Regulation of Epac by cAMP

Regulatie van Epac door cAMP

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Table of contents

Chapter 1:	General introduction	9
Chapter 2:	Mechanism of Regulation of the Epac Family of cAMP-dependent RapGEFs	25
Chapter 3:	Communication between the Regulatory and the Catalytic Region of the cAMP-responsive Guanine Nucleotide Exchange Factor Epac	35
Chapter 4:	Structure and regulation of the cAMP-binding domains of Epac2	43
Chapter 5:	Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac	51
Chapter 6:	Summarising discussion	62
	Summery	70
	Samenvatting	71
	Curriculum vitae	72
	List of publications	73
	Acknowledgements	74

1

General introduction

General Introduction

Cells are surrounded by a lipid membrane, keeping the cell organelles together and acting as a border between the cell and its environment. The exchange of both material and information between the cell and the environment is strictly controlled. Many signalling molecules acting as primary messengers in the communication of cells are not able to pass the cell membrane, but are detected by receptor proteins spanning the membrane. The information of ligand binding to the extra-cellular part of the receptor is translated into the cell as an alteration of the intra-cellular part. This change induces the action of intra-cellular proteins. Some of these actions result in the generation of so-called second messengers. These molecules, of which cAMP is a classical example, spread the extra-cellular primary signal within the cell.

cAMP – a second messenger

cAMP, first identified in 1957 (1), is synthesised by adenylate cyclase (2) from ATP by the release of pyrophosphate and degraded to AMP by phosphodiesterases (for details see Box1). The interplay of adenylate cyclases and phosphodiesterases allows a strictly local and temporal control of the cAMP concentration within the cell. cAMP mediates such different effects as kinase activity, transcription activation, ion flux over membranes and activation of G-proteins. This implies the involvement of a number of different proteins. However most, if not all cAMP effects observed in normal eukaryotic tissues were attributed to protein kinase A (PKA). The kinase activity of PKA is dependent on cAMP. In fact, the activation of PKA by cAMP generated by adenylate cyclase after stimulation of the cell was one of the first second messenger systems discovered in the late 1950s and early 1960s. In addition cAMP was known to act on cyclic nucleotide dependent ion channels.

In 1998 an additional target of cAMP was identified: Exchange protein directly activated by cAMP (Epac) (3;4). Epac is – like PKA – ubiquitously expressed in eucaryotes. Epac is an exchange factor specific for the small G-protein Rap. Rap is a molecular switch, that cycles between a GDP bound inactive and a GTP bound active state. In its active state Rap cause activation of signalling pathways in the cell. Epac induces in a cAMP dependent manner the transition from the GDP bound state to the GTP bound state by exchanging the nucleotides (Fig. 1B). Thus Epac regulates – like PKA – cellular signalling. It is of great interest to define the exact role of Epac in cAMP mediated signalling.

Epac – a target of cAMP

Epac consists of a regulatory and a catalytic part. The catalytic part contains a REM domain and a CDC25 homology domain (Fig. 1A). The latter meditates guanine nucleotide exchange activity (GEF-activity) specifically on the small G-proteins Rap1 and Rap2. This activity is dependent on cAMP binding to the regulatory part. The individual domains of Epac are discussed below, for an overview see Fig. 1A. In higher organisms two isoforms of Epac, Epac1 and Epac2, exist. The major difference between Epac1 and Epac2 is an additional N-terminal cAMP-binding domain in Epac2. In lower eukaryotes like *Drosophila* and *C. elegans*, only one form of Epac is identified, which is, due to the presence of an additional N-terminal cAMP-binding domain, more related to Epac2. In these lower organisms however, this domain is less conserved and lacks some sequence features required for cAMP binding. A splice variant of Epac2 lacking the N-terminal cAMP-binding domain as well as the DEP domain was identified in humans and mice (5). These findings, together with our observation that, *in vitro*, the N-terminal domain is not required to maintain Epac2 in its inactive state in the absence of cAMP

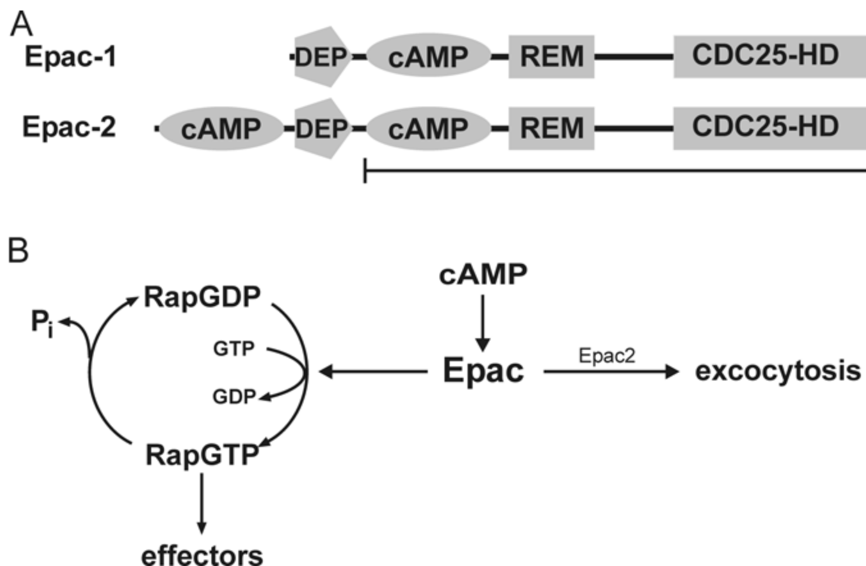
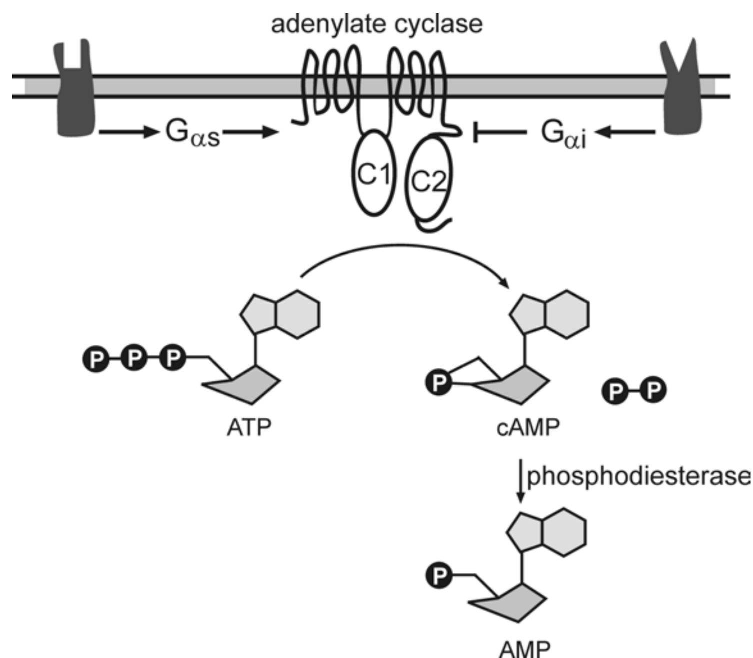


Fig. 1 A. **Domain organisation of Epac1 and Epac2**. cAMP, cAMP-binding domain; DEP, Dishevelled Egl-10 Pleckstrin; REM, Ras exchange motif; CDC25-HD, CDC25 homology domain. The splice variant of Epac2 (5) is indicated by a line. B. **Epac in the context of signal transduction**. cAMP leads to the activation of Epac, which catalyses the exchange of Rap-bound nucleotide. This process results in the formation of RapGTP. RapGTP in turn interacts with effector proteins, which cause a cellular response. A Rap-independent role of Epac2 in exocytosis was suggested.

Box1

Nine isoforms of adenylate cyclyase (also called adenylyl cyclase and adenyl cyclase) are described in humans by now(125;126). All mammalian adenylate cyclases are structurally homologous and consist of two 6-trans-membrane-helix-domains and of two cytosolic domains, C1 and C2. The C1 domains are highly homologous to the C2 domains, so that the protein sequence contains two similar repeats. The C1 domain forms an intramolecular heterodimer with the C2 domain. The (correct) formation of the dimer is required for the catalytic activity, since the active site is at the C1/C2 interface (127).

Forskolin, originally isolated from *Coleus Forskohlii*, an traditional Indian medical plant, can directly stimulate Adenylate Cyclase. Crystal structure analysis of a heterodimer formed from recombinant C1 and C2 domains had shown forskolin to be bound in the dimer interface but outside of the catalytic site. Thus forskolin seems to stabilise the dimer. Adenylate cyclase is mainly regulated by the G_{α}-subunits of heterotrimeric G-proteins. G_{α}-subunits with a stimulatory effect are called $G_{\alpha s}$ and those with an inhibitory effect $G_{\alpha i}$. The regulatory effect is mediated in both cases by a direct interaction of the G_{α} subunit and the adenylate cyclase. Although structural data of a complex containing C1, C2, $G_{\alpha s}$, forskolin and a non-hydrolysable ATP analogue are available it is not clear how the stimulatory effect is mediated (127). This is mainly due to the absence of any structural information of an inactive form of adenylate cyclase. Nevertheless, a conformational change after binding of $G_{\alpha s}$ was proposed. Whereas all isoforms of adenylate cyclase are stimulated by $G_{\alpha s}$ and almost all are inhibited by $G_{\alpha i}$, additional regulation by $G_{\beta\gamma}$, calmodulin, PKC and PKA are described for some isoforms (125;126).



Generation of cAMP by adenylate cyclase. Adenylate cyclase synthesises cAMP from ATP by the release of pyrophosphate. Phosphordiesterase converts cAMP to AMP by cleaving the phosphate. The interplay of adenylate cyclases and phosphodiesterases controls the level of cAMP in the cell. Adenylate cyclase itself is activated or inhibited by G-subunits of heterotrimeric G-proteins.

(chapter 2), indicates that the N-terminal cAMP-binding domain is most likely not involved in cAMP mediated regulation of Epac2.

Exchange factors mediate their effects through their respective small G-proteins (Fig. 1B). However, a possible Rap-independent role of Epac2 in exocytosis was suggested (see below). Epac is a member of a group of Rap-specific exchange factors. These exchange factors are regulated by a wide variety of stimuli. The first identified exchange factor for Rap was C3G (6). After activation of receptor tyrosine kinases, C3G is recruited to the plasma membrane with the help of docking proteins such as Crk (7). This mechanism resembles the activation of Ras by the Grb2●Sos complex (8). Three isoforms of GRP (CalDAG-GEF) are described, one of which is specific for Rap, one for Ras and the third for both Rap and Ras (9;10). The Ras specific GRP1 (CalDAG-GEF II) respond to the classical second messengers Ca^{2+} and Diacylglycerol (DAG). So far it has been not possible to demonstrate an allosteric regulation of these GEFs *in vitro* and it was suggested that enrichment of DAG in membranes results in recruitment of CalDAG-GEF to these membranes (11;12). Little is known about the regulation of PDZ-GEF. PDZ-GEF contains a degenerated cAMP-binding domain (13), but evidences for a cAMP or cGMP mediated regulation of PDZ-GEF are published (14). However, we found by *in vitro* experiments that the affinity of the potential cAMP-binding domain to cAMP is very low and therefore a regulation by cAMP is most likely of no physiological relevance (15;16). An interaction between synaptic scaffolding molecule (S-Scam) and PDZ-GEF was reported, although the physiological relevance is not

clear (17). Repac (Related to Epac), also called GFR (Guanine nucleotide exchange factor for Rap), (18) can be understood as Epac lacking the regulatory part. Repac is constitutively active and maybe regulated by interaction with other proteins. Along these lines an inhibition of Repac by M-Ras was shown (19). Thus cAMP dependent action on Rap by Epac takes place in the context of a complex regulation network.

Epac2 – a mediator of exocytosis

A pivotal role of cAMP controlled signalling in exocytosis is generally accepted. Whereas it was possible to attribute most effects to cAMP-induced PKA activation, others are described as PKA-independent. Recently it was shown that Epac2 activation is directly involved in the release of insulin from pancreatic β-cells (20-22). How this contribution of Epac2 is mediated is not known in detail, but it was demonstrated that Epac2 increases the intracellular Ca^{2+} concentration ($(Ca^{2+})_i$), most likely by the release of Ca^{2+} from the endoplasmatic reticulum via ryanodine sensitive Ca^{2+} channels (21;22).

Intriguingly, there is evidence that Epac2 acts at least in part independently of Rap. It was shown by two-hybrid-analysis as well as by pull-down assays that Epac2 interacts directly with Rim2, known as an effector of the small G-protein Rab3, which is itself involved in exocytosis. This interaction is mediated by the PDZ-domain of Rim2 (20) (Fig. 2). That this interaction between Epac2 and Rim2 is responsible for the effect of Epac2 in exocytosis is formally not shown, but implicated. Transfection of the single PDZ-domain of Rim2 blocks the Epac2 mediated effect in exocytosis. This blockage can be overcome at least partially by co-expression of Epac2 (23). A general function of Rim2 in exocytosis is well established. Since Rim2 is localised in the active zone of the targeted membrane, where vesicle fusion occurs, and since Rab is localised in the vesicle membrane, it was suggested that the interaction between Rim2 and Rab anchors the vesicle at the active zone in a ready-to-fuse-state (24). This distribution of Rim2 and Rab was also shown for pancreatic β-cells (25). Rim also interacts directly with SNAP25 and Synaptogamcin, which are both part of the SNARE complex (Fig 2). The SNARE complex is involved in anchoring the vesicles at the active zone and it undergoes subsequent rearrangements, required for the fusion process itself. At least two events contibute to these rearrangements: (a) a conformational change of syntaxin, a component of the SNARE complex. This conformational change was confirmed by NMR studies (26). It was shown genetically that Rim is upstream of unc13 and that unc13 is upstream of the conformational change of syntaxin (27). (b) an increase in $(Ca^{2+})_i$. Some protein-protein interactions in the SNARE complex are Ca^{2+}-dependent (28). The general requirement of an increase in $(Ca^{2+})_i$ was demonstrated for a multitude of model systems for exocytosis (29-35).

As mentioned above it was shown that Epac2 activation results in an increase of $(Ca^{2+})_i$ (21;22). There are different possibilities how Epac2 could cause this increase: (a) RIM is known to interact with N- and L-type Ca^{2+}-channels (28). (b) Epac was shown to interact with the SUR1 subunit of K_{ATP}-channels. These channels are involved in the regulation of depolarisation events, which lead to insulin secretion via a $(Ca^{2+})_i$ increase (36). (c) Rap was shown to

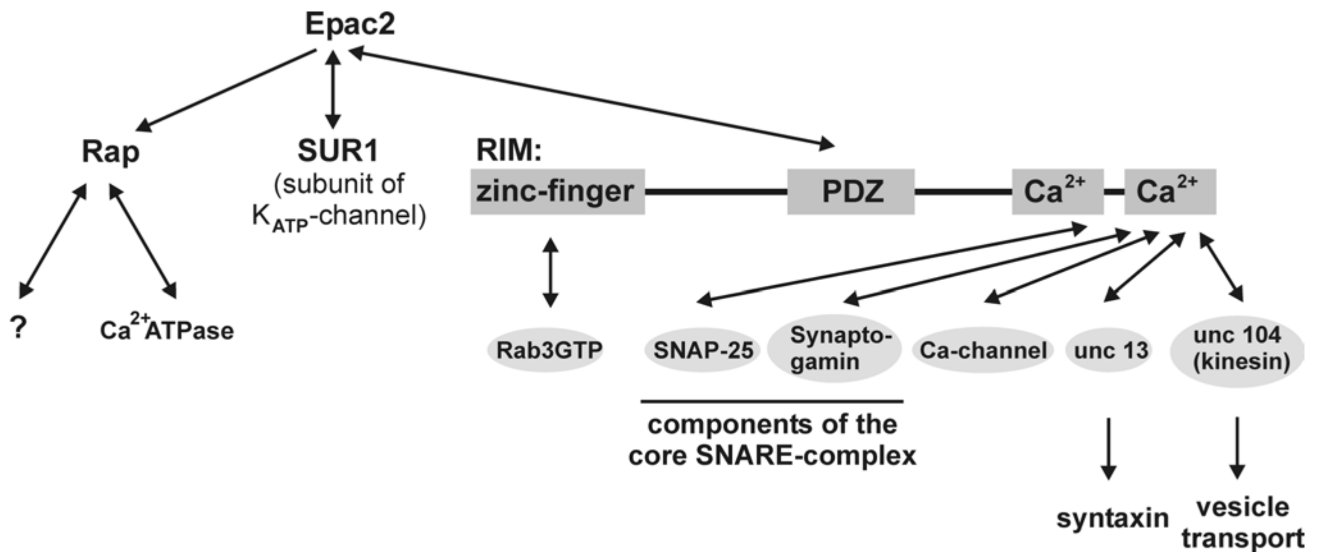


Fig. 2 **Epac2 is involved in exocytosis**. The different possibilities by which Epac2 could influence exocytosis are shown. Arrows with two arrowheads indicate interaction whereas arrows with only one arrowhead indicate activation. The individual pathways are discussed in the text. Ca^{2+} stands for Ca^{2+} binding domain.

interact with a Ca^{2+}-ATPase (37;38). The physiological relevance of all these interactions is unknown.
It is important to note, that the effect of Epac2 in exocytosis is cAMP dependent. So far it has not been shown that the interaction between Rim and Epac2 is cAMP dependent. Neither a cAMP dependent localisation of Epac2 is demonstrated. One possible explanation for these findings is that the Epac-Rim-pathway is necessary but not sufficient for exocytosis and that additional signals by RapGTP are required (Fig. 2).

Rap – a target of Epac

The small G-protein Rap cycles between a GDP bound inactive state and a GTP bound active state, and therefore acts as a molecular switch. The transition from the GDP to the GTP bound state is mediated by Guanine Nucleotide Exchange Factors, like Epac, which accelerate the intrinsically slow dissociation rate and cause thereby the loading with GTP (Fig. 1B, for details see Box 2). The transition of Rap back to the inactive state is achieved by the GTPase activity of Rap resulting in the hydrolysis of GTP to GDP. This activity is stimulated by GTPase activating proteins (GAP). GDP stays bound to the protein, whereas the phosphate is released immediately. Only in its GTP bound state Rap is able to interact with so-called effector proteins. Activation of the effector after binding to the G-protein is either due to a conformational change of the effector or to its relocalisaton. The term "activation of a G-protein" is often understood from the point of view of signalling, it refers therefore to the transition of the G-protein from the "inactive" GDP to the "active" GTP bound state. It does, however, not refer to the activation of the enzymatic activity of the G-protein, i.e. the hydrolysis of GTP.
Rap was originally identified in a screen for cDNAs able to revert the transforming phenotype of oncogenic K-Ras (39). For this reason it was called Krev (K-Ras reverting phenotype). From this initial observation, Rap was proposed to be an antagonist of Ras mediated effects. This model was supported by the finding that Rap is able to bind to most of the effectors of Ras (40-43). Thus it was proposed that RapGTP traps Ras effectors in an inactive complex. However, it was shown that Rap is able to induce cell transformation as well (44), and in the meantime it seems to be clear that Rap mediated signalling is independent of Ras. Most importantly a pivotal role of Rap in integrin signalling was shown (see below), (for review see (45)).

Rap in integrin signalling

Integrins are mediators of cell-cell-adhesion as well as of cell-matrix-adhesion and thus regulate such important processes as the correct cohesion of tissues, the interaction of immune cells with their targets, or the mobility of cells during development. Integrins are heterodimers consisting of a longer α- and a shorter β-chain. Both chains are transmembrane proteins that consist of a relatively short C-terminal cytosolic tail and of several extracellular N-terminal domains. The extracelluar domain interacts with cell surface proteins of other cells in the case of cell-cell-adhesion or with proteins of the extracellular matrix in case of cell-matrix-adhesion. The interaction partners are called ligands of integrins. The adhesion in physical sense is mediated by these interactions.
Adhesion of cells and thus integrin function is tightly regulated. The regulation occurs at different levels. The adhesion capability of integrins is determined by the "affinity" and the "avidity" state of the integrins. Both states are targeted by regulation processes. "Affinity" is defined as the affinity of a protein-ligand interaction in the thermodynamic sense. "Avidity" is less clear in definition. The term summarises phenomena of integrin density, often understood as local densities, clustering and accessibility. These properties are in part controlled by the attachment of integrins to the cytoskeleton. Induction of a highly adhesive state requires detachment, movement and reattachment of integrins. A signalling process is called inside-out signalling if a signal resulting in an alteration of the integrin state is generated in the cell (upon stimulation of the cell). On the other hand, integrins cause signalling events in the cell depending on their ligand-state, is called outside-in signalling. Thus adhesion by itself influences cellular events. The inhibition of proliferation by cell-cell contacts (contact inhibition) is well known example. The initial ligand binding leads to an amplification of adhesion.
Two possible experimental ways of inducing integrin activity and therefore adhesion have to be distinguished. (a) application of suitable factors leading to activation of receptors that control signalling which induces integrin activity (b) direct targeting of the integrins by application of Mn^{2+} or activating antibodies. The later activation mechanism is based on the notion that the interaction of integrins with their ligands is absolutely depending on divalent cations such as Mg^{2+} and Ca^{2+}. Each integrin chain contains four or five extracellular ion binding sites. Ion binding induces a high affinity binding state of the integrin. Similarly, some antibodies are able to induce this state.
The requirement for Rap in cell-adhesion induced by CD31 (46), CD14 (47), the β-adrenergic receptor (48), erythropoietin (49), interleukin-3 (49), CD98 (50), phorbol ester (51), the thrombin receptor (52) or G-CSF (53) was either shown directly or is strongly implicated. Although the importance of Rap is unambiguous, it is not clear how Rap mediates these effects. Establishment of the final adhesion state after initial receptor stimulation requires a set of signalling

events, each of which may be influenced by Rap. Signals caused by the receptor have to influence the affinity or avidity state of the integrin and may additionally control integrin-independent requirements for adhesion. It is likely that Rap is involved in these processes. The β_2-adrenergic receptor stimulates the activation of adenylate cyclase and thereby the generation of cAMP, which can activate Epac. An Epac-selective cAMP analogue is indeed able to induce adhesion (48). C3G and the adaptor protein CrkL are required for adhesion in many cellular systems (54-59). The C3G•CrkL complex becomes membrane-localised through interaction of SH2 domain of CrkL after activation of receptor tyrosine kinases. Thus, the formation of RapGTP is directly linked to receptor stimulation. The activation state of integrins can be monitored by monoclonal antibodies that recognise only the active state of the integrin. In this way it was shown, that Rap is required for receptor induced integrin activation (46).

Mn^{2+} and antibody induced cell adhesion requires Rap as well (46;60). Experiments with purified integrins have shown that Mn^{2+} (61) and activating antibodies (62;63) induce a high affinity state of the integrin without the requirement of any additional factors *in vitro*. On the other hand cells expressing integrins lacking the cytosolic tail are not able to establish a stable adhesion state. Thus Rap is requires for the ability of integrins to function as adhesion molecules *in vivo*, perhaps by regulating the connection of integrins to the cytoskelett.

Domain structure of Epac

The DEP domain – correct localisation of Epac may play a role in cell division

A certain sequence in Dishevelled was found to be conserved in other proteins as well and was therefore nominated a new domain (64). This domain was called DEP domain for Dishevelled, Egal-10 and Pleckstrin, the most prominent proteins identified in this search. Interestingly, also Epac (referred to as ymx5) was identified and recognised as a protein containing a DEP domain, a cAMP-binding domain and a CDC25-homology domain, but not further noticed (64). Since many of the found proteins were known to be membrane localised or otherwise connected to membrane functions, the DEP domain was suggested to be involved in membrane localisation. This prediction was later confirmed for the DEP domain of Dishevelled. It is not clear whether the DEP domain mediates localisation by a protein-protein interaction or by a direct membrane interaction, since interaction partners are hitherto unknown. Evidence for both scenarios is described in literature. Wang et al. (65), who solved the NMR structure of the DEP domain from Dishevelled, identified a positively charged surface area that may be involved in a direct but unspecific membrane interaction. On the other hand, it is possible to use the single DEP domain of Dishevelled as a dominant negative suppresser of Dishevelled mediated activation of the wnt signalling pathway (65). Introduction of point mutations in the DEP domain in the context of a full length Dishevelled represses wnt signalling (65). Both findings argue for protein-protein interactions.

The cellular localisation of Epac at the plasma membrane was shown to be dependent on the DEP domain (66). In addition, localisation of Epac1 at perinuclear membranes and a co-localisation with mitochondria was observed (66). The mitochondrial localisation is due to an N-terminal mitochondrial targeting sequence.

The localisation of Epac1 is dependent on the cell cycle. Whereas Epac1 is localised at the plasma membrane in quiescent cells, it is found to be co-localised with the mitotic spindle and the centrosomes during metaphase and around the chromosomes during anaphase (66). Whether Epac plays a role cell cycle progression is at the moment just a fascinating object of speculation.

In addition, during anaphase and telophase Epac is enriched in the area where the two daughter cells are separated from each other (66). Intriguingly it was shown that the correct formation of adherens junctions is effected in Rap-negative *Drosophila* cells (67). It was suggested that Rap is responsible for the correct deconstruction of the adherens junctions and the contractile ring after cell division (67). A putative link between Rap and adherens junctions is the nucleotide-dependent interaction of Rap with AF-6 (68;69), which was shown to be localised at adherens junctions (70;71) as well as at tight junctions (72;73). AF-6 in turn interacts with ZO-1 (74;75), which is associated with the adherens junction complex protein α-catenin (76).

These findings together imply an attractive model: Epac activates Rap in the interface of the dividing cell, where separation of the daughter cells occurs. This separation process is then completed by the action of Rap.

The CDC25-homology domain – structural basis of nucleotide exchange

Each family of the Ras superfamily has its own type of exchange factors, which are structurally not related to each other. This is evident from the crystal structures determined so far (Fig. 3). The structures of three exchange factors have been determined alone and in complex with the corresponding G-proteins: the DH/PH domain of Sos alone (77) and in complex with CDC42 (78), RCC1 (79) alone and in complex with Ran (80), the sec domain of ARNO (81) alone and in complex with Arf (82). The structures of three exchange factors have been determined only in

complex with their corresponding G-protein: the REM/CDC25 homology domain of Sos in complex with Ras (83) the DH/PH domain of Tiam in complex with Rac (84) and SopE in complex with CDC42 (85). SopE is a bacterial toxin and its structure does not correspond to the canonical DH/PH motif of the mammalian exchange factors for the Rho family. In addition the structure of Mess4 (86), an exchange factor for Rab (87), has been determined in the absence Rab.

The exchange activity of Epac is mediated by its CDC25-homology domain. This domain is found in all exchange factors for members of the Ras family, although each exchange factor is characterised by a profile of specificity towards the individual members of the Ras family. However, the general architecture of all CDC25 homology domains is identical, and the Ras•SOS complex is thus a model for the whole family. CDC25-homology domains are always found together with a REM domain, although the relative

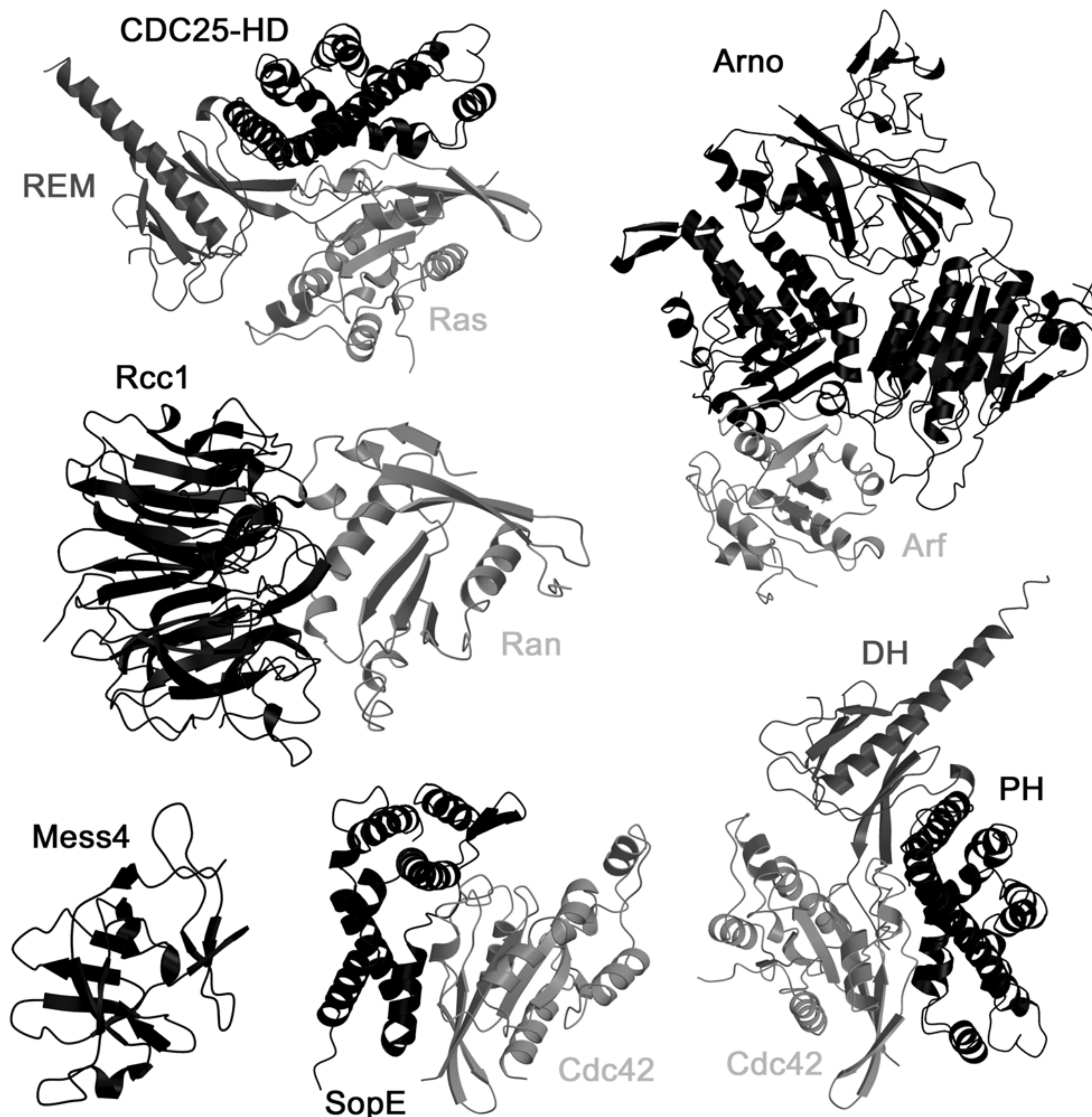
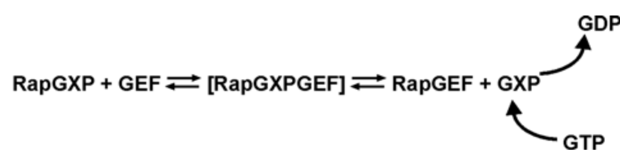


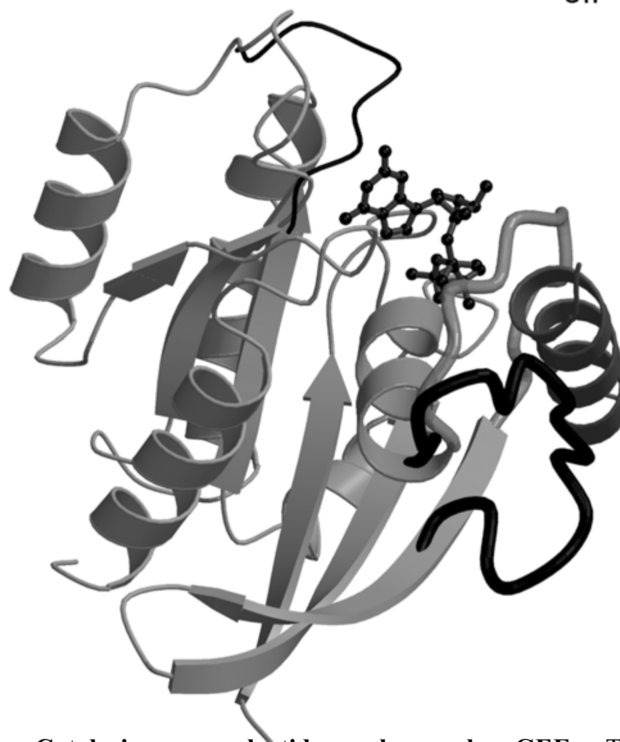
Fig. 3 **Guanine nucleotide exchange factors are structurally not conserved**. Representative structures for the different families of exchange factors are shown if data is available in complex with their small G-proteins. The G-protein is coloured in light grey, the catalytic domain in black and additional domains of the exchange factor are in dark grey. These additional domains for example the REM domain of Sos are not required for the catalysis itself. The figure was generated by the use of the programs molscript (128) and raster3d (129).

Box2

The sequence of the exchange reaction is summarised in the figure. The nucleotide affinity of the G-protein is very high, and the intrinsic dissociation rate of the nucleotide is very slow. Binding of the exchange factor to the nucleotide-bound G-protein leads to an intermediate trimeric complex. The trimeric complex decays either by the release of the exchange factor or of the nucleotide. The latter results in the nucleotide free G-protein bound to the exchange factor. This complex is stable, can be purified in the absence of nucleotide and can be used for structural studies. The trimeric complex can be regenerated by binding of a nucleotide and, in case it decays by the release of the exchange factor, the nucleotide which had been originally bound to the G-protein is exchanged for the nucleotide that had rebound to the complex of G-protein and exchange factor. Thus, the exchange factor does not exchange one type of nucleotide for another one, e.g. GDP for GTP, due to a selection or discrimination process. It just catalyses the release of any nucleotide bound to the G-protein. Due to its GTP hydrolysing activity the G-protein in the cell is predominately bound to GDP. However, the GTP concentration in the cell is one ore two orders of magnitude higher than the GDP concentration. Thus, most often GTP rebinds just by chance to the complex of nucleotide free G-protein and exchange factor. This mechanism requires that there is no competition between the nucleotide and the exchange factor for the G-protein. Competition in the classical sense means that the G-protein can bind to the nucleotide and the GEF, but the binding of the nucleotide excludes the binding of the GEF and the binding of the GEF excludes the binding of the nucleotide. Competition results therefore in displacement of one binding partner but not in the exchange of one binding partner. It is thus necessary that the nucleotide and the GEF can bind at the same time. On the other hand, a totally independent binding would result in a stable trimeric complex and again there would be no exchange. Thus a combination of “binding at the same time” and displacement is required. The known structures of G-proteins in complex with exchange factors demonstrate how this requirements are realised.

The following discussion will focus on the Ras•Sos complex. The binding of the nucleotide can be split into two major determinants, the contribution of the base and of the phosphate-sugar moiety. An Mg^{2+} ion is pivotal for binding of the nucleotide, by co-ordinating the negatively charged phosphates as well as residues of the G-protein. These moieties of the nucleotide bind to quite separated areas of the binding pocket. Binding of Sos interferes predominantly with the binding of the phosphate-sugar moiety. The so-called P-loop and the switch I region are bent by Sos in a way that opens up the binding pocket. This abolishes the binding of the phosphates and displaces the Mg^{2+}. The binding area for the base is more or less unperturbed (see figure). Thus the binding of the nucleotide is weakened sufficiently to allow a high dissociation rate of the nucleotide, but rebinding is possible: base first, displacement of Sos second.





A. **Catalysis on nucleotide exchange by GEFs**. The reaction scheme of the interaction between a GEF and a small G-protein (here Rap) is shown. For a detailed explanation see text. B. **Action of Sos on Ras**. The GppNHp bound Ras (light grey) (130), was superimposed to the nucleotide free Ras of the Ras•Sos complex (91). Only the elements of the nucleotide free Ras that are different from the nucleotide bound Ras are shown (black). The helix from Sos which bends switch I away to open the nucleotide binding pocket is shown in dark grey.

location of both domains within the protein sequence is highly variable (88). The REM domain is not required for the catalytic activity, since the single CDC25 homology domain is sufficient to stimulate nucleotide exchange *in vitro* (89;90). No direct interaction between the REM domain and Ras was observed in the structure of the Ras•SOS complex, and it was suggested that the REM domain stabilises the CDC25 homology domain by shielding hydrophobic residues against the solvent (91). More recently an activity modulating role of the REM domain has been suggested. Margarit et al. crystallised a Ras•Sos•Ras•GTP complex, in which an additional GTP-loaded Ras interacts with the REM domain and the CDC25 homology domain independently of the active site of Sos. They also demonstrated that the presence of Ras•GTP can accelerate the rate of nucleotide exchange (92). However, the structural data can not fully explain the observed biochemical effects.
Although the structure of different exchange factor families is evolutionary not conserved, the basic principle of the catalytic mechanism is the same. It was analysed in great detail for the Ran-RCC1 (93) and for the Ras-CDC25 system (90), the detailed mechanism of exchange is described in Box 2.

The cAMP-binding domain – and the experimental problem of targeting signalling pathways specifically

Such different functions as kinase activity, transcription activation, ion fluxes and activation of small G-proteins are all directly controlled by the cyclic nucleotides cAMP and cGMP. This implies the involvement of quite a number of different proteins. However, a common feature of all these proteins is a cyclic nucleotide binding domain (Fig. 4). These domains are homologous in sequence and share consensus motifs indicative of cyclic nucleotide binding (for review see (94)). The three-dimensional architecture of the domains is identical, as it is evident from the structures solved so far ((95-97), chapter 4). The classes of cAMP or cGMP regulated proteins, as shown in figure 3, will be described in the following section. (Epac is excluded from this short overview, since it is subject of this thesis.)

<u>*PKA:*</u>

The cAMP-dependent serine/threonine protein kinase A (PKA) was one of the first kinases identified and became a paradigm of second messenger systems. PKA is a heterotetramer, consisting of 2 regulatory and 2 catalytic chains (Fig. 4). Four different regulatory chains (RIα, RIβ, RIIα and RIIβ) and three different catalytic chains (Cα, Cβ and Cγ) are known. Each regulatory chain contains two cAMP-binding sites, a pseudosubstrate sequence blocking the kinase active site and a dimerisation sequence. Binding of cAMP results in the release of the catalytic chains from the complex. The free catalytic domain is active and phosphorylates a multitude of protein targets. An additional mode of regulation is realised by the protein kinase-A inhibitor protein (PKI) which contains a pseudosubstrate sequence and is able of binding and thus inhibiting the free catalytic domain. The regulatory subunits bind to A-kinase anchoring protein (AKAP) which is associated with the cytoskeleton and is thereby involved in the control of PKA localisation. (For review see (98))
PKA controls a lot of physiological processes, the most important ones of which are listened in the following:
Glycogen storage (an example for the involvement of PKA in metabolism): Activation of PKA results in decomposition of glycogen to glucose-1-phosphate, which is supplied to glycolysis or released as glucose from liver cells into the blood. This effect is propagated on three levels. First, glycogen-synthetase is phosphorylated and thus inactivated by PKA. Thus the (re)synthesis of glycogen is inhibited. Second, Phosphorylase-kinase is phosphorylated and thereby activated by PKA. Activated Phosphorylase kinase in

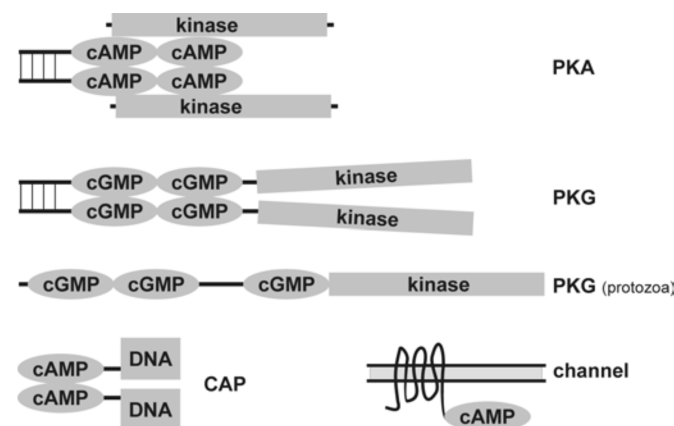


Fig. 4 **Domain architecture of proteins containing cAMP-binding domains**. PKA (protein kinase A) forms an inactive heterotetramer in the absence of cAMP, which consists of 2 regulatory and 2 catalytic subunits. Each regulatory chain contains two cAMP-binding and one dimerisation domain. The catalytic subunits containing a kinase domain are released from the inactive heterotetramer after cAMP binding. In PKG (protein kinase G) two cGMP-binding domains and one kinase domain are localised in one polypeptide chain. An additional cGMP binding domain is found in PKG from protozoa. CAP (catabolite gene activator protein) forms a homodimer, which interacts with DNA after cAMP binding and induces transcription, (DNA, DNA-binding domain). Cyclic nucleotide dependent ion channels are built from six transmembrane helices. The cyclic nucleotide binding domain is localised at the C-terminus.

turn phosphorylates and thus activates glycogen-phosphorylase, which cleaves glycogen to glucose-1-phosphate. Third, PKA phosphorylates phosphoprotein-phosphatase-inhibitor, which in its phosphorylated state inhibits phosphoprotein-phosphatase. Dephosphorylation and thus inhibition of glycogen-phosphorylase is thus prevented. (For review see (99;100))

Muscle relaxation: The hormonal control of muscle contraction is mainly mediated by cAMP. Myosin light chain kinase is activated by binding to the Ca^{2+}-Calmodulin complex, but inhibited by phosphorylation by PKA. Phosphorylation of myosin is required for muscle contraction. Activation of PKA results therefore in muscle relaxation.

Gene transcription: The cAMP response element (CRE) was identified as a consensus sequence in promoter regions of genes which are expressed in response to cAMP stimulation. Transcription factors of the cAMP response element binding protein family (CREB-family) are homodimers, which initiate gene transcription after phosphorylation by PKA. The free catalytic domain of PKA can directly enter the nucleus once released from the inactive complex.

Further direct effects in signal transduction: Many targets of PKA are elements of a wide variety of signalling pathways, which can be initiated, modulated or inhibited by phosphorylation. The review of the huge number of reports dealing with this subject is outside the scope of this thesis, which focuses more on the mechanistic aspects of the regulation of Epac. Isolated examples are discussed where required.

PKG:

In contrast to PKA, two cGMP binding sites and a kinase domain are combined within one polypeptide chain (Fig. 4), which forms homodimers mediated by a N-terminal leucine zipper. Binding of cGMP induces kinase activity, most likely due to a conformational change in the regulatory region which releases an intramolecular interaction between the regulatory and the catalytic part. Thus PKG imitates the regulation mechanism of PKA. The cyclic nucleotide binding domains of PKA and PKG are highly related. Three isoforms of PKG (PKGIα, PKGIβ and PKGII) exist. PKGIα and PKGIβ are splice variants, which differ only in the extreme N-terminus. PKGI is found in the cytosol and therefore termed soluble, whereas PKGII is membrane associated (for review see (101)). An important function of PKGII is the phosphorylation and thereby stimulation of the ion channel cystic fibrosis transmembrane conductance regulator (CFTR) ((102), for review see (103)). In addition PKG plays an important role in the cardiovascular system (for review see (104)). For a general review of cellular function of PKG see (101;105;106).

Recently a novel class of PKG was discovered in protozoa (107). These kinases contain a third cGMP binding domain "inserted" between the two N-terminal cGMP-binding domains and the C-terminal kinase domain (Fig. 4). Biochemical analysis have shown a functional relevance of all three domains (108;109).

CAP:

The catabolite gene activator protein (CAP) is found only in bacteria. It is a homodimer and binds to DNA only in the presence of cAMP (Fig. 4). CAP can initiate gene transcription by a direct interaction with RNA-polymerase. Best known is the function of CAP in initiating gene expression in the lac operon system.

Cyclic nucleotide regulated ion channels:

Some cation channels are directly regulated by a cytosolic cyclic nucleotide binding domain. Binding of cyclic nucleotide either modulates or controls the ion flux depending on the channel type. cAMP gated ion channels play a specialised role in the olfactory system and cGMP gated ion channels control the ion flux of the rods and cones in the visual system (for review see (110))

As can be seen from this summary, very different cAMP (and cGMP) binding proteins are known. However, most of these proteins are well separated from each other, either by tissue specific expression or by restriction to a relative small appanage of the kingdoms of life. Cyclic nucleotide gated ion channels are, apart from their specialised function as pacemaker channels in the heart, assumed to be restricted mainly to the visual and olfactory sensory systems. The catabolite gene activator protein is found in bacteria only but not in any higher organism.

The situation became more complex with the discovery of Epac. Both Epac and PKA are widely expressed. As discussed above Epac acts on the small G-protein Rap, a classic field of signal transduction. PKA regulates gene transcription and acts on many elements of signalling systems (The function of PKA in metabolism is not too important in this context). One has to assume that PKA and Epac mediated signalling interfere with each other. This dilemma is illustrated impressively by Rap itself. A cAMP dependent control of Rap and Rap-mediated effects was already noticed before the discovery of Epac (44;111). Rap1GAP was shown to be phosphorylated by PKA (besides CDC2), although without any influence on the *in vitro* activity (112;113). Rap is phosphorylated by PKA (114), although studies with a Rap mutant lacking the phosphorylation site in Drosophila show no abnormalities (115). These findings argued for a role of PKA in the regulation of Rap. However the much more direct link turned out to be Epac (3;4). In consequence, experiments have to be designed in a way that distinguishes between PKA and Epac mediated effects.

This could be achieved by the specific activation or inhibition of these pathways.
Two major ways to stimulate cAMP dependent pathways are in use practical. One is to activate adenylate cyclase and the other is to apply external cAMP or cAMP analogues. Stimulation of adenylate cyclase by Forskolin resembles the natural cellular generation of cAMP (Box 1), but clearly does not discriminate between PKA and Epac. The low permeability of the cell membrane for cAMP makes the simple addition of cAMP to cell cultures dishes ineffective. Various derivatives of cAMP, with an increased membrane permeability are available and widely used. The binding of these cAMP analogues to PKA is well characterised (116;117), and the binding to Epac is analysed in chapter 5 of this thesis. Only two cAMP based inhibitors of PKA are known, Rp-cAMPS and Sp-cAMPN(CH_3)$_2$, which compete with cAMP for the binding to the regulatory subunit (118;119) (for details see chapter 4 and 5). The usability of these analogues is limited due to their reduced affinity for PKA in comparison to cAMP (118;119). A common target for PKA inhibitors is the kinase domain itself. The kinase activity can be inhibited by substrates like peptides, which do bind to the active site but do not accept a phosphate group (120;121). The probably most widely used inhibitor for PKA is H89, which competes with ATP for binding to the kinase domain ((122), for review see (123)).
Thus, experiments have to distinguish carefully between PKA and Epac mediated effects. This is possible by the use of PKA specific inhibitors as well as the resently describe Epac specific activtor 8-pCPT-2'-O-Me-cAMP ((22;124) chapter 5).

Scope of this thesis

The more recently discovered Epac and the long known PKA are both major players in the intracellular cAMP mediated signalling. The activity of both proteins is controlled by a cAMP domain of very similar architecture. The effect of cAMP caused in the cell is therefore a result of both Epac and PKA activity.
The aim of this thesis was to elucidate the mechanism by which cAMP activates Epac.
We describe a biochemical characterisation of Epac in chapter 2 and 3. The inactive state of Epac is maintained by an intramolecular interaction between the cAMP-binding domain and the catalytic region and terminated by the release of this interaction upon cAMP binding. We identified a short conserved motif in Epac that is responsible for this interaction.
Structural information was only available of the cAMP-binding domains of PKA and CAP in complex with cAMP. We determined therefore in chapter 4 the crystal structure of the regulatory region of Epac2 containing two cAMP-binding domains and one DEP domain in the cAMP free state. In fact, this is the first of a cAMP-binding domain in the absence of ligand. From this data we deduce a model of a cAMP induce conformational change that is transferred throughout the cAMP-binding domain to the catalytic domain. This mechanism seems to be universal for all cyclic nucleotide binding proteins.
Finally we analysed in chapter 5 the molecular basis for the action of Epac specific cAMP analogues which allows to discriminate between Epac and PKA mediated effects *in vivo*.

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

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

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Epac1 (cAMP-GEFI) 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 particular the regulation of integrin activation by inside-out signaling (4). A large variety of extracellular stimuli, including growth factors, cytokines, and cell adhesion molecules, regulate Rap1(5–10). The intracellular pathways that activate Rap1 include common second messengers like cyclic adenosine monophosphate (cAMP), calcium, and diacylglycerol (DAG)[1] (11). A number of guanine nucleotide exchange factors (GEFs) have been identified that mediate the activation of Rap1. The first described Rap1GEF, C3G, is found in a complex with the proto-oncogene product c-Crk and may activate Rap1 as a consequence of complex formation and translocation induced by receptor tyrosine kinase signaling (12). CalDAG-GEFI has calcium binding EF-hands and a domain that resembles C1-type DAG binding domains and may explain the activation of Rap1 by these two second messengers (13). Recently, another type of GEF for Rap1, called PDZ-GEF1(14), nRapGEP (15), or RaGEF (16) was described. This GEF contains, in addition to the catalytic region, a Ras binding domain, which may interact directly with Ras and Rap1 *in vitro*, a PDZ domain that drives membrane association and a domain that is related to cAMP binding domains (RCBD) but does not bind cAMP. The most intriguing RapGEF, however, is Epac (exchange protein directly activated by cAMP), because this GEF represents a novel target for cAMP, independent from the classical target protein protein kinase A (PKA) (17, 18).

Epac was identified in the data base as a genomic sequence with homology to cAMP-binding sites as well as GEFs for Ras-like proteins (17). Independently, the same protein, named cAMP-GEFI, was found in a differential display screen for novel brain-enriched genes related to signaling in the striatum (18). Epac contains a C-terminal catalytic region that activates Rap1 but not Ras, Ral, or R-ras. This region comprises the enzymatic GEF domain and the Ras exchange motif (REM), which is needed for stability of the GEF domain. The N-terminal part of the protein contains a DEP domain, of which the function is unclear, and a cAMP binding domain that is similar to the cAMP binding domains in the regulatory subunit of PKA. cAMP is required for the activation of full-length Epac *in vitro*, and deletion of the regulatory N-terminal part containing the cAMP binding domain results in the constitutive activation of Epac, indicating that it serves as an auto-inhibitory domain. 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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[1] The abbreviations used are: DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; cAMP, cyclic adenosine monophosphate; PDZ, domains that occur in PSD-95, Dlg, and ZO-1; RCBD, related to cAMP binding domains; Epac, exchange protein directly activated by cAMP; PKA, protein kinase A; REM, Ras exchange motif; DEP, domains that occur in Dishevelled, Egl-10, and pleckstrin; Repac, related to Epac; GST, glutathione *S*-transferase; mantGDP, 2′,3′-bis(*O*)-*N*-methylantharanoloyl guanosine diphosphate; ITC, isothermal titration calorimetry; RD, regulatory domain; HA, hemagglutinin.

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 PMT2-SM-HA eukaryotic expression vector. Epac1 constructs are derived from human cDNA, and Epac2 constructs are derived from murine cDNA. HA-Epac1-DDEP contains Epac1 lacking amino acids 71–140, 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-RD) contains amino acids 2–329, the cAMP binding domain of Epac1 contains amino acids 149–318, and Epac1-DDEP 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-DDEP 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 used contained the R1a subunit of bovine PKA, lacking amino acids 1–91(20). Protein production was induced in Bl-21 bacteria using 100 nM isopropyl-1-thio-β-D-galactopyranoside for 20 h at room temperature. After protein production, bacteria were pelleted and lyzed 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 × *g* to remove insoluble material. GST fusion proteins we purified from the cleared lysate by batchwise incubation with glutathione-agarose beads (Sigma), eluted from the beads in buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 10 mM glutathione, and dialyzed for 20 h in the same buffer without glutathione. When indicated, proteins were cleaved from the GST tag by incubation with thrombin and purified by gel filtration. Small GTPases used for *in vitro* experiments were described elsewhere (21).

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 mM) and isobutylmethylxanthine (1 mM) for 10 min. The GTP-bound form of Rap1A was specifically isolated using GST-RalGDS as an activation-specific probe 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 [^{32}P]orthophosphate for 5 h. Rap2A was precipitated using 12CA5 antibodies, and nucleotides were eluted and separated on polyethyleneimine-cellulose F thin layer chromatography (TLC) plates. Labeled nucleotides were visualized using a phosphoimager, and GTP/GDP ratios were calculated using the program ImageQuant.

In Vitro Activation of Small GTPases—Experiments were performed as described (21). Briefly, 100 nM purified GTPase (Rap1A or Rap2A) loaded with fluorescently labeled 2′,3′-bis(*O*)-*N*-methylantharanoloyl guanosine diphosphate (mantGDP) was incubated in the presence of excess unlabeled GDP with 50 nM purified GEF unless indicated differently. Release of mGDP was measured in real time as a decrease in fluorescence. To calculate reaction rates, single exponential functions were fit using the program Grafit3.0 (Eritacus). 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.

Cell Fractionation—Cells were scraped in mild lysis buffer (20 mM Hepes, pH 7.4, 5 mM EGTA, 1 mM sodium vanadate, 1 μM leupeptin, 1 μM aprotinin) and homogenized through a 23-gauche 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 Epac1 in both fractions was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using 12CA5 monoclonal antibody directed against the HA tag. Distribution of the endogenous epidermal growth factor receptor was analyzed using a monoclonal antibody (Transduction Laboratories), and the presence of p42 mitogen-activated protein kinase was investigated using a polyclonal antiserum described earlier (22).

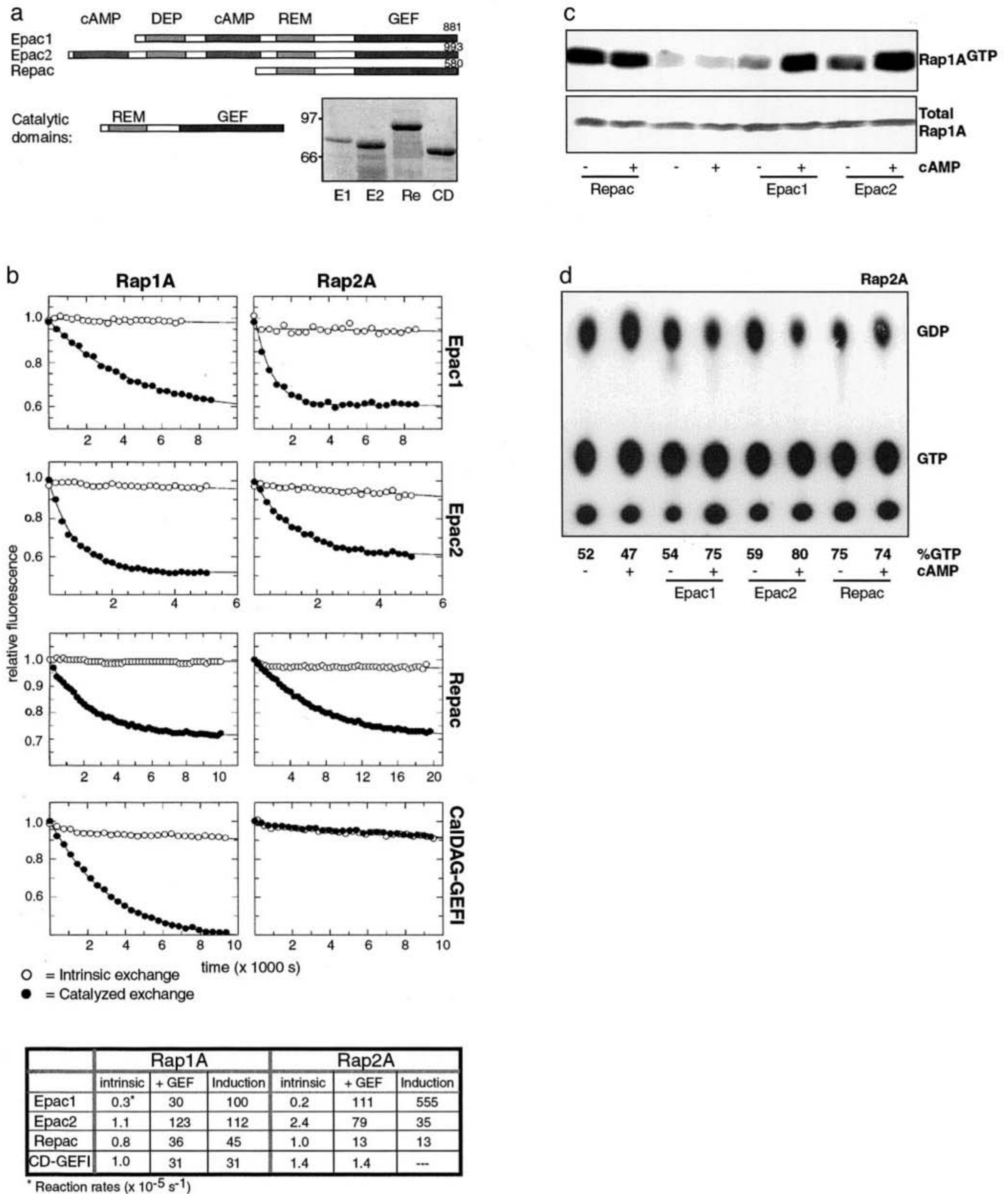
RESULTS

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. 1*a*). 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. 1*b*). Equal amounts (approximately 100 nM) of GST fusions of the catalytic domains (Fig. 1*a*) 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. 1*c*. Epac1 activated Rap2A five times more efficiently than Rap1A, whereas Epac2 and Repac activated Rap2A 3-fold less efficiently (Fig. 1*b*). Activation of Rap2 is not a common feature of all Rap1GEFs, since C3G (21) and CalDAG-GEFI (Fig. 1*B*) did not exhibit catalytic activity toward Rap2A *in vitro*.

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 isobutylmethylxanthine to raise the level of cAMP. Activation of Rap1A was measured using the previously described activation-specific probe assay, in which Rap1GTP is specifically precipitated with the Rap1 binding domain of RalGDS (5). As shown in Fig. 1*c*, all three GEFs activate Rap1A. The activation by Epac1 and Epac2 is enhanced by forskolin treatment.

The activation-specific probe assay is less suitable for measuring activation of Rap2A, due to the high basal level of Rap2GTP in cells (14). Therefore, we incubated the cells with ^{32}P-labeled orthophosphate followed by precipitation of Rap2A and separation of bound GDP and GTP. We observed that all three GEFs activate Rap2A *in vivo* (Fig. 1*d*). The activation of Rap2A by Epac1 and Epac2 is enhanced by forskolin treatment. From these results we conclude that all three members of the Epac family activate both Rap1 and Rap2.

Epac2 Has a Second, Low Affinity Binding Site for cAMP—In the completed sequence of the *Caenorhabditis elegans* genome, only one Epac-related gene could be found. This gene encodes a



	Rap1A			Rap2A		
	intrinsic	+ GEF	Induction	intrinsic	+ GEF	Induction
Epac1	0.3*	30	100	0.2	111	555
Epac2	1.1	123	112	2.4	79	35
Repac	0.8	36	45	1.0	13	13
CD-GEFI	1.0	31	31	1.4	1.4	---

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

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-GEFI (*CD*) were purified using glutathione-agarose beads, separated by SDS-polyacrylamide gel electrophoresis, and stained by 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 [^{32}P]orthophosphate, Rap2A was immunoprecipitated using 12CA5 monoclonal antibodies, nucleotides were eluted and separated by TLC, and GTP/GDP ratios were measured using a phosphoimager.

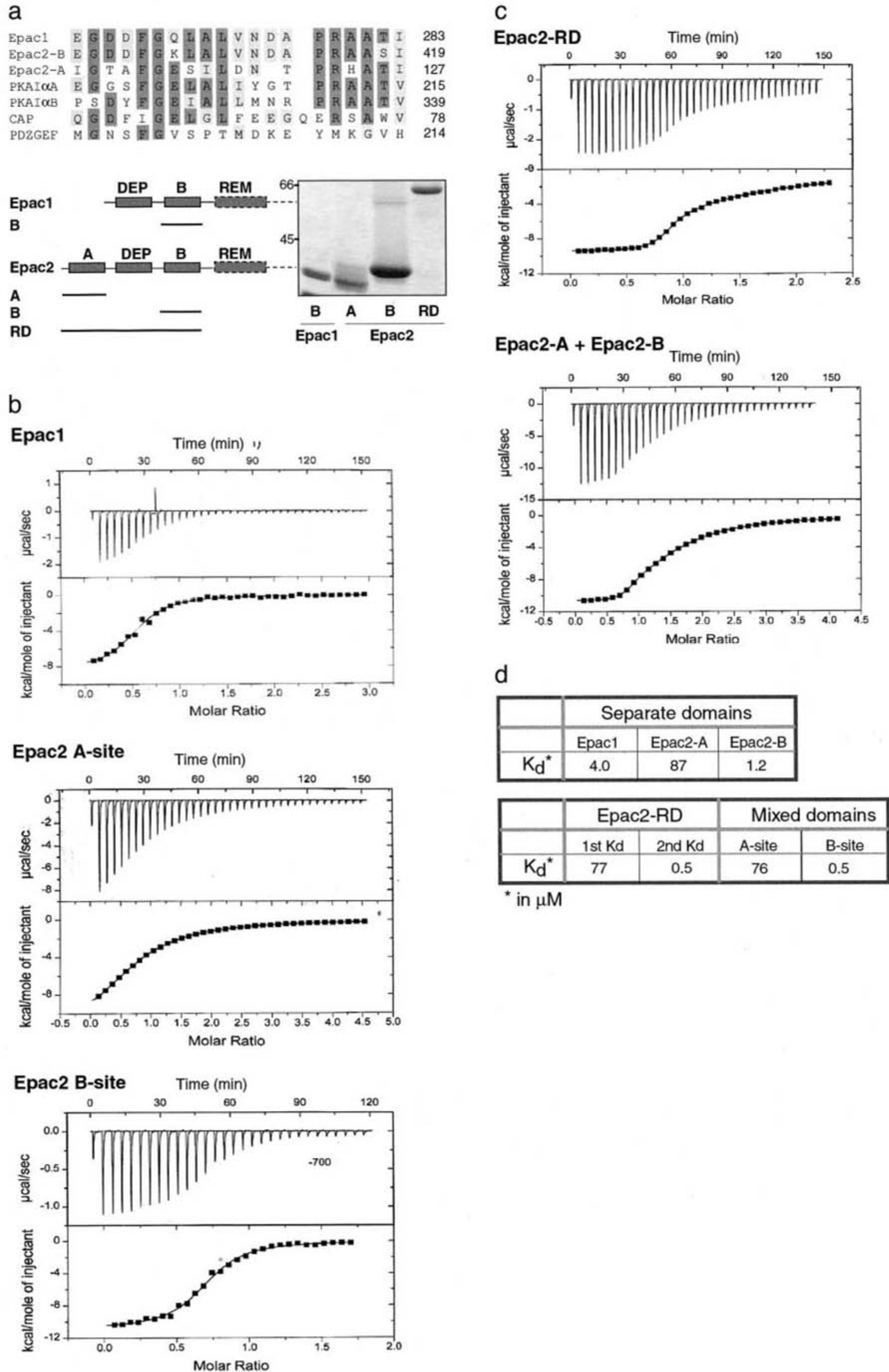


FIG. 2. **Different cAMP binding sites in Epac1 and Epac2.** *a*, alignment of different cAMP binding pockets (residues identical in more than 50% of the depicted domain are marked by *dark boxes*; *light boxes* indicate conserved residues) and schematic presentation and purification of GST fusion constructs containing the cAMP binding domains of Epac1 (*B*) and Epac2 (*A* and *B*) and the regulatory domain of Epac2 (*RD*) (Coomassie-

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. 1*a*). 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. 2*a*). 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. 2*a*). To compare these different sites, we analyzed purified domains (Fig. 2*a*) 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. 2*b*). 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. 2*c*), 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. 2*c*, *lower panel*). The data from these measurements are summarized in Fig. 2*d*. 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. 3*a*). As shown in Fig. 3*b*, 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. 3*a*, *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. 1*b*). 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. 3*c*, 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. 3*d*). 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. 4*a*). As shown in Fig. 4*b*, 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. 3*c*) nor a PKA construct containing both its cAMP binding domains (Fig. 4*b*) 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. 3*b* 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. 5*a*, 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. 5*a*, *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 59 μM cAMP binding domain of Epac1 titrated with 0.72 mM cAMP, 200 μM cAMP-binding site A of Epac2 titrated with 3.7 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.

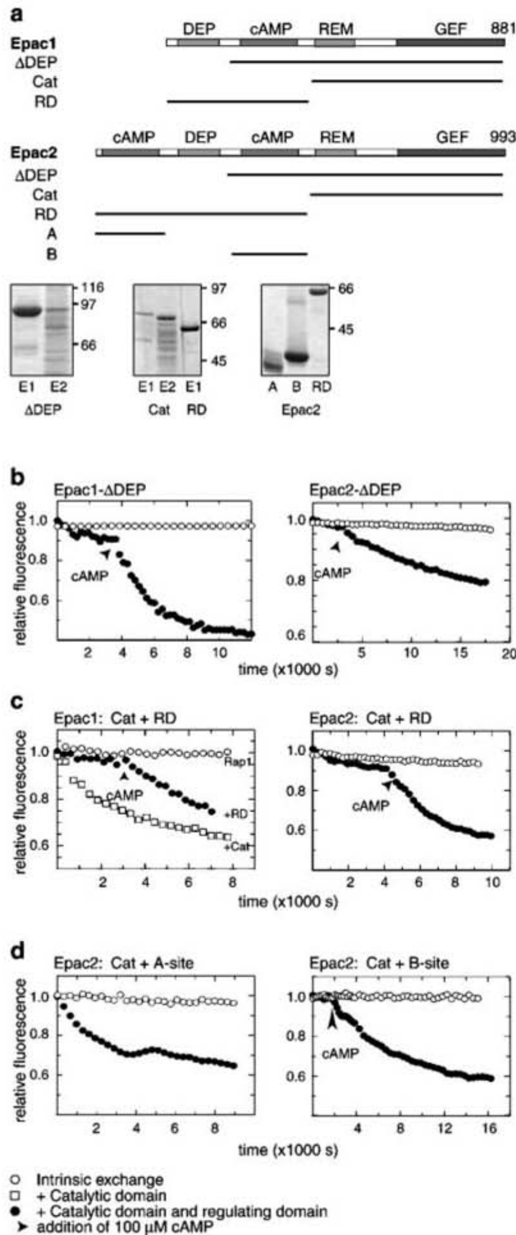


FIG. 3. **Regulation of Epac catalytic activity by isolated B-sites.** *a*, schematic representation and purification (Coomassie-stained gel) of the GST fusion constructs used in *b*, *c*, and *d*. *Cat*, catalytic domain. *b*, Epac1-ΔDEP or Epac2-ΔDEP was incubated at approximately 50 nM with purified Rap1A loaded with mantGDP (100 nM). cAMP (10 μM) was added at the indicated time points. *c*, isolated catalytic domains of Epac1 or Epac2 (50 nM) were incubated with their respective regulatory domains (Epac1-RD at 150 nM, Epac2-RD at 3 μM), and mantGDP loaded Rap1A (100 nM). cAMP (100 μM) was added at the indicated time points. In the *left panel* Rap1A in the presence of only the catalytic domain of Epac1 (*open squares*) is shown for comparison. *d*, the catalytic domain of Epac2 (50 nM) is incubated with the isolated cAMP binding domains of Epac 2 (A-site at 200 μM and B-site at 3 μM) and mantGDP-loaded Rap1A (100 nM). 100 μM cAMP was added to the incubation with the B-site at the indicated time point.

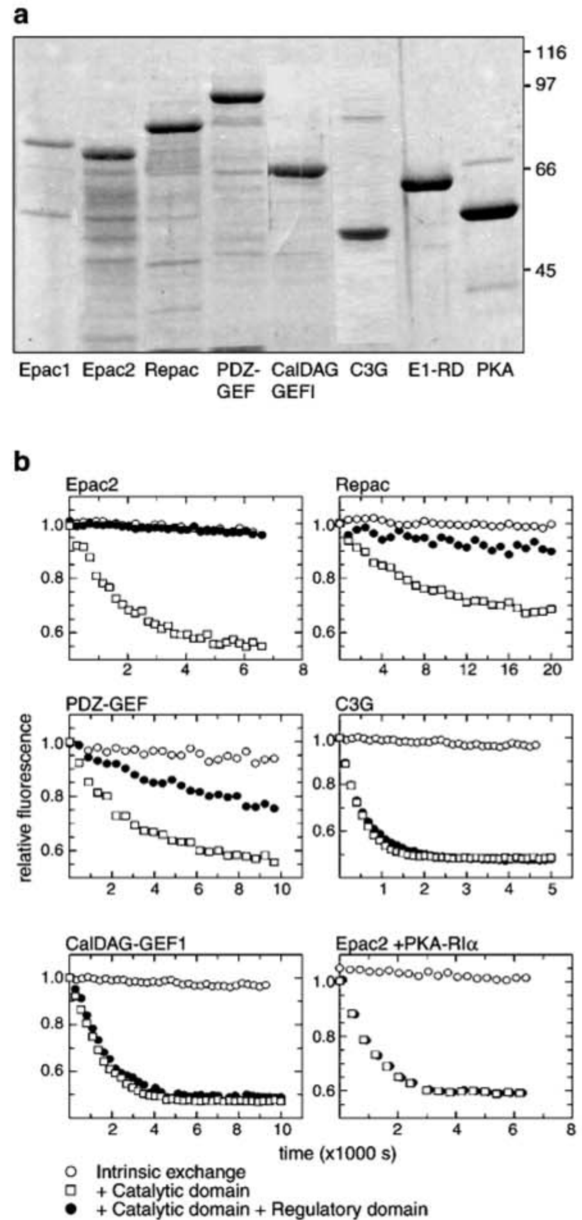


FIG. 4. **The regulatory domain of Epac1 can block the catalytic domain of closely related RapGEFs.** *a*, purification of GST fusions of the catalytic and regulatory domains used in *b*. C3G was cleaved from the GST tag and purified by gel filtration. *b*, intrinsic exchange of mantGDP-loaded Rap1A (100 nM) or Rap2A in the case of PDZ-GEF1 (*open circles*), activation by the indicated catalytic domains (50 nM) (*open squares*), and inhibition or lack of inhibition by the presence of Epac1-RD at 150 nM, 300 nM in the case of C3G and CalDAG-GEFI, or PKA-RIα at 500 nM (*black circles*).

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. 5*b*, 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 Rap1GEFs. 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.

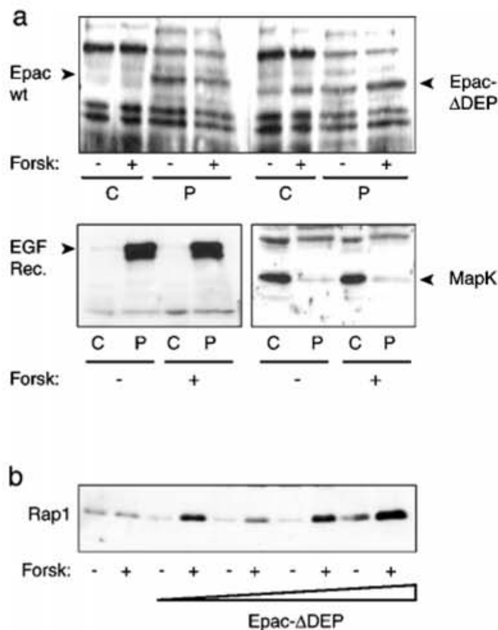


FIG. 5. **CAMP-independent membrane targeting of Epac1 by the DEP domain.** *a*, Cos-7 cells were transfected with HA-Epac1 or Epac1 lacking the DEP domain (HA-Epac1-ΔDEP). Cells were lysed in a syringe, and particulate fractions (*P*) and cytosolic fractions (*C*) were separated by centrifugation. Distribution of Epac in these fractions was analyzed by Western blotting using 12CA5 monoclonal antibodies directed against the HA tag. *Lower panels* show distribution of the epidermal growth factor receptor (*EGF Rec.*) as a transmembrane protein and mitogen-activated protein kinase (*Mapk*) as cytosolic protein for as a control for the fractionation. *wt*, wild type. *b*, Rap1A was cotransfected with increasing amounts of HA-Epac1-ΔDEP (30 ng, 100 ng, 300 ng, and 1 μg), and Rap1 activation was measured using activation-specific probes and 12CA5 monoclonal antibodies on a Western blot.

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-GEF1 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).[2] 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.[2]

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),[3] 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 able also 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

[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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Communication between the Regulatory and the Catalytic Region of the cAMP-responsive Guanine Nucleotide Exchange Factor Epac

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Communication between the Regulatory and the Catalytic Region of the cAMP-responsive Guanine Nucleotide Exchange Factor Epac*

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Epac1 is a guanine nucleotide exchange factor (GEF) for the small GTPase Rap1 that is directly activated by cAMP. This protein consists of a regulatory region with a cAMP-binding domain and a catalytic region that mediates the GEF activity. Epac is inhibited by an intramolecular interaction between the cAMP-binding domain and the catalytic region in the absence of cAMP. cAMP binding is proposed to induce a conformational change, which allows a LID, an α-helix at the C-terminal end of the cAMP-binding site, to cover the cAMP-binding site (Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., de Rooij, J., Bos, J. L., and Wittinghofer, A. (2003) *Nat. Struct. Biol.* 10, 26–32). Here we show that mutations of conserved residues in the LID region affect cAMP binding only marginally but have a drastic effect on cAMP-induced GEF activity. Surprisingly, some of the mutants have an increased maximal GEF activity compared with wild type. Furthermore, mutation of the conserved VLVLE sequence at the C-terminal end of the LID into five alanine residues makes Epac constitutively active. From these results we conclude that the LID region plays a pivotal role in the communication between the regulatory and catalytic part of Epac.

A multitude of cellular stimuli, including hormones, growth factors, and neurotransmitters, induce activation of the $G\alpha_s$ subunit of heterotrimeric G-proteins, which, in turn, activates adenylyl cyclase to synthesize cyclic AMP (cAMP) from ATP. cAMP acts as a second messenger that binds to and regulates PKA[1] and cyclic nucleotide-dependent ion channels (1). More recently, cAMP-responsive guanine nucleotide exchange factors (GEFs) for Rap proteins were identified, *i.e.* Epac (exchange protein directly activated by cAMP) (2) or cAMP-GEF (3). Rap proteins are members of the Ras family of small GTP-binding proteins. They act as molecular switches that cycle between a GDP-bound inactive and a GTP-bound active state. Nucleotides are bound tightly to the protein and are released with a very slow dissociation rate. GEFs act by accelerating the slow intrinsic nucleotide dissociation rate by several orders of magnitude. Because the GTP concentration in the cell is much higher then the GDP concentration, Rap is then loaded with GTP. In the GTP-bound form, Rap interacts specifically with proteins to activate downstream targets. Rap1 plays, among other things, a pivotal role in integrin-mediated cell adhesion (4–9). Rap-GTP is recycled into its inactive state by hydrolyzing GTP to GDP, a process that is catalyzed by Rap-specific GTPase-activating proteins (RapGAPs).

Two isoforms of Epac, Epac1 and Epac2, were found in mammalian cells, both consisting of a regulatory and a catalytic region (2, 3). The catalytic region contains a CDC25 homology domain and a REM domain, both of which are characteristic for GEF proteins of Ras-like small GTP-binding proteins such as Ras, Ral, and Rap (Fig. 1). Whereas the CDC25 homology domain contains the active site (10) and forms the interface with its substrate (11), the REM (12) domain stabilizes the CDC25 homology domain without being directly involved in catalysis (11). The regulatory region of Epac contains a cAMP-binding domain and a Dishevelled/Egl-10/pleckstrin (DEP) domain (13). The cAMP-binding domain is responsible for cAMP-mediated GEF activation (2, 3, 14), whereas the DEP domain is responsible for the membrane localization of Epac (15). Whether the DEP domain binds to proteins or lipids is still elusive. Epac2 contains an additional N-terminal cyclic nucleotide monophosphate-binding domain of still unknown function.

Activation of Epac as well as PKA and PKG (cGMP-regulated kinase) is mediated by the binding of cyclic nucleotide to the cyclic nucleoside monophosphate (cNMP)-binding domains. Whereas Epac and PKG consist of only one polypeptide chain, PKA is composed of catalytic (C) and regulatory (R) subunits. The R subunit contains two cAMP-binding domains and forms a homodimer that, in the absence of cAMP, binds to and inhibits the protein kinase activity of the two C subunits. Binding of cAMP leads to the release of the C subunits from the complex and its activation. PKG also contains a dimerization sequence in its regulatory part. Inactive PKG thus resembles inactive PKA, although it contains a different number of polypeptide chains. A chimera of PKG and PKA can be created that maintains the nucleotide specificity of the cyclic nucleoside monophosphate-binding domains (16). These findings suggest that, in PKA and PKG, the inhibition is realized in the same way by the binding of a regulatory domain to the kinase domain either in an intra or an intermolecular manner. For Epac, the regulatory region also inhibits GEF activity in the absence of cAMP, and cAMP relieves this inhibition (2). Indeed, *in vitro*, an isolated catalytic domain, which is constitutively active, is inhibited by an isolated regulatory domain, suggesting a direct

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[1] The abbreviations used are: PKA, protein kinase A; GEF, guanine nucleotide exchange factor; Epac, exchange protein directly activated by cAMP; REM, Ras exchange motif; DEP, Dishevelled/ Egal-10/pleckstrin (domain); PKG, cGMP-regulated protein kinase; mantGDP, 2′(3′)-*O*-(*N*-methylanthraniloyl) guanosine diphosphate; C subunit, catalytic subunit; R subunit, regulatory subunit; CAT, catalytic domain; RD, regulatory domain; AC_{50}, half-maximal activity.

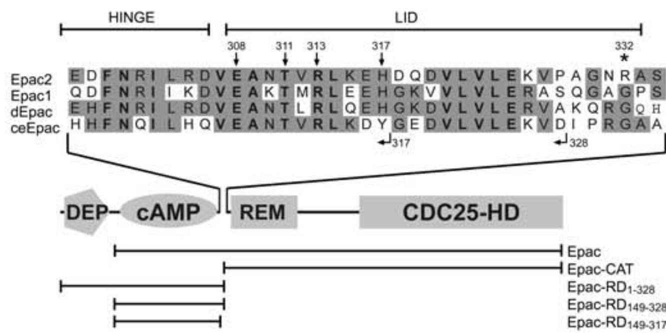

FIG. 1. **Schematic representation of Epac1.** Indicated is the domain structure of Epac1 with a DEP domain, a cAMP-binding domain (*cAMP*), a REM, and a CDC25 homology domain (*CDC25-HD*). The enlarged region is the C-terminal region of the cAMP-binding domain and is aligned with the homologous regions in Epac2, *Drosophila* Epac (*dEpac*), and *Caenorhabditis elegans* Epac (*ceEpac*). The region comprises part of the HINGE region and the LID region as defined from the structure of the regulatory region of Epac2 (21). Indicated by *arrows* are the conserved residues that have been mutated to Ala and analyzed in this study. The single nucleotide polymorphism G332S in Epac1, identified by Vanvooren *et al.* (23), is highlighted by an *asterisk*. Also indicated are the deletion mutants of Epac1 used in this study.

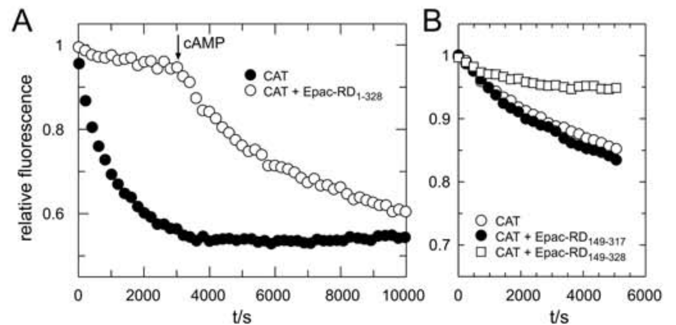

FIG. 2. **Rap1 activation by the catalytic domain of Epac1 in the presence or absence of different constructs of the regulatory domain.** *A*, 100 nM Epac-CAT and 300 nM Epac1-RD$_{1-328}$ were incubated with Rap1 loaded with mantGDP, and a decrease in florescence as a measure for exchange activity was recorded as described under "Experimental Procedures." cAMP was added when indicated. *B*, 100 nM of Epac-CAT and either 200 μM Epac-RD$_{149-317}$, 200 nM Epac-RD$_{149-328}$, or no additional protein was incubated with Rap1 loaded with 2′(3′)-*O*-(*N*-methylanthraniloyl) guanosine diphosphate (mantGDP). Note that, in this experiment, Epac-RD$_{149-317}$ was used at 1000-fold higher concentrations then was Epac-RD$_{149-328}$.

physical interaction between the regulatory and the catalytic regions of Epac. This inhibition is released by cAMP (14).

The kinase domain of the C subunit of PKA was structurally analyzed in great detail and was, in fact, the first protein kinase structure to be solved (17, 18). Structures of the different R subunits of PKA have been analyzed in the presence of bound cAMP (19, 20). However a structure of the holoenzyme is, to date, not available, and the interaction of the R and the C subunits is incompletely understood in molecular terms. Likewise, it is not understood in any detail how the binding of cAMP induces dissociation of the holoenzyme. Recently, we have solved the structure of the regulatory region of Epac2 in the cAMP-free conformation (21). When compared with the cAMP-bound regulatory subunits of PKA, the most obvious difference between these structures is a C-terminal helix, also called LID, which, in PKA, covers cAMP against the solvent and is unstructured in the determined Epac structure, most likely because it is flexible due to the absence of cAMP. It was argued that the HINGE region regulates the orientation of the LID. In turn, the conformation of the HINGE is directly regulated by the presence or absence of cAMP (21). Here we present results indicating that the region of Epac, which would correspondent to the LID region in PKA, is involved in the direct communication between the regulatory domain and the catalytic region. A mutation has been found that completely relieves the auto-inhibition of Epac.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—All Epac constructs were expressed as GST fusion proteins from the pGEX-4T2 vector in the *Escherichia coli* strain CK600K as described previously (22). These constructs are from human Epac1 and comprise the following amino acid residues: Epac, 149–881; Epac-RD$_{1-328}$, 1–328; Epac-RD$_{149-317}$, 149–317; Epac-RD$_{149-328}$, 149–328; and Epac-CAT, 324–881. Single/double amino acid and Ala$_5$ mutations were made in the Epac-(149–881) background. Mutations were introduced according to the QuikChange procedure (Stratagene). Because of low expression levels and poor solubility, only low and impure amounts of Epac-CAT and Epac(Ala)$_5$ were available. The concentration of these proteins was therefore determined after SDS-PAGE gel electrophoresis. The C-terminal truncated version of Rap1B was expressed as described (10). We refer to this construct as Rap1 in the text.

Isothermal Titration Calorimetry—To analyze the binding of cAMP to the isolated cAMP-binding domain, a microcalorimeter (MicroCal Inc.) was used. The protein was brought to 25 °C in the 1.38-ml cell of the apparatus, and cAMP was injected in 40 steps of 6 μl every 4 min up to a 2–3-fold molar excess. The concentration of the protein was typically 50 μM, and the concentration of the nucleotide solution was 10–20 times higher. The cAMP solution was in the same buffer as the purified protein. The data were analyzed using the manufacturer's software.

In Vitro Activation of Rap1—*In vitro* activation of Rap1 was performed as described previously (10). Briefly, 200 nM of Rap1B loaded with the fluorescent GDP analogue 2′(3′)-*O*-(*N*-methylanthraniloyl) guanosine diphosphate (mantGDP) were incubated in the presence of 20 μM GDP (Sigma) and either 100 nM (mutant) Epac or isolated domains as indicated. cAMP (Sigma) was added as indicated for the individual experiments. The nucleotide exchange was measured in real time as decay in fluorescence using a Spex1 spectrofluorometer (Spex Industries). The decay is caused by the release of protein-bound mantGDP, which shows higher fluorescence intensity in the hydrophobic environment of the protein than in the buffer solution. The obtained data were fitted to a single exponential decay, and the rate constants (k_{obs}) calculated were plotted against the cAMP concentration. The concentration dependence of the rate constants was treated as a normal titration experiment. All data analysis, fitting, and plotting were done with the Grafit 3.0 program (Erithacus Software).

RESULTS

Regulation of GEF Activity via the C-terminal Region of the cAMP Domain—The isolated recombinant catalytic domain of Epac1 (Epac-CAT) is active as a GEF and accelerates release of GDP bound to Rap1. The activity is independent of cAMP but can be inhibited by the addition of recombinant regulatory domain (Epac-RD$_{1-328}$) (14). The heterodimeric Epac-RD$_{1-328}$-Epac-CAT complex can be reactivated by cAMP (Fig. 2*A*). To investigate whether an isolated cAMP-binding domain was sufficient for *trans*-inhibition, we tested Epac-RD$_{149-328}$. As expected, this cAMP-binding domain was sufficient to inhibit Epac-CAT. Interestingly however, a cAMP-binding domain that is shorter by only eleven amino acids at the C terminus (Epac-RD$_{149-317}$) did not inhibit Epac-CAT, not even at much higher concentrations (Figs. 1 and 2*B*).

To exclude the possibility that Epac-RD$_{149-317}$ is not properly folded, we determined the affinity for cAMP, because an unfolded protein should not be able to bind cAMP. As determined by isothermal titration calorimetry, the cAMP affinities for Epac-RD$_{149-328}$ (4 μM) and for Epac-RD$_{149-317}$ (3 μM) are similar (Fig. 3). From these results we conclude that the additional eleven amino acids are required for the *trans*-inhibition of Epac-CAT and, thus, for the communication of the cAMP-binding domain to Epac-CAT.

The VLVLE Sequence Is Required for Auto-inhibition—Sequence comparison identifies a VLVLE motif within the additional eleven residues, which is conserved between different Epac proteins from man to nematode and fly (Fig. 1). To inves-

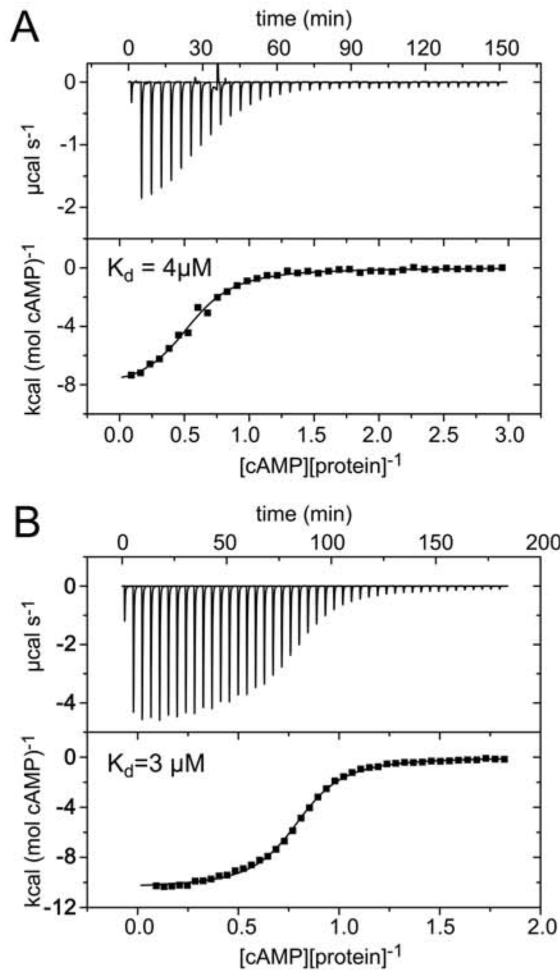

FIG. 3. **Affinity measurement of cAMP by isothermal titration calorimetry.** Epac-$RD_{149-317}$ (*A*) or Epac-$CAT_{149-328}$ (*B*) were titrated with cAMP by isothermal titration calorimetry as described under "Experimental Procedures." The *top section* of each diagram shows the heat produced during each titration step plotted against time. The *bottom sections* show the integrated heat release of each injection normalized to the added amount of cAMP.

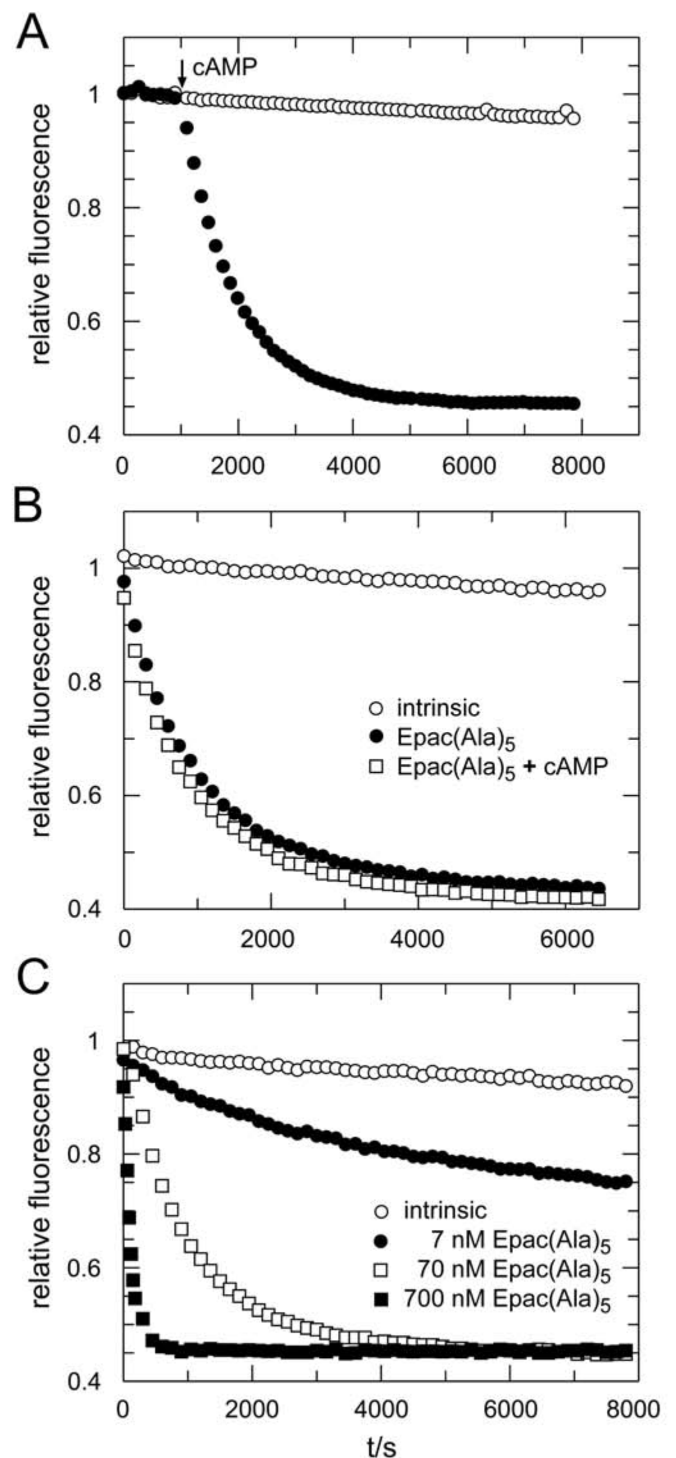

FIG. 4. **Epac(Ala)$_5$ is constitutively active.** Epac and Epac(Ala)$_5$ were incubated with Rap1 loaded with mantGDP, and a decrease in florescence as a measure for exchange activity was recorded. *A*, 200 nM Rap1-mantGDP in the absence (*open circles*) and presence (*closed circles*) of 100 nM Epac. 500 μM cAMP was added where indicated. *B*, 70 nM Epac(Ala)$_5$ in the presence and absence of 500 μM cAMP as indicated. *C*, various concentrations of Epac(Ala)$_5$ as indicated in the absence of cAMP. The concentration of the various constructs was estimated by SDS-gel analysis (see "Experimental Procedures").

tigate the function of these residues in the regulation of Epac1, we mutated the VLVLE motif in Epac to AAAAA creating Epac(Ala)$_5$. Epac is inactive in the absence of cAMP but becomes active after the addition of cAMP (Fig. 4*A*). In contrast, Epac(Ala)$_5$ is active in the absence of cAMP in a concentration-dependent manner (Fig. 4, *B* and *C*). Importantly, cAMP does not stimulate this activity any further (Fig. 4*B*). From these results we conclude that the VLVLE sequence is required for the inhibition of Epac by the regulatory domain.

To understand in more detail the contribution of the individual residues of the VLVLE motif, mutations were generated as indicated (Table II). First, residues were mutated in a pair-wise alanine scan generating Epac(VAALE), Epac(VLAAE), and Epac(VLVAA). The mutant proteins were characterized by determination of the cAMP concentration necessary to obtain half-maximal activity (AC_{50}) as well as the maximal GEF activity measured as the maximal rate constant k_{max} for dissociation of mantGDP from Rap1-mantGDP, which was achieved under standard experimental conditions and saturating concentrations of cAMP. The results are shown in Fig. 5 and summarized in Table I. All of the mutations were inactive in the absence of cAMP and show cAMP-dependent GEF activity. Importantly, AC_{50} and k_{max} values of the mutants did not differ more than 2-fold compared with the wild type protein. Whereas the k_{max} of Epac(VLVAA) is comparable with wild type, it shows an AC_{50} of 25 μM as compared with 45 μM for wild type. Epac(VAALE) and Epac(VLAAE) are characterized by a reduced k_{max} but an almost unchanged AC_{50}. Apparently, the VLVLE motif tolerates a number of mutational assaults.

In a second series of experiments, more drastic mutations to charged or bulky residues were introduced into the VLVLE

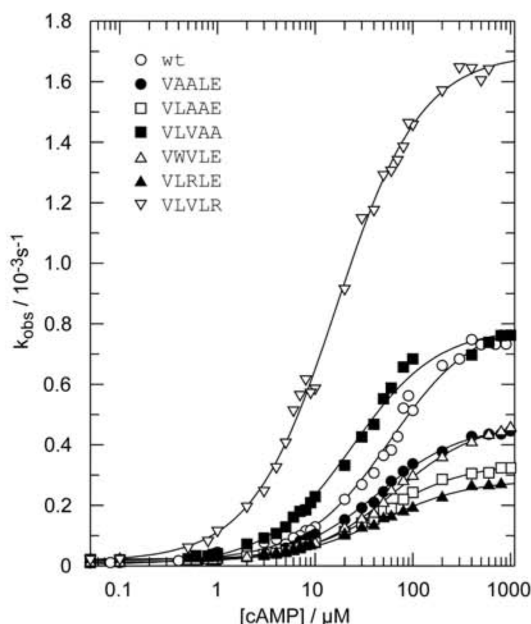

FIG. 5. **Activity of Epac mutated in VLVLE motif.** Various mutations in the VLVLE motif were generated. 200 nM Rap-mantGDP were incubated in the presence of an excess GDP with 100 nM Epac at different concentrations of cAMP. The exchange activity was monitored as a decrease in fluorescence and fitted to a single exponential decay. The obtained individual reaction rates (k_{obs}) were plotted against the cAMP concentration. *wt*, wild type.

TABLE I
Biochemical properties of mutations in the VLVLE region
The AC_{50} values and the relative k_{max} as determined from Fig. 5 are summarized.

	AC_{50}	Relative k_{max}
	μM	
Wild type	45	1
VAALE	45	0.6
VLAAE	50	0.4
VLVAA	24	1
VWVLE	65	0.6
VLRLE	50	0.4
VLVLR	17	2.3

sequence to generate Epac(VLRLE), Epac(VWVLE), Epac-(VLDLE), and Epac(VLVLR) (Fig. 5 and Table II). Replacing leucine with the more bulky hydrophobic residue tryptophane in Epac(VWVLE) is well tolerated, as is the positive charge in Epac(VLRLE). This latter mutant shows a 2.5-fold reduced maximal catalytic activity. Epac(VLDLE), wherein valine is replaced by the small negatively charged aspartate, is not soluble. Apparently, this alteration has a drastic effect on the structure of the protein. Replacement of the negative glutamate by alanine in Epac(VLVAA) showed no gross changes in properties. However, changing the negatively charged glutamate into the positively charged arginine in Epac(VLVLR) resulted in a 2-fold decrease in AC_{50} and, very surprisingly, a 2.3-fold increase in k_{max} as compared with wild type. Because the concentration of active protein is crucial for these experiments, we used different preparations of wild type and mutant proteins and obtained similar results, not deviating by more than 15% for both AC50 and k_{max}. From these measurements we conclude that the VLVLE sequence can tolerate extensive mutations without affecting the inhibition by the regulatory region. However, the mutations do modulate the ability of cAMP to activate Epac. Most dramatic is the 2.3-fold increase activity of Epac(VLVLR), indicating that wild type Epac is activated submaximally by cAMP, at least *in vitro*.

TABLE II
Biochemical properties of mutations in the LID region
The K_d values for the isolated cAMP binding domains (Epac-$RD_{149-328}$) were determined by isothermal titration calorimetry. The corresponding AC_{50} values and relative k_{max} for cAMP-mediated Epac activation are determined from Fig 6.

	K_d	AC_{50}	Relative k_{max}
		μM	
Wild type	3	45	1
E308A	6.3	15	3
T311A	5.5	15	2.3
R313A	6.2	40	1
H317A	3.6	50	0.2

Additional Conserved Amino Acids in the LID Region—From the comparison between the crystal structure of the cAMP-free regulatory domain of Epac2 and the cAMP-bound regulatory domain of PKA (19, 20), a model of the molecular mechanism of cAMP-induced activation was developed (21). It predicts that cAMP binding induces a conformational change in the phosphate binding cassette (PBC) and the HINGE of the cAMP-binding domain. This, in turn, induces the C-terminal helix of the cAMP-binding domain, the LID, to move toward the core structure and shield cAMP from the solvent. The VLVLE motif is located at the end of the LID (Figs. 1 and 8). A number of additional conserved residues in the LID (Fig. 1) were analyzed for their possible involvement in binding the adenine base of cAMP and/or mediating the interaction between the regulatory region and the catalytic region. Four mutants were made in Epac-$RD_{148-328}$ and analyzed by measuring the affinity of cAMP to the isolated cAMP-binding domain by isothermal titration calorimetry. Whereas H317A showed wild type affinity, E308A, T311A, and R313A showed a 2-fold reduction in affinity for cAMP, arguing that these residues make a small contribution to binding of cAMP. In addition, the same mutations were made in Epac to determine cAMP-dependent catalytic activity using the fluorescent GEF assay (Fig. 6 and Table II). The AC_{50} values of R313A and H317A are similar to wild type, whereas E308A and T311A show a 3-fold lower AC_{50}. Remarkably, both E308A and T311A showed a 2–3-fold increase in cAMP-induced GEF activity compared with wild type, whereas H317A has a 5-fold reduced maximal activity.

The G332S Polymorphism Does Not Affect the Regulation of Epac by cAMP—Interestingly, a G322S polymorphism is localized C-terminal to the cAMP-binding domain in close proximity to the VLVLE sequence (23). To investigate whether this mutation may affect the regulation of Epac by cAMP, this mutation was introduced in Epac, and cAMP-induced activation was analyzed (Fig. 7). However, G332S showed the same AC_{50} value as wild type, whereas the maximal activity was slightly increased by 20%.

DISCUSSION

A previous comparison of the structures of the cAMP-free (open) cAMP-binding domain of Epac and the cAMP-bound (closed) cAMP-binding domain of PKA revealed that the main difference between the open and the closed structure is the orientation of the LID. In the closed conformation of PKA, this LID covers the cAMP-binding site and interacts with the adenine group of cAMP. This suggests that this LID region may be the main determinant of the interaction between the cAMP-binding domain and the catalytic region of Epac (21). This would imply that, in the open conformation, the LID is able to interact with the catalytic region, resulting in an inhibition of the activity (see Fig. 8). Here we show that the LID region of Epac1 indeed plays a pivotal role in the communication between the regulatory and catalytic domain of Epac. Most nota-

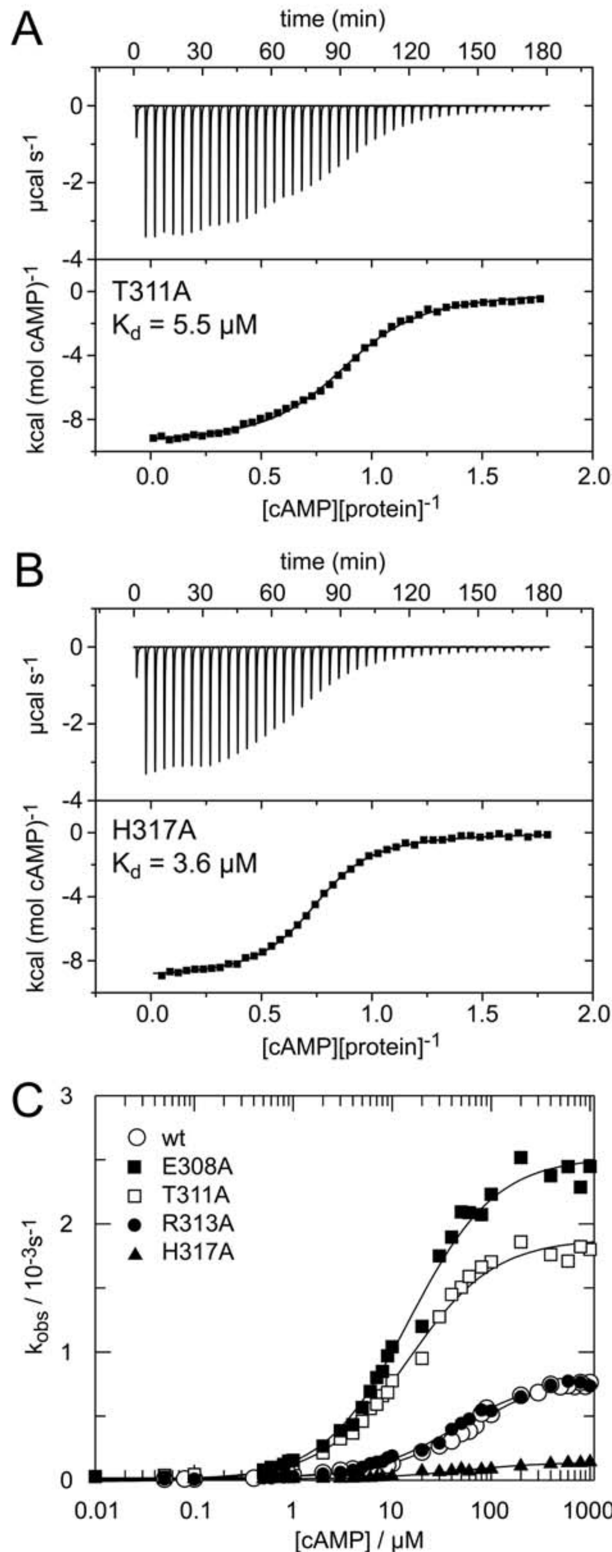

FIG. 6. **Mutations in the LID region change the activation properties of Epac.** *A* and *B*, the affinity of the isolated cAMP-binding domain for cAMP was analyzed by isothermal titration calorimetry. Representative measurements are shown for T311A (*A*) and H317A (*B*). The determined affinities are summarized in Table II. *C*, 200 nM RapmantGDP were incubated in the presence of excess GDP and different cAMP concentrations with 100 nM Epac and the indicated mutants, respectively. The reaction rates (k_{obs}) were determined by fitting analysis and plotted against the cAMP concentration. *wt*, wild type.

bly, deletion of the C-terminal region of the LID, containing a conserved VLVLE sequence, completely abolishes the ability of the regulatory domain to inhibit the exchange activity of Epac.

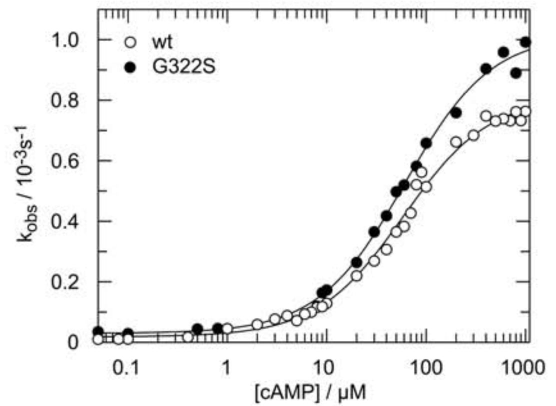

FIG. 7. **Characterization of the Single Nucleotide Polymorphism (SNP)G332S *in vitro*.** 100 nM Epac wild type (*wt*) and EpacG332S were analyzed in the fluorescence based GEF assay, respectively. The obtained k_{obs} values were plotted against the cAMP concentration.

This was shown in a *trans*-inhibition experiment in which an isolated cAMP-binding domain is capable of inhibiting an isolated catalytic region only when the region containing the VLVLE sequence is present. In addition, the Epac(Ala)$_5$ mutant, wherein the VLVLE sequence is replaced by alanine residues, is constitutively active, and cAMP cannot activate this mutant protein further. Thus, the VLVLE sequence is essential for inhibition of the catalytic region by the open cAMP-binding domain even when the two domains are separated. This strongly suggests that this region directly interacts with the catalytic region. Further analysis of the VLVLE sequence revealed that mutating individual residues into alanine had modifying effects on cAMP-induced activation. This is reflected by either a change in the AC_{50} for cAMP and/or a change in the maximal activity. Apparently, the sequence requirement for the VLVLE sequence to inhibit the catalytic domain is not very strict. Nevertheless the sequence is highly conserved. This may indicate that the small changes we observe can nevertheless not be tolerated *in vivo* or imply that the VLVLE sequence, in addition to inhibiting the regulatory domain, serves an additional function. Indeed, single amino acid changes did affect cAMP-induced regulation. This is reflected by either a change in the AC_{50} for cAMP and/or a change in k_{max}. Most surprisingly, mutating glutamate into the positively charged arginine resulted in a 2–3-fold higher k_{max} of Epac. From these results, we conclude that the VLVLE sequence also serves as an element that is responsible for translating cAMP binding in a correct activation response. Most likely, the VLVLE sequence is conserved to serve both in the inhibition of the catalytic region and the establishment of the correct conformational response to cAMP.

We have also identified conserved residues in the N-terminal part of the LID region that are involved in the regulation mechanism. From their positioning compared with PKA, we assumed that these residues might be involved in the interaction with the base of cAMP. Indeed, the affinity of cAMP for isolated cAMP domains of the E308A, T311A, and R313A mutants is reduced ~2-fold. This difference is relatively minor and suggests that the core structure and the phosphate-binding cassette mainly provide the affinity of cAMP for Epac. As for the single mutations in the VLVLE sequence, mutants with mutations in the N-terminal LID region also affect the AC_{50} of cAMP to activate Epac and the maximal activation at saturating cAMP levels. The H317A mutant is still responsive to cAMP with a wild type AC_{50} value but a k_{max} of only one-fifth of wild type. This indicates that H317 plays a key role in releasing Epac from auto-inhibition, perhaps by sensing the correct base

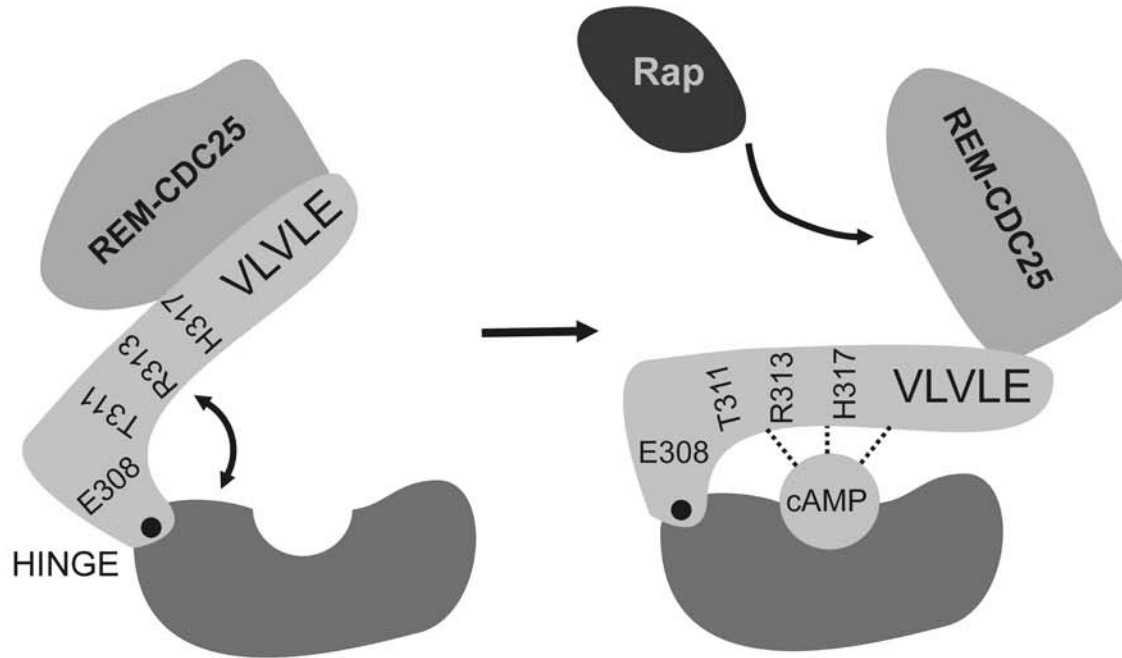

FIG. 8. **Model of the regulation of Epac by cAMP.** Schematic model for the involvement of the LID region and its VLVLE sequence in mediating the communication between the regulatory and catalytic regions of Epac. The LID region, in particular the VLVLE motif, is interacting with the catalytic region of Epac in the absence of cAMP. This prevents the interaction between Epac and Rap. Upon binding of cAMP, a conformational change is induced that allows the C-terminal helix at the hinge to move closer to the core structure of the cAMP-binding domain. This movement is, in turn, responsible for the flip of the LID region, including placement of the VLVLE motif over the cAMP-binding pocket. This conformation is stabilized by the interaction of the LID with cAMP.

structure of cAMP. A similar interaction is found between cAMP and tryptophane or a tyrosine residue in the LID of PKA (19, 20). In contrast, E308A and T311A show a reduced AC_{50} value and a higher k_{max}. Apparently, these mutants make Epac more active.

The observation that certain LID mutants have a higher or lower k_{max} than wild type is very intriguing and reminiscent of our findings that certain cAMP analogues also show either reduced (22) or increased maximal activity.[2] This indicates that the LID region in the presence of cAMP still influences the catalytic region and precludes a simple model as for PKA, wherein the activation of the catalytic region is caused by the dissociation of the regulatory region. For Epac, the interaction between the regulatory region and the catalytic region apparently remains after the binding of cAMP and imposes a restraint on the activity of Epac. One possible reason for this continuing restraint in the presence of cAMP is that Epac has an additional level of regulation that can modulate the effect of cAMP. This additional level of regulation may impinge on Epac through the binding of regulators to, for instance, the DEP domain, affecting the orientation of the LID region.

This explanation for the various levels of maximal activity, which is consistent with the mutational data and thermodynamic considerations, can be put forward by considering the four-state model of Epac activation (Fig. 9) proposed earlier (22). Regulation of Epac is thus described by a system of coupled equilibria between a bound and an unbound state and between an inactive and an active state. Whereas ligand-free Epac exists mostly (but not exclusively) in the inactive conformation, the binding of cAMP to the cAMP-binding domain shifts the equilibrium more (but not totally) to the active conformation. One should note that the AC_{50} values measured here reflect the overall equilibrium of cAMP binding and activation, whereas k_{max} reflects the equilibrium between the

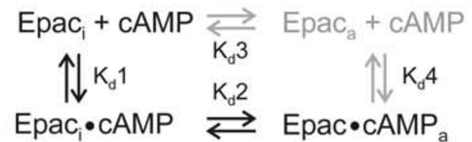

FIG. 9. **Coupled equilibria for Epac activation.** Epac exists in four states, *i.e.* in cAMP-bound and-unbound states as well as active (*subscript a*) and inactive (*subscript i*) states. The occupancy of the cAMP-free but -active state (*colored in gray*) is very low for Epac wild type and all analyzed mutations, except for $Epac(Ala)_5$. For wild type Epac, the equilibrium K_{d2} is not completely on the right side.

cAMP-bound inactive and active conformation The VLVLE-$(Ala)_5$ mutation would thus favor the active conformation even in the absence of ligand, described by K_{d3}. Other mutations would influence the conformational equilibrium K_{d2} between cAMP-bound inactive and active conformation. The fact that Epac(VLVLR), Epac(E308A), and Epac(T311A) have a higher maximal activity than wild type protein would indicate, keeping with the same model, that cAMP does not induce maximal activity even in the wild type protein and that Epac can therefore exist in a cAMP-bound but inactive conformation. The fact that maximal activity can be increased 2–3-fold indicates that the equilibrium described by K_{d2} is close to or even a bit lower than unity and that a low energy barrier for the conformational change (described by K_{d2}) might be advantageous for cAMP signaling or will be influenced by other cellular components. Preliminary NMR shows that, in the absence and presence of cAMP, there is indeed a fast dynamic inter-conversion between two conformations, which we assume corresponds to the active and inactive conformations.[3]

At this moment, the residues in the catalytic region in contact with the LID are elusive. It could be that the LID, in particular the VLVLE sequence, interacts with the CDC25 homology domain, thereby preventing binding of Rap (Fig. 8).

[2] H. Rehmann, unpublished observations.

[3] A. Shimada, unpublished observations

Alternatively, the LID region may induce a conformational change in the catalytic region, for instance through an interaction with the REM domain. Indeed, recently structural and biochemical evidences were presented that the REM domain could accelerate the intrinsic activity of the CDC25 homology domain of the Ras GEF Sos (24).

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Structure and regulation of the cAMP-binding domains of Epac2

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Structure and regulation of the cAMP-binding domains of Epac2

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Cyclic adenosine monophosphate (cAMP) is a universal second messenger that, in eukaryotes, was believed to act only on cAMP-dependent protein kinase A (PKA) and cyclic nucleotide-regulated ion channels. Recently, guanine nucleotide exchange factors specific for the small GTP-binding proteins Rap1 and Rap2 (Epacs) were described, which are also activated directly by cAMP. Here, we have determined the three-dimensional structure of the regulatory domain of Epac2, which consists of two cyclic nucleotide monophosphate (cNMP)-binding domains and one DEP (Dishevelled, Egl, Pleckstrin) domain. This is the first structure of a cNMP-binding domain in the absence of ligand, and comparison with previous structures, sequence alignment and biochemical experiments allow us to delineate a mechanism for cyclic nucleotide-mediated conformational change and activation that is most likely conserved for all cNMP-regulated proteins. We identify a hinge region that couples cAMP binding to a conformational change of the C-terminal regions. Mutations in the hinge of Epac can uncouple cAMP binding from its exchange activity.

Rap proteins are small guanine nucleotide–binding proteins closely related to Ras. They are reported to regulate a variety of cellular processes, most notably inside-out regulation of integrin-mediated cell adhesion (reviewed in ref. 1). Rap proteins are molecular switches cycling between a GDP-bound inactive and a GTP-bound active state. Activation of Rap is regulated by guanine nucleotide exchange factors (GEFs), which increase nucleotide dissociation by several orders of magnitude. GTPase activating proteins (GAPs) increase the hydrolysis of GTP and, thus, contribute to inactivation. Raps are regulated by a variety of extracellular stimuli, including growth factors, cytokines and cell adhesion molecules, some of which induce second messengers such as cyclic adenosine monophosphate (cAMP), classically known as the activator of cAMP-dependent protein kinase A (PKA)[2] and cyclic nucleotide-regulated ion channels[3], calcium and diacylglycerol (DAG). These second messengers act *via* several Rap-specific GEFs[1], including the Epac (exchange protein directly activated by cAMP)[4,5] family members Epac1 and 2, which contain a C-terminal catalytic region responsible for nucleotide exchange and an N-terminal inhibitory regulatory region (RR). RR of Epac1 consists of a DEP (Dishevelled, Egl, Pleckstrin) domain, which is responsible for membrane localization, and one cyclic nucleotide monophosphate (cNMP)-binding domain (Fig.1*a*), whereas Epac2 (refs. 6,7) has a DEP domain sandwiched between two cNMP-binding domains, cNMP-A and -B (Fig. 1*a*).

To gain molecular insights into the cAMP-regulated exchange activity, we have determined the 2.5 Å resolution crystal structure of the regulatory region of Epac2 (residues 1–463) in the absence of cAMP (Fig. 1*b*–*d*). Because the region 168–179 (dashed lines, Fig. 1*c*) connecting the cNMP-A and DEP domain is not visible in the electron density, it would be possible, in principle, to define two locations of the cNMP-binding domains relative to each other (arrangements 1 and 2, Fig. 1*e*). However, because the 12 missing residues would have to span 46 Å in arrangement 2 and only 18 Å in arrangement 1, barring any steric clash, arrangement 1 remains the only possibility. It shows a face-to-face organization of the cNMP-binding domains and buries a surface area of 2,200 Å^2. Assuming a similar binding mode for cAMP as in previous structures (see below), the binding sites for the cyclic nucleotide (yellow spheres, Fig. 1*c*) face each other, whereas the DEP domain is positioned away from the cAMP-binding domains.

The DEP domain

Membrane localization of Epac is mediated by the DEP domain[4], which consists of a core formed by three-helices (H1, H2 and H3), a β-hairpin arm between H1 and H2, and two short β-strands following H3. Superimposition (Fig. 2) of the DEP domains from Epac2 with that of Dishevelled (mDvl1) determined by NMR[8] reveals that although H2 and H3 can be superimposed easily (r.m.s. deviation <2.0 Å), H1 and the β-hairpin adopt different positions (r.m.s. deviation >3.2 Å). The long extra helix H4 (Fig. 1*c*), which connects the DEP domain to the second cNMP-binding domain, is not present in mDvl1. Although no binding partner, protein or otherwise, for DEP has been identified, Wong *et al.*[8] suggest that recognition and interaction of mDvl1 with as yet unknown proteins is mediated by a dipole formed by Lys434 (in the β-hairpin region of mDvl1) and Asp445 and Asp448 (in H2 of mDvl1). Interestingly, this dipole is also conserved in Epac2, as are additional residues of the β-hairpin (Fig. 2).

A ligand-free cNMP-binding domain

The ligand-free cNMP-A and -B domains of Epac2 feature a

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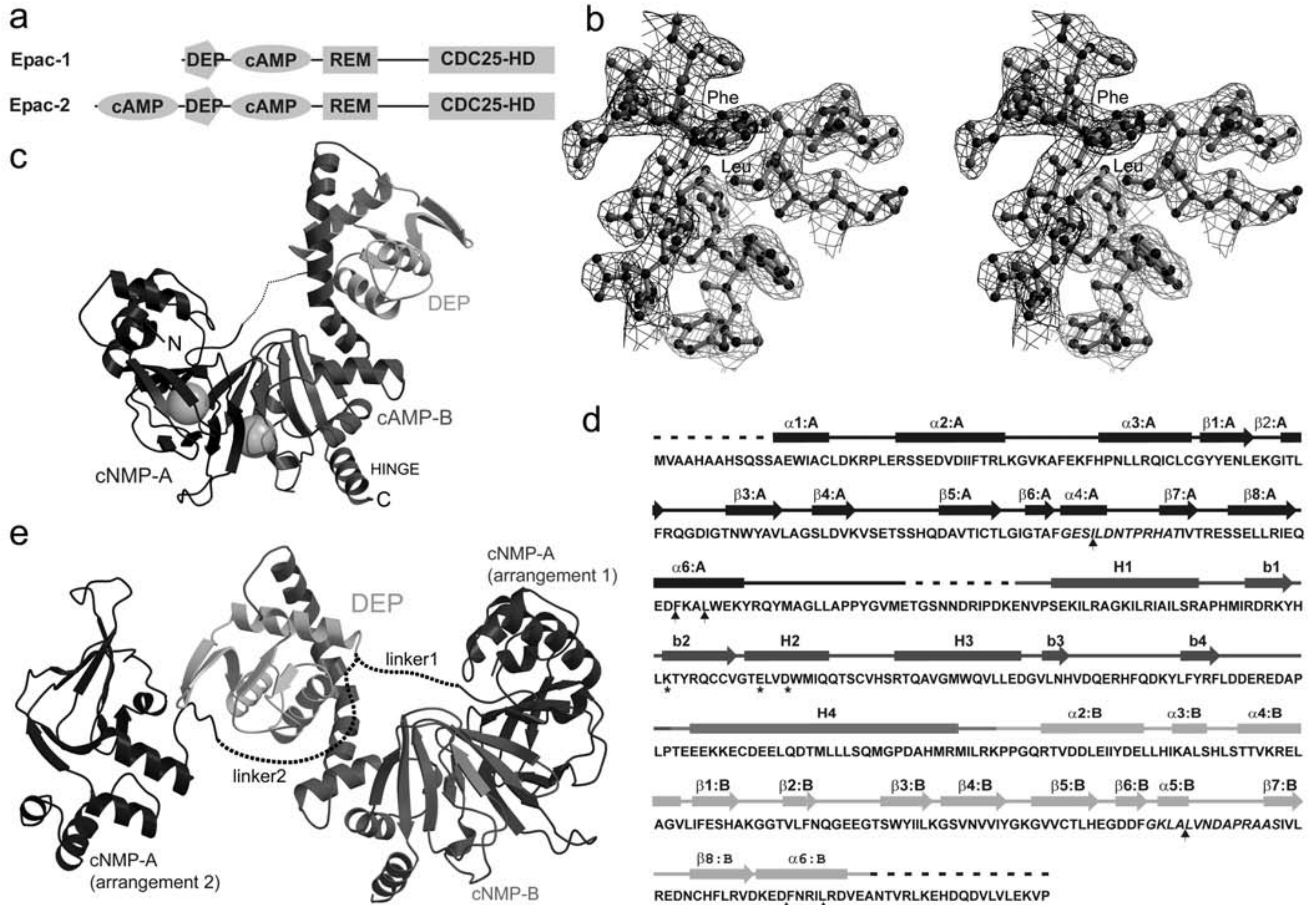

Fig. 1 Structure of Epac. ***a***, Domain organization of Epac1 and 2. Indicated color code is used throughout the figure. ***b***, Stereo view of a $2F_o - F_c$ composite omitted electron density map (contoured at 1.5 σ). The hydrophobic environment of Leu408 and Phe435 is shown. Different parts of the peptide chain are highlighted by individual colors. ***c***, Ribbon diagram of the regulatory domain of Epac2 with N and C termini as indicated. The C-terminal extra helix of the DEP domain is dark green. ***d***, Amino acid sequence, with secondary structure annotation. The phosphate-binding cassette (PBC) is indicated in red letters. Dashed lines indicate portions of the polypeptide chain not visible in the electron density. ***e***, The two possible arrangements (1 and 2) for the first cNMP-binding domain relative to the second (see text). Arrangement 1 corresponds to (*c*). Dotted lines, linker 1 and 2, indicate the minimal path of the polypeptide chain required to bridge the gap in both arrangements.

core structure similar to PKA[9,10] and the catabolite activator protein (CAP)[11]: an eight-stranded jellyroll β-barrel with one internal short α-helix. The core structure is flanked by one C-terminal and three N-terminal α-helices. The structure of the cNMP-binding domains of the regulatory subunit of PKA[9,10] and CAP[11] have been determined previously in the presence of cAMP. Superimposition of the ligand-bound cNMP-binding domains with ligand-free Epac domains (Fig. 3*a*) shows that the core structure defined by the β-sheets is similar, with an averaged r.m.s. deviation of 0.8 Å (excluding the phosphate-binding cassette (PBC) loop). This and the sequence conservation of relevant residues (see below) strongly argue for a conserved ligand-binding mode (Fig. 1*c*) and suggest that the observed differences in structure outside the core (shaded areas, Fig. 3*a*) are indicative of a ligand-induced conformational change.

The common feature of Epac 1 and Epac 2 is a catalytic domain preceded by a cNMP domain, which is responsible for cAMP-mediated activation. Deletion of the additional cNMP-binding domain (cNMP-A) of Epac2 does not appreciably modify its regulation by cAMP[6]. The relative importance of the second cNMP domain for regulation is supported by the affinities of the isolated cNMP-A and cNMP-B domains for cAMP, which are 70 μM and 1 μM, respectively[6]. The two domains interact face to face along the binding pockets with extensive contacts, an orientation that is incompatible with nucleotide binding because it would result in clashes of either the two adjacent cAMPs or of cAMP with the neighboring domain. Thus, large conformational changes upon ligand binding have to be assumed, as supported by our observations that crystals crack upon soaking with cAMP, the drastic reduction in solubility of Epac-ΔDEP upon cAMP addition[12] and an improved stability at 30 °C of the single cNMP-binding domain (from two days in the absence of cAMP to several weeks in the presence of cAMP).

In ligand-bound structures of PKA and CAP, the cAMP-binding site is closed by an α-helix that stabilizes the base and has been called the 'lid'[10]. A nonconserved base-binding region (BBR) also contacts the base of cAMP (Fig. 3*a*). Although striking differences in the lid architecture and its position have been found between the different classes of cAMP-regulated proteins and even between subclasses of PKA[10], a C-terminal helix or a helix from another cAMP domain always shields the binding site from solvent. Such a lid is not observed in the cNMP-binding domains of Epac2, presumably because of the different orientation of the C-terminal helix (see below) and because the 19 residues following helix α6B are invisible in the structure and, thus, presumably flexible. Biochemical studies have shown that

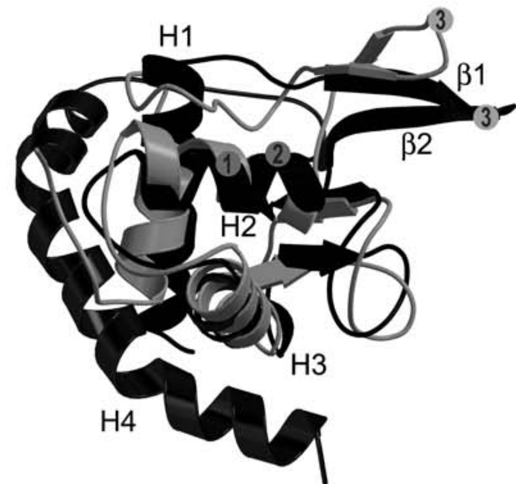

Fig. 2 Ribbon diagram of the DEP domains of Epac and Dishevelled. The DEP domains of Epac (yellow and green) and Dishevelled (gray) are superimposed on each other. Secondary structure elements are labeled as in Fig. 1c. N and C termini, as well as the position of the residues forming the dipole, are indicated as follows: '1' corresponds to Asp225[Epac] and Glu448[Dvl1]; '2' to Glu222[Epac] and Asp445[Dvl1]; and '3' to Lys212[Epac] and Lys434[Dvl1].

the lid is involved in mediating ligand-induced biological effects, such as release of the catalytic subunit of PKA[13] (reviewed in ref. 14), DNA binding of CAP[15], gating of cNMP-activated ion channels[16] or regulation of Epac activity (H.R., unpub. observations). The C-terminal helix of PKA has also been proposed to be responsible for the cooperative binding behavior and the communication between the cNMP-binding domains[17,18]. These data not only indicate the common features prevailing in the cAMP-binding domains, but also clearly highlight the importance of the lid region in regulation.

The cAMP-induced conformational change

The cAMP-binding pocket contains a highly conserved signature motif GELAL$(X)_{3-5}$PR(A/T)A(T/S) (Figs. 1*c*, 3*e*) termed the PBC[10,19], which provides interactions to the phosphate and ribose moieties of cAMP (Fig. 3*b*). The absence of nucleotide in the cNMP domains of Epac2 leads to significant changes around the PBC. The major differences between Epac and PKA are that the glutamic acid of the PBC is not conserved in the Epacs and that the corresponding residue is not required for binding cAMP (H.R., unpub. observations). Because the glutamic acid is necessary for tight binding in PKA, its absence may be responsible for the lower affinity of cAMP binding in Epac. Structural overlay (Fig. 3*b*) shows that the PBC around the PRAAS/T motif is already in position to interact with cAMP. Assuming a conserved binding mode for Epac, we would expect a small conformational change of the glycine (GELAL) to accept a hydrogen bond from the 2′ hydroxy group of the ribose. A much larger conformational change would have to be postulated for the alanine (GELAL) to donate a hydrogen bond to one phosphate oxygen, resulting in a local rearrangement of the neighboring residues (Figs. 1*b*, 3*a–c*).

In Epac, the latter movement would affect the position of Leu408, which in cNMP-B (Fig. 3*c*) forms a hydrophobic pocket with several residues from a region between the core and a region that we propose to call the 'hinge' (Fig. 3*a*,*c*). The corresponding region in PKA was identified by ϕ and ψ angle analysis as a hinge point for the variability of the different domain orientation in PKAIa and PKAIIb[10]. Both Leu408 and Phe435 from the hinge region of Epac are invariant in all cNMP-binding domains. In the ligand-bound state, the corresponding Leu353 in PKA (PKAIIβ-B, the second cNMP-binding domain of PKAIIβ) adopts a different position and stabilizes the contacts of the phosphate with the PBC. This puts Phe379 of PKA into the position that is occupied by Leu in the unbound state. We would envision that binding of cAMP attracts the PBC, reorients the invariant leucine and produces a large movement of the hinge and regions C terminal to it, including the lid, toward the nucleotide (Fig. 3*c*). In the unbound state seen in Epac2, the hinge movement is blocked because Phe435 would clash into Leu408. Thus, binding of cAMP would initiate a series of events in which the phosphate sugar moiety binds to the correctly oriented PRAAT motif in the first step, and a second step induces a conformational change in the FGELAL region for further contacts. This, in turn, allows the hinge to move toward the β-barrel and induces a rearrangement of the lid to stabilize the base-binding site. A similar binding sequence was suggested for the Ras proteins in which the binding of the base preceded the binding of the sugar and the phosphates[20]. Together, these data indicate that a small conformational change in the PBC region could be transmitted *via* the hinge into a large structural change of the C-terminal lid, similar to the lever arm movements found in motor proteins.

Biochemical evidence for the proposed mechanism

The proposed model of regulation is supported by the findings on the R_p-isomer of adenosine-3′, 5′-cyclic monophosphorothioate (R_p-cAMPS), which binds but does not activate PKA[21] or Epac (H.R. unpub. observations). One phosphate-oxygen of cAMP (marked 1, Fig. 3*b*) forms a hydrogen bond with the backbone and side chain NH of the invariant Gly and Arg residues, respectively, of the PBC. A sulfur at this position would alter the interactions, preventing the PBC from adopting its canonical conformation. In the PKA inhibitor R_p-cAMPN$(CH_3)_2$, the bulky dimethylamide group replaces O2 and would similarly prevent the movement of the PBC.

Our model may also explain the properties of the cNMP-A domain of Epac2, which shows a conformation more similar to the ligand-bound structure (Fig. 3*a*,*d*). It is shorter by one residue, resulting in a tighter PBC than observed in cNMP-B or the cNMP-binding domains of PKA (Fig. 3*d*,*e*). From the structural overlay, the missing residue corresponds to either alanine or the invariant leucine. The space for the phenylalanine from the hinge is therefore not blocked by the PBC, and the hinge is not prevented from adopting the active conformation. Because domain A has a glutamic acid but its cAMP affinity is low and the corresponding residues in Epac1 or domain B of Epac2 do not contribute significantly to cAMP binding (H.R., unpub. data), we conclude that the structural differences are not due to the absence or presence of glutamic acid in PBC. The different architecture of the PBC, together with its noncanonical sequence, is most likely the reason for the low affinity of the cNMP-A domain, which is unlikely to participate in Rap activation.

Because the invariant Leu from the PBC and the highly conserved Phe from the hinge mediate the conformational change of the hinge and the lid, we questioned whether mutating these residues would modify the cAMP-mediated conformational change and induction of GEF activity. To simplify the biochemical analysis and because deletion of the first domain in Epac2 does not greatly modify cAMP-induced activation, we chose Epac1, which has highly homologous PBC and hinge regions (Fig. 3*e*) but contains only a single cNMP-binding domain (Fig. 1*a*). We argued that replacing the invariant leucine (Leu273

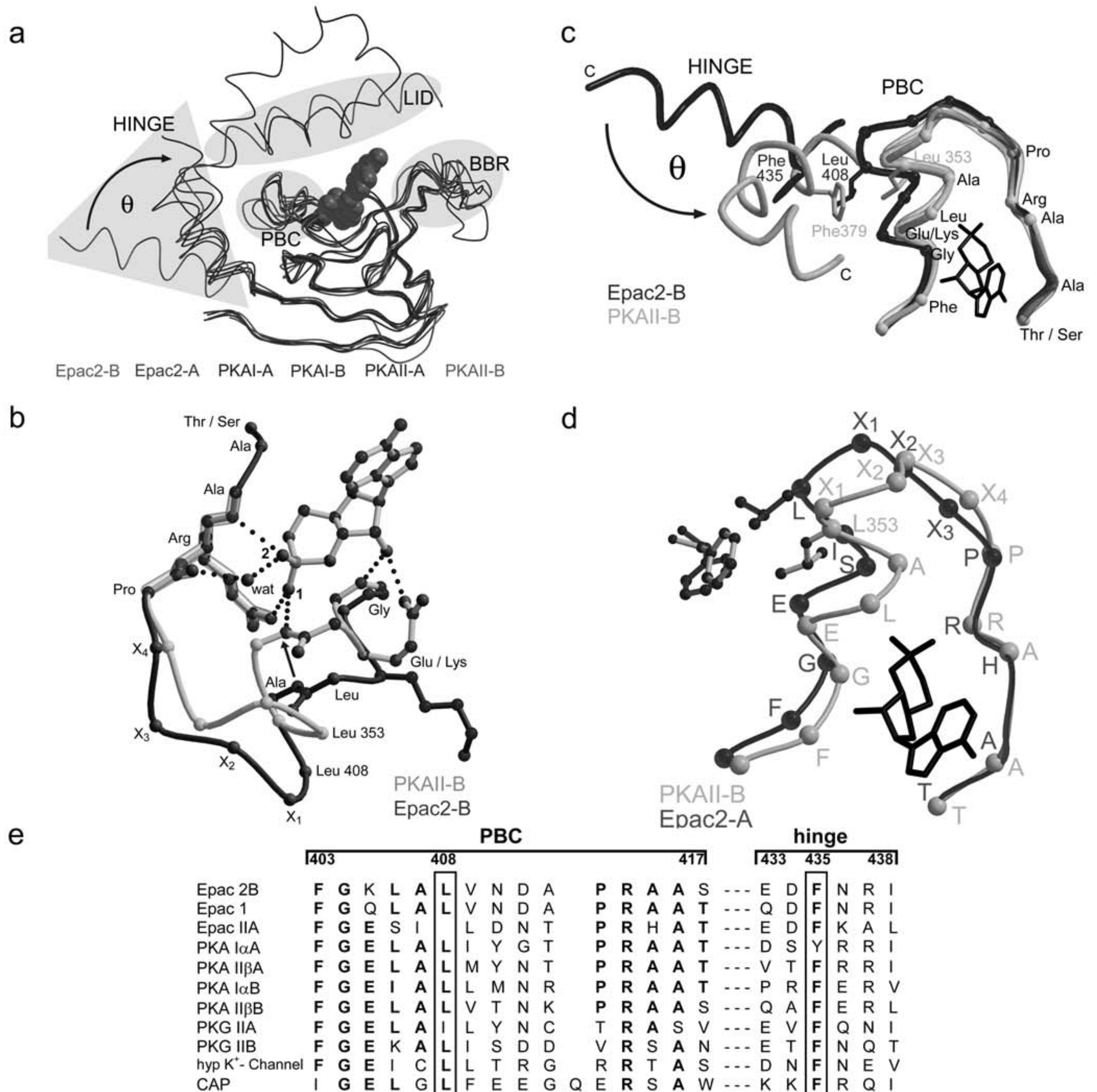

Fig. 3 Comparison of cAMP-binding domains of Epac (cAMP-free) and PKAs (cAMP-bound). ***a***, Superimposition of cNMP domains with the color coding as depicted. The four elements of cAMP binding and conformational change, PBC, BBR, hinge and lid, are shown within the gray background. The arrow indicates the proposed conformational change of the hinge by an angle θ. ***b***, Detailed view of the PBC with cAMP and interacting residues in ball and stick representation. Hydrogen bonds are indicated by dotted lines. The chiral oxygens of the phosphate are marked 1 and 2 (see text). The residues of the PBC are labeled according to the consensus sequence GELAL(X)$_{3-5}$PR(A/T)A(T/S). ***c***, View of the PBC and the hinge, with the invariant Phe and Leu residues considered to be crucial for the conformational change. The PBCs of PKAIIβ-A, PKAIα-A and PKAIα-B are represented in gray. ***d***, The conformation of the PBC from the cNMP-A domain of Epac2 in comparison with PKA. ***e***, Structure-based sequence alignment of the PBC and the hinge region (around Phe435) from Epac2 and from various cNMP binding domains. Highly conserved residues are shown in bold.

in Epac1 and Leu408 in Epac2) with a bulky tryptophan might stabilize the open conformation and prevent the phenylalanine from swinging inward. Indeed, the GEF activity of the L273W mutant of Epac1 cannot be activated with 500 μM cAMP (Fig. 4*a*). Because this could be the result of the inability to bind cAMP, we determined the binding affinity of the mutant by isothermal titration calorimetry (ITC). Although the K_d of the mutant (20 μM) is five-fold reduced compared with wild type[6,12], the binding site is saturated under the conditions of the activation experiment. In contrast to wild type protein, the affinity of the mutant full-length protein is similar to that of the isolated domain (Fig. 4*b*,*c*), and the protein does not precipitate upon the addition of cAMP[12]. These results indicate that, in the mutant, cAMP binding and conformational change are uncoupled from each other.

The F300A and F200T mutations of the highly conserved Phe300 (Phe435 in Epac2) in the hinge of Epac1 has an opposite effect on the activation process. Both mutations destabilize the ligand-free conformation, as determined by the concentration of cAMP necessary to achieve half-maximal activity; it decreased

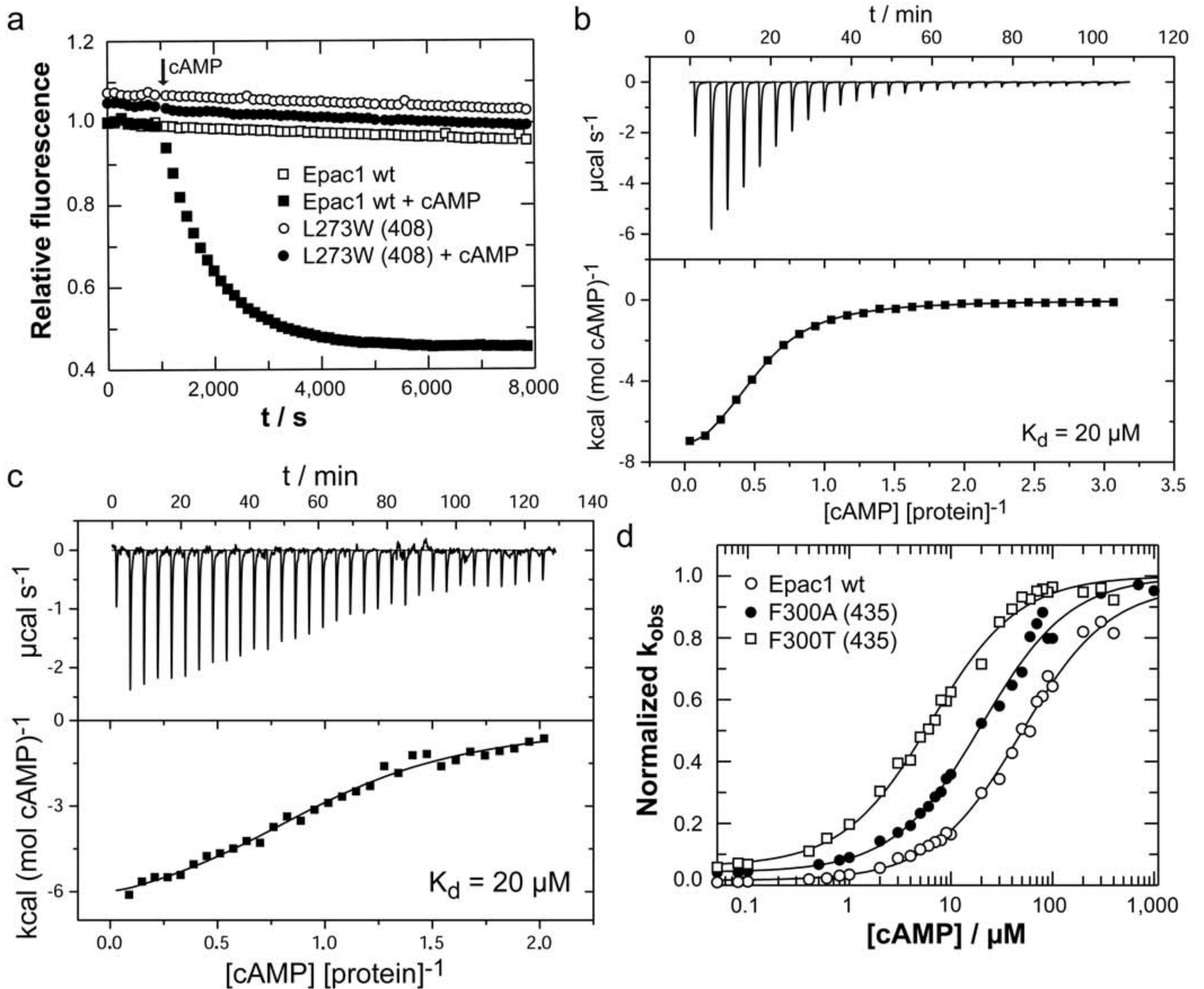

Fig. 4 Biochemical analysis of the regulation mechanism of Epac. ***a***, Exchange activity is measured in a fluorescence Rap–GEF assay by using the fluorescent analog methylanthraniloyl-GDP (mant-GDP) as described earlier[4]. The residue numbering is according to Epac1, and the numbers in parentheses indicate the numbers corresponding to Epac2. No GEF activity is seen for L273W Epac1 even in the presence of cAMP. The arrow indicates the time point at which cAMP was added. ITC measurements of the cAMP affinity for the L273W mutant in either ***b***, the isolated cNMP-binding domain or ***c***, the full-length protein (see Methods). ***d***, Dependence of Rap–GEF activity on cAMP concentrations for either wild type (wt) or mutant Epac1. Half-maximal activities are 46 µM, 20 µM and 6 µM for wt, F300A and F300T, respectively. (Methods are as in Kramer *et al.*[12], but half-maximal activity is 10× higher than reported.)

from 50 µM for wild type to 20 µM and 6 µM for the F300A and F300T mutants, respectively (Fig. 4*d*). These experiments reveal the significance of Leu273 and Phe300 of Epac1 (Leu408 and Phe435 of Epac2) for the cAMP-induced conformational change.

Conclusion

We have shown[4,6] earlier that the regulatory region of Epac binds to the catalytic domain, thereby masking the interaction with the GTP-binding protein Rap1. cAMP binding relieves this interaction. In accordance with the arguments presented here, we would propose that cAMP binding induces a change in the orientation of the PBC, which modifies the position of the invariant leucine and makes way for the highly conserved phenylalanine to flip into the vacant place. This, in turn, induces a movement of the hinge and lid and liberates the catalytic domain of Epac from intrasteric inhibition. Epac exists in a ligand-free and ligand-bound form and in an inactive and active conformation. Activation and ligand binding can be uncoupled from each other, as shown by using cAMP analogs[12] or by mutating residues involved in the conformational change as shown here. In the light of sequence (Fig. 3*e*) and structure conservation, we propose that the mechanism for the conformational change involving the PBC, hinge and lid regions is most likely conserved between the cyclic nucleotide binding domains of Epacs, protein kinases and ion channels.

Methods

Protein preparation. The RR (residues 1–463) of mEpac2 (AccNo. AF115480 (EMBL)), the single cAMP-binding domain (residues 149–328) and the ΔDEP version (residues 149–881) of hEpac1 (AccNo. AF103905) were expressed as GST-fusions (pGEX4T2 (Pharmacia)) in *Escherichia coli* strain CK600K and purified as described[12]. For selenomethionine (SeMet) labeling, the protein was expressed in the *E. coli* strain DL41 growing in LeMaster medium containing 0.1 g l^{-1} SeMet. Mutations were gen-

Table 1 X-ray data collection and refinement statistics for the RR of mEpac2

	λ0	λ1	λ2	λ3
Data collection				
Wavelength (Å)	0.934	0.9792	0.9795	0.9735
Resolution range (Å)[1]	25–2.5 (2.7–2.5)	25–3.15 (3.22–3.15)	25–3.25 (3.33–3.25)	25–3.2 (3.27–3.20)
Number of reflections				
Total	151,369	329,431	230,682	239,822
Unique	23,875	23,624	21,498	22,372
Completeness (%)[1]	96.8 (92.9)	98.8 (98.9)	98.9 (98.8)	98.5 (99.1)
I / σ[1]	24.4 (7.8)	11.3 (2.2)	12.3 (2.2)	11.5 (2.1)
R_{sym} (%)[1]	6.7 (17)	11.0 (36.3)	9.1 (40.0)	10.8 (36.4)
Overall figure of merit (FOM) for MAD phasing of Epac				
Acentric	0.53			
Centric	0.55			
Refinement				
Resolution range (Å)	25–2.5			
Reflections	23,875			
R_{cryst} (%)	24.4			
R_{free} (%)[2]	27.8			
Average B-factor (Å^2)				
Protein	63.4			
Solvent	58.3			
R.m.s. deviation from ideal values				
Bond lengths (Å)	0.007			
Bond angles (°)	1.3			

[1]Values in parentheses correspond to highest resolution shell.
[2]The R_{free} was calculated with 5% of the data omitted from structure refinement.

erated by QuikChange mutagenesis according to the Stratagene procedure.

Crystallography. Crystals of the mEpac2 RR were grown at room temperature by the hanging drop method using a reservoir solution containing 25 mM HEPES, pH 7.5, 1 M phosphate (250 mM NaH_2PO_4 and 750 mM K_2HPO_4), 10 mM dithiothreitol (DTT) and a protein concentration of 40 g l^{-1}. The crystals diffract to 2.5 Å resolution and belong to the orthorhombic space group $P2_12_12_1$, with one molecule per asymmetric unit (solvent content of 66%). For data collection at 100 K, crystals were shock frozen in liquid nitrogen with a solution containing the mother liquor and 23% (v/v) glycerol. SeMet crystals were grown and treated similarly.

MAD data were collected to 3.2 Å at 100 K using the BM30 beamline at ESRF at three wavelengths (Table 1). A 2.5 Å data set with high redundancy, collected at beamline ID14-1 of ESRF, was used for the final refinement. The data sets were processed with DENZO, SCALEPACK[22] and/or XDS[23]. Of the ten selenium sites, eight were found by SOLVE[24] or SnB[25]. SHARP[26] was used to estimate experimental phases (αMAD) at 3.2 Å (Table 1). The electron density map obtained upon density modification with SOLOMON[27] was used for initial model building.

This model was subjected to iterative rounds of building and refinement. O[28] was used to build the model into the $2F_o - F_c$ and $F_o - F_c$ map, and refinement was carried out with CNS[29], usually consisting of bulk solvent correction, positional torsion angle simulated annealing and B-factor refinement. In each cycle, model bias was avoided by calculating a composite simulated annealing omit map, which was regularly used to verify, correct or build the model. The final model, which shows good stereochemistry (Table 1), consists of 421 amino acids and 78 water molecules. The high B-factors for the model (Table 1) are also reflected in the high Wilson B-factors (57.8 Å^2). Residues 1–7, 168–179 and 452–463 are not seen in the electron density because of conformational disorder (Fig. 1). The Ramachandran map depicts 98.6% of main chain torsion angles in the most favored and allowed regions, with no residues in disallowed regions. Figures were generated using MolScript[30] and Raster3D[31].

Biochemistry. The affinity of mutant ΔDep-Epac1 and the isolated cNMP-binding domain of cAMP were determined by ITC (MicroCal). In this procedure, 50–100 μM protein was thermostated in the cell at 25 °C, cAMP was injected stepwise, the resulting heat was measured and the data was analyzed using software provided by the manufacturer. GEF assays were performed as described[4] using 200 nM of Rap-mGDP, 100 nM Epac and various concentrations of cAMP. The decrease in fluorescence with time was analyzed, and the observed rate constants were plotted as a function of cAMP concentration.

Coordinates. Coordinates have been deposited in the Protein Data Bank (accession code 1O7F).

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Competing interests statement
The authors declare that they have no competing financial interests.

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5

Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac

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Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac

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Running title: Ligand interaction of Epac

Summary

Epac is a cAMP dependent exchange factor for the small GTP-binding protein Rap. The activity of Epac is inhibited by a direct interaction between the C-terminal helical part of the cAMP-binding domain, called the LID, and the catalytic region, which is released after binding of cAMP. Here we show that the activation properties are very sensitive to modifications of the cyclic nucleotide. Some analogues are inhibitory whereas others are stimulatory, some are characterised by a much higher activation potential than normal cAMP. Mutational analysis of Epac allows insights into a network of interactions between the cyclic nucleotides and Epac. Mutations in the LID region are able to amplify or to attenuate selectively the activation potency of cAMP analogues. The properties of cAMP analogues previously used for the activation of the cAMP responsive protein kinase A (PKA) and of 8-pCPT-2'-O-Me-cAMP, an analogue highly selective for activation of Epac were investigated in detail.

Introduction

Epac[1] (exchange protein directly activated by cAMP) is a cAMP-responsive guanine nucleotide exchange factor (GEF) for the small GTP-binding proteins Rap1 and Rap2 (1;2). Two isoforms of Epac, Epac1 and Epac2, were described in mammalian cells, both containing a regulatory and a catalytic region (Fig. 1). The catalytic region contains a CDC25 homology domain and a Ras exchange motif (REM) domain. Whereas the CDC25-homology domain alone is sufficient to mediate the catalytic activity (3;4), the REM domain of SOS has been shown to stabilise the CDC25-homology domain structurally without being directly involved in the interaction with Ras (5). More recently an activity-modulating function of the REM domain was suggested (6).

The regulatory domain of Epac contains a cAMP-binding domain and a DEP domain. The DEP domain mediates membrane localisation of Epac and is not involved in regulation of the exchange activity in vitro. The cAMP binding domain inhibits the catalytic activity in the absence of cAMP. The inhibition is released after binding of cAMP. An additional N-terminal cAMP binding domain is present in Epac2. This domain is not required to maintain the inactive state of Epac2 in the absence of cAMP (7).

Insight in the structural basis of the regulatory mechanism was obtained from the x-ray structure of the regulatory region of Epac2 containing both cAMP binding domains and the DEP domain. The structure was solved in the absence of cAMP and was the first structure of a cAMP binding domain in the ligand-free state (8). Based on a structural comparison with cAMP bound regulatory subunits of PKA (9;10) it was proposed that cAMP binding induces a conformational change in the phosphate binding cassette (PBC) (8). This conformational change allows a C-terminal helix containing a HINGE region and a LID region to bend towards the cAMP binding site and to cover cAMP[2] (8). Considering the high conservation of residues involved in the conformational change, the proposed model seems to account for the activation not only of Epac, but also of PKAs and cAMP-regulated ion channels.

When analysing the role of the LID region of Epac in more detail, we observed that a conserved VLVLE sequence in the C-terminal end of the LID region is mediating the inhibition of the catalytic region. Indeed, mutation of this region resulted in a constitutively active Epac. Surprisingly, single point mutations in the LID region either abolished cAMP induced activity almost completely or resulted in an activity even higher then that of the wt protein. This indicated that after binding of cAMP the LID region regulates the activation status of Epac.[2]

[1] The abbreviations used are: Epac, exchange protein directly activated vy cAMP; GEF, guanine nucleotide exchange factor; REM, Ras exchange motif; DEP, Dishevelled, Egal-10, Pleckstrin; PKA, protein kinase A; 8-Br-cAMP, 8- Bromoadenosine-3', 5'-cyclicmonophosphate; 2-Cl-cAMP, 2- Chloroadenosine- 3',5'-cyclicmonophosphate; 8-Cl-cAMP, 8- Chloroadenosine-3',5'-cyclicmonophosphate; 2' dcAMP, 2'-Deoxyadenosine-3',5'-cyclicmonophosphate; cIMP, Inosine-3',5'-cyclicmonophosphate; 8-MA-cAMP, 8- Methylaminoadenosine-3', 5'-cyclicmonophosphate; N^6-phenyl-cAMP, N^6-Phenyladenosine-3',5'-cyclicmonophosphate; 2'-O-Me-cAMP, 2'-O-Methyladenosine-3',5'-cyclicmonophosphate; 8-pCPT-cAMP, 8-(4-Chlorophenylthio)adenosine-3',5'-cyclicmonophosphate; 8-pCPT-2'-O-Me-cAMP, 8-(4- Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclicmonophosphate; 8-PIP-cAMP, 8- Piperidinoadenosine-3',5'-cyclicmonophosphate; Rp-cAMPS, Adenosine-3',5'-cyclicmonophosphorothioate, Rp-isomer; Sp-cAMPS, Adenosine-3',5'-cyclicmonophosphorothioate, Sp-isomer; cXMP, Xanthosine-3', 5'-cyclicmonophosphate

[2] Rehmann et al. JBC in press

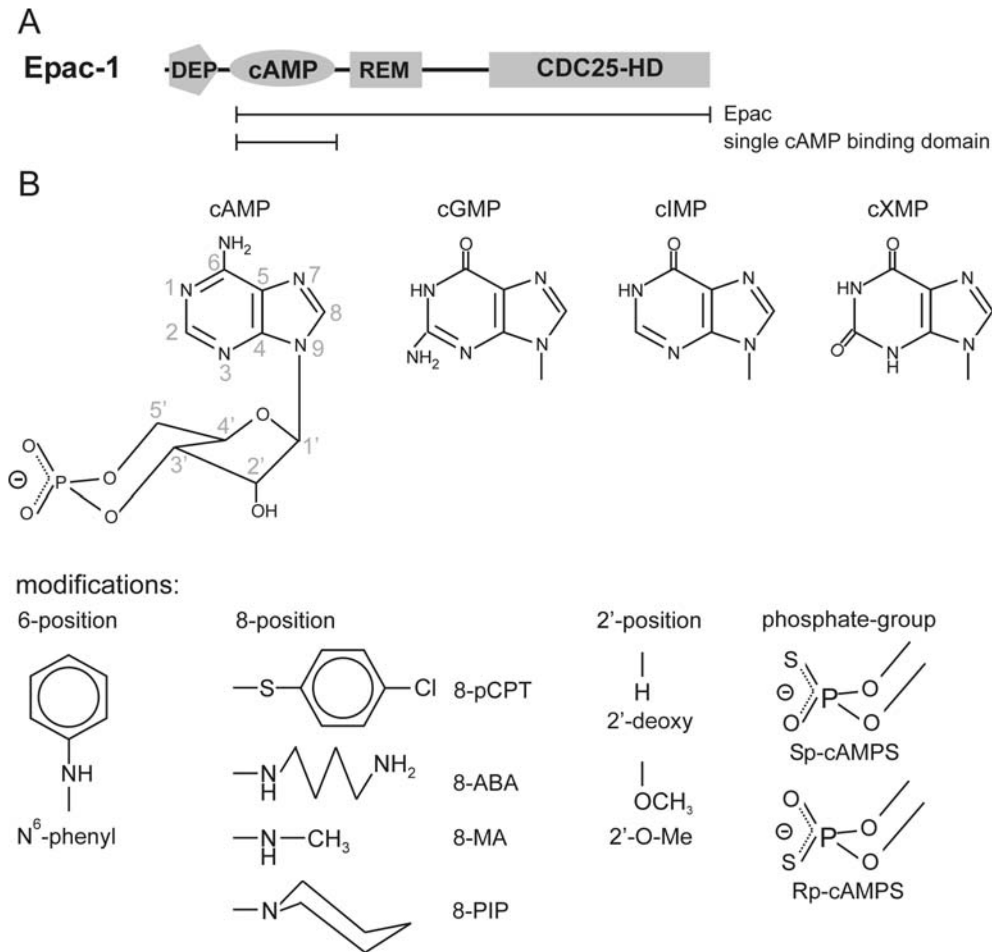


Fig. 1 Schematic representation of Epac1 and 2 and the structure of cyclic nucleotide analogues.
A. Indicated is the domain structure of Epac1 with a Dishevelled, Egl-10, Pleckstrin (DEP) domain, a cAMP binding domain (cAMP), a Ras exchange motif (REM) and a CDC25 homology domain (CDC25-HD). The deletion constructs used in this study are indicated. B. Chemical structures of the cyclic nucleotides and nucleotide analogues used in the present study, with numbering of the ring positions of the base and the ribose are indicated in the cAMP structure. All modifications are grouped according to the position of the substitutions.

Recently, we identified a novel cAMP analogue, 8-pCPT-2'-O-Me-cAMP, which binds and activates Epac efficiently both in vitro and in vivo. Most importantly, this compound was completely insensitive in activating PKA both in vitro and in vivo (11). Here we have characterised 8-pCPT-2'-O-Me-cAMP further and noted Epac that bound to this compound had a 3 fold higher v_{max} than Epac bound to cAMP. By testing other cAMP analogues and by mutational analysis we found that the 2'-OH atom of cAMP is responsible for the super- activation. We propose a model of how it communicates with the LID region.

Experimental Procedure

Preparation of proteins

All Epac constructs were expressed as GST-fusion proteins from the pGEX-4T2 vector in the *E.coli* strain CK600K as described previously (12). All constructs are human Epac1 and correspond to the following amino acids: Epac 149-881, $Epac_{149-328}$ 149-328. Mutations were introduced according to the Quickchange Procedure (Stratagene). The C-terminal truncated version of Rap1B was expressed and loaded with 2'(3')-O-(N-methylanthraniloyl) guanosine diphosphate (mantGDP) as described (13). We refer to this construct as Rap1B or simply Rap1 in the text.

Isothermal Titration Calorimetry

To analyse the binding of cAMP to the isolated cAMP-binding domain a Microcalorimeter (MicroCal Inc.) was used. For details see Rehmann et al.[2].

In vitro activation of Rap1

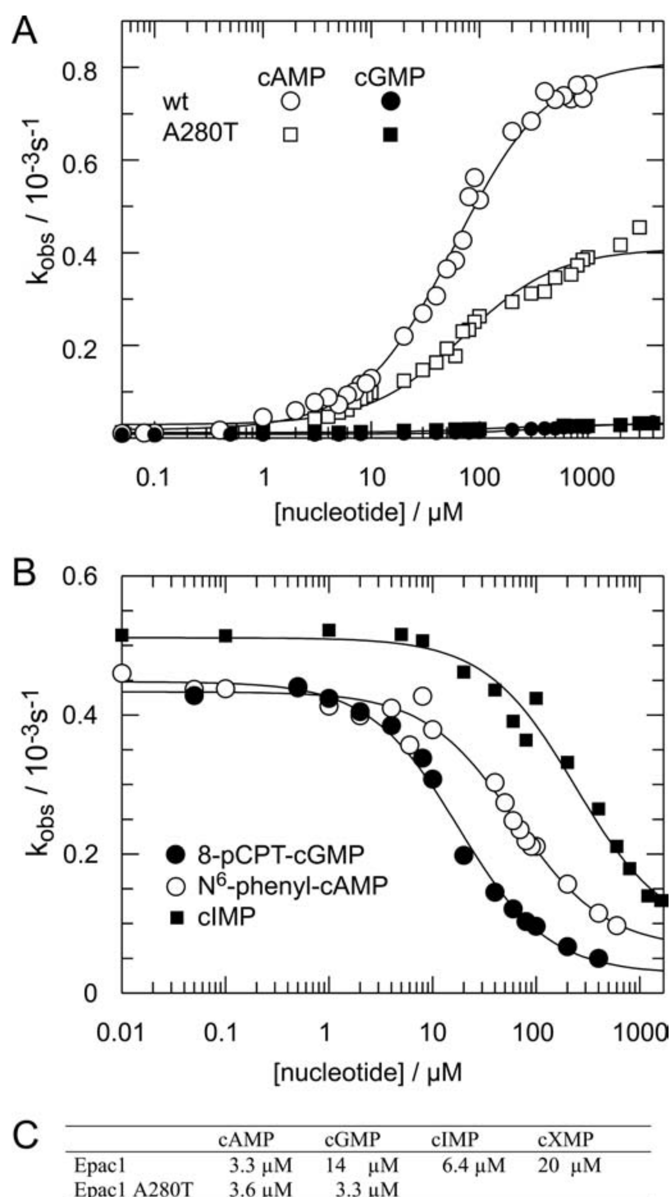
The experiments were performed as described earlier by Rehmann et al.[2]. Briefly, 200 nM of Rap1B loaded with the fluorescent GDP analogue mantGDP were incubated in the presence of 20 µM GDP (Sigma) and 100 nM Epac. cAMP (Sigma) and analogues thereof was added as indicated for the individual experiments. Cyclic nucleotides except camp itself were obtained from Biolog Life Science Institute (Bremen). The nucleotide exchange was measured in real time as a decay in fluorescence using a spectrofluorometer "spex1" (Spex Inc.). The origin of the decay is the release of the protein bound mGDP, that shows a higher fluorescence intensity in the hydrophobic environment of the protein the in the buffer solution. The obtained data were fitted to a single exponential decay and obtained rate constants (k_{obs}) were plotted against the cAMP concentration. The concentration dependency of the rate constants were treated as a normal titration experiment.

All data analysis, fitting and plotting were done with the Grafit 3.0 program (Erithacus Software).

Results

Interactions of Epac with various cNMPs

We have used the isolated cNMP-binding domain of Epac 1, Epac-$RD_{149-328}$, to determine the equilibrium dissociation constant K_d for the nucleotides cAMP, cGMP, cIMP and cXMP (Fig 1A) by isothermal titration calorimetry (ITC). Interestingly, although cAMP has the highest affinity (3.3µM) of the cyclic nucleotides tested, the affinities of cIMP (6.4µM), cGMP (14 µM) and cXMP (20µM) are of similar magnitude. Next we investigated whether these nucleotides are able to activate Epac using a fluorescence-based assay where the ability of Epac to act as a GEF for Rap1 is tested with increasing concentration of nucleotide (see experimental procedure and (13)). From this we derive the half-maximal concentration for activation, AC_{50}, and the maximal rate of exchange under saturating concentration of activator k_{max}. Whereas cAMP activated Epac with the expected AC_{50} of about 45 µM (Fig. 2A and (8)), neither cGMP (Fig. 2A) nor the other cyclic nucleotides (data not shown) were able to activate Epac significantly. To investigate whether the failure of cGMP, cIMP and cXMP to activate Epac is due to a failure to bind to full-length $Epac_{149-881}$ we measured whether these cNMPs were able to inhibit



C

	cAMP	cGMP	cIMP	cXMP
Epac1	3.3 µM	14 µM	6.4 µM	20 µM
Epac1 A280T	3.6 µM	3.3 µM		

Fig. 2 cIMP, cGMP and cXMP are inhibitors of Epac.
A. Rate constants for Epac-mediated GEF reactions were measured under standard conditions, using 200 nM Rap1•mantGDP in the presence of 100 nM wild-type or mutant Epac with excess GDP, at different concentration of cyclic nucleotide, from the rate of the decrease in fluorescence intensity (k_{obs}), as indicated in the Experimental Procedure. The k_{obs} values were plotted against the indicated cyclic nucleotide concentration. B. Inhibition of GEF activity was measured under standard conditions using a constant concentration of 60µM cAMP and the indicated concentrations of 8-pCPT-cAMP, N^6-phenyl-cAMP and cIMP, respectively. k_{obs} were determined from the single experiments and plotted against the concentration of cyclic nucleotide. C. The nucleotide affinity of the single cAMP binding domain ($EpacRD_{149-328}$) were determined by ITC as described in Experimental Procedure.

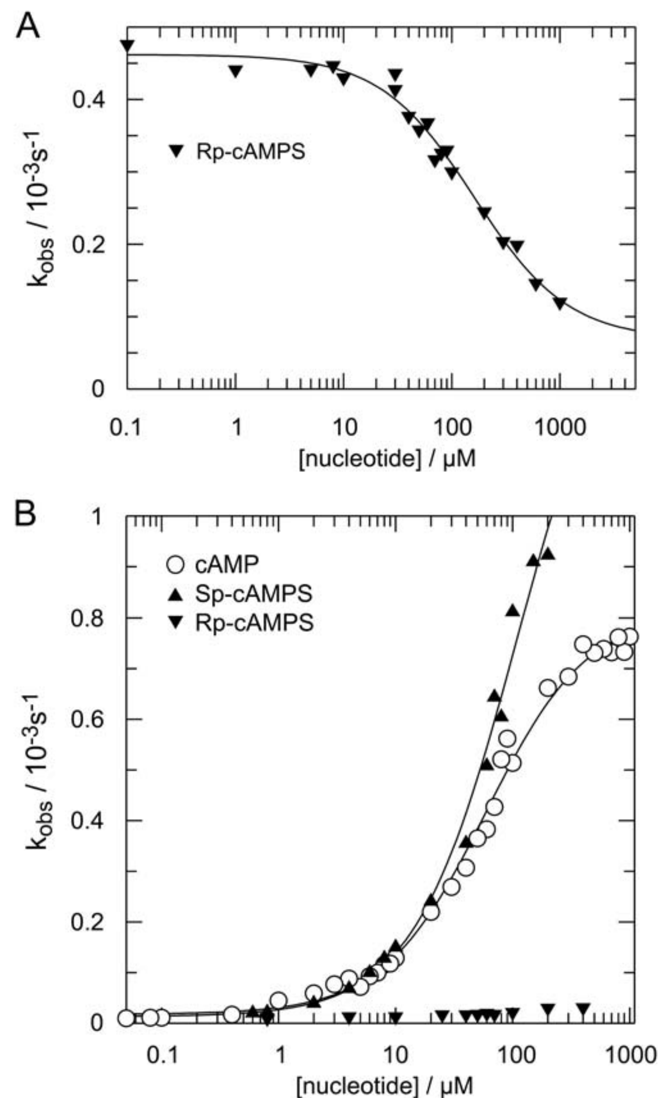


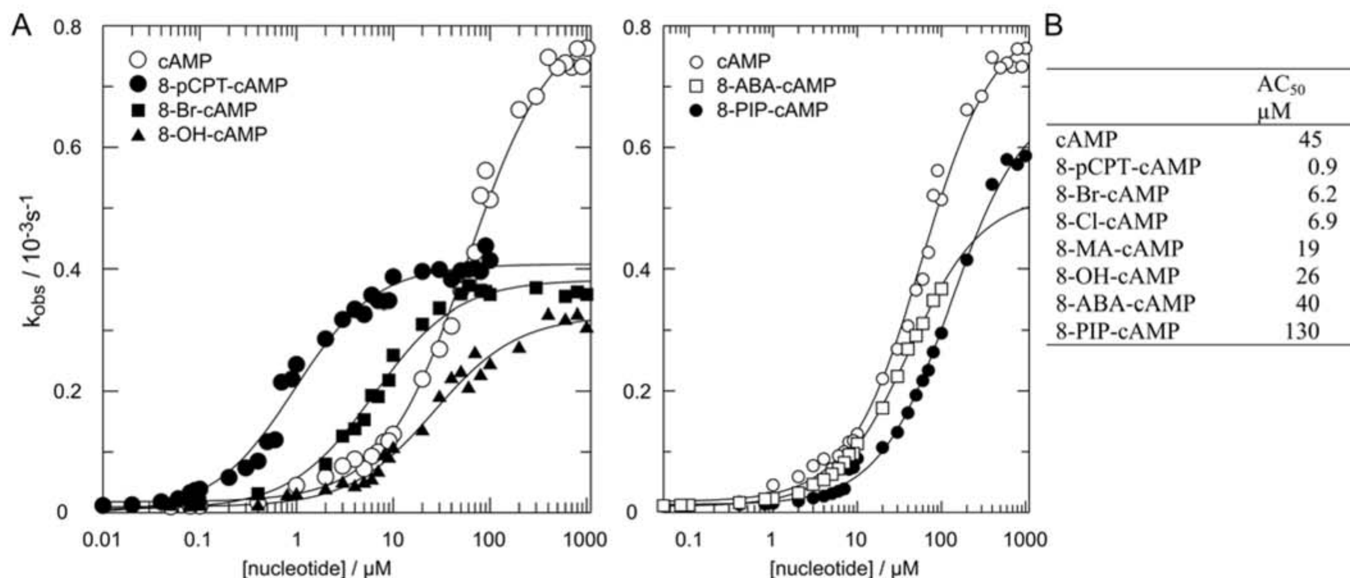
Fig. 3 Rp-cAMPS acts as an inhibitor of Epac.
A. The inhibitory properties of Rp-cAMPS were analysed as described in Fig. 2B. B. The dependency of the exchange activity on the sulphur substituted analogues Rp-cAMPS and Sp-cAMPS was analysed as described in Fig. 2A

the cAMP induced activation. As shown in Fig. 2B cIMP indeed inhibited cAMP induced activation of Epac. The IC_{50} of cIMP to inhibit Epac in the presence of 60µM cAMP is about 200µM, compatible with a 2-3 fold lower affinity of cIMP compared to cAMP. Similar results were obtained for cGMP and cXMP (data not shown).

The R**A**AT motif is conserved in the regulatory domains of cAMP dependent kinases and it is replaced by R**T**AT/N in cGMP dependent kinases. It was shown that the A to T mutations in PKA change the selectivity from cAMP to cGMP and vice versa (14-16). In the case of $EpacRD_{149-328}$ the A280T mutation does indeed increase the affinity for cGMP whereas the affinity for cAMP is not altered (Fig. 2C). Nevertheless, in the context of the auto-inhibited ΔDEP-Epac, the mutant does not show an improved activation behaviour after stimulation with cGMP, but rather a 2 fold reduction of k_{max} after activation by cAMP (Fig. 2A). From these results we conclude that although a number of cyclic nucleotide can bind to Epac, only cAMP is able to activate its GEF activity for Rap1. cAMP differs from the other three cNMP's by the structure of the nucleotide base, and cGMP, cIMP and cXMP have in common that the 6-amino group of the base in cAMP is replaced by oxygen. To investigate whether indeed the N6 group is crucial for the activation of Epac, we tested the activity of the commonly employed N6-phenyl-cAMP analogue *in vitro*. As expected, this compound did not activate Epac (data not shown), but inhibits rather efficiently cAMP induced activation *in vitro* (Fig. 2C).

Modification of the cyclic phosphate

The exocyclic oxygens of the phosphate group, which are not involved in the ring formation, can be replaced by sulphur in a stereo-specific manner generating Rp- and Sp-cAMPS isomers, respectively (Fig.1). Rp-cAMPS is an inhibitor of PKA, whereas Sp-cAMPS which has a strongly reduced affinity, is able to activate PKA (17;18). Similar to the findings for PKA, Rp-cAMPS does not active Epac but rather inhibits cAMP-induced activation of Epac



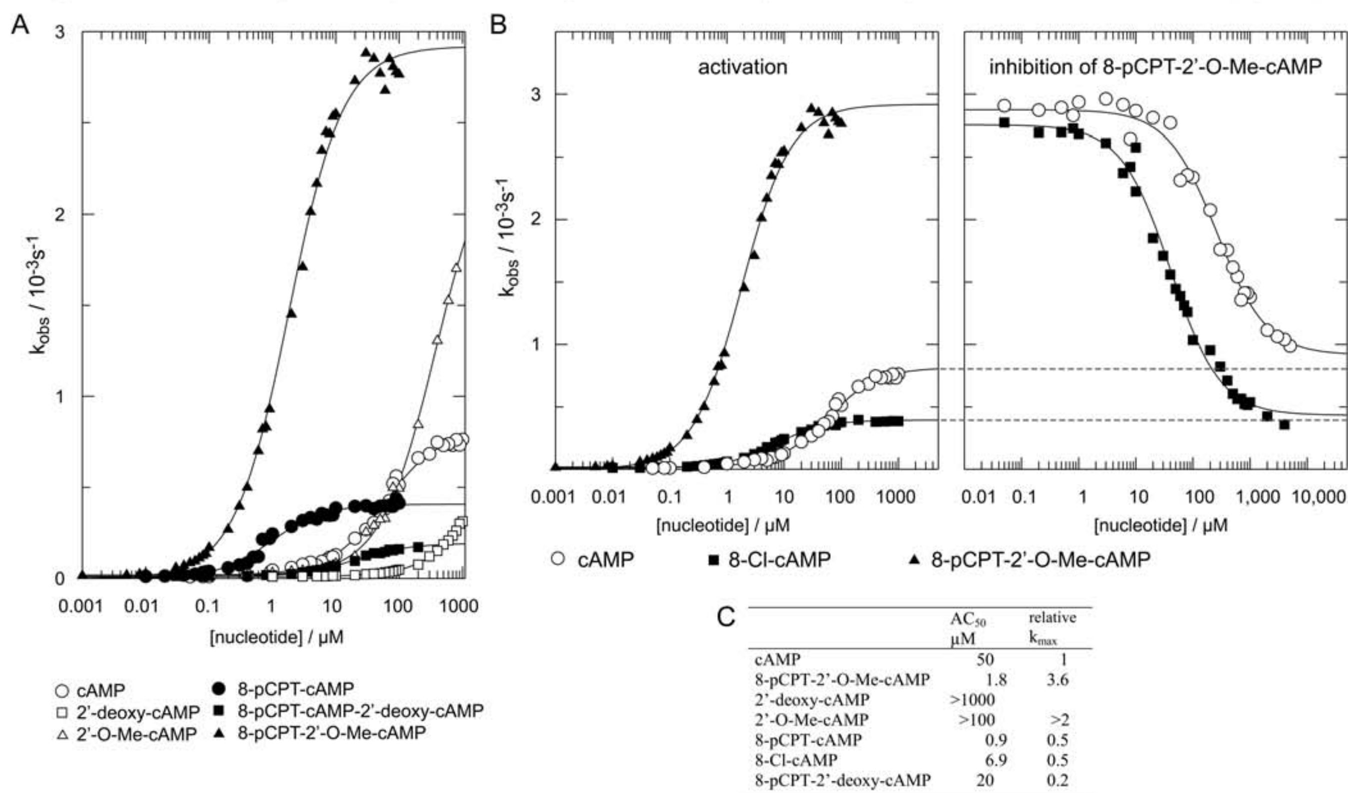
	AC50 μM
cAMP	45
8-pCPT-cAMP	0.9
8-Br-cAMP	6.2
8-Cl-cAMP	6.9
8-MA-cAMP	19
8-OH-cAMP	26
8-ABA-cAMP	40
8-PIP-cAMP	130

Fig. 4 Epac is activated at lower concentrations by several 8-modified cAMP analogues than by cAMP itself.
A. Several analogues with different substitutions at the 8-position were tested for their ability to activate the GEF activity of Epac under standard conditions, with different concentrations of cyclic nucleotides as indicated. k_{obs} values obtained were plotted against the concentrations of the analogues and the AC_{50} was determined from these plots. B. Table of the AC_{50} value of analogues determined from titration experiments as shown in A.

(Fig. 3A,B). In analogy to PKA, Sp-cAMPS is able to activate Epac. (Fig. 3B), with a slight modification of both AC50 and k_{max}.

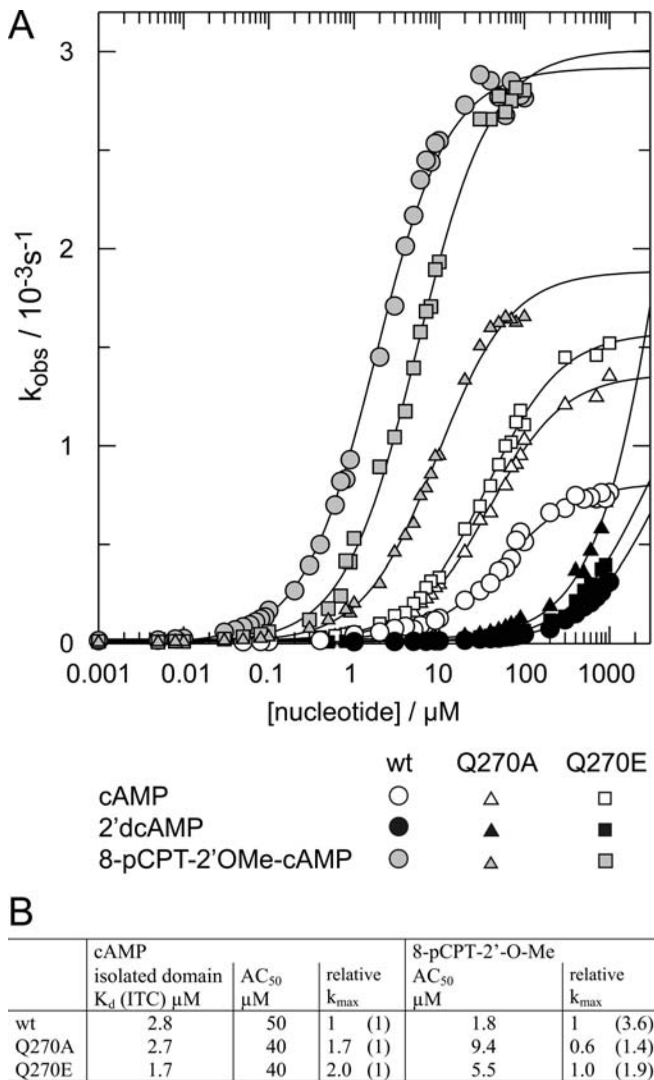
Modifications at the 8 and 2 positions of the base

To optimise the membrane permeability of cAMP, the 8-position of the base is often modified. We have tested a number of these analogues for their effect on the activation of GEF activity of Epac. All 8-modified analogues tested are active, but the maximal GEF activity at saturating concentrations of is reduced (Fig. 4A). In



	AC50 μM	relative k_{max}
cAMP	50	1
8-pCPT-2'-O-Me-cAMP	1.8	3.6
2'-deoxy-cAMP	>1000	
2'-O-Me-cAMP	>100	>2
8-pCPT-cAMP	0.9	0.5
8-Cl-cAMP	6.9	0.5
8-pCPT-2'-deoxy-cAMP	20	0.2

Fig. 5 "Super-activation" of Epac1 by 8-pCPT-2'OMe-cAMP.
A. Activation of Epac with the indicated analogues is measured under standard conditions, as shown previously. Data for 8-pCPT-cAMP are taken from Fig. 4. B. The left panel shows the concentration dependent activity of cAMP, 8-Cl-cAMP and 8-pCPT-2'-O-Me-cAMP on Epac1. The data for cAMP and 8-pCPT-2'-O-Me-cAMP are taken from A. The right panel shows the inhibition of 8-pCPT-2'OMe-cAMP mediated activity by cAMP and 2-Cl-cAMP, respectively. A fixed concentration of 80μM 8-pCPT-2'-O-Me-cAMP was added to the reaction mixture as described in A. and the concentration of the inhibitory nucleotides were as indicated. C. The table summarise AC_{50} and k_{max} values of the analogues used in A and B. The k_{max} values are normalised to the cAMP induced activity.



	cAMP isolated domain K_d (ITC) µM	AC_{50} µM	relative k_{max}	8-pCPT-2'-O-Me AC_{50} µM	relative k_{max}
wt	2.8	50	1 (1)	1.8	1 (3.6)
Q270A	2.7	40	1.7 (1)	9.4	0.6 (1.4)
Q270E	1.7	40	2.0 (1)	5.5	1.0 (1.9)

Fig. 6 Q270 plays an important role in the activation mechanism of Epac.
A. The mutants EpacQ270A and Q270E were generated and compared with wt with respect to there activation behaviour after stimulation with cAMP, 2'-deoxy-cAMP and 8-pCPT-2'-O-Me-cAMP. The cAMP and 8-pCPT-2'-O-Me-cAMP dependency of Epac wt is reproduced from Fig. 5A. B. The table summarises the A_{50} and k_{max} values. The k_{max} are normalised to the wt protein for the respective analogue (normalisation within the column). In addition the k_{max} are normalised to cAMP for the respective mutant (normalisation within the row), these values are given in brackets. The affinity of cAMP to $EpacRD_{149-328}$ as determined by ITC are given in the first column.

addition, these modifications affect the AC_{50}, many of them activating more efficiently than cAMP. Most notable is the 50 fold decrease in AC_{50} of 8-pCPT-cAMP compared to cAMP (Fig. 4). Whereas all 8-modifications activate Epac and affect both the k_{max} and the AC_{50}, 2-Cl-cAMP has a similar k_{max} and AC_{50} for Epac as cAMP (data not shown).

Modification of the 2'-position of the ribose

One of the most striking differences between the cNMP-binding domains of Epac and those of other proteins is the presence of a glutamine (Q) at position 270 of Epac, which corresponds to a glutamate (E) that is totally conserved in all other cAMP- or cGMP-binding proteins. Since in PKA this glutamate was shown to form a hydrogen bound with the 2'-OH group of the ribose (9) and was shown to be crucial for high affinity binding (19) we wondered, whether Epac would tolerate modifications of the 2'-OH group. Its "deletion" in 2'-deoxy-cAMP results in a drastic increase of the AC_{50} and k_{max} could not be reached even at 1000 µM nucleotide (Fig. 5A). This argues that the 2'-OH group is important for binding and activation. To maintain the oxygen we generate 2'-O-Me-cAMP and found a more moderate reduction of the AC_{50}, which h was estimated to be in the rage of 100 µM. Surprisingly, 2'-O-Me-cAMP induced guanine nucleotide exchange rates (k_{obs}) much higher then the k_{max} of cAMP, even though 2'-O-Me-cAMP was used at non-saturating conditions.

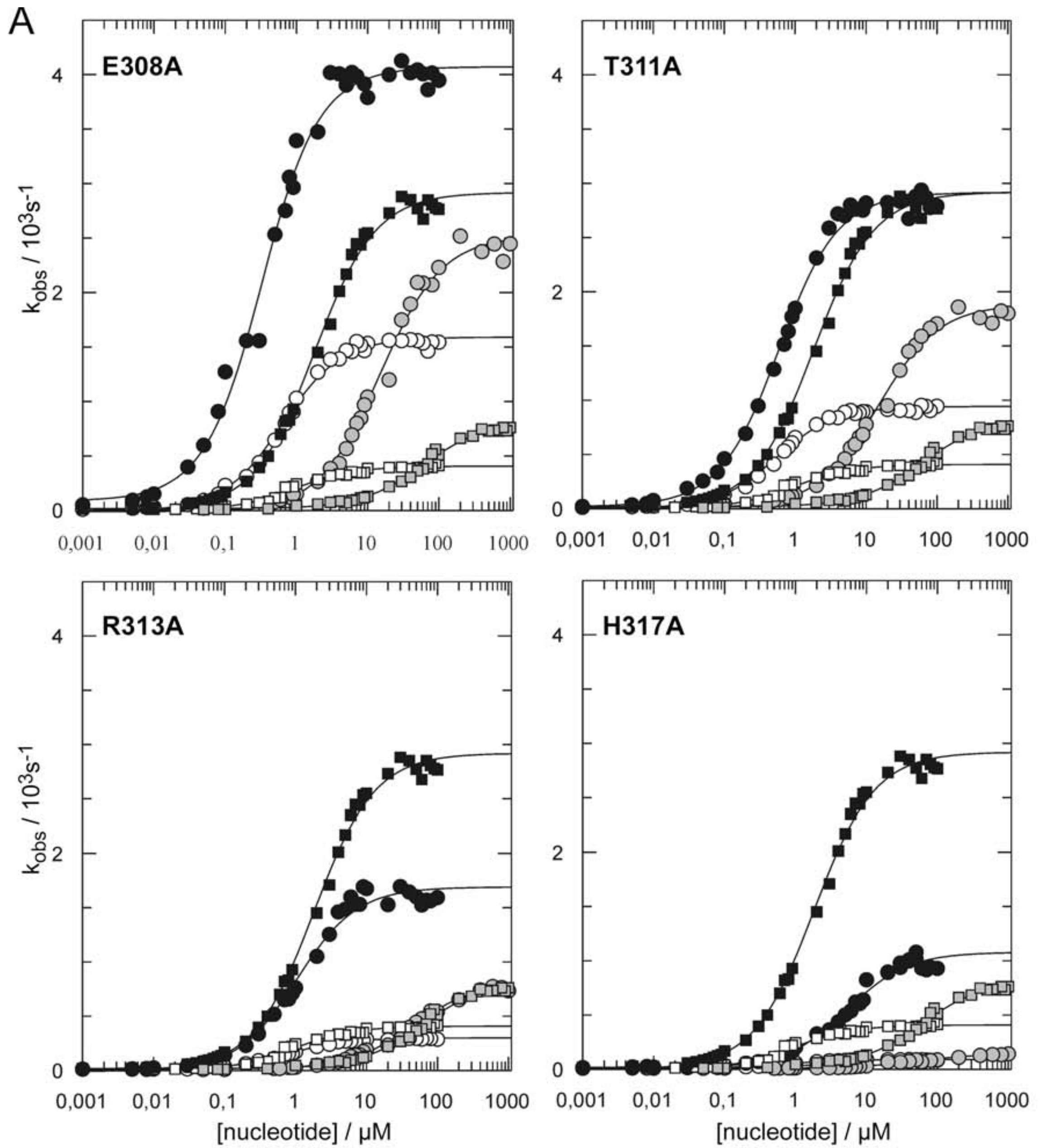
Since the 8-pCPT modification was found to decrease the AC_{50} (Fig. 4A), we wondered whether this positive effect on affinity could be combined with the positive effect on k_{max} of the 2'-O-Me substitution. Indeed, 8-pCPT-2'-O-Me-cAMP showed a decreased AC_{50} *and* an increased k_{max} (Fig. 5A,C). The AC_{50} of 8-pCPT-2'-O-Me-cAMP (1.8 µM) is only slightly lower than the AC_{50} of 8-pCPT-cAMP (0.9 µM) and still 25 times lower then the AC_{50} found for cAMP. In addition, the k_{max} of 8-pCPT-2'-O-Me-cAMP is 7.2 fold higher than 8-pCPT-cAMP and still 3.6 fold higher than cAMP. In order to demonstrate that this superactivation of Epac by 8-pCPT-2'-O-Me-cAMP is due to a change in equilibrium between an active an inactive conformation of Epac, and is not due to any different conditions of the assay, we incubated Epac induced by 8-pCPT-2'-O-Me-cAMP with increasing concentrations of cAMP. We find, that with increasing concentrations of cAMP, the GEF activity is reduced down to a level observed for cAMP alone. Similarly, 8-Cl-cAMP inhibits 8-pCPT-2'-O-Me-cAMP induced GEF activity to a level normally achieved by this analogue, which is half as much as that of cAMP. We can thus conclude that the diverse effects of the 8-pCPT and the 2'-O-Me modifications on inducing GEF activity can be combined to create a super-activating cAMP analogue which specifically activates Epac and can be used as a tool to decipher the role of Rap1 in signal transduction pathways (11;20).

Role of the Gln270 in binding and activation

To analyse the role of Q270 in the interaction with the 2'OH group of cAMP side we generated the mutants EpacQ270A and EpacQ270E. The mutation Q270E mimics the residue conserved in PKA. Importantly, the affinity of cAMP for the isolated cNMP-binding domains of both mutants was not affected demonstrating that unlike in PKA, this residue is not involved in binding of cAMP (Fig. 6B). The mutants were then further analysed, in the context of $Epac_{149-881}$, for their activation properties in response to stimulation by cAMP, 2'-deoxy-cAMP and 8-pCPT-2'-O-Me-cAMP (Fig. 6A). For cAMP a two fold higher k_{max} was observed for both mutants, whereas the AC_{50} remains unaffected, indicating that Gln270 is not crucial for the activation mechanism of cAMP and has, if anything, an inhibitory role on full activation. Activation by 8-pCPT-2'-O-Me-cAMP is differently affected by the two mutations: whereas the Q270E mutation had no effect on the k_{max} but slightly decreased the AC_{50}, the Q270A mutation decreased k_{max} and left the AC_{50} unaffected. The AC_{50} of 2'deoxycAMP is too high to determine k_{max} either for wild-type and mutant proteins (Fig. 6). From these results we conclude that the presence of a hydrogen bond donor or acceptor in the position of residue Q270 does contribute to normal activation by cAMP and super-activation by 8-pCPT-2'-O-Me-cAMP in a manner that is not immediately obvious in the absence of a cAMP-bound 3D structure.

The LID region is involved in super-activation

Recently we mutated conserved polar residues Glu308, Thr311, Arg313 and His317 in the LID of Epac1[2]. As isolated cAMP-binding domains these mutant proteins have a two fold lower affinity for cAMP except H317A for which the affinity for cAMP was unaffected. In addition these mutations affected cAMP-induced activation of Epac (Rehmann et al.[2] and Fig. 7). By comparing the effects of cAMP, 8-pCPT-cAMP and 8-pCPT-2'-O-Me-cAMP we observe that R313A and T311A have a roughly similar effect on the activation of Epac by both cAMP and 8-pCPT-cAMP, but surprisingly an opposite effect on the activation by 8-pCPT-2'-O-Me-cAMP (Fig. 7), R313A did not influence the k_{max} of 8-pCPT-cAMP and cAMP but reduced the k_{max} of 8-pCPT-2'-O-Me-cAMP almost two fold. T311A increased the kmax of cAMP and 8-pCPT-



□ wt + cAMP □ wt + 8-pCPT-cAMP ■ wt + 8-pCPT-2'-O-Me-cAMP

○ mutant + cAMP ○ mutant + 8-pCPT-cAMP ● mutant + 8-pCPT-2'-O-Me-cAMP

B

	cAMP			8-pCPT-cAMP		8-pCPT-2'-O-Me-cAMP	
	isolated domain K_d μM	AC_{50} μM	relative k_{max}		relative k_{max}	AC_{50} μM	relative k_{max}
wt	3.0	45	1 (1)	0.9	1 (0.5)	1.8	1 (3.6)
E308A	6.3	15	3.0 (1)	0.6	3.9 (0.6)	0.3	1.4 (1.6)
T311A	5.5	15	2.3 (1)	0.5	2.3 (0.5)	0.5	1.0 (1.5)
R313A	6.2	40	1.0 (1)	0.7	0.7 (0.4)	1.0	0.6 (2.1)
H317A	3.6	50	0.2 (1)	0.7	0.2 (0.6)	5.2	0.4 (7.7)

Fig. 7 The LID region is directly involved in the activation process.
A. Four conserved polar residues of the LID region were mutated to Ala and analysed for the dependency of their activity on the cyclic nucleotide concentration. The analysis of Epac wt is shown in each individual graph for comparison, values for cAMP-induced activation is taken from Rehmann et al.[2].
B. The table shows the AC_{50} and k_{max} values. The k_{max} are normalised to the wt protein for the respective analogue (normalisation within the column). In addition the k_{max} are normalised to cAMP for the respective mutant (normalisation within the row), these values are given in brackets.

2'-O-Me-cAMP two fold, but has no effect on the k_{max} of 8-pCPT-2'OMe-cAMP. For H317A the kmax achieved with 8-pCPT-2'OMe was greatly reduced compared to wild type, whereas for E308A, the kmax was even increased by about 25%. Also, the AC_{50} of the analogues was affected by the mutations. Most notably, although the affinity for cAMP for the isolated cAMP-binding domain of T311A and T311A was reduced, the AC_{50} was considerably lower. From these results we conclude that the LID region is involved in the super-activation of Epac by 8-pCPT-2'-O-Me-cAMP. Particularly the differential effect of 8-pCPT-2'-O-Me-cAMP compared to cAMP and 8-pCPT-cAMP on R313A and T311A indicates that the 2'-O-Me-group communicates with the LID to induce super-activation.

Discussion

We have analysed the effect of various cAMP analogues on their effects on Epac activation in vitro. We first noted that although cGMP, cIMP and cXMP do bind to Epac with an affinity similar to cAMP they are unable to activate Epac. This implies that the composition of the base, particularly the N6 position, is absolutely critical for the activation of Epac. In contrasts, cGMP, cIMP and cXMP are competitive inhibitors. Since the IC_{50} is the upper µM range it is unlikely that this inhibition is physiologically relevant. In this respect Epac differs from PKA. Both cGMP (14) and cIMP (21) are able to fully activate PKA, although only at high concentrations. Similarly, N^6-phenyl-cAMP inhibits Epac but activates PKA (21). All these nucleotides have an identical cyclic-phosphate ribose group and thus the interaction of the base, presumably with the LID determines the difference. Apparently alterations the N6 position of cAMP abolishes the ability of cyclic nucleotides to activate Epac but not PKA. This implies that suitably N6-altered cAMP-analogues may be able to differentiate between PKA and Epac.

With respect to cyclic nucleotide dependent protein kinases, the RAAT- and the RTAT/N-motif were identified to determine the cAMP and cGMP specificity, respectively. From modelling studies an extra hydrogen bound between the 2-NH_2-group of the guanine and the first threonine in R**T**AT/N was predicted (22). These theoretical considerations were confirmed by side directed mutagenesis for both PKA (16;23) and PKG (15), which demonstrated an increased cGMP affinity due to the presence of the threonine. Similarly, $EpacRD_{149-328}$A280T showed an increased affinity for cGMP as well. However, EpacA280T showed no improved activation by cGMP in terms of k_{max}. Thus the A/T conversion increased the affinity of cGMP for Epac, but has no effect on the activation of Epac by cGMP. Interestingly, as predicted from studies with PKA (14) and PKG (15) the A/T conversion did not or only marginally affect the affinity for cAMP. However it did reduce the maximal activation of Epac by cAMP, showing that the RAAT sequence is not only involved in conferring specificity in binding but also in transferring the cAMP binding information to the activation of Epac.

From the structural analysis of Epac and PKA it was clear that the cyclic phosphate group is critical for the activation of both proteins. Indeed Rp-cAMPS, in which one of the free oxygens of the phosphate group is replaced by a sulphur atom, is an inhibitor of PKA (17;18), whereas Rp-cGMPS inhibits PKG (24). Indeed, Rp-cAMPS also inhibits cAMP-induced Epac activation. This supports strongly the common activation mechanism of these proteins as proposed previously by us (8), i.e. the replacement of the equatorial oxygen perturbs the hydrogen bonding between the phosphate and an alanine in the PBC and prevents the reorientation of the PBC, the first step in the activation of both proteins.

Modifications at the 8-position of the base are commonly used as cNMP-analogues for PKA and PKG, since these modifications both increase the permeability for cells and reduce the AC_{50} to activate these proteins (21;25). In particular 8-pCPT-cAMP and 8-Br-cAMP are widely use for in vivo experiments. The 8-Br-substitution was shown to stabilise the syn conformation of cAMP, the conformation of cAMP adopted in the crystal structures of PKAI and PKAII (9;10), which provides a possible explanation for the decreased AC_{50}. The analogues also have a lower AC_{50} for Epac compared to cAMP, particularly the 8-pCPT-modifictaion resulted in a 50 fold reduction in AC_{50}. However, the maximal activation of Epac by these analogues is two fold lower compared to cAMP indicating that modifications at the 8 position also affect the k_{max} of the protein. However, not all modifications of the 8 positions affect the behaviour of cAMP on Epac. e.g 8-ABA-cAMP.

The highly conserved glutamate in the FG**E**LAL sequence of the PBC of PKA, PKG, cyclic nucleotide-gated ion channels and even the bacterial CAP protein, is replaced by a glutamine (Q270) in Epac1 and a lysine in Epac2. Glutamic acid was found to form a hydrogen bond with the 2'-OH group of the cyclic nucleotide and was shown to be absolutely essential for the binding of cAMP to PKA (19). While the Epac structure analysis (8) showed that the corresponding residue would be close to the 2'-OH-group, a potential interaction between Q270 would not significantly contribute to the binding affinity for cAMP. Neither the AC_{50} of EpacQ270A or EpacQ280E nor the K_d of $Epac_{149-328}$Q270A or $Epac_{149-328}$Q270E is altered. This suggested that the glutamine in Epac is not involved in the direct binding of cAMP and would question the necessity of the 2'-OH-group for activation of Epac. However, drastic effects on activation of GEF activity with respect to both AC_{50} and k_{max} were obtained by modifications of the 2'-OH group of the ribose. Replacement of the 2'-OH group by H or by OCH_3 leads to an increase of the AC_{50}, indicating that interactions of the 2'-OH-group contribute to the binding of Epac, but not via Q270. Indeed, the affinity of 2'-deoxy-cAMP for the cAMP binding domain was dramatically reduced. However, this reduction was much less, but still ten fold, when the oxygen was preserved by replacing the OH-group for an O-Me-group (Christensen, submitted for publication). This indicated that the OH group still confers binding energy for cAMP to Epac, presumably through its interaction the conserved glycine in the FGELAL sequence. Interestingly, 2'-O-Me- cAMP (and perhaps 2'-deoxy-cAMP) at non-saturated concentration induces k_{obs}-values that are higher then the k_{max} of cAMP mediated activation. Apparently, the 2'-O-Me-group, shifts the conformational equilibrium of the nucleotide bound Epac more efficient to the active site. Combined with the increased affinity of the 8-pCPT-group, both modifications resulted in a highly specific "superactivator" of Epac (11). The effect of the increased k_{max} is in part mediated by Q270. The k_{max} of 8-pCPT-2'-O-Me-cAMP mediated activation of EpacQ270A (but not EpacQ270E) is decreased significantly. On the other hand, the k_{max} of cAMP mediated activation for both mutants is increased in comparison to wild type. This indicates that Q270 disfavours the cAMP bound active state but favours the 8-pCPT-2-O-Me-cAMP bound active state.

Mutagenesis of the LID demonstrate a role of this region in the super-activation behaviour of 8-pCPT-2'-O-Me-cAMP, although the role of individual residues in this process is still unclear. A potential link between the LID and the OH/OCH_3-group and/or Q270 is H317. In all known PKA structures a hydrogen bond between the glutamate in the FGELAL sequence (corresponding to Q270 in Epac1) and a tyrosine/tryptophane in the LID is found. This tyrosine/tryptophane may corresponding to H317. Indeed, cAMP- as well as 8-pPCT-2'-O-Me-cAMP-mediated activation is drastically reduced in EpacH317A. R313 may be specifically responsive to the 2'-O-Me-group, since the k_{max} of cAMP is not altered, whereas the k_{max} of 8-pCPT-2'-O-Me-cAMP is reduced two fold. In contrast, T311 may be specifically responsive to the 2'-OH-group. Further structural and mutational analysis of both the LID and the catalytic domain are required to fully understand the molecular mechanism of Epac activation by cAMP and cAMP analogues.

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6

Summarising discussion

Summarising discussion

Different proteins are targeted by the second messenger cAMP, namely Epac, PKA and cyclic nucleotide regulated ion channels in eukaryotes, and the catabolite gene activator protein (CAP) in prokaryotes. To all these proteins, a cAMP binding domain of the same architecture is in common. This implies a common general regulation mechanism on the molecular level. This also implies an interference of the signalling events caused by these proteins as soon as they occur in the same cell. In this thesis, the regulation mechanism of Epac was investigated and it may now be possible to postulate a basic regulation mechanism of general validity for proteins regulated by cAMP- and cGMP-binding domains.

Regulation of Epac

Based on the comparison of the crystal structure of the cAMP free regulatory region of Epac (chapter 4), with the cAMP bound structures of PKA (1;2) and CAP (3), we suggest a model of cAMP regulation that is summarised in Fig. 1. The major difference between the cAMP free structure of Epac (dark grey) and the cAMP bound structure of PKA (light grey) is the arrangement of the C-terminal part of the cAMP-binding domain. This helical area shields cAMP against the solvent in cAMP bound structures, but is found in an open conformation in case of Epac, allowing free access for cAMP to the cAMP-binding site (part a of Fig. 1). According to the model, binding of cAMP rearranges this part of the protein by movement of a helix, called the hinge, closer to the core of the domain. How does cAMP cause this movement? Part b of figure 1 shows a magnification of the hinge region. It is not possible for the hinge of the cAMP free structure to adopt the same angle as in the cAMP bound structure, since a phenylalanine of the helix would clash with a leucine from a loop of the core domain, which is rearranged upon cAMP binding. This loop is tighter in PKA and thereby eaves space for the phenylalanine. The loop, called phosphate binding cassette (PBC) (4), is highly conserved in sequence in all cAMP binding domains and forms hydrogen bonds to the phosphate sugar moiety of the cyclic nucleotide

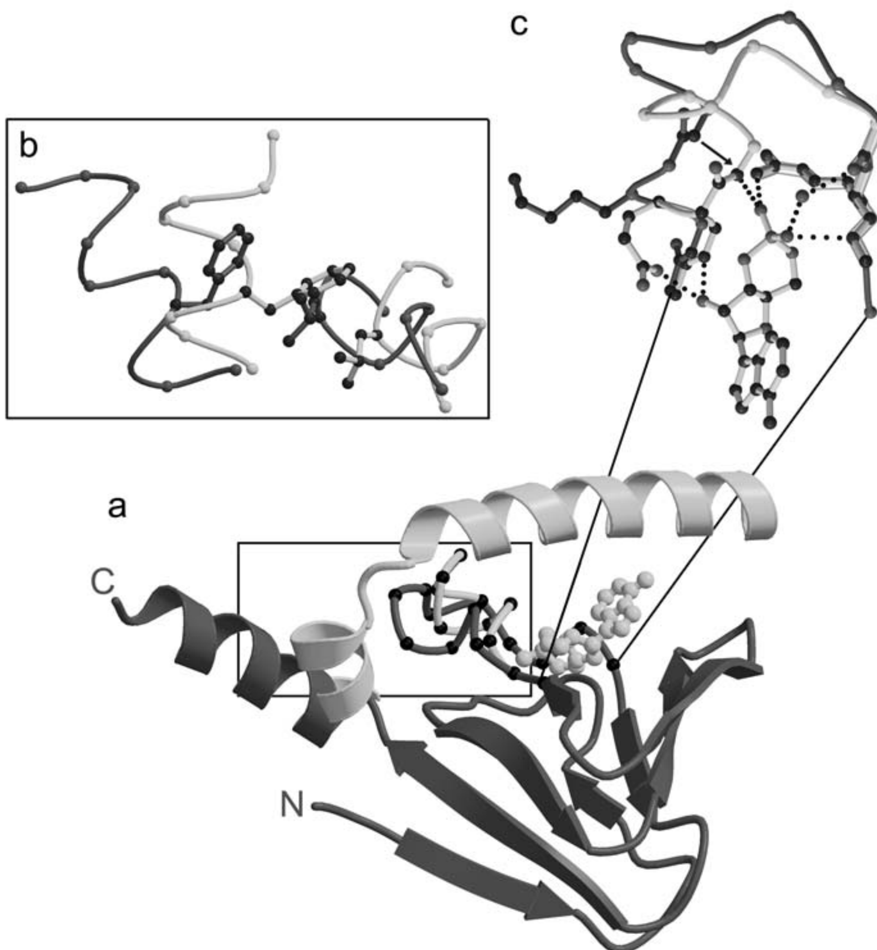


Fig. 1 **Regulation of Epac by cAMP**. A superimposition of the second cAMP-binding domain of Epac (dark grey) and PKA (light grey) is show in part a. Parts of PKA which are identical to Epac are omitted for clarity. The N- and C-terminus are indicated. Part b shows a magnification of the hinge region containing the conserved phenylalanine and leucine, which blocks the movement of the C-terminal helix closer to the core in the absence of cAMP. Part c shows a magnification of the Phosphate binding cassette (PBC). In the absences of cAMP parts of the PBC are in the correct position to accept hydrogen bonds from cAMP, whereas other parts have to undergo conformational rearrangements for cAMP binding. The parts of this figure and of all other figures containing plots of protein structures were generated using MolScript (30) and Raster3D (31).

(1;2). A magnification of the PBC is shown in part c of figure 1. Also in the absence of cAMP, some parts of the PBC are in the correct conformation to accept hydrogen bonds, others are not. These parts will move closer to cAMP to form the hydrogen bonds. The PBC is tightened by this rearrangement and the leucine is moved away to allow the helix to flip over. This mechanism was confirmed by mutagenesis studies (chapter 4).

It is possible to inhibit the isolated catalytic region by addition of the isolated regulatory region of Epac1 in trans. The inhibition could be released by the addition of cAMP (5). This experiment demonstrates a direct protein-protein interaction between the regulatory and the catalytic region of Epac, which is released upon cAMP binding. These studies were extended to Epac2 (chapter 2), where it was found that the second cAMP binding domain of Epac2 is sufficient to inhibit the catalytic domain in trans. Furthermore, we demonstrated the involvement of the VLVLE motif of Epac, a sequence that directly follows the C-terminal helix of the cAMP-binding domain, in the interaction with the catalytic region of Epac (chapter 3). The activation properties of Epac are very sensitive to mutations in the whole area of the C-terminal helix and the VLVLE-motif (chapter 3 and 5). Thus the C-terminal helix and the VLVLE stretch of the cAMP-binding domain is a pivotal element of the intramolecular binding interface of Epac.

Is it possible to extend this basic model of regulation to other cAMP binding proteins? The following section will focus on CAP and PKA, the only other cAMP-binding proteins for which structural information is available.

Regulation of CAP

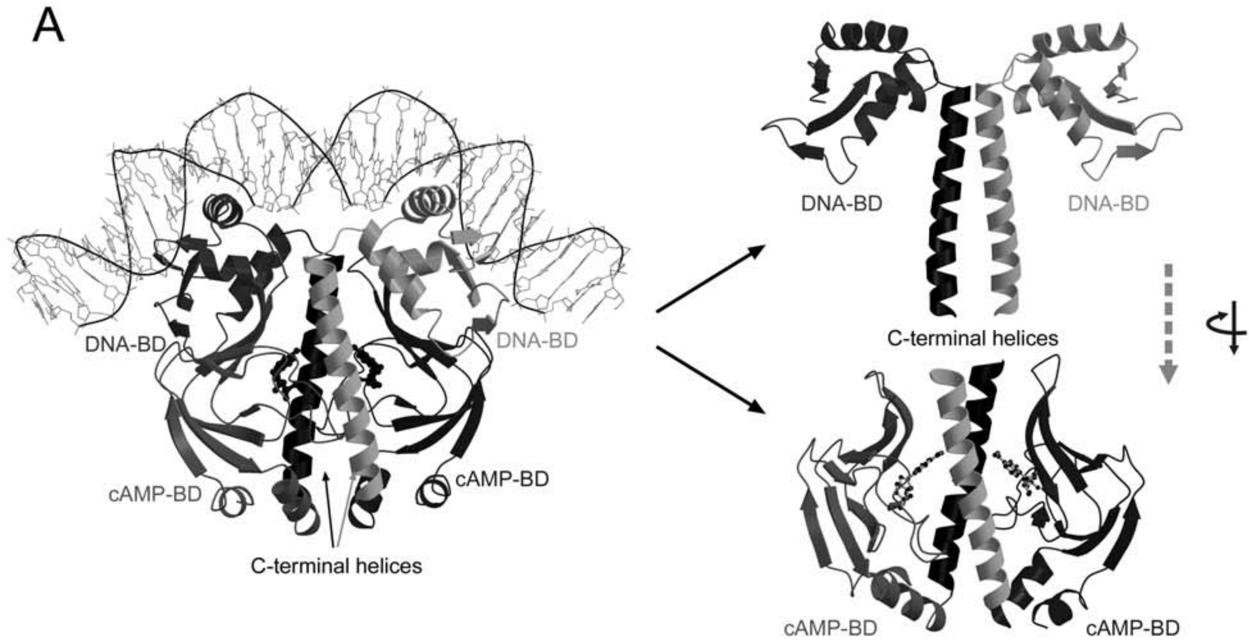
The cAMP-binding domain of CAP (catabolite gene activator protein) was the first one for which structural data was available, and thus it was used as a model for PKA and cyclic nucleotide gated ion channels. Different states of CAP were crystallised: the homodimer of CAP bound to cAMP (3) and the homodimer of CAP bound to cAMP in complex with DNA (6) (Fig. 2A). However, no structural information of the cAMP free state is available so far. The crystal structure of CooA was determined recently (7). CooA is a transcription factor of the CAP family and consists of a DNA-binding domain, which is structurally equivalent to the DNA-binding domain of CAP, and a heme containing domain, which is structurally equivalent to the cAMP-binding domain of CAP. The heme group is localised at the position corresponding to cAMP in the CAP structure. Since CooA is activated by the binding of carbon monoxide (CO) to heme and since the structure was determined in the absence of CO, this structure could be understood as a model for the inactive, ligand free state of CAP.

The major difference between these three structures is the relative orientation of the DNA-binding domain and the ligand-binding domain. The domains itself differs only marginally from each other. The orientation of the DNA-binding domain is incompatible with DNA binding in the CooA structure, since it does not allow a concomitant binding of both domains (compare Fig. 2B with Fig. 2D). The comparison with CAP suggest that ligand binding to transcription factors of the CAP family induces a flipping of the DNA binding domain by 180° relative to the cAMP-binding domain (compare Fig. 2B with Fig. 2C). The DNA-binding domain of chain A is in the correct orientation after ligand binding, whereas an additional induced fit upon DNA-binding is required in chain B (compare Fig. 2C with Fig. 2D).

Although the comparison of CooA and CAP gives deep insights into the behaviour of the DNA-binding domains, its use to draw conclusions for the ligand-binding domains is limited. Crucial residues involved in cAMP binding are not conserved between CAP and CooA. A movement of the cAMP binding domain relative to the C-terminal helix was suggested from the first cAMP bound structure of CAP. cAMP is buried deeply in the cAMP-binding pocket so that an exit and entry way for this cyclic nucleotide has to be generated (Fig. 2F). A solution of this problem is not evident from the CooA structure. Although the C-terminal helices are rotated relative to each other in comparison to CAP, there is still no easy access to the ligand binding site. This is easy to understand since the binding site of CooA has to be accessible only for the small CO. In conclusion, it is not clear how cAMP binding to the cAMP-binding domain is transferred to the rearrangement of the DNA-binding domains. Still, a movement of the cAMP-binding domains relative to the C-terminal helices would give cAMP access to the binding site, and this movement (as a movement of the C-terminal helix) is predicted from the model based on our analysis of Epac.

A role of the base binding region in regulation?

The base binding region (BBR) is situated in a loop between two β-sheets in the core structure of the cAMP-binding domain. Based on studies with CAP it was suggested to be involved in communication of cAMP binding to remote parts of the protein. In some crystal forms of CAP, hydrogen bonds between the DNA-binding domain and the BBR were observed (3;6). However, the BBR is very divergent and neither conserved between different types of cAMP-binding domains nor between cAMP-binding domains of the same family. The BBR often contains insertions, which differ in length by up to 12 residues. In fact, this loop is


A
DNA-BD
DNA-BD
cAMP-BD
cAMP-BD
C-terminal helices
DNA-BD
DNA-BD
C-terminal helices
cAMP-BD
cAMP-BD

B
CooA
DNA-BD
DNA-BD
cAMP-BD
cAMP-BD
ligand free
C
CAP without DNA
DNA-BD
DNA-BD
cAMP-BD
cAMP-BD
ligand bound, without DNA
D
CAP with DNA
DNA-BD
DNA-BD
cAMP-BD
cAMP-BD
ligand bound, with cAMP

Fig. 3 **A role of the base binding region in regulation**. The cAMP binding domains of Epac (chapter 4), PKA (1;2) and CAP (3) were structurally superimpose. In the figure only the BBR is shown (the complete superposition is shown in chapter 4 Fig. 3A). As can be seen, the BBR of the individual proteins differs in conformation and length. With the data available it is not possible to correlate a specific conformation with the nucleotide states.

the most divergent part of the core of all cAMP-binding domains solved so far (Fig. 3). The structural flexibility of the BBR is reflected by (a) high B-factors; the loop is not visible in the electron density in some structures and by (b) different conformations of the BBR in the single domains of the CAP dimer.
Independently of whether these considerations make a role of the BBR in regulation likely or not, it is not possible to draw any conclusion from a comparison of the cAMP free structure (Epac) with the cAMP bound structures (PKA and CAP). This is due to the fact, that it is not clear whether a different conformation has to be attributed to cAMP binding or to the different sequence of the BBR. In addition, we found no evidence for a role of the BBR for regulation of Epac activity during our biochemical analysis.

Regulation of PKA

The inactive complex of PKA consists of two regulatory subunits (R) and two catalytic subunits (C). cAMP binding to this complex results in the dissociation of the catalytic subunits from the complex. The free catalytic subunits are catalytically active kinases. Whereas structural information of the cAMP bound regulatory subunit(1;2) as well as of the catalytic subunit in complex with AppNHp and an inhibitory peptide (8;9) is available, no crystal structure for the inactive R_2C_2-complex was obtained so far.
A low resolution model for the R_2C_2-complex was derived from neutron and x-ray scattering experiments (10). This model is shown in a cartoon like fashion in Fig 4A. The regulatory domains interact with each other through an N-terminal dimerisation domain in an antiparallel orientation. This orientation is confirmed by NMR-studies of the dimerisation domain (11). The linker region, which contains the pseudosubstrate sequence, connects the dimerisation domain to the cAMP-binding domains. The catalytic subunits are bound to the pseudosubstrate sequences as well as to the cAMP-binding domains. An interaction of both catalytic subunits with each other is excluded by neutron scattering data (10). Thus the interaction between each R and the individual C is independent of the respective other R-C pair. This is confirmed by a deletion mutant of R, lacking the dimerisation domain still being able to bind and to inhibit the catalytic subunit by the formation of an RC-complex (Fig 4A) (12;13).
The binding interface of the R-subunit was narrowed down further by biochemical experiments. Despite deletion of the N-terminal dimerisation domain, deletion of the C-terminal cAMP-binding domain does not interfere with the inhibitory properties of the R-subunit (Fig. 4A) (12;14;15). The minimal requirement for proper binding and inhibition therefore is the pseudosubstrate sequence and the first cAMP binding domain. This indicates a less important role of the second cAMP binding domain. Since cAMP binding to the regulatory subunit is a cooperative process, it was suggested that cAMP first binds to the second cAMP

Fig. 2 **Regulation of CAP by cAMP.** A. The complete crystal structure of the CAP dimer in complex with cAMP and DNA is shown in the left panel. The right panel shows a separation of the DNA-binding and the ligand-binding domains for clarity of presentation. The C-terminal helix of the cAMP-binding domain, which connects the cAMP-binding domain to the DNA-binding domain is shown in both parts. To reconstruct the complete structure the DNA-binding domain has to be rotated by 90° and moved downwards. The C-terminal helices, shown for both parts, would be superimposed after the transformation. CAMP-BD, cAMP-binding domain; DNA-BD, DNA-binding domain. B, C, D. The separated DNA-binding domains (upper panel) the separated cAMP-binding domains (middle panel) and a schematic representation (lower panel) are shown for the ligand free structure of CooA (B) the ligand bound structure of CAP (C) and the ligand bound structure of CAP in complex with DNA (D). All domains are shown in the same orientation as obtained from a superimposition of the ligand binding domains of chain A.

binding domain and thereby primes cAMP binding to the first domain. However it is not shown, that binding necessarily has to occur sequentially.
The inhibition itself is mediated by the pseudosubstrate sequence, which binds to the active site instead of the substrate protein. PKA can be inhibited by peptides derived from the pseudo substrate sequence of PKA and PKI (16;17). However the affinity of the pseudo substrate sequence of R is only in the micro molar range (18), whereas a construct containing the pseudosubstrate sequence and the first cAMP-binding domain can bind the C-subunit with 10 nM affinity (14). Mutations in the pseudo substrate sequence lead to an R-subunit that can bind but not inhibit the C-subunit and thus forms an RC-complex with full kinase activity in the absence of cAMP (19). It is thus the first cAMP binding domain that binds to the catalytic subunit and thereby positions the pseudo substrate sequence in optimal vicinity to the active site.
The binding site in the first cAMP-binding domain was

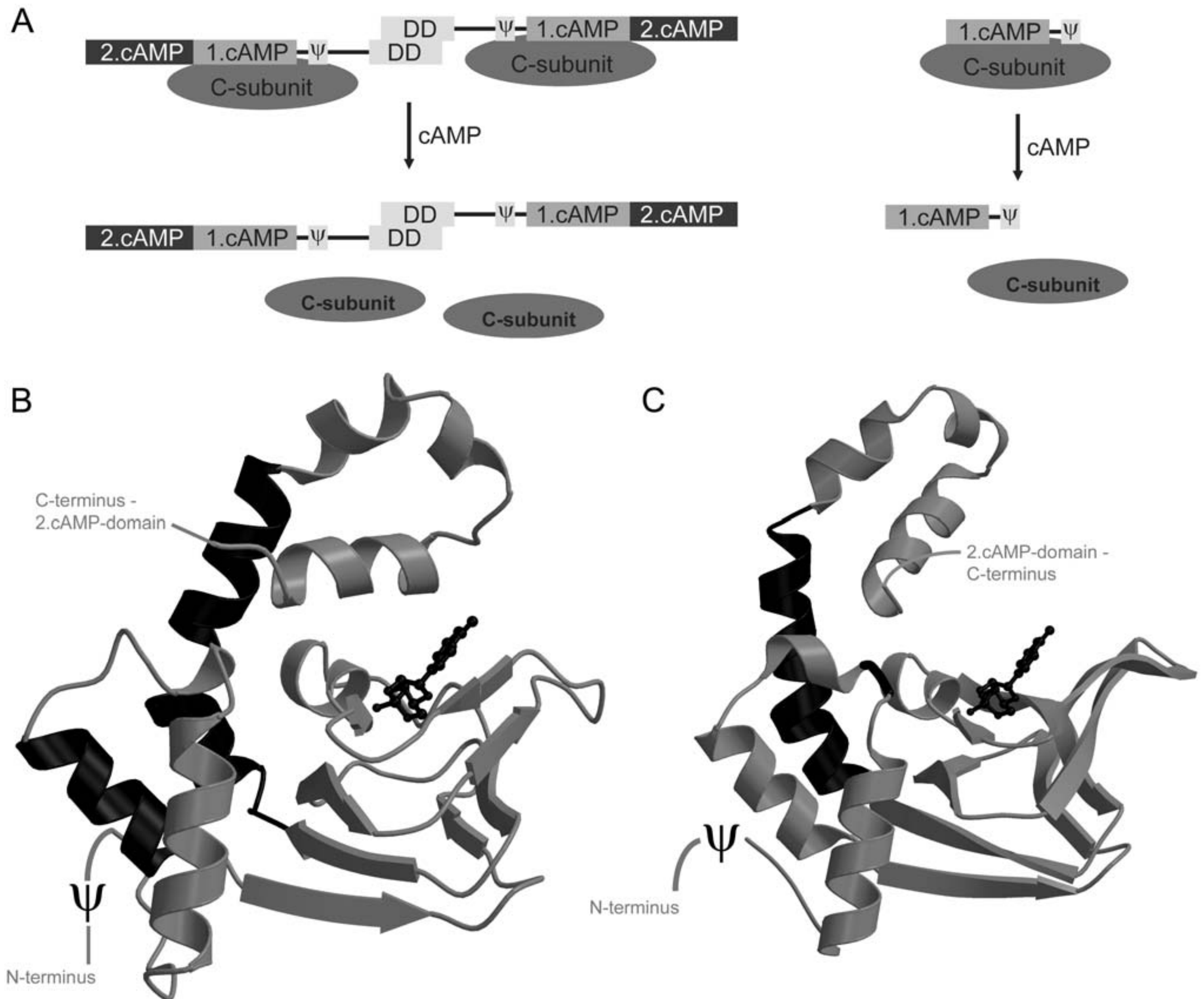


Fig. 4 **Regulation of PKA by cAMP**. A. Cartoon-like model of the quaternary structure of PKA. The model summarises data from neutron scattering experiments and from biochemical analysis. Each catalytic subunit (C-subunit) is bound independently to a regulatory chain. cAMP binding causes the dissociation of the R_2C_2-complex to $R_2(cAMP)_4$ and 2C. The released C-subunits became catalytically active. The right panel shows the minimal complex of a t truncated regulator chain that can still be regulated by cAMP. ψ pseudo substrate sequence. B,C. The first cAMP-binding domain of RI (B) and RII (C) are shown. The localisation of the N-terminal pseudo substrate sequence ψ, that is not visible in the electron density, is indicated. The second, C-terminal cAMP-binding domain is omitted for clarity. Region identified by $H/^2H$-exchange experiments as a potential binding interface for the catalytic subunit are coloured in black. The interface consist mainly of an helix N-terminal and C-terminal of to the core of the first cAMP-binding domain. A rearrangement of the C-terminal helix upon cAMP-binding would alter this interface.

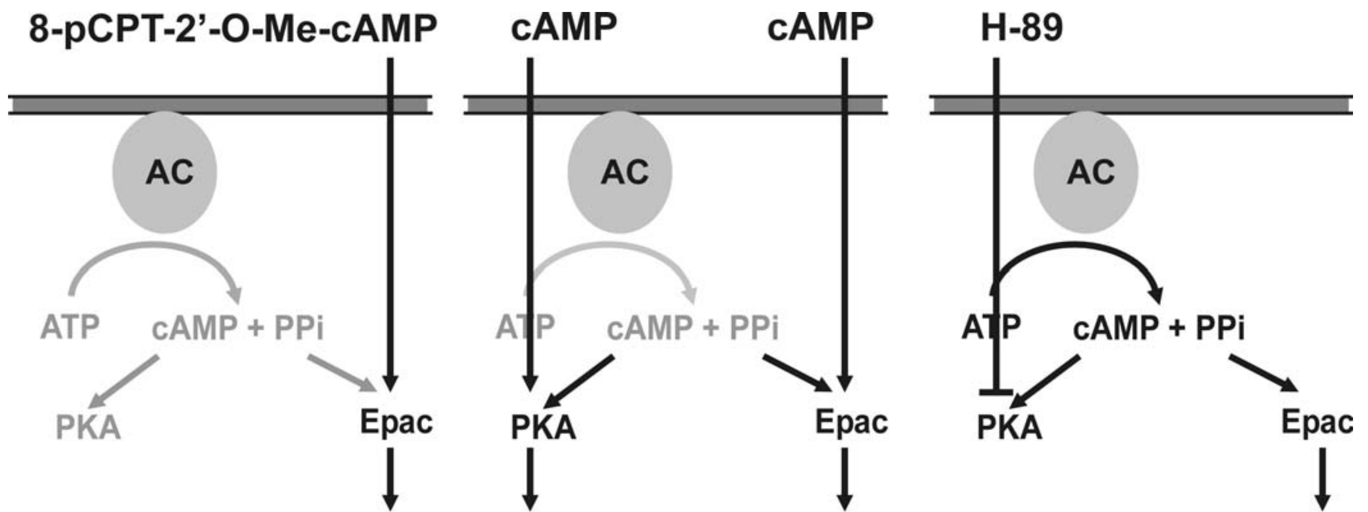


Fig. 5 **Specific activation of Epac**. 8-pCPT-2'-O-Me-cAMP was identified as an analogue that activates specifically Epac. It can be combined with experiments using unspecific cAMP stimuli or the PKA specific inhibitor H89. Thus it is possible to distinguish PAK and Epac mediated effects from each other.

mapped by $^1H/^2H$ exchange experiments (20;21). In this way, areas in the R-subunit were identified that are protected against ^2H-incorporation in the presence of the catalytic subunit. In addition to the pseudosubstrate sequence, a helix N-terminal to the core of the first cAMP-binding domain as well as the C-terminal helix of the first cAMP-binding domain were protected in RI (20) and in RII (21) (Fig. 4B,C). The importance of these region for the interaction with the catalytic subunit is in agreement with mutational studies (22).

Thus the C-terminal helix as well as an N-terminal helix of the first cAMP-binding domain is pivotal parts of the binding interface for the catalytic subunit. A movement of the C-terminal helix, as predicted from our analysis of Epac, would change the relative positions of the N- and the C-terminal helix. It is tempting to speculate that this movement would destroy the binding interface formed by these helices.

A common feature in the regulation mechanism of Epac and PKA is the mode of inhibition. In both cases it is mediated by a protein-protein interaction between the cAMP-binding domain and the catalytic region, which is released upon cAMP binding. Many evidences for this intramolecular interaction of Epac were presented in this thesis. Most likely it is possible to extend this mechanism to PKG. X-ray scattering experiments suggested a large size increase of PKG upon cGMP binding in one dimension (23). This is in agreement with the release of an intramolecular interaction between the cGMP-binding domains and the kinase domain.

For all three types of cAMP regulated proteins of which structural information is available an important role of the C-terminal helix is evident and also the regulatory properties of cyclic nucleotide gated ion channels are influenced by alterations in this helix (24). The cAMP-binding domain is used in all of these proteins as a switch module, which works with the same basic principle. cAMP-binding is converted into a movement of the C-terminal helix by a relatively small rearrangement of the PBC. The C-terminal helix differs in these proteins and allows therefore the connection of cAMP binding to such different functions as nucleotide exchange activity or kinase activity.

Specific targeting of Epac

The common cAMP-binding domain in different receptor proteins leads to an interference of the signalling pathways controlled by these proteins. This complicates the analysis of cAMP mediated effects in *in vivo* systems. The basis for the development of Epac specific cAMP analogues is the identification of Epac specific feature of cAMP binding. A combination of a mutational analysis of Epac1 and the analysis of a wide variety of cAMP analogues was undertaken (chapter 5). An individual feature of Epac is the absence of glutamic acid in the highly conserved consensus sequence FG**E**LAL found in other cAMP-binding proteins. In fact, the glutamic acid was expected to be totally conserved in all cAMP-binding domains and to be absolutely required for binding of cAMP to PKA (25). However, mutations of the corresponding glutamine in Epac to either glutamic acid or alanine do not influence the binding affinity for cAMP. The Epac specific activator 8-pCPT-2'-O-Me-cAMP makes use of this unique feature of Epac. The glutamic acid is known to form hydrogen bonds to the 2'-OH group of cAMP in PKA (1;2), and thus PKA does not accept modifications of the 2'-position. This is different in

Epac: The 2'-O-Me-group even increases the activation potency as compared to cAMP, although this is associated with a reduction of affinity. However, the reduction of affinity can be overcome by the addition of the 8-pCPT modification, and thus 8-pCPT-2'-O-Me-cAMP has higher activation potency and higher affinity for Epac1 than normal cAMP. These features combined with high membrane permeability due to the hydrophobic 8-pCPT modification make this analogue useable for *in vivo* experiments (26;27).

This now allows to distinguish between PKA and Epac mediated effects (Fig. 5). 8-pCPT-2'-O-Me-cAMP can be used to activate Epac specifically. A combined activation of Epac and PKA is possible by the application of cell permeable cAMP-analogues such as 8-Br-cAMP or 8-pCPT-cAMP. A specific inhibition of PKA is possible by the use of H89, an inhibitor that targets the catalytic subunit of PKA (28;29) and therefore does not interfere with Epac.

The studies presented in this thesis contribute to our understanding of how cAMP regulates proteins containing cAMP-binding domains. The model of a general mechanism for this regulation process was presented. The example of 8-pCPT-2'-O-Me-cAMP, a specific activator of Epac, demonstrate that it should be possible to develop Epac specific inhibitors and PKA specific activators as well.

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Summary

The role of cAMP as a second messenger in cellular signalling is well established. In the eukaryotic cell the targets of cAMP are PKA, cyclic nucleotide gated ion channels and Epac. All these proteins have a cAMP-binding domain of same architecture in common. Its function is to bind cAMP and thereby sense the presence of cAMP. Binding of cAMP to this sensor domain is transformed into an altered property of the whole protein. In spite of the important role of cAMP in regulating cellular events, the knowledge on a molecular level of how cAMP binding is translated into protein action was limited so far. For a long time structural information was available only for cAMP-bound (i.e. "active") cAMP-binding domains, but information of cAMP free (i.e. "inactive") cAMP-binding domains was missing.

We determined the cAMP free crystal structure of the regulatory region of Epac2 containing two cAMP-binding domains. The high conservation of both sequence and structure between Epac and PKA allows to compare these structures as representatives of the ligand bound and the ligand free state of cAMP-binding domains in general. From these analyses a general model was developed of how cAMP-binding is sensed by cAMP-binding domains. A relative small rearrangement of residues directly involved in cAMP binding is transduced to a reorientation of the C-terminal helical part of the cAMP-binding domain. The helices of the different classes of cAMP-regulated proteins have common features related to the rearrangements induced by cAMP and unique features to allow communication to the respective remote parts of the protein. By adaptation of the C-terminal helix to the individual requirements in the context of the regulated protein class, the cAMP-binding domain can be used as an universal switch unit.

The critical importance of this helix for the regulation of PKA, CAP and cyclic nucleotide gated ion channels is described in the literature and was shown for Epac by various approaches in this thesis. In Epac the helix forms an interface interacting with the catalytic region. This interaction blocks the catalytic centre in the absence of cAMP. This binding and therefore the blockage is terminated after binding of cAMP.

To complete the structural investigation, extensive biochemical analysis was undertaken. This confirmed already mentioned the importance of the C-terminal helix and corroborated the mechanism suggested for cAMP sensing. In addition, the requirements for productive cAMP-binding and activation were characterised. Thus, the action of Epac specific activators such as 8-pCPT-2'-O-Me-cAMP can be explained. This analogue is able to activate Epac with higher efficiency than normal cAMP, but can neither activate nor inhibit PKA. These studies showed also that it is in principle possible to inhibit Epac with certain cAMP analogues. Based on these findings it should be possible to develop Epac specific inhibitors. This will allow the specific investigation of Epac mediated effects.

Samenvatting in het Nederlands

cAMP heeft een welbekende rol als een ‘boodschappermolecuul’ in signaal transductie. In de eukaryotische cel zijn de doeleiwitten van cAMP PKA, cyclic nucleotide gated ion channels, en Epac. Al deze eiwitten hebben dezelfde structuur van hun cAMP bindend domein met elkaar gemeen. De functie van dit domein is het binden en daardoor waarnemen van cAMP. Binden van cAMP aan dit domein leidt tot een verandering van de eigenschappen van het gehele eiwit. Hoewel cAMP een belangrijke rol speelt in regulatie van cellulaire processen, is de kennis van hoe precies binding van cAMP wordt vertaald in verandering van de eigenschappen van het gehele eiwit op moleculair niveau beperkt. Gedurende lange tijd was alleen structurele informatie beschikbaar van cAMP-gebonden (‘actieve’) cAMP bindingsdomeinen, maar informatie over cAMP-vrije (‘inactieve’) cAMP bindende domeinen ontbrak.

Wij hebben de cAMP-vrije kristalstruktuur bepaald van het regulatiedomein van Epac2, welke twee cAMP bindende domeinen bevat. Omdat zowel de sequentie als de struktuur van de regulerende domeinen van Epac en PKA goed geconserveerd zijn, is het mogelijk de structuur van de ligand-gebonden en ligand-vrije cAMP bindende domeinen met elkaar te vergelijken. Zodoende stellen we een algemeen model voor waarmee verklaard kan worden hoe binding van cAMP waargenomen wordt door cAMP-bindende domeinen. Een relatief kleine plaatsverandering van aminozuren die direct betrokken zijn bij binding van cAMP leidt tot een plaatsverandering van de C-terminale helix van het cAMP bindende domein. De helixen van de verschillende soorten cAMP-gereguleerde eiwitten bezitten algemene eigenschappen die gerelateerd zijn aan de plaatsveranderingen die in gang gezet worden door cAMP, en unieke eigenschappen die communicatie met verderop in het eiwit gelegen domeinen toestaan. Door aanpassing van de C-terminale helix aan de individuele voorwaarden in de context van de gereguleerde eiwitklasse, kan het cAMP bindende domein gebruikt worden als een universele schakelaar.

Het belang van deze helix voor de regulatie van PKA, CAP, en cyclic nucleotide gated ion channels is beschreven in de literatuur, en is in dit proefschrift op verscheidene manieren ook aangetoond voor Epac. In Epac vormt de helix een ‘interface’ die een interactie aangaat met het katalytische domein. In afwezigheid van cAMP blokkeert beze interactie het katalytische centrum. Deze binding, en daarmee de blokkade, wordt opgeheven door binding van cAMP.

Om de studie van de structuur af te maken, is een intensieve biologische analyse gedaan. Dit bevestigde het boven genoemde belang van de C-terminale helix en sterkte het gesuggereerde model voor het waarnemen cAMP. Daarnaast werden de voorwaarden voor productieve cAMP binding en activatie gekarakteriseerd. Zodoende kan nu het effect van Epac-specifieke activatoren, zoals 8-pCPT-2’-O-Me-cAMP, worden verlaard. Deze analoog kan Epac activeren met hogere efficientie dan gewoon cAMP, maar kan PKA noch activeren, noch remmen. Bovendien toonden deze studies aan dat het in principe mogelijk is om Epac te remmen met bepaalde cAMP analogen. Gebaseerd op deze bevindingen zou het mogelijk moeten zijn om Epac-specifieke remmers te maken, waarmee beter onderzoek naar de effecten van Epac gedaan kan worden.

Curriculum vitae

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Dankword

The dankword is maybe the most difficult part in writing a thesis. There are many people I would like to thank and many things I would like to say. I have the feeling that what ever I will write, will be incomplete. I have to solve this dilemma practically: I am most worried about a missing name. Therefore, who ever feels that her or his name is missing, should tell me and she or he will be invited for a drink or for a dinner.
Hans, you gave me a lot of freedom in the last four years. I was able to do many experiments, that came in my mind, sometimes without any outcome, sometimes with success. That was a great time. I enjoyed the spirit – your spirit – of discussing science in your group; presentations, discussions and talks were always fun.
Des weiteren gilt mein besondere Dank Herrn Prof. Dr. Alfred Wittinghofer ... *(Fred: „Bin ich das?“)*, Hey Fred, weiterhin geile Forschung, haut den Bayern ein paar Bälle in den Kasten und sieh zu, dass Du hin und wieder zum Fliegenfischen kommst. *(„Bist Du es jetzt?“)*
To generate the order of the acknowledgments every single acknowledgment or acknowledgment unit was represented by and number. The numbers were then chosen stochastically to give the order presented here:
Andreas, braucht Du eine Anreisebeschreibung nach Utrecht? Über den Kanal zum Rhein, bei Utrecht in einen alten Arm abbiegen, zur Alten Gracht, Du kommst ohne das Boot zu verlassen bis auf 3 Meter an die Theke meiner Feier. Simone, Deine Kinetiken sind einfach ästhetisch schön und gehören in Marmor gemeißelt. Karin, you defined the level of Dutch language I should reach, I will do my very best. Peter Bayer, vielen Dank für die ein oder andere NMR Messung. Gretel, ich hoffe Amsterdam gefällt Dir so gut wie mir Utrecht. Marian, Mariandel, Großmeister der Hefen, wir müssen noch verschiedene Unterwäschefragen diskutieren, wuff. Armando, good luck with primary target identification, but do not destroy Utrecht for it. Dom, siehe zu, dass Du für den Notfall immer zwei bis drei halbe Scheine im Eis hast. Übrigens, 17 Tyrosine geben $2^{17} = 131072$ mögliche verschiedene Phosphorylierungsmuster. Christian P., ein Mediziner der erfolgreich Biochemie betreibt, Ausnahmen bestätigen die Regel. Brigitte und Norbert, Ihr seit das Laser-Fluoreszenz-Team des Institutes. Karelia, ich hoffe Epac wird Dir noch Freude bereiten. Knap vrouwen van het secretariat met name Beatrice, Saskia, Felicia and Marianne bedanked for your help with getting many many things organised in Utrecht. Ilme, Axel and Roman thanks for data collection. Martin W., der Trick ist, einfach was reinsocken. Boudewijn, you was introduced to me as the guy, who is just sitting in his office and just writing nature papers. Johan, discoverer of Epac, you handled over a very nice protein. I used neither the manuscript of your thesis, I had to bring to Fred, nor your car to set on a fire. Rocks, alter Zellkulturfritze und Spetseskämpe, sorry dass unsere in vitro Kultur Dich zur Emigration nach Heidelberg gezwungen hat. Astrid, noch heute sind Küvetten mit Deinem Namen beschriften und noch heute findet man Dich in den Spexes (wie geht da die Pluralbildung?). Sanne, you are living next to the fluorescence apparatus, we will meet often. Michael Seewald, das meeting Seewald et al. war eine nette Sache. Alexandra, achte darauf, dass der Wolf an seinem Schreibtisch mit seiner Doktorarbeit nicht faul und träge wird. Martin, lasse Dich niemals von einem Photomultiplier oder anderen „Kleinigkeiten“ abhalten. Mike, lets see what AF-6 will have for you, good luck, we will do our best. Pieter, go for the future, enjoy life. Lars, Lars & Lars, “Lars, hast Du, weißt Du, könntest Du,” ist immer eine gute Einleitung im Labor Ahmadian. Alex E., fange mit dem Drucken Deiner Doktorarbeit etwas früher an. Rado and Dennis good luck with some more structures. PD Dr. M. Reza Ahmadian, Du musst alle Deine Jungs zusammenhalten und sehen das der Laden läuft. Ah, Silke, Du machst das auch schon so. Patricia, vier Jahre war ich so zu sagen Gast bei Euch im Labor und Du hast eine Menge meiner Unordnung ertragen, wie auch immer, es hat mir gut gefallen. Delia, success with your projects. All die schönen Müller Mädels, Slava, Nadine, Anette, Sonja, Christina, Eva, Mareike, ach ja und Maria, so ist die Forschung. Alice, Du bist zwar bei Peter, aber gehörst doch auch hier genannt. Beate, singe weiter und allzeit freie Strecke zwischen Dortmund und Oberhausen. Taisa, you are working now on the bench, on which I started my PhD thesis four years ago. I hope you will enjoy it as much as I did. Jorrit, it is fun to combine our in vivos and in vitros and to go trough the different levels of pain of writing a thesis. A good start for your emigreren. Revathy, you appear the first time in Dortmund when I came back the first time from Utrecht, I will never forget the first experiment you, Gerrit and me did together: light scattering at the fluorometer, we had to change to second order for detection. Doro, Du bist die Geheimwaffe Freds, keine Klonierung zu schwer, keine Klonierung zu eilig und natürlich auch sonst universell einsetzbar. Lydia, you are the mother of the Bos-Lab. Gerrit, ich glaube ich habe Deinen Rekord, Doktorand mit der längsten Vergangenheit am Institute, geschlagen. Marita, you survived the cold and rainy Netherlands, enjoy the sunny Spain. I am sorry, that I never obtained positive in vitros for you. Marta B. maybe we should do a nice in vitro with your KIAA. Robert (der mit einem b), wir sollten hin und wieder mal was durchregulieren, Essen, Akroplois und solche Sachen. Dank für Deine Mühe mit meiner Thesis. Juliane, weiterhin viel Spaß mit Sequenzanalyse und Familienforschung, Du wirst schon noch beweisen, dass Ras die Urmutter aller GTPase ist. Martina, Mrs. BTS, die BTS hat mit Dir gelebt. Kim, it was fun to

measure activity the other way round. Jun, your antibodies are real nice, I hope your family will find a nice home in Hilversum. Thilo, mal wieder Lust auf eine kleine Dienstreise, vielleicht mal irgendwo hin wo es weniger Zement gibt? Das Institut schafft gerade neue Dienstwagen an. Caro, schöne Frau Körnchen, siehe mal zu, dass Du Deine Steuerstreitigkeiten mit der US-Regierung beilegst und nach Utrecht kommen kannst. Super-Giffey, pass immer schön auf Caro auf und tu was sie Dir sagt (z.B. Kaffee holen). Toshi, you get the eukaryotic expression system running, good luck with your kinase. Fried, real biologist and godfather of worms, I hope you will get some c. elegant papers for all your effort. Andreas T., von Dortmund nach München, immer in Bewegung bleiben. Michael, Michael und Michael, das wird in letzter Zeit etwas inflationär, aber ich wünsche einen ähnliche Vermehrung des Erfolges. Und den Spö gab es ja auch mal, gönn Dir hin und wieder mal ein gut gekühltes Weißbier. HABA, Du bist der einzige der die HPLC neu programmieren konnte, Stromausfälle sind jetzt eine Qual. Bea, maybe you will still find a PDZ-GEF regulator, small compound or protein. You managed, that I go for dinner in Dortmund. Marc, Du hast Standards gesetzt, wer immer von Dortmund aus in die Industrie wechselt, wird jetzt nach x mal Saric-Gehalt beurteilt. Miranda, nice to have you back in Utrecht, although if it is just Fridays. Fons and Piet, I travelled with you to many Lab events, bedanked. Robbert, Holländer aus Dortmund, Du hast mich in Kontakt mit Epac und Utrecht gebracht, dankt you well. Özkan, Deine Arbeitszeiten sind wahrlich beeindruckend, Du solltest Dir mal eine Blutprobe abnehmen und Deine clock proteine sequenzieren, gibt garantiert ein paper. Partha, fouier transformation spectroscopy, no GTPase you would not go for. Peter, Herwig, Du gehörst zum Urgestein des Institutes, nichts gegen Deine Arbeit, aber ich hoffe Du bekommst niemals meine Lunge zu sehen. 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Rolf, 1000 Höhenmeter plus Schneesturm, wir waren in der selben Leistungsklasse und immer gut mit Schirm ausgerüstet. Boriana, der Schritt Deiner Schuhe macht Dich unverkennbar, man hört am Wochenende gleich ob Du im Institute bist oder nicht. Daniel, noch ein alter Bochumer, den es nach Dortmund verschlagen hatte. Zhao, you was a room mate in my first office. I really liked this office. Savi, have a good start in the world of companies, but never forget how much fun research could be. Alex, alter Wolf, wir haben so mache S-Bahn Fahrt miteinander verbracht. Wie war das Toxikologe in Singapur oder Australien? Australien hat den Vorteil, das jedes Viech das da rumkrabbelt das giftigste seiner Art weltweit ist. Kris, nobody reaches your speed in presenting her or his results during work discussion. Alex B., ich habe nie jemanden getroffen, der mit größerer Zielstrebigkeit, Hartnäckigkeit und Ausdauer ein Protein aufzureinigen gesucht hat. 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Christine, vielleicht kannst Du Deine stopped flow Messungen so verbessern, dass Du sie morgens um 8 startest und mittags noch mal vorbeikommst, um das Gerät auszuschalten. Quacki, alter Hintersitzer und Stadtexpressfahrer, bleib Deiner Geige treu, und halte mich auf dem laufenden, wenn ihr wieder Konzert habt. Andrea, der kleine Italiener, wie Fred Dich seiner Zeit angekündigt hat, ich bin sicher, Du klärst den Mechanismus Deines Enzyms auf. Wim, you keep things running. You was on holiday and what happens: breakdown of the power supply and a lot door openers are not working anymore. Agni, I always enjoyed to sit with you and Viktor in the graphics room. You are a very special Indian. Christiane, frühe Epac Mitstreiterin, allezeit gute Fahrt mit Motorrad und Wohnmobil, gut bereift solltest Du ja immer sein. John and Roland, the last two species of homo sapiens semaiensis, I hope you will still survive in our lab. Christian G, Dein Roboter ist mal was anderes in unserem Labor. Ulla, was wäre die Abteilung ohne den ständigen Nachschub an sauberem Glas. Beni, was soll ich denn dem Stieglitz sagen ohne das er es albern fände? Wendy, a new "borne" worm in our lab would call you mother, if it could speak. You keep the Worm facilities running! Katja, auch wenn

die Kernpore klein in Armstrong ist, ist sie doch ein großes Vorhaben, viel Erfolg. Jens, schwinge Dich auf zu neuen Zielen, mit dem selben Elan, den ich erlebte als wir zusammen im Büro saßen. Vielen Dank, für so manche Diskussion. Marion, das Angebot steht, wenn Arno Dich zu nervös macht, schick in zu meiner Feier, das gibt Dir ein paar Stunde Ruhe und am nächsten Tag darf er auch beim Aufräumen helfen . Lara und Oli, die Nummerierung der ganzen Repeateeinheiten verwirren mich bis heute, however, man kann sie ins ITC stecken. Prof. (endlich) Dr. Herrmann, Dortmund verliert einen Thermodynamiker und Kinetiker in Dir, aber ich hoffe, Du baust Dir was schönes in Bochum auf. Paulien, our Grisu the dragon, if you need a fire, let me know. Arjan, vriend van het mas-spec, lets do some nice experiments. Jürgen, Du bist der eisernste Fahrradfahrer Dortmunds, Du könntest ohne weiteres niederländische Staatsbürgerschaft beantragen, und der Aufdruck „Rauche führt zu einem qualvollen Tod“ auf den Zigarettenschalten gefällt Dir doch sicher.
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