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P-Rex2, a new guanine-nucleotide exchange factor for Rac ☆

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Abstract We have identified a new guanine-nucleotide exchange factor, P-Rex2, and cloned it from human skeletal muscle and brain libraries. It has widespread tissue distribution but is not expressed in neutrophils. P-Rex2 is a 183 kDa protein that activates the small GTPase Rac and is regulated by phosphatidylinositol (3,4,5)-trisphosphate and the βγ subunits of heterotrimeric G proteins in vitro and in vivo. P-Rex2 has structure, activity and regulatory properties similar to P-Rex1 but has divergent tissue distribution, as P-Rex1 is mainly expressed in neutrophils. Together, they form an enzyme family capable of mediating Rac signalling downstream of G protein-coupled receptors and phosphoinositide 3-kinase.

Keywords: P-Rex2; P-Rex1; GEF; Rac; PI3K; Gβγ subunits

1. Introduction

The small GTPase Rac (isoforms 1, 2 and 3) regulates a wide range of cell functions, ranging from cytoskeleton-linked aspects such as cell shape, movement, secretion and phagocytosis to others such as transcription, translation or the production of reactive oxygen species [1,2]. Deletion of the ubiquitous Rac isoform Rac1 in mice is embryonic lethal [3], and loss of Rac function in neutrophils results in immune deficiencies in both human patients and in mouse models [4–7].

Rac, like all Rho-family GTPases, is regulated by guanine-nucleotide exchange factors (GEFs) [8]. These promote the dissociation of GDP from Rac, allowing free GTP to bind, thus rendering the GTPase active. More than a dozen Rac-GEFs have been identified so far, including for example the Vav, Sos and Tiam families. Some of these GEFs are specific for Rac, others more promiscuous [8]. Their GEF activities are tightly regulated, usually by protein kinases and/or by phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3), a lipid second messenger produced by class I phosphoinositide 3-kinase (PI3K) [9]. Phosphorylation stimulates, for example, the Rac-GEF activities of Vav1, Ras-GRF1 and Tiam1 [10–13], while PtdIns(3,4,5)P_3 strongly stimulates the Rac-GEF activities of P-Rex1 and SWAP-70 [14,15] and weakly those of Vav1, Sos1 and possibly Tiam1 [16–19]. The regulation of P-Rex1 is an exception: it is not only substantially activated by binding to PtdIns(3,4,5)P_3 but also by the βγ subunits of heterotrimeric G proteins, and these two mechanisms can either work independently of each other or in synergy [14]. P-Rex1 is mainly expressed in neutrophils and is necessary for full-scale reactive oxygen species formation in neutrophil-like HL60 and NB4 cells in response to stimulation of heterotrimeric G protein-coupled receptors [14].

Here, we report the identification and characterisation of Phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 2 (P-Rex2), a homologue of P-Rex1 that is also activated by PtdIns(3,4,5)P_3 and by Gβγ subunits and that is expressed in a wide range of tissues but not in neutrophils. P-Rex2 is likely to be an important mediator of Rac signalling that is able to act directly downstream of both G protein-coupled receptors and class I PI3K.

2. Materials and methods

2.1. Materials

Recombinant EE-Rac1, EE-G$\beta_1\gamma_2$ subunits and EE-tagged P-Rex1 were produced in Sf9 cells, purified, and stored as previously described [14]. Recombinant NH_2-terminal P-Rex2 was expressed in Sf9 cells, purified utilising the EE-tag and stored in PBS, 1 mM EGTA, 1 mM DTT, 0.01% Na azide, 50% glycerol and 2 mg/ml BSA. D/D-stearoyl-arachidonyl-PtdIns(3,4,5)P_3, the naturally occurring form of PtdIns(3,4,5)P_3, was synthesised by P. Gaffney [20].

2.2. Cloning of P-Rex2

The existence of P-Rex2 was predicted from searches of the EMBL database using the TBLASTN programme with the P-Rex1 peptide sequence [21]. Human skeletal muscle and brain marathon cDNA libraries from Clontech were used to clone P-Rex2 by PCR. For this, a battery of primers was designed on the basis of the sequence prediction from the EMBL database searches. A full-length clone was assembled in the pPCRScript vector from Stratagene and sequenced. Subcloning was done into pCMV3 [22] for expression in mammalian cells with either NH_2-terminal EE- or myc-epitope tags and into pAcoG1 for

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Abbreviations: P-Rex2, phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 2; GEF, guanine-nucleotide exchange factor; PtdIns(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphoinositide 3-kinase

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expression in Sf9 cells with an NH_2-terminal EE-epitope tag. Alignment of P-Rex2 and P-Rex1 was done using the CLUSTALX programme [23], and protein domain predictions were made using the SMART programme [24].

2.3. Northern blots

Northern blots were done with Human Multiple Tissue Northern blotting membranes from Clontech according to the manufacturer's instructions using a cDNA probe corresponding to nucleotides 2254–3403 of human P-Rex2.

2.4. In vitro Rac-GEF assay

Sf9 cell-derived purified human recombinant P-Rex2 was assayed for Rac-GEF activity in vitro essentially as previously described [14]. Briefly, GDP-loaded recombinant Sf9 cell-derived purified EE-tagged 100 nM Rac1 was incubated with 5 nM P-Rex2, [^{35}S]GTPγS and liposomes (consisted of PC, PS and PI) that either did or did not contain synthetic PtdIns(3,4,5)P_3 or recombinant Sf9 cell-derived purified $G\beta_1\gamma_2$ subunits. Rac was then immunoprecipitated using its EE-tag and its [^{35}S]GTPγS-loading was measured.

2.5. In vivo Rac-GEF assays

Sf9 cells were infected to express P-Rex2, p110γ/p101 PI3K and/or $G\beta_1\gamma_2$ subunits, serum-starved, and then endogenous GTP-Rac and GTP-Cdc42 were pulled down from cell lysates using the PAK-CRIB domain (that binds GTP-loaded but not GDP-loaded Rac and Cdc42 [25]) and visualised by anti-Rac or anti-Cdc42 Western blotting essentially as previously reported [14].

2.6. Immunofluorescence microscopy

Myc-tagged P-Rex2, P-Rex1 or DAPP1 were transiently expressed in pig aortic endothelial (PAE) cells by electroporation and plated onto glass coverslips. At 8 h after electroporation, cells were starved for 10 h in serum-free F12 medium containing 1% fatty-acid-free BSA. Medium was then replaced with fresh pre-warmed HEPES-buffered (pH 7.4, RT) serum-free F12 medium containing 1% fatty-acid-free BSA. After 60 min, cells were treated with or without 100 nM wortmannin for 10 min and then stimulated for 5 min with or without 10 ng/ml PDGF, fixed, permeabilised, and double-stained with anti-myc antibody followed by Alexafluor 488 anti-mouse antibody (Molecular Probes) and with TRITC-phalloidin (Sigma) essentially as previously described [22].

3. Results

3.1. Identification of P-Rex2

The existence of a homologue of P-Rex1 was predicted from database searches with P-Rex1 peptide. We named this homologue P-Rex2, for phosphatidylinositol (3,4,5)-trisphosphate–dependent Rac exchanger 2. The gene for human P-Rex2 lies on chromosome 8, q13.2. Like the gene for P-Rex1, the P-Rex2 gene is conserved throughout the vertebrates (Supplementary material Fig. A). No further homologues of the P-Rex family are predicted to exist.

For the cloning of human P-Rex2, we designed PCR primers on the basis of the predicted sequence and cloned the full-length cDNA from human skeletal muscle and brain marathon cDNA libraries. EMBL database accession code is AJ437636. The EMBL database also contains two related entries that hint to the possible existence of alternatively spliced versions of P-Rex2 (AY508997 and AY508996 and) (Supplementary material Fig. B and Fig. 1A).

Human P-Rex2 is a 183 kDa protein. It has an NH_2-terminal DH domain (the typical catalytic domain for GEFs of Rho-family GTPases) in tandem with a PH domain (phosphoinositide-binding domain) followed by two DEP and two PDZ domains (protein–protein interaction domains) and a weak homology over its C-terminal half to Inositol Polyphosphate 4-Phosphatase (Supplementary material Fig. B and Fig. 1A). P-Rex1 is very similar in size (185 kDa) and has the same domain structure [14]. Alignments show that P-Rex1 and P-Rex2 are 59% identical both on the nucleotide and amino acid levels (Supplementary material Fig. B).

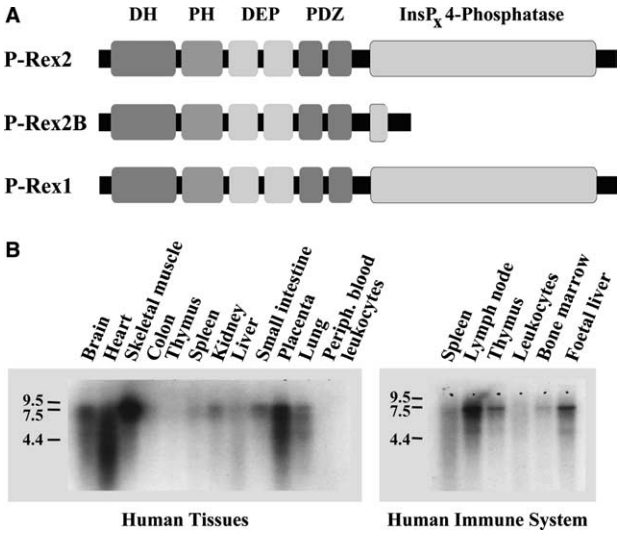

Fig. 1. Domain structure and tissue distribution of human P-Rex2. (A) Domain structure of human P-Rex2, P-Rex2B and P-Rex1 as predicted by SMART. The similarity to Inositol Polyphosphate 4-Phosphatase was added manually. P-Rex2B is predicted to exist by EMBL database entry AY508997. (B) Northern blots of P-Rex2 using Clontech Human Multiple Tissue Northern membranes with a cDNA probe made from nucleotides 2254–3403 of human P-Rex2.

3.2. Tissue distribution of P-Rex2

The tissue distribution of P-Rex2 was investigated by Northern blotting using Clontech Human Multiple Tissue Northern membranes with a cDNA probe corresponding to nucleotides 2254–3403 of human P-Rex2. This showed that P-Rex2 is widely expressed, most strongly in skeletal muscle, heart and placenta, but, notably, is absent from peripheral blood leukocytes (Fig. 1B).

3.3. P-Rex2 is a Rac-GEF in vivo

The similarity between P-Rex2 and P-Rex1 suggested that P-Rex2 is a Rac-GEF. To test this, we looked at the shape of PAE cells as a first read-out, as this depends on the activation status of their endogenous Rac (Fig. 2A and B). PAE cells are kite-shaped when basal and bear membrane ruffles and lamellipodia when Rac is active, while overexpression of Rac-GEFs or constitutively active Rac produces spreading and grossly exaggerated lamellipodia that make the cells look like fried eggs (denoted here "active-Rac shape") [14,22]. When P-Rex2 or, for comparison, P-Rex1 were expressed in PAE cells, some of the transfected cells were "active-Rac" shaped, whereas control cells were not (Fig. 2A and B), suggesting that P-Rex2 is a Rac-GEF in vivo.

To expand on this, we next tested whether P-Rex2 can be activated by PI3K in vivo, by looking for wortmannin-sensitivity of the "active-Rac" cell shape both in unstimulated and PDGF-stimulated PAE cells (Fig. 2A and B). Wortmannin-treatment reduced the percentage of P-Rex2-transfected cells displaying the "active-Rac" cell shape, whereas PDGF-stimulation increased it by 2-fold, and this was also sensitive to

wortmannin-treatment. These results suggest that P-Rex2 mediates the activation of Rac in a PI3K-dependent manner in vivo.

To test whether P-Rex2 Rac-GEF activity can be stimulated by Gβγ subunits as well as by PI3K in vivo, we used Sf9 cells which we infected with various combinations of P-Rex2, p110γ/p101 PI3K and/or $G\beta_1\gamma_2$ subunits. The activity of endogenous Rac was measured using the PAK-CRIB assay where GTP-loaded Rac is pulled down from cell lysates and visualised by Western blotting [25]. We used this approach previously to show that the Rac-GEF activity of P-Rex1 is synergistically stimulated by PI3K and Gβγ subunits [14]. We found here that, while expression of P-Rex2 with either PI3K or Gβγ subunits alone had only small effects on Rac GTP-loading (Fig. 2C) (on average 1.5- and 2-fold over basal, respectively, judged by densitometric scanning of Western blots from three separate experiments), P-Rex2 Rac-GEF activity was synergistically stimulated by PI3K and Gβγ subunits together (about 7-fold over basal) (Fig. 2C). Hence, P-Rex2 is a Gβγ- and PI3K-dependent Rac-GEF and regulated in a synergistic fashion in vivo just like P-Rex1.

Next, we used the same Sf9 cell system and PAK-CRIB assay to look for possible Cdc42-GEF activity of P-Rex2, as the small GTPase Cdc42 is very similar to Rac. However, P-Rex2 did not activate Cdc42 under these conditions (Fig. 2C), which is again the same as previously found for P-Rex1 [14].

3.4. P-Rex2 is activated by PtdIns(3,4,5)P_3 and by Gβγ subunits in vitro

To test whether P-Rex2 Rac-GEF activity can be stimulated by PtdIns(3,4,5)P_3 and by Gβγ subunits in vitro, we produced recombinant human P-Rex2 with an NH_2-terminal EE-tag in Sf9 cells and purified it using its epitope tag (Fig. 3A). For the in vitro GEF assay, we used this purified recombinant P-Rex2 with GDP-loaded Sf9 cell-derived purified recombinant EE-Rac1 as a substrate in a defined liposome environment and measured P-Rex2-dependent [^{35}S]GTPγS-loading of Rac.

Using this assay, we measured P-Rex2 Rac-GEF activity with increasing concentrations of synthetic PtdIns(3,4,5)P_3 in the liposomes. This showed that PtdIns(3,4,5)P_3 is sufficient to stimulate P-Rex2 Rac-GEF activity and that its effect is substantial, with an up to 12-fold activation and an EC_{50} of about 0.2 μM (Fig. 3B). We next verified that the effect of PtdIns(3,4,5)P_3 is through its direct binding to P-Rex2 (rather than, for example, by changing the liposome environment or through a charge effect). For this, synthetic PtdIns(3,4,5)P_3 was coupled to beads and incubated with Sf9 cell-derived purified human P-Rex2 using a method that we have previously employed to identify many PtdIns(3,4,5)P_3-binding proteins [26]. P-Rex2 bound directly to the PtdIns(3,4,5)P_3 beads and could be displaced from the beads only by an excess of the naturally occurring DD-form of PtdIns(3,4,5)P_3 but not by its LL-PtdIns(3,4,5)P_3 stereoisomer (data not shown).

Next, we assayed P-Rex2 Rac-GEF activity with increasing concentrations of recombinant Sf9 cell-derived $G\beta_1\gamma_2$ subunits. Like PtdIns(3,4,5)P_3, the Gβγ subunits were sufficient to stimulate the Rac-GEF activity of P-Rex2, and did so substantially, with a 15-fold activation over basal at 0.5 μM Gβγ subunits (Fig. 3C). These levels of stimulation of P-Rex2 Rac-GEF activity by PtdIns(3,4,5)P_3 and Gβγ subunits in vitro are very similar to those previously found for P-Rex1 [14].

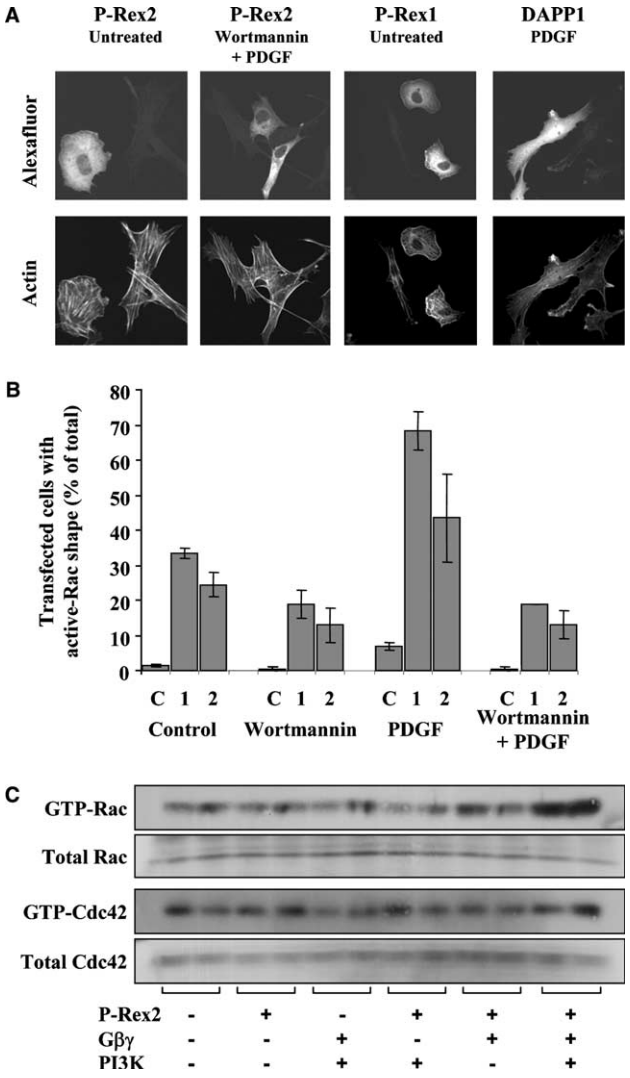

Fig. 2. P-Rex2 is a Rac-GEF in vivo. (A) Confocal immunofluorescence micrographs of PAE cells transfected with myc-tagged human P-Rex2, P-Rex1, or DAPP1, as described in Section 2. Serum-starved cells were pre-treated with or without 100 nM wortmannin for 10 min, stimulated with or without 10 ng/ml PDGF for 5 min, as indicated, and then double-stained with Alexafluor for myc-tagged proteins and with TRITC-phalloidin for polymerised actin. Confocal micrographs are from one experiment representative of three. (B) Quantification of results from confocal immunofluorescence micrographs of myc-tagged human P-Rex2 (2), P-Rex1 (1), or DAPP1 (c) obtained as in (A). One hundred transfected cells from each coverslip were assessed for their shape. Spread cells with grossly exaggerated lamellipodia were counted as "active-Rac". Data are means ± range from two separate experiments. (C) P-Rex2 Rac-GEF activity in Sf9 cells. Sf9 cells were infected to express human P-Rex2, p110γ/p101 PI3K and/or $G\beta_1\gamma_2$ subunits as indicated, serum-starved, and then endogenous GTP-Rac and GTP-Cdc42 were pulled down using PAK-CRIB and visualised by Western blotting (top panels; from 4×10^6 cells/lane) and compared to total-Rac and total-Cdc42 (bottom panels; lysates from 8×10^4 cells/lane), as detailed in Section 2. Data are from one experiment representative of three.

4. Discussion

The similarities between the P-Rex2 enzyme we have identified here and P-Rex1 [14] are extensive. Both are Rac-GEFs and regulated by PtdIns(3,4,5)P_3 and Gβγ subunits. A clue to

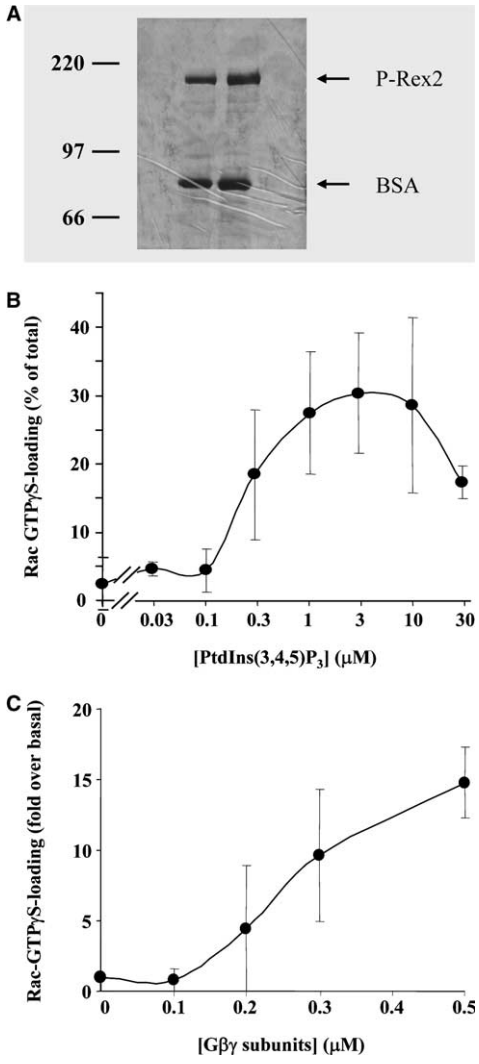

Fig. 3. P-Rex2 is directly activated by PtdIns(3,4,5)P_3 and by Gβγ subunits. (A) Coomassie-stained SDS–PAGE gel of purified P-Rex2. Recombinant human EE-P-Rex2 was produced in and purified from Sf9 cells as detailed in Section 2, and its purity assessed by Coomassie-staining (2 or 4 μg P-Rex2/lane). (B) PtdIns(3,4,5)P_3 dose–response of P-Rex2 Rac-GEF activity. Sf9 cell-derived purified recombinant P-Rex2 was subjected to an in vitro Rac-GEF assay as detailed in Section 2 with the indicated concentrations of PtdIns(3,4,5)P_3. Data are means ± range from two to four separate experiments. (C) Gβγ subunit dose–response of P-Rex2 Rac-GEF activity. As in (B), but with the indicated concentrations of Sf9 cell-derived purified recombinant $G\beta_1\gamma_2$ subunits instead of PtdIns(3,4,5)P_3. Each point has a different cholate concentration (from Gβγ subunit storage buffer), which has been corrected for by subtracting an appropriate cholate-only control. Data are means ± range from two separate experiments.

why both P-Rex1 and P-Rex2 exist lies obviously in their divergent tissue distribution. In neutrophils, where P-Rex1 is mainly expressed, P-Rex1 regulates the formation of reactive oxygen species by the NADPH oxidase [14], an enzyme complex that not only requires GTP-Rac as an activating signal but also as an integral subunit. As the NADPH oxidase is not expressed in other cell types, P-Rex2 governs presumably the more general cellular roles of Rac, such as regulation of the cytoskeleton, transcription and translation. P-Rex2 expression is highest in skeletal muscle. Interestingly, another Rac-GEF, Trio, is essential for skeletal muscle development, by regulating the formation of secondary myotubes [27].

Their divergent tissue distribution not only influences the cellular roles of P-Rex1 and P-Rex2, but also impacts on the way they are activated. In neutrophils, one extracellular signal is sufficient to generate both Gβγ subunits and PtdIns(3,4,5)P_3 and, hence, to fully activate P-Rex1, due to the presence of a Gβγ-regulated form of PI3K, class 1B PI3K [28]. In contrast, P-Rex2 is expressed in many cell types and tissues that are thought not to contain class 1B PI3K. Hence, two different extracellular signals are needed to fully activate P-Rex2 in these tissues, one that stimulates G protein-coupled receptors to generate Gβγ subunits and one that stimulates protein-tyrosine kinase-linked receptors to generate PtdIns(3,4,5)P_3 via the ubiquitous class 1A PI3K. The contribution of each activating pathway to the functional importance of P-Rex2 may even turn out to vary between cell types or receptor types. A comparable situation can be found for the Rac-GEF Vav1, regarding the contribution of tyrosine phosphorylation and PtdIns(3,4,5)P_3-binding to its regulation: in B cells, Vav1 Rac-GEF activity is stimulated by the protein-tyrosine kinase Syk, and this is not affected by PI3K, whereas in T cells, Fyn-mediated tyrosine phosphorylation cooperates with PI3K to activate Vav1 [29].

For P-Rex2, both activating signals, Gβγ subunits and PtdIns(3,4,5)P_3, are membrane-bound, and it is generally accepted that the membrane is the place of action for GEFs. However, the subcellular localisation of P-Rex2 is mostly cytoplasmic, and we did not find any large-scale membrane translocation upon PDGF-stimulation of PAE cells. The situation is the same for P-Rex1. We assume that low-level membrane association of the P-Rex enzymes in serum-starved cells is sufficient for Rac activation. However, the importance of membrane association and translocation is clearly a point of interest for future work on P-Rex family enzymes.

P-Rex1 and P-Rex2 are unique among GEFs in that they are directly activated by Gβγ subunits. The Rac-GEF activity of Ras-GRF1 has been reported to be stimulated by Gβγ subunits, but their effect is indirect by modulating the Rac-GEF's tyrosine phosphorylation state [12]. Dbl, a RhoA- and Cdc42-GEF, can bind Gβγ subunits directly via its NH_2-terminus, but this has no effect on its enzymatic activity [30]. Other classes of enzymes that are directly regulated by Gβγ subunits exist, the class 1B PI3K mentioned above [28] and the PLCβ family [31], but a common Gβγ-binding site, equivalent perhaps to the PH domain as a phosphoinositide-binding site, does not seem to exist. Future mutational analysis of the P-Rex family enzymes will be needed to define where the Gβγ subunits bind and their mechanisms of action.

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