P-REX2, a novel PI-3-kinase sensitive Rac exchange factor

Hans Rosenfeldt[a], José Vázquez-Prado[b], J. Silvio Gutkind[a,*]

[a]*Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, DHHS, Bethesda, MD 20892-4330, USA*
[b]*Department of Pharmacology, CINVESTAV-IPN Av. Instituto Politécnico Nacional 2508 Col. San Pedro Zacatenco, 07360 Apartado postal 14-740, 07000 México, D.F. Mexico*

Received 4 June 2004; accepted 22 June 2004

Available online 22 July 2004

Edited by Richard Marais

Abstract We have identified an activator of Rac, P-REX2, that is structurally related to the exchange factor PtdIns(3,4,5)-dependent Rac exchanger (P-REX1), but exhibits distinct tissue-specific expression. P-REX2 is spliced into two RNA species, ~3.5 and ~10 kb in size. The cDNA corresponding to the smaller transcript encodes a protein that exhibits strong similarity with P-REX1 within its N-terminal domains, but differs in the C-terminal region. P-REX2 promoted increased levels of GTP-bound Rac that could be further stimulated by enhancing PI-3K activity. Thus, P-REX2 may serve as a novel link between Rac activation and the PI-3 kinase pathway.

Keywords: Guanine nucleotide exchange factor; PI-3K; G-protein

1. Introduction

Guanine nucleotide exchange factors (GEFs) for small GTP-binding proteins of the Rho family, including Rho, Rac, and Cdc42, play an essential role in the spatio-temporal specific accumulation of active, GTP-bound forms of these GTPases. This coordinated activation of Rho GTPases is a vital component of important physiological and pathological processes as diverse as cell migration, cellular contraction, gene expression, cell proliferation, and transformation [1]. GEF proteins achieve this fine-tuning of small G-protein activity in response to specific signal transduction pathways by virtue of a large variety of protein–protein and protein–lipid interaction domains such as PDZ (PSD95/DLG/ZO1), PH (Pleckstrin-homology), SH3 (Src-homology-3), CH (Calponin Homology), and the FYVE (Fab-1, YGL023, Vps27, and EEA1) domains (reviewed in [1,2]).

Here, we report the identification of a guanine nucleotide exchange factor (GEF) for Rac containing strong sequence identity to PtdIns(3,4,5)-dependent Rac exchanger (P-REX1) [3]. We have designated this protein PtdIns(3,4,5)-dependent Rac exchanger-2 (P-REX2), based on this sequence similarity. The gene for P-REX2 encodes a protein that contains the same domain architecture as P-REX1, including DH (Dbl-homology), PH, Dep (Disheveled, EGL-10, Pleckstrin) and PDZ domains, but contains a much shorter C-terminus. P-REX2 overexpression stimulated Rac- and Cdc42-GTP loading, and serum response element (SRE) dependent transcription. Moreover, P-REX2 activation of Rac and the SRE was further stimulated by PI-3 kinase, suggesting that P-REX2 may function as part of a tissue-specific mechanism by which polypeptide growth factors and G protein-coupled receptors (GPCRs) regulate small GTPases.

2. Materials and methods

2.1. Materials

Bovine serum albumin (fatty acid-free), Dulbecco's modified Eagle's medium, trypsin/EDTA solution, and Fetal bovine serum were purchased from Sigma (St. Louis, MO); anti-mouse secondary HRP-linked antibody was from ICN Pharmaceuticals (Aurora, OH); and Glutathione–Sepharose beads were purchased from Amersham Biosciences (Piscataway, NJ).

2.2. DNA constructs

Clone FLJ12987, containing base pairs 646-2948 of P-REX2b, was obtained from the National Institute of Technology and Evaluation (NITE), Chiba, Japan. PCR using primers derived from chromosomal sequences aligning to the P-REX1 N-terminus were used to amplify base pairs 25–646. Alignment of P-REX2b cDNA sequence showed that an in-frame ATG directly preceded by an ACC translational start site occurred 24 bp upstream of the 5′ oligo. We designated this sequence as the putative start site. P-REX1 was obtained by ligating a 5′ RACE product to KIAA1415, thus reconstituting a full-length cDNA corresponding to P-REX1 (NM_020820).

2.3. Northern blotting

A Multiple Tissue Northern blot from pooled fetal human tissue (Clontech, Palo Alto, CA) was hybridized with [^{32}P]dCTP labeled P-REX1 and P-REX2-specific probes generated from their DH–PH domains. Blots were exposed 1–5 days at −70 °C with scintillation screen.

2.4. Small GTPase pull-down assay

Cytosolic extracts were prepared from HEK 293T cells co-transfected with vector or expression plasmids for tagged Rac, Cdc42, and RhoA. Pull-down assays using GST-Pak or rhotekin fusion proteins were performed essentially as previously reported [4].

2.5. Luciferase assays

HEK 293T cells were transfected with different expression vectors in combination with 50 ng of the pSRF luciferase reporter (Stratagene). Luciferase activity was detected using a Luciferase Assay Kit (Promega) and a Microliter plate luminometer (DINEX Tech).

* Corresponding author. Fax: +1-301-402-0823.
E-mail address: sg39v@nih.gov (J.S. Gutkind).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.06.097

3. Results

3.1. Identification of a P-REX1 related gene

NCBI blast analysis of the P-REX1 cDNA against human chromosomal sequence generated two sets of homologous sequences, one derived from chromosome 20, corresponding to P-REX1, and another from chromosome 8. Further blast analysis of these aligning sequences against EST databases indicated the presence of a P-REX1-related gene on chromosome 8. Based on sequence similarity to P-REX1, we designated this gene as P-REX2.

Since all aligning EST sequences contained 3′ portions of P-REX2 (based on the P-REX1 sequence), we used the MIT Scan gene prediction program [5,15] to generate a putative transcript from chromosome 8 sequence. The transcript predicted by this program contained about 1000 bp of sequence that did not align with P-REX1, and all attempts to amplify PCR products from a variety of cDNA sources failed. As an alternative strategy, we used the tn-blast program [6,7] to align the P-REX1 protein sequence to chromosome 8 DNA. The resulting alignment identified a total of 30 regions on chromosome 8 that contained >30% identity to P-REX1 protein when translated (Fig. 1). These stretches of alignment spanned over 265 kb in the 8q13.1 region of this chromosome. The identity between the P-REX1 search sequence and the chromosome 8 genomic DNA ranged from 30% to 80%, indicating that the search had identified exons from a putative gene similar to but not identical to P-REX1. We generated a series of PCR products derived from primers targeting the P-REX2 chromosomal sequence containing identity to P-REX1, starting from the most 5′ available EST FLJ12987 [8], obtained from NIST, Chiba, Japan, and proceeding to a fragment that terminates 25 bp short of the putative start site (Fig. 1A). The eight amino acids encoded by the predicted initial 25 base pairs align to the PREX1 N-terminus, and there is a strong translational initiation sequence immediately upstream of the first ATG, indicating that this methionine codon likely represents the beginning of the P-REX2 open reading frame.

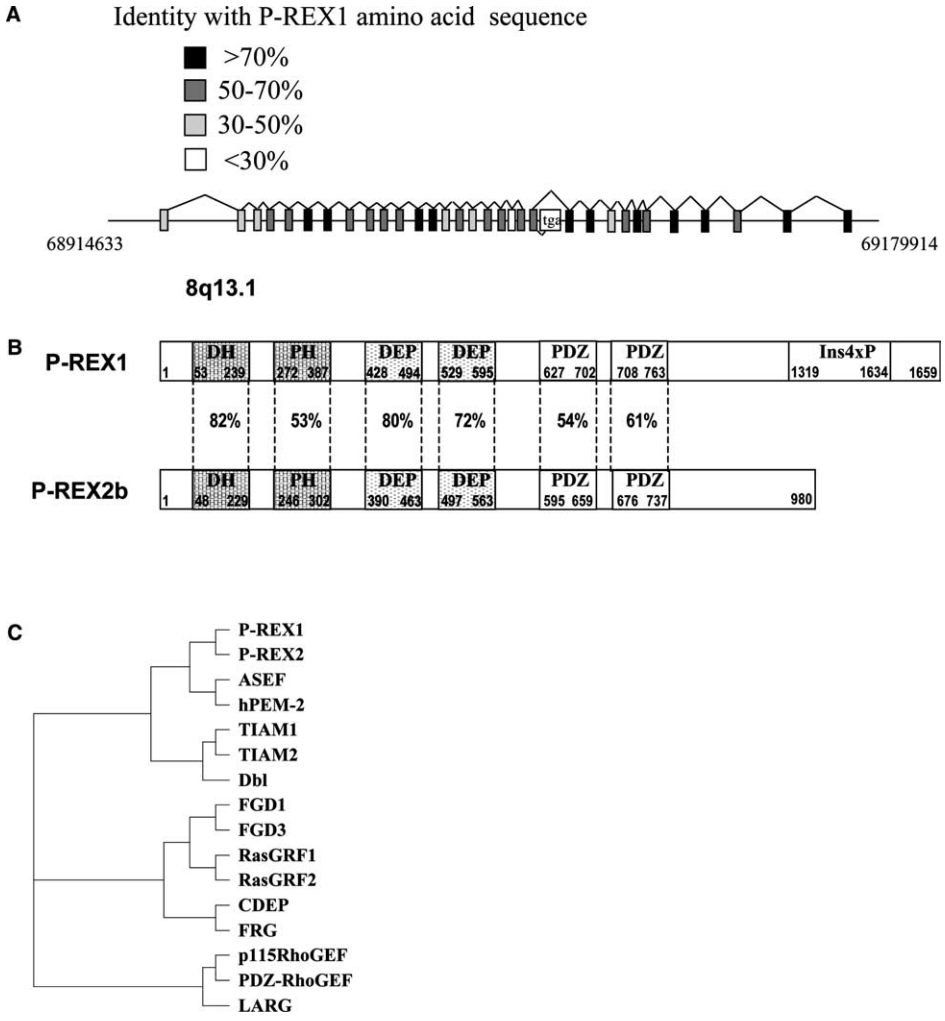

Fig. 1. Bioinformatics Analysis. (A) Alignment of the P-REX1 amino acid sequence to the translated sequences from a 250 kb region in chromosome 8q13.1 using the tnBlast program (NCBI). Chromosome 8 regions aligning to P-REX1 are depicted according to sequence identity, revealing the exon structure of a P-REX1-related gene. Sequences from these studies identified FLJ12987, an EST containing a non-PREX1-related exon that encodes an in-frame stop codon, suggesting the possibility that this P-REX1-related gene is alternatively transcribed. (B) Comparison of P-REX1 and P-REX2b amino acid sequences. Both proteins contain the same N-terminal domains in the same order, but differ in their C-terminus. Percent amino acid identity between the shared N-terminal domains is depicted in the alignment. (C) Dendogram derived from ClustalW [16] analysis of Dbl-homology (DH) domain amino acid sequences from a range of known Rac, Cdc42, and Rho-exchange factors.

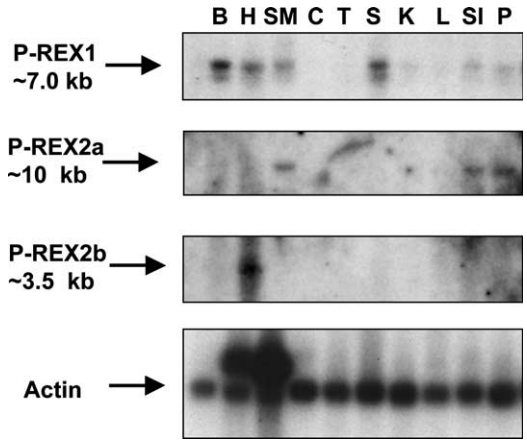

Fig. 2. Multiple tissue Northern blot analysis of P-REX1 and P-REX2 expression pattern. P-REX1 expression is focused primarily in peripheral blood leukocytes, but can also be detected in varying amounts in brain, heart, skeletal muscle, spleen, kidney, small intestine, and placenta. By contrast, there are two detectable P-REX2 transcripts. P-REX2b (~3.5 Kb) is mainly expressed in heart, while P-REX2a (~10 Kb) is expressed in skeletal muscle, small intestine, and placenta. B: brain, H: heart, SM: skeletal muscle, C: colon, T: thymus, S: spleen, K: kidney, L: lung, SI: small intestine, P: placenta.

Further, alignment of the predicted FLJ12987 protein sequence to 8q13.1 revealed two additional exons corresponding to bp 69059206–69059367 and bp 69067332–69067558 of chromosome 8 (gi 37626424). Both these exons exhibited <30% identity with P-REX1 and the second area of alignment contained an in-frame TGA stop codon (Fig. 1A). This stop codon-containing exon was present in both FLJ12987 and chromosome 8 sequences, but did not align to P-REX1. Sequencing of the 3′ end of the FLJ12987 EST indicated the presence of a poly-A tail, suggesting that this clone derived from a mature mRNA. Interestingly, additional P-REX1-aligning sequences downstream of this exon indicated the strong possibility that P-REX2 was alternatively transcribed. The larger transcript predicted from the homology of chromosome 8 to P-REX1 was designated P-REX2a (GenBank Accession No. BK005161) and the smaller transcript identified was designated P-REX2b (GenBank Accession No. BK005160).

Sequence comparison of P-REX2b to P-REX1 at the predicted protein level showed that there is an overall 69% identity between the two molecules (Fig. 1B). The predicted protein sequence of the P-REX2 clone that we identified is substantially smaller than P-REX1, with a molecular weight of 112 kDa. This form of P-REX2, however, did contain all but one of the identifiable protein motifs in P-REX1, such as the DH, PDZ, and DEP domains, with a missing InsPx4 phosphatase domain (Fig. 1B) as the sole exception. This difference is likely to be a result of differential splicing, since P-REX1 aligning sequences on chromosome 8 that occur after the TGA stop codon in the last exon of the P-REX2b clone contain this domain (Fig. 1A). Indeed, the predicted P-REX2a transcript also encodes an InsPx4 phosphatase domain (not shown). Sequence analysis of the DH domains from a variety of Rho, Rac and Cdc42-specific guanidine exchange factors indicated that the DH domains of P-REX2 and P-REX1 were closely related to the DH domains present in ASEF, a Rac-specific GEF [9], and hPEM-2, a Cdc42-specific GEF [10] (Fig. 1C). P-REX1 and P-REX2 related transcripts were also found in fish, chickens, rats, and mice but not in fruit flies or worms (not

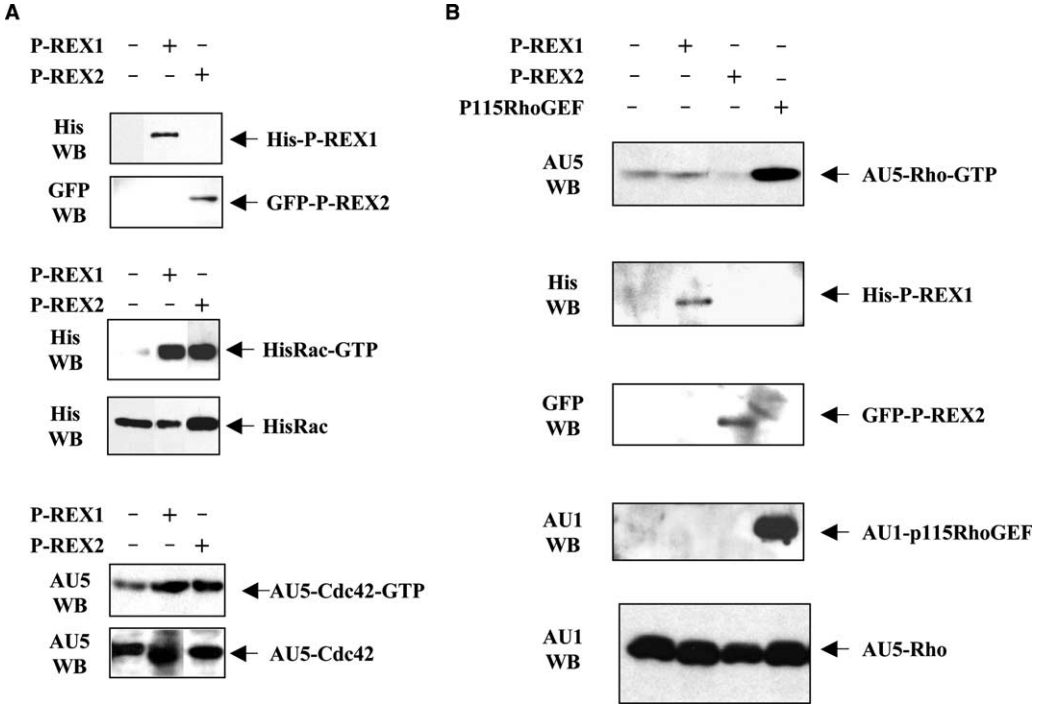

Fig. 3. P-REX1 and P-REX2 activate Rac and Cdc42 but not RhoA. His-tagged P-REX1 and GFP-tagged P-REX2 were co-expressed in HEK 293T cells with (A) His-tagged Rac, AU5-tagged Cdc42, or (B) AU5-RhoA. Cells were assayed for Rac, Cdc42, and RhoA-GTP levels by a GST-Pak or GST-Rhotekin pull down approach. WB: Western blot. As a control, AU1-tagged p115RhoGEF elevated levels of Rho-GTP.

shown). Interestingly, only one locus (ESTs BW059256 and BW061514) could be found in the sea squirt (*Ciona intestinalis*), a primitive chordate.

3.2. Tissue distribution of P-REX2 transcripts

In order to determine the expression pattern of P-REX2, we used a fragment corresponding to base pairs 141–877 of P-REX2, as a probe for multiple tissue Northern blot (Clontech). This analysis revealed the presence of two distinct P-REX2 transcripts expressed in different tissues (Fig. 2). P-REX2b was expressed in heart, was ~3.5 kb in size and matched the size of the P-REX2 clone that we generated from RT-PCR and the FLJ12987 EST clone. By contrast, P-REX2a was ~10 kb in size and was expressed in skeletal muscle, small intestine, and placenta, highlighting the possibility that a much larger version of P-REX2 was present in these tissues. Further, these data suggested that the P-REX2 transcript that is specifically expressed in heart corresponded to the cDNA clone presented herein. P-REX1 is most highly expressed in peripheral blood leukocytes, but both P-REX2 transcripts were not present in this tissue (data not shown).

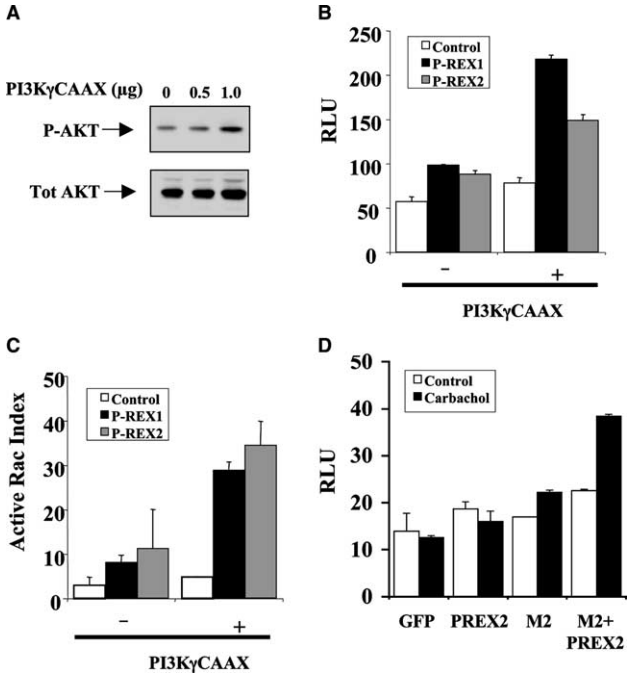

Fig. 4. P-REX1 and P-REX2 are sensitive to PI-3K stimulation. (A) A dominant active catalytic subunit of PI-3Kγ stimulates AKT phosphorylation. HEK 293T cells were transfected with varying concentrations of pcDNA-PI3KγCAAX. Elevated PI-3K activity was assessed using phospho-specific antibodies to detect AKT phosphorylation. (B) SRE stimulation by P-REX1 and P-REX2 is PI-3K-sensitive. HEK 293T cells were co-transfected with or without P-REX1 or P-REX2 and with or without PI-3KγCAAX (0.5 μg/sample) and a luciferase reporter plasmid. Luciferase activity reported as Relative Light Units (RLU). (C) Quantitation of Rac activation. Rac activation mediated by P-REX1 and P-REX2 was assayed in the presence or absence of PI-3K-CAAX co-transfection (0.5 μg/sample) and X-ray films scanned into image files. NIH Image was used to quantitate each band to calculate an "Active Rac Index". The "Active Rac Index" is the Rac signal from the pull-down assay divided by the signal of Rac in the total lysate multiplied by 100. (D) SRE stimulation by the G_i-coupled receptor M2 is potentiated by P-REX2 co-expression. Cells were treated for 6 h in the presence or absence of 100 μM carbachol.

3.3. P-REX2 stimulates Rac and Cdc42 but not RhoA

We next tested whether P-REX1 and P-REX2 could activate tagged forms of Rac, Cdc42, or RhoA. As shown in Fig. 3A, His-tagged P-REX1 and GFP-tagged P-REX2b were readily detected, each present at the expected molecular weight (186 and 136 kD, respectively), and both P-REX1 and P-REX2 promoted the accumulation of GTP-bound Rac. Both P-REX1 and P-REX2 could also stimulate Cdc42, albeit to a lesser extent. By contrast, there was no detectable RhoA stimulation by either P-REX1 or P-REX2, although RhoA was activated upon co-transfection with p115RhoGEF.

3.4. P-REX2 is activated by PI-3K

In order to test whether the sequence similarity between P-REX1 and P-REX2 is sufficient to make P-REX2 sensitive to PI-3K activity, we tested the ability of P-REX2 to stimulate Rac activation and SRE-dependent transcription in the presence of a dominant active p110 PI-3Kγ subunit (PI-3KγCAAX) [11]. As a control, PI-3KγCAAX increased AKT phosphorylation in a dose-dependent fashion (Fig. 4A). Expression of both P-REX1 and P-REX2 stimulated SRE-dependent transcription (Fig. 4B). This effect was enhanced by PI-3KγCAAX, although the P-REX2-mediated increase in luciferase activity in the presence of PI-3KγCAAX was only about half as much as that as P-REX1. Moreover, both P-REX1 and P-REX2 stimulated basal levels of active Rac when expressed in suboptimal amounts, but co-expression of low levels of PI-3KγCAAX dramatically increased this activation (Fig. 4C). Finally, since PI-3K is an integral part of the signaling pathway emanating from GPCRs, we tested whether co-expression of P-REX2 might potentiate the signaling capacity of a G_i-coupled receptor such as M2. Indeed, expression of P-REX2 increased M2 signaling to the SRE upon carbachol stimulation (Fig. 4D).

4. Discussion

4.1. A novel P-REX1-related gene

The amino acid sequence of P-REX1 [3] aligns to a stretch of sequences along chromosome 8q13.1, indicating the presence of closely related gene. We designate this P-REX1-related gene PtdIns(3,4,5)-dependent Rac exchanger 2 (P-REX2) based on this alignment. Sequences similar to those reported in this study have been recently deposited (GenBank Accession Nos. AJ437636, AY508996, and AY508997).

4.2. Sequence relationship to P-REX1

While P-REX2 contains the same N-terminal domains as P-REX1 (such as the DH and PH domains), the P-REX2b transcript reported herein differs most from P-REX1 in the C-terminal region. In fact, the C-terminal region of P-REX1 contains 890 aa after the second PDZ domain, while the P-REX2b protein only has 243 aa. This difference may reflect different functions. For example, P-REX1 contains a C-terminal InsPx4-phosphatase domain [3] that is not present in PREX2b. While there is no available data on whether P-REX1 possesses InsP phosphatase activity, it is possible that this domain is not used as a catalytic center, but as a sequence targeting the P-REX1 protein to InsP patches in the plasma membrane. Examples where a protein motif related to a catalytic domain can be used in this fashion include the GAP-

related domain (GRD) of IQGAP, which targets this protein to GTP-bound Cdc42 [12] and Rac [13], and the RGS domain present in some Rho GEFs such as PDZ RhoGEF [14].

4.3. Two transcripts

The presence of at least two transcripts suggests that there are at least two proteins generated from the P-REX2 gene, both containing the Rho-GTPase nucleotide exchange domain. Interestingly, these two transcripts are expressed in different tissues. The 3.5 kb P-REX2b transcript is expressed only in heart, while the larger P-REX2a transcript is present in skeletal muscle, kidney, small intestine, and placenta. Although the present study focuses only on P-REX2b, it is likely that the difference between these two transcripts lies in whether or not they include a C-terminal InsPx4-phosphatase domain.

4.4. PI-3K sensitivity

Both P-REX1 and P-REX2 stimulate Cdc42 and Rac-GTP accumulation, the latter being enhanced potently by an activated form of PI-3K. These data suggest that P-REX2 functions as a Rho-family GTP exchange factor that responds to the PI-3K pathway in a manner similar to P-REX1 [3]. As the PI-3K pathway is a downstream target of G_i-coupled GPCRs and receptor tyrosine kinases (RTKs), it is likely that P-REX2, like P-REX1, may serve as a link between GPCRs and RTKs and Rac and Cdc42 activation. This may, in turn, participate in increasing cell migration, ROS formation, and other biological effects that are dependent on these small GTPases.

Acknowledgements: The authors thank Dr. Paul A. Randazzo for support during the writing of the manuscript. J. V.-P. acknowledges support by the Fogarty International Center, NIH Research Grant #D43 TW06664 and Fundación Miguel Alemán.

References

[1] Schmidt, A. and Hall, A. (2002) Genes Dev. 16, 1587–1609.
[2] Zheng, Y. (2001) Trends Biochem. Sci. 26, 724–732.
[3] Welch, H.C. et al. (2002) Cell 108, 809–821.
[4] Servitja, J.M., Marinissen, M.J., Sodhi, A., Bustelo, X.R. and Gutkind, J.S. (2003) J. Biol. Chem. 278, 34339–34346.
[5] Burge, C.B. and Karlin, S. (1998) Curr. Opin. Struct. Biol. 8, 346–354.
[6] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[7] Madden, T.L., Tatusov, R.L. and Zhang, J. (1996) Methods Enzymol. 266, 131–141.
[8] Ota, T. et al. (2004) Nat. Genet. 36, 40–45.
[9] Kawasaki, Y., Senda, T., Ishidate, T., Koyama, R., Morishita, T., Iwayama, Y., Higuchi, O. and Akiyama, T. (2000) Science 289, 1194–1197.
[10] Reid, T., Bathoorn, A., Ahmadian, M.R. and Collard, J.G. (1999) J. Biol. Chem. 274, 33587–33593.
[11] Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R. and Wymann, M.P. (1998) Science 282, 293–296.
[12] Ho, Y.D., Joyal, J.L., Li, Z. and Sacks, D.B. (1999) J. Biol. Chem. 274, 464–470.
[13] Ruiz-Velasco, R., Lanning, C.C. and Williams, C.L. (2002) J. Biol. Chem. 277, 33081–33091.
[14] Fukuhara, S., Chikumi, H. and Gutkind, J.S. (2001) Oncogene 20, 1661–1668.
[15] Burge, C. and Karlin, S. (1997) J. Mol. Biol. 268, 78–94.
[16] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.