P-Rex1 – a multidomain protein that regulates neurite differentiation

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Summary

The Rac-GEF P-Rex1 promotes membrane ruffling and cell migration in response to Rac activation, but its role in neuritogenesis is unknown. Rac1 promotes neurite differentiation; Rac3, however, may play an opposing role. Here we report that in nerve growth factor (NGF)-differentiated rat PC12 cells, P-Rex1 localised to the distal tips of developing neurites and to the axonal shaft and growth cone of differentiating hippocampal neurons. P-Rex1 expression inhibited NGF-stimulated PC12 neurite differentiation and this was dependent on the Rac-GEF activity of P-Rex1. P-Rex1 inhibition of neurite outgrowth was rescued by low-dose cytochalasin D treatment, which prevents actin polymerisation. P-Rex1 activated Rac3 GTPase activity when coexpressed in PC12 cells. In the absence of NGF stimulation, targeted depletion of P-Rex1 in PC12 cells by RNA interference induced the spontaneous formation of β-tubulin-enriched projections. Following NGF stimulation, enhanced neurite differentiation, with neurite hyper-elongation correlating with decreased F-actin at the growth cone, was demonstrated in P-Rex1 knockdown cells. Interestingly, P-Rex1-depleted PC12 cells exhibited reduced Rac3 and Rac1 GTPase activity. This study has identified P-Rex1 as a Rac3-GEF in neuronal cells that localises to, and regulates, actin cytoskeletal dynamics at the axonal growth cone to in turn regulate neurite differentiation.

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Key words: Rac1, Rac3, Actin, Guanine nucleotide exchange factor, Neurite differentiation

Introduction

Neurite differentiation is a cellular process responsible for neuronal patterning and the formation of connections crucial for the development of the nervous system. The establishment of neuronal networks is regulated by actin cytoskeletal dynamics. In response to specific stimuli, the growth cone of the axon produces and retracts filopodia and membrane ruffles, events that require the temporal and spatial regulation of the actin cytoskeleton (Luo, 2002). The Rac family of small GTPases, which includes Rho, Rac and Cdc42, promote morphological changes during neuronal development, including neurite outgrowth, axonal guidance and dendritic development (Dickson, 2001; Govek et al., 2005; Luo, 2000). The Rho GTPases function as molecular switches, cycling between GTP-bound active forms and GDP-bound inactive forms. The temporal and spatial activation of Rho GTPases during neuronal differentiation in response to various extracellular cues is crucial for neurite outgrowth (Aoki et al., 2004).

Rac1 and Rac3 (Rac1B), but not Rac2, are expressed in brain. Rac1 stimulates the formation of membrane ruffles and is essential for neurite outgrowth, axonal growth, guidance and branching (de Curtis, 2008; Govek et al., 2005). Rac3 exhibits high identity with Rac1, with divergent sequences in its C-terminus (Malosio et al., 1997). A recent study using RNA interference (RNAi)-mediated depletion of Rac3 from mouse NIE-115 neuroblastoma cells revealed that Rac3 may exhibit an opposing function to Rac1, negatively regulating cell matrix adhesions and neurite outgrowth, activities dependent on its polybasic C-terminal region (Hajdo-Milasinovic et al., 2007). By contrast, studies analysing hippocampal neurons from Rac3−/− mice revealed no defects in neuronal differentiation or polarisation (Gualdoni et al., 2007).

The active/inactive states of Rac proteins are regulated by a variety of intracellular molecules that include guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs unlock the nucleotide-binding site of the GTPase, allowing GDP dissociation and binding of GTP, whereas GAPs stimulate the intrinsic activity of Rho family proteins to hydrolyse GTP. Several GEFs specific for Rac (Rac-GEFs), including STEF (Tiam2), Tiam1, kalirin, Trio, Vav2/3, GEFT and alsin (Als2), are strongly implicated in regulating neuritogenesis (Aoki et al., 2005; Bryan et al., 2004; Estrach et al., 2002; Matsuo et al., 2002; Penzes et al., 2001; Tanaka et al., 2004; Tudor et al., 2005).

The PtdIns(3,4,5)P3-dependent Rac exchanger (P-Rex) proteins (P-Rex1, P-Rex2 and P-Rex2b) are a novel family of Rac-GEFs. P-Rex1 depletion from neutrophil-related cell lines is associated with decreased G protein-coupled receptor-stimulated reactive oxygen species formation (Welch et al., 2002). P-Rex1 (BC067047) knockout mice have mild neutrophilia, and neutrophils exhibit decreased reactive oxygen species production (Dong et al., 2005; Welch et al., 2005). The P-Rex family of enzymes are directly activated by Gβγ subunits and PtdIns(3,4,5)P3 (Hill et al., 2005; Welch et al., 2002). P-Rex1 contains multiple domains including an N-terminal catalytic Dbl-homology (DH) domain, which contains the catalytic GEF motifs for Rac activation, followed by a pleckstrin homology (PH) domain, which binds PtdIns(3,4,5)P3, and tandem DEP (Dishevelled, EGL-10, pleckstrin homology) domains; the latter domains have recently been shown to interact with the mTor complex (Hernandez-Negrete et al., 2007). Following the DEP domains are two PDZ (post-synaptic density, disc-large, ZO-1 homology) domains; however, their function in P-Rex1 remains speculative. In the C-terminal region, P-Rex1 shows homology to...
in this study, we have investigated the role that P-Rex1 plays in regulating neuronal morphogenesis in rat primary embryonic hippocampal neurons and in NGF-stimulated PC12 cells. In hippocampal neurons, P-Rex1 localises to the distal tips of developing neurites, the axon shaft and growth cone. Ectopic expression of P-Rex1 and RNAi-mediated targeted depletion of P-Rex1 in PC12 cells demonstrate that P-Rex1 inhibits neurite elongation by directing actin cytoskeletal dynamics specifically at the growth cone. Furthermore, we present evidence that P-Rex1 may function as a novel Rac3-GEF in neuronal cells. These studies have revealed that the relatively uncharacterised Rac-GEF, P-Rex1, contributes to the complex network of signalling events that regulate neuronal morphogenesis.

**Results**

**P-Rex1 distribution during neuronal differentiation**

to determine the functional role of P-Rex1 in regulating neuronal differentiation, we first investigated the localisation of P-Rex1 in hippocampal neurons and PC12 cells. P-Rex1-specific polyclonal antibodies were raised to a unique P-Rex1 peptide sequence (Fig. 1A) and immune serum affinity-purified on a peptide-coupled thiopropyl Sepharose column. In immunoblots, P-Rex1 antibodies detected a ~180 kDa protein consistent with P-Rex1 in mouse brain lysates, rat E18 hippocampal neurons and PC12 cells (Fig. 1B). Some proteolytic P-Rex1 immunoreactive peptides migrating at 110 and 80 kDa were also detected in hippocampal neurons and PC12 cells. Two PEST sites, predicted to be susceptible to calpain-mediated cleavage (DICE, 1987), are predicted in the P-Rex1 sequence. However, mouse brain immediately harvested and boiled in SDS-PAGE reducing buffer exhibited only the single, 180 kDa immunoreactive polypeptide.

Rat pheochromocytoma PC12 cells provide an experimentally accessible model system in which to study neurite outgrowth. NGF stimulation allows PC12 cells to initiate and extend neurites; however, these processes do not form polarised axons or dendrites, or specialised synapses (Green and Tischler, 1976). Indirect immunofluorescence of undifferentiated serum-starved PC12 cells using affinity-purified P-Rex1 antibodies revealed punctate cytosolic staining (Fig. 1C, top row). No immunoreactivity was detected using

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**Fig. 1.** P-Rex1 localises to the shaft and distal tip of PC12 neurite growth cones. (A) Domain structure of P-Rex1 showing the Dbl homology (DH), pleckstrin homology (PH), Dishevelled, EGL-10, pleckstrin homology (DEP), post-synaptic density, disc-large, ZO-1 homology (PDZ), PEST sequences (P) and 4-phosphatase homology (4ptase) domains. The peptide sequence used as an immunogen to generate the P-Rex1-specific antibody is indicated. (B) Cell lysates from mouse brain, rat E18 hippocampal neurons and undifferentiated PC12 cells (30 μg) were immunoblotted with affinity-purified P-Rex1 antibodies. Immunoreactive endogenous P-Rex1 in PC12 cells and primary hippocampal neurons demonstrate that P-Rex1 inhibits neurite elongation by directing actin cytoskeletal dynamics specifically at the growth cone. Furthermore, we present evidence that P-Rex1 may function as a novel Rac3-GEF in neuronal cells. These studies have revealed that the relatively uncharacterised Rac-GEF, P-Rex1, contributes to the complex network of signalling events that regulate neuronal morphogenesis.

Undifferentiated PC12 cells were also stained with P-Rex1 peptide-adsorbed antibody as control (right-most panel). Scale bars: 10 μm.
peptide-adsorbed serum (Fig. 1C). Preimmune sera were also non-reactive (not shown). Following brief NGF stimulation (6 minutes), intense P-Rex1 staining was detected at the tips of lamellipodia and filopodia co-localising with phalloidin-stained F-actin (Fig. 1C, arrows). Following 8-hour NGF-stimulated differentiation, P-Rex1 was detected at the PC12 cell periphery, neurite shaft and at the distal end of the growth cone, the specialised motile tip of a neuronal process that contains an actin-rich peripheral (P) zone and a central (C) zone of bundled microtubules. High-magnification images revealed that P-Rex1 was localised in the central regions of the growth cone (Fig. 1C, see ‘C’ in bottom row), but was absent from the actin-rich P zone (see ‘P’ in bottom row). P-Rex1 consistently localised to growth cone tips, distal to the actin-rich zone (Fig. 1C, arrowheads, bottom row), the site of actin polymerisation within the P zone of the growth cone (Lin and Forscher, 1995).

Primary hippocampal neurons provide a well-characterised model for the development of neuronal polarity, with cells progressing through five developmental stages characterised by the outgrowth of short, unpolarised neurites during stage 2, axonal specification in stage 3 and dendritic differentiation and synaptogenesis during stages 4 and 5 (Dotti et al., 1988). In early stage 2 hippocampal neurons, P-Rex1 was detected at the cell periphery, the developing neurite shaft and at the tips of the nascent growth cone (Fig. 2A, arrows, growth cone staining). Rac1 exhibited a patchy distribution in the developing neurite, being most intensely localised at the distal ends of the nascent growth cone, and was co-localised with P-Rex1 (Fig. 2A, arrow in upper row), but not in the body of the emerging growth cone or the neurite shaft. F-actin at the developing growth cone co-localised with P-Rex1, but only at the most-distal end of the neurite tips (Fig. 2A, arrows in middle row). P-Rex1 exhibited partial co-distribution with β-tubulin across the span of the neurite shaft and at the distal neurite tips (Fig. 2A, arrows in bottom row).

In early stage 3 neurites, P-Rex1 was detected at the cell periphery, prominently in the primary neurite shaft and developing growth cone, as illustrated by the intensity of staining in the neurite shaft (Fig. 2B, open arrows in ‘glowover’ images). P-Rex1 co-localised with actin at the growth cone and with β-tubulin at the neurite shaft (Fig. 2B, second panel, arrowhead). In differentiated stage 4-5 neurons, P-Rex1 localised to the perinuclear region of the cell body and intensely in the growth cone and shaft of one process (Fig. 2C, ‘glowover’ images). Intense P-Rex1 antibody staining of Tau1 (Mapt)-positive axons was detected, with only faint staining in MAP2 (Mtap2)-positive dendrites, indicating that P-Rex1 exhibits a polarised axonal distribution (Fig. 2B, see arrows for Tau1-positive, MAP2-negative axons).

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Ectopic expression of P-Rex1 inhibits neurite differentiation via Rac-GEF activity

We examined the effect of ectopic expression of P-Rex1 on NGF-mediated PC12 neurite differentiation for 3 days. For each construct,
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The length of the longest neurite was measured and growth cone F-actin was examined by Texas Red-phalloidin staining (see Fig. 3A). Cells were classified as differentiated upon the acquisition of one or more neurites that were greater in length than the diameter of the cell body. By this criterion, 50% of cells expressing vector-only controls were differentiated. Ectopic expression of P-Rex1 resulted in a 3-fold decrease in differentiation (Fig. 3C). P-Rex1-expressing cells initiated actin-rich projections, but these did not differentiate into elongated neurites as shown by scoring the number of neurites/actin-rich projections of any length. However, P-Rex1 cells initiated ~2-fold more actin-rich projections than did vector controls (Fig. 3B, upper row, arrows; supplementary material Fig. S1). Cells were treated with low-dose cytochalasin D, which inhibits actin polymerisation. Under these conditions, >40% of HA-P-Rex1-expressing PC12 cells exhibited differentiated neurites (Fig. 3C), suggesting that P-Rex1 inhibition of neurite differentiation is mediated by actin. A P-Rex1 mutant (P-Rex1GEFdead) that is GEF-dead as a consequence of two point mutations, E56A and N238A, inhibits neurite differentiation (Fig. 3C).
in the Rac-GEF domain (Hill et al., 2005), did not inhibit neurite differentiation indicating that Rac activation is crucial for P-Rex1 function. Mutant P-Rex1 that contained only the central 4-phosphatase homology domains (P-Rex1ΔAN) had no effect, when at low dose (1 μg), on neurite differentiation, but at high levels (5 μg) resulted in a similar phenotype to wild-type P-Rex1, with a 3-fold inhibition of differentiation resulting in short projections with greatly increased F-actin (Fig. 3A-C). This might be due to at least unidentified molecular mechanisms because this construct does not contain the Rac-GEF activating domain, but does contain the central and 4-phosphatase domains. HA-P-Rex1Δ4P, which lacks the 4-phosphatase homology domain but contains the Rac-GEF domain, did not inhibit neurite differentiation, although we noted that F-actin growth cone content appeared reduced (Fig. 3A-C). This result suggests that the 4-phosphatase domain might contribute to P-Rex1 inhibition of neurite differentiation and actin polymerisation by unknown mechanisms. In control studies, expression of mutant and wild-type P-Rex1 was shown to be intact by immunoblot analysis (Fig. 3A).

Recent studies have revealed that the Rac proteins, Rac1 and Rac3, although highly related, may exert distinct functions in the developing nervous system (Hajdo-Milasinovic et al., 2007). P-Rex1 activates Rac1 in purified-component assays, but in neutrophils it exhibits preference for Rac2, a haematopoiesis-specific Rac (Dong et al., 2005; Welch et al., 2005). The ability of P-Rex1 to function as a Rac3-GEF has not been reported. To address this, PC12 cells were co-transfected with vector, P-Rex1 or P-Rex1ΔGEFdead, and with Rac1 or Rac3, and an ELISA-based Rac activation assay (G-LISA) performed. In response to NGF, only a small increase in Rac1 activation in the P-Rex1-expressing cells was detected in each of four experiments and this trend only approached significance (P=0.057). By contrast, Rac3 activation in P-Rex1-expressing cells was consistently higher, and statistically significant (P=0.011), in response to NGF as compared with vector controls. The P-Rex1ΔGEFdead construct did not activate either Rac1 or Rac3 (Fig. 4A). P-Rex1 can therefore activate Rac3 in neuronal cells and this is dependent on its Rac-GEF activity. Rac3 localises to the plasma membrane of stimulated cells, but in contrast to Rac1, it also exhibits a perinuclear localisation (Hajdo-Milasinovic et al., 2007). There was minimal co-localisation of P-Rex1 and Rac1 at the plasma membrane of PC12 cells following a 3-minute NGF stimulation (Fig. 4B, arrowheads, middle row). Interestingly, however, P-Rex1 co-localised with Rac3 in a perinuclear distribution (Fig. 4B, arrow, bottom row).

We also examined the role P-Rex1 plays in the differentiation of primary neurons, by transfecting rat embryonic hippocampal cells after 1 day of differentiation in vitro (1 d.i.v) with plasmids encoding HA-P-Rex1. However, no neurons expressing P-Rex1 could be identified, for reasons unknown (results not shown). To overcome this, differentiated hippocampal neurons were transfected at 7 d.i.v., then grown for an additional 2 days. HA-P-Rex1 was detected at the cell body, the distal half of the axon shaft and growth cone (Fig. 5A), co-localising with Tau1, an axonal marker (Fig. 5B, arrowhead). Significantly, P-Rex1 expression resulted in enlargement of the Tau1-positive axonal growth cones (2.1-fold) associated with prominent F-actin accumulation; however, MAP2-positive dendritic growth cones were not affected (Fig. 5A, lower row, Fig. 5C). Expression of the P-Rex1ΔAN mutant, which lacks the Rac-GEF, PH, DEP and PDZ domains, resulted in loss of the enlargement of axonal growth cones seen with wild-type P-Rex1 expression (Fig. 5C).

P-Rex1 RNAi-mediated depletion promotes spontaneous neurite initiation

The effects on neuronal differentiation of RNAi-mediated depletion of P-Rex1 were investigated. Oligonucleotides designed to unique P-Rex1 sequences (P-Rex1 RNAi), or the same sequence scrambled (scram RNAi), were cloned into the psiRNA-hH1 vector, stably transfected into PC12 cells (Fig. 6A) and individual clones isolated. Immunoblot analysis demonstrated a reduction in P-Rex1 protein (58 and 70% in P-Rex1 RNAi clones 1 and 5, respectively), relative to scrambled RNAi clones (3 and 4) (Fig. 6B). RT-PCR analysis confirmed a ~50% reduction in P-Rex1 mRNA, consistent with the immunoblot analysis (Fig. 6C). In the absence of NGF stimulation, conditions not normally associated with neurite formation in controls, P-Rex1 depletion resulted in the loss of PC12 rounded cell morphology (Fig. 6D). Additionally, P-Rex1 RNAi clones
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**Fig. 5.** Hippocampal neurons ectopically expressing P-Rex1 show enlarged growth cones with prominent F-actin accumulation. Embryonic rat hippocampal cells were transiently transfected at 7 d.i.v. with plasmids encoding HA-P-Rex1. At 9 d.i.v., cells were co-stained with HA antibodies (red or green) and either (A) Alexa Fluor 594-conjugated phalloidin (green) or (B) Tau1 (red) antibodies. (A) Representative images of differentiated neurons (upper row), with higher-magnification images of the boxed areas (1, 2) shown in the two lower rows; growth cones are outlined. The relative intensity of F-actin staining is shown on the left in glowover images, in which blue indicates high-intensity staining (see scale). Scale bars: 10 μm. (B) Co-localisation of HA-P-Rex1 and Tau1. In the merged image, axon is indicated by an arrowhead. Scale bar: 20 μm. (C) The fold increase in growth cone area in HA-P-Rex1 or HA-P-Rex1ΔN relative to mock-transfected cells. Axonal and dendritic growth cones were identified by Tau1 and MAP2 staining, respectively. Thirty cells were scored for each construct for two independent transfections. Bars indicate the mean ± s.e.m. of the growth cone area; *P<0.05.

We next examined Rac1 versus Rac3 activation in PC12 cells expressing control or P-Rex1 RNAi. Cells were transfected with Rac1 or Rac3 and Rac activation assays performed (Fig. 6E). Recent studies have revealed that in P-Rex1+/− neutrophils, Rac1 and Rac2 peak activation is reduced by 25 and 50%, respectively (Welch et al., 2005). In another study, Rac1 activation was not altered in P-Rex1+/− neutrophils, whereas Rac2 activation was almost completely abrogated, revealing that although P-Rex1 can activate both Rac isoforms in vitro, in haematopoietic cells it functions as a Rac2-specific GEF (Dong et al., 2005). In response to NGF stimulation, peak Rac1 activation was decreased by ~20% in P-Rex1 RNAi cells as compared with the control, but this was not statistically significant. By contrast, we noted a significant, ~40% decrease in Rac3 activation in NGF-stimulated P-Rex1 knockdown cells. These studies suggest that the P-Rex1 RNAi-mediated phenotype might be a consequence of decreased Rac3 activity.

P-Rex1 RNAi-mediated depletion enhances neurite elongation

P-Rex1 RNAi clones were stimulated with NGF for 3 days. The percentage of cells bearing differentiated neurites increased >1.8-fold upon targeted depletion of P-Rex1, relative to scrambled or vector controls (Fig. 7A and see supplementary material Fig. S2 for a comparison of vector versus scrambled RNAi). Although the majority (>70%) of differentiated scrambled RNAi cells exhibited short neurites that were less than two cell diameters in length, less than 40% of differentiated P-Rex1 RNAi cells carried short neurites. By contrast, ~30% of P-Rex1 RNAi versus <5% of scrambled RNAi PC12 cells displayed neurites that were longer than three diameters of the cell body, a 6-fold increase (Fig. 7B). Analysis of the length of the longest neurite per cell after 3 days of NGF stimulation revealed that P-Rex1 RNAi neurites were ~1.4-fold longer than those of scrambled RNAi controls (Fig. 7C).

Rac and its effectors regulate axonal guidance and direction (Dickson, 2001; Kubiseski et al., 2003; Ng et al., 2002). Although this cannot be evaluated in PC12 cells, we noted that P-Rex1 RNAi neurites exhibited a ‘wandering neurite’ phenotype, with a 5-fold increase in the number of neurites showing multiple changes in direction, or one significant change in direction with an angle of less than 130°, compared with scrambled RNAi neurites (Fig. 7A, representative images). Actin instability has been implicated in mediating defects in neuronal elongation and pathfinding (Marsh and Letourneau, 1984; Zhou et al., 2002). Rac and its effectors regulate axon branching and suppress ectopic axon/neurite formation. P-Rex1 RNAi PC12 cells exhibited a >2-fold increase in the number of branch points per neurite (data not shown).
Because siRNAs can exert off-target effects, we investigated whether co-transfection of increasing amounts of P-Rex1 cDNA could rescue the phenotype mediated by P-Rex1 RNAi knockdown. To this end, P-Rex1 RNAi clones were transfected with increasing amounts of wild-type HA-P-Rex1 (1, 2 or 5 μg), or vector (5 μg), and neurite length assessed. A dose-dependent reduction in the percentage of cells bearing long neurites correlated with transfection of wild-type P-Rex1, but not vector cDNA (Fig. 7D). P-Rex1 also rescued the ‘wandering neurite’ phenotype (not shown). The P-Rex1GEFdead construct only partially rescued the hyper-elongated phenotype. Interestingly, unlike wild-type P-Rex1, expression of HA-P-Rex1Δ4P did not rescue the phenotype of P-Rex-1 RNAi-
transfected cells (Fig. 7D); instead, it enhanced the phenotype and an increased number of cells with hyper-elongated neurites was observed (Fig. 7D). Previous studies have indicated that the 4-phosphatase domain of P-Rex1 is required to maintain the protein in the cytosol with basal levels of Rac activity, and that the N-terminal GEF and PH domains mediate membrane localisation (Barber et al., 2007; Hill et al., 2005). Loss of the 4-phosphatase domain might allow unimpeded translocation of P-Rex1 to the membrane where the predominant Rac isoform, Rac1, localises. As Rac1 GEF activity promotes neurite outgrowth and, as we have shown, P-Rex1 exhibits GEF activity for both Rac1 and Rac3, this might lead to unopposed neurite elongation. However, we cannot exclude the possibility that this construct might homodimerise with the residual endogenous P-Rex1 and act as a dominant-negative construct. HA-P-Rex1ΔN did not rescue the phenotype in P-Rex1 RNAi cells. Therefore, both the Rac-GEF and 4-phosphatase-homology domains contribute to P-Rex1 regulation of neuronal differentiation.

To assess the effect of P-Rex1 knockdown in hippocampal neurons, cells at 1 d.i.v. were transfected with the P-Rex1 RNAi constructs and with eGFP-expressing vector (ratio of 0.5 μg eGFP:1.5 μg RNAi plasmid to identify RNAi-transfected neurons). Cells were differentiated for 2 days before fixation and staining for F-actin (Fig. 8A, representative images). As with P-Rex1-depleted PC12 cells, P-Rex1-depleted neurons exhibited neurites that were 1.3-fold longer than controls, with an average of two neurites per cell regardless of the construct expressed (data not shown). Interestingly, the growth cones of P-Rex1 RNAi neurons were ~30% smaller than those of controls and exhibited less F-actin (Fig. 8A, enlarged images of growth cones), the opposite phenotype to that observed with ectopic P-Rex1 expression. The actin-rich P zone of the growth cone is composed of radially aligned, filopodial bundles of F-actin with lamellipodial-meshed F-actin situated between the bundles. Dynamic pioneer microtubules advance from the C zone into the P domain and, via interactions with F-actin bundles, promote neurite outgrowth and growth cone turning. The advance of dynamic microtubules into the growth cone P domain is inhibited by retrograde flow of F-actin (Lin and Forscher, 1995). F-actin is polymerised from monomeric G-actin subunits at the neurite membrane where the predominant Rac isoform, Rac1, localises. As Rac1 GEF activity promotes neurite outgrowth and, as we have shown, P-Rex1 exhibits GEF activity for both Rac1 and Rac3, this might lead to unopposed neurite elongation. However, we cannot exclude the possibility that this construct might homodimerise with the residual endogenous P-Rex1 and act as a dominant-negative construct. HA-P-Rex1ΔN did not rescue the phenotype in P-Rex1 RNAi cells. Therefore, both the Rac-GEF and 4-phosphatase-homology domains contribute to P-Rex1 regulation of neuronal differentiation.

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P-Rex1 activates Rac3

In this study, we made the unanticipated finding that P-Rex1 activates Rac3 in neuronal cells. This conclusion is further supported by the fact that the phenotype of the P-Rex1 knockdown neurites resembles that described for Rac3, but not Rac1, knockdown in neuroblastoma cells. Rac1, which is ubiquitously expressed, and Rac3, which is neuron-specific, may function differently in neuronal development despite their closely related sequences (Hajdo-Milasinovic et al., 2007). These functional differences might be a consequence of the divergent sequences in the C-terminal region, which might target distinct effectors. Although Rac3 exhibits a developmentally regulated distribution in mammalian brain, the physiological function of Rac3 in mammalian neurons remains poorly understood. A recent study demonstrated that hippocampal neurones from Rac3<sup>−/−</sup> mice develop normally in culture, although it was acknowledged that long-term depletion of Rac3 in these neurons may lead to a compensatory upregulation of Rac1 activity (Gualdoni et al., 2007). Initial studies demonstrated that the avian homologue of Rac3 (Rac1B) promoted neurite outgrowth in dissociated avian neurons (Albertinazzi et al., 1998). However, Rho GTPases exhibit species-, cell type- and matrix-dependent effects on neurite outgrowth (Govek et al., 2005). Recent studies have shown that RNAi-mediated depletion of Rac1 promotes cell rounding in unstimulated neuronal N1E-115 cells, whereas Rac3 depletion stimulates the outgrowth of neurite-like extensions, suggesting that Rac3 exhibits an opposing function to Rac1 in promoting neurite outgrowth (Hajdo-Milasinovic et al., 2007). Here, we have presented several lines of evidence that P-Rex1 activates Rac3 in neuronal cells. First, P-Rex1 activated Rac3 when P-Rex1 was ectopically expressed in PC12 cells and co-localised with Rac3 at the peri-Golgi region and plasma membrane. Secondly, RNAi-mediated depletion of P-Rex1 promoted the spontaneous formation of projections in PC12 cells, a phenotype similar to that obtained upon knockdown of Rac3 in N1E-115 neuroblastoma cells. Consistent with this, P-Rex1-depleted cells exhibited reduced activation of Rac3. Although P-Rex1 activates Rac1 in purified-component assays, neutrophils from P-Rex1<sup>−/−</sup> mice showed little change in Rac1-GEF activity, but significant decreases in Rac2-GEF activity (Welch et al., 2005). Given that the P-Rex1 RNAi knockdown phenotype mimics the Rac3 knockdown, we propose that P-Rex1 contributes to Rac3 activation in some neuronal cells.

P-Rex1 knockout mice appear healthy with no gross neuronal phenotype (Dong et al., 2005; Welch et al., 2005). However, detailed analysis of the brain and neural tissue has not been described and neither cognitive function nor behavioural analysis of these mice has been reported. Rac1<sup>−/−</sup> mice are embryonic lethal with no mice surviving to E10 (Sugihara et al., 1998). By contrast, Rac3<sup>−/−</sup> mice are healthy with no gross brain abnormalities; however, specific behavioural differences to wild type, including superior motor coordination and learning, were noted (Corbetta et al., 2008; Corbetta...
et al., 2005). Rac3 gene expression is highest postnatally in areas of the brain that contain projection neurons involved in long and complex neuronal networks, such as the hippocampus and cerebral cortex (Corbetta et al., 2005). Therefore, P-Rex1 might activate Rac3 in a specific subset of neuronal cells.

P-Rex1 regulates actin dynamics at the growth cone

Co-ordinated neurite outgrowth is essential for both normal nervous system development and for nerve regrowth following injury. P-Rex1 expression inhibited neurite differentiation and elongation, a phenotype corrected by inhibition of actin polymerisation. By contrast, P-Rex1-depleted neurites exhibited increased F-actin, smaller growth cones and a reduction in lamellipodial veils, correlating with neurite hyper-elongation and abnormal, ‘wavy’ neurites. These results support the contention that targeted depletion of P-Rex1 leads to decreased Rac activation and, thereby, decreased growth cone actin polymerisation and membrane ruffling, which in turn allows the unopposed activity of microtubules to promote neurite elongation. Rac activity regulates the cycling of actin polymerisation and depolymerisation at membrane ruffles, and the balance between too much versus too little Rac activity governs neurite/axon guidance and elongation. Extensive membrane ruffling strongly attenuates neurite expansion, process formation and directional growth as a consequence of the inhibition of microtubule-mediated neurite extension (Tanaka and Sabry, 1995). Growth cone F-actin, which is not incorporated into actin bundles, may retard the dynamic advance of microtubules into the peripheral region (Zhou et al., 2002). The density of actin in the nascent growth cone of PC12 cells overexpressing P-Rex1, and the failure of these neurites to differentiate, did not allow clear visualisation of F-actin in the growth cone in these cells. However, P-Rex1-mediated inhibition of neurite differentiation was rescued by low-dose cytochalasin D treatment, which, by decreasing actin polymerisation at membrane ruffles, may enable neurite extension.

P-Rex1 is a multi-domain protein

The relative roles that Rac and its various Rac-GEFs play in neurite outgrowth, guidance and differentiation are dependent on the cell type, stimulus and matrix upon which the neurons differentiate. Many neuronal Rac-GEFs have a multi-domain structure that allows the activation of Rac exchange activity at specific subcellular regions to be regulated by extracellular signalling and to link with appropriate downstream effectors (Rossman et al., 2005). Domains other than the Rac-GEF domain can modulate exchange activity, often acting as scaffolds so that both the Rho GTPase and its other than the Rac-GEF domain can modulate exchange activity, regions to be regulated by extracellular signalling and to link with actin filament dynamics. For example, the Rac-GEF domain of P-Rex1 is known to bind to the Rac-GEF domain of P-Rex1 (Chhatriwala et al., 2007). Additionally, many of these proteins have GEF activity-independent functions; for example, kalirin is able to regulate actin to induce lamellipodia via both Rac1-dependent and Rac1-independent mechanisms (Schiller et al., 2005). Here, we examined the P-Rex1 domains that regulate its capacity to inhibit neurite differentiation. Rac-GEF activity was crucial for P-Rex1 function, as a P-Rex1GEFdead construct that could activate neither Rac1 nor Rac3 did not inhibit neurite differentiation when overexpressed. Interestingly, however, this construct could only partially rescue the P-Rex1 RNAi knockdown phenotype, suggesting that other domains might contribute to P-Rex1 function. In this regard, it is of interest that expression of a P-Rex1 mutant lacking the 4-phosphatase domain (P-Rex1Δ4P) in RNAi P-Rex1-depleted cells was unable to rescue the hyper-elongation phenotype, but instead exacerbated it. Cells overexpressing a 4-phosphatase domain deletion mutant (P-Rex1Δ4P) developed neurites of a similar length and F-actin content to vector-transfected cells. These results suggest that the 4-phosphatase domain of P-Rex1 might play a role in regulating neurite elongation, by promoting actin polymerisation independent of the Rac-GEF domain and/or by inhibiting de-polymerisation, or by another as yet unidentified mechanism.

Recent studies by Welch’s group using in vitro binding and stimulation of purified proteins has revealed that the isolated 4-phosphatase domain does not modulate the ability of PtdIns(3,4,5)P3 or Gβγ subunits to stimulate P-Rex1 Rac-GEF activity (Hill et al., 2005). However, in the absence of the 4-phosphatase homology domain, the level of basal and/or stimulated P-Rex1 Rac-GEF activity is significantly attenuated, suggesting a functional interaction between the DH/PH domains and the 4-phosphatase homology domain that prevents P-Rex1 GEF enzyme inactivation at high concentrations of PtdIns(3,4,5)P3. Indirect evidence also suggests that the 4-phosphatase domain might contain a second PtdIns(3,4,5)P3-binding domain (Hill et al., 2005). Collectively, our analyses indicate that P-Rex1 regulation of neurite elongation requires both an intact Rac-GEF domain and a 4-phosphatase domain, although the function of the 4-phosphatase domain remains elusive.

In summary, this study has identified P-Rex1 as a Rac3-GEF in neuronal cells that regulates neurite differentiation. As Rac3 knockout mice exhibit enhanced motor skills and learning ability, it would be of great interest in future studies to determine the cognitive function of P-Rex1+/− mice.

Materials and Methods

Reagents

DNA-modifying and restriction enzymes were from Fermentas, New England Biolabs or Promega. Texas Red-conjugated phalloidin, Alexa Fluor 488-conjugated phalloidin and Alexa Fluor 594-conjugated deoxyribonucleoside I (DNaseI) were from Molecular Probes (Eugene, OR). Antibodies specific for Rac were from Upstate (Lake Placid, NY), β-tubulin from Zymed (San Francisco, CA), MAP2 from Sigma (St Louis, MO), Tau1 from Chemicon (Temecula, CA), actin from Santa Cruz (Santa Cruz, CA) and hemagglutinin (HA) from Covance (Berkeley, CA). All hippocampal neuron culture reagents were purchased from Gibco BRL (Gaithersburg, MD). Oligonucleotides were obtained either from Micromon (Monash University, Australia) or GeneWorks (Adelaide, Australia). PC12 cells were from the American Type Culture Collection. Synthetic peptides were obtained from Chiron Mimotopes (Melbourne, Australia). Unless otherwise stated, all other reagents were from Sigma. pCGN vector was a gift from Tony Tiganis (Monash University, Australia).

Cloning of human P-Rex1

A human P-Rex1 (PREX1) partial cDNA (KIAA1415, residues 359-4890) was a gift from Dr T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan). A retained intron of 1100 bp contained within this clone was removed using a PCR-based mutagenesis protocol with overlapping primers, 5′-CTATGAACCACAGCTTA-GCTTAGAATTCCCGTGTGCGGCC-3′ and 5′-CTCTTCTCTTGTTTAAACCTCCTGG-3′. This fragment was amplified from a human EST (BFI10873) using primers 5′-TCCTAGAACCACAGCTTA-GCTTAGAATTCCCGTGTGCGGCC-3′ and 5′-CTATGAACCACAGCTTA-GCTTAGAATTCCCGTGTGCGGCC-3′. An additional 254 bp of 5′ sequence was amplified from a human EST (BF110873) using primers 5′-GCTTTACATTTCCCCGTGCGGCC-CGGGATCCCGCGCGC-3′ and 5′-ACATTTCCCCGTGCGGCC-CGGGATCCCGCGCGC-3′. This fragment was ligated into a unique ß site within the P-Rex1 cDNA. To generate HA-tag fusion constructs, full-length P-Rex1 cDNA, or various P-Rex1 mutants, were amplified by PCR and subcloned into the pXud site of pCGN in-frame with the N-terminal HA tag.

Production of a P-Rex1-specific anti-peptide antibody

A P-Rex1-specific anti-peptide antibody was generated to a peptide corresponding to a unique sequence (1346LGYRYNNNGEYEESS1360) within human P-Rex1. The peptide, conjugated to diphtheria toxoid, was injected into New Zealand White rabbits and the anti-peptide antibodies were affinity purified from immune sera by chromatography using the peptide coupled to thiopropyl--Sepharose according to manufacturer’s instructions (Chiron Mimotopes, Melbourne, Australia). Peptide-
P-Rex1 antibodies were prepared by incubating the P-Rex1-specific antibody with the peptide-coupled column for 2 hours at room temperature. 

PC12 cell culture 

PC12 cells were maintained in PC12 medium (DMEM supplemented with 10% FCS, 5% horse serum, 2 mM l-glutamine, 100 units/ml penicillin and 0.1% streptomycin). For transient transfections, cells were suspended in 200 μl of PC12 medium with the addition of a 50 μl DNA mix comprising 1-5 μg DNA in 0.15 M NaCl, then electroporated at 0.2 kV, 975 μF. Cells were added to 8 ml of PC12 medium and incubated for 48 hours at 37°C in 5% CO₂. For indirect immunofluorescence, cells were plated onto 0.01% poly-L-lysine (PLL)-coated coverslips. For differentiation assays, cells were incubated in low-serum medium (DMEM supplemented with 1% horse serum, 2 mM l-glutamine, 100 units/ml penicillin and 0.1% streptomycin) containing 50 or 100 ng/ml NGF for the indicated times.

Preparation of hippocampal neurons 

Hippocampal neuron cultures were prepared from pregnant Sprague-Dawley rats (Monash University Animal Ethics Project No. BAM/B/2004/47) at day 18 of gestation as described (Banker and Goslin, 1991), with slight modifications. Briefly, following dissection, hippocampi were digested in 0.25% trypsin in Hanks Balanced Salt Solution at 37°C for 15 minutes. Cells were manually dissociated by trituration using a fire-polished Pasteur pipette and plated onto 0.01% PLL-coated coverslips at a density of 5 x 10⁵-1 x 10⁶/cm². Following adherence of cells to coverslips, plating medium (MEM, 10% FCS, 0.6% glucose, 100 units/ml penicillin and 0.1% streptomycin) was replaced with Neurobasal medium supplemented with B27 and 0.5 mM glutamine, and cells were allowed to differentiate for 1-7 days d.i.v. as indicated. For transient transfection of hippocampal neurons, cells were transfected at 1 or 2 days i.v. with 1 μg of DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Neurons were fixed in PBS containing 4% paraformaldehyde and 0.12 M sucrose for 20 minutes.

Generation of P-Rex1 RNAi clones 

Stable shRNA-expressing P-Rex1 and scrambled control clones were generated using the psiRNA-hH1neo Kit (InvivoGen). The following oligonucleotide pairs were annealed and ligated into the BbvI site of the psiRNA-hH1neo vector (as per manufacturer’s instructions), generating P-Rex1-psirna-hH1neo and scrambled-psiRNA-hH1neo (P-Rex1-specific or scrambled equivalent sequence underlined, loop sequence (Cytoskeleton, CO) according to the manufacturer's instructions. PC12 cells transiently expressing P-Rex1, or stably RNAi P-Rex1-depleted, were transiently transfected with the pcDNA3.1/myc-Rac1 or with pEF-BOS HA-Rac1, a kind gift from Dr Collard (Netherlands Cancer Institute). Cells were serum starved, or serum starved and then briefly NGF-stimulated, before whole-cell lysates were prepared using the cell lysis buffer provided, clarified by centrifugation and snap-frozen until required. Following determination of protein concentration according to the manufacturer’s protocol, cell lysates were equilibrated by protein concentration, to 1 or 1.5 mg/ml, and then incubated in the Rac-GTP affinity plate for 30 minutes. Bound, activated Rac1, 2 or 3 was recognised using the pan-Rac antibody provided and a colorimetric reaction measured by absorbance at 490 nm. Assays were performed in duplicate. Protein expression of Rac1 versus Rac3 or P-Rex1 in transfected PC12 cells was quantitated using pan-Rac or P-Rex1 antibodies, respectively, by immunoblot analysis of each transfection.

Examination of P-Rex1 RNAi knockdown by RT-PCR 

RNA was extracted from P-Rex1 or scrambled RNAi clones using the RNeasy Miniprep Kit (Qiagen, AL) according to the manufacturer’s instructions. RT-PCR was performed using a QuantiTect SYBR green RT-PCR Kit (Qiagen) with P-Rex1- or G-actin-specific QuantiTect Primer Assays (Qiagen). Reactions (25 μl) containing 10 ng RNA, 12.5 μl x QuantiTect SYBR Green RT-PCR Master Mix, 2.5 μl 10 x QuantiTect Primer Assay and 0.25 μl Quant iTect RT Mix were performed in triplicate in a Corbett 3000 Rotor Gene Cycler using the following conditions: 50°C for 30 minutes, 95°C for 15 minutes, then 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Relative expression of P-Rex1 as compared with Gapdh was calculated using the ΔΔCt method as described (Dussault and Pouliot, 2006).

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