublet from Sf9 cells that was batch phosphorylated or de-phosphorylated on a small scale (10–50 μl) and then used directly for P-Rex1 activity assays. Using these small scale assays, we optimized the conditions for P-Rex1 de-phosphorylation and phosphorylation (see Fig. 2). Small scale de-phosphorylation reactions using λ-PPase were carried out for 10 min at 30°C in a buffer containing 50 mM Tris, pH 7.5, 0.1 mM Na2EDTA, 5 mM phenylmethylsulfonyl fluoride, 0.01% Brij 35 (v/v), and 2 mM MnCl2. The ability of various concentrations of the λ-PPase to de-phospho-rylate P-Rex1 was tested on 80 ng/ml P-Rex1 in a 50-μl reaction volume. The reactions were terminated using either a final concentration of 2–4 mM sodium orthovanadate (33) or 25 μl of 2× Laemmli sample buffer. Control reactions were carried out in the absence of the λ-PPase and the reactions were supplemented with the appropriate λ-PPase storage buffer. To test the P-Rex1 activity of de-phosphorylated P-Rex1, the reactions were terminated with sodium orthovanadate, placed on ice, and assayed for activity as described under “P-Rex1 Activity Assay.” Unless otherwise specified, λ-PPase-treated P-Rex1 was diluted 10-fold into the P-Rex1 activity assay to achieve a final P-Rex1 concentration of 30 nM (Fig. 1).
Small-scale PKA phosphorylation reactions were carried out at 30 °C for 30 min in a buffer containing 20 mM Hepes, pH 7.5, 200 mM micr

...sion of the activation of Rac by PKA. The reactions were initiated by the addition of ATP at a final concentration of 125 μM. The reactions were terminated by the addition of the PKA inhibitor peptide (PKI residues 5–24) to a final concentration of 100 nM or by addition of 2× Laemmli sample buffer. Control reactions were carried out in the absence of the kinase, and the reactions were supplemented with the appropriate kinase storage buffers. The PKA storage buffer contained 2 mM ATP, 250 mM KPO4, and 1 mM phenylmethylsulfonyl fluoride. The PKA enzyme was stored in small aliquots and used only once. To test the P-Rex1 activity of phosphorylated P-Rex1, the reactions were terminated with the appropriate peptide inhibitor, placed on ice, and diluted 10-fold into the assay, and P-Rex1 activity was determined as above (Fig. 1). Reactions to determine the concentration of PKA needed for effective phosphorylation of P-Rex1 were carried out as above with varying concentrations of PKA in the presence of 600 ng of P-Rex1 in a volume of 10 μl for 5 min at 3 °C. Radiolabeled phosphorylation reactions were carried out as described above with the addition of [γ-32P]ATP (2.2 × 107 cpm/μl, final).

Once the de-phosphorylation and phosphorylation protocols for P-Rex1 were optimized, large-scale purifications of de-phosphorylated and phosphorylated P-Rex1 were undertaken using treatment of P-Rex1 on the EE-antibody column with λ-PPase and/or PKA. Purification of de-phosphorylated and phosphorylated P-Rex1 from the column allows isolation of highly pure P-Rex1 free of the λ-PPase or PKA, as these enzymes are washed away before elution of P-Rex1 from the column. This preparation of de-phosphorylated and phosphorylated P-Rex1 began with the initial steps of the purification of the P-Rex1 protein from Sf9 cells (17) with the following modifications. The starting column volume was 2 ml and the loading and washing protocols were carried out as described (17) until the point of elution. At this stage, about 500 μl of the resin was processed to obtain the native (Sf9) P-Rex1 doublet as described (17). The remaining resin (~1.5 ml) was resuspended in 6 ml of the above λ-PPase reaction buffer containing 12 ng/μl of λ-PPase and incubated at 30 °C for 5 min. The resin was washed twice using 2–4 column volumes of the standard column wash buffers (17). Then, the de-phosphorylated P-Rex1 was eluted from a 500-μl aliquot of the λ-PPase-treated resin using 5 sequential elutions (500 μl each) containing 400 ng/μl of EE peptide (17). The remaining 1 ml of the λ-PPase-treated and washed resin was divided equally and treated with or without 0.5 units of PKA. Phosphorylation reactions were carried out for 5 min at 30 °C, using the buffer described above. After the incubation, 500 μl of PKA-treated resin was washed with 2–4 column volumes of buffer. Then, the PKA-phosphorylated P-Rex1 was eluted with 400 ng/μl of EE peptide as described above (17). The remaining 500 μl of λ-PPase-treated resin treated in the absence of PKA was washed as above, the P-Rex1 was eluted and used as the PKA reaction control (consisting of de-phosphorylated P-Rex1).

P-Rex1 Activity Assay (P-Rex1-mediated Rac Guanine Nucleotide Exchange) Using synthetic lipid vesicles of defined composition (200 μM phosphatidylcholine, 200 μM phosphatidylglycerol, 200 μM phosphatidyserine, 10 μM PIP2), P-Rex1 activity assays were performed in the presence of 100 nM GST-Rac1 (Rac), either at room temperature for 4 min or at 30 °C for 1 min. The liposomes were prepared as described (17) and the total reaction volume was 10 μl (for reactions at 30 °C) or 25 μl (for reactions at room temperature). Unless otherwise specified, all assays were performed in the presence of 0.001% CHAPS as described (17).

Affinity Precipitation of Activated-Rac from HEK 293T Cells—HEK 293T cells were transfected with DNAs for different Rac-GEFs as follows: (a) a pcDNA3 mammalian expression plasmid encoding EE-tagged P-Rex1 (1); (b) a pcDNA3 plasmid encoding a hemagglutinin-tagged Tiam1 C-1199 mutant (30, 31); (c) a pEGFP mammalian expression plasmid encoding wild-type Vav2 (Vav2-WT) (29); and (d) a pEGFP plasmid encoding oncogenic Vav2 (Vav2-onco) (29). Cells were transfected using Lipofectamine 2000 per the manufacturer’s protocols with the following variations. The cells were plated overnight in 10-cm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and no antibiotics. The following day, the cells were treated with the plasmid DNAs of interest. The transfection conditions were optimized to ensure that all the Rac-GEFs tested were expressed at levels where they promoted comparable levels of Rac activation. Thus the cells were transfected with 24 μg of P-Rex1 plasmid DNA, 10 μg of Vav2-WT, 10 μg of Vav2-onco, or 10 μg of Tiam1 C-1199, in the presence of 24 or 10 μl of Lipofectamine (1 μg of DNA/1 μl of Lipofectamine), respectively. Forty-eight hours post-transfection the cells were serum-deprived for 4 h prior to treatment with the following agonists: 100 μM S-cAMPS, 100 mM rolipram followed by incubation with 100 μM isoproterenol, 50 mM lysophosphatidic acid (LPA18:1), or 100 μM isoproterenol plus 50 mM LPA (see Figs. 5 and 6). Control plates were treated with water. After treatment, the reactions were stopped by quickly washing the cells twice with cold phosphate-buffered saline and freezing them with liquid nitrogen. The plates were stored at −80 °C until use.

The activated Rac precipitation assays were performed using either the Activated Rac Assay kit or PKA PBD-GST beads that were produced in our laboratory; both worked equally well. The cells were lysed in 800 μl of Mg2+ lysis buffer containing 25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM microcystin, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 100 μg/ml benzamidine, 500 μg/ml Pefabloc SC Plus. The plates were scraped on ice and the lysate was pre-cleared using 20 μl of GST-agarose beads for each sample. The supernatant (~800 μl) was collected and 500 μl of the supernatant was incubated with 5–10 μl of PAK1 PBD-agarose beads for 1 h at 4 °C. The beads were washed three times in the above buffer by centrifugation and resuspension and finally eluted in 25 μl of 2× Laemmli sample buffer and boiled for 5 min. The samples were resolved on a 12% SDS-PAGE and blotted for Rac using a Rac-specific antibody (Upstate catalog number 05-389).

Immunoprecipitation of EE-tagged P-Rex1 from HEK 293T Cells—For the P-Rex1 immunoprecipitation assays, cells were lysed and harvested as described for the activated Rac precipitation assays. Fifteen μl of EE epitope-tagged agarose beads were added to 300 μl of supernatant, which had been diluted 1:1 with Buffer A (25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 μM microcystin, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 100 μg/ml benzamidine, 500 μg/ml Pefabloc SC Plus). The plates were scraped on ice and the lysate was pre-cleared using 20 μl of GST-agarose beads for each sample. The supernatant (~800 μl) was collected and 500 μl of the supernatant was incubated with 5–10 μl of PAK1 PBD-agarose beads for 1 h at 4 °C. The beads were washed three times in the above buffer by centrifugation and resuspension and finally eluted in 25 μl of 2× Laemmli sample buffer and boiled for 5 min. The samples were resolved on a 12% SDS-PAGE and blotted for Rac using a Rac-specific antibody (Upstate catalog number 05-389).

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FIGURE 1. PKA can phosphorylate pure P-Rex1. P-Rex1 (500 ng) was incubated with 50 units of PKA and the reactions were carried out as described under “Experimental Procedures.” Aliquots containing about 100 ng of P-Rex1 were run on an 8% SDS-PAGE, stained with silver, dried, and the autoradiograph prepared. A, the autoradiograph; and B, the silver-stained gel of the P-Rex1 phosphorylated with PKA. C, the effect of phosphorylation on the GEF activity of P-Rex1. The Rac guanine nucleotide exchange assays to monitor P-Rex1 GEF activity were carried out on de-phosphorylated and phosphorylated P-Rex1, as described under “Experimental Procedures” in a 10-μl volume in the presence of 0.0025% CHAPS.

with 0.25 mCi of 32P at 37 °C for 2 h in a 0.5-ml volume. After treatment with LPB, isoproterenol, or p-cAMPS, the cell media was removed and cells were lysed in 0.3 ml of the Rac Mg2+ lysis buffer containing 1 mM sodium orthovanadate, 1 μM microcystin, 10 μg/ml leupeptin, 10 μg/ml apro tinin, 100 μg/ml benzamidine, and 500 μg/ml Pefabloc SC Plus at 4 °C. The lysate was collected in tubes containing 20 μl of GST-agarose beads. The lysate was centrifuged at 14,000 × g in a microcentrifuge at 4 °C and the supernatant collected and processed for EE-tagged P-Rex1 immuno precipitation as described above.

Gel Electrophoresis and Protein Concentration Determination—The identity and purity of protein samples were confirmed by gel electrophoresis on 8 or 12% SDS-polyacrylamide gels (SDS-PAGE) followed by silver staining and/or Coomassie Blue staining. Protein concentrations were determined using densitometric analysis of Simply Blue-stained SDS gel (17). Phosphorylation of P-Rex1 with 50 units of PKA caused a 4–6-fold (Fig. 1B) on an 8% SDS gel. Each lane contains ~250 ng of P-Rex1.

SDS gel (17). Phosphorylation of P-Rex1 with 50 units of PKA caused a shift in the migration position of P-Rex1, producing a single band that co-migrates with the upper band of the P-Rex1 doublet (arrows) on an 8% SDS gel. B, PKA phosphorylation reactions were carried out in the presence of 0–50 units of PKA in a 10-μl volume for 5 min at 30 °C. Phosphorylation of P-Rex1 produced a single band that co-migrated with the upper band of the doublet (arrows) on an 8% SDS gel. Each lane contains ~250 ng of P-Rex1.

FIGURE 2. Treatment of pure P-Rex1 with varying concentrations of λ-phosphatase and PKA. Each reaction contained a total of 4 μg of P-Rex1 purified from Sf9 cells that was treated with the indicated amounts of λ-phosphatase and PKA, as described under “Experimental Procedures.” A, de-phosphorylation of P-Rex1 by λ-phosphatase. P-Rex1 de-phosphorylation was carried out in the presence of 0.3–300 ng of λ-phosphatase in a 50-μl volume for 10 min at 30 °C. De-phosphorylation of P-Rex1 produced a single band that co-migrated with the lower band of the P-Rex1 doublet (arrows) on an 8% SDS gel. B, PKA phosphorylation of P-Rex1. PKA phosphorylation reactions were carried out in the presence of 0–50 units of PKA in a 10-μl volume for 5 min at 30 °C. Phosphorylation of P-Rex1 produced a single band that co-migrated with the upper band of the doublet (arrows) on an 8% SDS gel.

RESULTS

Phosphorylation of P-Rex1 by PKA—When incubated with [γ-32P]ATP and 50 units of PKA for 30 min at 30 °C, P-Rex1 is clearly phosphorylated (+PKA in Fig. 1A). Control reactions performed in the absence of PKA reveal that this preparation of P-Rex1 does not undergo autophosphorylation (−PKA in Fig. 1A). As seen in the left panel of Fig. 1B, the P-Rex1 purified from Sf9 cells migrates as two bands in an 8% SDS gel (17). Phosphorylation of P-Rex1 with 50 units of PKA caused a shift in the migration position of P-Rex1, producing a single band that co-migrates with the upper band of the P-Rex1 doublet (right panel, Fig. 1B), which is co-incident with the phosphorylated band shown on the autoradiograph in Fig. 1A. This suggests that P-Rex1 may be phosphorylated during expression in Sf9 cells and that the doublet represents partially phosphorylated P-Rex1. Interestingly, protein kinase C also phosphorylates P-Rex1 although this event does not cause a shift in the migration of either band in the P-Rex1 protein (data not shown).

The phosphorylation of P-Rex1 by PKA does not require the presence of PIP2 or βγ, however, it markedly changed the ability of P-Rex1 to catalyze PIP2-dependent nucleotide exchange on Rac as shown in Fig. 1C. Note that under control conditions, using untreated P-Rex1 (control) or P-Rex1 incubated at 30 °C in the absence of the kinase (no PKA), 0.5 μM βγ is able to stimulate the GEF activity of P-Rex1 at least 4–6-fold (Fig. 1C, dark bars) compared with P-Rex1 alone (Fig. 1C, white bars). The phosphorylation of P-Rex1 by PKA appeared to inhibit PIP2-mediated P-Rex1 activity although the differences were not statistically significant, as determined using the t test (compare white bars in the left and right panels of Fig. 1C). Importantly, the phosphorylation of the protein completely blocked the ability of βγ to stimulate the GEF activity of P-Rex1, suggesting that this event interferes with the Gβγ interaction of P-Rex1.

De-Phosphorylation of P-Rex1 by λ-Phosphatase—The shift in migration observed upon PKA phosphorylation of P-Rex1 (Fig. 1B), suggested that the lower band of the P-Rex1 doublet purified from Sf9 cells might be a de-phosphorylated form of the protein. To test this possibility, we treated P-Rex1 with increasing concentrations of λ-Phosphatase, an efficient serine/threonine/tyrosine phosphatase (33), resolved the protein on an 8% SDS gel and stained it with Coomassie Blue. Treatment of P-Rex1 with λ-Phosphatase does indeed produce a form of the protein that migrates as a single band at the position of the lower band of the P-Rex1 doublet (top panel, Fig. 2A). Note that as little as 3 ng of the λ-phosphatase per 100 ng of P-Rex1 efficiently de-phosphorylated P-Rex1. Higher concentrations ranging from 30 to 300 ng were only slightly more effective.

Having established that the P-Rex1 as purified from Sf9 cells consists of partially phosphorylated P-Rex1, we determined the concentrations of PKA needed to fully shift the P-Rex1 doublet into the single, slower
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The ability of concentrations of Gs squares significantly different from the activity of the native P-Rex1 (Student’s t test; p < 0.05).

studies. The [35S]GTPγS binding of Rac was measured as described under “Experimental Procedures.” The [35S]GTPγS binding of Rac was measured with no additions (gray bar) or in the presence of 30 nM native P-Rex1 (black bar), λ-PPase-treated P-Rex1 (white bar), or PKA-treated P-Rex1 (striped bar). The bar graph presents the mean ± S.D. from four separate experiments. The activities of λ-PPase-treated and PKA-treated P-Rex1 were significantly different from the activity of the native P-Rex1 (Student’s t test; p < 0.05).

Effects of De-phosphorylation and Phosphorylation on PIP3-dependent P-Rex1 Activity—Once the conditions for the phosphorylation and de-phosphorylation of P-Rex1 were optimized, we used modifications of these protocols to isolate three forms of P-Rex1 (native, de-phosphorylated, and phosphorylated) during its purification on the EE-antibody column. As described under “Experimental Procedures,” treatment of P-Rex1 with the phosphatase or kinase during its purification allowed us to obtain highly pure, native de-phospho and phospho forms of P-Rex1 for biochemical studies. Fig. 3 shows the GEF activity of these three forms of P-Rex1 in vesicles containing PIP3. Note that the native (Sf9) form of P-Rex1 stimulates the Rac guanine nucleotide exchange well (black bar). The de-phosphorylated form of P-Rex1 is much more active (white bar) and phosphorylation of this form of P-Rex1 with PKA inhibits Rac guanine nucleotide exchange significantly (striped bar). The activity of the λ-PPase-treated and PKA-treated P-Rex1 were significantly different from that of the native P-Rex1 doublet, as determined using the t test (p < 0.05). These data provide the result that, in the presence of PIP3, the activity of P-Rex1 is modulated by its phosphorylation state.

Stimulation of PIP3-mediated P-Rex1 Activity by Gβγγ—Next we explored the ability of Gβγ to stimulate the three forms of P-Rex1. The activity of the native (Sf9) P-Rex1 is markedly stimulated by increasing concentrations of Gβγ with an EC50 of 16 nM (Fig. 4A, open squares), a value consistent with that observed previously (17). The ability of concentrations of Gβγ ranging from 0.1 to 300 nM to stimulate the activity of the de-phosphorylated P-Rex1 (closed triangles) was compared with their ability to stimulate native (open squares) and phosphorylated (closed squares) forms of the P-Rex1 protein (Fig. 4, A and B, respectively). As expected from the data in Fig. 3, the λ-PPase-treated P-Rex1 was more active at all Gβγ concentrations tested; however, the EC50 values for Gβγ stimulation was unchanged at 16 nM. The data in Fig. 4B demonstrate that the ability of Gβγ to stimulate the GEF activity of P-Rex1 is markedly blunted by phosphorylation with PKA (compare closed triangles and closed squares). The activation curve for PKA-treated P-Rex1 was greatly right-shifted and the calculated EC50 value was 763 nM. There was a marked difference in the Gβγ activation curve between the de-phospho and phospho forms of the enzyme, as reflected by the 47-fold difference in the EC50 values observed with the two forms of the enzyme. Similar effects were observed with the Gβγ dimer (data not shown), which stimulates P-Rex1 activity to a lesser extent compared with Gβγ (17). These results suggest the following: (a) P-Rex1 is partially phosphorylated in Sf9 cells; (b) de-phosphorylation of P-Rex1 enhances its activity in the presence of PIP3; and (c) phosphorylation of P-Rex1 inhibits both PIP3- and Gβγ-mediated P-Rex1 activity. These results imply that PKA phosphorylates a site (or sites) within P-Rex1 that may be involved in interacting with Gβγ.

Having established that P-Rex1 could be phosphorylated in vitro, we undertook experiments to determine whether activation of receptors coupled to Gi and Gs could elicit similar effects in intact cells. Accordingly, HEK293T cells were transfected with an expression plasmid encoding for an EE-tagged P-Rex1 and stimulated via their endogenous LPA and β-adrenergic receptors, which can couple to Gi and Gs, respectively (35–37). The data presented in Fig. 5 show that the EE-tagged P-Rex1 could be efficiently immunopurified from the transfected cells (Fig. 5A, lane 1). Interestingly, the P-Rex1 protein rapidly isolated from these cells also migrated as a doublet in an 8% SDS gel (Fig. 5, A and B, lane 1), suggesting that P-Rex1 is also partially phosphorylated in HEK293T cells. The reasons for this finding have not been explored but it may be because of overexpression of the protein. Importantly, the existence of both forms of P-Rex1 in HEK293T cells facilitates monitoring of its phosphorylation state because the phosphorylated form of the P-Rex1 protein migrates as a higher band compared with the de-phosphorylated form (Figs. 1 and 2).

To explore how receptor activation affected the activity and phosphorylation state of P-Rex1 in HEK293T cells, P-Rex1 transfected cells were stimulated with LPA and isoproterenol to activate the Gi and Gs α subunits, respectively. These stimuli would be expected to cause the release of the Gβγ dimer and to activate PKA (via Gs-coupled receptors), and mimic the effects of the in vitro experiments shown in Fig. 4. As expected, the P-Rex1 immunoprecipitated from cells stimulated with 50 nM LPA migrated as a doublet (Fig. 5A, lane 2), as this treatment does not lead to the activation of PKA. Treatment of cells with the phosphodiesterase inhibitor rolipram (Fig. 5, R) alone also produced immunoprecipitated P-Rex1 that migrated as a doublet (Fig. 5A, lane 6). However, treatment of cells with rolipram followed by isoproterenol or isoproterenol alone led to all the immunoprecipitated P-Rex1 migrating in the upper band (Fig. 5A, lanes 4 and 5). This result is consistent with the stimulation of PKA in cells via the activation of Gi-coupled β-adrenergic receptors. To confirm this point, we treated the cells with SncAMPs, a specific and potent activator of PKA (38). The results demonstrated that P-Rex1 immunoprecipitated from cells treated with SncAMPs migrated in a single band at the position of the upper band in the doublet (Fig. 5A, lane 3).

To determine whether treatment of HEK cells with LPA, rolipram, isoproterenol, isoproterenol + rolipram, or SncAMPs lead to phos-
Phosphorylation of P-Rex1, we labeled the cells with 32P for 2 h prior to immunoprecipitating the P-Rex1 bands shown in Fig. 5A. Interestingly, the autoradiograph presented in Fig. 5B shows that both the upper and lower bands of the P-Rex1 doublet incorporate 32P during the control incubation (Fig. 5B, lane 1), suggesting that multiple sites are phosphorylated in this protein. Importantly, treatment of the cells with either S3-cAMPs or isoproterenol alone or a combination of isoproterenol and rolipram to activate PKA leads to increased phosphorylation of P-Rex1 and collapses the doublet into the single, upper band (Fig. 5B, lanes 3–5). These results clearly indicate that P-Rex1 is important for the interaction with the Gβγ dimer and the latter by phosphorylation of a region of P-Rex1 important for the interaction with the Gβγ dimer.

To demonstrate that the inhibition of Rac activation in cells upon treatment with PKA activators, such as S3-cAMPs, was specifically because of P-Rex1 phosphorylation and not because of other secondary effects, we performed experiments in the presence of a number of other Rac-GEFs. Accordingly, we measured levels of activated Rac in cells transfected with Vav2-WT, constitutively active Vav2-onco, or Tiam1 C-1199. As noted under “Experimental Procedures” the transfection conditions were optimized to ensure that all the Rac-GEFs tested were expressed at levels where they promoted comparable levels of Rac activation. Cells were treated in the absence or presence of S3-cAMPs (Fig. 6D) and as expected, treatment with S3-cAMPs had a great inhibitory effect on Rac activation in cells expressing P-Rex1 compared with untreated cells (Fig. 6D, lanes 9 and 10). Treatment with S3-cAMPs did not affect Rac activation in cells expressing P-Rex1.
not have any effect on Rac activation in cells expressing constitutively active Vav2-onco and Tiam1 C-1199 (Fig. 6D, lanes 5–8). Rac activation is slightly diminished in cells expressing Vav2-WT but not as dramatically as in cells expressing P-Rex1.

**DISCUSSION**

Rac is a small monomeric G protein that plays an important role in several different processes such as leukocyte migration, chemotaxis, platelet aggregation, and superoxide production (39). As a key player in so many different cellular and physiological processes, Rac activation is very tightly regulated by proteins such as GEFs. GEFs can stimulate Rac activation by catalyzing the exchange of GDP for GTP on Rac. Thus far, several different GEFs have been identified for Rac including Sos1, Vav1–3, Trio, Ost, Bcr, Abr, Ect2, Tiam1, P-Rex1, P-Rex2a, and P-Rex2b (1, 3, 4, 6). Most GEFs contain multiple functional domains consisting at least of a core DH/PH tandem domain, which apparently confers GEF activity. The other functional domains contained within GEFs, such as DEP and PDZ domains, are thought to be involved in intramolecular regulation of GEF activity or in coupling GEFs to specific upstream signals or in mediating other cellular functions (6, 7).

Phosphorylation is an important mode of regulation for a number of Rac-GEFs. Of all the GEFs identified for Rac, Vav1–3, Ect2, and Tiam1 have been shown to be stimulated by phosphorylation by certain kinases, such as protein kinase C and calmodulin-dependent protein kinase II (18–23). The P-Rex proteins are the newest members of the diverse Rac-GEF family and they have been shown to be synergistically stimulated by PIP2 and Gβγ subunits (1, 3, 4, 17); however, their regulation by phosphorylation has not been studied. Neutrophil chemotaxis is known to be inhibited by the stimulation of G, coupled receptors that are thought to stimulate PKA (13, 16, 40–42). Because P-Rex1 is highly expressed in neutrophils and contains 28 predicted PKA phosphorylation sites (using NetPhos (43)), we determined the effects of PKA phosphorylation of P-Rex1 on its GEF activity.

This study reports four major findings in the regulation of P-Rex1 GEF activity by PKA. First, P-Rex1 is a substrate for PKA. An upward shift in the migration of the P-Rex1 doublet was observed upon PKA phosphorylation of P-Rex1 (Fig. 1B). A similar shift in migration has been observed with Tiam1 (19) and Ect2 (22), two other Rac-GEFs, however, their sites of phosphorylation have not been determined.

Second, de-phosphorylation and phosphorylation of P-Rex1 affects its PIP2-mediated Rac GEF activity. De-phosphorylation of the P-Rex1 doublet by λ-PPase lead to enhanced Rac activity and produced a downward shift in the migration of the P-Rex1 protein (Figs. 2 and 3). This is in direct contrast from the results found with Ect2, which has been shown to be inhibited upon de-phosphorylation with λ-PPase (22). Moreover, PKA phosphorylation of P-Rex1 leads to the inhibition of PIP2-mediated P-Rex1 GEF activity (Fig. 3). Both of these findings are novel and the latter finding is strikingly different from the positive regulation of other Rac-GEFs by phosphorylation (18–23). However, these results are consistent with the ability of G, coupled receptors to inhibit the effects of Rac in intact myeloid cells, such as chemotaxis and superoxide release (13, 14).

Third, PKA phosphorylation of P-Rex1 diminishes its modulation by Gβγ2γ2. The EC50 of PKA-phosphorylated P-Rex1 is 763 nM, which is more than 47-fold lower than the EC50 of de-phosphorylated or doublet P-Rex1 for Gβγ2γ2. These results suggest that the site of PKA phosphorylation of P-Rex1 might be within the Gβγ interaction domain of P-Rex1. A study done by Hill et al. (5) has shown that the P-Rex1 mutant containing the isolated DH/PH tandem domain can still be modulated by Gβγ2γ2, suggesting that the Gβγ interaction domain lies somewhere within the first 400 amino acids of the P-Rex1 protein, although the other domains may serve a minor modulatory role (5). Thus it is possible that the PKA phosphorylation site(s) that confers inhibition of P-Rex1 GEF activity mediated by Gβγ5 may lie within this region of the P-Rex1 protein. Future studies will determine the exact sites of phosphorylation on P-Rex1.

Last, the results demonstrate that phosphorylation of P-Rex1 occurs in cells upon stimulation of the G, coupled β-adrenergic receptor (Fig. 5), suggesting that the phosphorylation of P-Rex1 is a physiologically relevant process. Moreover, the phosphorylation of P-Rex1 diminished
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its ability to activate Rac in cells (Fig. 6). This directly corroborates our in vitro data, and suggests a novel mechanism for Gαi-coupled receptor-mediated inhibition of Rac activity via the phosphorylation of a Rac-GEF, P-Rex1, which diminishes its ability to be stimulated by its co-activators, PIP3 and Gβγ (Figs. 3 and 4). We have noted that not all Gβγ dimers stimulate P-Rex1 equally (17). Thus, the intriguing possibility exists that stimulation of Gαi-coupled receptors not only leads to the phosphorylation of P-Rex1, rendering it less active, but that the Gβγ dimers released following activation of Gαi-coupled receptors are less effective in stimulating P-Rex1. This would ensure efficient inhibition of Rac activation at two distinct levels. Further studies need to be done to confirm this notion.

There are still many unanswered questions regarding the regulation of P-Rex1 by phosphorylation. For instance, the number and identity of the sites of phosphorylation of P-Rex1 have yet to be determined. Because both the upper and lower band of the native P-Rex1 appears to be phosphorylated in HEK293T cells (Fig. 5B) it is likely that P-Rex1 will have a complex pattern of phosphorylation. Indeed, initial mass spectrometry analysis of the P-Rex1 phosphorylated with PKA in vitro shows that many serine residues are phosphorylated. It will also be important to ascertain if P-Rex2a and P-Rex2b, the other members of the P-Rex family, are regulated by similar mechanisms.

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