P-Rex1, a PtdIns(3,4,5)P3- and Gβγ-Regulated Guanine-Nucleotide Exchange Factor for Rac

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Summary

Rac, a member of the Rho family of monomeric GTPases, is an integrator of intracellular signaling in a wide range of cellular processes. We have purified a PtdIns(3,4,5)P3-sensitive activator of Rac from neutrophil cytosol. It is an abundant, 185 kDa guanine-nucleotide exchange factor (GEF), which we cloned and named P-Rex1. The recombinant enzyme has Rac-GEF activity that is directly, substantially, and synergistically activated by PtdIns(3,4,5)P3 and Gβγs both in vitro and in vivo. P-Rex1 antisense oligonucleotides reduced endogenous P-Rex1 expression and C5a-stimulated reactive oxygen species accumulation in the plasma membrane (not all PH domains bind PtdIns(3,4,5)P3; Lemmon and Ferguson, 2000). In cells, type 1 PI3Ks have been shown to be necessary for receptor-driven stimulation of Rac, and activated type 1 PI3Ks are sufficient to activate Rac (Hawkins et al., 1995; Reif et al., 1996). These pathways are widely important and underpin responses such as lamellipodia formation and associated membrane ruffling and possibly ROS formation. Despite this, the mechanisms by which type 1 PI3Ks and/or PtdIns(3,4,5)P3 can activate Rac are unclear in many cellular contexts. This is partly a consequence of the fact that no Rac-GEFs have been purified and identified on the basis of their activity and, relevantly here, from a cellular context that displays PI3K-dependent activation of Rac. On the basis of studies subsequent to their original discovery and characterization, four subgroups of the currently known Rac-GEFs have been claimed to be regulated in a PI3K-dependent fashion, namely Tiam, Vav, Sos, and PIX. However, these effects of PI3K and/or PtdIns(3,4,5)P3 are, where direct, small or, where indirect, via modulation of unknown or phosphorylation-based mechanisms (Han et al., 1998; Rameh et al., 2001; Fleming et al., 2000; Buchanan et al., 2000; Yoshii et al., 1999; Scita et al., 1999; Das et al., 2000; Nimnual et al., 1998).

In neutrophil-like cells, Rac plays important roles in a variety of signaling pathways, particularly activation of PAK kinases and phospholipase D and, further downstream, responses such as chemotaxis, phagocytosis, and ROS formation (Roberts et al., 1999; Dorseuil et al., 1992). Its roles in coordinating receptor-stimulated ROS formation are probably best understood. Rac (Rac2 in most species) is, along with p47phox, p67phox, gp91phox, and gp22phox, a component of the catalytically active NADPH oxidase complex that is assembled on the phagosomal/endoosomal or plasma membrane of appropriately stimulated cells (Babior, 1999). This process has been correlated with activation of Rac (Akasaka et al., 1999; Benard et al., 1999). It has been demonstrated to be inhibited by Rac-GTPase activating proteins (GAPs) in vitro (Geiszt et al., 2001), augmented in Rac-GAP knockouts (Bcr) (Roberts et al., 1999), and inhibited in some immunodeficient patients that carry key mutations in Rac2 (Ambruso, 1999).
et al., 2000), and GTP bound but not GDP bound Rac can both bind p67phox and p91phox and activate PAK kinases that are claimed to phosphorylate p47 and p67phox (Babior, 1999). However, some work suggests that receptor-stimulated ROS formation can occur without activation of Rac (Geijser et al., 1999), implying basal levels of GTP-Rac are sufficient, or simply not necessary, for some regulatory mechanisms.

There is evidence that PI3Ks play a key role in neutrophils in mediating signaling between activation of G protein-linked receptors and stimulation of ROS formation. Type 1B PI3K nullizygotes fail to produce Rac and PtdIns(3,4,5)P3, indicating that a G protein (either recombinant SF9-derived Gs, the added We found the PtdIns(3,4,5)P3-stimulated Rac-GEF activation of ROS formation in response to inflammatory mediators (Condille and Hawkin, 2000). The mechanism, however, by which PI3Ks contribute to driving ROS formation is unclear. We have shown that PtdIns3P (a potential breakdown product of PtdIns(3,4,5)P3) regulates ROS formation via binding to the PX domain of p40phox, an effect that can be detected in the presence of GTPγS-Rac and hence cannot involve Rac activation (Ellson et al., 2001). Some data supports the idea that in neutrophils, type 1 PI3Ks may be upstream of activation of Rac. The PI3K inhibitors LY294002 and wortmannin have been shown to significantly reduce activation of Rac in response to inflammatory mediators (Akasaki et al., 1999; Benard et al., 1999). However, one paper has presented convincing data showing that FMLP-stimulated activation of Rac is resistant to PI3K inhibitors, apparently contradicting the work described above (but see Discussion) (Geijsen et al., 1999), and has instead, along with the precedent set by p115Rho-GEF that is activated by Gα13 (Hart et al., 1998), lead to the suggestion that a Gα subunit may activate one or more neutrophil Rac-GEF activities (Geijsen et al., 1999).

The identity of the Rac-GEF(s) that are involved in receptor-stimulated activation of Rac and/or ROS formation in neutrophils remain unknown. In the context of the distributions and properties of the known Rac-GEFs and the types of receptors that can drive ROS formation, it seems plausible that Vav and/or Sos proteins could be downstream of the protein-tyrosine-linked receptors while there are no clear candidates for a similar role downstream of the G protein-linked receptors.

Results

PI3K, Gβγ, and PtdIns(3,4,5)P2—Regulation of Rac Activation and ROS Formation

Mixtures of cytosol and low-density membranes from neutrophils can be stimulated to produce ROS by the addition of amphiphiles such as SDS and arachidonic acid. We tested the idea that type 1 PI3Ks could operate upstream of ROS formation by adding combinations of purified, recombinant p101/p110γγ-P13K and purified Gβγ (either recombinant SF9-derived Gβγ or bovine brain Gβγ, both of which can substantially activate p101/p110γγ-P13K) in the presence of MgATP and GTP. We have shown previously that under similar conditions PtdIns(3,4,5)P2, PtdIns(3,4)P2, and PtdIns3P are synthesized in these assays (Pacold et al., 2000). Although the assays contain endogenous PI3Ks and Gβγ, the added recombinant proteins independently activated ROS formation but acted synergistically when added together (Figure 1A). These effects were all inhibited by the potent PI3K inhibitor wortmannin, suggesting they were the result of PI3K activity, although we noted that the effects of Gβγ alone were surprisingly less wortmannin sensitive. Addition of chemically synthesized phosphoinositides in the form of liposomes showed that the biological diastereoisomer of PtdIns(3,4,5)P2 could potently activate ROS formation (Figure 1B). The stereoselectivity of these results suggests these effects are not the result of a physico-chemical property of the added PtdIns(3,4,5)P2 and support the results obtained above where the PI3K phospholipase membrane lipids and hence creates membrane-localized lipid products.

In contrast, the literature defining an important role for PI3Ks in activation of Rac and the important part Rac plays in the assembly of the NADPH oxidase complex, we asked the question, do these effects depend on activation of Rac? By adding small amounts of pure, recombinant, posttranslationally lipid-modified EE of Gα13 and PμPPG into these assays, we could show p101/p110γγ-P13K and Gβγ can independently and, in combination, synergistically activate Rac (Figure 1C). p101/p110γγ-P13K, both alone and when in synergistic combination with very low concentrations of Gβγ, stimulated activation of Rac1 in a significantly wortmannin-sensitive fashion. In contrast, higher concentrations of Gβγ stimulated Rac1 activation in a wortmannin-resistant fashion. PtdIns(3,4,5)P2 alone also stimulated activation of Rac1. These results suggested Rac could be acting downstream of p101/p110γγ-P13K in this system. We sought to test this by attempting to inhibit activation of Rac by preincubating our cytosolic and membrane fractions with pure, lipid-modified dominant-negative N17-Rac1. This treatment significantly inhibited p101/p110γγ-P13K- and Gβγ-mediated activation of ROS formation (Figure 1A). Further increases in N17-Rac1 concentration did not result in any greater inhibition (not shown). PtdIns(3,4,5)P2-stimulated ROS formation was also inhibited by N17-Rac1, although less efficiently than for PI3K-stimulated ROS formation (mean of 35%, data not shown).

The above data suggest that Rac can act downstream of p101/p110γγ-P13K, Gβγ, and PtdIns(3,4,5)P2 in stimulation of ROS formation in these assays. We tested whether purified, recombinant, lipid-modified Rac could stimulate ROS formation. GTPγS-Rac1 stimulated ROS formation substantially more effectively than GDP-Rac1 or GTPγS-treated N17-Rac1 (Figure 1D). The implication of this result is that activation of Rac can be sufficient to stimulate ROS formation. However, this is in the context of complex mixtures of neutrophil cytosolic and membrane fractions and hence, these effects may in fact depend on other signals. The PtdIns(3,4,5)P2-dependent Rac1 activation we observed in this system suggested to us that this effect is mediated by PtdIns(3,4,5)P2-sensitive Rac-GEF(s) present in either the neutrophil cytosol or membrane fractions.

Purification of a PtdIns(3,4,5)P2-Sensitive Rac-GEF from Neutrophil Cytosol Fractions

We found the PtdIns(3,4,5)P2-stimulated Rac-GEF activity was recovered in cytosol fractions and attempted to...
Figure 1. PI3K and Gβγ Regulate Rac Activation and ROS Formation

(A) PI3K and Gβγ synergistically stimulate Rac activation and ROS formation. Neutrophil cytosol and low-density membranes were incubated with 45 nM recombinant p101/p110 PI3K and/or 54 nM bovine brain Gβγ, either with wortmannin (200 nM, gray bars) or without (black bars) or with dominant-negative N17-Rac (200 nM, white bars), and Rac activation (SPC, single photon counts in 0.1 min) was measured. Data are means (n = 4) ± SD from two experiments.

(B) PtdIns(3,4,5)P3 stimulates ROS formation. Neutrophil cytosol and low-density membranes were incubated with isomers of PtdIns(3,4,5)P3, PtdIns(3,4)P2, or PtdIns(4,5)P2 (S/A, stearoyl-arachidonyl; P/P, dipalmitoyl), and ROS formation was measured. Data are means (n = 2–6) ± range.

(C) PI3K and Gβγ synergistically stimulate Rac activity. Neutrophil cytosol and low-density membranes were incubated with PI3K (50 nM), PtdIns(3,4,5)P3 (30 μM), and/or Gβγ (40 nM at left, 200 nM at right) either with (gray bars) or without (black bars) wortmannin (200 nM), and incorporation of [35S]GTP into EE-Rac1 was quantified (means ± SD from four experiments).

(D) Active Rac induces ROS formation. Neutrophil cytosol and low-density membranes were incubated either with wt-Rac (black bars) or dominant-negative N17-Rac (200 nM, white bars), preloaded and incubated with the indicated guanine nucleotides and, for wt-Rac, with wortmannin (200 nM, gray bar), and ROS formation was measured. Data are means (n = 2–6) ± range from three experiments.

purify the enzyme(s) responsible from this source (Figure 2). The assay used during the purification was a modification of the assay used above and quantitated Rac-GEF activity in terms of enhanced [35S]GTP-S binding to pure, recombinant, lipid-modified EE-Rac1 in the presence of mixed phospholipid liposomes (PtdCho, PtdS, PtdIns) either with or without PtdIns(3,4,5)P3 (10 μM final). Fractionation of cytosol on fast-flow Q-sepharose resolved a major peak of PtdIns(3,4,5)P3-sensitive Rac-GEF activity. This peak of activity was further purified via SP-sepharose, heparin sepharose, gel-filtration, and Mono S (Figure 2A) to yield a preparation that contained only two detectable proteins, a major band of 196 kDa and a minor band of 142 kDa, both of which perfectly correlated with the elution profiles of Rac-GEF activity during the last two columns (Figure 2B). Both proteins were transferred to nitrocellulose and digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF and N-terminal sequencing. This established that the 142 kDa minor band was almost certainly a proteolytic fragment of the major band. We named the protein P-Rex1, for PtdIns(3,4,5)P3-dependent Rac-GEF exchange.

Cloning and Expression of Human P-Rex1

We cloned the relevant human gene using a combination of library screening from random-primed human U937 cell and spleen cDNA libraries and PCR from a human leukocyte marathon-ready cDNA library. Together these approaches yielded a full-length ORF of 4980 bp (EMBL and GenBank accession number AJ320261), with the start ATG being preceded by a passable Kozak sequence and a CG-rich region at the N terminus but no upstream stop codon, leaving a small possibility that we have not identified the true start ATG. Underlying genomic sequence showed that the coding sequence
of P-Rex1 is arranged into 41 exons, stretched over more than 300 kb of chromosome 20 at q13.13 (AL131078, AL048641, AL445192, AL035106, AL133342). It also revealed the potential existence of a splice variant and a potential homolog on chromosome 8 (see database entry EST BAB14375). The P-Rex1 protein sequence is 1659 amino acids long, predicting a protein of 185 kDa, and harbors all five tryptic peptides obtained from the purified pig enzyme to greater than 95% purity in one step using a monoclonal anti-EE antibody crosslinked to protein G sepharose (Figure 3A). The protein contains a tandem DH/PH domain typical of Rho family GEFs, two DEP and two PDZ domains, and significant similarity over its C-terminal half to Inositol Polyphosphate 4-Phosphatase (Figure 3B).

We have studied P-Rex1 mRNA expression by probing human multiple-tissue Northern blots from Clontech with a probe made from 673 bp of the P-Rex1 coding sequence. The Northern blots revealed a major band of approximately 6 kb, which is consistent with the expected size of full-length P-Rex1 mRNA, and a minor band just below. They show that P-Rex1 is expressed mainly in peripheral blood leukocytes and brain, less in spleen and lymph nodes, and much weaker in most other tissues (Figure 4A).

We transiently expressed P-Rex1 with an N-terminal EE-epitope tag in Cos-7 cells, and anti-EE Western blots revealed a protein with an apparent MW of 197 kDa in the cell lysates (Figure 4B).

**PtdIns(3,4,5)P3 and Gβγ-Dependent Activation of Rac by P-Rex1 In Vitro**

We expressed P-Rex1 with an N-terminal EE-tag in SF9 cells. The protein expressed well and could be purified to greater than 95% purity in one step using a monoclonal anti-EE antibody crosslinked to protein G sepharose. Recombinant P-Rex1 displayed PtdIns(3,4,5)P3-sensitive Rac-GEF activity very similar to that of the purified protein. We tested the specificity of P-Rex1 for various Rho family GTPases and Rac proteins that were with or without posttranslational lipid modifications or carried different epitope tags. P-Rex1 displayed similar PtdIns(3,4,5)P3-sensitive activity against Rac1, Rac2, and CDC42 and low activity against RhoA (Figure 4C). Interestingly, the Rac1 protein did not need to be lipid modified to serve as a substrate in the context of these assays (Figure 4C).

Further analysis of the Rac-GEF activity of P-Rex1 showed that PtdIns(3,4,5)P3 had a 50% maximal effect at 0.3 μM (Figure 5A), at which concentration P-Rex1
Figure 3. Structure of Human P-Rex1

(A) Amino acid sequence of human P-Rex1. Tryptic peptides obtained from purified P-Rex1 are underlined in black. Protein homology domains are highlighted.

(B) Schematic representation of the domain structure of P-Rex1.

was significantly selective for the biological D-diastereo-isomer of PtdIns(3,4,5)P3 compared to its other diastereoisomers (Figure 5B). When different phosphoinositides were compared at 10 μM, P-Rex1 was still selective for PtdIns(3,4,5)P3, with a weak activation by the biological diastereoisomer of PtdIns(3,4)P2, but not by other phosphoinositides (Figure 5B). We observed that P-Rex1 was activated by dipalmitoyl PtdIns(3,4,5)P3 in an apparently more stereo-selective fashion than by stearoyl-arachidonyl lipids. We have observed a similar effect with ARAP3 (Krugmann et al., 2002); however, these lipid preparations activate PKB completely stereospecifically and PDK-1 with equivalent partial selectivity. We assume this reflects a fatty acid-sensitive interaction between the phosphoinositides and the proteins that bind them.

We have analyzed the interaction between soluble P-Rex1 and PtdIns(3,4,5)P3 or PtdIns(3,4)P2-containing phospholipid vesicles, immobilized on a dextran-coated L1 gold chip, utilizing surface plasmon energy transfer technology (Biacore). P-Rex1 binding to phospholipid vesicles was substantially augmented by the inclusion.
Figure 4. Expression and Substrate Specificity of Human P-Rex1

(A) Northern blots. Multiple-tissue Northern blots from Clontech were probed for P-Rex1 mRNA expression.

(B) Western blot. EE-epitope-tagged P-Rex1 was transiently expressed in Cos-7 cells, then extracted with 1% Triton-X100, containing buffer, and aliquots of a 10,000 g supernatant (equivalent to 5 x 10^9, 5 x 10^8, 5 x 10^7 cells/lane) were immunoblotted with anti-EE antibody.

(C) Recombinant human P-Rex1 GEF activity was assayed using liposomes (PtdCho, PtdIns, 200 μM each) with (dark bars) or without (light bars) PtdIns(3,4,5)P_3 (10 μM) and the indicated purified GTPases (100 nM). Data are duplicate means ± range from one of three experiments.

of PtdIns(3,4,5)P_3 and to a lesser extent by PtdIns(3,4)P_2 (Figure 5C). The presence of PtdIns(3,5)P_2, PtdIns(4,5)P_2, PtdIns3P, PtdIns4P, or PtdIns5P in the phospholipid vesicles increased P-Rex1 binding only weakly. These results are consistent with the idea that PtdIns(3,4,5)P_3 has a direct effect on P-Rex1 in our assay rather than indirect effect by, for example, influencing the distribution of Rac or the ability of Rac to interact with P-Rex1.

Although we had no direct assay data to support the possibility that Gβγ could activate P-Rex1 directly, the presence of the DEP domains, which commonly occur in proteins that interact with heterotrimeric G-proteins, and our earlier results with neutrophil cytosol/membrane mixtures encouraged us to test the effects of Gβγ on P-Rex1 Rac-GEF activity. Pure Gβγ from bovine brain and prepared as recombinant EE-Gβγ from coinfected SF9 cells both activated P-Rex1 Rac-GEF activity in vitro (Figures 5D and 5E). The effects of Gβγ were abolished by preheating (95°C for 30 min), substantially inhibited by prebinding with purified GDP bound Gα and, in the case of the recombinant Gβγ, dependent on their posttranslational lipid modifications (Figure 5E). Gα alone or in the presence of AIF did not activate P-Rex1 Rac-GEF activity. It should be noted that cholate (the detergent we use in the storage buffer for our preparations of Gβγ proteins) strongly inhibits P-Rex1 Rac-GEF activity; consequently, the scale of the effects of Gβγ in an experiment are a combined function of the Gβγ and the total cholate concentrations. We tested the effects of combinations of Gβγ and PtdIns(3,4,5)P_3 (Figure 5F). P-Rex1 was activated synergistically by Gβγ and PtdIns(3,4,5)P_3, suggesting that their effects are mediated via distinct mechanisms.

PtdIns(3,4,5)P_3- and Gβγ-Dependent Activation of Rac by P-Rex1 In Vivo

To address questions about the selectivity of P-Rex1 for Rac versus CDC42 in cells and the physiological
Figure 5. Regulation of Recombinant Human P-Rex1 Rac-GEF Activity by PtdIns(3,4,5)P₃ and Gᵦ₁γ In Vitro

(A) PtdIns(3,4,5)P₃ dose response. P-Rex1-dependent activation of EE-Rac1 was assayed in the presence of liposomes containing 200 µM each of PtdCho, PtdS, PtdIns, and the indicated concentrations of PtdIns(3,4,5)P₃ (final P-Rex1 concentration was 100 nM). Data are means (n = 2–4) ± range from two pooled experiments.

(B) Lipid specificity of P-Rex1-dependent activation of Rac was measured in the presence of liposomes (as in [A]) with either 10 µM (dark bars) or 0.3 µM (white inset bars) of the indicated phosphoinositides (S/A, stearoyl-arachidonyl; P/P, dipalmitoyl). Data are duplicate means ± range obtained from one of two separate experiments.

(C) Phosphoinositide-dependent binding of P-Rex1 (100 nM) to liposomes containing PtdCho, PtdS, PtdE (330 µM each), and the indicated phosphoinositides (6 mol-%) was measured by Biacore. Data are means ± SD from four pooled experiments.

(D) Gᵦ₁γ dose response. P-Rex1-dependent activation of Rac was assayed using the indicated concentrations of purified bovine brain Gᵦ₁γ. Final cholate concentration was 0.0072% except for 1 µM Gᵦ₁γ samples (0.0104%). Data are duplicate means ± range from one of three experiments.

(E) Controls for Gᵦ₁γ effects. P-Rex1-dependent activation of Rac was assayed using, where indicated, Gᵦ₁γ (0.3 µM, bovine brain-derived except where indicated to be prenylated or nonprenylated, which were derived from SF9 cells), mixed Gα subunits (0.23 µM), Aif (10 µM), boiled bovine brain Gᵦ₁γ (0.5 µM), recombinant prenylated Gᵦ₁γ (0.5 µM), or recombinant nonprenylated Gᵦ₁γ (0.5 µM). For combinations of Gᵦ₁γ and Gα, these (or control buffers) were preincubated for 30 min on ice. Final cholate concentration was 0.012%. Data are duplicate means ± range from one experiment.

(F) Synergy between PtdIns(3,4,5)P₃ (0.2 µM) and bovine brain Gᵦ₁γ (0.3 µM) in the regulation of P-Rex1 Rac-GEF activity. Final cholate concentration was 0.0048%. Data are duplicate means ± range from one of three experiments.

significance of the effects of Gᵦ₁γs and PtdIns(3,4,5)P₃, we have observed in the test tube, we prepared the relevant baculoviruses to allow us to study the activation of endogenous Rac and CDC42 in SF9 cells. We found that SF9 cells infected with baculoviruses driving P-Rex1, p101/p110−PI3K, and Gᵦ₁γ production showed substantial increases in the levels of endogenous GTP-Rac but no change in the levels of endogenous GTP-CDC42, suggesting that in vivo P-Rex1 acts as a Rac-GEF (Figure 6A). Furthermore, the pattern of activation of Rac by p101/p110−PI3K and Gᵦ₁γ production was consistent with our in vitro results, showing synergistic activation of P-Rex1 Rac-GEF activity (Figures 6A and 6B). Although coexpression of PI3K and Gᵦ₁γ led to significantly higher PtdIns(3,4,5)P₃ production than PI3K expression alone, direct comparison of the increases in PtdIns(3,4,5)P₃ and GTP-Rac clearly showed that Rac activation in the presence of Gᵦ₁γs and PI3K was substantially bigger than could be accounted for by the increase in PtdIns(3,4,5)P₃ alone (Figure 6C).

P-Rex1 Induces a Phenotype Like Constitutively Active Rac in PDGF-Stimulated PAE Cells

We sought evidence that P-Rex1 can be regulated by signaling pathways downstream of cell-surface receptors and act as a Rac-GEF in mammalian cells. We used a porcine aortic endothelial (PAE) cell line that stably overexpresses the PDGFB receptor. In these cells, PDGF stimulates PtdIns(3,4,5)P₃ accumulation, wortmannin-sensitive activation of Rac, and Rac-dependent membrane ruffling and lamellipodia formation, and stable overexpression of constitutively active V12-Rac
Figure 6. Rac-GEF Activity of Human Recombinant P-Rex1 In Vivo

(A) Western blots of Rac activation by P-Rex1 in vivo. Aliquots of 5 × 10⁶ Sf9 cells in 6 cm dishes were infected with combinations of viruses encoding P-Rex1, Gγ1, Gγ2, p101, p110, or control viruses where indicated. After 42.5 hr in growth medium, then 4 hr serum-free, the cells were subjected to a PAK-Crib pull-down assay. Immunoblots were probed with anti-Rac (top and second gels) or anti-CDC42 (third gel) antibodies. The equivalent of 0.18 dishes of cells was loaded from PAK-Crib pulldowns and 0.05 dishes for total lysates. The bottom gel shows the second panel filter after staining with Coomassie.

(B) Synergistic PI3K and Gγ-dependent activation of Rac by P-Rex1 in vivo. Sf9 cells were infected with the above viruses as indicated, then treated as in (A). ECL-exposed films were digitized, and the data shown are means ± range (n = 4) from two pooled experiments.

(C) Gγ and/or PI3K-induced formation of PtdIns(3,4,5)P₃ in Sf9 cells was measured (data are means [n = 5] ± range) and plotted against P-Rex1-dependent Gγ and/or PI3K-induced activation of Rac (data from [B]).

causes the formation of strongly exaggerated lamellipodia (“fried eggs”; Figure 7A; Hawkins et al., 1995; Welch et al., 1998).

PAE cells were transiently transfected with N-terminally myc-tagged P-Rex1 and serum starved, and the effects of PDGF stimulation on cell shape and the distribution of myc-P-Rex1 were analyzed by indirect immunofluorescence microscopy (Figure 7B). In unstimulated cells, P-Rex1 localization was mainly cytosolic, although some seemed plasma membrane bound, and the cells had the typical basal shape. However, in about 30% of unstimulated cells, P-Rex1 expression resulted in the formation of strongly exaggerated lamellipodia (fried eggs) that appeared identical to those induced by constitutively active V12-Rac. The proportion of cells showing the V12-Rac-like phenotype was reduced in cells treated with wortmannin (Figures 7B and 7C), suggesting that it was induced by basal PI3K activity. PDGF stimulation resulted in a wortmannin-sensitive increase of V12-Rac-like phenotype to 80% of P-Rex1-expressing cells. This phenotype was specific to P-Rex1-positive cells and not induced by DAPP1 in parallel experiments. In cells showing the V12-Rac-like phenotype, P-Rex1 localization was mainly cytosolic, but there was significant costaining with the subcortical actin ring at the edge of the lamellipodia and a small variable accumulation of P-Rex1 in the plasma membrane. However, the plasma membrane translocation was weaker and less clear than for DAPP1 in parallel experiments. Similar experiments, in which we examined the distribution of GFP-tagged P-Rex1 in control and PDGF-stimulated PAE cells by live imaging with a scanning confocal microscope, gave the same results (not shown). Therefore, although PI3K activation does not cause a large-scale translocation of P-Rex1 to the plasma membrane, it is sufficient to induce strong P-Rex1-mediated lamellipodia formation. These results suggest that P-Rex1 can act as a Rac-GEF and can be regulated by signaling pathways downstream of cell surface receptors in mammalian cells.

Agonist-Stimulated ROS Formation in a Neutrophil-like Cell Line Is Dependent on P-Rex1

We treated a promyelocytic cell line (NB4) with retinoic acid and either phosphorothioate antisense oligonucleotide targeted against P-Rex1 or a randomized control oligonucleotide. After 2 days, both populations of cells had differentiated normally and displayed indistinguishable MapK activation in response to C5a (Figure 8C) and expression of β-COP (not shown). In contrast, the antisense-treated cultures specifically, the levels of P-Rex1 fell by 80%–85% (Figure 8B), and C5a-stimulated ROS formation fell by about 40%–45% (Figure 8A). Finally, as the C-terminal half of P-Rex1 has substantial homology with Inositol Polyphosphate 4-Phosphatase, we attempted to determine whether the protein possessed Inositol Polyphosphate 4-Phosphatase activity. In the case of the Rac-GEFs,
P-Rex1, a PI3K- and Gβγ-Regulated Rac-GEF

Figure 7. P-Rex1 Induces a Phenotype Like Activated Rac in PAE Cells

(A) Immunofluorescence micrographs of serum-starved normal (first and second images) or stably V12-Rac transfected (third image) PAE cells after stimulation with 10 ng/ml PDGF for 5 min (second image) or without (first and third images). Fixed cells were labeled with FITC-phalloidin to stain filamentous actin.

(B and C) Expression of P-Rex1 in PAE cells. Myc-tagged P-Rex1 or DAPP1 were transiently expressed in PAE cells. These were grown (10 hr), serum starved (8 hr), treated with wortmannin (100 nM, 10 min) or not, and then stimulated with a range of PDGF concentrations for 5 min, as indicated. Cells were fixed and stained with anti-myc antibody followed by FITC secondary antibody to label P-Rex1 or DAPP1 and with TRITC-phalloidin to label filamentous actin.

(B) Immunofluorescence micrographs.

(C) Quantification of immunofluorescence microscopy data. Results were obtained by counting 100 P-Rex1-positive cells (dark bars) or DAPP1-positive cells (light bars) per coverslip. P-Rex1 data are from duplicate coverslips (means ± range) from one of two independent experiments. DAPP1 data are from one coverslip per condition from one experiment.

this means that activities in lysates or those involved in specific signaling events have only rarely been attributed to a specific GEF and further, that the contributions any GEF makes toward total cellular GEF activities are unclear. We have resolved neutrophil cytosol by chromatography on Q-Sepharose and found a major peak of PtdIns(3,4,5)P3-sensitive Rac-GEF activity (in the presence of PtdIns(3,4,5)P3, it represented about 65% of total Rac-GEF activity), which we have purified, cloned, and named P-Rex1. P-Rex1 is a surprisingly abundant protein, about 0.1% of cytosolic protein (cf. 0.001% for the type 1B PI3K purified from similar fractions).

Our results show that PtdIns(3,4,5)P3 can substantially activate P-Rex1 Rac-GEF activity in vitro and that cell surface receptors can activate P-Rex1 in a PI3K-dependent fashion in vivo. Further, we demonstrate P-Rex1 can selectively bind PtdIns(3,4,5)P3-containing phospholipid vesicles. However, P-Rex1 does not substantially translocate from the cytosol to the sites of PtdIns(3,4,5)P3 accumulation in vivo; rather, the enzyme is partially localized to the membrane in serum-starved cells. The implication of these results is that PtdIns(3,4,5)P3, is able to activate the enzyme by inducing a catalytically significant conformational shift or by reorientating P-Rex1 at the membrane surface rather than by targeting it to the membrane. This is totally compatible with the emerging view of the role of the PH domain in the tandem DH/PH domains of Rho family GEFs as a phosphoinositide-inhibited repressor of DH domain GEF activity (Worthylake et al., 2000; see Introduction). This is quite distinct to the generally accepted view of the role of the PH domain in proteins such as PLCβ where lipid binding acts purely as a membrane-targeting device.

Some data has suggested that activation of heterotrimeric G proteins in neutrophil-like cells can stimulate...
to independent sites, although we have not identified these sites. P-Rex1, hence, becomes one of a growing list of effector proteins that are regulated by G protein subunits in neutrophils, including p101/110γ-PI3K and PLCγs (Sternweis and Smrcka, 1992; see Introduction). G protein-mediated signaling pathways in neutrophils respond rapidly, e.g., maximal activation of Rac can occur within 10 s. The fact that both p101/p110γ-PI3K and P-Rex1 appear to be partially membrane localized in serum-starved cells and are activated at the level of the membrane without any requirement for translocation from the cytosol (Krugmann et al., 2002) probably contributes to this rapidity.

Our data are consistent with the existence of a signaling pathway in neutrophils from G protein-linked receptors and via Gαγ, type 1B PI3K, PtdIns(3,4,5)P3, and activation of Rac to enhanced ROS formation. There is significant work that has also suggested these links; however, this appeared weak in the absence of an appropriate Rac-GEF. The literature also contains high-quality work that is apparently inconsistent with this model: activation of Rac by ligands such as fMLP has been shown to be resistant to PI3K inhibitors (Geijsen et al., 1999; see Introduction). Our results offer a possible explanation; they suggest that at the earliest times of stimulation with ligands like fMLP, Gαγ activation of P-Rex1 may be more important than activation via PtdIns(3,4,5)P3, as the levels of PtdIns(3,4,5)P3 rise due to Gαγ stimulation of p101/p110γ-PI3K. Moreover, this phenomenon would be exaggerated in unprimed neutrophils, which produce up to 20 times less PtdIns(3,4,5)P3 in response to fMLP (Condliffe and Hawkins, 2000). This is exactly what is observed in the literature: workers with demonstratedly unprimed neutrophils who also stimulate for the shortest times (10 s) found Rac activation by fMLP to be largely resistant to PI3K inhibitors (Geijsen et al., 1999). Those workers who tested PI3K inhibitors at later times of stimulation (1 min) find that PI3K inhibitors substantially, but not completely, inhibit activation of Rac (Akasaki et al., 1999; Benard et al., 1999).

Figure 8. P-Rex1 Is Necessary for ROS Formation
Human promyelocytic NB4 cells were differentiated for 2 days with 1 μM all-trans retinoic acid and treated with 10 μM of either P-Rex1 antisense oligonucleotide or randomized control oligonucleotide and then subjected to the experiments below. (A) Oligonucleotide-treated NB4 cells were stimulated with 0.15 nM C5a, and ROS formation (SPC, single photon counts) was measured. Data are mean ± SD (n = 4) from one of three experiments. (B) Total lysates of P-Rex1-transfected or control Cos7 cells, human neutrophils, or oligonucleotide-treated NB4 cells were analyzed for P-Rex1 expression level by Western blot using a polyclonal anti-P-Rex1 antibody. (C) Oligonucleotide-treated NB4 cells were serum-starved and then stimulated with C5a as indicated for 3 min at RT. MapK activation was measured by Western blot using a phospho-MapK antibody and densitometric scanning of the blots. Data are mean ± range (n = 2) from two experiments.

Rac-GEF activities (Geijsen et al., 1999), and furthermore, a small (about 40% above control) effect of Gβγ on Rac-GEF activity in neutrophil lysates has been reported (Arcaro, 1998). P-Rex1 is an example of a Rac-GEF that can be activated directly by Gβγ. The ability of Gβγs and PtdIns(3,4,5)P3 to synergistically activate P-Rex1 suggests that these regulators can bind simultaneously
particular cell contacts in view of the key role Rac plays in, for example, neutrite outgrowth (Luo et al., 1997).

**Experimental Procedures**

**Materials**

Monomeric GTPases (EE-Rac1, GST-Rac1, EE-N17 Rac1, EE-Rac2, GST-CDCC42, and GST-RhoA) were purified from S9 cells in the GDP bound state to >95% purity and were stored in 1% (w/v) cholate, 5 μM GDP, 5 mM MgCl2, 1 mM EGTA, 1 mM DTT, 100 mM NaCl, 40 mM HEPES/NaOH (pH 7.4; 4°C), and stored in 1% cholate, 1 mM DTT, 2 mM EGTA, 10 mM 1,10-phenanthroline (P-phenanthroline), and 1 mM EDTA. GST-Rac was derived from bacteria.

Glutathione-π were purified from bovine brain (Sternweis and Robishaw, 1984) or from S9 cells (both wild-type EE-ij1,2; and nonprolyl mutated EE-ij,1,C186S–2) and stored in 1% cholate, 1 mM DTT, 20 mM HEPES/NaOH (pH 8.0; 4°C), 5 μM GDP (for bovine brain Glu,γ), and 1 mM EDTA. Glu subunits (a mixture of αs, αg, and αz) were purified from bovine brain (Sternweis and Pang, 1990) and stored in 50 mM HEPES/NaOH (pH 8.0; 4°C), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1% cholate, and 10 μM GDP. Phosphorothioate antisense oligonucleotides and controls directed to P-Rex1 have been designed and manufactured by BIONOSTIK, Göttingen, Germany (antisense, TCA ATG ATG AAG ATC; randomized control, ACT ACA CTA GAC TAC).

Recombinant EE-p101/Hexa-His-p110 PI3K was produced from S9 cells (Stephens et al., 1997). Recombinant NH-terminally EE-tagged P-Rex1 was purified from S9 cells utilizing the EE-tag and stored in PBS, 1 mM EGTA, 1 mM DTT, 0.1% Na azide. Stearyl-arachidonyl (S/A)-PtdIns(3,4,5)P3 stereoisomers were synthesized by P. Gaffney (Gaffney and Reese, 1997). All dipalmitoyl (P/P)-phosphoinositides were made by G. Painter (Painter et al., 1999). In this manuscript, the term PtdIns(3,4,5)P3 refers to D/D-(S/o) were purified from bovine brain (Sternweis and Pang, 1990) and stored in 50 mM HEPES/NaOH (pH 8.0; 4°C), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1% cholate, and 10 μM GDP. Phosphorothioate antisense oligonucleotides and controls directed to P-Rex1 have been designed and manufactured by BIONOSTIK, Göttingen, Germany (antisense, TCA ATG ATG AAG ATC; randomized control, ACT ACA CTA GAC TAC).

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**Cloning of Human P-Rex1**

A typtic digest of purified pig PtdIns(3,4,5)P3-dependent Rac-GEF yielded five peptides (T14, T30, T44, T69, and T72) that were analyzed by MALDI-TOF and N-terminal sequencing. T72 was identical to mouse Est AA796530 (homologous to Tiam). T14 and T69 were near identical to mouse Est A1468041 (homologous to Inositol Polyphosphate 4-Phosphatase). T30 and T44 were uncharacterized. Underlying human genomic sequence placed T44 into the Inositol Polyphosphate 4-Phosphatase homology region and T72 near the N terminus of a predicted protein. A predicted partial sequence encompassing these regions has been published (Nagase et al., 2000), Est AA796530 was cut with Bgl2 and labeled with cDNA library and a human spleen random prime GT11 cDNA library, yielding 24 Rac1 were quantified by scintillation counting (blanks without EE-Rac1 were subtracted).

**Rac-GEF Assay for P-Rex1 Purification and Recombinant P-Rex1**

Liposomes (phosphatidycholine [PtdCho], phosphatidylserine [PtdS], phosphatidylinositol [PtdIns]; final assay concentration 200 μM each) were sonicated in lipid buffer (20 mM HEPES/NaOH [pH 7.5; 4°C], 100 mM NaCl, 1 mM EGTA) with or without PtdIns(3,4,5)P3 (final assay concentration 10 μM) and were incubated for 10 min on ice with 2 μl of purified, GDP-loaded, recombinant, lipid-modified EE-Rac1 in 5 mM MgCl2, 50 mg/ml BSA, 5 mM DTT, 20 mM HEPES/NaOH (pH 7.5; 4°C), 100 mM NaCl, 1 mM EDTA (final assay concentration 100 nM EE-Rac1, 0.0024% cholate). Then, 4 μl of Rac-GEF activity (cytosol, column fractions, or recombinant P-Rex1) was added, followed by 2 μl GTP/S (in lipid buffer, final assay concentration 5 μM, including 1 μl (C/GT)S). After 10 min at 30°C, the reaction was stopped and EE-Rac1 was pulled down using anti-EE antibodies coupled to protein G sepharose, and [32P]GTP-S-loading of Rac was detected by scintillation β counting. Recombinant P-Rex1 was diluted in “buffer A” (20 mM HEPES/NaOH [pH 7.5; 4°C], 1 mM β-glycerophosphate, 1% betaine, 0.01% Na azide, 0.5 mM EGTA, 200 mM KCl, 10% ethylene glycol) to a final assay concentration of 50 nM. In assays with Glu,γ, 2 μl of Glu in buffer A were added to the liposome/Rac mix before the 10 min on ice, and P-Rex1 was added as 5X.
and 36 clones of varying lengths, respectively. In parallel, PCR primers based on underlying genomic sequence were used to screen a marathon-ready human leukocyte cDNA library (Clontech).

The full-length sequence was obtained from three fragments and cloned into pBluescript (Stratagene) as follows. Clone 1 was cut SalI/SphI to yield pBluescript with the N terminus of P-Rex1 up to the first SphI site. Clone 2 was cut SphI/BclI to give the middle of P-Rex1, and clone 3, the PCR-derived C terminus, was cut BclI/SalI out of the T tail vector. The fragments were three-way ligated. The resulting polylinker of pBluescript had additional SpeI, NotI, and PstI sites 5' of SalI. The 5' overhang was replaced by PCR, creating an in-frame EcoRI/startATG. A 60 bp 3' overhang was kept. Full-length P-Rex1 was subcloned into pCMV mammalian expression vectors with N-terminal myc or EE-epitope tags (Welch et al., 1998) or pAcO1G1 Sf9 cell expression vector with N-terminal EE-tag by ligating P-Rex1 from EcoRI/SpeI-cut pBluescript-P-Rex1 into EcoRI/XbaI cut vectors.

Northern Blots
The same probe as described above for library screening was used to hybridize Clontech multiple-tissue Northern blots as specified by the manufacturers.

Surface Plasmon Resonance
Assays were conducted as described (Ellison et al., 2001) using mixed phospholipid vesicles (PtdCho, PtdS, phosphatidyethanolamine [PtdE]), 330 μM each final concentration) with or without added phosphoinositides (6 mol-% final concentration) to load the L1 vesicle capture chip (Biacore) prior to the injection of 100 nM purified recombinant Sf9 cell-derived P-Rex1.

Rac and CDC42-GEF Assays and Measurement of PtdIns(3,4,5)P2 Formation in Sf9 Cells
Rac and CDC42-GEF in vivo assays were performed as PAK-Crib pull-down assays (based on the fact that only activated GTP bound but not GDP bound Rac and CDC42 bind to the Crib domain of PAK), as described (Sander et al., 1998), with endogenous Rac and CDC42 from Sf9 cells that were infected to produce combinations of P-Rex1, Gq5-y8, and P123. Measurement of PtdIns(3,4,5)P2 formation in Sf9 cells was done by radioligand displacement assay essentially as described (Van der Kaay et al., 1996).

Immunofluorescence Microscopy
Pig aortic endothelial (PAE) cells were transiently transfected with pCMV3-myc-P-Rex1 or pCMV3-myc-DAPP1 by electroporation, grown on coverslips for 10 hr, and then serum starved for 6 hr. They were then treated or not with 100 nM wortmannin for 10 min followed by stimulation with varying doses of PDGF for 5 min. Cells were fixed and prepared for immunofluorescence microscopy by staining of P-Rex1 and DAPP1 with anti-myc epitope tag primary and FITC-goat anti-mouse secondary antibodies and filamentous actin with TRITC-phalloidin, as previously described (Welch et al., 1998).

NB4 Cell Culture, MapK and ROS Formation Assays
NB4 cells (from M. Lanotte, Paris) were cultured and differentiated 12753–12758. NB4 Cell Culture, MapK and ROS Formation Assays

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