Mechanism of Regulation of the Epac Family of cAMP-dependent RapGEFs*

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Johan de Rooij‡§, Holger Rehmann‡§†, Miranda van Triest‡§, Robert H. Cool‡‡**, Alfred Wittinghofer†, and Johannes L. Bos‡ ‡‡

From the ‡Department of Physiological Chemistry and Centre for Biomedical Genetics, University Medical Centre Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands and §Max-Planck-Institut für Molekulare Physiologie, 44227, Dortmund, Germany

Epac1 (cAMP-GEF1) and Epac2 (cAMP-GEFII) are closely related guanine nucleotide exchange factors (GEFs) for the small GTPase Rap1, which are directly regulated by cAMP. Here we show that both GEFs efficiently activate Rap2 as well. A third member of the family, Repac (GFR), which lacks the cAMP dependent regulatory sequences, is a constitutive activator of both Rap1 and Rap2. In contrast to Epac1, Epac2 contains a second cAMP binding domain at the N terminus, as does the Epac homologue from Caenorhabditis elegans. Affinity measurements show that this distal cAMP binding domain (the A-site) binds cAMP with much lower affinity than the cAMP binding domain proximal to the catalytic domain (the B-site), which is present in both Epac1 and Epac2. Deletion mutant analysis shows that the high affinity cAMP binding domains are sufficient to regulate the GEFs in vitro. Interestingly, isolated fragments containing the B-sites of either Epac1 or Epac2, but not the A-site from Epac2, inhibit the catalytic domains in trans. This inhibition is relieved by the addition of cAMP. In addition to the cAMP binding domains, both Epac1 and Epac2 have a DEP domain. Deletion of this domain does not affect regulation of Epac1 activity but affects membrane localization. From these results, we conclude that all three members of the Epac family regulate both Rap1 and Rap2. Furthermore, we conclude that the catalytic activity of Epac1 is constrained by a direct interaction between GEF and high affinity cAMP binding domains in the absence of cAMP. Epac1 becomes activated by a release of this inhibition when cAMP is bound.

Rap1 is a small GTPase closely related to Ras and implicated in the regulation of a variety of cellular processes including the control of platelet activation, T-cell anergy, B-cell activation, and neuronal differentiation (1, 2). Very recently, Rap was shown to be involved in the control of cell adhesion (3), in addition to Epac (from now on called Epac1), a second, closely related protein has been identified named Epac2 (or cAMP-

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** Present address: Dept. of Molecular Microbiology, Rijksuniversiteit Groningen, Postbus 14, 9750 AA Haren, The Netherlands. Supported by a grant from the European Community.

†† To whom correspondence should be addressed. Tel.: 31-30-2539977; Fax: 31-30-2539035; E-mail: j.l.bos@med.uu.nl.

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GEFII (18) as well as a related protein named Repac (for related to Epac) (or GRF (19)), which lacks the regulatory sequences present in Epac1 and Epac2. Here we have studied the regulation and function of the different Epac family members in more detail. First, we observe that all three members activate, in addition to Rap1, the close relative Rap2. Second, we identified an additional cAMP-binding site in Epac2, located N-terminal to the DEP domain. Third, mutant analysis revealed that the cAMP binding domains proximal to the catalytic domains in Epac1 and Epac2 (the B-sites) function as inhibitors of the GEF domains in the absence of cAMP. Finally, we show that the DEP domain is involved in membrane localization of Epac1 independent from cAMP signaling.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—** Constructs used for expression of GEFs and small GTPases in mammalian cells are cloned in the PPTM-5HMA eukaryotic expression vector. Epac1 constructs are derived from human cDNA, and Epac2 constructs are derived from murine cDNA. HA-Epac1 and HA-Epac2-DEDP contain Epac1 lacking amino acids 1-140, which span the DEP domain. For purification of glutathione S-transferase (GST) fusion constructs, all cDNAs were cloned in pGEX bacterial expression vectors. The catalytic domain of Epac1 contains amino acids 324–881, the regulatory domain of Epac1 (Epac-RE) contains amino acids 2–329, the CAMP binding domain of Epac1 contains amino acids 149–318, and Epac1-DEDP contains amino acids 149–881. The catalytic domain of Epac2 contains amino acids 460–993, the regulatory domain contains amino acids 1–463, the CAMP-binding site A contains amino acids 1–160, the B-site contains amino acids 280–463, and Epac2-DEDP contains amino acids 280–993. The GST fusion construct of Repac contains amino acids 2–580. The catalytic domain CalDAG-GEFI contains amino acids 3–422. The catalytically active PDZ-GEF1 construct contains amino acids 251–1001. The GST-PKA fusion construct contained the R1α subunit of bovine PKA, lacking amino acids 1–91(20). Protein production was induced in Bl21 bacteria using 100 μM isopropyl-1-thio-D-galactopyranoside for 20 h at room temperature. After protein production, bacteria were pelleted and lysed in ice-cold phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors. The lysate was sonicated three times for 10 s and centrifuged at 10,000 rpm, 30 min at 4 °C. The supernatant was collected, filtered through a 0.22 μm filter, and stored at −80 °C.

To validate the results obtained in vitro, we investigated whether the Epac family members also activate Rap1 and Rap2 in vivo. Cells were transfected with Epac cDNAs together with either Rap1A or Rap2A and stimulated with forskolin and forskolin-related agonists together with forskolin and forskolin-related agonists.

**RESULTS**

**Epac Family Members Activate Both Rap1 and Rap2—** Currently, the Epac family of GEFs consists of three members, Epac1 and Epac2, which are regulated by cAMP and Repac (related to Epac), which lacks any apparent regulatory sequences (Fig. 1a). Previously, it was shown that these GEFs activate Rap1 in vivo as well as in vitro but not the closely related GTPases Ras, R-ras, or Ral. We have extended these experiments and found that all three GEFs can directly activate Rap2A as well (Fig. 1b). Equal amounts (approximately 100 nM) of GST fusions of the catalytic domains (Fig. 1a) were incubated with fluorescent mantGDP-loaded Rap1A or Rap2A (100 nM) in the presence of excess unlabeled GDP, and exchange of guanine nucleotides was followed in real time as a decrease in fluorescence. To compare the activity of the different GEFs toward Rap1A and Rap2A, single exponential curves were fit from which the exchange reaction rates were calculated. These rates were compared with the intrinsic exchange reaction rates of the GTPases measured in the same experiment. From these calculations a fold induction of guanine nucleotide exchange on Rap1A and Rap2A was derived, which is depicted in Fig. 1c. Epac1 activated Rap2A five times more efficiently than Rap1A, whereas Epac2 and Repac activated Rap2A 3-fold less efficiently (Fig. 1b). Activation of Rap2 is not a common feature of all RapGEFs, since C3G (21) and CalDAG-GEFI (Fig. 1b) did not exhibit catalytic activity toward Rap2A in vitro.

**In Vivo Activation of Rap—** Cells were transfected with HA-tagged Rap1A or Rap2A and serum-starved for 20 h before the activation experiments. Cells were stimulated with forskolin (20 μM) and isobutylmethylxanthine (1 mM) for 10 min. The GTP-bound form of Rap1A was specifically isolated using GST-RalGDS as an activation-specific probe assay, as described (5). Detection on Western blot was by 12CA5 monoclonal antibodies directed against the HA tag. In vivo labeling experiments for Rap2 were performed as described (22). Briefly, serum-starved cells were labeled with [3H]Phtophosphate for 5 h. Rap2A was precipitated using 12CA5 antibodies, and nucleotides were eluted and separated by electrophoresis on a 10% polyacrylamide gel and visualized by autoradiography. These experiments were performed as described (22). Briefly, 100 nm purified GTPase (Rap1A or Rap2A) loaded with fluorescently labeled 2.3′bis-O-Methylxanthanaroxylnosuanine diphasphosphate (mantGDP) was incubated in the presence of excess unlabeled GDP with 50 nm purified GEF unless indicated otherwise. Release of mantGDP was measured in real time as a decrease in fluorescence. To calculate reaction rates, single exponential functions were fit using the program Grafit3.0 (Erutics). In all in vitro experiments, the Rap1A and Rap2A proteins were used.

**Isothermal Titration Calorimetry—** Binding of cAMP was investigated by isothermal titration calorimetry (ITC) (MicroCal Inc.). The isolated cAMP binding 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 contained 1.36 ml of protein solution, and typically, the nucleotide was added in steps of 6 μl every 4 min. The data were analyzed using the manufacturer’s software.

**Fractionation—** Cells were scraped in mild lysis buffer (20 mM Hepes, pH 7.4, 5 mM GTP-γS, 1 mM sodium vanadate, 1 μM leupeptin, and 1 μM aprotinin) and homogenized through a 23-gauge syringe. Intact cells and nuclear components were removed by two rounds of centrifugation at 6000 rpm for 1 min in an Eppendorf table centrifuge. Next, cytosolic and particulate fractions were separated by centrifugation at 50,000 × g at 4 °C for 90 min. The particulate fraction was dissolved in buffer containing 1% Triton X-100, 50 mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, 1 μM leupeptin, and 1 μM aprotinin. Distribution of the endogenous epidermal growth factor receptor was analyzed using an anti-EGFR monoclonal antibody (Transduction Laboratories), and the presence of p42 mitogen-activated protein kinase was investigated using a polyclonal antiserum described earlier (22).
**FIG. 1. Epac family members activate both Rap1 and Rap2.**

**a.** Bacterially expressed GST fusion proteins containing the catalytic domains of Epac1 (E1), Epac2 (E2), Repac (Re), and CalDAG-GEF1 (CD) were purified using glutathione-agarose beads, separated by SDS-polyacrylamide gel electrophoresis, and stained with Coomassie. **b.** Purified catalytic domains were incubated at approximately 100 nM with purified Rap1A or Rap2A proteins (100 nM) loaded with mantGDP. A decrease in fluorescence was measured at intervals of 15 or 20 s. Data points shown represent the mean of 20 subsequent measurements. **c.** Rap1A was cotransfected in Cos-7 cells with the indicated full-length GEF constructs. Cells were stimulated with forskolin (20 μM) and isobutylmethylxanthine (1 mM) for 10 min, and Rap1 activation was measured using GST-RalGDS-Ras binding domain as an activation-specific probe. The lower panel depicts a Western blot probed for total Rap1A present in the lysates. **d.** HA-Rap2A was cotransfected in Cos-7 cells with the indicated full-length GEF constructs. Cells were labeled with [32P]orthophosphate, Rap2A was immunoprecipitated using 12CA5 monoclonal antibodies, nucleotides were eluted and separated by TLC, and GTP/GDP ratios were measured using a phosphoimager.

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|       | Rap1A    | Rap2A    |
|-------|----------|----------|
| Epac1 | 0.3'     | 30       |
| Epac2 | 1.1      | 123      |
| Repac | 0.8      | 36       |
| CD-GEF1| 1.0     | 31       |

* Reaction rates ($x 10^{-5} \text{ s}^{-1}$)

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**Fig. 2. Different cAMP binding sites in Epac1 and Epac2.**

- **a.** Alignment of different cAMP binding pockets (residues identical in more than 50% of the depicted domain are marked by dark boxes; light boxes indicate conserved residues) and schematic presentation and purification of GST fusion constructs containing the cAMP binding domains of Epac1 (B) and Epac2 (A and B) and the regulatory domain of Epac2 (RD) (Coomassie-}

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protein that has a putative second cAMP binding domain at the N terminus apart from the reported cAMP binding domain proximal to the catalytic region. This domain is not present in Epac1, but in Epac2, a similar cAMP-binding site is present (Fig. 1a). As judged from primary sequences, this site is similar to the genuine cAMP-binding sites of Epac1, Epac2, and PKA but distinct from the RCBD domain in PDZ-GEF, a RapGEF that does not respond to cAMP (Fig. 2a). We named the N-terminal cAMP binding domain present in Epac2 and C. elegans Epac the A-site, and we named the cAMP binding domain proximal to the catalytic domains, which is present in Epac1, Epac2, and C. elegans Epac the B-site (Fig. 2a). To compare these different sites, we analyzed purified domains (Fig. 2a) for in vitro binding to cAMP by ITC. We found that the A-site of Epac2 binds cAMP with an apparent affinity of 87 μM, whereas the B-site has an affinity of 1.2 μM, which is comparable with the affinity of 4 μM, observed for the cAMP binding domain of Epac1 (Fig. 2b). Apparently, the A-site has a much lower affinity for cAMP as compared with the B-site.

In the regulatory subunits of PKA two cAMP binding domains are present that cooperatively bind to cAMP, meaning that the binding of cAMP to one site influences the affinity of the second site for cAMP (20). To investigate whether sites A and B in Epac2 may also act cooperatively, we measured cAMP binding affinity to the complete regulatory region of Epac2. Best-fit analysis revealed two binding sites with \( K_d \) values of 0.5 and 76 μM (Fig. 2c), which are in the same range as the affinities of the isolated domains. As a final control, a mixture of the separately purified A and B cAMP-binding sites of Epac2, in which no cooperativity can occur, was analyzed in the same assay. This yielded exactly the same result as the titration of the complete regulatory domain of Epac2 (Fig. 2c, lower panel). The data from these measurements are summarized in Fig. 2d. We conclude that no cooperativity occurs in binding of cAMP to the regulatory domain of Epac2 and that also in the full-length Epac2 protein, the B-site has a much higher affinity for cAMP that the A-site.

**Isolated B-sites Inhibit the Exchange Activity of Epac Catalytic Domains**—To investigate the role of the different N-terminal domains in the regulation of Epac1 and Epac2 activity by cAMP, we made several deletion constructs (Fig. 3a). As shown in Fig. 3b, mutant Epac1 (Epac1-ΔDEP) and Epac2 (Epac2-ΔDEP) proteins containing, next to the catalytic domain, only the B-site cAMP binding domain respond to cAMP in vitro like full-length Epac1. The fact that Epac2-ΔDEP responded less strongly than Epac1-ΔDEP is most likely due to difficult purification of this construct (Fig. 3a, left panel), which resulted in less GEF being present in the reaction. As a negative control in these experiments we used AMP. This closely related small molecule did not activate Epac1-ΔDEP or Epac2-ΔDEP (data not shown), confirming the specificity of the cAMP binding domains for the cyclic nucleotide. Whereas Epac mutants that contain the B-site are regulated by cAMP, proteins that lack the B-sites as well as the other N-terminal domains are constitutively active (see Fig. 1b). This implies that the B-sites serve as auto-inhibitory domains.

Next we investigated whether a direct covalent linkage between the catalytic domain and the B-site is essential for this regulation or whether they can function as separate domains. We therefore isolated the regulatory domains of both Epac1 and Epac2 and incubated them with the corresponding catalytic domains. As shown in Fig. 3c, both regulatory domains completely inhibit the catalytic activity of the corresponding GEF domains, showing that they can form a stable complex that prevents GEF activity. The addition of cAMP abolishes the inhibitory effect. To dissect the role of the two cAMP-binding sites in the regulatory domain of Epac2, purified domains of the A- and the B-site of Epac2 were incubated with the catalytic domain of Epac2. Only the B-site and not the A-site (even at high concentration) inhibits the catalytic domain of Epac2 (Fig. 3d). The use of cAMP binding domain constructs containing also the DEP domain did not alter the ability of the A-site or B-site to inhibit the catalytic activity (data not shown).

**The Mechanism of Epac Regulation Is Conserved in a Subset of RapGEFs**—To investigate whether the cAMP binding domain of Epac1 can regulate only the catalytic domain of Epac1, we incubated the regulatory domain of Epac1 with the catalytic domains of the other RapGEFs (Fig. 4a). As shown in Fig. 4b, Epac1-RD inhibited the catalytic activity of both Epac2 and Repac. Interestingly, also the catalytic activity of PDZ-GEF was inhibited. In contrast, the GEF activity of the catalytic domains of C3G and CalDAG-GEFI was not inhibited. From these results we conclude that the isolated regulatory domain of Epac1 can act as an inhibiting structure for a specific subset of RapGEFs. This indicates that this mechanism of regulation is conserved between Epac and PDZ-GEF. In PDZ-GEF, a structure related to cAMP binding domains (RCBD) is present that probably plays a similar role as the B-sites of Epacs in the regulation of GEF activity. Furthermore, this property is specific for certain cAMP binding domains only, because neither the A-site of Epac2 (Fig. 3c) nor a PKA construct containing both its cAMP binding domains (Fig. 4b) affected the activity of the catalytic domain of Epac2. Thus we conclude that a specific sequence or structure in the B-sites of Epacs enables these domains to form an inhibitory interaction with the catalytic domains of a subset of RapGEFs.

**The DEP Domain Localizes Epac to the Membrane Fraction**—In addition to the cAMP binding domains, both Epac1 and Epac2 have a DEP domain. Such a domain was previously recognized in Disheveled, Egl-10, and pleckstrin (hence the name DEP domain) (23), and in the case of Disheveled, it was found to be involved in Frizzled-induced membrane localization (24). In Fig. 3b we showed that the DEP domain is not required for regulation of Epac1 by cAMP. We therefore tested the possibility that the DEP domain is involved in membrane localization of Epac1. Cos-7 cells expressing Epac1 or Epac1-ΔDEP were separated in particulate and cytosolic fractions, and the distribution of Epac1 in these fractions was determined. As shown in Fig. 5a, full-length Epac1 is only observed in the particulate fraction, whereas Epac1-ΔDEP is to a large extent present in the cytosolic fraction. As a control for this fractionation experiment, the distribution of the epidermal growth factor receptor, a transmembrane protein, and p42 mitogen-activated protein kinase, an exclusively cytosolic protein, in the same Epac-transfected fractions, was analyzed (Fig. 5a, lower panel). Both control proteins were detected almost exclusively in the expected fractions, proving a clear separation of membrane and cytosolic fraction. This result indicates that stained gel). b and c, the affinities of the isolated cAMP-binding sites in Epac1 and Epac2 were determined by ITC (see “Experimental Procedures”). The upper parts of the graphs show the time-dependent heating power detected after each injection of cAMP. In the lower part, the integrated heating power is normalized to the concentration of injected cAMP and plotted against the molar ratio of the nucleotide and the protein. The conditions used for the different constructs were 50 μM cAMP binding domain of Epac1 titrated with 0.72 μM cAMP, 200 μM cAMP binding site A of Epac2 titrated with 5.7 mM cAMP, 34 μM cAMP binding site B of Epac2 titrated with 0.36 mM cAMP, 68 μM complete regulatory domain of Epac2 titrated with 0.68 mM cAMP, and in the mixture, 200 μM each site-A and site-B of Epac2 titrated with 3.7 mM cAMP. d, \( K_d \) values (μM) that are calculated from the ITC measurements in b and c are summarized in a table.
the DEP domain is involved in the localization of Epac1 to membrane structures. Importantly, stimulation of these cells with forskolin to induce cAMP did not have any effect on the distribution of Epac1 over the two fractions, indicating that the intracellular localization of Epac1 by the DEP domain is not dependent on cAMP. Next we investigated whether the DEP domain is required for in vivo regulation of Epac1 by cAMP. As shown in Fig. 5a, in NIH3T3-A14 cells, Epac1-ΔDEP strongly activates Rap1A in response to forskolin. From these results we conclude that the DEP domain is not directly involved in the cAMP-induced regulation of Epac1.

**DISCUSSION**

We have extended the characterization of the Epac family of cAMP-dependent RapGEFs. We found that all three members, Epac1, Epac2, and Repac, can activate the closely related GTPase Rap2A. Furthermore, we clarified the mechanism by which Epac1 and Epac2 are regulated by cAMP. The DEP domain targets Epac1 to membrane structures independent of cAMP signaling. The high affinity cAMP binding domains in Epac1 and Epac2 (B-sites) inhibit the catalytic activity of the GEF domains in the absence of cAMP. If cAMP levels rise, the auto-inhibition is relieved.
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The RapGEFs identified thus far can be placed in four different families. Whereas C3G and CalDAG-GEFI show little or no exchange activity toward Rap2(21), the Epac family members and PDZ-GEFI can efficiently activate Rap2 as well. This means that, next to Rap1, Rap2 proteins are also targets for PKA-independent cAMP-signaling routes. Presumably, the difference in GTPase specificity can be explained by the presence of specific sequence properties in the catalytic domains of Epacs and PDZ-GEF, which are absent from CalDAG-GEF and C3G. From sequence alignments, however, we were not able to identify such sequences, indicating that subtle differences may be sufficient.

Rap1 and Rap2 share 70% homology and differ in one residue in the effector domain. Like Rap1, Rap2 proteins have a threonine at the 61 position, at which a glutamine is present in Ras. As a consequence, the Rap proteins have a relatively low intrinsic GTPase activity (25). Our finding that Rap2 shares some but not all GEFs with Rap1 indicates that Rap2 is regulated to a certain extent by the same signals as Rap1. However, the basal level of Rap2GTP is much higher than that of Rap1GTP in a number of cell lines that have currently been tested (14). This may be explained by the fact that Rap1GAP, a ubiquitously expressed GAP for Rap1, has a 40-fold lower activity toward Rap2(26). Indeed, overexpression of Rap1GAP leads to complete inactivation of Rap1, even in the presence of overexpressed, active Rap1GEFs, whereas the basal and GEF-induced GTP-levels of Rap2 are only marginally reduced.

Epac1 and Epac2 are both regulated by cAMP. However, some clear differences exist between the regulatory domains of these GEFs. Epac1 has a single cAMP binding domain, and Epac2, as well as C. elegans Epac, has a second domain that is homologous to described cAMP binding domains. The affinity of this second domain, which we called the A-site, is much lower (87 μM) than that of the cAMP binding domains proximal to the GEF domains of Epac1 and Epac2 (the B-sites, 4 and 1.2 μM, respectively). The affinity of the PKA holoenzyme for cAMP was determined at approximately 0.8 μM in cells (27). This is in the same range as the affinity of the B-sites of Epac1 and Epac2 for cAMP. Furthermore, in hepatocytes, which have relatively high levels of cAMP, it was calculated that the intracellular concentration of cAMP ranges from 0.3 to maximally 36 μM (27), which makes it likely that the B-sites and not the A-site of Epac2 are regulated by cAMP in cells. Perhaps the A-site interacts with a different cAMP-like small molecule or an unrelated compound, which together with cAMP, is responsible for full activation of Epac2. At present the function remains elusive.

The mechanism by which cAMP regulates the activity of Epacs was studied in vitro using deletion mutants. We observed that the B-sites alone are sufficient to provide cAMP-dependent activation. Importantly, isolated B-sites can inhibit the activity of the isolated GEF domains, indicating that the regulation is mediated by an interaction between these domains, which is strong enough to survive separation of the two domains in different protein constructs. This interaction leads to the inactivation of the GEF domain in the absence of cAMP. The binding of cAMP either abolishes the interaction or changes it in such a way that it no longer prevents GEF activity.

The ability of the B-sites to inhibit the catalytic activity as a separate domain prompted us to investigate whether an overexpressed B-site, mutated in its cAMP binding pocket, could function as an interfering mutant in cAMP-induced, Epac-mediated Rap1 activation. Although we were able to find mutants that inhibit the catalytic domains even in the presence of cAMP, these mutant domains could not inhibit cAMP-induced activation of Rap1, which was mediated by Epac1-ΔDEP (containing the B-site) in vitro or full-length Epac1 in vivo. This indicates that a separate B-site cannot compete with the intrinsic B-site. Possibly, steric hindrance by the cAMP-bound intrinsic B-site prevents the interaction of the ectopic, mutated B-site with the catalytic domain.

Interestingly, Epac1-RD was also able to inhibit the catalytic domains of Epac2, Repac, and PDZ-GEF. This indicates that the mechanism by which the B-site interacts with the catalytic domain is rather conserved. It is obvious that the presence of both domains in one protein facilitates this mode of regulation, but it could be hypothesized that originally, in early evolution, the two domains were expressed as separate proteins. This question is particularly interesting with respect to Repac, which lacks any intrinsic regulatory domain. It is well possible that a separate regulatory domain, which has not yet been identified, regulates this GEF. Alternatively, Repac is a constitutively active GEF, which is responsible for basal levels of Rap1GTP and Rap2GTP.

As shown by the in vitro experiments with mutants lacking the DEP domain, this domain is not involved in the regulation of GEF activity. Instead it is involved in the membrane localization of Epac1, presumably by binding to a membrane-associated protein, a function that is also assigned to the DEP domain of Disheveled (24). So far, however, no proteins interacting with a DEP domain have been identified. Alternatively, DEP domains may interact with specific lipid molecules. Since Epac is completely membrane-associated both in the absence

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2 J. de Rooij, unpublished observations.

3 S. O. Doskeland, personal communication.
and presence of cAMP, we conclude that cAMP does not regulate translocation. This is in contrast to many other GEFs, for instance for Ral, Ras, and members of the Arf family, where membrane translocation may be the most important mechanism of activation.

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