Subcellular localisation of Epac

Subcellulaire lokalisatie van Epac

(met een samenvatting in het Nederlands en Chinees)

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Jun Zhao

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Promotor: Prof. Dr. Johannes L. Bos

Department of Physiology Chemistry
and Centre for Biomedical Genetics
University Medical Center Utrecht,
Utrecht, The Netherlands

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博士毕业论文

Epac 蛋白的亚细胞定位

Subcellular localisation of Epac

Subcellularie lokalisatie van Epac

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导师：Johannes L. Bos 教授

乌特勒之大学，生理化学系，信号转导研究组

二零零六年九月十二日

谨以此书献给

养育我的父母

以及我的丈夫和女儿

for my family

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Abbreviations:

AC: Adenylate cyclase
AF-6: Acute lymphoblastic leukemia 1 fusion partner from chromosome 6
AKAP: A-kinase anchoring protein
ATF1: Activating transcription factor 1
ATP: Adenosine trisphosphate
cAMP: Cyclic adenosine 3',5'monophosphate
CAT: Catalytic domain of Epac
CBP: CREB binding protein
CDC25: Cell division cycle 25
CFP: Cyan fluorescence protein
CICR: Ca^{2+}-induced Ca^{2+} release
CNG channels: Cyclic nucleotide-gated cation channels
cNBD: cNMP binding domain
CREB: cAMP responsive element-binding protein
CREM: cAMP response element modulator
DAG: Diacylglycerol
DEP: Disheveled, Egl-10, Plekstrin
E6TP1: High-risk human papilloma viruses E6 oncoproteins targeted protein 1
EBP50: ERM binding phosphoprotein 50
EGF: Epidermal growth factor
EM: Electron microscope
Epac: Exchange protein directly activated by cAMP
ERM: Ezrin, Radixin and Moesin
FERM: Four-point-1 ERM
ERK(5): Extracellular signal regulated kinase (5)
EzB: Ezrin binding
FRET: Fluorescence resonance energy transfer
GAPDH: Glyceraldehyde phosphate dehydrogenase
GAPs: GTPase activating proteins
GEF: Guanine nucleotide exchange factor
GFP: Green fluorescence protein
Gi: Inhibitory G protein
007: 8-pCPT-2'OMe-cAMP
GLP-1: Glucagon-like peptide-1
Gs: Stimulatory G protein
GST: Glutathion S-transferase
HA: Haemagglutinin
HGF : Hepatocyte growth factor
ICAM-1: Intercellular adhesion molecule 1
IP3-R: Inositol triphosphate receptor
LFA-1:Leukocyte function- associated molecule-1
mAbs: Monoclonal antibodies
MAGI-1: MAGUK with inverted domain structure-1
mAKAP: Muscle-specific A-kinase anchoring protein
mantGDP:2'.3'-bis(O)-N-methy-lantharanoloyl guanosine diphosphate
MAPK: Mitogen activated protein kinase
NHE3: Sodium-proton exchanger 3
NLS: Nuclear localization signal
PBC: Phosphate binding cassette
PDEs: phosphodiesterases
PDZ: PSD-95/DlgA/ZO-1
PKA: Protein kinase A
PKB: Protein kinase B
PLCζ: Phospholipase Cζ
pAb: Polyclonal antibody
R9AP: RGS9 anchoring protein
RA: Ras association
RanBP2: RAN binding protein 2
RBD: Ras binding domain
RD: Regulatory domain of Epac
REM: Ras exchange motif
RGS9: Regulator of G-protein signaling 9
Rim2 : Rab3-interaction molecule 2
RYR: Ryanodine receptor
S1P: Sphingosine-1-phosphate
siRNA: Small interfering RNA
SPA1: Signal induced proliferation association protein 1
YFP: Yellow fluorescence protein

1
General Introduction

Cyclic AMP

Cyclic adenosine 3', 5'- monophosphate (cAMP) was the first second messenger to be identified[1] and plays an integral role in various physiological processes such as gene transcription, neuronal functions, cardiac muscle contraction, vascular relaxation, and cell proliferation[2,3,4,5]. It is synthesized from ATP by a plasma membrane-bound enzyme, adenylate cyclase, and is rapidly hydrolyzed by one or more cyclic AMP phosphodiesterases (PDEs) into adenosine 5'-monophosphate (5'AMP)[6,7]. Adenylate cyclase is a large multipass transmembrane protein with its catalytic domain at the cytosolic side of the plasma membrane. It can be activated by a stimulatory G protein (Gs) and inhibited by an inhibitory G protein (Gi). There are at least eight isoforms in mammals, most of which are regulated by both G proteins and Ca^{2+}. Each isoform consists of two hydrophobic domains and two catalytic cytoplasmic domains (C1 and C2). The C1 domains are highly homologous to the C2 domains and can form an intramolecular heterodimer with C2 domain. The correct formation of the dimer is required for the catalytic activity, since the active site is located at the C1/C2 interface[8]. PDEs can be activated among others by calcium through calcium calmodulin[9], the insulin receptor[10] and PKA[11]. Inhibition of the catalytic activity of PDE is achieved among others by mitogen activated protein kinase (MAPK)-dependent phosphorylation[12] (Fig1).

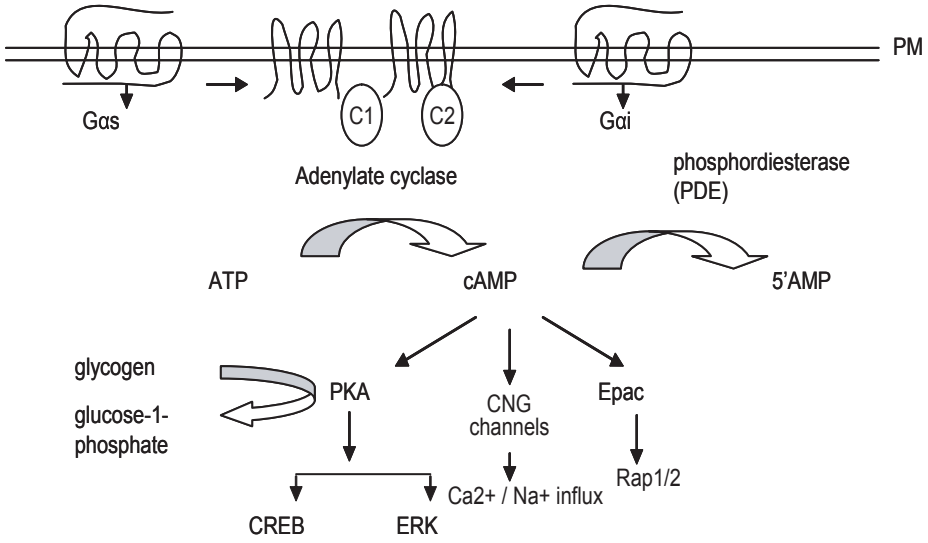

Figure 1. Outline of cAMP generation and its related signaling pathway.
Adenylate cyclases synthesize cAMP from ATP. Phosphodiesterases converts cAMP to 5'AMP. PKA, Epac and CNG channels are cAMP targets, however, they have different downstream effectors and are involved in different signaling pathways.
cAMP: Cyclic adenosine3', 5'- monophosphate, 5'AMP: Adenosine 5'-monophosphate, CREB: cAMP responsive element-binding protein, Epac: Exchange protein directly activated by cAMP, ERK: Extracellular signal-regulated kinase, PM: Plasma membrane.

PKA

The cAMP–dependent serine/threonine protein kinase (PKA) was one of the first kinase to be identified [13]. This protein consists of two catalytic subunits (C) and two regulatory subunits (R)[14]. Each regulatory subunit contains an N-terminal dimerisation domain, two cAMP binding sites (A and B) and a hinge region. The cAMP-A domain interacts directly with the catalytic subunit and is essential for the stable binding of the regulatory subunit to the catalytic subunit. The cAMP-B domain does not contact the catalytic subunit, but it modulates access of cAMP to the cAMP-A domain. The hinge region contains an autoinhibitory sequence which can directly contact the C subunit and prevent substrate binding[15]. Binding of cAMP to the cAMP-B domain induces a conformational change, upon which the cAMP-A domain becomes accessible to cAMP. This causes the dissociation of the complex, thus releasing the catalytic subunits. The released catalytic subunits subsequently phosphorylate their substrates. A-kinase anchoring proteins (AKAPs) are scaffolds that organize complexes to determine the precise location and timing of signal transduction events upon the activation of cell surface receptors[16]. They provide a platform for the coordination of phosphorylation and dephosphorylation events by sequestering enzymes such as protein kinases and phosphatases with their appropriate substrates. AKAPs can bind to PKA by forming a dimer between the hydrophobic face of a conserved amphipathic helix within AKAPs and an N-terminal four-helix bundle in the regulatory subunit (R) of PKA[17,18]. Additionally, a distinct region of AKAP contains a targeting sequence that serves to tether the complex to a specific subcellular compartment[19,20]. Anchoring of the kinases not only facilitates localized activation of the PKA catalytic subunit (C) following elevation of the second messenger cyclic AMP (cAMP) [21], but also facilitates interactions of PKA with its specific substrates. In addition, AKAPs can assemble proteins involved in fine-tuning of the signal event. For instance, muscle-specific A-kinase anchoring protein (mAKAP) can tether PKA, PDE4D3, ERK5 and Epac1, leading to the local control of cAMP levels[22-24]. Besides targeting PKA in certain compartments, AKAPs also can maintain substrate-specific complexes in association with a variety of ion channels[25-27] and serve to position distinct signaling complexes at or on a given organelle[28-30] (Fig2).

PKA controls many physiological processes, with the conversion of glycogen to glucose-1-phosphate being a typical example of a PKA short-term regulatory effect [31,32]. Another important effect of PKA is the regulation of gene transcription, a long-term regulatory effect. The CREB (cAMP response element binding protein) family of transcription factors are well-known targets of PKA. This family includes the cAMP responsive element-binding protein (CREB), the cAMP responsive element modulator (CREM), and the activating transcription factor (ATF1). As a result of phosphorylation by PKA, the CREB dimer interacts with DNA at the cAMP response element (CRE). Gene transcription starts upon binding of co-activators such as CREB binding protein (CBP) and p300, enabling both proteins to interact with the phosphorylated form of CREB and direct the transcriptional machinery situated at the TATA box[33].

PKA can also regulate cell growth via mitogen–activated protein (MAP) kinase, also known as extracellular signal-regulated kinase (ERK). ERK signaling couples growth factors to

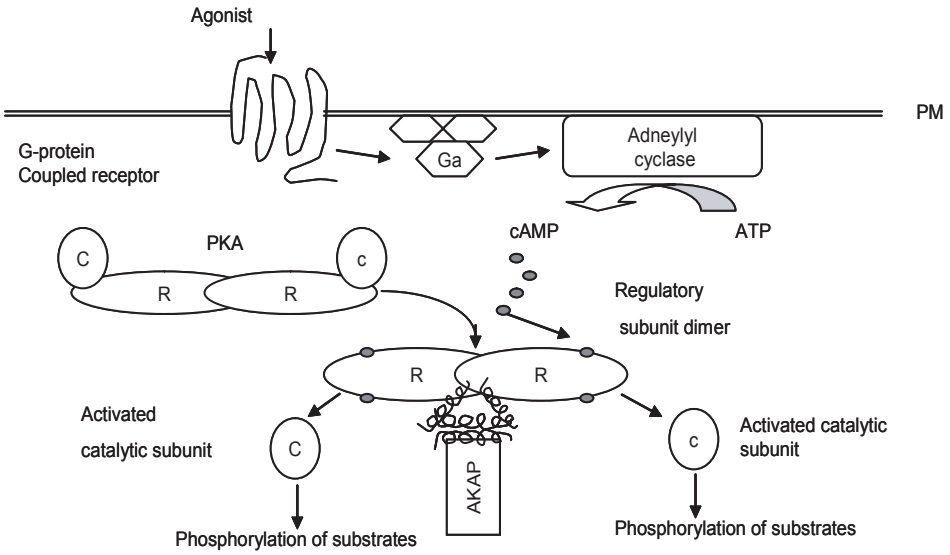

Figure 2. Overview of PKA activation

PKA is a cAMP–dependent serine/threonine protein kinase. It contains two regulatory (R) and two catalytic (C) subunits. Each regulatory subunit contains two cAMP binding sites (cAMP-A and cAMP-B). Binding of cAMP to the cAMP sites induces the activation of PKA. As a consequence the catalytic subunit is released and can interact with its substrate. A-kinase anchoring proteins (AKAPs) dock PKA at the plasma membrane by interacting with the regulatory part of PKA.

PKA: Protein kinase A, C: Catalytic subunit, R: Regulatory subunit, PM: Plasma membrane,

AKAP: A-kinase anchoring proteins.

cell proliferation through the GTPase Ras. Activated Ras binds to and activates its effector Raf1. Activated Raf1 phosphorylates and activates MEK, which in turn phosphorylates and activates ERK[34].

It has long been appreciated that cAMP inhibits cell growth by blocking growth factor-mediated activation of ERKs. cAMP and PKA have been linked to inhibition of the ERK cascade in many cell types[35-39]. PKA can block Ras-dependent signals to ERKs by blocking Raf activation[37] and PKA phosphorylation seems to inhibit Raf activity directly[40]. For example, phosphorylation of serine 43 on Raf-1 by PKA prevents Raf-1 binding to Ras in fibroblasts[38]. Recently, PKA-induced phosphorylation events were also implicated in the inhibition of Ral[41,42].

In contrast, cAMP can also stimulate ERKs in diverse cell types[43-45]. When cAMP activates ERK, it stimulates cell differentiation as well as proliferation within the same cell. In Schwann and kidney cells, lower concentrations of cAMP induced proliferation through ERKs, whereas higher concentrations induces sustained activation ERKs as well as expression of markers of differentiation[46,47]. In neuronal cells, the coupling of cAMP/PKA to ERKs might also depend on the developmental stage and they are also required for changes in synaptic plasticity induced by neuronal activity and depolarization[48,49].

However, cAMP induced ERK activation that occurs independently of PKA has also been described in retinal cells, FRTL5 cells and bone cells[35,50,51]. This suggested the involvement of Epac and Rap1 as mediators in cAMP-induced PKA-independent signaling pathway.

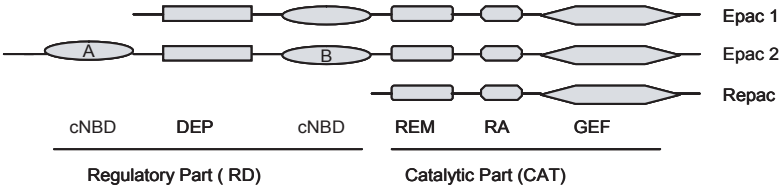

Figure 3. Structure of Epac proteins
DEP: Disheveled, Egl-10, Plecktrin domain; cNBD: cNMP binding domain; REM: Ras-exchange motif; RA: Ras association domain; GEF: guanine nucleotide exchange factor.

Protein kinase A (PKA) was the first target of cAMP to be discovered[13,52] and it was thought to be responsible for all the cAMP mediated effects. However, identification of another cAMP target, exchange protein directly activated by cAMP (Epac) opened a new window for understanding cAMP mediated PKA independent biological effects.

Epac

The Epac proteins were first identified in 1998[53,54]. This family consists of three members: Epac1, Epac2, and Repac. Epac1 is widely expressed and is enriched in human kidney, ovary, brain, and skeletal muscles, Epac2 is mainly expressed in the brain and adrenal gland[54,55], and Repac is strongly expressed in the human brain[56]. Both Epac1 and Epac2 consist of a regulatory and a catalytic part, however, Repac only contains a catalytic part and is kept in a constitutively active conformation[55]. The regulatory part of the Epac protein contains a cNMP binding domain and a Disheveled, Egl-10, Plekstrin (DEP) domain. The catalytic part consists of a REM domain, RA domain, and a CDC25 homology domain. The CDC25 domain mediates guanine nucleotide exchange activity towards the small GTPase Rap1 and Rap2. The REM domain is not required for catalytic activity of Epac, but it was thought to stabilize the CDC25 domain[57]. The RA domain of Epac2 has been demonstrated to associate with active H-Ras, thus facilitating Epac2 targeting to the plasma membrane[58]. The RA domain of Repac has been found to bind to GTP-bound M-Ras in vitro[59]. Nevertheless, the function of the RA domain of Epac1 is still elusive (Fig 3).
The activity of Epac depends on the binding of cAMP to the regulatory domain[53,55]. This induces a conformational change of Epac proteins, thereby opening up the catalytic domain of Epac to allow binding of Rap[60-62]. The crystal structure of the Epac2 regulatory domain in the absence of cAMP revealed that the phosphate binding cassette (PBC; a highly conserved structure in cNMP binding domain which interacts with the phosphate-sugar region of cAMP) and the orientation of the hinge region (which connects the cAMP binding pocket and the lid which covers the cAMP binding pocket) suggested a large conformational change after cAMP binding[60]. In the absence of cAMP binding, a conserved leucine residue within the PBC restricts the orientation of the hinge in the regulatory domain of Epac2, thereby preventing the hinge from moving closer to the cAMP binding domain. In contrast, binding of cAMP attracts the PBC, reorients the invariant leucine residue and induces a large movement of the hinge and also the C terminal regions. Consequently, the C terminal region of the hinge forms a lid to interact with cAMP to cover and stabilize the cAMP base-binding stie. So, the small conformational change within the PBC region could be

transmitted via the hinge region into a large and extended structural change of the C-terminal lid, thereby exposing the catalytic domain of Epac and facilitating activation of Rap.
There is an extra cNMP binding domain (cNMP-A) in the Epac2 protein structure compared to Epac1 (Fig. 3), but it has lower binding affinity (70uM) for cAMP compared to the other cNMP binding domain (cNMP-B domain) of Epac2 (1uM) and it is not required for the regulation of Epac2 by cAMP[55]. The function of cNMP-A in Epac2 is still unclear.
Both Epac1 and Epac2 proteins contain a DEP domain, which was supposed to play a role in the correct targeting of the Epac protein to certain membrane compartments. Indeed, deletion of the DEP domain of Epac abolished the membrane association of Epac[55,63] and also reduced its nucleotide exchange activity toward Rap1[64,65]. Moreover, Martemyanov et al. showed that the DEP domain of a photoreceptor-specific signaling protein, RGS9 (the regulator of G-protein signaling 9), plays an essential role in its delivery to the intracellular compartment where it is functional. In addition, deletion of the DEP domain of RSG9 abolished the interaction with a transmembrane protein, R9AP (RGS9 anchoring protein), known to anchor RGS9 at the surface of photoreceptor membranes. These findings indicate that a novel function of the DEP domain is targeting signaling proteins to a specific compartment of highly polarized cells. Interestingly, sequence analysis of R9AP reveals the presence of a conserved R-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) motif, this predicts the possibility that DEP domains might serve to target various DEP-containing proteins to the sites of their intracellular action via interactions with the members of extended SNARE protein family[66]. Whether the DEP domain of Epac also targets Epac at its correct position by association with specific membrane anchors remains elusive.

Cyclic nucleotide-gated cation channels (CNG channels)

CNG channels were discovered in the plasma membrane of the outer segment of rod photoreceptors in vertebrates, in which they are essential for generation of the primary electric signal in photoreceptor response to light[67]. However these channels are widely expressed in the central nervous system and also in some tissue types such as kidney, heart muscle and liver[68,69]. CNG channels are nonselective cation channels that mediated Ca^{2+} and Na^{+} influx in response to the direct binding of intracellular cyclic nucleotides[70]. The channels include pacemarker voltage-gated potassium channels or other channels which are involved in the transduction of sensory signals[71,72]. Very low concentrations of cAMP or cGMP are sufficient to directly bind and modulate the activity of ion channels[73,74].

Rap

The Ras superfamily of small GTPases consists of 13 families, which includes over 100 proteins. These members share similarities in their GTP binding domains. Ras-like small GTPases function as molecular switches, cycling between GTP- and GDP- bound forms. The switch is activated by a guanine nucleotide exchange factor (GEF) which interacts with the GTPase and induces the release of GDP and the binding of GTP. Binding of GTP causes a conformational change of the protein and allows the binding of downstream effectors. The cycling between GDP and GTP also results in the translocation of proteins from the

cytoplasm to cytoplasmic surface of the membrane and active effectors, thus inducing the activation of signaling cascades[75,76]. The switch can be turned off through hydrolysis of GTP by the intrinsic GTPase activity, which is stimulated by GTPase activating proteins (GAPs)[77-79]. Consequently, the interaction between GTPase and its effectors is abolished and results in the termination of the signaling cascade.
Ras is the best studied member of the Ras superfamily, it is found to be mutated in 15% of all human tumors[80]. Rap1 was originally identified as a protein which reversed the morphologic transformation of the v-Ki-ras-expression NIH3T3 cell line[81]. Because the effector domain of Rap1 was virtually identical to that of Ras, it was suggested that Rap1 inhibited the effect of Ras through the formation of an inactivated complex with the effectors of Ras[82].
The Rap family includes four genes, encoding Rap1A, Rap1B, Rap2A, and Rap2B. Rap1A and Rap1B are about 95% identical in amino acid sequence, whereas Rap2A and Rap2B are 90% similar[83]. Rap1 and Rap2 proteins share approximately 70% amino acids[84]. Subcellular localization of Rap family members was studied in fibroblasts using a specific anti-Rap1 affinity-purified antibody. Both Rap1A and Rap1B are located at late endocytic compartments (late endosome / lysosome), whereas Rap2A colocalized with several markers of the Golgi complex[85]. Both Rap1A and Rap1B were also observed within nuclei in a human oropharyngeal SCC (squamous cell carcinoma) cell line[86]. Rap1 is activated by a large variety of stimulus, including stimulus that activate receptor tyrosine kinases and serpentine receptors. Common second messenger like calcium, diacylglycerol, and cAMP are frequently mediating this activation[87]. Using FRET-based Rap1 activation probe, it was found that upon epidermal growth factor (EGF) stimulation, Rap is activated at intracellular perinuclear region in COS-1 cells, and the timing of Rap activation at perinuclear regions is dependent on Rap GEFs. However, RapGAP dictates the spatial activation of Rap[88,89]. By using GFP-tagged Rap, another group showed that Rap is enriched at the plasma membrane (PM) and endosomes instead of the Golgi apparatus. Furthermore, activated Rap was located predominantly at the plasma membrane[90].

Besides Epac, Rap can also be activated by a number of other GEFs. C3G was the first RapGEF to be identified[91]. This GEF can constitutively associated with Grb2, Crk1 and CrkII and other Crk-like protein through its proline-rich sequence[92], and expression of Crk enhances the GEF activity of C3G toward Rap[93]. C3G is regulated by complex formation and membrane localization[91]. It has been suggested to be involved in cell adhesion and cell migration through Rap1[91,94-96]. Recently, C3G was also found to be involved in E-cadherin mediated cell-cell contact formation[97]and cAMP/PKA-induced Rap1 and ERKs activation[98].

PDZ-GEFs (PDZ-GEF1 and PDZ-GEF2) are also Rap GEFs that contain a domain which resembles the cNMP binding domain of Epac, but this domain does not bind to cAMP[99,100]. They can specifically activate Rap1 and Rap2[59,100,101], but the signal(s) that activate PDZ-GEFs are still unknown. More recently, it was shown that the MAGI-1/PDZ-GEF1 complex is involved in Rap activation upon cell-cell contact, thus enhancing the vascular endothelial cadherin –mediated cell adhesion[102].

RasGRPs (CalDAG-GEFs) contain putative calcium and DAG binding domains and may also be regulated by these second messengers. CalDAG-GEF1 has exchange activity towards R-Ras and Rap1, CalDAG-GEF2 towards Ras and R-Ras, whereas CalDAG-GEF3 towards H-Ras, R-Ras, and Rap1[103]. The last discovered CalDAG-GEF RasGRP4 only has exchange activity toward Ras[104,105].

The last RapGEF that will be addressed here is DOCK4, which can specifically activate Rap[106]. DOCK4 is a member of the CDM (ced-5 of Caenorhabditis elegans, DOCK180 [downstream of Crk with molecular weight of 180 kDa] of humans, and myoblast city of Drosophila melanogaster) gene family. The CDM proteins significantly differ at their C terminus, which provide specificity in cellular signaling. The divergent, proline-rich C terminus contributes to the localization of the DOCK signaling complex to distinct subcellular destinations via binding to specific adaptor proteins[107]. DOCK180, for instance, is recruited to the cell membrane and activated following integrin signaling, leading to the formation of a complex including the adaptor protein CrkII and the scaffold protein p130cas[108,109].
DOCK4 contains an N-terminal SH3 domain, a region of extended homology with other DOCK family members (35% amino acid identity with DOCK180, 39% with DOCK2, and 54% with DOCK3), and a C-terminal proline-rich region that appears to be unique for each family member. The SH3 binding domain and a second C-terminal proline-rich motif is only present in DOCK4 and CED-5, another CDM family member. The proline-rich C-terminal domain of DOCK4 predicts a role for this protein in CrkII binding and GTPase signaling pathways. This is mainly based on the function of the C-terminal proline-rich domain of DOCK180. Dock180 was reported to form a complex with CrkII and p130 (Cas) via its C-terminal proline-rich domain and this complex can be recruited by integrin receptor alphavbeta5 (αvβ5) heterodimers, which in turn triggers Rac activation and phagosome formation[110]. DOCK2, another CDM family member whose expression is restricted to lymphocytes, also appears to regulate Rac signaling and cell migration[111].

RapGEFs keep Rap in an active state (GTP bound conformation). In contrast, GAPs increase the GTPase activity of Rap, thereby switching the Rap-GTP bound form into a Rap-GDP bound form. The first RapGAP to be identified is RapGAP. This GAP has stronger catalytic activity towards Rap1 than Rap2. Through mutagenesis, it has been shown that only amino acids 75 to 416 of this 663 amino acids-containing protein are necessary for full GAP activity. The two serine residues (490 and 499) outside of the catalytic domain of RapGAP can be phosphorylated by PKA both in vitro and vivo, probably this does not affect the GAP activity of RapGAP[112,113]. Unlike the GAPs of the other Ras-like GNBPs (guanine-nucleotide-binding proteins), in which the insertion of a catalytic arginine of the GAP into the activate site is the main mechanism to downregulate Ras-like proteins[114], the downregulation of Rap by RapGAP is achieved by the insertion of a catalytic asparagine instead of an arginine into the active site[115]. Other RapGAPs include the Spa family (Spa1, SPAL, SPAR, and E6TP1), which also stimulate the GTPase activity of Rap1, but how their activity is regulated is still unknown[116-119] (Fig 4). A recent report suggested that Spa1 and Rap can be recruited by the scaffolding protein AF6 through its PDZ domain and RBD, subsequently down regulating Rap1 activation and therefore inhibiting adhesion of cells to fibronectin[120].

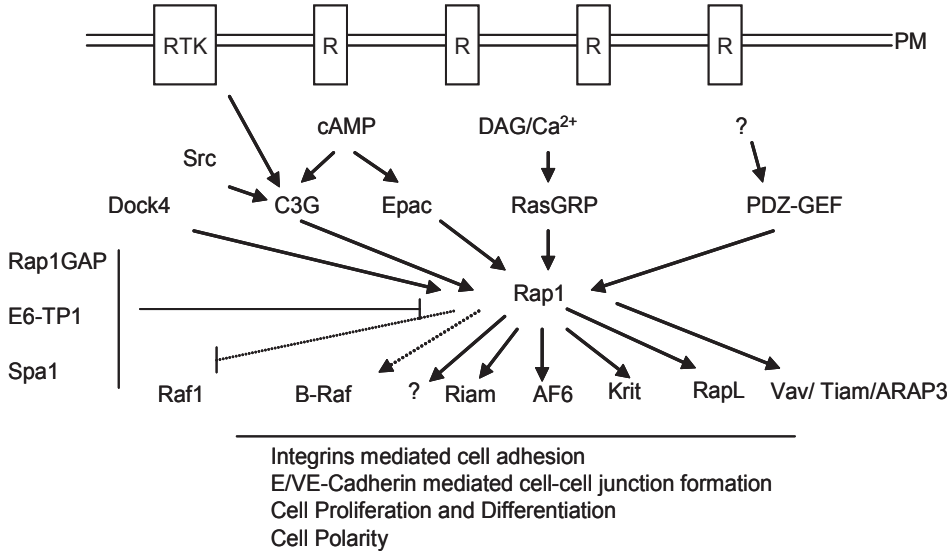

Figure 4. Regulation of Rap GTPase and its downstream signaling pathways
cAMP, calcium (Ca^{2+}) and diacylglycerol (DAG) can activate Rap guanine nucleotide exchange factors, thus, leading to the activation of Rap. GTP bounded Rap can be down-regulated by RapGAP, SPA1 and E6-TP1. Rap may regulate different signaling pathways via different downstream effectors.

Function of Rap signaling pathway

The Rap pathway has been implicated in a number of physiological processes including cell proliferation, integrin-mediated cell adhesion[121,122], E-/VE-cadherin mediated cell-cell junction formation[97,102,123,124,125] , regulation of sodium proton exchange activity[126], secretion[127] and many other functions[64,128-131]. A detailed description on the role of Rap in these processes is given in the following part.

Cell proliferation

Much attention has been placed on Rap1 after it was identified in a screen for genes that could revert the transformation of cells by oncogenic K-Ras[81]. The hypothesis was that Rap1 could antagonize Ras signaling by trapping Ras effectors in an inactive complex, since it was shown that Rap could trap Raf1, one of the Ras downstream effectors, in an inactive complex[132-134]. This hypothesis was confirmed by the observation that Rap1A interferes with Ras-dependent Raf-1 activation by inhibiting the binding of Ras to Raf-1[135], and overexpression of GTP-bound Rap can interfere with ERK activation in fibroblasts by competitive interference with Ras-induced c-Raf1 activation[134]. On the other hand, Rap1 was reported to stimulate cell growth in Swiss 3T3 cells irrespectively of the presence of insulin[136] and it was able to activate the Ras effector B-Raf independently of Ras in certain cell types[137,138]. This is in accordance with the observation that SPA-1 null mice display deregulated activation of endogenous Rap1 in hematopoietic progenitors, resulting in ERK activation and enhanced proliferation independently of Ras[139,140]. Rap1 was also shown to

be responsible for the activation of the MKK3/6-p38MAPK pathway by cell stretching and contraction stimuli in both mouse fibroblastic L-929 cells and human embryonic kidney-derived 293T cells[141].

In contrast, using a specific activator of Epac, endogenous Rap1 does not seem to be involved in the ERK signaling pathway both in NIH-3T3-A14 cells and CHO cells[142]. Rap is likely to play a role independently of Ras. More recently, PKA-independent and PKA-dependent Rap1and ERK activation were analysed in detail. PKA-independent activation of Rap involved Epac, but this perinuclear pool of Rap activated by Epac does not result in ERK activation. On the other hand, PKA-dependent Rap activation is achieved on the plasma membrane via the Rap exchange factor C3G, and this activation required the GTP-dependent association of Rap1 with B-Raf, leading to ERK activation. B-Raf is a physiological target of Rap1, but its utilization as a Rap1 effector is GEF specific[98]. Overall, all data on the cross talk between Rap and Ras signaling suggests that regulation of ERKs by Rap is cell type specific.

Integrin-mediated inside out signalling

Integrins is a family of cell-surface molecules that regulate cell adhesion to specific extracellular-matrix components such as fibronectin, or to specific receptors on neighboring cells. It is involved in many important processes such as the interaction of immune cells with their targets, mobility of cells during development, and metastasis of tumor cells[143,144]. Integrins contain a longer (α-) and a shorter (β-) chain. Both chains are transmembrane proteins that can form heterodimers. The extracellular domain interacts with ligands coming from the surface of other cells or with proteins of the extracellular matrix. Rap is involved in the integrin signaling pathway by affecting the "avidity" and "affinity" of integrins. Avidity is the ability of clustering of integrins, whereas affinity is the binding ability of integrins to its ligands[87]. The first indication of an involvement of Rap in integrin-mediated cell adhesion was the reduced cell adhesion induced by the granulocyte colony stimulating factors after introduction of the RapGAP Spa1[145].Then, several reports showed that Rap fulfills roles in inside-out signaling of integrins. Firstly, in Jurkat cells, overexpression of Rap1 induced integrin αLβ2 (LFA1)-mediated adhesion to the intercellular adhesion molecules via the cytoplasmic tail of αL. However, the cytoplasmic part of β2 is only involved in endocytosis of LFA-1[146]. Secondly, introduction of the Rap1 dominant negative mutant RapN17 inhibits T-cell receptor-mediated LFA-1 activation in Jurkat T cells[129]. Thirdly, in a murine macrophage cell line (J774A.1), complement-mediated phagocytosis, which requires activation of αMβ2, was abolished by inhibition of Rap signaling[147]. Moreover, αLβ2 can be activated by ligation with adhesion molecule CD31 and this can be inhibited by blocking Rap signaling pathway[148]. Additionally, Rap1 was also indicated to be involved in integrins with a β1 chain, i.e. α5β1[122,149]and β3 chain, i.e. αIIbβ3[150] mediated adhesion. For instance, in an ovarian tumor cell line (OVCAR3), β1 integrin-mediated cell adhesion is induced by the cAMP-Epac-Rap pathway instead of the cAMP-PKA pathway[121]. All these findings firmly suggested that Rap plays an important role in integrin-mediated inside-out signaling.

E-/VE-cadherin mediated cell-cell junction formation

More recently, Rap1 as well as Epac1 were found to be the regulators of E-cadherin and VE-cadherin-mediated cell-cell junctions. Cadherins are components of adherens junctions and they can form calcium-dependent, homotypic interactions to stabilize cell-cell contacts. The cytoplasmic tails of cadherins bind to a number of proteins, including β-catenin, α-catenin and p120ctn, to form a connection with the actin cytoskeleton[151,152]. The first indication that Rap1 is involved in the regulation of cadherins came from studies in Drosophila[153]. In clones of Rap1-deficient wing cells the even distribution of DE-cadherin around the cell was disrupted and condensed to one side of the cell. In addition, cell-cell contacts were disrupted and the cells were dispersed in between the wild-type epithelial cells. This result was confirmed in mammalian cells as well. For instance, inhibition of the RapGEF DOCK4 resulted in the disruption of adherent junctions, whereas introduction of DOCK4 as well as Rap1 resulted in the restoration of these junctions[106]. In addition, inhibition of Rap1 resulted in a disappearance of E-cadherin from the cell surface and the disruption of cell junctions in MDCKs, whereas scattering of MDCK cells was inhibited by activation of Rap. Additionally, activation of endogenous Rap via the Rap exchange factor Epac1 also antagonized hepatocyte growth factor (HGF)-induced disruption of adherens junctions [123]. Interestingly, the Rap1GEF C3G directly binds to E-cadherin and might regulate E-cadherin-mediated cell adhesion through Rap1[97]. PDZ-GEF1, one of GEFs for Rap, was found to form a complex with MAGI-1, and involved in Rap activation upon cell-cell contact, thus enhancing the vascular endothelial cadherin–mediated cell adhesion[102]. Finally, in human umbilical vascular endothelial cells (HUVEC), the Rap pathway is also involved in the regulation of VE-cadherin mediated cell-cell contacts, thus decreasing endothelial cell permeability[124,125,154,155]. Activation of Epac results in markedly enhanced basal endothelial barrier function by increasing cortical actin and subsequent redistribution of adherens and tight junction molecules to cell-cell contacts. Activation of Epac also counteracts thrombin-induced hyperpermeability through down-regulation of Rho GTPase activation, suggesting cross-talk between Rap and Rho GTPases. Thus, Epac/Rap activation represents a new pathway for regulating endothelial cell barrier function.

Function of Epac in exocytosis

The main function of Epac is to activate Rap1 and this is consequently involved in the regulation of cell proliferation, inside-out integrin signaling and cadherin-mediated cell adhesion. In addition, Epac2 has been implicated in exocytosis. In insulin–secreting pancreatic β-cells, intracellular calcium levels are elevated by a process called calcium-induced calcium release (CICR), and this process can lead to exocytosis in certain cell lines. Normally, calcium is stored in the endoplasmic reticulum and gated by inositol triphosphate receptor (IP3-R) or the ryanodine receptor (RYR). In pancreatic β-cells, mobilization of calcium stores is regulated by glucagon-like peptide-1(GLP-1) in a cAMP dependent manner, probably via the ryanodine receptor, and this effect can be blocked by a dominant negative form of Epac2 and not by the PKA inhibitor H89. This suggested that Epac mediate CICR[156]. Indeed, 8-pCPT-2'-O-Me-cAMP, which specifically activates Epac but not PKA, acts in human pancreatic β-cells and Ins-1 insulin-secreting cells to mobilize Ca^{2+} from intracellular Ca^{2+} stores via Epac-mediated CICR[157,158]. All of these observations

suggest that cAMP also exerts its effects on secretion via Epac. The finding that Rim2 (Rab3-interaction molecule 2) can interact with Epac2 also gives a reasonable explanation for the data which indicted that Epac2 is involved in different aspects of insulin secretion and the release of calcium from internal stores[157,159]. Rim2 mediates cAMP-dependent, PKA-independent insulin secretion in pancreatic β-cells by interacting with Epac2[158]. The formation of the complex consisting of Epac2, Rim2 and Piccolo (a Ca^{2+} sensor in pancreatic β-cells) is important in cAMP-induced insulin secretion[160]. However, insulin secretion is also achieved through the cAMP induced regulation of ryanodine sensitive calcium channels. It is not clear whether these two processes are regulated by the Rap signaling pathway. Recently, it was reported that both PKA and Epac are involved in exocytosis[161]. For example, in mouse melanotrophs (from pituitary tissue slices) the stimulation of cAMP production by the application of oestrogen increases the efficiency of the hormonal output through both PKA and Epac2-dependent pathways. This suggests that cAMP regulates and modulates exocytosis by coordinating both PKA-dependent and PKA-independent mechanisms and this may be achieved through cAMP compartmentalization[161,162].

Epac is also implicated in several other biochemical and biological processes. For example, Epac and PKA mediate opposing effects of cAMP on PKB regulation. Proper localization and activation of Epac lead to a phosphatidylinositol 3-kinase-dependent PKB activation, while stimulation of PKA inhibits PKB activity[64]. Epac is also predicted to play a role in the regulation of phospholipase Cζ (PLCζ) via Rap2B[163], the regulation of the H^+, K^+-ATPase in rat kidney cortical-collecting-dut cells[164] and also the regulation of sodium proton exchange activity in kidney cells[126]. Overall, the discovery of Epac added a new dimension to the cAMP research field.

Scope of this thesis

The aim of my thesis is to determine the subcellular localization of Epac1 and the analysis of functional domains responsible for Epac1 localization.

To confirm the activation model of Epac1 upon cAMP binding, we generated a FRET probe of Epac1 by sandwiching Epac1 between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) and measured fluorescence resonance energy transfer (FRET) between the two fluorescent moieties. Using this probe, we confirmed the closed-open model (inactivate – activate conformation) of Epac1 upon cAMP binding. Additionally, compared to the PKA FRET probe, the Epac1 sensor showed a much larger dynamic range upon sequential increase in cAMP, and this allows the Epac1 probe to measure rapid changes of physiological cAMP level which the PKA probe failed to record (Chapter 2).

To unravel more functions of Epac, we generated and characterized antibodies against Epac1. 5D3 was chosen for further characterizations based on its ability to recognize the activate conformation of Epac1. Epitope mapping was also performed in vitro. The epitope region of 5D3 was mapped within the cAMP binding domain. The flanking region surrounding Leucine 273 of Epac1 is the precise site for 5D3 targeting (Chaper 3). Immunofluorescence labeling of Epac1 with the 5D3 Ab and additional characterization data of both Epac1 and Epac2 mAbs were shown in the Addenum of Chaper 3.

The subcellular localization of Epac was investigated by using Epac Abs. We observed that the perinuclear region and the plasma membrane, especially the microvilli, are the main targeting sites of Epac1. Functional parts responsible for the correct localization of Epac were also analyzed in detail. Both the first 49aa, also called the Ezrin binding (EzB) domain, and the DEP domain are required for the proper localization of Epac1 and also for Epac-mediated Rap activation. But the localization in the microvilli is only dependent on the EzB domain (Chapter 4). Importantly, the microvillar localization is achieved through binding to Ezrin/Radixin proteins that function as linkers between the actin cytoskeleton and the apical membrane of polarized cells and as scaffold protein for protein complexes. Epac1 only binds to the active conformation of Ezrin/Radixin, indicating that the activation state of Ezrin/Radixin is crucial for the spatial regulation of Epac1 (Chapter 4).

Removing the EzB domain (the first 49 aa) released Epac from the microvilli and redistributed Epac to the plasma membrane and nucleus. HGF or RapV12 induced-cell scattering or cell spreading also resulted in the accumulation of Epac inside the nucleus. It is suggested that cell polarity is very important to maintain the apical localization of Epac and also its function. However, the function of nuclear located Epac remains a question mark (Addenum of Chapter 4).

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2

Detecting cAMP-induced Epac activation by fluorescenceresonance energy transfer: Epac as a novel cAMP indicator

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Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator

Bas Ponsioen[1,2]*, Jun Zhao[3]*, Jurgen Riedl[3], Fried Zwartkruis[3], Gerard van der Krogt [1], Manuela Zaccolo[4], Wouter H. Moolenaar[2], Johannes L. Bos[3+] & Kees Jalink[1++]

[1] Division of Cell Biology, and [2] Division of Cellular Biochemistry andCentre of Biomedical Genetics, TheNetherlands Cancer Institute, Amsterdam, The Netherlands, [3] Department of Physiological Chemistry and Centre of Biomedical Genetics, UMCU, Utrecht, The Netherlands, and [4] Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, Padova, Italy

Epac1 is a guanine nucleotide exchange factor for Rap1 that is activated by direct binding of cAMP. In vitro studies suggest that cAMP relieves the interaction between the regulatory and catalytic domains of Epac. Here, we monitor Epac1 activation in vivo by using a CFP–Epac–YFP fusion construct. When expressed in mammalian cells, CFP–Epac–YFP shows significant fluorescence resonance energy transfer (FRET). FRET rapidly decreases in response to the cAMP-raising agents, whereas it fully recovers after addition of cAMP-lowering agonists. Thus, by undergoing a cAMP-induced conformational change, CFP–Epac–YFP serves as a highly sensitive cAMP indicator in vivo. When compared with a protein kinase A (PKA)-based sensor, Epac based cAMP probes show an extended dynamic range and a better signal-to-noise ratio; furthermore, as a single polypeptide, CFP–Epac–YFP does not suffer from the technical problems encountered with multisubunit PKA-based sensors. These properties make Epac-based FRET probes the preferred indicators for monitoring cAMP levels in vivo.

Keywords: Epac; PKA; cAMP; FRET

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[1]Division of Cell Biology, and [2]Division of Cellular Biochemistry and Centre of Biomedical Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, The Netherlands
[3]Department of Physiological Chemistry and Centre of Biomedical Genetics, UMCU, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands
[4]Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, Via Orus 2, 35124, Padva, Padova, Italy

*These authors contributed equally to this work
[+]Corresponding author. Tel: +31 30 253 8988; Fax: +31 30 253 9035;
E-mail: j.l.bos@med.uu.nl
[++]Corresponding author. Tel: +31 20 512 1933; Fax: +31 20 512 1944;
E-mail: k.jalink@nki.nl

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INTRODUCTION

Cyclic AMP is a common second messenger that activates protein kinase A (PKA), cyclic nucleotide-regulated ion channels and Epac (for exchange proteins directly activated by cAMP). Epacs are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2 (de Rooij et al, 1998). Rap GTPases cycle between an inactive GDP-bound and an active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. Rap proteins are involved in many biological processes, most notably the regulation of cell adhesion through integrins and cadherins (Bos, 2003). The GEF Epac1 consists of a C-terminal catalytic domain characteristic of exchange factors for Ras family GTPases and an N-terminal regulatory domain. The latter domain contains a cAMP-binding site similar to those of protein kinase A (PKA) and, in addition, a DEP domain that mediates membrane attachment (de Rooij et al, 1998; Rehmann et al, 2003a). In vitro studies have shown that cAMP is absolutely required for the activation of Epac (de Rooij et al, 1998). It has been hypothesized that the regulatory domain of Epac functions as an auto-inhibitory domain, which is relieved from inhibition by cAMP, but direct proof for this notion is lacking. In this model, Epac is folded in an inactive conformation at low cAMP levels, thereby preventing Rap binding due to steric hindrance. cAMP binding unfolds the protein, allowing Rap to bind. This is somewhat analogous to the mechanism of PKA regulation by cAMP; in its inactive conformation, two regulatory subunits are bound to two catalytic subunits. On binding of cAMP, this complex falls apart, resulting in the release of active enzymes. In the present study, we set out to measure Epac

activation in vivo by sandwiching Epac between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) and then measure fluorescence resonance energy transfer (FRET) between the two fluorescent moieties. FRET, the radiationless transfer of energy from a fluorescent donor to a suitable acceptor fluorophore, depends on fluorophore orientation and on donor–acceptor distance at a molecular scale. We show that in mammalian cells, CFP–Epac–YFP displays significant energy transfer, which rapidly diminishes following a rise in intracellular cAMP and increases again in response to a fall in cAMP. This indicates that cAMP causes a significant conformational change in vivo and supports the unfolding model for Epac activation. Taking advantage of this property, we characterized CFP–Epac–YFP as a FRET sensor for cAMP and generated cytosolic, catalytically dead mutants. We show that the Epac-based cAMP indicators outperform the previously reported PKA-based cAMP sensor (Adams et al, 1991; Zaccolo et al, 2000; Zaccolo & Pozzan, 2002) in several aspects.

RESULTS AND DISCUSSION

cAMP induces a conformational change in Epac

To monitor cAMP-induced conformational changes in Epac, we generated a construct in which Epac1 was fused amino terminally to CFP and carboxy terminally to YFP, as shown in Fig 1A. Using a GST–RalGDS assay (supplementary information online), it was confirmed that this construct was able to activate Rap1. CFP–Epac–YFP was transiently expressed in human A431 cells, where it localized to membranes and the cytosol (see below). Fluorescence spectra of these cells revealed significant FRET (Fig 1B, red line), indicating that CFP and YFP are in close proximity (B3–4 nm). Stimulation with forskolin, a direct activator of adenylyl cyclase, significantly decreased FRET (green line). Similar responses were observed in other cell types, including HEK293, N1E-115 and MCF-7 cells. Thus, cAMP induces a significant conformational change in Epac, in support of the unfolding model (Fig 1A). We next analysed the kinetics of cAMP-induced FRET changes by ratiometric recording of CFP and YFP emission using a dualphotometer set-up (see Methods). Within seconds after addition of forskolin, FRET started to decrease, usually dropping to a minimum level in 2–3 min (Fig 1C). In the presence of the phosphodiesterase inhibitor IBMX (100 mM), forskolin evoked an average decrease of 30+3% in CFP/YFP emission ratio. This reflects near-complete saturation of cAMP binding to Epac, as deduced from experiments where cells were subsequently permeabilized with digitonin (10 mg/ml) in the presence of 2mM extracellular cAMP (Fig 1D). This caused at most a moderate (on average, ~3%) further drop in FRET.

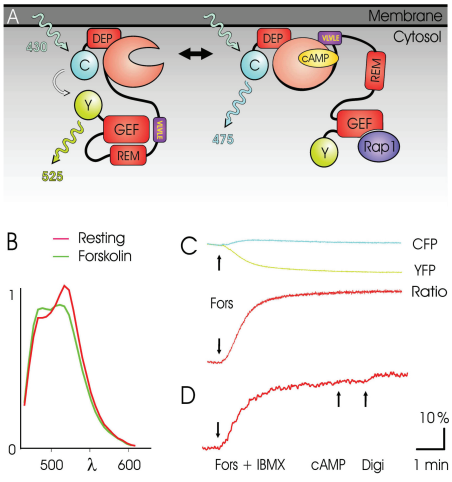

Figure 1 | cAMP-induced conformational change in Epac detected by FRET.

(A) Model for the conformational change following binding of cAMP to the regulatory domain of Epac (adapted from Bos, 2003). Following cAMP binding, the VLVLE sequence can interact with the regulatory domain, releasing the inhibition of the GEF domain by the REM domain. FRET between the CFP and YFP tags allows detection of this conformational change. (B) Emission spectra of CFP–Epac–YFP, excited at 430 nm. Red line, resting level; green line, 3 min after forskolin treatment (25 mM). (C) Time course of cAMP-induced CFP–Epac–YFP activation, monitored in A431 cells by FRET. Increases in the ratio CFP/YFP reflect unfolding of Epac. The arrow indicates addition of forskolin (Fors, 25 mM). (D) Cells were treated with forskolin (25 mM) and IBMX (100 mM) and subsequently permeabilized using digitonin (Digi, 10 mg/ml) in the presence of 2mM cAMP.

Epac activation is independent of subcellular localization

CFP–Epac–YFP localized to the cytosol and to membranes, in particular to the nuclear envelope and to perinuclear compartments. We confirmed proper targeting of CFP–Epac–YFP by comparing its distribution with that of immunolabelled endogenous Epac in OVCAR3 cells. Identical localization patterns were observed (Zhao et al, unpublished data), in agreement with a previous report (Qiao et al, 2002). Thus, CFP–Epac–YFP can be used as a FRET probe to image Epac activation. As activation of its downstream target Rap1 is membrane-delimited (Mochizuki et al, 2001; Bivona et al, 2004), we set out to visualize Epac activation throughout the cell by two different imaging FRET techniques (supplementary information online). The results show that, at least in these cells, agonists induce homogeneous FRET changes throughout the cell. Thus, Epac activation is not confined to membranes, indicating that cAMP binding is the main determinant of Epac activation.

CFP–Epac–YFP as a novel fluorescent cAMP indicator

Having shown that FRET changes in CFP–Epac–YFP reflect cAMP binding, we next investigated how well the Epac construct performs as an in vivo sensor for cAMP. We first tested whether CFP–Epac–YFP is insensitive to cGMP, given that cGMP binds to Epac with an affinity similar to that of cAMP, but fails to activate the enzyme (Rehmann et al, 2003b). In N1E-115 neuroblastoma cells, which express soluble guanylyl cyclase, a massive increase in intracellular cGMP levels ensued following stimulation with the NO donor sodium nitroprusside, as recorded by the cGMP-sensitive FRET sensor Cygnet-1 (Honda et al, 2001). In contrast, the Epac FRET signal was not affected by nitroprusside treatment (Fig 2A). We conclude that cGMP does not detectably affect the conformation of Epac. We next tested two cAMP analogues that are specific for either Epac or PKA. As shown in Fig 2B, the Epac-specific compound 8-p-CPT-2'-O-Me-cAMP (Enserink et al, 2002)

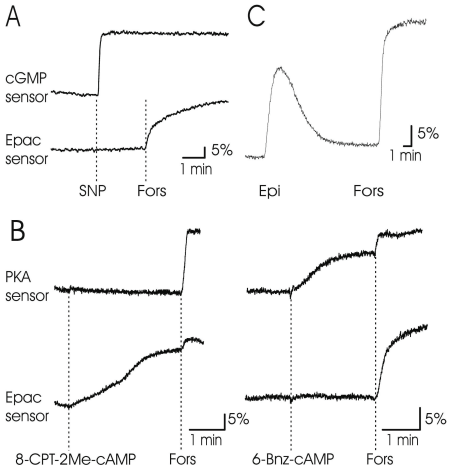

Figure 2 |CFP–Epac–YFP is a specific sensor for cAMP**.** (A) N1E-115 cells expressing either the cGMP sensor (Cygnet 2.1, upper trace) or the EpaccAMP sensor (lower trace) were treated with sodium nitroprusside (SNP, 1mM) and forskolin (25 mM). The traces depict cAMP- or cGMP-induced loss of FRET as an upward change in CFP/YFP ratio. (B) The PKA- and the Epac-cAMP sensor were tested for their sensitivity to 8-*p*-CPT-2'-OMe- cAMP (8-CPT-2Me-cAMP, 100 mM), a specific activator of Epac, and 6-benzoyl-cAMP (6-Bnz-cAMP, 1mM), which specifically activates PKA. In accordance with biochemical data (not shown), the slow and incomplete increases in CFP/YFP ratio in the upper right and lower left panels are caused by limited diffusion of these compounds over the plasma membrane. (C) Typical example of an agonist-induced cAMP response recorded with CFP–Epac–YFP in a Rat-1 fibroblast. Epi, epinephrine (250nM); forskolin (25 mM) is added to calibrate the response.

reduced FRET in the Epac-cAMP sensor but not in the PKA-cAMP sensor. Conversely, the PKA-specific compound 6-Bnz-cAMP (Christensen et al, 2003) specifically diminished the FRET signal only in cells expressing the PKA-based sensor (Fig 2B). Thus, the Epac-cAMP sensor preserves its specificity for cAMP analogues. We further tested the Epac FRET construct in various cell types, including Rat-1 and NIH3T3 fibroblasts, mouse GE11 epithelial cells, mouse N1E-115 neuroblastoma and human MCF7 breast carcinoma cells. Addition of various cAMP-raising agents and receptor agonists, including forskolin, epinephrine, prostaglandin E1 and neurokinin A, caused robust FRET decreases in all cases. In general, forskolin induced a

sustained decrease in FRET, whereas in most cell types, receptor agonists such as PGE1 and epinephrine (adrenaline) elicited transient signals lasting for 10–15 min (Fig 2C and data not shown). The transient nature of the epinephrine-induced signal is due to homologous receptor desensitization, as a second but distinct stimulus is still capable of decreasing FRET. We conclude that CFP–Epac–YFP is a specific, highly sensitive and reliable indicator of both transient and sustained changes in intracellular cAMP levels.

Inactive, cytosolic mutants have increased FRET responses

To generate a cytosolic variant, we next deleted the DEP domain (amino acids 1–148), which is the main determinant of membrane localization (Qiao et al, 2002; Bos, 2003). Indeed, this chimaera, CFP–Epac(δDEP)–YFP, located almost exclusively in the cytosol (Fig 3A) in HEK293 and other cells. This mutation also diminished Epac's ability to activate Rap1 significantly (supplementary information online). We further introduced mutations (T781A, F782A) to render the indicator catalytically dead. These residues were predicted to affect Rap1 binding based on the crystal structure of SOS, a closely related GEF (Boriack-Sjodin et al, 1998). The resulting construct, CFP–Epac(δDEP-CD)–YFP, showed no detectable Rap1 activation (supplementary information online). Spectral analysis revealed that the basal FRET level in the cytosolic variants was significantly above that of the full-length chimaera (Fig 3B). FRET in CFP–Epac(δDEP-CD)–YFP -expressing cells reliably decreased after stimulation with cAMP-raising agonists. Importantly, maximal changes in CFP/YFP ratio outperformed that of the full-length chimaera by about 50% in magnitude (B45 versus B30%), significantly increasing the signal-to-noise ratio (Fig 3C). Because selectivity remained unaltered when compared with CFP–Epac–YFP (not shown), the cytosolic localization, catalytic inactivity and improved signal-to-noise ratio make CFP–Epac(δDEP-CD)–YFP the indicator of choice for monitoring cytosolic cAMP levels.

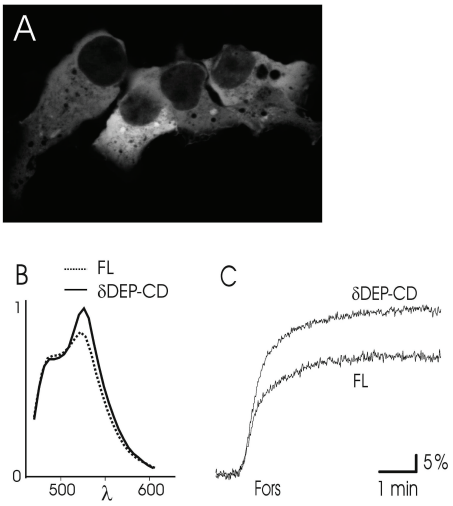

Figure 3 | CFP–Epac(δDEP-CD)–YFP is cytosolic, catalytically inactive and has improved signal-to-noise ratio.
(A) Confocal micrograph of HEK293 cells expressing CFP–Epac(δDEP-CD)–YFP shows absence of membrane labelling. (B) Emission spectra of CFP–Epac–YFP (dashed line) and CFP– Epac(δDEP-CD)–YFP (solid line), excited at 430 nm. (C) Comparison of forskolin-induced change in CFP/YFP ratio in cells expressing CFP– Epac–YFP (FL) and CFP-Epac(δDEP-CD)–YFP. Representative traces from seven experiments each

Epac cAMP sensors display an extended dynamic range

Previously described PKA-based cAMP sensors are tetramers consisting of two catalytic and two regulatory domains. These probes contain four cAMP-binding sites and have submicromolar (B300 nM) affinity in vivo (Bacskai et al, 1993). cAMP binding in PKA shows cooperativity with an apparent Hill coefficient of 1.6 (Houge et al, 1990). As a consequence, this probe has a steep dose–response relationship that rapidly reaches saturation. In contrast, in vitro studies have shown that the affinity of the single cAMP-binding site in Epac is at least an order of magnitude lower (de Rooij et al, 2000). We determined the affinities of the different fluorescent Epac constructs for cAMP in vitro by fluorescence ratiometry (supplementary information online). The results

showed affinities of B50, B35 and B14 mM for CFP–Epac–YFP, CFP–Epac(δDEP)–YFP and CFP–Epac(δDEP-CD)–YFP, respectively. Thus, the Epac-cAMP sensors should display right-shifted and extended dynamic ranges. To test this notion in vivo, cells expressing either CFP–Epac–YFP or the PKA-cAMP sensor were cocultured on coverslips, and neighbouring cells expressing comparable amounts of Epac and PKA, respectively, were analysed for FRET changes. Dosed photorelease of NPE-cAMP, a membrane-permeable caged cAMP analogue, was used to evoke identical incremental changes in intracellular cAMP in the two neighbouring cells (Fig 4A). Sequential increases in cAMP caused a rapid decrease in FRET and subsequent apparent saturation of the response in the PKA sensor, whereas the Epac sensor showed a much larger dynamic range. In line with these observations, the responses to forskolin-induced robust cAMP increases (Fig 4B) were rapid and saturating for the PKA-based sensor, whereas FRET in the Epac-based sensor changed more gradually and often did not saturate completely (Fig 1D). The shifted and extended dynamic range of Epac for cAMP has important consequences for measuring physiological cAMP levels. As shown in Fig 4C, in GE11 cells, isoproterenol (isoprenaline) triggers a rapid and rather sustained FRET change (B30%). In isoproterenol-pretreated cells, addition of lysophosphatidic acid (LPA) resulted in a rapid recovery of the FRET signal, as one would expect for a Gi-coupled receptor agonist that lowers cAMP levels (van Corven et al, 1989). It is to be noted that the PKA-based sensor failed to record this rapid effect of LPA, apparently due to saturation of the probe, but rather reported a substantial lag period (up to several minutes; Fig 4C, middle trace). That it fails to record the true kinetics of the LPA-induced cAMP response becomes evident when the Epac-based sensor is used. As shown in Fig 4C, CFP–Epac–YFP detects the initial fall in cAMP levels within seconds after LPA addition.

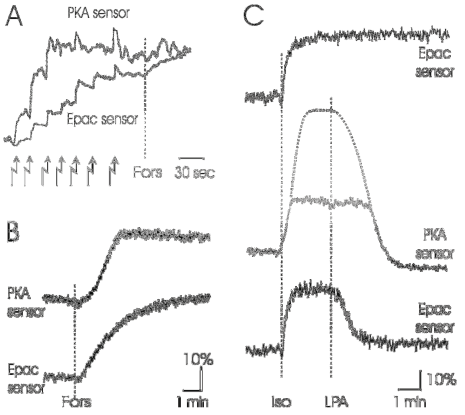

Figure4| The Epac-cAMP sensor exhibits an extended dynamic range as compared with the PKA-cAMP sensor.

(A) Flash photolysis (thin arrows, 1/15 s; thick arrows, 1/4 s) of NPE-caged cAMP in neighbouring A431 cells expressing either PKA-cAMP or Epac-cAMP sensor (as recognized by partial decoration of membranes). Forskolin (50 mM) was added to further increase cAMP levels. Traces are normalized for comparison. (B) Typical responses to forskolin (50 mM), recorded with the PKA- and the EpaccAMP sensor in A431 cells. Response rise times (10–90%) differed significantly (3475 s for PKA, n¼49; 248738 s for Epac, n¼49; Po0.005). Note the sharp transition from the dynamic response range to the saturated plateau phase in the PKA sensor trace. (C) Upper trace: sustained cAMP elevation evoked by isoproterenol (isoprenaline; 10 mM) in a GE11 epithelial cell. Middle and lower traces: registration of cAMP decreases induced by subsequent addition of LPA (5 mM), visualized with the PKA probe and the Epac probe, respectively. Note that Epac reveals the immediate LPA effect, whereas it is obscured by saturation of the PKA-cAMP sensor.

CONCLUSIONS

Our results support a model in which cAMP binding to the regulatory domain of Epac releases an inhibitory conformation that prevents binding to Rap1 (de Rooij et al, 2000). Importantly, the FRET signal not only reflects binding of cAMP but also activation of Epac because cGMP, which binds with a similar affinity but fails to activate Epac (Rehmann et al, 2003b), is without effect. We used this property to show that the local, membrane-delimited activation of Rap1 (Mochizuki et al, 2001; Bivona et al, 2004) is not due to local activation of Epac. The uniform Epac activation here observed contrasts with the findings of Zaccolo & Pozzan (2002), who detected subcellular cAMP gradients in cardiac myocytes with the PKA-based cAMP sensor. This

is probably explained by cell-type-specific differences in activity and intracellular distribution of the phosphodiesterases that shape such cAMP gradients, because we failed to detect gradients of cAMP using the PKA probe in our cells. It is to be noted that our in vivo data on the basis of photolysis of NPE-caged cAMP (Fig 4A) strongly support the notion that cAMP differentially regulates its effectors, that is, low cAMP concentrations signal mainly through PKA, whereas at higher doses cAMP exerts additional effects through Epac activation (Zwartkruis et al, 1998). This study further shows that Epac-based FRET constructs are ideally suited as cAMP sensors in that they exhibit improved characteristics compared with the commonly used PKA-based sensors. First, the moderate affinities of our Epac constructs (14–50 mM) result in a right-shifted dose–response relationship that matches physiological cAMP levels (Fig 4). During the review of this manuscript, a Kd of 2.3 mM was reported for a FRET sensor based on Epac's isolated cAMP-binding domain (Nikolaev et al, 2004). Thus, Epac-based sensors provide a wide range of affinities that allows matching the sensors to the anticipated cAMP levels. Second, the PKA regulatory subunits each contain two cAMP-binding sites that exhibit cooperative binding (Hill coefficient of 1.6), resulting in a very steep response. In contrast, the single cAMP-binding domain of Epac1 results in an extended dynamic range. Third, Epac needs only a single cAMP molecule for a 30% FRET change, while four molecules of cAMP are needed to cause a comparable change in two donor–acceptor pairs in PKA. Together with the lower affinity of Epac, this results in reduced buffering of cytosolic cAMP. This is not trivial, as expression levels of cytosolic FRET probes commonly are in the micromolar range (0.1–5 mM; van der Wal et al, 2001), that is, at cAMP levels found in the cytosol following receptor stimulation. Fourth, the Epac-cAMP sensor is a single polypeptide, eliminating expression- and stoichiometry-related problems encountered with the PKA-based versions. For instance, unbalanced expression levels of regulatory and catalytic subunits of PKA hamper quantitative analyses of FRET changes. Furthermore, a single cDNA construct allows easy generation of stably transfected cell lines, which is often a problem with the PKA-based sensor (unpublished observations). Fifth, monomeric Epac sensors show faster activation kinetics than the slowly dissociating PKA-based sensors (Nikolaev et al, 2004). In addition, the cytosolic CFP–Epac(δDEP-CD)–YFP construct exhibits even larger cAMP-induced FRET changes, resulting in a superior signal-to-noise ratio. Together, these properties make Epac-based FRET probes the preferred fluorescent indicators for monitoring elevated cAMP levels in living cells.

METHODS

Cell culture, transfections and live cell experiments.

Cells were seeded on glass coverslips, cultured and transfected with constructs as described (van Rheenen et al, 2004). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 23 NaHCO3, with 10mM HEPES added), pH 7.2, kept under 5% CO2, at 37℃. Agonists and inhibitors were added from concentrated stocks. Expression levels of fluorescent probes were estimated as described (van der Wal et al, 2001).

Dynamic FRET monitoring.

Cells on coverslips were placed on an inverted NIKON microscope and excited at 425 nm. Emission of CFP and YFP was detected simultaneously through 470 ± 20 and 530 ± 25nm band-pass filters. Data were digitized and FRET was expressed as ratio of CFP to YFP signals, the value of which was set to 1.0 at the onset of the experiments. Changes are expressed as per cent deviation from this initial value of 1.0. Loading and flash photolysis of NPE-caged cAMP. Cells were loaded by incubation with 100 mM NPE-caged cAMP for 15 min. Uncaging was with brief pulses of UV light (340–410 nm) from a 100W HBO lamp using a shutter. For comparison, traces were normalized with respect to baseline and final FRET values.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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Supplementary information

Ponsioen *et al.,* Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator

Methods

Materials. Isoproterenol, 1-oleoyl-LPA, prostaglandin E1, epinephrine and sodium nitroprusside were from Sigma Chemical Co. (St. Louis, MO); IBMX, forskolin, and neurokinin A were from Calbiochem-Novabiochem Corp. (La Jolla, CA); 1-(2-nitrophenyl)ethyl adenosine-3,-5-cyclic monophosphate (NPE-caged cAMP) was from Molecular Probes Inc.Eugene, OR); 8-p-CPT-2-*O*-Me-cAMP and N6-Benzoyladenosine-3,-5-cyclic monophosphate were kindly provided by Hans Gottfried Genieser (Biolog Life Sciences Bremen, Germany).

DNA Constructs. eCFP("non-sticky", A206K (Zacharias *et al.*, 2002), a multiple cloning site with BglII/EcoRV/NheI/SacI restriction sites, and eYFP (A206K) were cloned in-frame, and inserted in pCDNA3 (Invitrogen) using HindIII/XbaI. Full-length Epac1 was generated by PCR using human Epac1 (#AF103905) and cloned in-frame into the restriction sites EcoRV/NheI of the MCS using the primers 5'-TTGATATCTGATGGTGTTGAGAAGGATGCACC-3'and5'-GGGGCTAGCTGGCTCCAGCTCTCG GG-3'. The resultant construct contained the linker SGLRSRYL, separating eCFP from Epac1, and ASEL, separating Epac1 from eYFP.

CFP-Epac1(δDEP)-YFP was generated using the upstream primer 5'-TTGATATCAGCC CGTGGGAACTCATG-3' instead, deleting aa 1-148. The latter construct was rendered catalytically dead (CFP-Epac1(δDEP-CD)-YFP) by pointmutating T781A and F782A in the GEF domain.The chosen residues were predicted to affect Rap1-binding based on the crystal structure of the Son of Sevenless (SOS) protein, a GEF for H-Ras and a close family member of Epac (Boriack-Sjodin *et al.*, 1998).

The PKA-based cAMP sensor, consisting of two expression vectors encoding the YFP-tagged catalytic and CFP-tagged regulatory domain of PKA, was as published (Zaccolo *et al.*, 2002; Zaccolo and Pozzan, 2002). The FRET-sensor for cGMP, termed Cygnet-2.1 for *cy*clic *G*MP i*n*dicator using *e*nergy *t*ransfer, consists of a truncated form of the cGMP-dependent protein kinase sandwiched between CFP and YFP and was used as published (Honda *et al.*, 2001).

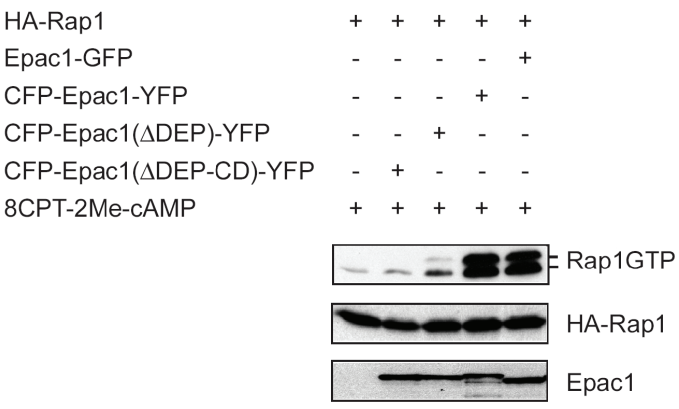
Fluorescence Lifetime Imaging. FLIM experiments were performed on a Leica inverted DMIRE2 microscope equipped with Lambert Instruments (Leutingewolde, the Netherlands) frequency domain lifetime attachment, controlled by the vendors EZflim software. CFP was excited with ~4 mW of 430 nm light from a LED modulated at 40 MHz and emission was collected at 450-490 nm using an intensified CCD camera. Calculated CFP lifetimes were referenced to a 1 μM solution of Rhodamine-G6 in medium, set at 4.11 ns lifetime. CFP-Epac-YFP expressing cells were cocultured with reference cells that expressed CFP only.

Confocal FRET imaging. We recently described FRET imaging by sensitized emission on a Leica TCS-SP2 confocal microscope (Mannheim, Germany) in detail (van Rheenen *et al.*, 2004). Briefly, reference cells expressing only CFP or YFP were seeded together with the CFP-Epac-YFP expressing cells and simultaneously imaged in the same field of view. Three images were collected: the donor image (CFP, excited at 430 nm and detected from 460-510 nm), sensitized emission image (YFP, excited at 430nm and detected from 528-603 nm) and the acceptor image (YFP, excited at 514 nm and detected from 528-603 nm). All images were shading-corrected. Donor leakthrough in the sensitized emission channel and false acceptor excitation that occurred at 430nm were corrected using correction factors derived from the reference cells as described (van Rheenen *et al.*, 2004). Fret efficiency was expressed by dividing the sensitized emission image with the donor image. Fluorescence spectra were recorded with the λ-scan functionality of the Leica confocal microscope from living cells, excited at 430 nm. Spectra are the mean of 10 scans from different cells.

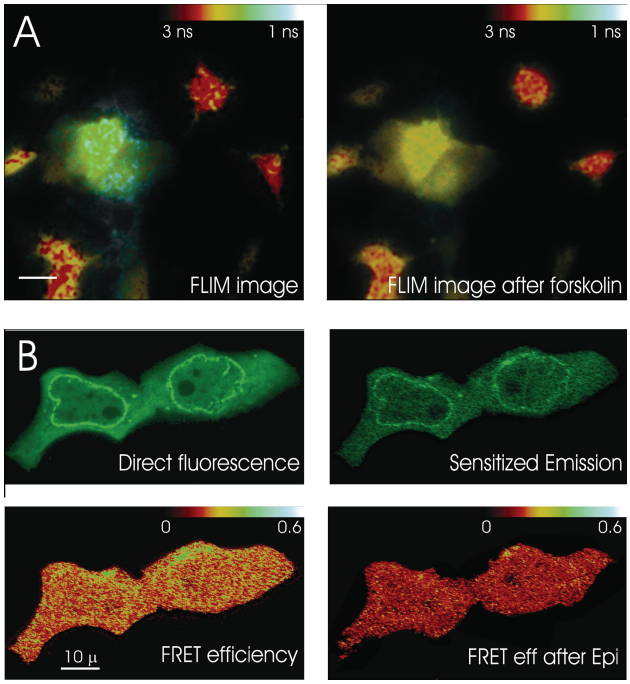
Supplementary figure 1. *In vivo* guanine nucleotide exchange (GEF) activity of Epac-based FRET probes

Different fluorescently tagged Epac constructs were tested for their guanine exchange activity towards Rap1. Indicated constructs were transfected in NIH3T3 cells, which do not express detectable amounts of endogenous Epac1. After 48 hours, cells were stimulated with 100 µM 8-p-CPT-2'-*O*-Me-cAMP for 15 min. Cells were lysed and assayed for GTP-bound Rap1 using GST-RalGDS as an activation-specific probe (de Rooij *et al.*, 1998). **Upper panel**, pull-down samples were probed with an antibody against Rap1 (Santa Cruz, SC-65). The upper band is HA-tagged Rap1, the lower band is endogenous Rap1. **Middle panel**, expression of HA-Rap1 as detected with an anti-HA monoclonal antibody (12CA5). **Lower panel**, expression of Epac1 constructs was verified using an Epac1 specific mouse monoclonal antibody (5D3).

Note that in line with the reported dependence of Epac signaling on correct subcellular localization, loss of the DEP domain significantly interferes with Rap1 activation. Residual activity is completely lost in CFP-Epac(δDEP-CD)-YFP, the mutant that lacks Rap1 binding.



Supplementary figure 2. Epac activation is independent of subcellular localization
Activation of the downstream target of Epac1, Rap1, reportedly is membrane-delimited, but conflicting views exist on whether this predominantly occurs at endomembranes or at the plasma membrane (Mochizuki *et al.*, 2001;Bivona *et al.*, 2004). We therefore set out to visualize Epac activation throughout the cell by two different FRET techniques. Initially, we confirmed that tagging of Epac with GFPs does not interfere with its proper localization by comparing the cellular distribution of CFP-Epac-YFP to that of immunolabeled endogenous Epac in OVCAR3 cells. In good agreement with published data for untagged Epac (Qiao *et al.*, 2002), CFP-Epac-YFP localized in the cytosol as well as to membranes (the nuclear envelope, perinuclear membranes, and to a lesser extent the plasma membrane). Details will be published elsewhere (Zhao *et al.,* in preparation).

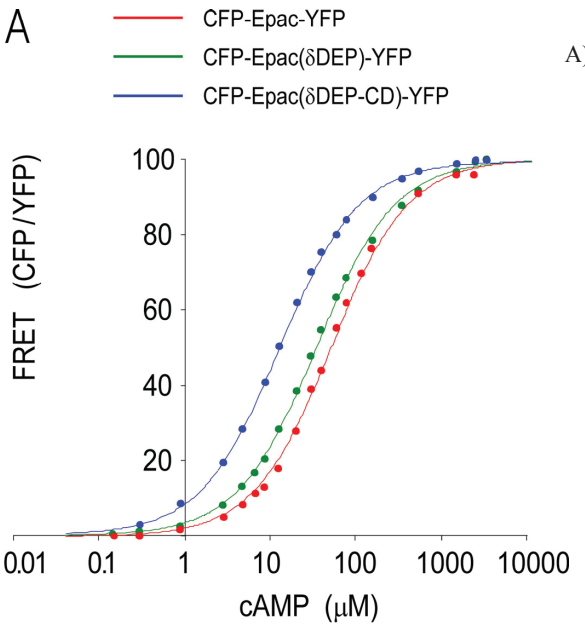


Supplementary Figure 2. Spatial distribution of Epac activity as detected by fluorescence resonance.
A) (left panel) FRET in CFP-Epac-YFP expressing A431 cells as detected by FLIM. The homogeneous lifetime of ~1.7 ns throughout the cell indicates ~30% FRET efficiency. For reference, CFP in control cells displays a lifetime of ~2.4 ns. (right panel) Stimulation with forskolin (1 µM) decreases FRET, causing the lifetime to increase to ~2.2 ns. **B)** Confocal images of an A431 cell expressing CFP-Epac-YFP. Upper left, YFP fluorescence; upper right, sensitized emission, i.e. calculated YFP emission resulting from FRET; lower left, calculated FRET efficiency in resting cell; lower right, FRET efficiency after epinephrine treatment (250 nM).

Widefield Fluorescence Lifetime IMaging (FLIM; see Methods) reports FRET quantitatively as a decrease in the excited-state lifetime of the fluorescent donor molecule. For reference, A431 cells expressing CFP-Epac-YFP were imaged along with HEK293 control cells expressing cytosolic CFP. In resting cells, our FLIM analysis failed to reveal spatial differences in FRET efficiency (Fig. 2A, left panel). Furthermore, activation of Epac with cAMP-raising agonists caused a similar FRET decrease throughout the cells (Fig. 2A, right panel).To better resolve subcellular details, we employed a recently developed, highly corrected confocal laser scanning FRET microscopy approach (van Rheenen *et al.*, 2004) that allows discrimination of CFP-Epac-YFP activation in the cytosol and at membranes. In the cell types studied, Epac activation state as deduced from FRET did not depend on membrane localization (lower left panel). cAMP-raising agonists such as epinephrine (250 nM) caused similar FRET changes at membranes and in the cytosol (lower right panel). The homogeneous FRET values determined for CFP-Epac-YFP throughout the cells are likely due to the rapid diffusion of cAMP in the cytosol. Taken together, our data demonstrate that Epac activation is not localized to membranes and further indicate that binding to cAMP is the main determinant of Epac activation.

Supplementary figure 3. Fluorescently tagged Epac constructs bind cAMP with micromolar affinities

To determine dissociation constants (Kd) towards cAMP, five 15-cm petridishes of HEK293 cells were transfected for each of the constructs. Cells were harvested 24h post transfection, washed in PBS and homogenized in hypotonic medium (PBS:H2O, 1:2) with a Downs piston.The homogenate was cleared by high-speed centrifugation for 10 minutes and subsequently ionic concentrations were corrected towards intracellular levels



A) Dose-response relationship for cAMP-induced FRET changes for CFP-Epac-YFP (red), CFPEpac(δDEP)-YFP (green), and CFP-Epac(δDEP-CD)-YFP (blue) *in vitro*. Apparent dissociation constants were 50 +/- 3 μM, 35 +/- 3 μM, and 14 +/- 2 μM, respectively (N=3). Hill coefficients did not differ significantly from 1 (0.97, 0.95 and 0.94, respectively). Shown are data and fitted curve of a representative example.

(in mM: 140 KCl, 5 NaCl, 1 MgCl2 and 10 HEPES for pH 7.2). FRET changes caused by consecutive additions of cAMP were recorded in the stirred cuvet of a PTI Quantamaster dual channel spectrofluorimeter (Lawrenceville, NJ). FRET was expressed as the ratio of the YFP channel (530 +/-10 nm) and the CFP channel (490 +/- 10 nm), when excited at 420 +/-3 nm. For analysis, we used the Hillfunction:
FRET([cAMP]) FRETmax*([cAMP]^n / (Kd^n + [cAMP]^n)).

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3

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

(To be submitted)

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

Jun Zhao, Joost Das, Fried J.T. Zwartkruis, Johannes L. Bos and Holger Rehmann

Department of Physiological Chemistry UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

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).

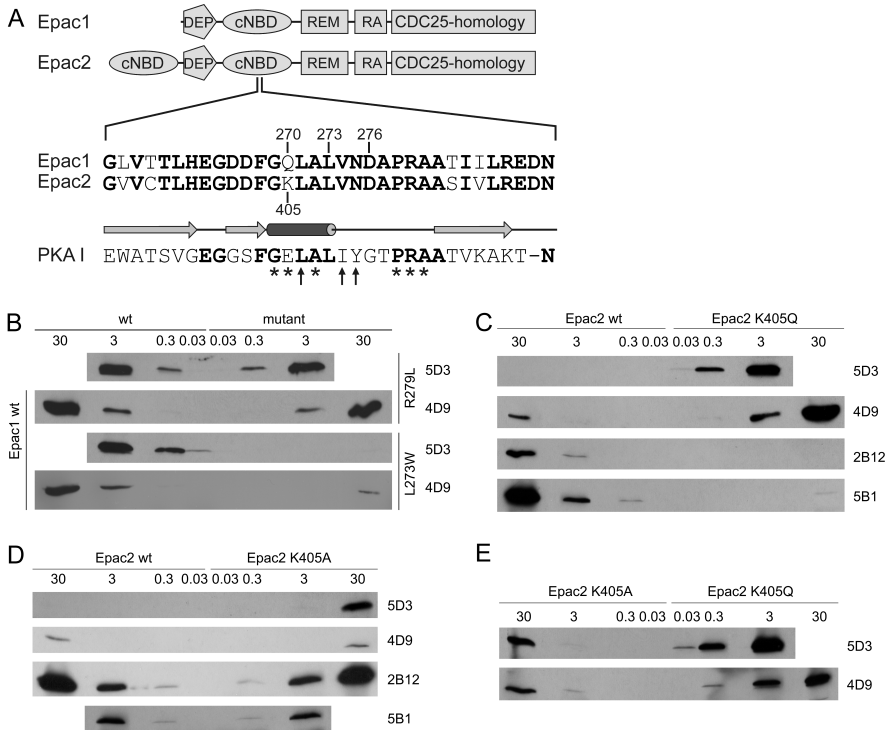

Fig.1 Epitope mapping.
(A) Domain organisation of Epac1 and Epac2. (DEP, Dishevelled, Egl-10, Pleckstrin; cNBD, cyclic nucleotide binding domain; REM, Ras exchange motif; RA, Ras association domain; CDC25-HD, CDC25-homololgy domain). A sequence alignment of the region, which is recognised by the antibodies, 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).

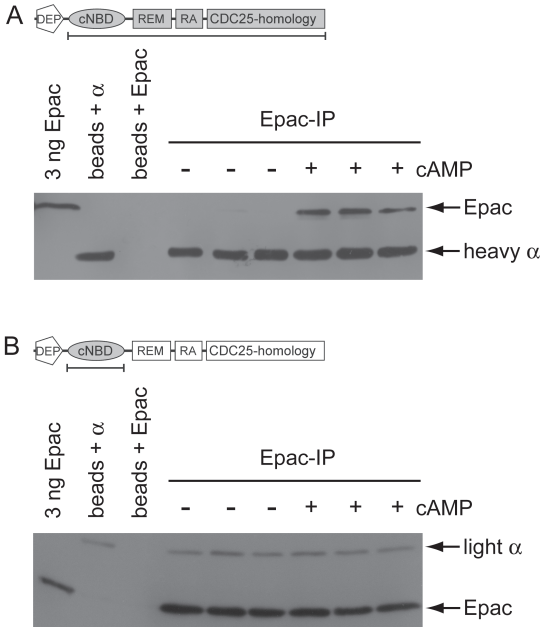

Fig.2 cAMP dependent interaction between 5D3 and Epac1.
Recombinant EpacΔDEP (A) or the isolated cNBD (B) were immuno-precipitated with 5D3, either in the presence or in the absence of cAMP. Epac protein was incubated with 5D3 immobilised on proteins A beads for 5 minutes. Experiments were done in triplicates. As a controls; lane 1, recombinant protein directly loaded to SDS-PAGE; lane 2, protein A beads precoupled 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

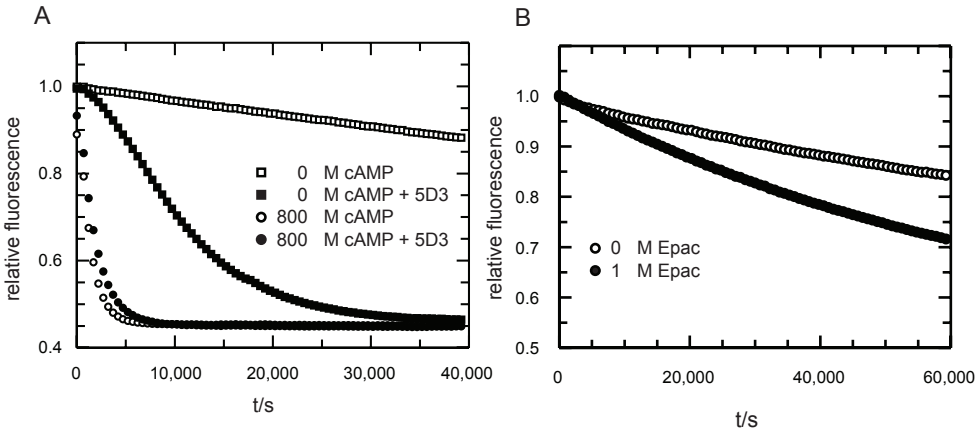

Fig.3 5D3 induces activation of Epac1.
The exchange activity of Epac (100 nM) towards Rap is measured in vitro. (A) 5D3 is added in the absence and presence of 800 uM cAMP to the exchange reaction. The cAMP induced activity of Epac is not altered in the presence of 5D3. In the absence of cAMP, 5D3 induces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

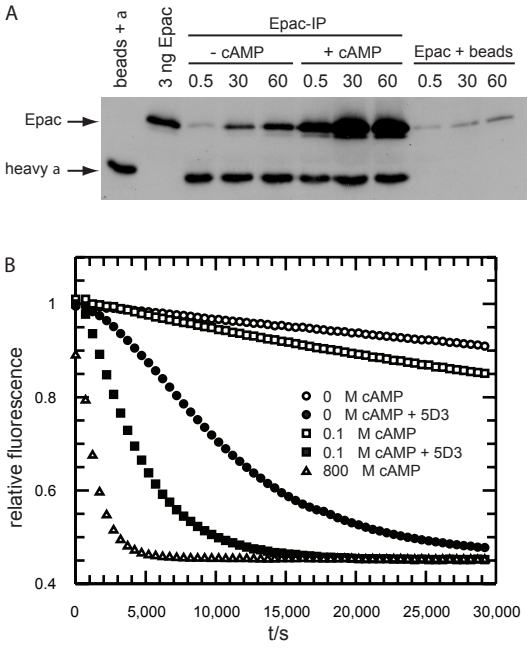

Fig.4 Slow kinetics of 5D3 binding to Epac1 in the absence of cAMP.

(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.

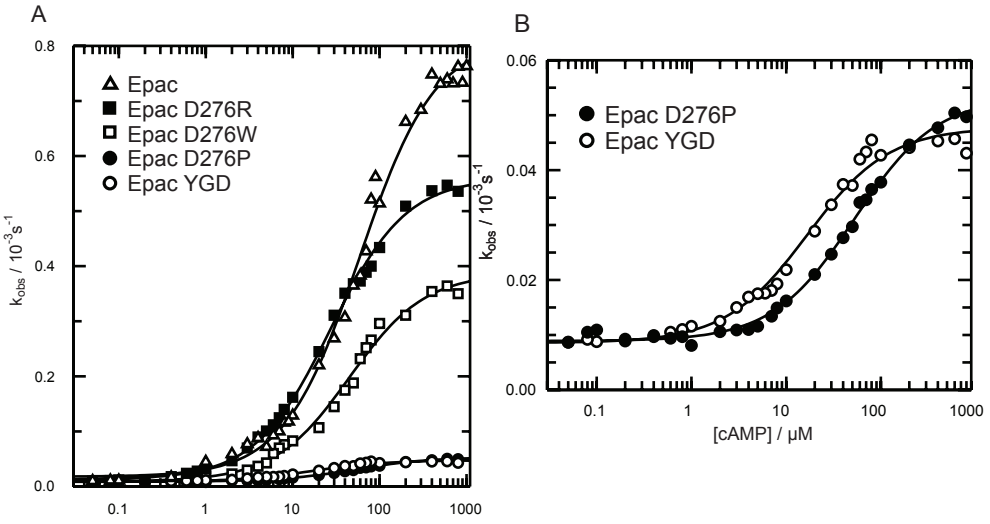

Fig.5 Mutations in the epitope region interfere with cAMP mediated regulation.
The cAMP dependent activity of Epac and Epac mutated in the epitope region were analysed. Exchange activity is plotted against the cAMP concentration. Epac YGD, Epac1ΔDEP N275Y/D276G/A277D.

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

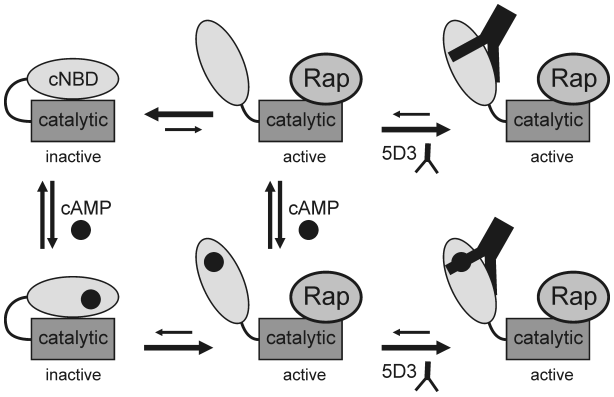

Fig.6 Equilibria in Epac activation.
5D3 can bind Epac only, if Epac is in the active open conformation. In the absence of cAMP only a minor fraction of Epac is in the active open conformation. After binding of cAMP the open active conformation is favoured. The open active conformation of Epac is trapped 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

Jun Zhao, Fried Zwartkruis, Holger Rehmann and Johannes L.Bos

Department of Physiology Chemistry and Centre of Biomedical Genetics, UMCU,
Universiteitsweg 100, 3584 CG, Utrecht, the Netherlands

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).

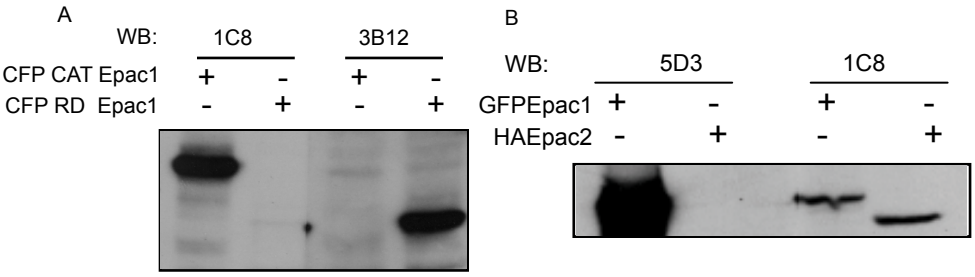

Figure 1. Specifity of Epac1 mAbs on Western blot.
(A) Total cell lysates from A14 cells, which had been transfected with either the regulatory or the catalytic region of Epac1 were blotted and probed with the mAb 1C8 (left strip) or 3B12 (right strip). (B) Total cell lysates from A14 cells, which had 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).

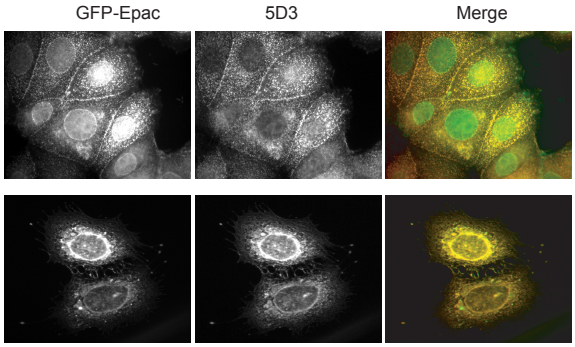

Figure 2. Immunofluorescence of MCF7 cells, stably expressing GFPEpac1 using the monoclonal antibody 5D3.
Upper panels: visualization of GFP tagged Epac1 by direct fluorescence of GFP or indirect using 5D3 in cells, which have been fixed with paraformaldehyde. Note that 5D3 does not stain the nuclei. 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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4

Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling

(Submitted)

Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling

Jun Zhao[1,4], Zhongchun Zhang[1,4], Matthijs R.H. Kooistra[1], Sander Basten[1], Viola Oorschot[2], John de Koning[1,3], Judith Klumperman[2], Fried J. Zwartkruis[1], Holger Rehmann[1], Johannes L. Bos[1] and Leo S. Price[1]

[1]Department of Physiological Chemistry and Centre of Biomedical Genetics, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.
[2]Department of Cell Biology, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.
[3] Hybrigenics SA, 3-5 Impasse Reille, 75014 Paris, France

[4]these authors contributed equally to this work.

Corresponding authors: Johannes L. Bos and Leo S. Price. Department of Physiological Chemistry and Centre of Biomedical Genetics, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands,
Tel: 31 30 2538989; Fax: 31 30 2539035
Email: J.L.Bos@med.uu.nl; L.S.Price@med.uu.nl

Abstract

Epac is a cAMP-regulated exchange factor for the small GTPase Rap, which is involved in various cAMP-dependent processes including cell adhesion and secretion. Here we show that Epac1 is particularly abundant at the apical surface of epithelial cells. In search for an apical anchor protein for Epac1 we identified the membrane-cytoskeleton linker protein Ezrin as an Epac1-interacting protein. Indeed Epac1 colocalised with Ezrin at the apical membrane of polarised epithelial cells and at the apical, lumenal surface of kidney tubule epithelial cysts grown in collagen gels. The interaction between Ezrin and Epac1 was dependent on the Ezrin FERM domain and was promoted by receptor activation, suggesting that Epac1 interacts with the open conformation of Ezrin. For Epac1, the N-terminal 49 amino acids are both necessary and sufficient for apical localisation. Importantly, either deletion of Ezrin by RNA interference or disruption of the Epac-Ezrin interaction with the N-terminal 49 amino acids displaced Epac1 from the apical membrane and inhibited Rap1 activation following β-adrenergic receptor stimulation. From these results we conclude that Ezrin is an anchor protein for Epac1 that regulates the apical localisation of Epac1 and couples β-adrenergic receptor stimulation to Rap1 signalling.

Epac1 (exchange protein directly activated by cAMP) is a widely expressed guanine nucleotide exchange factor (GEF) for the small GTPase Rap1 and Rap2[1-4]. The N-terminal regulatory region of the protein consists of a DEP (Dishevelled, Pleckstrin, Egl-10) domain, involved in membrane localization and a cAMP-binding domain. The cAMP-binding domain maintains Epac in an auto-inhibited state in the absence of cAMP[5]. The cAMP analogue 8-pCPT-2'-O-Me-cAMP (007) that specifically activates Epac but not protein kinase A[6], has allowed a number of biological processes to be assigned to Epac1. Most notably, Epac1 has been implicated in integrin-mediated cell adhesion[7], E-cadherin[8,9] and VE-cadherin-mediated cell junction formation[10-13], regulation of sodium proton exchange activity[14] and regulation of secretion[15,16]. The presence of several protein or lipid interaction domains in Epac1 indicates that Epac1 is recruited to specific regions in the cell. Indeed Epac1 is reported to be located in mitochondria[17], in the perinuclear region[18], in cell-cell junctions[8] and in the brush border of kidney tubules[14].

Ezrin is a member of the ERM (Ezrin Radixin Moesin) family of proteins, which link integral plasma membrane proteins with the actin cytoskeleton[19]. ERM proteins possess an N-terminal FERM (band 4.1 ERM) domain and a C-terminal domain which includes an actin binding domain and residues that mediate an intra-molecular interaction with the FERM domain which prevents interaction with the membrane and F-actin[20]. Ezrin is regulated by phosphorylation and by phospholipid binding, which contribute to release of auto-inhibition and thus promote its crosslinking function[21-23]. Ezrin is expressed principally at the apical domain of epithelial cells, where it enhances microvilli formation[24-26]. Ezrin is also involved in the regulation of adherens junctions and focal adhesions and membrane ruffles and consequently plays a role in adhesion and migration[27,28]. In addition to stabilising membrane-actin interactions, Ezrin also functions as a scaffold for cell signalling, associating with the β-adrenergic receptor and PKA (protein kinase A) and thus bringing together a source of cAMP with its target kinase[29,30]. By linking up with PKA-regulated membrane channels, such as NHE3, Ezrin targets this signalling complex to its effector proteins[31]. A recent report showed that Epac also regulates NHE3 function[14].

We show here that Ezrin binds to Epac1 and is responsible for the targeting of Epac1 to the apical membrane. Binding of Epac1 requires the active conformation of Ezrin. Indeed, receptor stimulation induces this association. Furthermore, the Epac-Ezrin interaction is required to mediate β-adrenergic receptor-dependent activation of Rap. From these results we conclude that Ezrin is an anchor protein for Epac1, that regulates the apical localisation of Epac1 and couples β-adrenegic receptor stimulation to Rap1 signalling.

Results

Apical localisation of Epac1

We showed previously that Rap1 is activated by the Epac1-specific cAMP-analogue 007 in the ovarian carcinoma cell line OvCar3[6]. We therefore examined the expression of Epac1 in these cells. We observed that the monoclonal antibody 5D3, which was raised against Epac1, recognized a double band of the predicted size of Epac1. These bands were absent in cells treated with siRNA against Epac1 (Fig. 1A). Two additional Epac1 siRNAs also suppressed

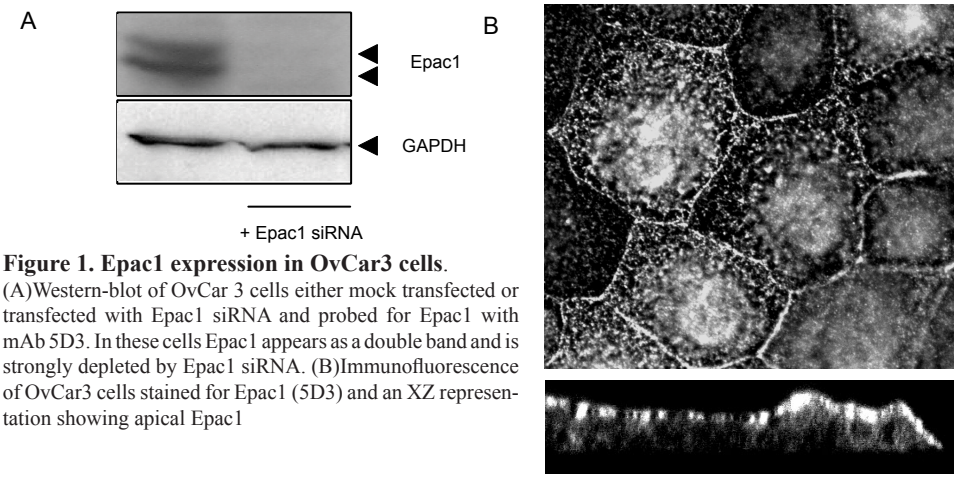

Figure 1. Epac1 expression in OvCar3 cells.
(A)Western-blot of OvCar 3 cells either mock transfected or transfected with Epac1 siRNA and probed for Epac1 with mAb 5D3. In these cells Epac1 appears as a double band and is strongly depleted by Epac1 siRNA. (B)Immunofluorescence of OvCar3 cells stained for Epac1 (5D3) and an XZ representation showing apical Epac1

Epac1 expression (not shown). Moreover, a polyclonal antibody raised against Epac1, α-Epac1-1, recognized the same two bands (not shown). Confocal analysis of OvCar3 cells showed that Epac1 was located predominantly in punctate structures and at sites of cell-cell contact, a staining that was abolished after treating the cells with Epac1 siRNA (Fig. 1B upper panel, and data not shown). Interestingly, Z-stack analysis of OvCar3 cells demonstrated a predominantly apical plasma membrane localisation of Epac1, suggesting that the punctate structures represented microvilli (Fig 1B lower panel).
To study the apical localisation of Epac1 further, we used Madin Darby Canine Kidney cells (MDCK), which develop a polarised 'cobblestone' morphology with distinct apical and basal-lateral domains. Since MDCK cells do not express detectable levels of Epac1, we stably expressed human Epac1 in these cells by retroviral transduction[8]. The localisation of Epac1 in these cells was similar to that observed in OvCar3 cells (Fig. 2A). Z-stack analysis of cells co-stained for Epac1 and the tight junction marker ZO-1 showed that Epac1 is located predominantly at the apical surface. This result was corroborated by immunoelectron microscopy (EM) showing that the majority of Epac1 is located in microvilli at the apical surface of the cell. In addition to the apical localisation, Epac1 was found in the perinuclear region, including the Golgi (Fig. 2B), particularly in less polarized cells. We conclude that in polarized epithelial cells, the majority of Epac1 is present in microvilli at the apical plasma membrane.

Epac1 interacts with the apical membrane-cytoskeleton linker protein, Ezrin
To identify possible apical anchor proteins for Epac1 we performed a yeast 2-hybrid screen of a human placenta cDNA library using full length Epac1 as bait. This screen identified Ezrin and Radixin as Epac1-interacting proteins (9 and 5 colonies respectively). Ezrin and Radixin are members of the Ezrin-Radixin-Moesin (ERM) family of proteins, that exist either in a closed, auto-inhibited state whereby a C-terminal region interacts with the FERM domain, or in an open conformation which permits association with interacting proteins (see introduction). Importantly, Ezrin is predominantly localized in apical membranes[32] and thus is a prime candidate for being the apical anchor protein for Epac1. Co-immunoprecipitation experiments were performed to test whether Epac1 interacts with

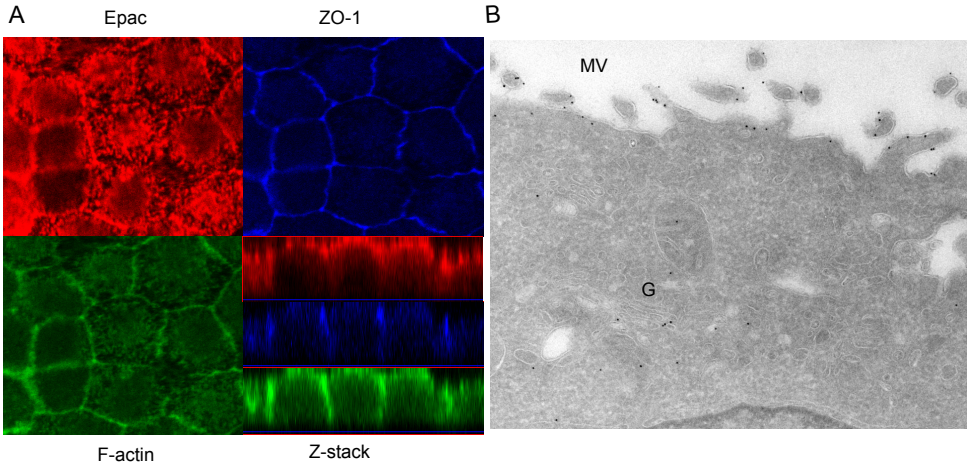

Figure 2. Apical Epac1 expression in polarised MDCK cells.
(A) Immunofluorescent micrographs of confluent MDCK cells stably expressing Epac1 stained with anti-Epac1 monoclonal antibody (5D3), showing a predominantly apical localisation of Epac1. Tight junctions are labelled with anti-ZO-1 polyclonal antibodies and Alexa-488 phalloidin to label filamentous actin. XZ representations are shown in the lower right panel. (B) Transmission electron microscopy image of MDCK-Epac1 cells labelled with a-Epac1-1. The microvilli (MV) and the Golgi aparatus (G), which show most Epac1 labelling are indicated.

Ezrin *in vivo*. HA-tagged Epac1 was transfected into 293T cells together with flag-tagged forms of either full-length Ezrin or with the N-terminal 492 amino acids of Ezrin (Ezrin-N3) lacking the auto-inhibitory C-terminus (Fig. 3A). Immunoprecipitation of Ezrin-N3, resulted in co-precipitation of Epac1 (Fig. 3B). In contrast, full-length Ezrin only weakly associates with Epac1 suggesting that Epac1 interacts preferentially with the FERM domain-containing N-terminal region of Ezrin and not with auto-inhibited Ezrin. Similarly, Epac1 also interacted with Radixin-N3 but not full-length Radixin (not shown). To confirm that the endogenous proteins interact as well, we examined the interaction of Epac1 and Ezrin in ACHN human kidney carcinoma cells. Indeed, Ezrin co-immunopreciptates with Epac1 (Fig. 3C). S1P induces activation of the small GTPase Rho, and the subsequent synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2)[33], which promotes Ezrin association with the plasma membrane and acquisition of an open conformation[23,34]. We therefore tested whether stimulation of ACNH cells with S1P would result in an increased association between Epac1 and Ezrin. Indeed, co-immunoprecipitation of Ezrin by Epac1 was enhanced when ACNH cells were stimulated with sphingosine-1-phosphate (S1P) (Fig. 3C). Co-immunoprecipitation of endogenous Epac1 and Ezrin was also observed in OvCar3 cells following S1P stimulation (not shown). We conclude from these results that Epac1 interacts with the open conformation of Ezrin *in vivo*. These results further imply that the interaction is regulated by stimuli that activate Ezrin.
Epac1 contains a number of domains that mediate protein-protein interactions, nucleotide exchange and membrane localisation (Fig. 3D). We therefore examined a series of deletion mutants of Epac1 to identify the region of interaction with Ezrin. We observed that deletion of the N-terminal 49 amino acids (Epac1-Δ49) strongly reduced co-immunoprecipitation with Ezrin-N3 (Fig. 3E), suggesting that Ezrin interacts with the N-terminal 49 amino acids of Epac1.

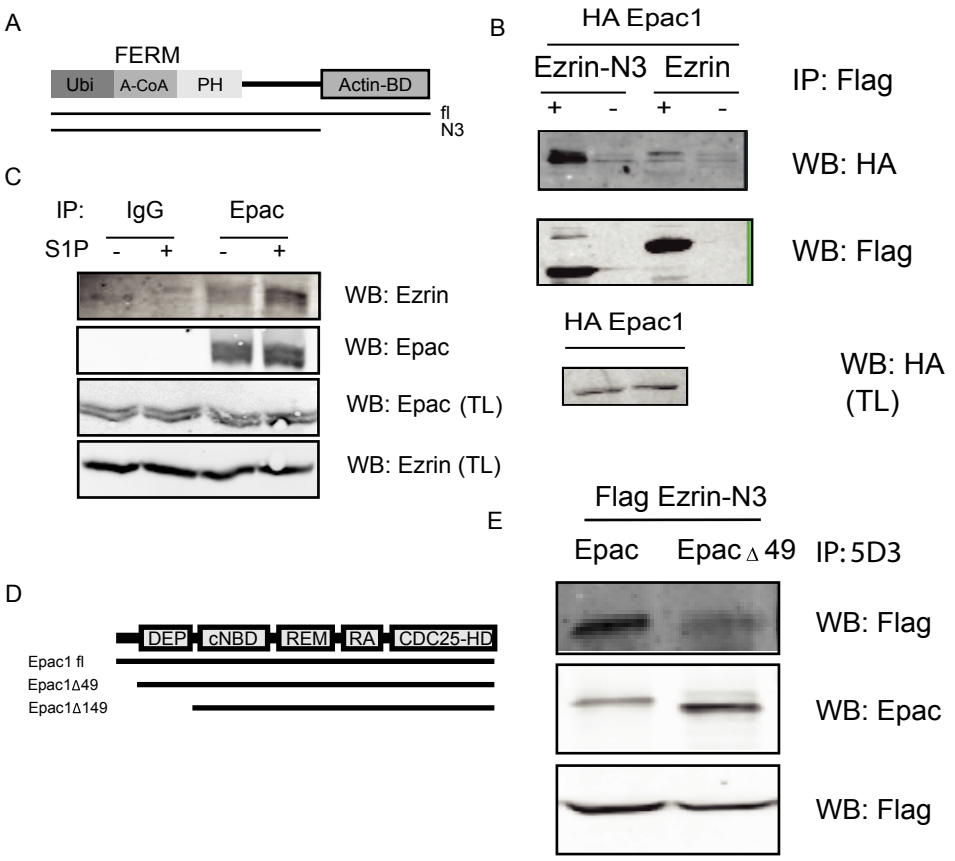

Figure 3. Epac1 interacts with ezrin in vitro.
(A) Domain structure of Ezrin, showing the N-terminal FERM domain, which consists of a ubiquitin-like fold, acyl Co-A binding region and a pleckstrin homology (PH) domain. The C-terminal contains an actin-binding domain and residues required for interaction with the N-terminal. (B) Anti-HA western blot (top panel) shows co-immunoprecipitation of flag-Ezrin with HA-Epac1 from lysates of 293T cells. Flag-tagged Ezrin-N3 but not flag-tagged full length Ezrin precipitates HA-Epac1 using beads coupled to anti-flag antibodies (+) but not beads coupled to anti-myc control antibody (-). Western blots also show that equal amounts of flag-tagged Ezrin were precipitated (middle panel) and HA-Epac1 was equally present in the total lysates, which were subsequently divided for the flag and control IP's (lower panel). (C) Co-immunoprecipitation of endogenous Ezrin with anti-Epac1 antibodies (5D3) from lysates of ACHN cells stimulated with sphingosine 1-phosphate (S1P), but not from unstimulated cells (upper panel). Epac1 was equally precipitated by 5D3 antibodies, but was not precipitated by control anti myc antibodies (IgG). Ezrin and Epac1 were present equally in the cell lysates (lower two panels). (D) Domain structure of Epac1: DEP domain also contributes to membrane targeting, nucleotide binding domain (NBD), Ras Exchange Motif (REM), Ras association (RA) domain and CDC25 homology catalytic domain. The deletion mutants used in this study are also shown. (E) Anti-flag blot showing co-immunoprecipitation of flag-Ezrin-N3 by myc-tagged full length Epac1 (Epac), but less by myc-tagged Epac1Δ49 (EpacΔ49). The lower panel shows approximately equal expression of Ezrin-N3 in original lysates.

The N-terminal tail of Epac1 is required for co-localization with ezrin at the apical plasma membrane.

We next investigated whether Epac1 colocalizes with Ezrin in the apical membrane. We observed that transiently expressed Myc-tagged Epac1 colocalises with Ezrin at punctate structures (Fig. 4A, upper panels). We also examined co-localisation of these proteins in cells grown in 3-dimensional collagen gels, which allows a more developed level of apical-basolateral polarisation. Under these conditions, stably expressed Epac1 shared an almost

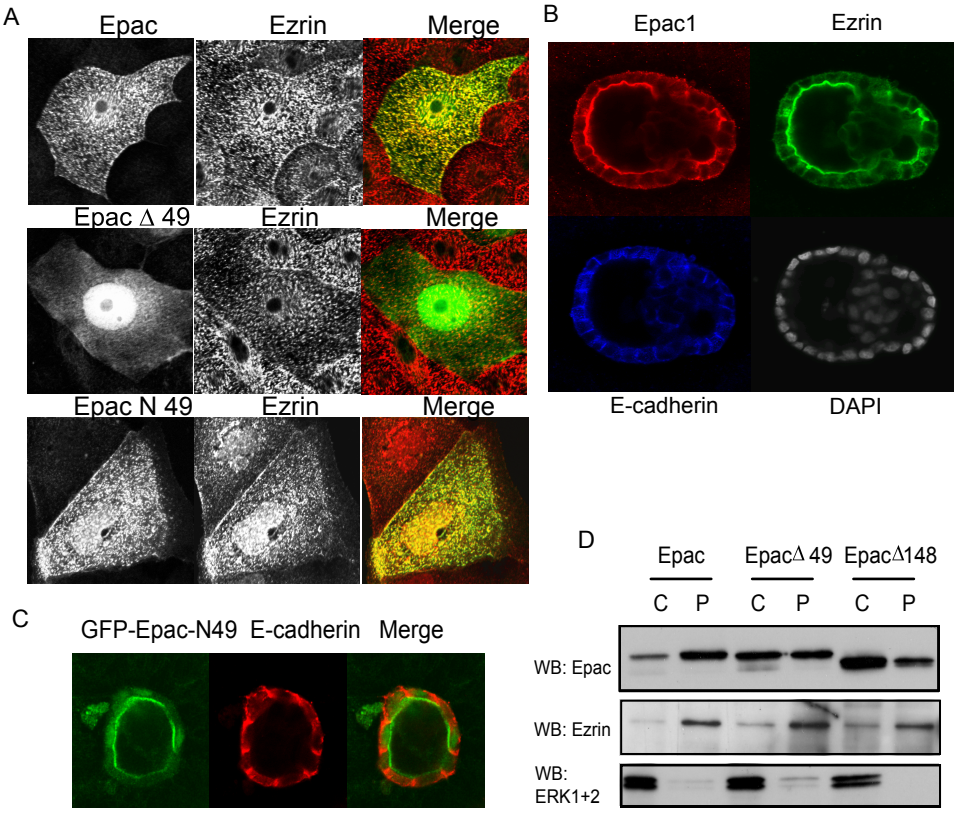

Figure 4. Ezrin colocalisation with Epac1.

(A) Confocal micrographs of MDCK cells transiently transfected with myc-tagged full length Epac1, myc-tagged Epac1Δ49 (lacking the N terminal 49 residues) and CFP fused to the N-terminal 49 amino acids of Epac1 (CFP-Epac1-N49). Cells were labelled with anti-Ezrin antibodies. Myc-tagged Epac1 proteins are labelled with anti-Epac1-1 polyclonal antibodies. CFP fluorescence reveals CFP-Epac1-N49 localisation. Note, cells were fixed 40 h after transfection and seeding on coverslips and are therefore quite flat. (B) Epac1 expressing MDCK cells grown in a 3-dimensional collagen gel and stained with anti-Epac1 polyclonal antibody, anti-Ezrin monoclonal antibody, anti E-cadherin rat monoclonal antibody (DECMA) and DAPI to label nuclei. (C) Confocal micrographs of MDCK cells stably expressing GFP fused to the N-terminal 49 residues of Epac1 (GFP-Epac1-N49), showing an apical distribution. E-cadherin staining (DECMA) is also shown. (D) Fractionation of MCF-7 cells expressing full length or deletion mutants of Epac1 showing that both the N49 tail and DEP domain contribute to membrane localisation of Epac1. C: cytosol, P: particulate fraction. Erk1/2 and Ezrin are localised predominantly in the cytosol and particulate fractions respectively.

identical distribution with Ezrin at the apical lumenal surface of MDCK epithelial cell cysts (Fig. 4B), supporting the notion that these proteins interact. Interestingly, non polarised clusters of cells showed a disordered distribution of both Ezrin and Epac1. In agreement with the failure of Epac1-Δ49 to interact with Ezrin, this mutant did not show the punctate microvillar distribution and did not co-localise with Ezrin. Instead, Epac1-Δ49 showed a cytoplasmic and nuclear distribution, while the distribution of Ezrin remained punctate (Fig. 4A, middle panel). Interestingly, a fusion protein of CFP with the isolated N-terminal 49 amino acids of Epac1 (CFP-Epac1-N49) did colocalise with Ezrin at microvilli (Fig. 4A, lower panel). Similarly, GFP-Epac1-N49 also localised to the lumen of epithelial cysts (Fig. 4C). The microscopy findings were confirmed by cell fractionation experiments; deletion of N49 resulted in an increase in Epac1 present in the cytosolic fraction. Deletion of an extended region of the N terminus of Epac1 which includes the DEP domain (Epac1Δ148) resulted in a further increase in cytosolic Epac1, confirming a previous report that the DEP domain contributes to the membrane localisation of Epac1 (Fig. 4D and[2]). Together, these results show that the majority of Epac1 colocalizes with Ezrin and that the N-terminal region of Epac1 is responsible for the colocalization.

Ezrin targets Epac1 to the apical membrane.

The above findings indicate that Ezrin targets Epac1 to the apical plasma membrane. To test this further, we treated OvCar3 cells with siRNA to deplete Ezrin and examined the localisation of Ezrin and Epac1 by immunofluorescence. The efficiency of Ezrin knockdown varied between cells producing a mosaic of Ezrin expression. We observed that in cells where Ezrin was strongly depleted, Epac1 is localised to the nucleus (Fig. 5 upper panels). We also examined the effect of simultaneous depletion of Ezrin, Radixin and Moesin on Epac1 distribution. Western blot analysis confirmed that all three proteins were depleted by at least 50% (not shown). We observed a similar effect on knockdown of Ezrin and mislocalisation of Epac1 as when Ezrin alone was depleted (Fig. 5 lower panels). From these results we conclude that indeed Ezrin is required for the apical localisation of Epac1 and suggest that in OvCar3 cells, Radixin (and Moesin) do not contribute significantly to this localisation.

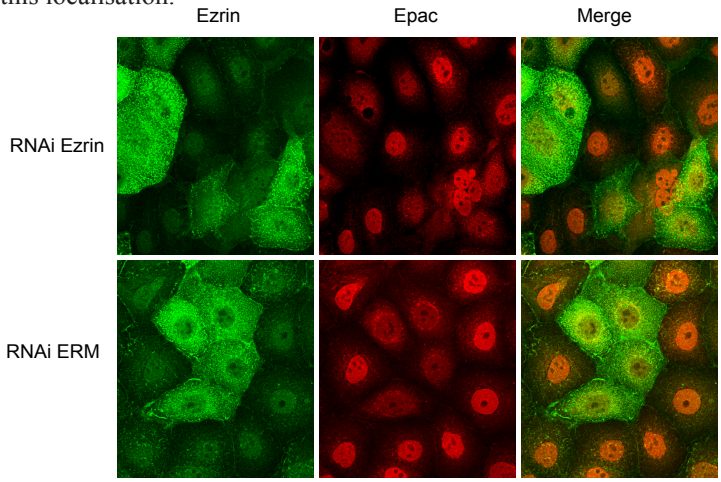

Figure 5. Ezrin is required for Epac1 localisation and function. Confocal microscopy of OvCar3 cells labelled with anti-Epac1 and anti-Ezrin antibodies following treatment with siRNAi oligos for Ezrin (upper panels) and Ezrin, Radixin and Moesin combined (lower panels). Note that in Ezrin depleted cells, the corresponding localisation of Epac1 is disrupted, resulting in strong nuclear localisation.

Apical targeting of Epac1 is required for efficient Rap1 activation.

To determine whether the apical localisation of Epac1 was important for its function in activating Rap, we expressed deletion mutants of Epac1 in A14 NIH 3T3 fibroblasts and examined Rap1 activation using 'pull-down' assays. Overexpression of full length Epac1 was sufficient to induce HA-Rap1 activation in the absence of additional cAMP-inducing stimulation. We observed that deletion of N49 from Epac1 strongly reduced its capacity to activate Rap1. Deletion of N148, which also includes the DEP domain, completely abolished the capacity of Epac1 to activate Rap1 (Fig. 6A). Thus, the ability of Epac1 to activate Rap1 correlated with its localisation to the plasma membrane (compare with Fig. 4D). Previous studies showed that deletion of the N-terminal 148 amino acids of Epac1 does not affect the regulation of Epac1 activity *in vitro*[2]. Taken together, these findings suggest that localisation of Epac1 via its N-terminal 49 amino acids are required for efficient activation of Rap1 in cells.

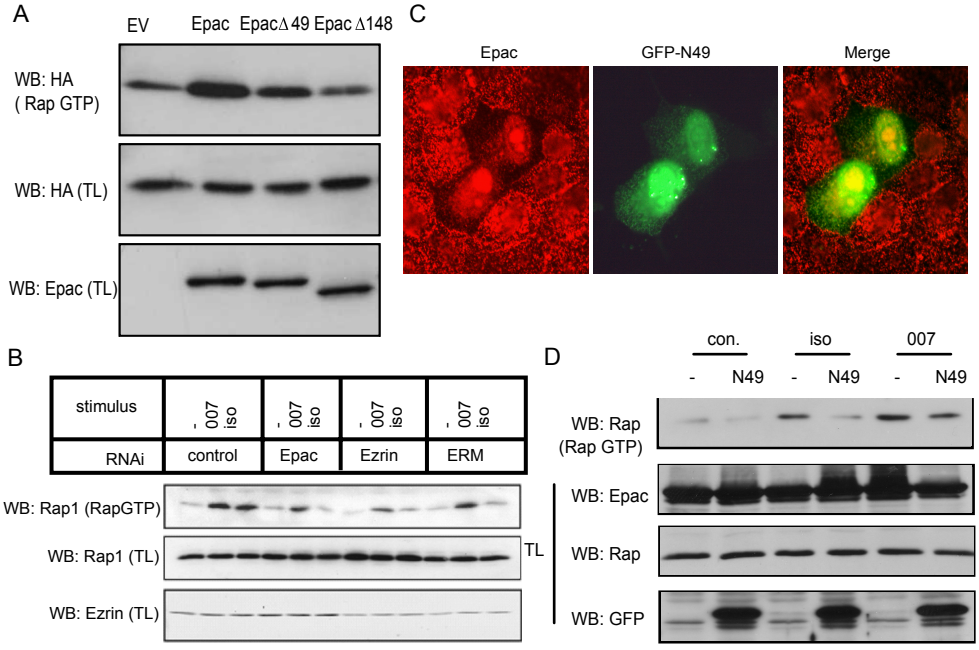

Figure 6. Ezrin-Epac1 interaction is required for Rap1 activation.

(A) Deletion of the N-terminal 49 amino acids and DEP domain of Epac1 impair their capacity to activate Rap. Empty vector, full length myc-Epac1, myc-Epac1-Δ49 and myc-Epac1-Δ148 were transfected into A14 NIH 3T3 cells together with HA-Rap1. Precipitated active HA-Rap1, and expression of HA-Rap1 and myc-Epac1 constructs in total cell lysates are shown. (B) Pull-down assay of active Rap1 from OvCar3 cells treated with siRNA duplexes for Epac1, Ezrin and Ezrin, Radixin and Moesin combined or control oligos. Cells were treated with 007 (100 uM) for 15 minutes, isoproteronol (100 uM) for 2 minutes or buffer control. The upper panel shows the amount of active Rap1 precipitated from cells. The middle panel shows that equivalent levels of Rap1 were present in cell lysates. Ezrin levels were reduced both by Ezrin and by pooled ERM siRNA oligos (Lower panel). (C) Confocal microscopy showing the displacement of Epac1 in cells transiently expressing GFP-Epac1-N49. (D) Pull down assay of activate Rap from MDCK-Epac1 cells with or without stable expression of GFP-Epac1-N49 and stimulated with 007 (100 uM, 15 minutes) or isoproteronol ('iso', 100 uM, 2 minutes). Rap, Epac1 and GFP-Epac1-N49 expression in total lysates are also shown.

Ezrin is required for efficient activation of Rap1 by Epac.
Ezrin is reported to associate both directly and indirectly with the β-adrenergic receptor (β-AR), one of the stimuli that activate Rap1 through Epac[7]. We therefore hypothesized that Ezrin brings Epac1 into the proximity of β-AR-induced local increases in cAMP concentrations and is thus required for efficient coupling of β-AR signalling to Rap1. To test this hypothesis, we examined the effect of RNAi-mediated depletion of Ezrin on the activation of Rap1 by β-AR stimulation. Stimulation of OvCar3 cells with isoproteronol to activate β-AR results in activation of Rap1 in OvCar3 cells and also induced Rap1 activation in cells which had been treated with scrambled siRNA. However, Rap1 activation induced by isoproteronol treatment was inhibited in cells depleted of Ezrin (Fig. 6B). Rap1 activation induced by the Epac-specific analog 007 was also inhibited by Ezrin RNAi, although to a lesser extent. Simultaneous depletion of Ezrin, Radixin and Moesin had a similar effect on Rap1 activation as depletion of Ezrin alone. These findings suggest that Ezrin couples adrenergic receptor signalling to Epac-Rap1 activation. Since the effect of Ezrin depletion on Rap1 activation could also be due to more general disruption of microvilli, we examined the effect of inhibiting the Ezrin-Epac1 interaction using the N-terminal 49 amino acids of Epac1, which we predicted would function as a competitive inhibitor of the interaction. Indeed, when overexpressed, GFP-Epac1-N49 results in the release of Epac1 from the microvilli (Fig. 6C). In addition, GFP-Epac1-N49 potently inhibited isoproteronol-induced Rap1 activation and also induced moderate inhibition of 007-induced Rap1 activation (Fig. 6D). We conclude from these experiments that Ezrin-dependent targeting of Epac1 to the apical plasma membrane is required for efficient coupling of β-AR stimulation to Epac-Rap1 signalling.

Discussion

We show here that Epac1 is localised predominantly on microvilli at the apical plasma membrane of various epithelial cell lines. Furthermore, we identified the apical membrane-cytoskeleton cross-linking protein, Ezrin, as a binding partner of Epac1. Ezrin is a member of the Ezrin-Radixin-Moesin (ERM) family of proteins that contain a C-terminal actin binding domain and an N-terminal FERM domain, which interacts with integral membrane proteins. ERM proteins exist either in a closed, auto-inhibited state whereby a C-terminal region interacts with the FERM domain, or in an open conformation which permits association with interacting proteins. The conclusion that Epac1 interacts with Ezrin is based on a number of observations. Firstly, Ezrin binds to Epac1 in a yeast two hybrid screen. Secondly, endogenous Epac1 co-immunoprecipates with endogenous Ezrin. Thirdly, Epac1 and Ezrin co-localize at the apical site of cells. Fourthly, the N-terminal 49 amino acids of Epac1 are required both for apical localisation and for interaction with Ezrin. Finally, knock-down of Ezrin results in a release of Epac1 from the apical surface. We therefore concluded that indeed Ezrin is a *bona fide* interaction partner of Epac1 that targets Epac1 to the apical plasma membrane. An apical localisation of Epac1 was previously described in proximal tubes of the kidney[14], a result confirmed by us using our antibodies (data not shown). This implies that the apical localisation is physiologically relevant.
We mapped the region required for Epac1 binding to the first 492 amino acids of Ezrin,

which contains the FERM domain. This region is also required for interaction with other Ezrin binding partners, such as EBP50/NHERF, ICAM-2, CD44 and FAK[35-37]. Further deletions of either the N or C termini disrupted interaction with Epac1 – presumably due to disruption of the tertiary structure of the FERM domain. Epac1 did not bind to full length Ezrin, suggesting that the interaction was suppressed by the C-terminal auto-inhibitory region. Indeed, addition of the C-terminal region of Radixin inhibits Epac1 association with the N-terminal part of Radixin (not shown). Consistent with a requirement for an open Ezrin conformation, the interaction between endogenous Epac1 and Ezrin was induced upon stimulation with sphingosine-1-phosphate (S1P), which via Rho activation induces PIP2 synthesis, which has been shown to promote Ezrin conformational activation. This indicates that Epac1 activity is subject to dual regulation – firstly by agonists that induce increases in intracellular cAMP and thus Epac1 activation and secondly, by agonists that regulate Ezrin conformational activation and thus Epac1 targetting.

We showed previously that stimulation of the β-AR results in Epac-mediated activation of Rap1[7]. Interestingly, Ezrin also associates both directly and indirectly via EBP50 with the β-AR[29,38], which after stimulation induces cAMP through activation of adenylate cyclase. We therefore tested whether association of Epac1 with Ezrin is required for an efficient coupling of the β-AR to Epac-Rap1 signalling. We observed that depletion of Ezrin by siRNA inhibited Rap1 activation induced by isoproteronol and to a lesser extent by the Epac1 specific analogous 007. This was unlikely to be a consequence of disruption of the apical membrane due to Ezrin loss, since expression of Epac1-N49, which functions as an interfering peptide and releases Epac1 from the apical site, also inhibited isoproteronol-induced Rap1 activation. Importantly, we observed only a partial inhibition of Rap1 activation when the cells were incubated with 007. Also, when we expressed Epac1 lacking the first 49 amino acids, we observed a residual Rap1 activation, indicating that Epac1 that is not linked to the apical membrane can still activate Rap1 although less efficiently. This decreased efficiency is not due to an intrinsic defect of the Epac1 protein, since deletion of the first 148 amino acids has no apparent effect on the ability of Epac1 to activate Rap1 in vitro[2]. We conclude from these results that the targeting of Epac1 to the apical plasma membrane by Ezrin is required for β-AR-mediated activation of Epac1 and subsequent activation of Rap1.

The localisation of Epac1 at the lumenal surface is consistent with recent reports describing a role for Epac1 in the regulation of cytoplasmic pH via the Na^+/H^+ exchanger (NHE3) at the brush border of proximal tubules[14] and exocytosis in the collecting ducts in the kidney[16]. NHE3 also binds directly to Ezrin[39], suggesting that the Ezrin-Epac1 interaction that we describe here is important for NHE3 regulation. Unfortunately, we were thus far unable to make this connection.

Not all functions of Epac can be attributed to a localisation at the apical site of cells. For instance, we have previously reported that Epac-Rap1 signalling enhances integrin-mediated cell adhesion[7] and the formation of E-cadherin-mediated cell-cell contacts[8,9,40]. Interestingly, Ezrin also localises to and regulates cell-cell contacts in epithelial cells[41] and via binding to FAK, mediates focal adhesion formation[27]. Whether the interaction of Epac with Ezrin plays a role in these processes remains to be investigated. It should be noted however, that Epac1 is found at other locations as well, including the plasma membrane and

the perinuclear region. We did not observe significant co-staining with the mitochondrial marker mitotracker (not shown) a result which is at variance with a previous report[17]. Epac1 has been observed at the nuclear envelope in other cell types cells[42]. These differences may reflect cell type variation, but more specifically, may be a consequence of differences in apical-basolateral polarity and the lack of competition for Epac1 by apically localised or conformationally active Ezrin. Interestingly, deletion of the N-terminus of Epac1, depletion of Ezrin by RNA interference or overexpression of the isolated N-terminus of Epac1 all resulted in the nuclear localisation of Epac1. The factors regulating the nuclear localisation of Epac1 and the intriguing possibility that Epac1 performs a signalling function in the nucleus require further investigation.

The interaction of Epac1 with Ezrin is also interesting with respect to the observation that Ezrin binds PKA[30]. In addition, Epac1 was found to interact with phosphodiesterase 4D3[18]. This intriguing network of interactions suggests that Ezrin may play host to an entire cAMP-dependent signalling pathway, including the activating β-AR. A similar role was recently described for mAKAP, a muscle specific adaptor protein which coordinates a complex between PKA, Epac1 and PDE4D3[18]. Coordinated activation of PKA and Epac1 on AKAPs may turn out to be a common feature in cAMP-dependent signalling.

We have shown here that Ezrin targets Epac1 to the apical plasma membrane of polarised epithelial cells, coupling adrenergic receptor activation with the Rap signalling pathway. This gives insight into the mechanism of Epac1 control. It was shown previously that Epac, through the cAMP binding domain, interacts with the light chain of microtubule associating protein[43]. Further proteins domains that may mediate interactions are the DEP domain and the RA-domain. Although the interaction partners of these domains are currently unclear, one can envision complexity in the regulation of Epac1, a feature that seems to be common for GEFs of small GTPases.

Methods

Constructs and antibodies

To generate vectors containing Epac1 full length (aa 2-881), Epac1Δ148 (aa 149-881), Epac1Δ49 (aa 50-881) and Epac1-N49 (aa 1-49), the corresponding coding sequences of human Epac1 (GI:3978530) were introduced into a donor vector, pDONR201 (Invitrogen) to allow sub-cloning by the "Gateway"-procedure (Invitrogen). The constructs were then recombined in pcDNA3-Flag-His, pcDNA3-Myc-, pcDNA-meGFP-, pcDNA-meCFP- and pcDNA3-meYFP-destination vectors according to standard protocols (Invitrogen). Ezrin was obtained as clone IRALp962A182.1 from the Deutsches Ressourcenzentrum für Genomforschung (RZPD) (Berlin, Germany). Using Gateway adapted PCR, we amplified Ezrin and Ezrin-N3 (residues 1 to 492) and cloned these into pDONR201 and then into pcDNA3-flag as above. pcDNA-meCFP and pcDNA-meYFP were kindly provided by O. Rocks and P. Bastiaens (EMBL, Heidelberg, Germany). Ezrin-GFP was kindly provided by Erik Sahai (Cancer Research UK, London).

The mouse Epac1 monoclonal antibody (mAb) 5D3 and the rabbit Epac1 polyclonal antibody (pAb) (α–Epac1-1) were raised against recombinant Epac1Δ148. Rabbit pAb against ZO-1 was from Zymed. Anti-Rat-E-cadherin mAb (DECMA) was from Sigma. Ezrin was from Becton Dickinson. Anti-flag was from Kodak. Anti-HA monoclonal antibody 12CA5

was purified by our laboratory. Anti-ERK1/2 rabbit pAb was generated by our laboratory. Goat-anti-mouse/rabbit/rat Alexa 405,488,568 and 647 secondary antibodies were from Molecular Probes. Fluorescein and Rhodamine phalloidin was from Sigma.

Cell culture:
MDCK-II, MCF7, 293T and A14 cells were routinely cultured in DMEM provided with 10% FCS, 0.5% glutamine, penicillin and streptomycin (all from BioWhittaker, Belgium) at 37 °C in 6.0 % CO2. OvCar3 cells were from ATCC and were cultured as above in RPMI. ACHN cells were cultured in Eagle's MEM contained 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The MDCK Epac1 cell line was generated and cultured as described before8.

Yeast 2-hybrid screening
A human placenta random primed library was custom-screened with Epac1 full length and Epac1 residues 1-328 (Epac1: RapGEF 3, homo sapiens, GI: 3978530) by Hybrigenics S.A. (Paris, France) as previously described[44].

Transfection.
MCF7 were transfected with the indicated constructs using FuGENE 6 (Roche, Diagnostics). MDCK cells were washed with PBS containing 1 mM EGTA, detached by trypsinisation (PBS + 0.2% trypsin, 1 mM EDTA), resuspended in complete medium at a density of $1X10^7$ cells/ml and transfected with 20ug of the indicated constructs by electroporation (1000F, 260v). A14 cells were transfected with using the calcium phosphate method. Cells were lysed or fixed 40 h after transfection.
For si-RNA transfection of OvCar3 cells, siRNA duplexes were diluted in Optimem (Gibco). Oligofectamine (Invitrogen) was added and the mix added to cells according to manufacturers instructions. siRNA oligos were as follows: Epac1 (No.1 GACCGGAAGTACCACCTTA; No.2 CCATCATCCTGCGAGAAGA; No.3:GCACCTACGTCTGCAACAA) were from Dharmacon, siRNA's against Ezrin (No.1GAACAGACCTTTGGCTTGGAGTTGA; No.2 TGGCCTCCA CTATGTGGATAATAAA; No.3 CCTCAAAGAGTGATGGACCAGCACA), against Radixin (GCCAGGCTACCTGGCTAATGATAGA), and Moesin (TGGCCTCGTATGCTGTCCAGTCTAA) were from Invitrogen. Ezrin siRNA is the mixture of No.1, 2 and 3; ERM siRNAi is the mixture of Ezrin No.1, Radixin and Moesin oligos).

Co-immunoprecipitation
293T cells were transiently transfected with 10 µg of plasmid DNA as indicated in the figure legends. After culturing for 40 h, cells were lysed in 1 ml of 1% Triton X-100 buffer containing additionally 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, aprotinin (1 µg/ml), and pepstatin (1 µg/ml). Cell lysates containing equal amounts of total protein were incubated for 2 h at 4°C with 10 µl of antibody pre-coupled protein A-Sepharose beads (Pharmacia). The beads were washed extensively with lysis buffer and bound proteins were

analysed by SDS-PAGE and Western blotting. For immunoprecipitation of Epac1 from ACHN cells, cells were lysed in a buffer containing 20 mM Tris pH 8.0; 1% Triton X-100, 0.5% Na-DOC; 10 mM EDTA; 150 mM NaCl and protease inhibitors. After clearing the lysate, lysates were incubated in the presence of 1 mM 8-Br-cAMP (MP biochemicals) with anti-Epac1 monoclonal antibody 5D3 and protein A beads for 2 h. 8-Br-cAMP is included in the IP since binding of Epac1 5D3 is dependent on an open (cAMP-bound) conformation (JZ unpublished findings). Precipitates were washed and subjected to SDS-PAGE and Western blotting.

Subcellular fractionation
MCF7 cells were treated with hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 1 mM $NaVO_3$, 1 µM leupeptin, 1 µM aprotinin) and homogenized through a 23G 11/4 microlance**.** Trypan blue staining of the cell lysate showed that more than 90% of the cells were broken. The lysate was centrifuged directly at 100,000g at 4°C for 90 minutes and the cytosol-containing supernatant was removed and SDS-sample buffer added (C). The pellet, containing all non-cytosolic material, was washed in lysis buffer and dissolved in sample buffer (P).

Immunofluorescence of cells cultured in 2-dimensions and in 3-dimensional collagen gels.
Cells were cultured on glass coverslips. Before fixation, medium was removed and cells were washed three times with ice-cold PBS. Fixation was performed with ice-cold methanol for 2 min for OvCar3 cells or with 3.8% formaldehyde for 20 min for MDCK cells, followed by permeabilisation with 0.2% TritonX-100 for 10 min. The samples were incubated with blocking buffer containing 4% goat serum and 0.2% bovine serum albumin (BSA) in PBS for 1 h. Cells were labelled with primary antibody for 2 h followed by washing 3 times with PBS. Alexa-conjugated secondary antibodies were applied for 1h. MDCK cells were cultured in collagen I gels as previously described[45]. Briefly, MDCK-II cells stably expressing Epac1 were suspended in a solution containing in 1% collagen and 3.7g/l $NaHCO_3$ in complete medium and added to 24-well plates and allowed to polymerise at 37°C for 20 min. Complete medium was added and replaced every 3 days. For tubulogenesis experiments, to prepare gels for microscopy, gels were incubated with collagenase (Sigma), (5ug/ml), then fixed for 30 minutes in a solution containing 3.7% formaldehyde, 10 mM PIPES pH 6.8, 0.3 M sucrose, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.1% Triton X-100. Gels were washed, permeabilised further with 0.2% Triton X-100 for 5 min , blocked for 1 h with 10% FBS with 0.01 M glycine in PBS. Gels were incubated overnight in primary antibody, washed 3 times in PBS and then incubated for at least 3 h with secondary antibody. Gels were washed extensively with PBS and mounted between glass slide and cover slip in Immu-mount (Shandon). Images were recorded using a LSM510 laser scanning confocal microscope (Zeiss Microimaging). All pictures were obtained by confocal microscopy.

Electron Microscopy

MDCK Cells were fixed by adding 4% freshly prepared formaldehyde and 0.4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 to an equal volume of culture medium for 10 min, followed by post-fixation in 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 without medium. Cells were stored until further processing in 1% formaldehyde at 4ºC. Processing of cells for ultrathin cryosectioning and immuno-labeling according to the protein A-gold method was done as described[46]. In brief, fixed cells were washed with 0.05 M glycine in PBS, scraped gently from the dish in PBS containing 1% gelatin and pelleted in 12% gelatin in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose and afterwards mounted on aluminum pins and frozen in liquid nitrogen. To pick up ultra thin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used[47].

Rap1 pull down and Western blotting
OvCar3 cells were seeded in 10 cm dishes at 40% confluence the day before treatment with siRNA duplexes as described above. A14 cells were transiently transfected with the constructs as indicated. Active Rap1 was determined by 'pull-down' assay as described previously[48]. In brief, cells were stimulated as described, washed with ice-cold PBS and lysed in a buffer containing 1%NP40, 150 mM NaCl, 50 mM Tris-Cl pH 7.4, 10% glycerol, 2 mM $MgCl_2$ with leupeptin and aprotinin. After clearing by centrifugation, active Rap1 was precipitated with the recombinant Rap1 binding domain (RBD) of RalGDS fused to GST immobilized on glutathione beads. Active Rap1 precipitates and samples of total cell lysate were resolved by SDS-PAGE, transferred to PVDF membrane and probed with the appropriate primary and secondary antibodies.

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Addendum

Cell scattering induces nuclear accumulation of Epac1

Jun Zhao and Johannes L. Bos
Department of Physiological Chemistry and
Centre of Biomedical Genetics, UMC Utrecht,
Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.

In chapter 5, we showed that the EzB domain (the first 49aa) is required and sufficient for association to Ezrin/Radixin and for localization in microvilli. Indeed, removing the EzB domain (EpacΔ49) abolished the ability of Epac1 to bind Ezrin/Radixin and microvillar localization. Surprisingly, EpacΔ49 accumulates in the nucleus (Fig. 1). This promoted us to investigate whether disruption of microvillar structures, for instance in non-polarized cells, may result in a translocation of Epac to the nucleus. We therefore pretreated MDCK-Epac1 cells with HGF which induces cell scattering and consequently disrupts the structure of the microvilli and determined the localization of Epac. After staining with Epac1 Ab, a clear nuclear signal was observed (Fig 2. A). To confirm this result, we used MDCK cells stably expressing GFP-tagged Epac, and indeed also GFP-Epac translocates to the nucleus upon HGF treatment (Fig 2. B). Since overexpression of RapV12 induces cell spreading[1,2] and consequently, destroys the microvilli, we predicted that overexpression of RapV12 in MDCK-Epac1 cells also resulted in the accumulation of Epac1 in the nucleus. Indeed in all cells that do express RapV12 we observe Epac1 in the nucleus (Fig. 3). From these results we conclude that Epac1 accumulates in the nucleus once the microvillar structure was destroyed. One plausible explanation for the release of Epac1 from the microvilli is that Ezrin/Radixin is inactivated as a prerequisite or a consequence of scattering. However, why Epac translocates into the nucleus is unclear. There are a number of explanations. It may be that Epac always needs to be anchored either to the microvilli, or to the perinuclear region (Chapter 4) and that nuclear translocation is an artifact of overexpression. However, we do see some nuclear staining of Epac1 in OvCar 3 cells which expresses Epac1 endogenously after depleting Ezrin with siRAN oligos (Chapter 4 Fig. 5). Alternatively, the nucleus serves as a sink for Epac1 to keep it away from Rap1. However, more challenging is whether Epac does have a function in the nucleus. It has been reported that Rap1 may be in the nucleus

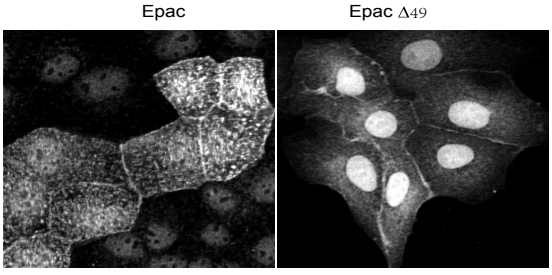

Figure 1. Removing the N-terminus tail induces Epac shifting from microvilli to the nucleus
HA-tagged Epac1 and Epac1Δ49 were transfected into MDCK cells, followed by staining with Epac1 mAb 5D3 (1:500).

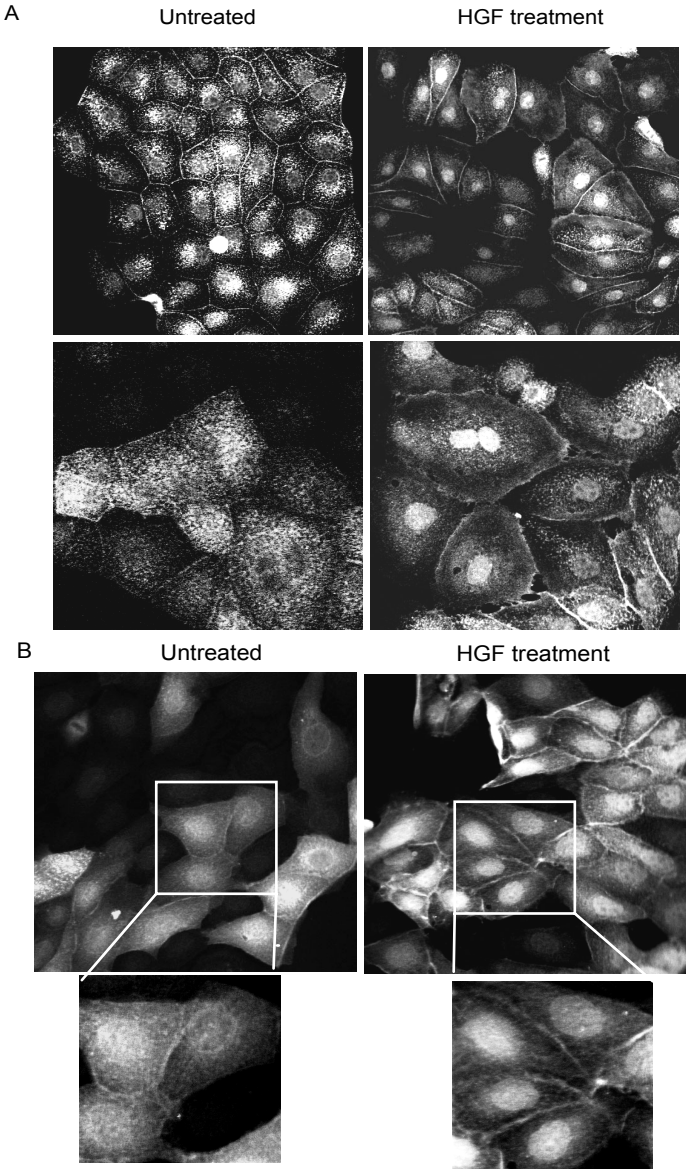

Figure 2. HGF induced nuclear accumulation of Epac
MDCK-Epac1 (A) or MDCK-GFP-Epac1 cells (B) were seeded on cover slides and treated with HGF (21ng/ml) for 18h, followed by fixation with 3.8% formaldehyde and labeled with Epac1 pAb (2293) (1:250) (A) or directly rinsed with PBS and mounted on glass slides after fixation with 3.8% formaldehyde (B). In Fig.1 (A), the magnification of upper panel of is 40X; the lower panel is 63X. In Fig1 (B), the lower panel is the enlargement of the upper panel.

as well[3], but whether that is indeed the case remains to be analyzed, particularly since Rap1 needs to be membrane-bound for proper functioning. Alternatively, nuclear Epac1 has a function distinct from Rap1.

How is Epac translocated to the nucleus? Sequence analysis did not reveal a clear nuclear localization signal. Recent mutational analysis of Epac indicated that a region in the catalytic domain (between amino acids 764 and 838) is responsible for the association to the nuclear fraction, but this fraction also contains the perinuclear membranes[4].

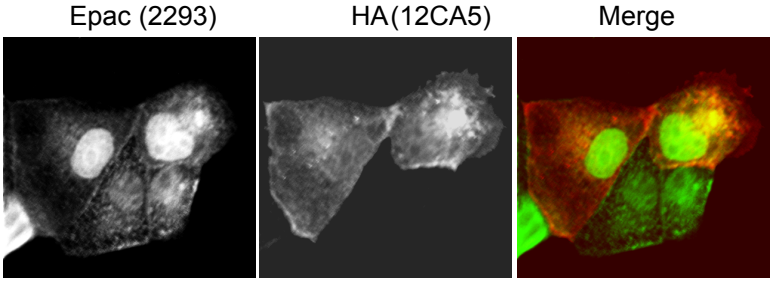

Figure 3. Overexpressed RapV12 induced nuclear accumulation of Epac
MDCK-Epac1 cells were transiently transfected with HA-tagged RapV12, followed by fixation and label with both anti-HA mAb (12CA5 tissue culture supernatant 1:50) and Epac1 pAb (2293 1:250).

In this respect, it is interesting to note that in a yeast two hybrid screen Epac1 was found to interact with RanBP2 (Zhang Z and Bos J.L., unpublished observation), a giant scaffold protein in the nuclear pore which is involved in nuclear translocation. This interaction between Epac1 and RanBP2 may also explain the frequently strong staining of Epac1 with the nuclear envelope (see for instance Fig. 2 of Addendum of Chapter 3.), but may also be instrumental in the translocation of Epac to the nucleus.

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5
General Discussion

General discussion:

Cyclic AMP, the first second messenger discovered, regulates a wide variety of cellular processes in response to hormones and various other stimuli[1]. PKA (cAMP–dependent serine/threonine protein kinase) was the first target of cAMP identified and many cAMP effects were thought to be executed through it[2]. However, the identification of other cAMP targets such as cyclic nucleotide regulated ion channel (CNG channel) and Epac1 (exchange protein directly activated by cAMP) promoted researchers to reconsider the cAMP effects mediated by PKA[3-5]. Epac1 is an exchange factor for the small GTPase Rap1 and Rap2 that can be activated directly by binding of cAMP to its cAMP binding domain[6,7]. Recently, by using the cAMP analogue 8-pCTP-2-O-Me-cAMP (007) which can specifically activate Epac1 but not PKA[8], a number of physiological processes has been assigned to Epac. Most notably, Epac1 has been implicated in integrin mediated cell adhesion[9,10], E-/VE-cadherin mediated cell-cell junction formation[11-15], regulation of insulin secretion[16,17] and sodium proton exchange activity[18].

Activation of Epac1

The Epac1 family consists of two members, Epac1 and Epac2. Both consist of a regulatory and a catalytic domain[19]. In the absence of cAMP, the protein is in the inactive conformation. The regulatory domain contains a DEP (Disheveled, Egl-10, Pleckstrin) domain which is responsible for proper localization of Epac1[19-21] and a cAMP-binding domain which is involved in activation of Epac1 via binding of cAMP[6,7]. Epac2 has a second cyclic nucleotide binding site of which the function is unclear. The catalytic domain contains a REM domain and a CDC25 homology domain; both of which are required for the catalytic function of Epac. In between the REM domain and the catalytic domain a Ras-association (RA) domain is present. In Epac2 this domain was found to bind to Ras[22]. Recently Rehmann et al., reported the crystal structure of inactive Epac2, clearly showing that the regulatory domain is folded over the catalytic domain, thereby preventing access of Rap1 to the catalytic domain. The auto-inhibition of the regulatory domain is thus by steric hindrance[7]. Upon binding of cAMP, it is predicted that the regulatory domain has to back fold. This conformational change was visualized by fluorescence resonance energy transfer (FRET) using an Epac1 protein sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Chapter2). CFP-Epac-YFP displays significant FRET that was rapidly diminished following a rise of intracellular cAMP, and increased again in response to a fall of cAMP level. This indicates that cAMP causes a significant conformational change of Epac1 in vivo and supports the unfolding model of Epac1 activation.

Localisation of Epac

One of the aims of my project was to determine the subcellular localization of Epac1 to get further insight in the function of Epac1 and the possible formation of signaling complexes. To this end, antibodies against Epac1 and Epac2 were generated and characterized, but the main focus was on Epac1. Using immunofluorescence microscopy, we observed that Epac1 is mainly located around the perinuclear region including the Golgi apparatus and the endoplasmatic reticulum (ER), and the plasma membrane. Interestingly, in fully polarized

cells we observe that Epac1 accumulates in the apical membrane, including the microvilli. Mutational analysis revealed that the N-terminal first 49 amino acids, also called the Ezrin/Radixin binding (EzB) domain, are responsible for microvillar localization of Epac. This suggested that most likely, Epac1 is bound to a microvillar protein. At the same time, Z. Zhang from our lab found in a yeast two hybrid screen that Epac1 interacts with two microvillar proteins: Ezrin and Radixin. Also for this interaction the first 49 amino acids (EzB domain) are required, strongly suggesting that indeed Ezrin/Radixin is the microvillar anchor for Epac1. Ezrin and Radixin are ERM (Ezrin, Radixin, Moesin) proteins and part of a large superfamily of proteins, whose prototype is protein 4.1 (also called red blood cell band 4.1). All members of this group of proteins contain a conserved FERM domain, mostly at the N-terminus[23,24]. ERM proteins are kept inactive by an intramolecular association of their N-terminal and C-terminal domains that masks protein-protein binding sites. Upon activation, the molecule unfolds allowing binding of membrane proteins such as CD44, ICAM[25-28] and EBP50 (ERM-binding phosphoprotein 50) to the N-terminal FERM domain and of polymerized F-actin to the C-terminal domain[29,30]. Therefore one of the functions of these proteins is to link the actin cytoskeleton to the plasma membrane. Another function is that they serve as scaffold proteins for protein complexes that function at the apical membrane of polarized cells. As such they are involved in the regulation of cell polarity, cell adhesion and cortical morphogenesis[31-33]. The interaction between Epac1 and Ezrin/Radixin was confirmed both in co-immunoprecipitation and by subcellular colocalisation. Importantly, Epac1 interacts only with the activated form of Ezrin/Radixin, indicating that localization of Epac1 is regulated by the regulation of Ezrin/Radixin. The consequence of this interaction for Epac1 function is still unclear. However, we do observe that Epac1 lacking the EzB domain is less efficient in the activation of Rap1 than full length Epac. This may imply that Ezrin/Radixin recruits Epac1 to its site of action. This action may be the activation of the apical sodium proton exchanger 3 which forms a complex with Ezrin [34-37] and is regulated by Epac1[18]. The formation of Epac-containing protein complexes was previously shown in cardiomyocytes. In these cells Epac1 participates in a cAMP responsive signaling complex that includes PKA, phosphodiesterase 4D3 (PDE 4D3) and extracellular signal regulated kinase 5 (ERK5) and this signaling complex is maintained by muscle-specific A-kinase anchoring protein (mAKAP). In this complex, Epac1 is involved in the activation of PDE to switch off the PKA signal at high concentration of cAMP[38]. Interestingly, PKA also regulates Erzin through Ser66 phosphorylation[39,40], and since PDE 4D3 interacts directly with Epac1[38], a similar complex may be formed in microvilli.

In addition to its localization in the plasma membrane/microvilli, we observed Epac1 in the perinuclear membranes including the Golgi, but also in the nucleus itself. This nuclear staining was particularly apparent for Epac1 that lacks the EzB domain, suggesting that binding to Ezrin/Radixin serves as a mechanism to keep Epac1 out of the nucleus. To test this idea we have induced cell scattering, which results in the loss of polarization of cells and disruption of the microvillar structure. Indeed, to our surprise, Epac1 accumulates in the nucleus (Adendum of Chapter 4). This preliminary finding is important since it indicates that Epac1 is translocated to the nucleus upon HGF stimulation. We have no clue yet what the function of Epac1 is in the nucleus. It has been reported that Rap1 may be in the nucleus as well [41], but whether that is indeed the case remains to be analysed, particularly since

Rap1 needs to be membrane-bound for proper functioning. Alternatively, nuclear Epac1 may have a function distinct from activating Rap1 or the nucleus may serves as a sink for Epac1 to keep it away from Rap1. The nuclear localization is further supported by the notion that in a yeast two hybrid screen Epac1 was found to interact with RanBP2 (Zhang Z and Bos J.L., unpublished observation), a giant scaffold protein in the nuclear pore and involved in nuclear translocation. This interaction between Epac1 and RanBP2 may also explain the frequently strong staining of Epac1 with the nuclear envelope (see for instance Fig. 2 of Addenum of Chapter 3). Since Epac1 has no clear nuclear localization signal, it is unclear how the protein is transported into the nucleus, but it may be mediated by binding to proteins like RanBP2.
A role for Epac1 at sites distinct form the plasma membrane or microvilli was previously suggested by two studies. First, using a FRET probe to monitor Rap1 activation, cAMP activates Rap1 at the perinuclear region in COS-1 cells[42] and secondly, in AtT20 cells expression of Epac1 resulted in the activation of Rap1 around perinuclear region[43]. However, which of the various biological functions of Epac1 is mediated by perinuclear Epac1 needs further investigation.

Multiple anchoring domains
In addition to the EzB domain, Epac1 has two additional membrane anchoring domains, the DEP domain[3,20,21] and the RA domain[22]. Both fractionation and immunofluroscence results suggested that the DEP domain is required for membrane association of Epac1[19,20] (Chapter 2 and 4) but not for microvillar localization. How the DEP domain confers membrane localization is unclear. But the DEP domain may be involved in binding to either a lipid or a membrane protein like the DEP domain of RGS9, which interacts with the membrane anchoring protein R9AP[44]. Deletion of the EzB domain or the DEP domain results in a reduced ability of Epac1 to activate Rap1. This indicates that the EzB domain and the DEP domain cooperate in the proper localization of Epac1 to the plasma membrane in microvilli. The function of the RA domain in Epac1 is still unclear. However, for Epac2 a similar domain was found to interact with Ras and involved in EGF-induced translocation of Epac2[22] . This suggests that the RA domain of Epac1 may also bind to one of the Ras like small GTPases. But, which one is the binding partner of RA domain of Epac1 is still a question mark.

Activation specific antibody
One of our Epac1 specific monoclonal antibodies 5D3 was shown to recognize an epitope close to the cAMP binding site. Interestingly, 5D3 recognizes Epac1 particularly in the presence of cAMP and thus in the active conformation. When 5D3 is added to Epac1 it results in a slow cAMP-independent activation of Epac, indicating that when Epac1 unfolds, 5D3 can trap it in the active conformation. This is further supported by the notion that very low concentrations of cAMP are already sufficient to rapidly and fully activate Epac1 in the presence of 5D3 (Chapter 3). This provides a proof-of-principle that there are ways to activate Epac1 independent of cAMP. It is noteworthy that 5D3 only recognizes a subfraction of Epac1 in cells fixed with 4% paraformaldehyde. For instance in MCF7 GFP-

Epac cells, 5D3 did not recognize Epac1 present in the perinuclear region. This may be due to the fixation procedure, but alternatively it may indicate that in the fraction of Epac1 that is not recognized, the epitope is shielded. Another possibility is Epac1 keeps inactive conformation around perinuclear region, but addition of cAMP did not restore staining. Whatever the explanation is, it may point to a further complexity in Epac1 localization.

Function of Epac

Our studies have shown that Epac1 is particularly involved in the control of cell junction formation [14,45]. For instance, 007 strongly reduce the permeability of the endothelial cell layer, a process mediated by VE-cadherin[11,13,14]. However our studies did not reveal any localization of Epac1 in mature junctions. This may imply that Epac1 in the junction is below our detection limit. Alternatively, Epac1 may direct the process from the apical site of the cell. Perhaps Epac1 triggers the process resulting in a cascade of events inside the junction. This may involve other Rap1GEFs like C3G and PDZ-GEF, both are found in complex with E-cadherin (C3G) or E-cadherin associating proteins (PDZ-GEF)[12,46]. It is clear that understanding the connection between the localisation of Epac1 and the various functions of Epac1 is one of the future challenges.

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Summary

Cyclic adenosine 3', 5'- monophosphate (cAMP) is a second messenger that functions through binding to its downstream targets protein kinase A (PKA), cyclic-regulated ion channels (CNG channels) and Epac (exchange protein directly activated by cAMP). Epac (Epac1 and Epac2) is a guanine nucleotide exchange factor towards both Rap1 and Rap2. It is kept in its inactive conformation by an intramolecular interaction between the regulatory and catalytic domain. Binding of cAMP to the cAMP-binding domain within the regulatory region liberates the catalytic domain, resulting in the activation of Epac. In order to visualize the conformational change between the inactive- and active-state of Epac, a CFP-Epac-YFP probe was generated and fluorescence resonance energy transfer (FRET) between the two fluorescent moieties was monitored in vivo. The FRET signal rapidly decreased in response to cAMP-raising agents, whereas it fully recovers after addition of cAMP-lowering agonists (Chapter 2). This indicates that cAMP causes a significant conformational change in vivo and supports the unfolding model for Epac1 activation. In addition, compared with the PKA-FRET probe, the Epac-FRET probe has a much larger dynamic range for cAMP, and this allows the Epac-FRET probe to measure changes in physiological cAMP levels which PKA-FRET probe failed to record (Chapter 2).

8-pCPT-2'-O-Me-cAMP (007), a cAMP analogue which can specifically activate Epac1 but not PKA has put Epac1 in a pivotal position in many biological processes such as VE-/E-cadherin-mediated junction formation, integrin-mediated cell adhesion, insulin secretion and sodium proton exchange activity. However, the lack of suitable antibodies against Epac1 limited a detailed analysis of Epac. Therefore, a series of Epac1 antibodies (Abs) were generated and characterized. 5D3, one of the Epac1 monoclonal Abs, was further characterized. Both in vivo (data not shown) and in vitro experiments demonstrated that 5D3 can specifically recognize the active conformation of Epac1, and the epitope of 5D3 was mapped within the cAMP binding domain, in particular around Leucine 273. This region is hidden during autoinhibition (Chapter 3 and its Addenum).

To determine the subcellular localization of Epac1 was the main goal of my project. Using Epac1 Abs, we observed that Epac1 is mainly distributed around the perinuclear region, especially in the endoplasmatic reticulum and the Golgi apparatus, as well as in the plasma membrane, especially at microvilli in fully polarized cells (Chapter 4). Functional domains which are responsible for the proper localization of Epac1 were also analyzed in detail. Our results revealed that both the DEP domain and the EzB domain (the first 49 aa) are required for the correct localization of Epac1 and the activation of Rap. The EzB domain is not only responsible but also sufficient for targeting Epac1 at microvilli. In contrast the DEP domain is only responsible for membrane localisation (Chapter 4). Importantly, the microvillar localization is through binding to Ezrin/Radixin, proteins that function as linkers between the actin cytoskeleton and the apical membrane of polarized cells and as scaffold protein for protein complexes. The functional relevance of this interaction is still unclear, but the mere fact that Epac1 only binds to the active form of Ezrin/Radixin indicates that activation of these proteins is a crucial step in the spatial regulation of Epac1.

An interesting and surprising observation was that Epac1 accumulates in the nucleus when the EzB domain of Epac1 was deleted. This effect could be mimicked by stimulation of the cells with HGF, which induces cell scattering, and with overexpression of RapV12 which induces cell spreading. These results suggest that upon loss of cell polarity and thus disruption of the apical structures, part of Epac1 translocate to the nucleus. However, the function of Epac1 in the nucleus remains unclear (Addendum of Chapter 4).

Samenvatting in het Nederlands

cAMP heeft een belangrijke rol in cellen als 'boodschappermolecuul', doordat het PKA, cyclisch-nucleotide gereguleerde ion-kanalen en Epac kan binden en activeren. Epac (Epac1 en Epac2) op zijn beurt kan de eiwitten Rap1 en Rap2 activeren. Deze twee kleine GTPasen zijn inactief als ze GDP gebonden hebben en worden actief na binding van GTP. Epac zorgt ervoor dat GDP los komt van Rap zodat Rap GTP kan binden, hetgeen in hogere mate aanwezig is in de cel.

Epac wordt in zijn inactieve conformatie gehouden door een interactie tussen de regulatoire en catalytische regio van het eiwit. Binding van cAMP aan de regulatoire regio zorgt voor een verandering van de structuur van Epac, waardoor het catalytische deel toegankelijk wordt voor Rap. Om deze conformatie-verandering te visualiseren is een CFP-Epac1-YFP probe gemaakt. De 'fluorescence resonance energy transfer' (FRET) tussen de twee fluorescente moleculen is afhankelijk van hun onderlinge afstand, en daardoor afhankelijk van de conformatie van Epac. In cellen met deze probe neemt het FRET-signaal snel af na toevoeging van cAMP-verhogende reagentia, wijzend op een vergrote afstand tussen de CFP en YFP moleculen, waarna het snel weer toeneemt in aanwezigheid van cAMP-verlagende stimuli (hoofdstuk 2). Dit geeft aan dat cAMP een significante conformatie-verandering van Epac teweegbrengt en ondersteunt het model dat Epac opengevouwen wordt bij activatie. Een voordeel van de Epac1-FRET probe is het grotere dynamisch bereik voor cAMP ten opzichte van de al eerder beschreven PKA-FRET probe. Hierdoor is het mogelijk deze Epac1-FRET probe te gebruiken om veranderingen in cAMP levels waar te nemen die buiten het bereik van de PKA-FRET probe vallen (hoofdstuk 2).

Een analoog van cAMP, 8-pCPT-2'-O-Me-cAMP (007), kan specifiek Epac activeren zonder de activiteit van PKA en de cyclisch-nucleotide gereguleerde ion-kanalen te activeren. Met behulp van deze analoog is de rol van Epac in verscheidene biologische processen reeds aangetoond, zoals de integrine-gemedieerde adhesie van cellen en de secretie van het hormoon insuline. Om de functie van Epac1 in meer detail te analyseren zijn antilichamen tegen het eiwit gegenereerd. Karakteristisatie van één van deze antilichamen tegen Epac1, 5D3 genaamd, heeft aangetoond dat dit antilichaam specifiek de actieve vorm van Epac1 kan herkennen. De epitoop herkend door dit antilichaam bevindt zich in het cAMP-bindende domein van Epac1, met name rond aminozuur Leucine 273, welke niet toegankelijk is wanneer Epac inactief is (hoofdstuk 3 en Addendum).

Verschillende antilichamen tegen Epac1 laten zien dat het eiwit zich in cellen voornamelijk bevindt in het gebied rond de celkern, met name het Endoplasmatisch Reticulum en het Golgi-apparaat, en nabij het plasma membraan (hoofdstuk 4). In volledig gepolarizeerde cellen bevindt Epac1 zich grotendeels in de microvilli. Twee domeinen in het regulatoire deel van Epac1 zijn verantwoordelijk voor de correcte localizatie van Epac1 en de activatie van Rap (hoofdstuk 4). Het DEP domein is verantwoordelijk voor membraan localizatie. De N-teminale staart van Epac daarentegen is noodzakelijk en tevens voldoende om Epac1 te localiseren in de microvilli. De aanwezigheid van Epac1 in de microvilli is afhankelijk

van de binding van Epac1 aan het eiwit Ezrin of Radixin. Deze eiwitten koppelen het apicale membraan van gepolarizeerde cellen aan het actine cytoskelet. Tevens kunnen Ezrin en Radixin verschillende andere eiwitten binden, waardoor ze meerdere eiwitten bij elkaar brengen. Epac1 kan alleen aan de actieve vorm van Ezrin en Radixin binden, wat aangeeft dat de activatie van Ezrin en Radixin waarschijnlijk een cruciale stap is in de regulatie van de localizatie van Epac1.

Epac1 zonder de N-terminale staart bevindt zich niet in de microvilli, maar hoopt zich verrassend genoeg op in de celkern. Ook stimulatie van cellen met het hormoon HGF, welke cellen uiteen drijft, of verhoogde aanwezigheid van actief Rap in cellen hetgeen spreiding van cellen veroorzaakt, zorgt voor de relocalizatie van Epac1 naar de kern. Dit suggesteert dat na verlies van polariteit van cellen en daardoor de verstoring van apicale structuren van de cel, Epac1 gedeeltelijk transloceert naar de celkern. Vooralsnog is de functie van Epac1 in de celkern nog onduidelijk (Addendum).

结 论

Cyclic adenosine 3', 5'- monophosphate (cAMP) 是细胞内的一个非常重要的第二信使分子，它可以通过其下游的作用原件比如：PKA，cyclic-regulated ion channels (CNG channels)和 Epac (exchange protein directly activated by cAMP) 来发挥其功能。Epac 是小 G 蛋白 Rap 的激活因子之一。它包括一个调控区和催化区，并可以通过调控区和催化区之间的分子内结合（autoinhibition）而使 Epac 蛋白呈现非激活态（闭合状态）。当 cAMP 结合在其调控区的 cAMP 结合域时，可以破坏其自体抑制作用，从而使其催化区暴露出来，利于 Rap 小蛋白的结合, 此时，Epac 则呈现了激活态（开放状态）。为了证明 Epac 蛋白的激活模型，我们构建了 Epac 的 FRET 载体，即将 YFP 和 CFP 银光蛋白融合在其氮端和碳端，检测 Epac 在从非激活态转变到激活态时，是否会降低从 CFP 到 YFP 的能量转移。试验结果表明，Epac 在非激活态时，氮端和碳端之间的距离非常接近以致于使 CFP 的辐射能量转移到邻近的 YFP，从而可以检测到 YFP 的辐射光谱。当 Epac 被激活时，氮端和碳端之间的距离由近变远，从而不能检测到由 CFP 辐射而转移到 YFP 的辐射光谱。因此，利用 Epac 的 FRET 载体，我们证实了 Epac 的激活模型就是一个由闭合到开放的过程（第二章）。

8-pCPT-2'-O-Me-cAMP (007)是 cAMP 的一个同源物，它可以特异地激活 Epac，而不影响 PKA 的活性。利用这一人工合成物，研究者已将 Epac 蛋白定位在 cAMP 介入的信号转导途经的一个非常重要的位置上。例如，Epac 已被证明介入了细胞增殖过程；VE-/E-cadherin 介导的细胞间的连结的形成过程；整合蛋白介导的细胞和基质的附着过程；胰岛素的分泌过程以及钠质子交换活性的调控过程。但是，为了了解 Epac 蛋白的更多的功能，我们制备了并鉴定了 Epac 的单克隆和多克隆抗体。由于 5D3，Epac 的单克隆抗体之一，可以识别激活状态的 Epac 蛋白，因此，在第三章我们详细地鉴定了这个单克隆抗体并把它的表抗原位置定位在了 Epac 调控区的 cAMP 结合域内，而第 273 亮氨酸的位置附近则是这一抗体表原位的精细位置，而这一位置正好处于 Epac 蛋白自体结合区。

Epac 的亚细胞定位是本论文的重要部分。而且对 Epac 进行亚细胞定位对于了解 Epac 的功能也很重要。利用我们制备的 Epac 单克隆和多克隆抗体，我们发现 Epac 主要分部在细胞核膜周围，特别是高尔基和内质网上，同时，细胞膜也是 Epac 蛋白的主要的分布区，特别是在极性化细胞的微乳突上（Microvilli）。为了了解对 Epac 亚细胞定位起重要作用的功能域，我们利用一系列的 Epac 缺失突变体发现 Epac 的正确定位需要两个膜定位信号，一个是 DEP 作用域，另一个是 N 末端的 49 个氨基酸。这两个膜定位信号不仅在 Epac 正确定位是起非常重要的作用，而且也是 Epac 激活小 G 蛋白 Rap 所不可缺少的功能区。N 末端的 49 个氨基酸只在 Epac 定位在微乳突上发挥作用，而且仅仅是 49 个氨基酸足以牵引 Epac 蛋白定位到细胞的微乳突上。DEP 作用域尽管在 Epac 定位在微乳突上没有发挥作用，但细胞组分的分离结果表明 DEP 作用域仍然在 Epac 的膜定位过程中发挥一定的功能。利用 Epac 作为诱饵，ERM 家族蛋白 Radixin 和 Ezrin 从酵母双杂库（Y2H）中被筛选出来。Radixin 和 Ezrin 是一个支架蛋白（Scaffold），它们定位在细胞的微乳突上并在连结细胞骨架和极性细胞膜之间起作用。Epac 通过它的 N 末端的 49 个氨基酸和 Radixin 和 Ezrin 的 N 端 结合在一起，但是这一个蛋白复合体的功能还是一个问号。同时在免疫共沉淀的试验中，我们发现 Epac 只能和激活态的 Radixin 和 Ezrin 免疫共沉淀，这一现象预示这些 ERM 蛋白可以调控 Epac 区域性功能的发挥（第四章）。在研究 Epac 蛋白的亚细胞定位过程中，我们发现当 Epac 的 N 末端的 49 个氨基酸缺失后，Epac 常常聚积在细胞核中。这一现象也在 HGF（细胞分散因子）诱导的细胞分散和 RapV12 引起的细胞扩散试验中得到了证实。这一结果预示着细胞极性或者细胞的微乳突结构的破坏可以诱导 Epac 蛋白转移到细胞核中，但是转移到细胞核中的 Epac 将发挥什么样的功能仍然有待于进一步的研究（第四章的 Addendum）。

Curriculum Vitae

Jun Zhao was born on May 27, 1969 in BaoTou, Inner Mongolia, and P.R. China

1987 – 1991 Studying for Bachelor Degree in Agronomy Department of Inner Mongolia Agricultural University. Specialization: Plant Protection

1991 – 1997 Lecturer at the Agronomy Department of Inner Mongolia Agricultural University.

1997 – 2000 Studying for Master Degree in the Key Lab of National Agricultural Biotechnology, Department of Biology, and China Agricultural University.
Specialization: Molecular Genetics
Supervisor: Prof. Guoying Wang
The thesis is mainly focused on Inheritance of resistance to Maize Yellow Spots caused by Cuvularia Lunata and localization the resistant gene on maize genetic map by using RFLP and SSR markers.

2000 – 2002 Exchange Program Scholar in Laboratory of Molecular Biology, Department of Plant Science, and Wageningen University
Supervisor: Prof. Ton Bisseling
The research mainly focused on Cloning Sym2 gene by Microsynteny between Pea and Medicago truncatula.

2002 – 2006 Research assistant (AIO) in Department of Physiological Chemistry and Center of Biomedical Genetics, Medical Center of Utrecht University
Supervisor: Prof. Johannes L. Bos
The thesis is mainly focused on Subcellular localization of Epac

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