Differential Sensitivity of P-Rex1 to Isoforms of G Protein βγ Dimers*

Received for publication, June 2, 2005, and in revised form, November 1, 2005 Published, JBC Papers in Press, November 21, 2005, DOI 10.1074/jbc.M506034200

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

P-Rex1 is a specific guanine nucleotide exchange factor (GEF) for Rac, which is present in high abundance in brain and hematopoietic cells. P-Rex1 is dually regulated by phosphatidylinositol (3,4,5)-trisphosphate and the GPγ subunits of heterotrimeric G proteins. We examined which of the multiple G protein α and βγ subunits activate P-Rex1-mediated Rac guanine nucleotide exchange using pure, recombinant proteins reconstituted into synthetic lipid vesicles. AlF4−-activated Gαi, Gαq, Gα12, or Gα13 βγ subunits were unable to activate P-Rex1. GPγ dimers containing GPβ1–4 complexed with γ2 stimulated P-Rex1 activity with EC50 values ranging from 10 to 20 nM. GPβ1–2γ2 was not able to stimulate P-Rex1 GEF activity. Dimers containing the β1 subunit complexed with a panel of different γ subunits varied in their ability to stimulate P-Rex1. The β1γ1, β1γ7, β1γ10 and β1γY15HA dimers all activated P-Rex1 with EC50 values ranging from 20 to 38 nM. Dimers composed of β1γ12 had lower EC50 values (≈112 nM). The farnesylated γ11 subunit is highly expressed in hematopoietic cells; surprisingly, dimers containing this subunit (β1γ11) were also less effective at activating P-Rex1. These findings suggest that the composition of the GPγ dimer released by receptor activation may differentially activate P-Rex1.

Heterotrimeric G proteins (G proteins),2 monomeric G proteins (small G proteins), and the p110-γ isoform of phosphatidylinositol 3-kinase (PtdIns 3-kinase) all play important roles in chemotaxis of neutrophils and other cells of hematopoietic lineage (1–4). Chemotaxis is a complex process that involves the functional coordination of a diverse array of proteins to produce cellular movement via rearrangements of the actin cytoskeleton (5). The activation of Rac, a small G protein, is a key step in chemotaxis (6). Rac is a member of the Rho subfamily of small G proteins. Like the other small G proteins, Rac is a binary switch that is inactive in the GDP bound state and active in the GTP bound state. The GDP to GTP exchange activity of Rac is regulated by at least three groups of proteins: GDP dissociation inhibitors, GTPase activating proteins, and GEFs (guanine nucleotide exchange factors). GDP dissociation inhibitors stabilize the GDP bound state of Rac, whereas GTPase activating proteins enhance the intrinsic GTPase activity of Rac. Although Rac activation can occur as a result of inhibition of GDP dissociation inhibitors and/or GTPase activating proteins, the stimulation of Rac-GEFs is thought to be the most biologically important mode of Rac activation (1).

The P-Rex family of proteins is a recently identified group of Rac-GEFs that has been shown to be dually modulated by the G protein βγ subunit and the lipid messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3) (7–9). The P-Rex family consists of three members: P-Rex1, P-Rex2a, and P-Rex2b (7–9). P-Rex1 is the only member of the P-Rex family that is expressed in hematopoietic cells, whereas P-Rex2a and P-Rex2b are found mainly in skeletal muscle and heart, respectively (7–9). All three members of the P-Rex family share a similar domain topology and exhibit GEF activity. They are all multimodular proteins containing an N-terminal tandem Dbl homology and pleckstrin homology domains followed by two DEP and two PDZ domains (7–9). P-Rex1 and P-Rex2a also contain an inositol polyphosphate 4-phosphatase (InsP4, 4-phosphatase) domain that is not present in P-Rex2b; however, neither of these proteins demonstrates inositol polyphosphate 4-phosphatase activity (7, 8). Thus the function of this latter domain still remains elusive.

P-Rex proteins apparently serve as a link between phospholipid and G protein-coupled receptor (GPCR) signaling and act as coincidence detectors for signals arising from these two pathways (10). The activation of GPCRs leads to the release of GPγ subunits, which have been shown to activate the p110-β and p110-γ isoforms of PtdIns 3-kinase leading to the production of PIP3 in hematopoietic cells such as neutrophils (11–13). Therefore, the stimulation of GPCRs in hematopoietic cells leads to the production of both activators of P-Rex1. Although the specific GPCR(s) responsible for P-Rex1 activation in hematopoietic cells has not been identified, the stimulation of Gγ coupled receptors expressed in neutrophils, such as the Met-Leu-Phe receptor, is likely to lead to the activation of P-Rex1 via the liberation of GPγ subunits. However, an unanswered question is: does the composition of the GPγ dimer released upon receptor activation differentially stimulate P-Rex1?

This issue is of central importance as it has become apparent that not all GPγ subunits are equivalent at interacting with receptors or effectors. For example, there are large differences in the ability of certain isoforms of the GPγ dimer to couple the β-adrenergic and the adenosine A2A receptors to the Gα subunit (14), the α2-adrenergic receptor to the Gα subunit (15), and the adenosine A1 or 5-HT1A receptors to the Gα subunit (16). There are also clear differences in the ability of dimers to regulate effectors (14, 17–23). Interestingly, only the β2γ2 dimer is able to inhibit the T-type calcium channel (18) and the β2γ2 dimer inhibits the muscarinic K+ channel (Kir3), whereas dimers containing the β1γ11 subunits activate the K+ channel (19). In addition, we have demonstrated that the p110γ isoform of PtdIns 3-kinase is differentially regulated by certain isoforms of GPγ (20). Given that both PtdIns 3-kinase and P-Rex1 are found in cells of hematopoietic origin (7, 11) and have been shown to be regulated by G protein βγ subunits, we hypothesized that P-Rex1 might have a GPγ sensitivity profile similar to that of PtdIns 3-kinase. Therefore, we undertook a study to test the...
ability of a panel of Gβγ dimers to activate P-Rex1. Moreover, as several guanine nucleotide exchange factors (p115 RhoGEF, LARG, and PDZ-RhoGEF) have been shown to be stimulated by the heterotrimeric G protein Gaβγ (24–27), we also explored the ability of a panel of G protein α subunits to regulate P-Rex1. We found that P-Rex1 is not modulated by activated Ga subunits but that P-Rex1 is selectively regulated by Gβγ subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—The reagents used for Sf9 cell culture and purification of G protein α and βγ subunits have been described (28, 29). GDP, imidazole, HEPES, Triton X-100, and cyanogen bromide-activated agarose were from Sigma. CHAPS and GTPγS were from Roche Molecular Biochemicals; Genapol C-100 was from Calbiochem; Ni2+-nitrilotriacetic acid Superflow resin was from Qiagen; [35S]GTPγS was from PerkinElmer Life Sciences; Source® 15Q ion exchange resin and the glutathione-Sepharose 4B (GST) resin were from Amersham Biosciences; Centricon 30 concentrators were from Millipore; and Ultra 30 and 100 concentrators were from Amicon. Porcine brain-extracted L-α-phosphatidylserine and L-α-phosphatidylinositol and PIP3 were from Avanti Polar Lipids. Bovine liver L-α-phosphatidylinositol and PIP3 were purchased from Sigma. All other materials were of the highest available purity.

**Recombinant Protein Constructs**—The protocols for constructing the G protein baculoviruses used in this study have been published: Gaα, Gaα (30), Gaα (31), βγ, γ (14, 32), βγ (33), γ (32), and γ (34, 35). The baculovirus for γ was the kind gift of Dr. David Siderovski, University of North Carolina (36). The baculovirus for γ and γ were the kind gifts of Dr. Janet D. Robishaw, Weiss Center for Research, and Dr. Pat Casey, University of North Carolina, respectively. The baculoviruses for Gaα and Gaα were gifts from Dr. T. Kozasa and Dr. P. Sternweis, respectively (24, 25). The cDNA encoding EE-tagged P-Rex1 and the recombinant baculovirus expressing EE-tagged P-Rex1 were kindly provided by Dr. Leonard R. Stephens, Cambridge University, United Kingdom (7). The bacterial expression plasmid encoding GST-Rac was obtained from Dr. Ian Macara at the University of Virginia.

**Culture and Infection of Sf9 Cells—**Spodoptera frugiperda cells (Sf9 insect cells) were cultured and maintained at 27 °C (20, 28, 29). Sf9 cells were infected at a multiplicity of infection of 3 with recombinant baculoviruses expressing G protein α and/or βγ subunits or with virus for the EE-tagged P-Rex1 and prepared as described in Kerchner et al. (20). Briefly, the cells were harvested 60–80 h post-infection by centrifugation at 100 × g. All cell pellets were washed three times in insect cell phase-buffered saline (IPBS: 7.3 mM Na2HPO4, pH 6.2, 58 mM KCl, 47 mM NaCl, 5.0 mM CaCl2) and re-suspended in an ice-cold buffer composed of 25 mM HEPES, pH 7.5, 1 mM MgCl2, 120 mM NaCl, 1 mM 2-mercaptoethanol supplemented with a mixture of freshly prepared protease inhibitors (aprotinin, leupeptin, and pepstatin (at 2 μg/ml), benzamidine at 20 μg/ml, and Pefabloc SC Plus at 100 μg/ml) and frozen at −80 °C until use.

**Preparation of the EE-antibody Column**—The hybridoma for the EE-epitope antibody was a gift of Dr. Gernot Walter at the University of California, San Diego (37), and the EE-antibody was produced at the core hybridoma facility of the University of Virginia. The mouse monoclonal EE-antibody was dialyzed against three changes of borate saline (30 mM boric acid, 150 mM NaCl, pH 7.8), concentrated using an Amicon Ultra 30 concentrator, and stored at −20 °C at a concentration of ~3 mg/ml, as determined by A260 (A260 = 1.5 for 1 mg/ml). Cyanogen bromide-activated agarose (CNBr-agarose) was prepared as follows: 1 g of CNBr-agarose was swollen and washed in 250 ml of 1 mM HCl for 15–30 min. About 3.5 ml of swollen resin was washed with 10–20 ml of borate saline prior to the addition of 3 ml of concentrated EE-antibody. The resin and antibody were incubated overnight at 4 °C with constant agitation. After the incubation, the resin was collected by centrifugation at 8–10 × g and the supernatant discarded. The resin was then incubated for 2 h in 0.1 M Tris, pH 8.0 (2 ml per ml of resin), washed twice with 10–20 ml of borate saline, and stored in borate saline containing 0.02% sodium azide at 4 °C until further use.

**Purification of P-Rex1—**Sf9 cells were infected for 60–80 h with the P-Rex1 virus and harvested via low speed centrifugation. The cells were lysed by nitrogen cavitation at 700 p.s.i. for 20–30 min in a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, and protease inhibitors. The cell lysate (60 ml) was centrifuged at 1,000 × g prior to the cytosol (60 ml) being loaded onto a 1-ml EE antibody-agarose column by gravity flow. To purify the protein, the EE column was washed with 10 ml of 25 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, and 1% Triton, followed by a 10-ml wash with 25 mM HEPES, pH 7.5, 500 mM NaCl, 2 mM EGTA. P-Rex1 was eluted in 1-ml fractions with 25 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, and 0.4 mg/ml EE peptide (CxEEEYMPME). The EE-P-Rex1 prepared using this protocol is highly pure as demonstrated in Fig. 1A. Approximately 2–3 ml of 0.4–0.8 μg/ml P-Rex1 was obtained from 500 ml of Sf9 culture.

**Purification of Rac—**The recombinant GST-Rac1 (Rac) protein was purified using a GST resin per the manufacturer’s protocols (Amersham Biosciences). One liter of DH5α bacterial cell culture expressing Rac was harvested and lysed using a French Press in 30 ml of buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM MgCl2, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors. To extract the Rac that was membrane associated, 1% Triton X-100 was added to the cell lysate and 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 at least an hour at 4 °C with constant mixing. After the incubation, the beads were washed twice with 10 ml of phosphate-buffered saline containing 1% Triton X-100 and three times with 10 ml of phosphate-buffered saline alone, using low speed centrifugation (3,000 × g for 2 min) and gentle resuspension. The beads containing the last wash were poured into a column and allowed to drain by gravity flow. Rac was eluted off the beads in 1-ml fractions with the following buffer: 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, and 5 mM reduced glutathione. The high purity of the Rac obtained using this method can be seen in Fig. 1B. Approximately, 3 ml of 2–3 mg/ml Rac was obtained from 1 liter of DH5α bacterial culture.

**Purification of G Protein βγ Subunits—**The βγ dimers were purified using Gaα affinity chromatography as described by Kerchner et al. (14, 20). The βγ dimers were purified as described (31). All βγ subunits, except βγ, were stored in 20 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, and 0.1% CHAPS. βγ was stored in 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% CHAPS, and protease inhibitors. Verification of the proper post-translational processing of the γ subunit in the dimers was accomplished using matrix-assisted laser desorption ionization mass spectrometry as described (38). In keeping with our previous experience (35, 38), the γ subunits in the dimers used in this study were fully and properly modified with the geranylgeranyl (γ, γ) and farnesy1 (γ) isoprenoid groups. The βγ dimers produced using these protocols were highly pure as published (14, 20, 31).

**Purification of G Protein α Subunits—**The procedure for purifying the α subunits of Ga, Ga, and Ga has been described (14, 20, 31). Viruses
encoding either Gα12 or Gα13 and β1 and γ2 subunits engineered to have a hexahistidine and FLAG tag at their N termini (β1N and γ2F), were used to express specific heterotrimeric G proteins in Sf9 cells. Purification of Gα12 and Gα13 was accomplished using minor variations of the methods of Kozasa (39). For Gα12, the Ni⁺² column was equilibrated and washed with G12 buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 0.5% (v/v) Genapol, 10 mM β-mercaptoethanol, 10 μM GDP, 15 mM imidazole, and protease inhibitors. For the elution step, G12 buffer was modified by lowering the NaCl and imidazole to 50 and 10 mM, respectively; MgCl₂ and GDP were raised to 50 mM and 20 μM, respectively, and 10 mM NaF and 30 μM AlCl₃ were added. The eluted fractions were pooled and further purified as described (39).

For Gα13, the Ni⁺² column was equilibrated with G13 buffer containing 20 mM Tris, pH 8.0, 300 mM NaCl, 1 mM MgCl₂, 0.2% (v/v) Genapol, 1 mM β-mercaptoethanol, 10 μM GDP, 5 mM imidazole, 0.5% n-octyl-glucoside, and protease inhibitors. The Gα13 column was washed with G13 buffer containing 1% n-octylglucoside and the Gα13 protein was eluted using G13 buffer containing 1% n-octylglucoside, 50 mM MgCl₂, 10 mM NaF, and 30 μM AlCl₃. The eluted fractions were pooled and further purified as described (39).

All Gα subunits were eluted with AlCl₃ and NaF (AlF₄⁻) ensuring that the proteins were properly folded and functional, as determined by their ability to be activated by AlF₄⁻. The Gα subunits were stored in the following buffers: 1) Gαi in 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10 μM GDP, and 0.1% CHAPS; 2) Gαi in 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 10 μM GDP, and 0.2% CHAPS; 3) Gαi in 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 10 μM GDP, and 0.4% cholate; 4) Gαi in 20 mM Tris, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 1 mM GDP, 10% glycerol and 0.1% CHAPS; and 5) Gαi in 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 10 μM GDP, and 0.1% CHAPS.

As another test of functionality, we monitored the ability of each of the Gα subunits to bind [35S]GTPγS at 30 °C for 60 min. The reactions were carried out in the presence of 20 mM Hepes, pH 7.5, 100 mM NaCl, and 1 mM EGTA, 5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 1 mM GTPγS, and 2 μl of [35S]GTPγS (1250 Ci/mmol) for an 80-μl reaction. GTP binding to Gαi was carried out in the presence of 0.5 μM GTPγS. The reactions were initiated by the addition of [35S]GTPγS and terminated by dilution with 1.5 ml of wash buffer containing 20 mM Hepes, pH 7.5, 100 mM NaCl, and 10 mM MgCl₂ and filtration over HAWP nitrocellulose filters in a Millipore filtration apparatus. The filters were washed three times with a total of 7.5 ml of wash buffer and the amount of [35S]GTPγS-Gαi bound to the filter was counted in a liquid scintillation counter.

**Activation of G Protein α Subunits**—The Ga subunits were activated prior to reconstitution into the phospholipid vesicles to monitor their effect on P-Rex1-mediated Rac guanine nucleotide exchange. Each Ga subunit was diluted 10-fold into activation buffer containing 20 mM Hepes, pH 7.5, 100 mM NaCl, and 1 mM EGTA, 5 mM MgCl₂, 10 mM NaF, and 30 μM AlCl₃ and incubated at room temperature for 30 min. The activated Ga subunits were not directly added to the lipid vesicles, as were the Gβγ subunits, because the AlF₄⁻ had a detrimental effect on the liposomes. Instead, the activated Ga subunits were diluted 10-fold more (a final dilution of 100-fold) into the assay and added to the reaction at 20-s intervals (~2 min after the addition of Rac). The a subunits (Gα12, Gα13, G12, and G13) were diluted a 100-fold to maintain optimal detergent levels between 0.001 and 0.004% CHAPS or cholate and were tested at concentrations ≥0.5 μM (Fig. 3).

**Gel Electrophoresis and Protein Concentration Determination**—The identity and purity of protein samples were confirmed by gel electrophoresis on 8 or 12% SDS gels followed by silver staining and/or by Coomassie Blue staining. Protein concentrations were determined using densitometric analysis of Simply Blue-stained gels using phosphoimager and ovalbumin to generate standard curves for P-Rex1 and the G proteins (Rac, α and βγ), respectively.

**Calculations, Statistics, and Expression of Results**—The data presented under “Results” are representative of 3 or more experiments. The data representing the effect of the various GβγS on P-Rex1-mediated Rac guanine nucleotide exchange activity were fitted to sigmoidal curves using the routines provided in GraphPad Prism. The EC₅₀ values presented in Table 1 were obtained by fitting normalized values to sigmoidal dose-response curves using the routines provided in GraphPad Prism. The data were normalized to percent of maximal Rac GTPγS.
binding. Three Gβγs (β2γ2, β1γ1, and β1γ2) were very poor stimulators of P-Rex1 GEF activity. These Gβγs were not normalized, rather they were plotted in comparison to an active βγ run in the same experiment. Thus EC_{50} values are not reported for these Gβγs in Table 1. The statistical significance between the fits of different data sets was determined on normalized data using the F statistic (40). Linear regression routines in GraphPad Prism were used to plot the straight lines in Figs. 2, B and C, and 4 (β2γ2).

RESULTS

Preparation of Purified P-Rex1, Rac, and G Proteins—We prepared highly purified recombinant proteins to test the specificity of interaction of G protein α and βγ subunits with P-Rex1. The purity of the Rac, P-Rex1, and the Gα13 and Gα13 subunits prepared for this study are shown in Fig. 1. Note that the EE-antibody column purified recombinant P-Rex1 essentially to homogeneity from the supernatant fraction of SF9 cells in a single step (Fig. 1A). The bacterial Rac was also purified to homogeneity using its GST tag in a single step (Fig. 1B). Fig. 1C shows that the G12 and Gα13 subunits prepared by elution from a βγ column are also highly pure. The Gα1, Gα12, Gα13 subunits and the Gβγ dimers used in this study are of equivalent purity, as described (14, 20, 31, 33, 35).

P-Rex1 Activity—To determine the ability of various G protein α and βγ isomers to activate P-Rex1, we performed P-Rex1 activity assays in synthetic vesicles containing phosphatidylserine, phosphatidylinositol, and phosphatidylinositol with/without phosphatidylinositol (3,4,5)-trisphosphate as described under “Experimental Procedures.” In liposomes devoid of PIP3, neither P-Rex1 nor βγ alone stimulated Rac guanine nucleotide exchange very well (Fig. 2A, white bars). Welch et al. (7) have shown that P-Rex1 combined with Gβγ can stimulate Rac guanine nucleotide exchange in the absence of PIP3. Using our preparations of P-Rex1 and GST-Rac, we did not observe much stimulation in the absence of PIP3. The reason for this discrepancy is not clear. One difference between our assay conditions and the previous studies (7, 8, 41) is that we used GST-Rac as opposed to EE-tagged Rac as the target of P-Rex1. Thus, one potential explanation for the differences observed may be that the larger GST tag is interfering with the PIP3 independent activation of Rac by P-Rex and the Gβγ dimer. This possibility can be explored in future studies of the interactions between Rac, P-Rex1, and Gβγ.

In the presence of liposomes containing 10 μM PIP3, Rac guanine nucleotide exchange was stimulated by P-Rex1 alone and was further

FIGURE 1. Purification of EE-tagged P-Rex1, GST-Rac, and G protein α subunits. A, recombinant, EE-tagged P-Rex1 was isolated from SF9 cells as described under “Experimental Procedures.” The purified protein was separated on an 8% SDS-PAGE and visualized by silver staining. B, recombinant, GST-tagged Rac (48 kDa) was isolated from bacterial cells as described under “Experimental Procedures.” The purified GST-Rac protein was separated on a 12% SDS-PAGE and visualized by silver staining. The band represents 112 kDa (GST-Rac). C, recombinant Gα13 and Gα13 (~40 kDa) were purified as described under “Experimental Procedures.” The purified proteins (400–600 ng of total protein) were resolved on a 12% SDS-PAGE and visualized with Coomassie staining.

FIGURE 2. Effect of PIP3 and Gβγ on P-Rex1 activity. A, PIP3 dependence of P-Rex1 activity. A bar graph representing Rac guanine nucleotide exchange in the presence of liposomes containing 0 (white bars) or 10 μM PIP3 (dark bars) in a 10-μl volume at 30 °C for 5 min. The data are representative of three separate experiments. B, a 4-min time course of Rac guanine nucleotide binding at room temperature in vesicles containing PIP3. P-Rex1 activity was measured as the amount of [35S]GTP·S bound to GST-Rac as described under “Experimental Procedures.” Rac exchange activity was tested with no additions (■), with 50 nM β1γ2 (▲), with 30 nM P-Rex1 (▼), and with 30 nM P-Rex1 and 30 nM Gβ1γ2 (●). C, effect of increasing concentrations of β1γ2 on P-Rex1 activity. Rac guanine nucleotide exchange activity of 100 nM Rac was measured in the presence of increasing concentrations of β1γ2 in the presence or absence of 30 nM P-Rex1, as described under “Experimental Procedures.” To calculate the percent increase in Rac GTP·S binding upon addition of β1γ2, Rac GTP·S binding in the absence of any additions and presence of 30 nM P-Rex1 were subtracted from the β1γ2 (●) and the P-Rex1+β1γ2 (□) curves, respectively. The P-Rex1+β1γ2 data represents the mean ± S.D. of 10 experiments.
enhanced by addition of $\beta_1\gamma_2$ (Fig. 2A, black bars). Having established assay conditions, which allowed a marked effect of G$\beta\gamma$ on P-Rex activity, all future experiments were performed in the presence of PIP$_3$ containing liposomes.

Fig. 2B shows the time dependence of Rac guanine nucleotide exchange activity. Note that the Rac guanine nucleotide exchange activity of pure Rac alone was low in the absence of P-Rex or G$\beta\gamma$ (closed squares). Addition of 50 nM $\beta_1\gamma_2$ to Rac had no effect on the guanine nucleotide exchange activity of Rac (closed triangles). This was the case for all 13 G$\beta\gamma$ dimers tested in this study (data not shown). As anticipated, addition of 30 nM P-Rex1 alone increased the amount GTP$_\gamma$S bound to Rac (inverted closed triangles). The addition of 50 nM $\beta_1\gamma_2$ further stimulated the activity of P-Rex1 (closed diamonds). As expected, in the presence of both P-Rex1 (30 nM) and $\beta_1\gamma_2$ (50 nM), Rac guanine nucleotide exchange was stimulated to a much greater extent than in the presence of either P-Rex1 or $\beta_1\gamma_2$ separately, illustrating the synergistic interaction between P-Rex1 and $\beta_1\gamma_2$ (7).

Fig. 2C demonstrates that $\beta_1\gamma_2$ was able to modulate P-Rex1 activity in a concentration-dependent manner in the presence of 30 nM P-Rex1 (open squares). The dimer had little effect in the absence of P-Rex1 (closed circles). The EC$_{50}$ of the $\beta_1\gamma_2$-stimulated P-Rex1 activity was 20 nM, which is consistent with the EC$_{50}$ values noted for the effect of G$\beta\gamma$ on other effectors, such as type II adenyl cyclase, phospholipase C-\(\beta\), and PtdIns 3-kinase (14, 20) and more potent than previously reported for P-Rex1 (7).

FIGURE 3. Lack of activation of P-Rex1 by G$\alpha$ subunits. The guanine nucleotide exchange activity of 100 nM GST-Rac was assayed in the presence and absence of 30 nM P-Rex1 with the following concentrations of G$\alpha$ subunits: A, 160 nM G$_{12}\alpha$; B, 195 nM G$_{13}\alpha$; C, 400 nM G$_q\alpha$; D, 861 nM G$_i\alpha$; E, 50 nM G$_{12}\gamma_2$, and F, 100 nM G$\beta_1\gamma_2$. Because of the inhibitory effect of the Ga activation buffer on the P-Rex GEF activity assay each Ga subunit was diluted 100-fold (final) into the activity assay, as described under “Experimental Procedures.” The Rac and Rac + P-Rex1 reactions were carried out in the presence of appropriate control buffers for each Ga. The data are representative of three separate experiments.

PIP$_3$-dependent P-Rex1 Activity in the Presence of Various G$\alpha$ Subunits—We tested the ability of representatives of the different Ga families to modulate P-Rex1 activity using five distinct Ga subunits (G$_s$, G$_i$, G$_q$, G$_{12}$, and G$_{13}$). Although we used concentrations of G$\alpha$ subunits that are supermaximal (>50 nM) in other assays, such as activation of adenyl cyclase or phospholipase C-\(\beta\) (14, 20), none of the G$\alpha$ subunits tested were able to stimulate Rac guanine nucleotide exchange activity above that seen with P-Rex1 alone (Fig. 3, A–E). It should be noted that the Ga activation buffer containing AlF$_4$ was inhibitory to the P-Rex1 activity assay (Fig. 3F). However, under these conditions, P-Rex1 alone was still able to catalyze nucleotide exchange on Rac and this effect was further enhanced in the presence of 100 nM G$\beta_1\gamma_2$ (Fig. 3F). Finally, as described under “Experimental Procedures,” all $\alpha$ subunits showed activity in at least two other assays, the ability to dissociate from the $\beta\gamma$ dimer in the presence of aluminum fluoride and the ability to couple to receptors or activate adenyl cyclase (G$_s$) or phospholipase C-\(\beta\) (G$_i$). Thus, the current data suggest that P-Rex1 responds selec-
The GBγ Sensitivity of P-Rex1

tively to the βγ dimer, a property shared with ion channels and PtdIns 3-kinase (17–20).

**Table 1** P-Rex Activity in the Presence of Various GBγ Dimers—P-Rex1 is highly expressed in hematopoietic cells (7) and GBγ subunits play a central role in the activation of these cells (20), especially by receptors such as the fMet-Leu-Phe, which couples to the Goα subunit (42). In keeping with this mechanism, P-Rex1 is markedly activated by GBγ. However, this isoform represents only one of 84 possible GBγ dimer combinations (43). As most GBγ subunits are ubiquitously expressed and numerous studies have demonstrated that certain GBγ isoforms selectively regulate effectors (14, 17, 19, 44), we tested a panel of 12 different βγ dimers to determine which GBγ isoforms could stimulate the activity of P-Rex1 *in vitro*.

The panel of G protein βγ isoforms tested was based on our experience with PtdIns 3-kinase, another GBγ-sensitive effector in hematopoietic cells (20). The effect of the 5 different GBγ subunits on P-Rex1 activity was determined using a series of pure GBγγ dimers. Fig. 4 shows that β1γ1 (open squares), β2γ2 (closed triangles), and β3γ3 (closed inverted triangles) are all stimulated P-Rex1 activity with similar potencies and efficacies. Interestingly, although β1γ1 (closed diamonds) was equally as potent as β1γ3, it was not as efficacious (Fig. 4 and Table 1). β1γ1HF (closed circles) was not an effective stimulator of P-Rex1 activity (Fig. 4). The data in Fig. 4 was normalized and re-plotted as the percent maximal effect of GBγ and fit to a sigmoidal curve to generate EC50 values and the data presented in Table 1. The EC50 values shown in Table 1 for the β1γ1γ3 dimers range from 10 to 20 nM; values that agree well with the results obtained in a study of the GBγ sensitivity of PtdIns 3-kinase (20).

We also determined the effect of seven different Gγ subunits using a series of pure GBγγ dimers, because β1 forms dimers with most Gγs (14, 20). Fig. 5A shows that four of the different GBγγ dimers tested were equally as effective as β1γ3 in promoting P-Rex1-mediated Rac guanine nucleotide exchange. As with the β series of dimers, each curve was normalized and fit to a sigmoidal curve to generate EC50 values and the data presented in Table 1. The β1γ2, β1γ3, β1γ10, and β1γ13ΔA dimers activate P-Rex1 with EC50 values ranging from 20 to 38 nM, values similar to those seen with the β-series. Fig. 5B shows that β1γ2 dimer-stimulated P-Rex1 GEF activity to a lesser extent with an EC50 of 112 nM. Dimers containing β1γ11 are also less effective and were 3-fold less potent than β1γ3 in stimulating P-Rex1 GEF activity (Fig. 5C). Interestingly, even though the γ11 subunit is highly expressed in hematopoietic cells, the β1γ11 dimer is not as effective at stimulating P-Rex1 or PtdIns 3-kinase (20). The γ11 subunit is similar to γ11 in its primary amino acid sequence and also contains the farnesyl lipid at its C terminus (43). Accordingly, the β1γ11 dimer was tested as another representative of a farnesylated Gγ. The data in Fig. 5C show that the β1γ11 dimer was also ineffective at stimulating P-Rex1 activity. A similar lack of effect was observed with the β1γ111 dimer (Table 1).

**Discussion**

The P-Rex proteins (P-Rex1, P-Rex2a, and P-Rex2b) have been shown to function as GEFs for the Rho subfamily of monomeric G-proteins (7–9, 41). These small GTP-binding proteins have been implicated in cytoskeletal reorganization and gene expression (5, 6). Of the six members of the Rho family of GTPases (5), P-Rex1 has been shown to stimulate nucleotide exchange on Rac1, Rac2, and Cdc42 and to a lesser extent on Rho, *in vitro* (7). However, in cells P-Rex1 has been shown to be a Rac-specific GEF (7). P-Rex1 is a PIP3-dependent Rac-GEF that can be synergistically activated by GBγ subunits and is highly expressed in cells of hematopoietic lineage such as neutrophils (7). It is a modular protein consisting of several functional domains: one tandem Dbll homology/pleckstrin homology domain, two DEP and two PDZ
domains, and an InsP₄, 4-phosphatase domain. The specific regions of P-Rex1 involved in mediating its PIP₃ and Gₛ dependence have not been determined. However, studies using deletion mutations suggest that the tandem Dbl homology/pleckstrin homology domain is necessary for P-Rex1 to be modulated by both PIP₃ and Gₛγ (41).

PI₃ is a lipid messenger produced in cells upon stimulation of PtdIns 3-kinases (45). Interestingly, similar to P-Rex1, the p110γ isoform of PtdIns 3-kinases is highly abundant in hematopoietic cells and is stimulated by Gₛγ subunits (11, 20, 46–48). The activation of GPCRs leads to the release of Gₛγ subunits that can stimulate PtdIns 3-kinase leading to the production of PIP₃, which can activate P-Rex1 along with the Gₛγ dimers that were initially released upon agonist binding of the receptor. Thus both co-activators of P-Rex1 are produced in cells upon GPCR activation.

There are 22 different α subunits that can be expressed from 17 genes in cells (49) and seven isoforms of Gₛ and 12 isoforms of Gᵧs that can constitute a specific Gₛγ dimer (43). The primary goal of this study was to determine whether P-Rex1 was differentially regulated by the large number of G protein isoforms known to exist in cells expressing this exchanger. Using purified recombinant proteins reconstituted into synthetic lipid vesicles, the results demonstrate a number of important points. The purified, activated Gₛ, Gₛ, Gₛ, Gₛ, and Gₛ α subunits were unable to modulate P-Rex1 activity. It is likely that each α subunit was a functional and properly folded protein because: (a) each Gₛ subunit was purified from an immobilized By column and eluted via activation with AlCl₃ and NaF, which only releases active protein; (b) each of the Gₛ subunits tested was able to bind GTPγS; and (c) the α subunits were effective in receptor coupling assays or able to activate effectors (14, 20, 31).

Thus, the current data suggest that P-Rex1 responds selectively to the Gₛ dimer, a property shared with ion channels and PtdIns 3-kinase (17–20). However, it would be useful to overexpress constitutively active Gₛ subunits in cells to examine their potential for regulating P-Rex1 activity in a cellular context.

Interestingly, the profile of Gₛγ dimers able to activate P-Rex1 mirrors that of PtdIns 3-kinase (20). Like PtdIns 3-kinase, P-Rex1, was markedly activated by Gₛγ subunits containing the Gₛ₃₁₄ subunits complexed with the Gᵧ₂ subunit with relatively similar EC₅₀ values. As expected (20), the Gₛγ₃₄ dimer did not activate P-Rex1. Gₛ₅ is the most divergent member of the Gₛ family of proteins, shows only 50% similarity to Gₛ, (43), and is unable to regulate adenylyl cyclase I, adenylyl cyclase II, and PtdIns 3-kinase (14, 20, 50, 51). However, even though both the Gₛ and P-Rex1 are highly expressed in the brain (7, 52, 53), the observations that Gₛ can bind to certain RGS proteins with high affinity (54, 55) make it difficult to speculate about the potential roles of Gₛγ in these tissues. Lastly, as was the case with PtdIns 3-kinase, the isoform of the Gᵧ subunit in the Gₛ dimer had a major effect on the ability of the dimer to activate P-Rex1. Specifically, dimers containing Gᵧ₁ were less effective at activating P-Rex1 (Fig. 5) and the Gₛγ₁ dimer had a lower EC₂₀ than dimers containing Gᵧ₂, Gᵧ₃, Gᵧ₇, and Gᵧ₁₃. Interestingly, the Gₛγ₁ dimer has also been shown to be less effective at activating adenylyl cyclase II, phospholipase C-β (35), and PtdIns 3-kinase (20). Similarly, Gᵧ₁, a Gᵧ subunit with a similar amino acid sequence and lipid modification as Gᵧ₁ (43, 56, 57), was also less effective at stimulating P-Rex1 (Fig. 5C). Both of these Gᵧ subunits, Gᵧ₁ and Gᵧ₁₃, are farnesylated, suggesting that the isoprenoid moiety may play a role in determining the activity of the Gₛγ-effector complex at this effector. These results add to the growing body of evidence arguing that the identity of the Gₛγ subunit released upon receptor activation plays a major role in their downstream effectors (14, 15, 18, 19).

With the discovery of the P-Rex family of proteins, a direct link has been established between the activation of heterotrimeric G proteins and the stimulation of PtdIns 3-kinase and Rac (7–9). As noted earlier, the activation of G protein-coupled receptors in hematopoietic cells leads to the release/production of both co-activators of the Rac-specific GEF (7), P-Rex1, one important step in neutrophil activation and chemotaxis. Interestingly, the stimulation of all receptors does not lead to Rac activation. In neutrophils and other cells of hematopoietic origin, the activation of Gₛ-coupled receptors, such as the fMet-Leu-Phe or the C₅a receptors leads to neutrophil chemotaxis (58). In contrast, the stimulation of Gₛ-coupled receptors, such as the A2a receptor, inhibit neu-
trophy chemotaxis (59, 60). Thus, it is clear that activation of heterotrimeric G proteins can both stimulate and inhibit neutrophil function. Intriguingly, activation of both Gα and Gβγ subunits will release Gβγ dimers that potentially activate two important steps in neutrophil chemotaxis: PtdIns 3-kinase and P-Rex1. Based on our findings, we hypothesize that Gβγ dimer combinations that do not stimulate P-Rex1 may be coupled to receptors such as the adenosine A2a receptor, which inhibit neutrophil function. The finding that both P-Rex1 and PtdIns 3-kinase respond to a similar profile of Gβγ dimers strengthens this notion. Naturally, release of Gβγ dimers of differing composition is just one of several possibilities to explain why activation of receptors coupled to Gα may not be able to stimulate neutrophil function. Another possibility is that the dimers are released in too low a concentration to be effective or that they are compartmentalized and not able to reach certain effectors. Negative regulation of P-Rex1 or other components of the Gα signaling pathway via phosphorylation by the CAMP-dependent protein kinase is another intriguing possibility that is discussed in the accompanying article (61).

Acknowledgments—We thank Dr. L. Stephens for the gift of the cDNAs encoding EE-P-Rex1, Dr. I. Macara for the gift of the GST-Rac1 plasmid, Dr. G. Worley for the gift of the EE-antibody column. We are grateful to Melinda Ring and Gavin MacCleery for excellent assistance in culturing SP9 insect cells and for the purification of Gβγ dimers.

REFERENCES
1. Welch, H. C., Coadwell, W. J., Stephens, L. R., and Hawkins, P. T. (2003) FEBS Lett. 546, 93–97
2. Fenteany, G., and Glogauer, M. (2004) Cell 116, 9–22
3. Procko, E., and McColl, S. R. (2005) Bioessays 27, 546–552
4. Blake, B. L., Wing, M. R., Zhou, Y. J., Lei, Y., Hillmann, J. R., Behe, C. L., Morris, R. A., Harden, T. K., Bayliss, D. A., Müller, R. J., and Siderovski, D. P. (2001) J. Biol. Chem. 276, 49267–49274
5. Grussmeyer, T., Scheidermann, K. H., Hutchinson, M. A., Eckhart, W., and Walter, G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7952–7956
6. Jonsson, G., and Nurnberg, B. (2003) J. Biol. Chem. 278, 50533–50539
7. Snow, B. E., Betts, L., Mangion, J., Sondek, J., and Siderovski, D. P. (1999) EMBO J. 18, 513–523
8. Procko, E., and McColl, S. R. (2005) Bioessays 27, 546–552
9. Hill, K., Krugmann, S., Andrews, S. R., Coadwell, W. J., Finan, P., Welch, H. C., Hawkins, P. T., and Stephens, L. R. (2005) Cell 120, 809–822
10. Motulsky, H. J., and Ransnäs, I. A. (1987) FASEB J. 1, 365–374
11. Hill, K., Krugmann, S., Andrews, S. R., Coadwell, W. J., Finan, P., Welch, H. C., Hawkins, P. T., and Stephens, L. R. (2005) J. Biol. Chem. 280, 4166–4173
12. Wenzel-Seifert, K., Arthur, J. M., Liu, H. Y., and Seifert, R. (1999) J. Biol. Chem. 274, 15801–15809
13. Richardson, M., and Robishaw, J. D. (2002) J. Biol. Chem. 277, 19573–19578
14. Hart, M. J., Jiang, X., Kozaza, T., Roscoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Science 280, 2112–2114
15. Kozaza, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) Science 280, 2109–2111
16. Fukuhara, S., Tsuruoka, M., Yamazaki, T., and Gotoh, J. (1999) J. Biol. Chem. 274, 5868–5879
17. Fukuhara, S., Chikumiti, H., and Gotoh, J. C. (2000) FEBS Lett. 485, 183–188
18. Nunn, T., Perrin, C., Schrieber, L. D., and Strobl, W. (1998) Nature 392, 687–689
19. Hill, K., Krugmann, S., Andrews, S. R., Coadwell, W. J., Finan, P., Welch, H. C., Hawkins, P. T., and Stephens, L. R. (2005) Cell 120, 809–822
20. Luo, Z., and Bollag, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7963–7968
21. Luo, Z., and Bollag, G. (1998) Science 280, 2112–2114
22. Snow, B. E., Betts, L., Mangion, J., Sondek, J., and Siderovski, D. P. (1999) EMBO J. 18, 513–523
23. Stuehr, D. J., and Holmgren, A. (1994) Annu. Rev. Immunol. 12, 455–485
24. Stuehr, D. J., and Fernandez, L. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4177–4181