Cell, Vol. 108, 809–821, March 22, 2002, Copyright ©2002 by Cell Press

P-Rex1, a PtdIns(3,4,5)P_3- and G$\beta\gamma$-Regulated Guanine-Nucleotide Exchange Factor for Rac

Heidi C.E. Welch,[1] W. John Coadwell,[2] Christian D. Ellson,[1] G. John Ferguson,[1] Simon R. Andrews,[2] Hediye Erdjument-Bromage,[3] Paul Tempst,[3] Phillip T. Hawkins,[1] and Len R. Stephens[1,4]

[1]Inositide Laboratory
[2]Bioinformatics Group
Signalling
The Babraham Institute
Cambridge CB2 4AT
United Kingdom
[3]Molecular Biology
Memorial Sloan-Kettering Cancer Center
New York, New York 10021

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)P_3-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)P_3 and G$\beta\gamma$s both in vitro and in vivo. P-Rex1 antisense oligonucleotides reduced endogenous P-Rex1 expression and C5a-stimulated reactive oxygen species formation in a neutrophil-like cell line. P-Rex1 appears to be a coincidence detector in PtdIns(3,4,5)P_3 and G$\beta\gamma$ signaling pathways that is particularly adapted to function downstream of heterotrimeric G proteins in neutrophils.

Introduction

Monomeric GTPases are key regulators of intracellular signaling (Bourne et al., 1990). Rac proteins (Rac1, 2, and 3) are a subfamily of the Rho family of monomeric GTPases involved in receptor-regulated responses such as transcriptional activation, lamellipodia formation, and stimulation of reactive oxygen species (ROS) production (Tapon and Hall, 1997). Rho family monomeric GTPases are molecular switches that are either "on" and can activate effector proteins when GTP bound or "off" when they are GDP bound. The GTPases can be activated by guanine-nucleotide exchange factors (GEFs) that act to accelerate nucleotide exchange by prising open the binding site of specifically the GDP bound form of the GTPases (Worthylake et al., 2000).

There is a large family of Rac-GEFs (though some can also act as GEFs for other monomeric GTPases). These include Vav (1, 2, 3), Tiam (1, 2), PIX (α, β), Ras-GRF (1, 2), and Sos (Manser et al., 1998; Scita et al., 1999; Stam and Collard, 1999). Protein kinases currently seem the major direct regulators of Rac-GEF activity. For example, Vav1 can be phosphorylated on tyrosine 174 and activated by Lck (Crespo et al., 1997; Han et al., 1997). Similarly, Ras-GRF1 has to be tyrosine-phosphorylated to display Rac-GEF activity (Kiyono et al., 1999), and Tiam1 is phosphorylated and regulated possibly by Ca^{2+}/calmodulin-dependent protein kinase II (Fleming et al., 1999). Other regulators of Rac-GEFs, for example, phosphoinositide 3-kinases (PI3Ks) and G$\beta\gamma$s, largely work by affecting these phosphorylations (Han et al., 1998; Kiyono et al., 1999).

Type 1 PI3Ks can be activated by cell-surface receptors to synthesize the intracellular messenger phosphatidylinositol(3,4,5)P_3 (PtdIns(3,4,5)P_3). The signaling targets of PtdIns(3,4,5)P_3 typically possess a PH domain that can bind the lipid and drive translocation of the host protein to the site of PtdIns(3,4,5)P_3 accumulation in the plasma membrane (not all PH domains bind PtdIns(3,4,5)P_3; Lemmon and Ferguson, 2000). In many 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)P_3 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)P_3 are, where direct, small or, where indirect, via modulation of unknown or phosphorylation-based mechanisms (Han et al., 1998; Rameh et al., 1997; 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 $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$, and $gp22^{phox}$, a component of the catalytically active NADPH oxidase complex that is assembled on the phagosomal/endosomal or plasma membrane of appropriately stimulated cells (Babior, 1999). This process has been correlated with activation of Rac (Akasaki 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

[4]Correspondence: len.stephens@bbsrc.ac.uk

et al., 2000), and GTP bound but not GDP bound Rac can both bind $p67^{phox}$ and $p91^{phox}$ and activate PAK kinases that are claimed to phosphorylate p47 and $p67^{phox}$ (Babior, 1999). However, some work suggests that receptor-stimulated ROS formation can occur without activation of Rac (Geijsen 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, and PI3K inhibitors block, ROS formation in response to inflammatory mediators (Condliffe and Hawkins, 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)P_3) regulates ROS formation via binding to the PX domain of $p40^{phox}$, 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 $G\alpha_{13}$ (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 is (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-, G$\beta\gamma$-, and PtdIns(3,4,5)P_3-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/p110γ-PI3K and purified G$\beta\gamma$s (either recombinant SF9-derived $G\beta_1\gamma_2$ or bovine brain G$\beta\gamma$s, both of which can substantially activate p101/p110γ-PI3K) in the presence of MgATP and GTP. We have shown previously that under similar conditions PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdIns3P are synthesized in these assays (Pacold et al., 2000). Although the assays contain endogenous PI3Ks and G$\beta\gamma$s, 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 G$\beta\gamma$s 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)P_3 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)P_3 and support the results obtained above where the PI3K phosphorylates membrane lipids and hence creates membrane-localized lipid products.

In context of 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-Rac1 and [α-^{32}P]GTP into these assays, we could show p101/p110γ-PI3K and G$\beta\gamma$ can independently and, in combination, synergistically activate Rac (Figure 1C). p101/p110γ-PI3K, both alone and when in synergistic combination with very low concentrations of G$\beta\gamma$s, stimulated activation of Rac1 in a significantly wortmannin-sensitive fashion. In contrast, higher concentrations of G$\beta\gamma$s stimulated Rac1 activation in a wortmannin-resistant fashion. PtdIns(3,4,5)P_3 alone also stimulated activation of Rac1. These results suggested Rac could be acting downstream of p101/p110γ-PI3K 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/p110γ-PI3K- and G$\beta\gamma$-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)P_3-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/p110γ-PI3K, G$\beta\gamma$, and PtdIns(3,4,5)P_3 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 cytosol and membrane fractions and hence, these effects may in fact depend on other signals. The PtdIns(3,4,5)P_3-dependent Rac1 activation we observed in this system suggested to us that this effect is mediated by PtdIns(3,4,5)P_3-sensitive Rac-GEF(s) present in either the neutrophil cytosol or membrane fractions.

Purification of a PtdIns(3,4,5)P_3-Sensitive Rac-GEF from Neutrophil Cytosol Fractions

We found the PtdIns(3,4,5)P_3-stimulated Rac-GEF activity was recovered in cytosol fractions and attempted to

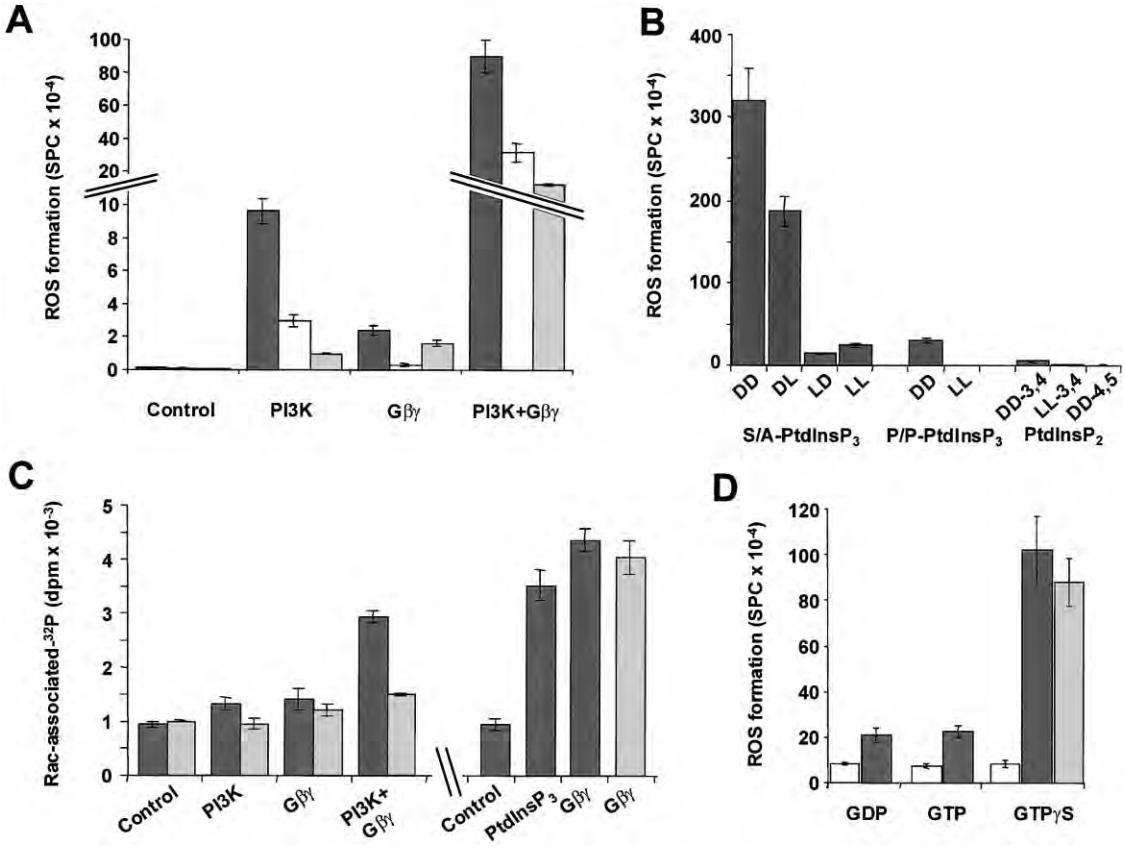


Figure 1. PI3K and Gβγ Regulate Rac Activation and ROS Formation

(A) PI3K and Gβγ synergistically stimulate 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 ROS formation (SPC, single photon counts in 0.1 min) was measured. Data are means (n = 4) ± SD from two experiments.

(B) PtdIns(3,4,5)P_3 stimulates ROS formation. Neutrophil cytosol and low-density membranes were incubated with isomers of PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, or PtdIns(4,5)P_2 (S/A, stearoyl-arachidonyl; P/P, dipalmitoyl), and ROS formation was measured. Data are means (n = 2–6) ± range.

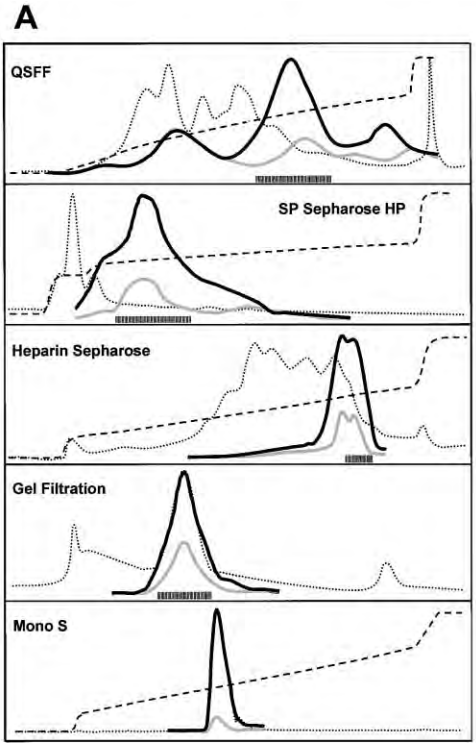
(C) PI3K and Gβγ synergistically stimulate Rac. Neutrophil cytosol and low-density membranes were incubated with PI3K (50 nM), PtdIns(3,4,5)P_3 (30 μM), and/or Gβγ (40 nM at left, 200 nM at right) either with (gray bars) or without (black bars) wortmannin (200 nM), and incorporation of [α-^{32}P]GTP into EE-Rac1 (30 nM) was quantified (means [n = 4–8] ± SD from four experiments).

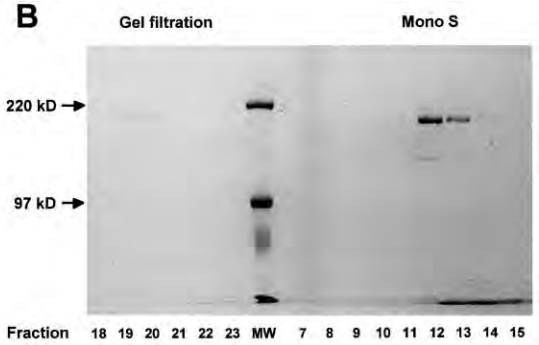
(D) Active Rac induces ROS formation. Neutrophil cytosol and low-density membranes were incubated either with wt-Rac (black bars) or dominant-negative N17-Rac (200 nM, white bars), preloaded and incubated with the indicated guanine nucleotides and, for wt-Rac, with wortmannin (200 nM, gray bar), and 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 [^{35}S]GTPγS 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)P_3 (10 μM final). Fractionation of cytosol on fast-flow Q-sepharose resolved a major peak of PtdIns(3,4,5)P_3-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)P_3-dependent Rac exchanger.

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





C

Column	Activity (%)	Protein (mg)	Volume (ml)	Rel. spec. activity	Fold purification
Cytosol	100	7500		0.013	1
QSFF	39.0	415	336	0.094	7.2
SP-Seph HP	11.1	19.5	84	0.57	43.9
Heparin-Seph	10.5	1.5	10	7	538
Gel filtration	10.5	0.6	10	17.5	1346
Mono S	8.7	0.53	1.2	16.4	1261

Figure 2. Purification of a PtdIns(3,4,5)P_3-Dependent Rac-GEF Activity from Pig Leukocyte Cytosol

(A) Chromatography profiles. The PtdIns(3,4,5)P_3-dependent Rac-GEF activity was purified from 90 l pigs' blood using this column sequence. The dotted line represents absorbtion at 280 nm; the dashed line shows salt concentration. Column fractions were assayed for Rac-GEF activity using liposomes either with (thick black line) or without (thick gray line) PtdIns(3,4,5)P_3. Gray bars represent the fractions selected for the following purification step.

(B) Silver-stained SDS-PAGE of fractions, including the peak of PtdIns(3,4,5)P_3-dependent Rac-GEF activity recovered from columns at the gel filtration (1% fraction vol.) and Mono S (1.67% fraction vol.) purification steps.

(C) Purification summary. The absolute activity of the starting material (100%) was calculated to be stimulating the loading of 1.4 pmol of GTPγS onto Rac (above Rac alone) min^{-1} mg^{-1} protein, in the presence of PtdIns(3,4,5)P_3, under the conditions described in Experimental Procedures.

of P-Rex1 is arranged into 41 exons, stretched over more than 300 kb of chromosome 20 at q13.13 (AL131078, AL049541, 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 (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)P_3- and G$\beta\gamma$-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)P_3-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)P_3-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)P_3 had a 50% maximal effect at 0.3 μM (Figure 5A), at which concentration P-Rex1

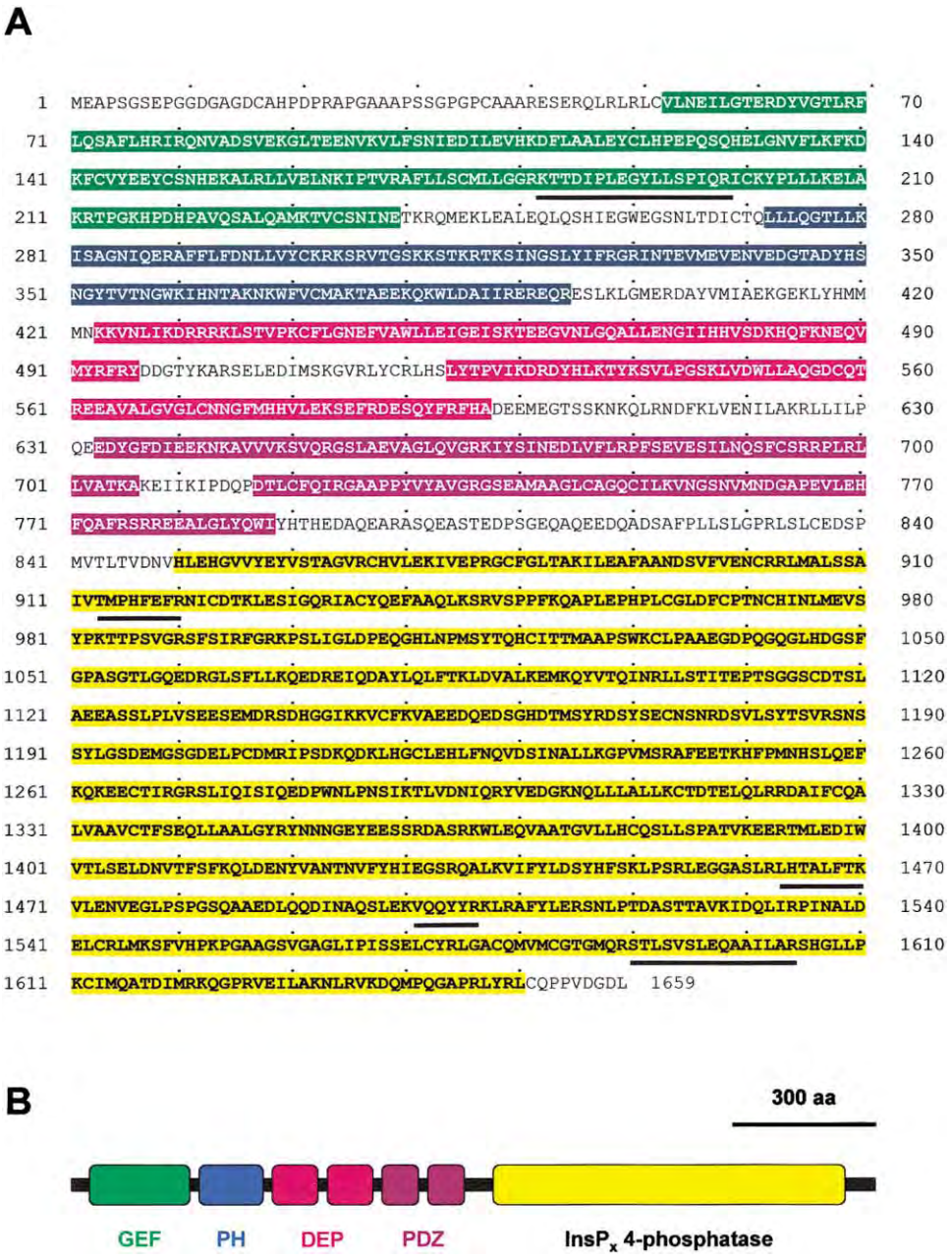


Figure 3. Structure of Human P-Rex1

(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-diastereoisomer of PtdIns(3,4,5)P_3 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)P_3, with a weak activation by the biological diastereoisomer of PtdIns(3,4)P_2, but not by other phosphoinositides (Figure 5B). We observed that P-Rex1 was activated by dipalmitoyl PtdIns(3,4,5)P_3 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 (Stephens et al., 1998). 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)P_3- or PtdIns(3,4)P_2-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

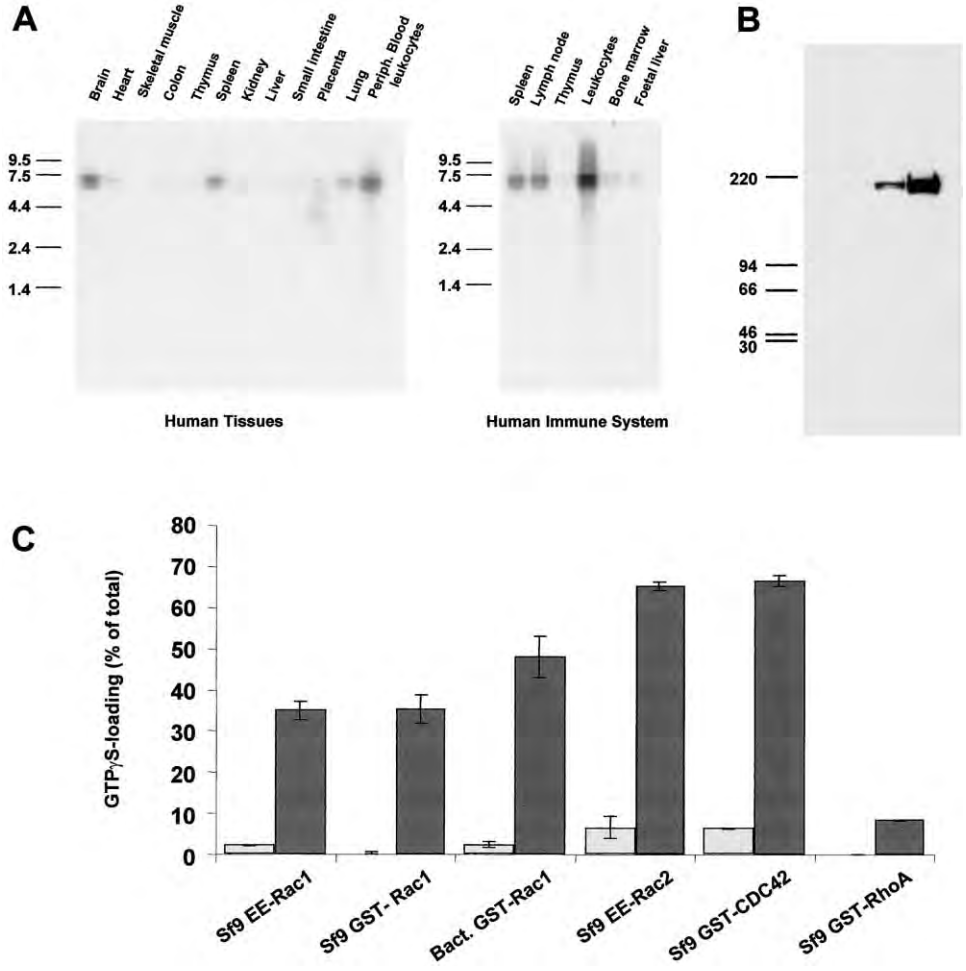


Figure 4. Expression and Substrate Specificity of Human P-Rex1

(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 5×10^3, 5×10^4, 5×10^5 cells/lane) were immunoblotted with anti-EE antibody.

(C) Recombinant human P-Rex1 GEF activity was assayed using liposomes (PtdCho, PtdS, 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 Gβγs 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 Gβγs on P-Rex1 Rac-GEF activity. Pure Gβγs from bovine brain and prepared as recombinant EE-G$\beta_1\gamma_2$ from coinfected SF9 cells both activated P-Rex1 Rac-GEF activity in vitro (Figures 5D and 5E). The effects of Gβγs 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 Gβγs, dependent on their posttranslational lipid modifications (Figure 5E). Gα alone or in the presence of AlF 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 Gβγs and Gα proteins) strongly inhibits P-Rex1 Rac-GEF activity; consequently, the scale of the effects of Gβγs in an experiment are a combined function of the Gβγ and the total cholate concentrations. We tested the effects of combinations of Gβγs and PtdIns(3,4,5)P_3 (Figure 5F). P-Rex1 was activated synergistically by Gβγs 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

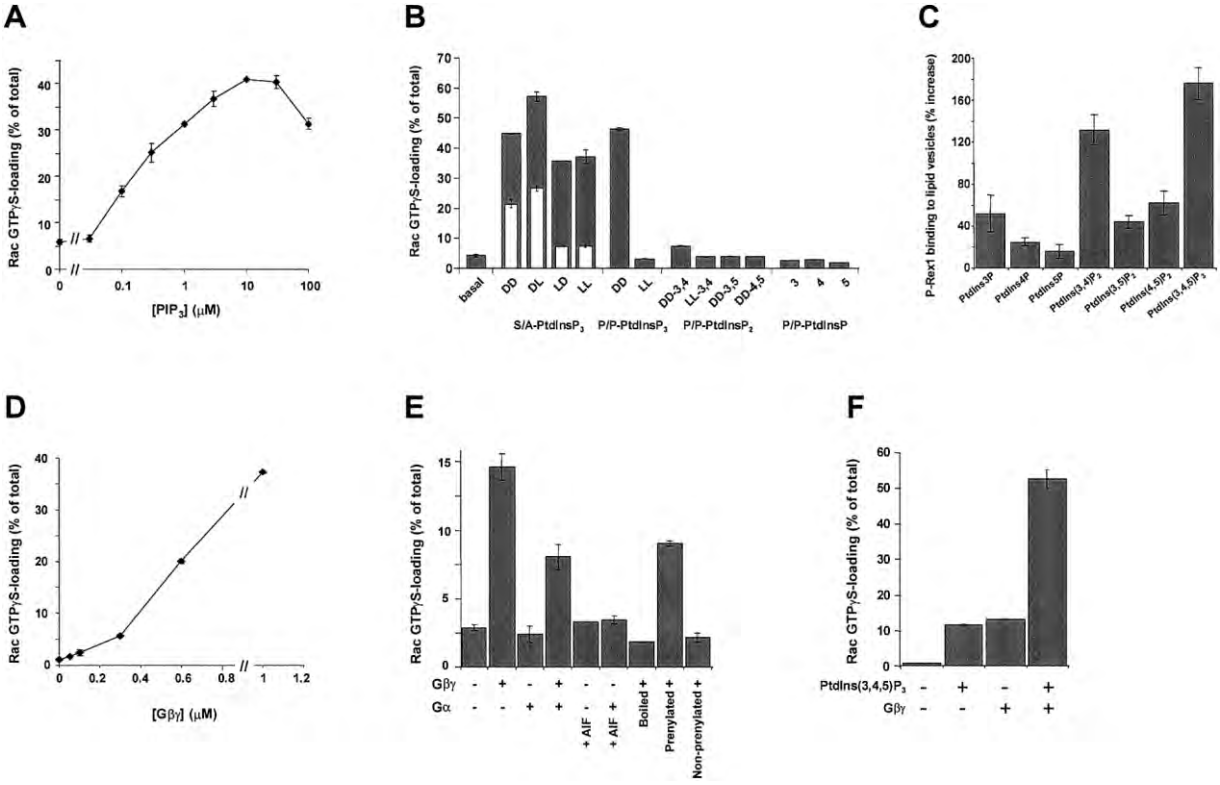


Figure 5. Regulation of Recombinant Human P-Rex1 Rac-GEF Activity by PtdIns(3,4,5)P_3 and Gβγ In Vitro

(A) PtdIns(3,4,5)P_3 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)P_3 (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 measuresd by Biacore. Data are means ± SD from four pooled experiments.

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

(E) Controls for Gβγ effects. P-Rex1-dependent activation of Rac was assayed using, where indicated, Gβγ (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), AlF (10 μM), boiled bovine brain Gβγ (0.5 μM), recombinant prenylated Gβγ (0.5 μM), or recombinant nonprenylated Gβγ (0.5 μM). For combinations of Gβγ 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)P_3 (0.2 μM) and bovine brain Gβγ (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 Gβγs and PtdIns(3,4,5)P_3 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/p110γ-PI3K, and $G\beta_1\gamma_2$ 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/p110γ-PI3K and $G\beta_1\gamma_2$ 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 Gβγ led to significantly higher PtdIns(3,4,5)P_3 production than PI3K expression alone, direct comparison of the increases in PtdIns(3,4,5)P_3 and GTP-Rac clearly showed that Rac activation in the presence of Gβγs and PI3K was substantially bigger than could be accounted for by the increase in PtdIns(3,4,5)P_3 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 PDGFβ receptor. In these cells, PDGF stimulates PtdIns(3,4,5)P_3 accumulation, wortmannin-sensitive activation of Rac, and Rac-dependent membrane ruffling and lamellipodia formation, and stable overexpression of constitutively active V12-Rac

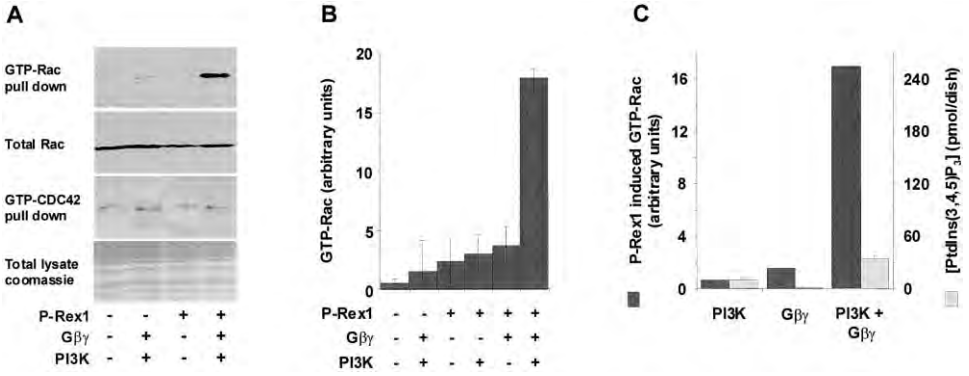


Figure 6. Rac-GEF Activity of Human Recombinant P-Rex1 In Vivo

(A) Western blots of Rac activation by P-Rex1 in vivo. Aliquots of 5×10^6 Sf9 cells in 6 cm dishes were infected with combinations of viruses encoding P-Rex1, $G\beta_1$, $G\gamma_2$, p101, p110γ, 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 G$\beta\gamma$-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) G$\beta\gamma$ and/or PI3K-induced formation of PtdIns(3,4,5)P_3 in Sf9 cells was measured (data are means [n = 5] ± range) and plotted against P-Rex1-dependent G$\beta\gamma$ 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 still 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, in 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).

P-Rex1 Has No Obvious Inositol Polyphosphate 4-Phosphatase Activity

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 using ^{32}P-PtdIns(3,4,5)P_3 and ^{32}P-PtdIns(3,4)P_2 as substrates and Inositol Polyphosphate 4-Phosphatase and SHIP-1 as controls. We also used paranitrophenolphosphate as a broad spectrum substrate in a protein phosphatase assay, using calf intestinal alkaline phosphatase and MEG-2 tyrosine phosphatase as controls. At P-Rex1 concentrations of up to 1.45 μM and 192 nM, respectively, in these assays, the enzyme exhibited no lipid or protein phosphatase activity.

Discussion

To our knowledge, no Rho family or Ras family GEFs have been successfully purified and identified on the basis of their GEF activity. In the case of the Rac-GEFs,

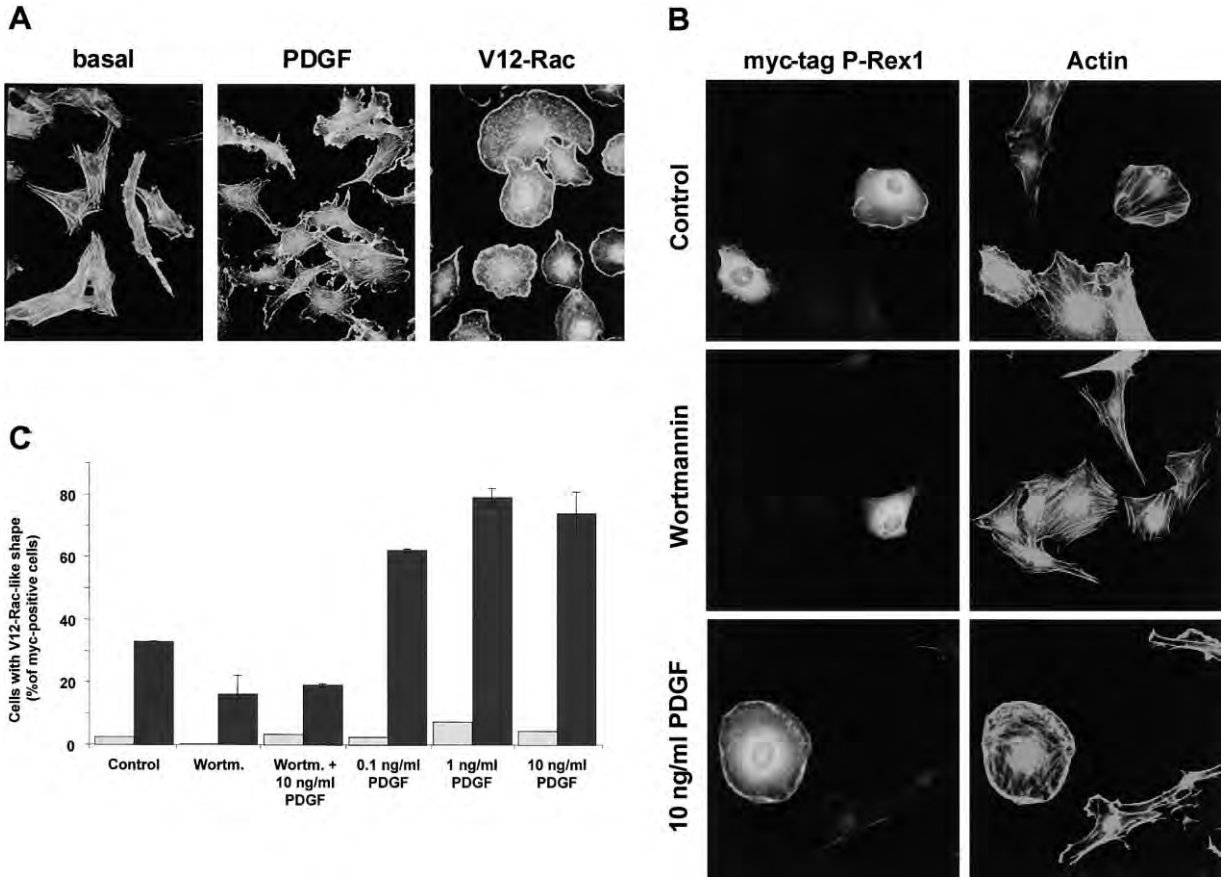


Figure 7. P-Rex1 Induces a Phenotype Like Activated Rac in PAE Cells

(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)P_3-sensitive Rac-GEF activity (in the presence of PtdIns(3,4,5)P_3, 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)P_3 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)P_3-containing phospholipid vesicles. However, P-Rex1 does not substantially translocate from the cytosol to the sites of PtdIns(3,4,5)P_3 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)P_3 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 PLCδ 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

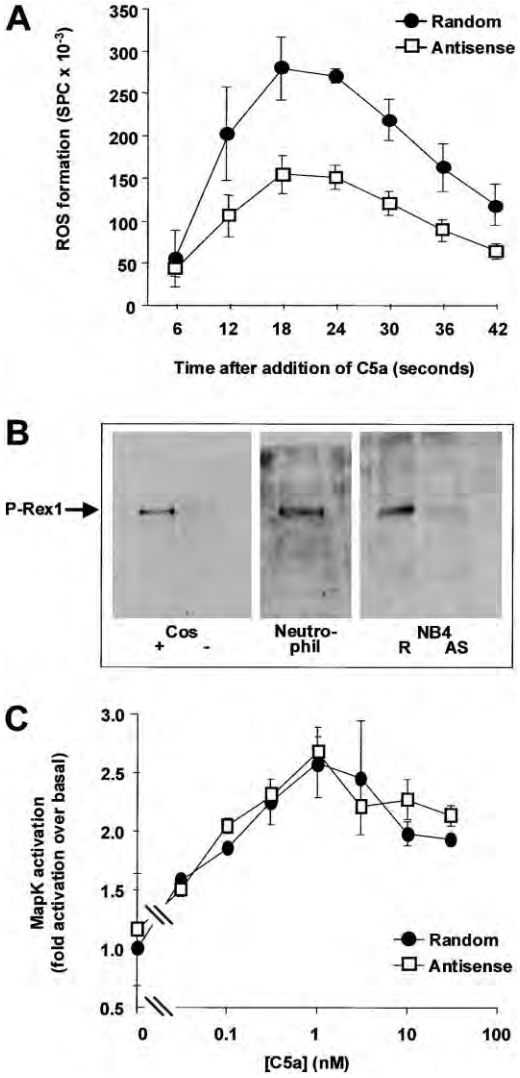


Figure 8. P-Rex1 Is Necessary for ROS Formation

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)P_3 to synergistically activate P-Rex1 suggests that these regulators can bind simultaneously 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 Gβγ subunits in neutrophils, including p101/110γ-PI3K and PLCβs (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 Gβγs, type 1B PI3K, PtdIns(3,4,5)P_3, 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, Gβγ activation of P-Rex1 may be more important than activation via PtdIns(3,4,5)P_3, as the levels of PtdIns(3,4,5)P_3 rise due to Gβγ 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)P_3 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).

P-Rex1 is a coincidence detector apparently designed to respond to the combined versus isolated appearance of PtdIns(3,4,5)P_3 and Gβγ, probably in the same membrane. In neutrophils, this set of signals is naturally delivered by activation of G protein-linked receptors in the context of a large population of G_i proteins in the plasma membrane and Gβγ-sensitive p101/110γ-PI3K (that is particularly enriched in hematopoietically-derived cells), which drives accumulation of PtdIns(3,4,5)P_3 in the membranes actually harboring Gβγs (Stephens et al., 1997). The importance of this synergy is possibly reflected in the fact that ligands like GM-CSF that activate type 1A PI3Ks primarily via protein-tyrosine kinase-based mechanisms do not cause detectable activation of Rac (Geijsen et al., 1999), despite the fact that they cause significant accumulation of PtdIns(3,4,5)P_3 (Corey et al., 1993). In other cellular contexts, perhaps in the brain, it is easy to imagine that P-Rex1 could be relevant in the detection of specific patterns of signaling that deliver coincident activation of type 1A PI3Ks and activation of G_i/G_o proteins. This type of regulation could be particularly significant in forming or strengthening

particular cell contacts in view of the key role Rac plays in, for example, neurite outgrowth (Luo et al., 1997).

Experimental Procedures

Materials

Monomeric GTPases (EE-Rac1, GST-Rac1, EE-N17 Rac1, EE-Rac2, GST-CDC42, and GST-RhoA) were purified from Sf9 cells in the GDP bound state to >95% purity and were stored in 1% (w/v) cholate, 5 μM GDP, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.15 M NaCl, 40 mM HEPES/NaOH (pH 7.4; 4°C). Non-lipid-modified GST-Rac was derived from bacteria.

Gβγ were purified from bovine brain (Sternweis and Robishaw, 1984) or from Sf9 cells (both wild-type EE-β1,γ2 and nonprenylated mutants EE-β1,C186S-γ2) and stored in 1% cholate, 1 mM DTT, 20 mM HEPES/NaOH (pH 8.0; 4°C), 5 μM GDP (for bovine brain Gβγs), and 1 mM EDTA. Gα subunits (a mixture of α_{i1}, α_{i2}, and α_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 BIOGNOSTIK, Göttingen, Germany (antisense, TCA TTG ATG GAG TAG ATC; randomized control, ACT ACT ACA CTA GAC TAC).

Recombinant EE-p101/hexa-His-p110 PI3K was produced from Sf9 cells (Stephens et al., 1997). Recombinant NH_2-terminally EE-tagged P-Rex1 was purified from Sf9 cells utilizing the EE-tag and stored in PBS, 1 mM EGTA, 1 mM DTT, 0.01% Na azide.

Stearyl-arachidonyl (S/A)-PtdIns(3,4,5)P_3 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)P_3 refers to D/D-(S/A)-PtdIns(3,4,5)P_3 unless otherwise stated.

Two independent affinity-purified sheep polyclonal anti-P-Rex1 antibodies (raised against conjugated peptides based on P-Rex1 sequence, CLHPEPQSQHE and CAAARESERQLRLR) were pooled and used to detect endogenous and heterologous P-Rex1 by immunoblotting.

ROS Formation Assay with Neutrophil Cytosol and Membrane Fractions

Neutrophil-enriched leukocytes were isolated from pigs' blood, sonicated in 0.25 M sucrose, 0.1 M KCl, 50 mM HEPES/NaOH (pH 7.2; 4°C), 1 mM DTT, 2 mM EGTA, 0.1 mM PMSF, and 1X antiproteases (20 μg/ml each of antipain, aprotinin, pepstatin, and leupetin), and centrifuged (100,000 × g, 1 h, 4°C) to yield cytosol and light membrane fractions (4.5 mg/ml protein, collected between 0.60 and 1.35 M sucrose and washed in sonication buffer). Light membranes (3 μl) were premixed with Gβγ subunits, p101/p110γ-PI3K, and/or N17-Rac (or boiled controls) in 6 μl containing 5 mM ATP, 8 mM $MgCl_2$, 20 mM HEPES/NaOH (pH 7.5; 4°C), 2 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM ortho-vanadate, 0.1 M KCl, 1 mM DTT, 0.01% (w/v) Na azide. After 25 min on ice, 20 μl was added containing 15 μl cytosol and 1 mM MgGTP, 20 μM FAD, 400 μM NADPH, and 200 μM luminol. After 8 min at RT, single photon counts (SPC) per 0.1 min were quantitated in a scintillation counter at intervals of 3–5 min for up to 20 min (blanks without cytosol were subtracted). When lipids were added, they were preincubated with the membranes, and 10 μM GTPγS replaced MgGTP. Where wortmannin was added, it was preincubated with both cytosol and membrane fractions for 15 min on ice. Where EE-Rac1 was added, it was preloaded with different guanine nucleotides at a 5-fold excess over bound GDP.

Rac-GEF Assay with Neutrophil Cytosol and Membrane Fractions

This assay was essentially as for ROS production, except pure, lipid-modified EE-Rac1 (30–50 nM final concentration) was added to the cytosol, and GTP, GTPγS, NADPH, FAD, and luminol were omitted. Three minutes after mixing membrane and cytosol fractions, [α-^{32}P]GTP (20 μCi per sample) was added. Four minutes later, the reaction was stopped and EE-Rac1 was pulled down using anti-EE antibody coupled to protein G sepharose. The total dpm on EE-Rac1 were quantified by scintillation counting (blanks without EE-Rac1 were subtracted).

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

Liposomes (phosphatidylcholine [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)P_3 (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 $MgCl_2$, 50 mg/ml BSA, 5 mM DTT, 20 mM HEPES/NaOH (pH 7.5; 4°C), 100 mM NaCl, 1 mM EGTA (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 GTPγS (in lipid buffer, final assay concentration 5 μM, including 1 μCi [^{35}S]GTPγS). 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 [^{35}S]GTPγS-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% 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 Gβγ, 2 μl of Gβγ in buffer A were added to the liposome/Rac mix before the 10 min on ice, and P-Rex1 was added as 5X.

Purification of PtdIns(3,4,5)P_3-Dependent Rac-GEF

Neutrophil-enriched leukocytes prepared from 90 l pigs' blood were sonicated in 30 mM Tris/HCl (pH 7.8; 4°C), 0.1 M NaCl, 4 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 0.5X antiproteases. The cytosol (100,000 × g supernatant) was diluted to 16.7 mM NaCl in "buffer B" (0.5 mM EGTA, 10% ethylene glycol, 1% betaine, 0.01% Na azide, 1 mM DTT, 50 μM PMSF, and 0.1X antiproteases) containing 10 mM Tris/HCl (pH 7.8; 4°C) applied to a 400 ml Q-sepharose fast-flow column equilibrated in buffer B containing 30 mM Tris/HCl (pH 7.8; 4°C) and 0.1 mM EDTA, and was eluted with a 0.1–0.6 M NaCl gradient over 3 l. The peak of PtdIns(3,4,5)P_3-dependent Rac-GEF activity eluted between 0.43 and 0.52 M NaCl, was desalted on a 1.4 l G25-fine column equilibrated in buffer B containing 20 mM HEPES/NaOH (pH 6.8; 4°C), and then was applied to a 50 ml SP-sepharose-HP column equilibrated in the same buffer and eluted with a 0.25–0.75 M KCl gradient over 500 ml. The activity was recovered between 0.31 and 0.375 M KCl, desalted on a 300 ml G25-fine column equilibrated in buffer B containing 20 mM HEPES/NaOH (pH 7.2; 4°C), applied onto a 12 ml Heparin sepharose column equilibrated the same buffer, and eluted with a 0.1–0.7 M KCl gradient over 150 ml. The activity was recovered between 0.55 and 0.69 M KCl. A fraction corresponding to 0.60–0.65 M salt, selected for good fold purification, was concentrated, pH adjusted, and applied to a 200 ml HPLC size exclusion column equilibrated in buffer B containing 20 mM HEPES/NaOH (pH 6.9; 4°C) and 120 mM NaCl. The activity was recovered after 104 ml, corresponding to an apparent size of 203 kDa, loaded onto a 1 ml Mono S FPLC column equilibrated in buffer B containing 20 mM HEPES/NaOH (pH 7.0; 4°C), and eluted with a 0.1–0.7 M KCl gradient over 54 ml. The pure, PtdIns(3,4,5)P_3-dependent Rac-GEF activity eluted between 0.375 and 0.425 M KCl.

Cloning of Human P-Rex1

A tryptic digest of purified pig PtdIns(3,4,5)P_3-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 A1466041 (homolgous 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 [α-^{32}P]dCTP using the prime-a-gene system (Promega) to make a 673 bp probe for screening a human U937 cell random prime λ-Zap2 cDNA library and a human spleen random prime λGT11 cDNA library, yielding 24

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 (Stratgene) as follows. Clone 1 was cut Sal1/Sph1 to yield pBluescript with the N terminus of P-Rex1 up to the first Sph1 site. Clone 2 was cut Sph1/Bcl1 to give the middle of P-Rex1, and clone 3, the PCR-derived C terminus, was cut Bcl1/Sal1 out of the T tail vector. The fragments were three-way ligated. The resulting polylinker of pBluescript had additional Spe1, Not1, and Pst1 sites 5′ of Sal1. 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 pCMV3 mammalian expression vectors with N-terminal myc or EE-epitope tags (Welch et al., 1998) or pAc0G1 Sf9 cell expression vector with N-terminal EE-tag by ligating P-Rex1 from EcoR1/Spe1-cut pBluescript-P-Rex1 into EcoR1/Xba1 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 (Ellson et al., 2001) using mixed phospholipid vesicles (PtdCho, PtdS, phosphatidylethanolamine [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)P_3 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, Gβγs, and PI3K. Measurement of PtdIns(3,4,5)P_3 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 8 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 filamentuous 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 in the presence of 1 μM all-trans retinoic acid as described (Lanotte et al., 1991) in the presence of either control or P-Rex1 antisense oligonucleotides for 2–3 days. MapK activation in response to C5a was monitored by immunoblotting with an anti-phospho-MapK antibody (from Cell Signaling Technology; used as recommended; 5×10^4 cells per sample). ROS formation was monitored using a luminol-based detection in a scintillation counter in single photon count mode (3×10^4 cells per sample).

Acknowledgments

We thank Pierce Gaffney for making S/A-phosphoinositides, Gavin Painter for making D/D-phosphoinositides, John Collard for providing us with the PAK-Crib construct, and Karen Cadwallader for assaying PtdIns(3,4,5)P_3 levels in Sf9 cells. The Isaac Newton Trust supports the L.R.S. laboratory. H.C.E.W. is the recipient of a Medical Research Council (MRC) Research Training Fellowship, and P.T.H. receives a Biotechnology and Biological Sciences Research Council (BBSRC) Advanced Senior Research Fellowship.

Received: July 6, 2001
Revised: February 12, 2002

References

Akasaki, T., Koga, H., and Sumimoto, H. (1999). Phosphoinositide 3-kinase-dependent and -independent activation of the small GTPase Rac2 in human neutrophils. J. Biol. Chem. *274*, 18055–18059.

Ambruso, D.R., Knall, C., Abell, A.N., Panepinto, J., Kurkchubasche, A., Thurman, G., Gonzalez-Aller, C., Hiester, A., deBoer, M., Harbeck, R.J., et al. (2000). Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. Proc. Natl. Acad. Sci. USA *97*, 4654–4659.

Arcaro, A. (1998). The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. J. Biol. Chem. *273*, 805–813.

Babior, B.M. (1999). NADPH oxidase: an update. Blood *93*, 1464–1476.

Benard, V., Bohl, B.P., and Bokoch, G.M. (1999). Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J. Biol. Chem. *274*, 13198–13204.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature *348*, 125–132.

Buchanan, F.G., Elliot, C.M., Gibbs, M., and Exton, J.H. (2000). Translocation of the Rac1 guanine nucleotide exchange factor Tiam1 induced by platelet-derived growth factor and lysophosphatidic acid. J. Biol. Chem. *275*, 9742–9748.

Condliffe, A.M., and Hawkins, P.T. (2000). Cell biology. Moving in mysterious ways. Nature *404*, 135–137.

Corey, S., Eguinoa, A., Puyana-Theall, K., Bolen, J.B., Cantley, L., Mollinedo, F., Jackson, T.R., Hawkins, P.T., and Stephens, L.R. (1993). Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src-related tyrosine kinase(s) in human myeloid derived cells. EMBO J. *12*, 2681–2690.

Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S., and Bustelo, X.R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature *385*, 169–172.

Das, B., Shu, X., Day, G.J., Han, J., Krishna, U.M., Falck, J.R., and Broek, D. (2000). Control of intramolecular interactions between the pleckstrin homology and Dbl homology domains of Vav and Sos1 regulates Rac binding. J. Biol. Chem. *275*, 15074–15081.

Dorseuil, O., Vazquez, A., Lang, P., Bertoglio, J., Gacon, G., and Leca, G. (1992). Inhibition of superoxide production in B lymphocytes by rac antisense oligonucleotides. J. Biol. Chem. *267*, 20540–20542.

Ellson, C.D., Gobert-Gosse, S., Anderson, K.E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, J.W., Cooper, M.A., Lim, Z.-Y., Holmes, A.B., et al. (2001). PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of $p40^{phox}$. Nat. Cell Biol. *3*, 679–682.

Fleming, I.N., Elliott, C.M., Buchanan, F.G., Downes, C.P., and Exton, J.H. (1999). Ca^{2+}/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation. J. Biol. Chem. *274*, 12753–12758.

Fleming, I.N., Gray, A., and Downes, C.P. (2000). Regulation of the Rac1-specific exchange factor Tiam1 involves both phosphoinositide 3-kinase-dependent and -independent components. Biochem. J. *351*, 173–182.

Gaffney, P.R.J., and Reese, C.B. (1997). Synthesis of 1-O-stearoyl-2-O-arachidonoyl-sn-glycer-3-yl-D-myo-inositol 3,4,5-trisphosphate and its stereoisomers. Bioorg. Med. Chem. Lett. *7*, 3171–3176.

Geijsen, N., van Delft, S., Raaijmakers, J.A., Lammers, J.W., Collard,

J.G., Koenderman, L., and Coffer, P.J. (1999). Regulation of p21rac activation in human neutrophils. Blood *94*, 1121–1130.

Geiszt, M., Dagher, M.C., Molnar, G., Havasi, A., Faure, J., Paclet, M.H., Morel, F., and Ligeti, E. (2001). Characterization of membrane-localized and cytosolic Rac-GTPase-activating proteins in human neutrophil granulocytes: contribution to the regulation of NADPH oxidase. Biochem. J. *355*, 851–858.

Han, J., Das, B., Wei, W., Van Aelst, L., Mosteller, R.D., Khosravi-Far, R., Westwick, J.K., Der, C.J., and Broek, D. (1997). Lck regulates Vav activation of members of the Rho family of GTPases. Mol. Cell. Biol. *17*, 1346–1353.

Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A., and Broek, D. (1998). Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. Science *279*, 558–560.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C., and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. Science *280*, 2112–2114.

Hawkins, P.T., Eguinoa, A., Qiu, R.G., Stokoe, D., Cooke, F.T., Walters, R., Wennström, S., Claesson-Welsh, L., Evans, T., Symons, M., and Stephens, L.R. (1995). PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. Curr. Biol. *5*, 393–403.

Kiyono, M., Satoh, T., and Kaziro, Y. (1999). G protein beta gamma subunit-dependent Rac-guanine nucleotide exchange activity of Ras-GRF1/CDC25(Mm). Proc. Natl. Acad. Sci. USA *96*, 4826–4831.

Krugmann, S., Anderson, K.E., Ridley, S.H., Risso, N., McGregor, A., Coadwell, J., Davidson, K., Eguinoa, A., Ellson, C.D., Lipp, P., et al. (2002). Identification of ARAP3, a novel PI3K effector regulating both Arf and Rho GTPases, by selective capture on phosphoinositide affinity matrices. Mol. Cell *9*, 95–108.

Lanotte, M., Martin-Thouvenin, V., Najman, S., Balerini, P., Valensi, F., and Berger, R. (1991). NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood *77*, 1080–1086.

Lemmon, M.A., and Ferguson, K.M. (2000). Pleckstrin homology domains: phosphoinositide-regulated membrane tethers. In Biology of Phosphoinositides, S. Cockcroft, ed. (Oxford: Oxford University Press), pp. 131–164.

Luo, L., Jan, L.Y., and Jan, Y.N. (1997). Rho family GTP-binding proteins in growth cone signalling. Curr. Opin. Neurobiol. *7*, 81–86.

Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell *1*, 183–192.

Nagase, T., Kikuno, R., Ishikawa, K.I., Hirosawa, M., and Ohara, O. (2000). Prediction of the coding sequences of unidentified human genes. XVI. The complete sequences of 150 new cDNA clones from brain which code for large proteins in vitro. DNA Res. *7*, 65–73.

Nimnual, A.S., Yatsula, B.A., and Bar-Sagi, D. (1998). Coupling of Ras and Rac guanosine triphosphatases through the Ras exchanger Sos. Science *279*, 560–563.

Pacold, M.E., Suire, S., Perisic, O., Lara-Gonzalez, S., Davis, C.T., Walker, E.H., Hawkins, P.T., Stephens, L., Eccleston, J.F., and Williams, R.L. (2000). Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. Cell *103*, 931–943.

Painter, G.F., Grove, S.J.A., Gilbert, I.H., Holmes, A.B., Paithby, P.R., Hill, M.L., Hawkins, P.T., and Stephens, L.R. (1999). General synthesis of 3-phosphorylated myo-inositol phospholipids and derivatives. J. Chem. Soc. Perkin Trans. *1*, 923–935.

Rameh, L.E., Arvidsson, A.K., Carraway, K.L., III, Couvillon, A.D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., et al. (1997). A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. J. Biol. Chem. *272*, 22059–22066.

Reif, K., Nobes, C.D., Thomas, G., Hall, A., and Cantrell, D.A. (1996). Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. Curr. Biol. *6*, 1445–1455.

Roberts, A.W., Kim, C., Zhen, L., Lowe, J.B., Kapur, R., Petryniak, B., Spaetti, A., Pollock, J.D., Borneo, J.B., Bradford, G.B., et al. (1999). Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. Immunity *10*, 183–196.

Sander, E.E., van Delft, S., ten Klooster, J.P., Reid, T., van der Kammen, R.A., Michiels, F., and Collard, J.G. (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J. Cell Biol. *143*, 1385–1398.

Scita, G., Nordström, J., Carbone, R., Tenca, P., Giardina, G., Gutkind, S., Bjarnegard, M., Betsholtz, C., and Di Fiore, P.P. (1999). EPS8 and E3B1 transduce signals from Ras to Rac. Nature *401*, 290–293.

Stam, J.C., and Collard, J.G. (1999). The DH protein family, exchange factors for Rho-like GTPases. Prog. Mol. Subcell. Biol. *22*, 51–83.

Stephens, L.R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A.S., Thelen, M., Cadwallader, K., Tempst, P., and Hawkins, P.T. (1997). The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell *89*, 105–114.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., McCormick, F., Tempst, P., et al. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science *279*, 710–714.

Sternweis, P.C., and Pang, C.H. (1990). Preparation of G proteins and their subunits. In Receptor Effector Coupling. A Practical Approach. E.C. Hulme, ed. (New York: Oxford University Press), pp. 1–30.

Sternweis, P.C., and Robishaw, J.D. (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J. Biol. Chem. *259*, 13806–13813.

Sternweis, P.C., and Smrcka, A.V. (1992). Regulation of phospholipase C by G proteins. Trends Biochem. Sci. *17*, 502–506.

Tapon, N., and Hall, A. (1997). Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr. Opin. Cell Biol. *9*, 86–92.

Van der Kaay, I., Cullen, P.J., and Downes, C.P. (1998). Phosphatidylinositol (3,4,5) trisphosphate mass measurement using a radioligand displacement assay. In Methods in Molecular Biology, Volume 105, I.M. Bird, ed. (Totowa, NJ: Humana Press), 109–125.

Welch, H., Eguinoa, A., Stephens, L.R., and Hawkins, P.T. (1998). Protein kinase B and Rac are activated in parallel within a phosphoinositide 3OH-kinase-controlled signaling pathway. J. Biol. Chem. *273*, 11248–11256.

Worthylake, D.K., Rossman, K.L., and Sondek, J. (2000). Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. Nature *408*, 682–688.

Yoshii, S., Tanaka, M., Otsuki, Y., Wang, D.Y., Guo, R.J., Zhu, Y., Takeda, R., Hanai, H., Kaneko, E., and Sugimura, H. (1999). alphaPIX nucleotide exchange factor is activated by interaction with phosphatidylinositol 3-kinase. Oncogene *18*, 5680–5690.

Accession Numbers

The EMBL and GenBank accession number for human P-Rex1 is AJ320261.