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An activation specific antibody for the cAMP responsive guanine nucleotide exchange factor Epac

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An activation specific antibody for the
cAMP responsive guanine nucleotide exchange factor Epac

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 Abstract
Epac1 is a guanine nucleotide exchange factor for the small G-protein Rap. It consist of a regulatory region, which contains a cyclic nucleotide binding domain (cNBD), and a catalytic region, which contains a CDC25-homology domain (CDC25-HD). In the absence of cAMP the cNBD blocks the access of Rap to the catalytic side. Upon binding of cAMP the cNBD changes its position and liberates the catalytic side. Thus the inactive state of Epac can be considered as a closed conformation and the active state as an open one. Here we have generated an antibody against Epac1, which can bind only to active Epac. Surprisingly, this antibody can activate Epac in the absence of cAMP. The epitope in Epac recognised by the antibody was identified. The detailed characterisation of the antibody gave insights in the mechanism by which Epac is regulated.

In eukaryotes three classes of proteins exist, which contain cNBDs and whose activity is regulated either by cAMP or by cGMP. These are the cAMP and the cGMP dependent protein kinases PKA and PKG, respectively, cyclic nucleotide regulated ion channels and exchange proteins directly regulated by cAMP (Epac). Epac proteins are guanine nucleotide exchange factors (GEF) specific for Rap1 and Rap2[1,2]. Rap proteins belong to the Ras subfamily of small G-proteins. G-proteins cycle between a GDP-bound state, inactive in terms of signal transduction, and a GTP-bound state, which is active. Transition to the active state occurs by binding to GTP, which in the cell is more abundant than GDP, as soon as GDP dissociates from the G-protein. The dissociation of bound nucleotides is catalysed by GEFs, such as Epac. Transition back to the inactive state is a consequence of hydrolysis of GTP to GDP by the intrinsic GTPase activity of the G-protein. This intrinsic activity can be enhanced by GTPase activating proteins (GAP). Rap proteins are implicated in a variety of cellular processes, most notably integrin mediated cell adhesion[3-9] and cadherin mediated cell junction formation[10-13].

Two isoforms of Epac, Epac1 and Epac2 exist in mammalians. The C-terminal catalytic region of Epac1 and Epac2 consists of a REM-domain, a RA-domain and a CDC25-HD. The CDC25-HD is found in GEFs for G-proteins of the Ras family[14] and as an isolated domain is sufficient to catalyse nucleotide exchange[15]. CDC25-HDs are always accompanied by a REM domain[14], which has mainly a structural function in stabilising the CDC25-HD[16]. RA-domains are found in effector proteins of the Ras family. They bind specifically to the GTP-bound form of Ras family G-proteins[17]. Recently it was shown

that the RA domain of Epac2 binds H-Ras and mediates a specific membrane localisation of Epac2[18]. The regulatory region of Epac1 consists of a Dishevelled, Egl-10, Pleckstrin (DEP) domain and a cNBD. The DEP domain contributes to the membrane localisation of Epac, whereas the cNBD keeps Epac in an auto-inhibited state in the absence of cAMP[1]. In Epac2 an additional cNBD is found at the N-terminal of the DEP domain.

Insight in the mechanism, by which cAMP binding induces activation, was obtained from structural[19,20] and biochemical[21-23] characterisations of the protein. The cNBD acts as an auto-inhibitory domain, since constructs lacking the cNBD are constitutively active[1,22]. Furthermore the isolated cNBD is able to reversibly inhibit the activity of the catalytic region in trans. Based on these observations it was proposed, that the cNBD competes with Rap for binding to the catalytic side[22]. This model was to a large extent confirmed by the crystal structure of Epac2 in its auto-inhibited state[20]. Indeed, the cNBDs of Epac2 block the access of Rap to the catalytic side. The cNBDs are hardly directly contacting the catalytic side, which is involved in Rap binding. Instead, they are hanging over the catalytic side and are occupying the space, which would be required for Rap binding. The position of the regulatory region relative to the catalytic region is fixed by two contact points. Both points are likely to be affected by cAMP binding and thus upon cAMP binding the cNBD is liberated to leave its inhibitory position.

Here we have generated monoclonal antibodies against both Epac1 and Epac2. Well characterised antibodies are valuable tools for the investigation of biological functions of proteins. We found that one of our antibody directed against Epac1 detects only active Epac. The detailed characterisation of this antibody gave more detailed insights in the regulation mechanism of Epac proteins.

Material and Methods

Preparation of protein:
The following constructs of Epac1 (RapGEF 3, homo sapiens, GI: 3978530) and Epac2 (RapGEF 4, mus musculus, GI: 4185566) were expressed as GST-fusion (pGEX4T2 (Pharmacia)) in bacteria and purified as described previously[24]: Epac1ΔDEP (aa 149-881), cNBD of Epac1 (aa 149-328) and Epac2ΔDEP (aa 280-993). Mutations were introduced by QuickChange mutagenesis according to the procedure of Stratagene. For simplicity we are referring to Epac1ΔDEP and to Epac2ΔDEP as Epac1 and Epac2, respectively, unless indicated otherwise.

Rap was purified as described[24].

Antibody generation
Eight week old BALB/c mice were intraperitoneally injected with 50 μg of Epac1ΔDEP or Epac2ΔDEP, respectively, mixed with an equal volume of Freund's adjuvant complete (Sigma). In three week interval mice were boosted twice with the same amount of protein in Freund's adjuvant incomplete (Sigma). Two weeks after the last injection mice were boosted with 50 μg protein dissolved in PBS. Four days later the mice were scarified, the spleen were isolated and fused to myeloma SP2/0 cells. Single cell clones were isolated and identified by ELISA following standard protocols.

Western Blotting of recombinant protein (epitope mapping)
Recombinant proteins were purified to 95% (see above) as monitored by SDS-PAGE and Comassie staining. The concentrations of the proteins were determined by the method of Bradford. Defined amount of protein were subjected to SDS-PAGE, transferred to PVDF membrane, blocked in PBS containing 2% milk powder and 0.5% BSA, processed according to standard procedures and analysed with enhanced chemiluminescence (ECL) (Amersham Pharmacia).

Immuno-precipitation of recombinant protein
5D3 was immobilised on protein A beads in buffer containing 50mM TrisHCl, pH 7.5, 50 mM NaCl, 5% glycerol and 0.5% BSA. 50 µl of preloaded beads were incubated at room temperature in the presence of 30ng Epac proteins in the presence or absence of cAMP (for duration of incubation see figure legend). After incubation the beads were washed three times with the same buffer, re-suspended in loading buffer, boiled for 2 minutes and separated from the buffer by centrifugation. The obtained samples were subjected to SDS-PAGE and Western-Botting with 5D3 as primary antibody if not indicated otherwise.

Determination of Epac activity in vitro
Epac exchange activity was determined as described earlier[24]. In brief, Rap1B (aa 1-167) was loaded with the fluorescent analogue 2'-/3'-O-(Nmethylanthraniloyl)-guanosinediphosphate (mGDP) (BioLog Life Science, Germany) and incubated in the presence of Epac and varying concentrations of cAMP. The fluorescence signal was monitored in real time by the use of a Carry Eclipse (Varian). The fluorescence properties of mGDP are depending on the local chemical environment, to which the fluorescent group is exposed. Thus the intensity of the emitted fluorescence is roughly twice as high if mGDP is bound in the hydrophobic environment of a G-protein as if it is exposed to water molecules in the buffer solution. By incubating Rap loaded with mGDP in the presence of an excess of normal GDP the release of the bound mGDP is causing a drop in the fluorescence intensity, which can be measure in real time. The decay in the fluorescence signal (k_{obs}) is equal to the rate of nucleotide dissociation was plotted as a measure of Epac activity against the cyclic nucleotide concentration.

Results
Monoclonal antibodies against Epac1 and Epac2 were generated (material and methods). For each protein independent hybridoma cell lines were isolated, resulting in antibodies 5D3 and 4D9 against Epac1 and 2B12 and 5B1 against Epac2. To identify the epitopes recognised by the antibodies, recombinant Epac proteins containing point mutations were subjected to Western blot analysis. Most mutants were recognised with very similar efficiency (Fig. 1 and data not shown). Interestingly, Epac1ΔDEPL273W is neither detected by 5D3 nor by 4D9, indicating that both antibodies recognise similar epitopes surrounding L273 (Fig. 1B). The flanking sequence is fully conserved in Epac1 and Epac2 with the exception of Q270 in Epac1 that correspond to K405 in Epac2. Indeed, both 4D9 and 5D3 are able to detect Epac2ΔDEP K405Q with similar efficiency as Epac1ΔDEP (Fig. 1C).
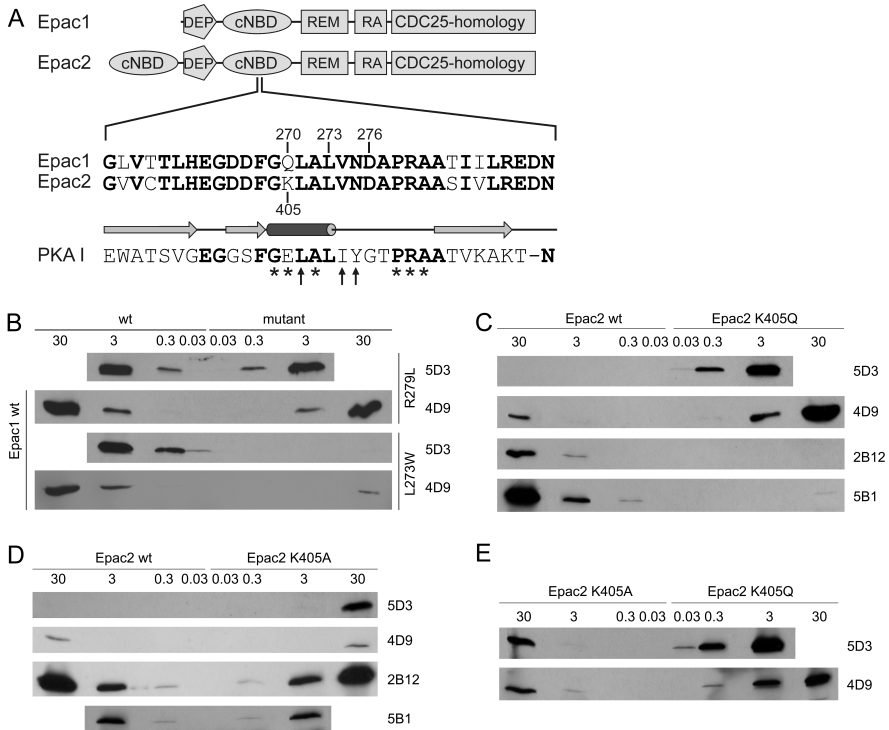
Fig.1 Epitope mapping.
(A) Domain organisation of Epac1 and Epac2. (DEP, Dishevelled, Egl-10, Pleckstrin; cNBD, cyclic nucleotide binding domain; REM, Ras exchange motif; RA, Ras association domain; CDC25-HD, CDC25-homololgy domain). A sequence alignment of the region, which is recognised by the antibodies, is shown (Epac1, homo sapiens; Epac2, mus musculus). Secondary structure elements are indicated below the alignment together with the corresponding sequence of protein kinase A (PKA I, first cNBD). Residues, which were subjected to mutagenesis are indicated by numbers. Residues, which are directly involved in interaction with cAMP, are marked by asterisk. Those residues of PKA, which interact with the kinase domain are highlighted by arrow. (B), (C), (D) and (E) 30, 3, 0.3 and 0.03 ng of recombinant protein were subjected to SDS page and Western Blotting with antibodies as indicated. In each blot, wt and mutated protein were loaded for comparison.

Surprisingly, the Epac2 specific antibodies 2B12 or 5B1 do not detect Epac2ΔDEP K405Q. Thus the epitopes of the Epac2 antibodies cover a very similar region as the epitope of the Epac1 antibodies. In Epac2ΔDEP K405A the positive charged lysine is replace by the chargeless and sterically simple alanine. Both, Epac1 and Epac2 antibodies detect this protein (Fig. 1D). Nevertheless 5D3 and 4D9 detect Epac2ΔDEP K405Q more efficiently than Epac2ΔDEP K405A (Fig. 1E). Thus the charge of the residue at position 270 (Epac1 numbering) is determining the specificity of the antibodies.

Next it was tested whether the antibodies are suitable for immunoprecipitation. 5D3 turned out to be the only antibody, which is able to precipitate Epac. Interestingly, if incubated for short times, 5D3 precipitates Epac1ΔDEP only in the presence of cAMP (Fig. 2A).
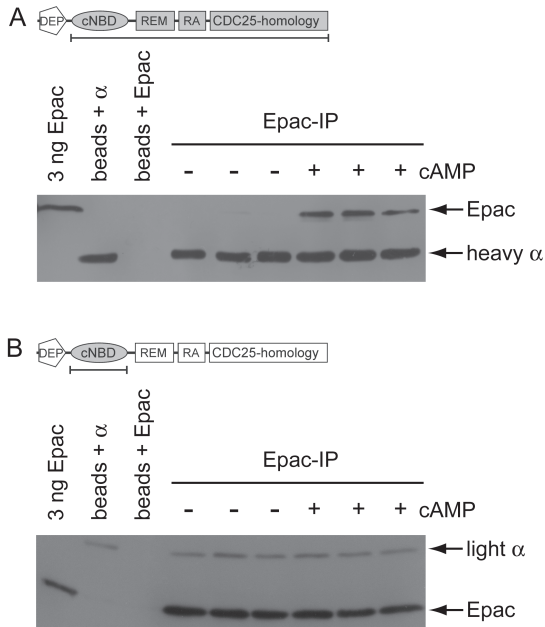
**Fig.2 cAMP dependent interaction between 5D3 and Epac1.**
Recombinant EpacΔDEP (A) or the isolated cNBD (B) were immuno-precipitated with 5D3, either in the presence or in the absence of cAMP. Epac protein was incubated with 5D3 immobilised on proteins A beads for 5 minutes. Experiments were done in triplicates. As a controls; lane 1, recombinant protein directly loaded to SDS-PAGE; lane 2, protein A beads pre-coupled with antibody prior to incubation with Epac are loaded to SDS-PAGE; lane 3, a precipitation reaction was carried out with protein A beads in the absence of antibody.

Thus 5D3 specifically bind to the active conformation of Epac. In contrast to Epac1ΔDEP the isolated cNBD is precipitated by 5D3 in the absence and the presence of cAMP with similar efficiency (Fig. 2B).

To gain more insight in the cAMP dependent interaction between 5D3 and Epac, the influence of 5D3 binding on Epac activity was measured. In the presence of saturating concentrations of cAMP the addition of 5D3 does not alter exchange activity of Epac
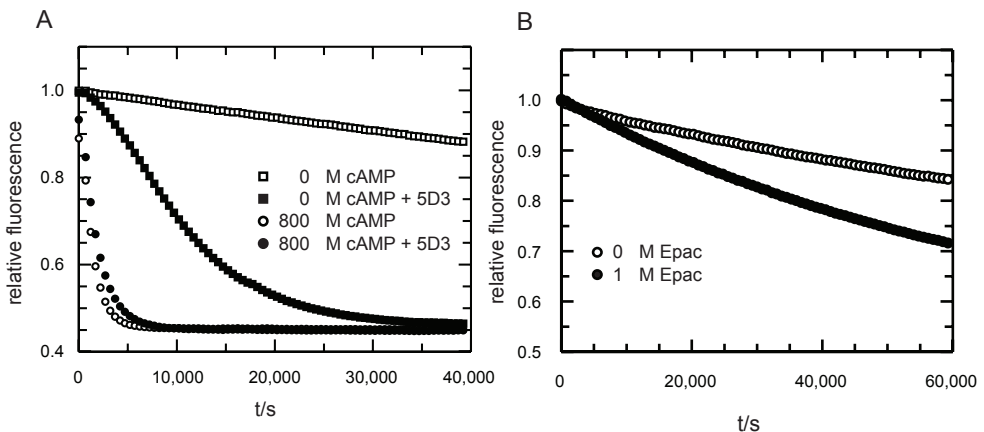

Fig.3 5D3 induces activation of Epac1.
The exchange activity of Epac (100 nM) towards Rap is measured in vitro. (A) 5D3 is added in the absence and presence of 800 uM cAMP to the exchange reaction. The cAMP induced activity of Epac is not altered in the presence of 5D3. In the absence of cAMP, 5D3 induces Epac activation itself. (B) Epac activity in the absence of cAMP.

towards Rap (Fig. 3A). However, in the absence of cAMP 5D3 induces Epac activity (Fig 3A). Epac exist in equilibrium between an inactive closed and an active open conformation as well as a cAMP free and cAMP bound state[23]. Thus, even in the absence of cAMP, a small fraction of Epac is in the open active conformation, as can be seen from the basal exchange activity of Epac in the absence of cAMP shown in Fig 3B. We hypotheses, that 5D3 can only bind to the open conformation and thus traps Epac in the active state. Indeed, 5D3 is able to precipitate Epac if both proteins are incubating together for one hour. Fig 4A shows an increase in amounts of Epac precipitated by 5D3 with increasing incubation time. Furthermore, addition of 5D3 to low concentrations of cAMP results in an amplification of the cAMP effect (Fig. 4B).

The results presented so far have shown that the epitope is covered by catalytic region in the absence of cAMP. Thus the epitope might contribute directly to the interaction between the regulatory and the catalytic region. Mutations introduced in the epitope region should therefore interfere with the activation behaviour of the protein. The epitope covers the phosphate binding cassette (PBC). The PBC is highly conserved in cNBDs and interacts with the phosphate sugar moiety of the cAMP[25]. Thus only those residues can be mutated, which are not directly involved in cAMP binding. The mutations Epac1ΔDEP D276R, D276P, D276W and N275Y/D276G/A277D were generated. These mutations do not abolish recognition by 5D3 or 4D9 (not shown), indicating that these mutations are not localised in the core of the epitope. The activation characteristics of these mutants were analysed in vitro (Fig. 5). Whereas cAMP binding is hardly influenced by the mutations as demonstrated by an AC_{50} very similar to that of wt Epac1, the maximal activity induced by cAMP, k_{max}, is drastically reduced. Thus, in these mutants cAMP binding is decoupled from activation.
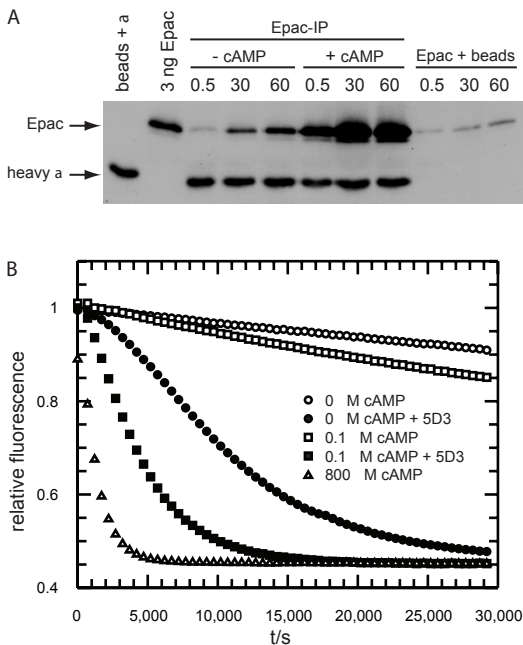
Fig.4 Slow kinetics of 5D3 binding to Epac1 in the absence of cAMP.

(A) EpacΔDEP was incubated in the presences of 5D3 immobilised on protein A beads. As the indicated points in time aliquots were taken and the beads were washed immediately. (B) Activity of Epac was monitored as in Fig.3. 5D3 was added to EpacΔDEP in the presence and absence of different concentration of cAMP.
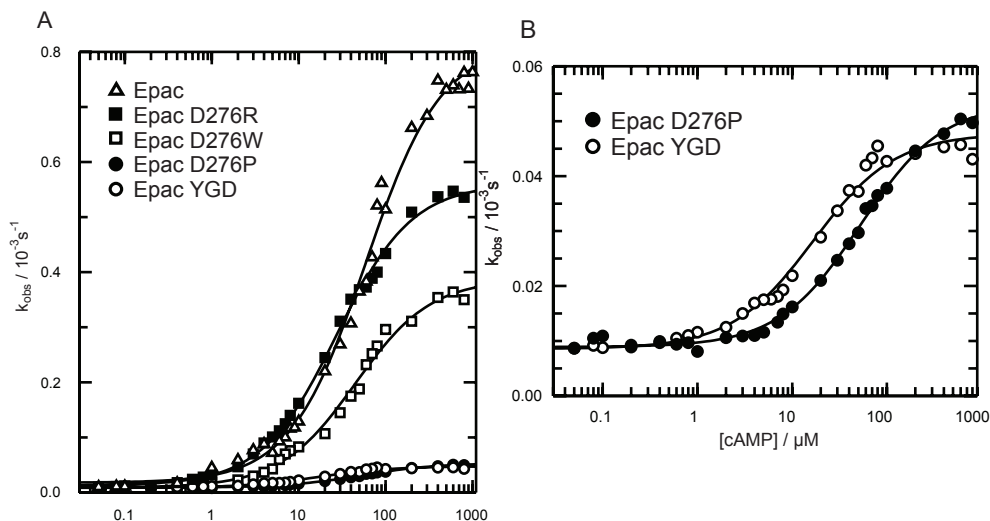
Fig.5 Mutations in the epitope region interfere with cAMP mediated regulation.
The cAMP dependent activity of Epac and Epac mutated in the epitope region were analysed. Exchange activity is plotted against the cAMP concentration. Epac YGD, Epac1ΔDEP N275Y/D276G/A277D.

Discussion

Several monoclonal antibodies against Epac1 and Epac2 were generated and characterised. The Epac1 specific antibody 5D3 is sensitive to the activation state of Epac. Whereas efficient precipitation of EpacΔDEP requires cAMP, the isolated cNBD precipitated with the same efficiency in the presence and absence of cAMP. Thus, 5D3 binds to that part of the surface in the cNBD, which is masked in the absence of cAMP by the catalytic region. Current models of Epac activation predict large intramolecular rearrangements associated with changes in the accessible surface area. Structural insights in this process were obtained from the crystal structure of Epac2 in its auto-inhibited state. The regulatory region of Epac2, namely cNBD1 and cNBD2, are blocking the access of Rap to the catalytic site in the CDC25 homology domain[20]. Upon cAMP binding the regulatory region rearranges and moves away from its inhibitory position.

The ability of 5D3 to trap Epac in the active or open conformation and to induce thereby Epac activation in the absence of cAMP is in agreement with the following model of Epac activation. Epac exist in equilibrium between a closed inactive and an open active conformation[23] (Fig. 6). Cyclic nucleotide binding shifts this equilibrium to the active conformation. Also in the absence of cAMP a small fraction of Epac exist in the active conformation, as demonstrated by a low activity of Epac in the absence of cAMP (Fig 3). In this small fraction the epitope is accessible to 5D3. Binding of 5D3 to this fraction has two consequences. (i) The antibody bound Epac is trapped in the active conformation since the bulky antibody prevents it from closing. (ii) The antibody does not interfere with the interaction between Epac and Rap and thus 5D3 bound Epac is catalytically active (Fig. 6). In agreement with this model, 5D3 induced nucleotide exchange can not be described as
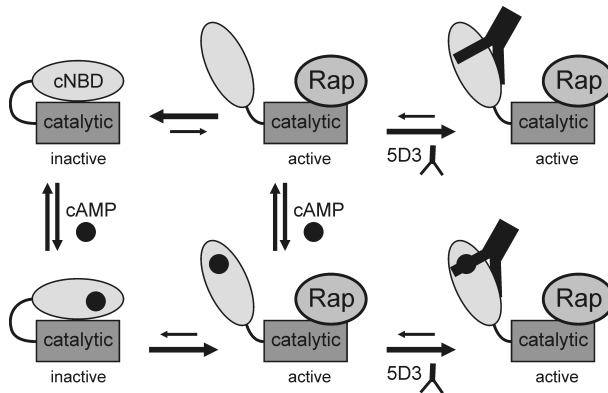
Fig.6 Equilibria in Epac activation.
5D3 can bind Epac only, if Epac is in the active open conformation. In the absence of cAMP only a minor fraction of Epac is in the active open conformation. After binding of cAMP the open active conformation is favoured. The open active conformation of Epac is trapped by 5D3.

a single exponential decay (Fig. 3A) as it is the case for normal cyclic nucleotide induced Epac activation[24]. Instead it is characterised by an initial lag phase. The lag phase reflects the slow increase in the concentration of active Epac, which is determined by the slow opening of Epac. This experiment demonstrates that cAMP acts by increasing the opening rate of Epac rather than inhibiting the closing process. Addition of cAMP results in a fast binding process and once cAMP bound Epac opens quickly, since no lag phase is observed as in the case of 5D3.

The epitope of 5D3 was identified by point mutations, which abolish the interaction with the antibody. The epitope partially covers the phosphate binding cassette (PBC) of the cNBD. The PBC is a highly conserved sequence motive in cNBSs[25]. It is directly involved in the interaction with the phosphate sugar moiety of the bound cyclic nucleotide[26]. Interestingly, the data presented here suggest, that the PBC is not solvent accessible in the absence of cAMP and that the PBC might be directly involved in an interaction with the catalytic region. To investigate the function of the PBC in the activation process in more detail, we generated Epac1 point mutations and analysed their activation characteristic in vitro. The choice of residues subjected to mutational analysis was limited to those, which are not directly involved in cAMP binding. Unaffected cAMP binding was demonstrated by AC_{50}-values similar to wt protein. However, k_{max} is for some mutants drastically reduced. k_{max} is a measure for the ability of the bound nucleotide to shift the equilibrium to the active conformation[23]. Thus, independent of cAMP binding itself, the PBC plays an important role in the translation of cAMP binding into activation.

It is interesting to correlate these findings with the structural data available. Structural information on Epac is limited to the crystal structure of the auto-inhibited Epac2, whereas the study presented here deals mainly with Epac1. The major difference between Epac1 and Epac2 is the additional N-terminal cNBD in Epac2. The cNBD of Epac1 corresponds to the second (C-terminal) cNBD of Epac2. The region in Epac2 corresponding to the epitope of 5D3 is covered by the N-terminal cNBD and indeed the first cNBD is expected to move relative to the second cNBD upon cAMP binding. However, Epac1 does not contain a N-terminal cNBD and thus an other part of the protein must shield the epitope in the absence of cAMP. This seems to be the catalytic region, since the cNBD alone is equally well assessable to the antibody in the absence and the presence of cAMP (Fig. 2). As already

discussed, the cNBDs of Epac2 exclude the access of Rap to the catalytic side, whereby the first cNBD has a major contribution. It might well be that in Epac1 the cNBD is more tilted towards the catalytic region to block additional space, which is occupied in Epac2 by the first cNBD. Interestingly, it was shown for protein kinase A (PKA), that the PBC is directly interaction with the catalytic subunit[27]. Thus it seems likely that the relative orientation between the regulatory and the catalytic region varies in detail between Epac1 and Epac2.

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Addendum

Characterization of monoclonal antibodies against Epac1

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cAMP is produced as a common second messenger from ATP by adenylate cyclase in response to a variety of extra-cellular signals, including hormones, growth factors and neurotransmitters. It regulates many cellular processes such as cell division, growth, differentiation, secretion and neoplastic transformation [1-5]. Protein kinase A (PKA) is a direct downstream target of cAMP and it was long thought that the majority of cAMP-dependent effects were executed by PKA. However, the identification of Epac (exchange protein directly activated by cAMP) opened a new window for cAMP research and prompted us to reconsider the effects of PKA. Epac is a guanine nucleotide exchange factor for the small GTPase Rap1 and Rap2 [6,7]. By using the cAMP analogue 8-pCPT-2'-O-Me-cAMP (007) that specifically activates Epac but not PKA[8], a number of biological processes have been assigned to Epac1, such as integrin-mediated inside-out signaling, E-/VE-cadherin-mediated cell-cell contact formation, insulin secretion and regulation of sodium-proton exchange activity[9-13].

In order to provide tools for further analysis of Epac, a series of Epac1 and Epac2 mAbs were generated and characterized. In chapter 3, one of these Epac1 mAbs, 5D3, which specifically recognizes the active conformation of Epac1, has been characterized extensively. Here we present a brief overview of the characterization of the other antibodies, which were simultaneously generated as well as some additional analysis of the 5D3 antibody.

RESULTS

A series of mAbs were generated by injection of recombinant Epac1ΔDEP or Epac2ΔDEP protein into mice (see chapter 3, materials and methods for further details). Four positive wells with Epac1 and five wells with Epac2 hybridomas were chosen for further sub-cloning and characterization using tissue culture supernatants. Cell lysates of A14 cells transfected with either the regulatory or the catalytic region of Epac1 were subjected to Western blotting. In agreement with the results described in chapter 3, most Epac1 antibodies strongly recognize the regulatory region of Epac1. Interestingly, 1C8 recognizes the catalytic region (fig. 1A and table 1). However 1C8 and 4D9 are not Epac1 specific since they cross react with Epac2 (Fig. 1B and Chapter 3 Fig. 1C). All the other antibodies are either Epac1 or Epac2 selective and can be used to detect Epac from different species (table 1).
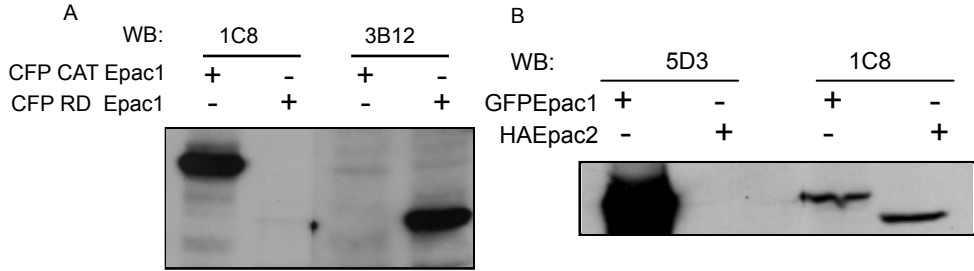
Figure 1. Specifity of Epac1 mAbs on Western blot.
(A) Total cell lysates from A14 cells, which had been transfected with either the regulatory or the catalytic region of Epac1 were blotted and probed with the mAb 1C8 (left strip) or 3B12 (right strip). (B) Total cell lysates from A14 cells, which had been transfected with either GFP tagged Epac1 or HA tagged Epac2 were blotted and probed with the mAb 5D3 (left strip) or 1C8 (right strip).

In our original experiments, to test the capacity of 5D3 to recognize Epac1 in immunofluorescence studies we made use of MCF-7 cells, stably transfected with GFP-tagged Epac1. We observed that GFP-tagged Epac1 is mainly distributed around the perinuclear region and the plasma membrane. However, in paraformaldehyde fixated specimens, 5D3 can only detect GFP-tagged Epac1, when located at the plasma membrane (Fig. 2 upper panel). Strikingly, both plasma membrane and perinuclear localized GFP-Epac1 was detected with 5D3 after fixation of the cells with acetone (Fig. 2 lower panel), which denatures proteins more dramatically compared to paraformaldehyde. Since no GFP degradation products were observed on Western blots, we conclude that mAb 5D3 is suitable for immunofluorescence studies and that depends on the fixation method used, distinct pools of Epac1 can be discerned. Whether recognition of Epac1 by 5D3 in paraformaldehyde fixated specimens reflects activation of Epac1 as shown in chapter 4, is at this point hard to judge for in FRET studies (chapter 2) no indications were found for

Table 1. Overview on the specificity and isotype of the different Epac monoclonal antibodies.

| Antigen	MAbs	Cross-reactivity	Epitope localization	Isotype #	WB				IP	IF
					Human	Mouse	Rat	Dilution		
Epac1	1C8	Yes	C- part	IgG2a	+	+	ND	1:1000	-	-
	3B12	ND	R-part	IgG2a	+	ND	ND	1:1000	-	-
	4D9 *	Yes	R-part	IgG2a	+	ND	ND	1:1000	-	-
	5D3 *	No	R-part	IgG2a	+	+	ND	1:10000	+ 1ul/ip	+ 1:500
Epac2	1D5	ND	R-part	IgG1	ND	+	ND	1:1000	-	-
	2B12 *	No	R-part	IgG2a	ND	+	+	1:1000	-	-
	3C12	No	R-part	IgG2a	ND	+	ND	1:1000	-	-
	4A3	ND	R-part	IgG2a	ND	+	ND	1:1000	-	-
	5B1 *	No	R-part	IgG2a	ND	+	+	1:1000	-	-
Notes: C-part/R-part: Catalytic/Regulatory part of Epac; +: Ab works; -: Ab does not work; WB: western blotting; IP: Immunoprecipitation; IF: Immunofluorescence. ND: not dertermined										

* For a more detailed characterisation of these Epac1/Epac2 mAbs, please check chapter 3.
# Isotype identification was performed by using Mouse Monoclonal Isotyping reagents kit (Sigma).

Figure 2. Immunofluorescence of MCF7 cells, stably expressing GFPEpac1 using the monoclonal antibody 5D3.
Upper panels: visualization of GFP tagged Epac1 by direct fluorescence of GFP or indirect using 5D3 in cells, which have been fixed with paraformaldehyde. Note that 5D3 does not stain the nuclei. Lower panels: visualization of GFP tagged Epac1 by direct fluorescence of GFP or indirect using 5D3 in cells, which have been fixed with acetone. Note that here 5D3 staining includes the nuclear membrane.
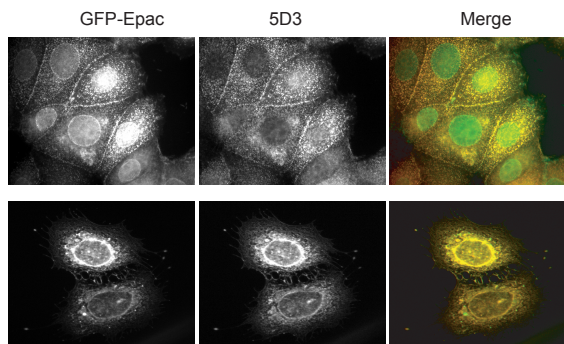
subcellular differences in the activation state of Epac. An alternative and perhaps more likely explanation could be that in the perinuclear region the Epac1 epitope is shielded by interaction with the other proteins, also located at the perinuclear membrane.

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