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Characterisation of PDZ-GEFs, a family of guanine nucleotide exchange factors specific for Rap1 and Rap2☆

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Abstract

PDZ-GEF1 (RA-GEF/nRapGEP/CNrasGEF) is a guanine nucleotide exchange factor (GEF) characterised by the presence of a PSD-95/DlgA/ZO-1 (PDZ) domain, a Ras-association (RA) domain and a region related to a cyclic nucleotide binding domain (RCBD). These domains are in addition to a Ras exchange motif (REM) and GEF domain characteristic for GEFs for Ras-like small GTPases. PDZ-GEF1 efficiently exchanges nucleotides of both Rap1 and Rap2, but has also been implicated in mediating cAMP-induced Ras activation through binding of cAMP to the RCBD. Here we describe a new family member, PDZ-GEF2, of which we isolated two splice variants (PDZ-GEF2A and 2B). PDZ-GEF2 contains, in addition to the domains characteristic for PDZ-GEF1, a second, less conserved RCBD at the N-terminus. PDZ-GEF2 is also specific for both Rap1 and Rap2. We further investigated the possibility that PDZ-GEF2, like PDZ-GEF1, is a cAMP-responsive GEF for Ras. However, in contrast to previous results, we did not find any effect of either PDZ-GEF1 or PDZ-GEF2 on Ras in the absence or presence of cAMP. Moreover, affinity measurements by isothermic calorimetry showed that the RCBD of PDZ-GEF1 does not bind cAMP with a physiologically relevant affinity. We conclude that both PDZ-GEF1 and 2 are specific for Rap1 and Rap2 and unresponsive to cAMP and various other nucleotides.

Keywords: cAMP; Ras; Rap; Guanine nucleotide exchange factor

1. Introduction

Rap1 is a Ras-like small GTPase that may function in a variety of different cellular processes, like integrin-mediated cell adhesion, cell proliferation and differentiation, and platelet activation [1,2]. A variety of extracellular stimuli (e.g. growth hormones and cytokines) are able to activate Rap1 [3]. Activation is mediated by guanine nucleotide exchange factors (GEFs) that substitute the bound GDP for GTP. Four types of Rap-specific GEFs have been identified, some of which are regulated directly by second messengers. CD-GEF1 and 3 are presumably regulated by calcium and diacylglycerol (DAG), since both contain calcium and DAG-binding domains [4–6]. Epac1 and 2, are GEFs directly regulated by cAMP through binding of cAMP to a cAMP-binding domain very similar to those present in the regulatory domain of protein kinase A (PKA) [7–9]. C3G is a GEF that is activated by receptor tyrosine kinases by binding to the activated receptor through the adapter protein Crk [10,11]. The most recently identified GEF for Rap1 is PDZ-GEF1.

PDZ-GEF1 (also called RA-GEF-1, nRap GEP or CNrasGEF) [12–15] is characterised by the presence of a PSD-95/DlgA/ZO-1 (PDZ) domain, a Ras-association (RA) domain and a region related to a cyclic nucleotide binding domain (RCBD). In addition, it contains a Ras exchange

Abbreviations: GEF, guanine nucleotide exchange factor; PDZ, PSD-95/DlgA/ZO-1; RA, Ras-association; RCBD, related cyclic nucleotide binding domain; REM, Ras exchange motif; DAG, diacylglycerol; PKA, protein kinase A; ORF, open reading frame; mantGDP, 2′,3′-bis(*O*)-*N*-methylantharanoloyl-guanosinediphosphate

☆ The nucleotide sequences for PDZ-GEF2A and 2B have been deposited in the GenBank database under GenBank Accession Number AF478468 and AF478469, respectively.

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motif (REM) and a GEF domain characteristic for GEFs for Ras-like small GTPases. At the C-terminus of PDZ-GEF1, a proline-rich region and a PDZ-binding motif are found. The GEF domain of PDZ-GEF1 efficiently exchanges nucleotides of both Rap1 and its close relative Rap2. The various other domains likely play a role in the regulation of activity or localisation of the protein.

The RA domain interacts with active Rap1 and also contributes to membrane localisation of PDZ-GEF1 [16]. This domain may function in a positive feedback loop. The PDZ-binding motif was found to interact with the scaffolding protein S-SCAM (or MAGI-2) and MAGI-1 [14,17]. These scaffolding proteins are localised to synaptic structures and cell adherens junctions, respectively, implying that PDZ-GEF1 might be localised there as well. Indeed, PDZ-GEF1 has been found to co-localise with β-catenin and ZO-1 at sites of cell–cell contact [18]. Finally, the PDZ domain also plays a role in localisation, as deletion of a critical part of the PDZ domain affects plasma membrane localisation [15]. PDZ domains are protein–protein interaction domains, usually interacting with the C-terminus of membrane proteins containing a PDZ-binding motif, but for PDZ-GEF1 the partner of the PDZ domain has not yet been identified. The stability of the PDZ-GEF protein is regulated by the E3 ligase Nedd4, which can bind to the proline-rich regions of PDZ-GEF1. Nedd4 ubiquitinates PDZ-GEF1, leading to degradation by the proteasome [19]. The RCDB of PDZ-GEF1 is closely related to the cAMP-binding domain of Epac. However, it lacks several critical residues involved in cAMP binding. The function of this domain in the regulation of PDZ-GEF1 with respect to GEF activity towards Rap1 and Rap2 is still unclear, although deletion of the RCBD results in vitro in more GEF activity [12]. Intriguingly, Pham et al. [15] reported that CNrasGEF, a protein identical to PDZ-GEF1, is a cAMP-responsive GEF for the small GTPase Ras.

We describe here the identification of PDZ-GEF2, a close relative of PDZ-GEF1, which is also a GEF specific for Rap1 and Rap2. No activity towards Ras was observed in vitro. PDZ-GEF2 contains in comparison to PDZ-GEF1 an extra, less conserved, RCBD at the N-terminus. We identified two splice variants of PDZ-GEF2 (2A and 2B). Recently, a third splice variant of PDZ-GEF2 (RA-GEF-2) was described [20]. In addition, we characterised both PDZ-GEF1 and 2 in further detail and found no evidence for cAMP binding by the RCBD, nor exchange activity towards Ras. We conclude that both PDZ-GEFs are specific for Rap1 and Rap2 and unresponsive to cAMP.

2. Materials and methods

2.1. Cloning of PDZ-GEF2A and 2B

Open reading frames (ORFs) of human PDZ-GEF2A and 2B were predicted on the basis of ESTs, using a blast program (NCBI) [21], and on basis of intron/exon predictions for the genomic sequence by NIX analysis (http://www.hgmp.mrc.ac.uk/). Total RNA was isolated from umbilical cord tissue and Jurkat T cells using RNAzol, according to the manufacturer's protocol (Campro Scientific). cDNA was generated from these total RNA extracts with poly-dT primers, using the Promega reverse transcription system. Three sets of primers were designed to amplify the whole coding sequence: N-for (gtcgacGAACTCACCCGTGGACC) and N-rev (TAAGTTGGATCCACGATGG) for the N-terminal part, M-for (gtcgacAGAGGGAGAAATTGTTATGG) and M-rev (gcggccgcAAGGTACCATATGCAGG) for the middle part, C-for (TTGGGAAAAGTTACCAAGC) and CA-rev (gcggccgcAAATAGGTCATCCAAAGG) and CB-rev (gcggccgcTTCATCAGAGTGTCTTCC), respectively, for the C-terminal part of PDZ-GEF2A and 2B. The PCR products were subcloned in pGEM-T vectors (Promega) and sequenced. Full-length PDZ-GEF2A, PDZ-GEF2A-Δ*RCBD* (amino acid 393–1601) and PDZ-GEF2-Δ*C* (amino acid 1–1141) were cloned using the unique internal *Bam*HI and *Bgl*II sites and the by PCR created *Sal*I and *Not*I sites. The RCBD of PDZ-GEF2 (amino acid 1–417) was subcloned in the *Sal*I and *Bam*HI sites of pBluesript (Stratagene), using the by PCR introduced *Sal*I site and a PDZ-GEF2 internal *Bcl*I site. For expression in mammalian cells, these construct were subcloned in the *Sal*I and *Not*I sites of pMT2-HA, in frame with the HA-tag. For protein purification PDZ-GEF2-Δ*C*, PDZ-GEF2-Δ*RCBD*-Δ*C* (amino acid 393–1141) and the RCBD of PDZ-GEF2 were cloned in the *Xho*I and *Not*I sites of pGEX-4T3 (Pharmacia) in frame with the GST-tag, using the *Sal*I and *Not*I sites.

2.2. Protein expression and purification

GST-tagged PDZ-GEF proteins were expressed in *E. coli* BL21 by induction with 0.1 mM IPTG for 20 h at room temperature. The bacteria were collected and lysed in ice-cold phosphate-buffered saline containing 0.5% Triton X-100 and protease inhibitors and sonicated six times for 20 s. Lysates were cleared by centrifugation at $10{,}000 \times g$ and glycerol was added at 10% final concentration. The GST fusion proteins were purified by incubation of the lysate with glutathione-agarose beads (Sigma) and eluted with 10mM glutathione in buffer containing 50 mM Tris, pH 7.5; 100 mM NaCl; 10% glycerol and 2mM $MgCl_2$. Purification of small GTPases and the proteins used for the experiment shown in Fig. 5 were described elsewhere [22,23].

2.3. Cell culture, transfection and stable cell lines

NIH-3T3-A14 cells and Rat1 fibroblasts were cultured in DMEM, supplemented with 10% fetal bovine serum and 0.05% glutamine. Cells were transfected, using the calcium-phosphate precipitation method [24]. Stable cell lines were made by transfecting NIH-3T3-A14 cells and

Rat1 cells with the vector pBabe, which contains the puromycin resistance gene, or with pBabe together with a HA-tagged PDZ-GEF construct (ratio 1:10). Two days after transfection, medium containing 2 μg/ml puromycin (Sigma) was added. Single colonies were picked and tested for expression. Monoclonal cell lines were made from clones expressing full-length PDZ-GEF1, by limiting dilution.

2.4. Northern and Western blotting

A multiple tissue northern with poly(A) RNA from various human tissues (CLONTECH) was probed, according to the protocol of the manufacturer, with a ^{32}P-radioactive labeled PDZ-GEF2 probe, coding for the sequence spanning the GEF domain. Western blotting of total lysates and protein samples, isolated with an activation specific probe, was performed using polyvinylidene difluoride membranes (NEN™). Antibodies used to detect the proteins were anti HA (12CA5 and sc-805, Santa Cruz), anti Rap-1/Krev-1 (sc-65, Santa Cruz), anti Ras (Transduction Laboratories) and anti phospho-CREB (Ser133, Cell Signaling).

2.5. In vitro activation of small GTPases

GEF activity was measured in vitro as described in Ref. [22]. Briefly, 100–400 nM of the purified small GTPase loaded with fluorescent labeled 2′,3′-bis(*O*)-*N*-methylanthraloyl-guanosinediphosphate (mantGDP), was incubated with 20–200 nM of purified GEF in the presence of excess unlabeled GDP. Nucleotides were added at 100 μM final concentrations. Release of mantGDP was measured in real time as a decrease in fluorescence. Reaction rates were calculated from fitted, single exponential curves. Cell extracts were made as follows. Cells were sheared 20 times through a 23 gauge needle in buffer containing 50 mM Tris, pH 7.5; 100 mM NaCl; 10% glycerol; 2mM $MgCl_2$. Lysates were cleared by centrifugation at 10,000 × *g* and molecules smaller than 3 kDa were collected by spinning the lysates through a 3 kDa size column (Centricon).

2.6. In vivo activation of small GTPases

NIH-3T3-A14 cells were transiently transfected with HA-tagged PDZ-GEF constructs in combination with HA-tagged Rap1 (cloned in pMT2-HA vector). Alternatively, cell lines stably expressing HA-tagged PDZ-GEF1 or PDZ-GEF2-Δ*C* were used. For the experiment shown in Fig. 3E, cells were serum-starved for 20 h prior to lysis. Cells were stimulated with forskolin (20 μM) in combination with IBMX (1 mM) for 5 or 15 min or with EGF (25 ng/ml) for 2 or 5 min. GTP-bound Rap was isolated using purified GST-RalGDS-RBD protein as an activation-specific probe as described [25] and for GTP-bound Ras purified GST-Raf-RBD was used [26].

2.7. Isothermal titration calorimetry (ITC) measurements

ITC measurements were done for cAMP binding to the RCBD domain of PDZ-GEF1 (amino acid 1–268) and to the cAMP-binding domain of Epac1. The isolated domains (cleaved from the GST-tag) were thermostatted in the cell of the apparatus to 25 °C and cAMP was injected from a syringe, in 40 steps, up to a 2–4-fold molar excess. The cell contains 1.36 ml protein solution (0.63 mM PDZ-GEF1-RCBD or 66.7 μM Epac1 cAMP-binding domain) in buffer containing 50 mM Tris–HCl pH 7.6; 50 mM NaCl; 5% glycerol and 5 mM DTE. Typically, cAMP was added in steps of 6 μl every 4 min. The data were analysed using the manufacturers software.

3. Results

3.1. Cloning of PDZ-GEF2A and 2B

The human PDZ-GEF2 gene was identified in a genomic sequence of chromosome 5 by homology searches with PDZ-GEF1 [12]. The intron/exon structure was predicted and primers were designed to amplify cDNA from human umbilical cord cDNA and from Jurkat T cell cDNA. Two different mRNAs were identified, which differ in their C-terminus due to alternative splicing. Like PDZ-GEF1 [12,17], PDZ-GEF2 is rather ubiquitously expressed (Fig. 1E). The transcript is about 9 kb and slightly larger than the PDZ-GEF1 transcript. Using a probe specific for PDZ-GEF2B mRNA we did not obtain a clear signal, indicating that the expression of this splice isoform is low or restricted. PDZ-GEF2A encodes a protein of 1601 amino acids, whereas PDZ-GEF2B encodes a protein of 1391 amino acids. The two proteins differ after amino acid residue 1249. PDZ-GEF2A is most homologous to PDZ-GEF1, with all the domains conserved. PDZ-GEF2B lacks the C-terminal proline-rich sequences and the PDZ binding motif, which are replaced by a sequence with no apparent homology (Fig. 1A and B). Unlike PDZ-GEF1, PDZ-GEF2 contains an additional, N-terminal domain, which is distantly related to the RCBD (Fig. 1D). This domain is also present in the single PDZ-GEF from *C. elegans*. This situation is reminiscent to that for Epac, in that Epac1 contains a single and Epac2 a double cAMP-binding motif (Fig. 1A).

3.2. PDZ-GEF2 specifically activates both Rap1 and Rap2 in vitro

To investigate the specificity of PDZ-GEF2 in vitro, a GST fusion protein named PDZ-GEF2-Δ*RCBD*-Δ*C* (Fig. 3A) was purified. This PDZ-GEF2 fusion protein lacks both the N-terminus, including the RCBD domains (1–391) and the C-terminus (1142–1601). For comparison, we used a similar construct for PDZ-GEF1 [12]. The

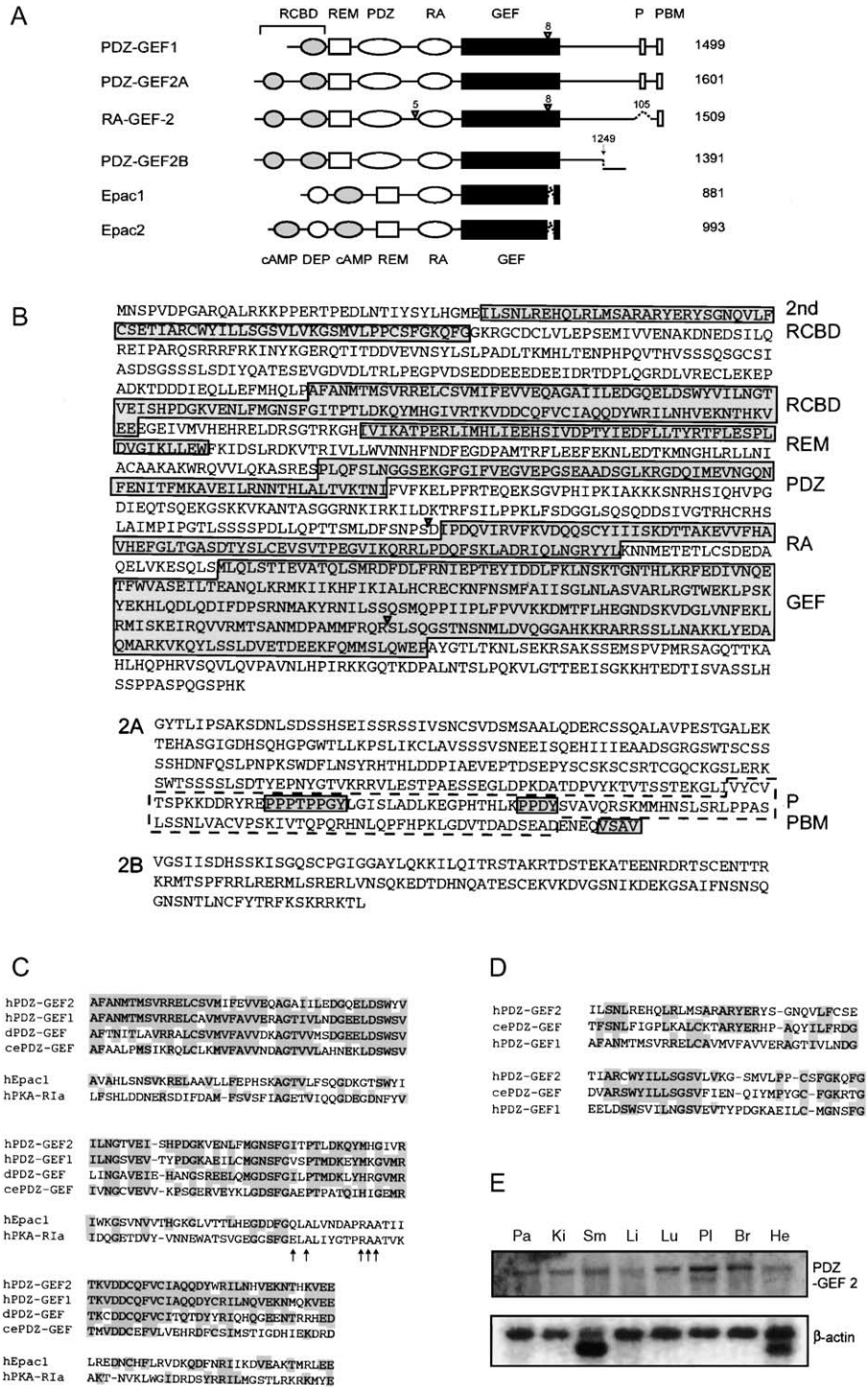
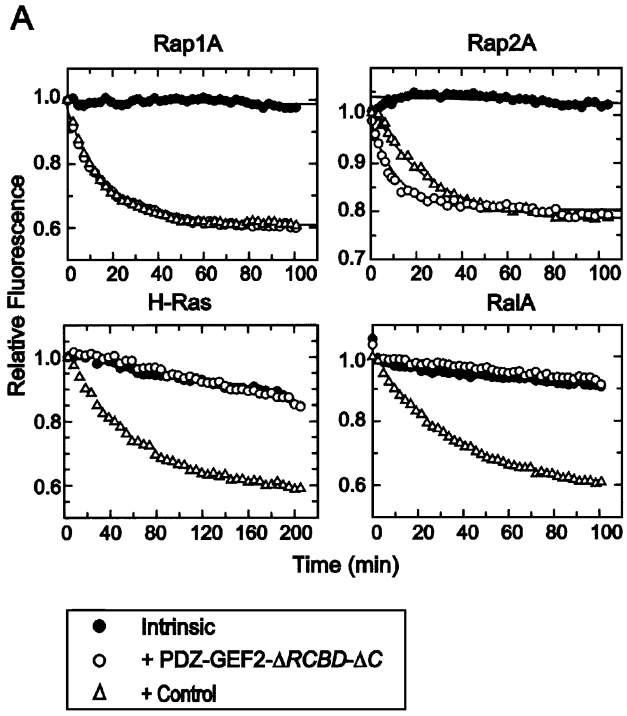


Fig. 1. PDZ-GEF organisation. (A) Domain organisation of PDZ-GEFs in comparison to Epac. The arrow with amino acid number 1249 indicates the site from which PDZ-GEF2A and 2B differ. Extra or deleted exons, compared to PDZ-GEF2A are indicated with amino acid counts in PDZ-GEF1 and RA-GEF-2, a sequence recently published by Gao et al. [20]. P stands for proline-rich region and PBM stands for PDZ binding motif. (B) Complete amino acid sequence of PDZ-GEF2A and 2B. The C-terminal ends where they differ are given separately, indicated by '2A' and '2B'. Domains are lifted out with grey boxes. Sites where RA-GEF-2 has small extra exons are indicated with arrowheads, and the exon that is not present in RA-GEF-2 is in the PDZ-GEF2A C-terminal part shown by a box with dashed sites. (C) Alignment of the RCBD of PDZ-GEFs from different species and the cAMP-binding domain of Epac1 and PKA. Identical amino acids are in a grey background. Amino acids in PKA that are involved in cAMP binding are indicated with arrows. (D) Alignment of the less conserved second RCBD domain of human PDZ-GEF2 and *C. elegans* PDZ-GEF with part of the RCBD of human PDZ-GEF1. (E) Tissue distribution of PDZ-GEF2. A Northern blot containing poly(A) mRNA from tissue of the pancreas (Pa), kidney (Ki), smooth muscles (Sm), liver (Li), lung (Lu), placenta (Pl), brain (Br) and heart (He) was probed with a PDZ-GEF2 sequence, spanning the catalytic domain.

proteins were incubated with different small GTPases loaded with mantGDP, in the presence of an excess of unlabeled GDP. Exchange activity was measured in real time as a decrease in fluorescence. PDZ-GEF2 clearly showed exchange activity towards both Rap1 and Rap2, but not to H-Ras or RalA (Fig. 2A). Also, no effect of PDZ-GEF1 and 2 on N-Ras was observed (data not shown). The catalytic activities were quantified as reaction rates calculated from fitted single exponential curves and compared to the intrinsic GTPase activities. PDZ-GEF2, like PDZ-GEF1, increased nucleotide exchange several hundred-folds and was slightly more active towards Rap2 than to Rap1 (Fig. 2B) [12]. From these results, we conclude that in vitro, both PDZ-GEF1 and 2 are GEFs for Rap1 and Rap 2, but not for Ras.



B

	Rate (per min)	Fold induction
Rap1A intrinsic	0.0002	
+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0623	310
Rap2A Intrinsic	0.0002	
+ PDZ-GEF1-Δ*RCBD*-Δ*C*	0.0413	210
+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0849	420

Fig. 2. In vitro activation of Rap1 and Rap2 by PDZ-GEF2. (A) Release of mantGDP from the loaded small GTPases, measured in vitro in real time in the presence or absence of exchange factors. The intrinsic activity of the relevant small GTPases (black dots) and the activity of PDZ-GEF2-Δ*RCBD*-Δ*C* towards the GTPase (open circles) are shown. As a control, Rap1 was incubated with Epac1-Δ*DEP* in the presence of cAMP, Rap2 with PDZ-GEF1-Δ*RCBD*-Δ*C*, H-Ras with CalDAGGEF3 and RalA with EDTA (open triangles). (B) Reaction rates for panel A are given for fitted curves for Rap1 and Rap2 together with PDZ-GEF, and the fold induction was calculated.

3.3. PDZ-GEF2 does activate Rap1 in vivo, but not Ras

NIH-3T3-A14 cells were transiently transfected with increasing amounts of HA-tagged PDZ-GEF1, PDZ-GEF2A or PDZ-GEF2A-Δ*RCBD* (Fig. 3A) together with HA-tagged Rap1A. Activation of HA-Rap1 was measured using GST-RalGDS-RBD as an activation-specific probe [25]. All the PDZ-GEF constructs used were able to activate Rap1 in vivo in a concentration dependent manner (Fig. 3B). To further study the regulation of Rap1 and possibly Ras by PDZ-GEF1 and 2, we constructed Rat1 and NIH-3T3-A14 cell lines stably expressing different PDZ-GEF constructs, i.e. full-length PDZ-GEF1, PDZ-GEF2-Δ*C*, PDZ-GEF1-Δ*RCBD*-Δ*C* and PDZ-GEF2-Δ*RCBD* (Fig. 3A). Strikingly, the cell lines expressing a PDZ-GEF construct lacking the RCBD domain (PDZ-GEF1-Δ*RCBD*-Δ*C* and PDZ-GEF2-Δ*RCBD*) were unstable. Analysis of a number of Rat1 cells expressing full-length PDZ-GEF1 did not show enhanced Rap1 activity (Fig. 3C). Similar results were obtained for PDZ-GEF1 expressing NIH-3T3-A14 cells (data not shown). These results suggest that full-length PDZ-GEF1, when stably expressed, is normally inactive. Interestingly, cells expressing PDZ-GEF2-Δ*C* did show elevated levels of Rap1-GTP (Fig. 3D), raising the possibility that the C-terminus might play an inhibitory role in the regulation of catalytic activity.

The cell lines stably expressing PDZ-GEF1 were used to investigate whether extracellular stimuli or second messengers could enhance PDZ-GEF activity. However, using EGF or endothelin, two known activators of Rap1 [3], we did not detect more Rap1-GTP in cells expressing full-length PDZ-GEF1 compared to the parental cell line (data not shown). Since it was previously reported that PDZ-GEF1 is responsive to cAMP with respect to Ras activation, we measured this effect using forskolin to activate adenylate cyclase. However, forskolin did not cause activation of Ras, but as expected, did induce CREB phosphorylation (Fig. 3E). Also in transient transfection experiments, using full-length PDZ-GEF1, Rap1 was not further activated upon stimulation with cAMP (data not shown).

3.4. RCBD of PDZ-GEF1 and 2 did not respond to cAMP or other small molecules

To further investigate whether PDZ-GEF is responsive to cAMP, we measured the effect of cyclic nucleotides on PDZ-GEF activity in vitro. However, neither cAMP nor 8-Br-cGMP was able to stimulate PDZ-GEF2 catalytic activity towards Rap2 or H-Ras. Both Rap2 and H-Ras could be activated in this type of assay, as shown in Fig. 2A. In contrast, Epac1-Δ*DEP*, which shows hardly any activity towards Rap2 by itself, was strongly stimulated by cAMP (Fig. 4C). From these and previous results with

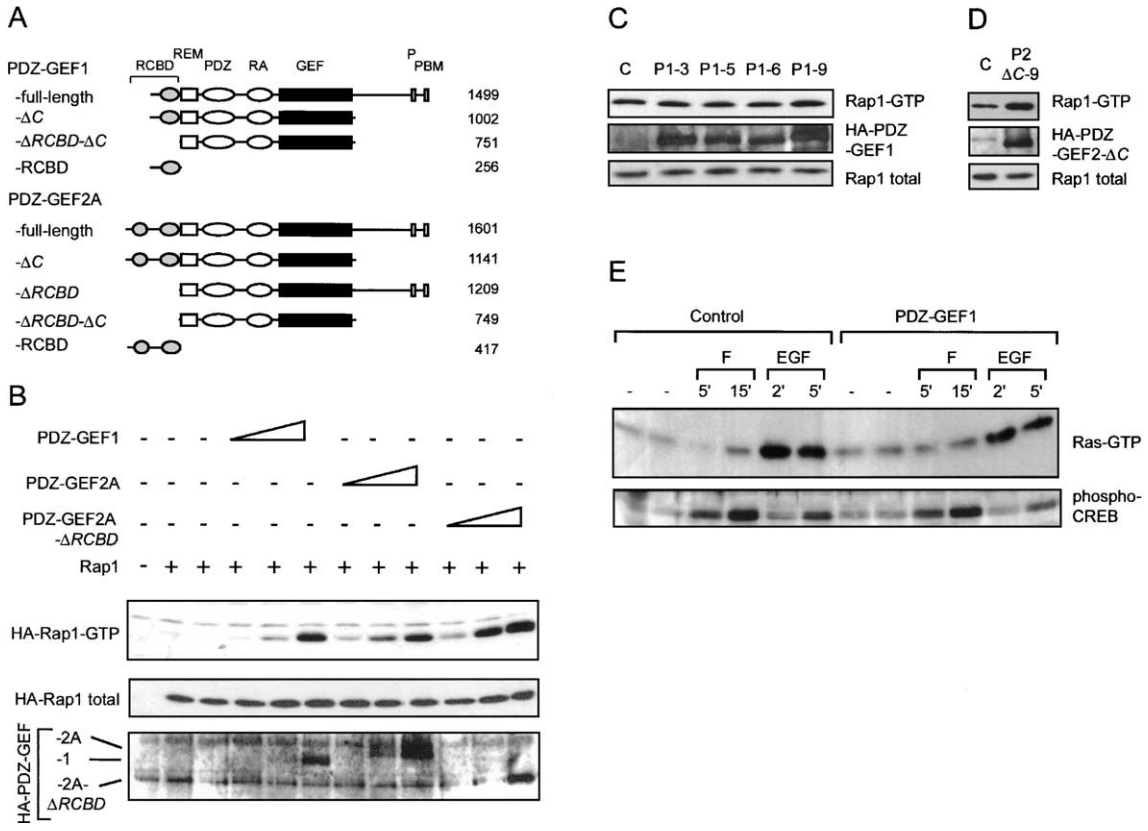


Fig. 3. Activation of Rap1 and Ras in vivo by PDZ-GEF1 and 2A. (A) Schematic drawing of PDZ-GEF constructs that were used in the different experiments. (B) NIH-3T3-A14 cells were transfected with HA-tagged constructs as indicated. HA-Rap1 activation (HA-Rap1-GTP) is shown, measured using RalGDS-RBD as an activation specific probe. Total levels of transfected proteins were visualized. (C) Activity of stable expressed full-length PDZ-GEF1 in Rat1 cells on endogenous Rap1. C stands for control and P1–3, -5, -6 and -9 are different Rat1 clones, stably expressing PDZ-GEF1. (D) PDZ-GEF2-Δ*C*, stably expressed in NIH-3T3-A14 cells (clone P2-ΔC-9), activity towards Rap1, compared to the parental cell line (C). (E) A Rat1 cell line, stably expressing PDZ-GEF1, and a control cell line were stimulated with forskolin (F) and EGF for the indicated time scales. Ras activation (Ras-GTP) is shown, measured using Raf-RBD as an activation specific probe. The amount of phosphorylated CREB upon stimulation in the same protein samples was visualized as a control.

PDZ-GEF1 [12], we conclude that in vitro, both PDZ-GEFs are unresponsive to cAMP. To further corroborate these findings, we determined the affinity of the RCBD of PDZ-GEF1 for cAMP in comparison to the cAMP-binding domain of Epac1 (Fig. 5). For PDZ-GEF1 the affinity was is in the mM range, whereas for Epac the affinity was in the μM range. Since the 1 mM affinity is very unlikely to be physiologically relevant, we conclude that cAMP is not a binding partner for the RCBD of PDZ-GEFs.

A large part of the RCBD of PDZ-GEF is very homologues to the Epac cAMP-binding domain, with the notable exception of cAMP interacting amino acids (Fig. 1C). It may be that other, perhaps cAMP-like, second messengers can interact with the RCBD and activate PDZ-GEF. We therefore tested several different nucleotides in the in vitro assay using GST-PDZ-GEF1-Δ*C* (Fig. 3A) and Rap2A-mantGDP. However, AMP, ADP, GDP, ATP, GTP, ADP-ribose, cyclic ADP-ribose, β-NAADP, adenine, adenosine and guanosine did not affect PDZ-GEF1 activity (Fig. 4D, data not shown and Ref. [12]). To more generally test second messengers for their ability to activate PDZ-GEFs, Jurkat T cells were stimulated with lipopolysaccharide (LPS) or the phorbolester TPA, both known to activate Rap1. Extracts containing molecules smaller than 3 kDa were used in the assay in vitro. No effect was observed on PDZ-GEF1 activity (Fig. 4E). Also, lysates from fMLP-stimulated neutrophils did not affect PDZ-GEF activity (data not shown). In contrast, a similar extract from forskolin-stimulated Jurkat cells did stimulate Epac1-Δ*DEP*, most likely due to the presence of cAMP (Fig. 4F), while lysates of cells stimulated by for instance serum did not stimulate Epac1 activity (data not shown).

3.5. The RCBD of PDZ-GEF2 is in vitro not auto-inhibitory

Previously, it was shown that the cAMP-binding domain of Epac clearly plays an inhibitory role, which is released in the presence of cAMP [7,9]. Besides, it was shown that PDZ-GEF1-Δ*C* in vitro is less active than PDZ-GEF1-Δ*RCBD*-Δ*C* and that the PDZ-GEF1-Δ*RCBD*-Δ*C* protein could be

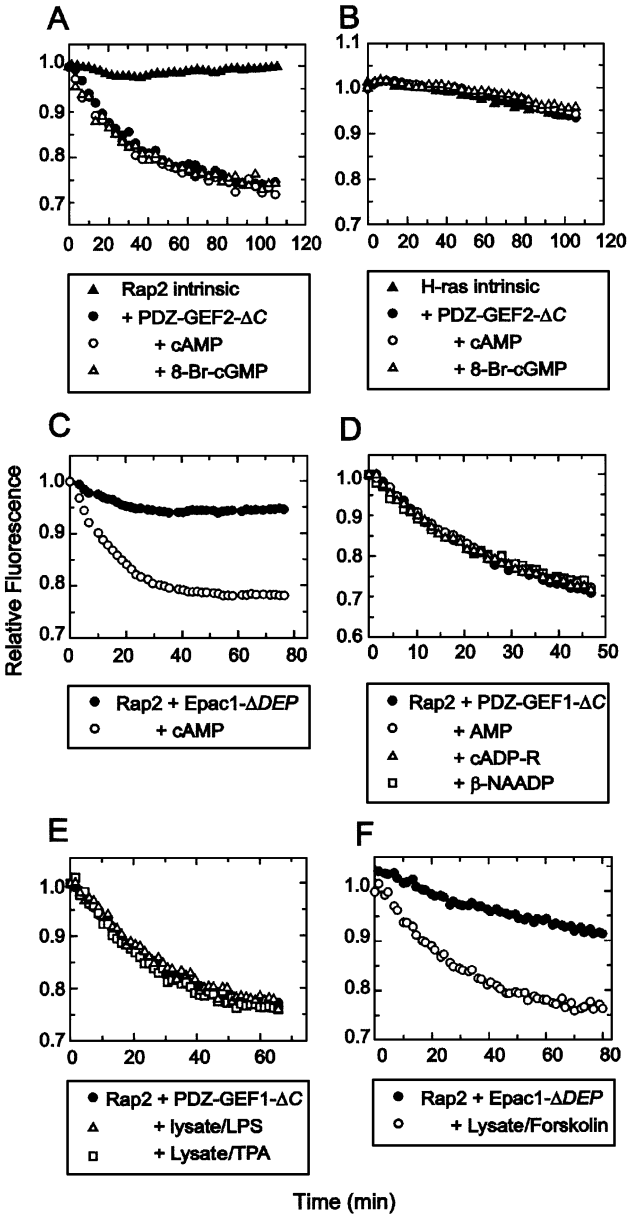
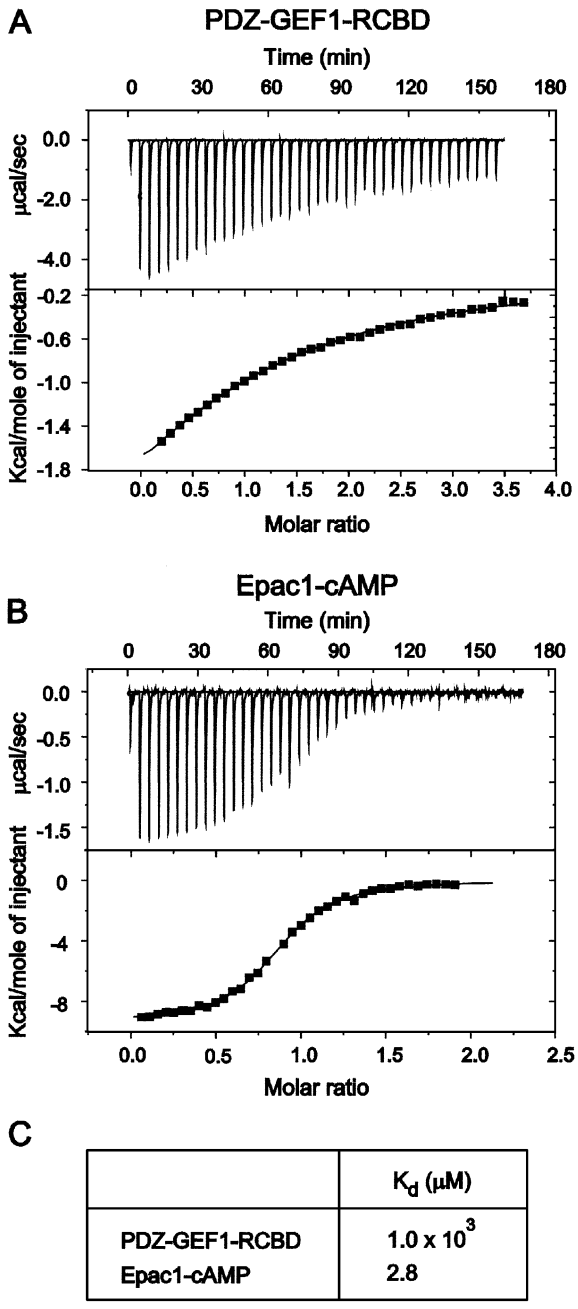


Fig. 4. Role of second messengers in PDZ-GEF activation. MantGDP loaded small GTPases were in vitro incubated with PDZ-GEF1-Δ*C*, 2-Δ*C* or Epac1-Δ*DEP* in the presence or absence of second messengers. Intrinsic GTPase activity is shown by black triangles (A, B). Black dots indicate the activity of the exchange factor alone towards the GTPase. Open circles, triangles and squares show the activity of the exchange factor in the presence of the second messengers as indicated below. (A, B) Respectively, Rap2A and H-Ras activation by PDZ-GEF2-Δ*C* in the presence of cAMP (open circles) or 8Br-cGMP (open triangles). (C) Rap2A activation by Epac1-Δ*DEP* in the presence of cAMP. (D) Rap2A activation by PDZ-GEF1-Δ*C* in the presence of AMP (open circles), cADP-ribose (open triangles) or β-NAADP (open squares). (E, F) Rap2A activation by, respectively, PDZ-GEF1-Δ*C* and Epac1-Δ*DEP* in the presence of molecules smaller than 3 kDa from Jurkat T cell lysates. Lysates from non-stimulated cells (black dots) were added in both cases and lysates from cells stimulated for 5 min with LPS (open triangles) or TPA (open squares) were added (E) and lysates from cells stimulated for 10 min with forskolin (open circles) were added (F).

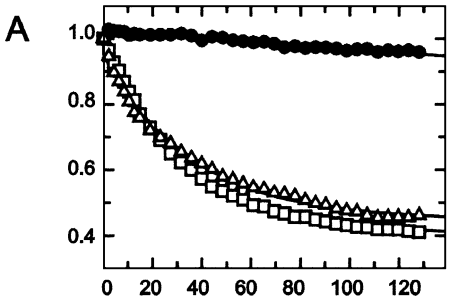


C

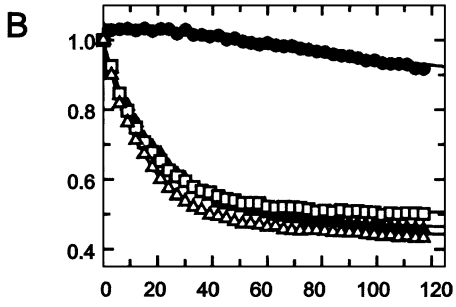
	K_d (µM)
PDZ-GEF1-RCBD	1.0×10^3
Epac1-cAMP	2.8

Fig. 5. Affinity of the PDZ-GEF1-RCBD for cAMP. The affinities of the isolated RCBD domain of PDZ-GEF1 (A) and the cAMP-binding domain of Epac1 (B) were determined by ITC. The upper parts of the graphics show the time-dependent heating power detected after each injection of cAMP. In the lower parts, the integrated heating power is normalised to the concentration of injected cAMP and plotted against the molar ratio of the nucleotide and the protein. (C) Calculated K_ds from the graphics above are plotted in a table.

inhibited by the cAMP-binding domain of Epac1 [9,12]. Based on these previous experiments, we predicted an auto-inhibitory role for the RCBD of PDZ-GEF2. As shown by the calculated reaction rates, deletion of the RCBD had only a minor effect on the activity of the protein (Fig. 6A). In agreement with this, the GST-PDZ-GEF2-Δ*RCBD*-Δ*C* pro-



		Rate (per min)	Fold induction
●	Rap1A intrinsic	0.0011	
□	+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0319	29
△	+ PDZ-GEF2-Δ*C*	0.0280	25



		Rate (per min)	Fold induction
●	Rap1A intrinsic	0.0016	
▲	+ PDZ-GEF2-Δ*RCBD*-Δ*C*	0.0457	29
□	+ 15x Epac1-cAMP	0.0578	36
△	+ 15x PDZ-GEF2-RCBD	0.0584	37

Fig. 6. Effect of the RCBD on PDZ-GEF2 activity in vitro. (A) Catalytic activity of PDZ-GEF2-Δ*C* (open triangles) and PDZ-GEF2-Δ*RCBD*-Δ*C* (open squares) measured in vitro as release of mantGDP from Rap1A. Intrinsic Rap1 activity is shown by black dots. Fold inductions were calculated from reaction rates of the fitted curves and plotted in a table. (B) Catalytic activity of PDZ-GEF2-Δ*RCBD*-Δ*C* (black triangles) measured as in panel A in the presence of 15-fold excess in molar concentrations of the PDZ-GEF2-RCBD (open triangles) or the Epac1 cAMP-binding domain (open squares). Fold inductions were calculated from reaction rates of the fitted curves and plotted in a table.

tein (Fig. 3A) could not be inhibited by the cAMP-binding domain of Epac1, or by the PDZ-GEF2-RCBD (Fig. 6B). We conclude from this that the influence of the RCBD on the activity of PDZ-GEF1 and 2 is different.

4. Discussion

Here we describe the identification of two splice variants (2A and 2B) of a new PDZ-GEF family member, PDZ-GEF2. Like PDZ-GEF1 [12], PDZ-GEF2 is a guanine nucleotide exchange factor specific for Rap1 and Rap2. PDZ-GEF2 differs from PDZ-GEF1 in that it contains an N-terminal extension that has a distant similarity to the RCBD of PDZ-GEF1. This region is conserved in the single orthologue of PDZ-GEF in *C. elegans* (Ce-RA-GEF [13]) (Fig. 1D). Another difference resides in the RA domains, since the RA domain of PDZ-GEF1 interacts with GTP-bound Rap1, whereas the RA domain of PDZ-GEF2 interacts with GTP-bound M-Ras [16,20]. The main differences between the PDZ-GEF2 isoforms are found at the C-terminus. PDZ-GEF2B lacks the proline-rich region and the PDZ binding motif, which are present in PDZ-GEF2A and PDZ-GEF1. Recently a third splice variant of PDZ-GEF2, RA-GEF-2, was identified [20]. This splice variant has small additional sequences just upstream of the RA domain and at the end of the GEF region, but it lacks the proline-rich region of PDZ-GEF2A (Fig. 1A and B). These differences between the splice variants might be important for subcellular localisation and stability. For example, both PY motifs in the proline-rich region of PDZ-GEF2A may function as Nedd4 binding sites. For PDZ-GEF1 it has been reported that the E3 ligase Nedd4 regulates its degradation, by binding to these sites [19]. Second, the PDZ binding motif at the C-terminus, which is absent in PDZ-GEF2B, can bind to a PDZ domain of S-SCAM (or MAGI-2) and MAGI-1, resulting in a location at sites of cell–cell contact [14,17]. This localisation may have an inhibitory effect on the activity of PDZ-GEFs since in general PDZ-GEFs lacking the C-terminus have a higher activity than full-length PDZ-GEFs (Fig. 3D). The RA domain is another part of PDZ-GEF1 that was found to influence the activity of PDZ-GEF1 [16]. Surprisingly, we did not find elevated PDZ-GEF activity upon EGF and endothelin stimulation in cell lines expressing PDZ-GEF1. Since it is known that both stimuli activate Rap1, which can bind to the RA domain of PDZ-GEF1, it appears that GTP loaded Rap1 is not sufficient to activate this exchange factor. Indeed, in vitro Rap1 could also not activate PDZ-GEF1, although the RA domain in vivo was required for optimal activity [16]. This suggests that either another small GTPase may activate PDZ-GEF1 via the RA domain, or that an additional signal is required.

One intriguing but controversial issue is the function of the RCDB in PDZ-GEFs. This domain is closely related to the cAMP-binding domain of Epac, PKA and cyclic nucleotide gated ion channels. However, residues that are critically involved in cAMP binding in PKA, like the 'PRAAT' sequence, are lacking (Fig. 1C). This suggests that PDZ-GEFs do not bind cAMP (or cGMP). Indeed, we and others did not find any evidence for the regulation of PDZ-GEFs by cAMP in vitro, or in vivo [12–14], (this study). Here we demonstrate by direct affinity measurements using isothermic calorimetry that the affinity of the RCDB of PDZ-GEF1 for cAMP is too low to be physiologically relevant (Fig. 5). This result is at variance with observations by Pham et al. who suggested that PDZ-GEF1 (CNrasGEF) mediates cAMP-induced Ras activation through direct binding of cAMP to PDZ-GEF1. Activation of Rap1 however was not induced by

cAMP in their experiments. Strikingly, in the same study, no effect of cAMP was observed in vitro on the activation of Ras by immuno-precipitated PDZ-GEF1. This and our results indicate that indeed PDZ-GEFs are not responsive to cAMP and that the reported effect on Ras is indirect.

We previously hypothesised that the RCBD of PDZ-GEF1 has an auto-inhibitory function, which would be relieved by an unidentified second messenger [12]. This was based on analogy to Epac, in which the cAMP-binding domain is clearly an auto-inhibitory domain that completely blocks Epac activity in vitro. Only in the presence of cAMP or after deletion of the cAMP-binding domain, Epac is active [7,9]. For PDZ-GEF1 it was found that the RCBD has some inhibitory effect [12]. However, we have not been able to identify a second messenger that could enhance the activity of the RCBD-containing protein (Fig. 4). Furthermore, deletion of the RCBD from our PDZ-GEF2 protein did not significantly affect its in vitro activity (Fig. 6). Together, these data indicate that the RCBD does not function as an inhibitory domain. On the other hand, it should be realised that our studies in tissue culture cells still support our initial hypothesis. Of note, we completely failed to isolate cell lines stably expressing PDZ-GEF-Δ*RCBD*s, although we could easily establish cell lines expressing full-length PDZ-GEF1. Moreover, full-length PDZ-GEF1 expressing cell lines did not show any enhanced Rap1 activity. To our knowledge, no other stable cell lines expressing activated RapGEFs have been reported. This suggests that cells cannot support highly active PDZ-GEFs and select against it. This may imply that the RCBD requires an additional protein for its regulation.

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