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).

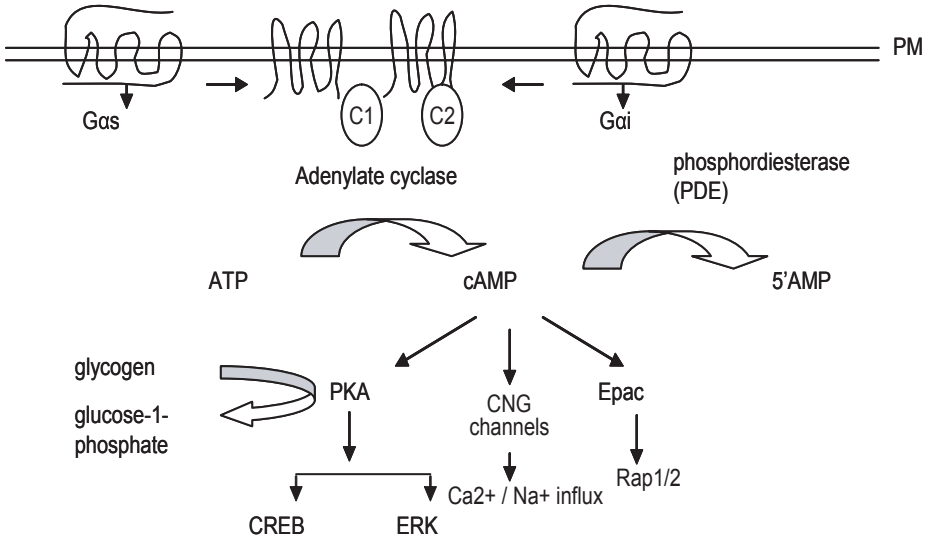

Figure 1. Outline of cAMP generation and its related signaling pathway.
Adenylate cyclases synthesize cAMP from ATP. Phosphodiesterases converts cAMP to 5'AMP. PKA, Epac and CNG channels are cAMP targets, however, they have different downstream effectors and are involved in different signaling pathways.
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

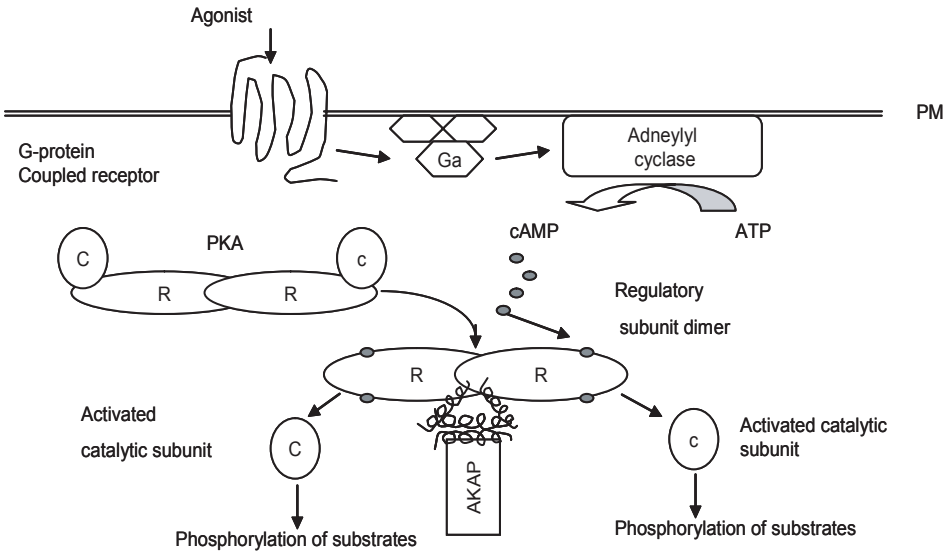

Figure 2. Overview of PKA activation

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.

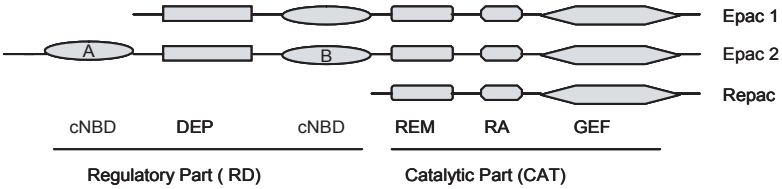

Figure 3. Structure of Epac proteins
DEP: Disheveled, Egl-10, Plecktrin domain; cNBD: cNMP binding domain; REM: Ras-exchange motif; RA: Ras association domain; GEF: guanine nucleotide exchange factor.

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].

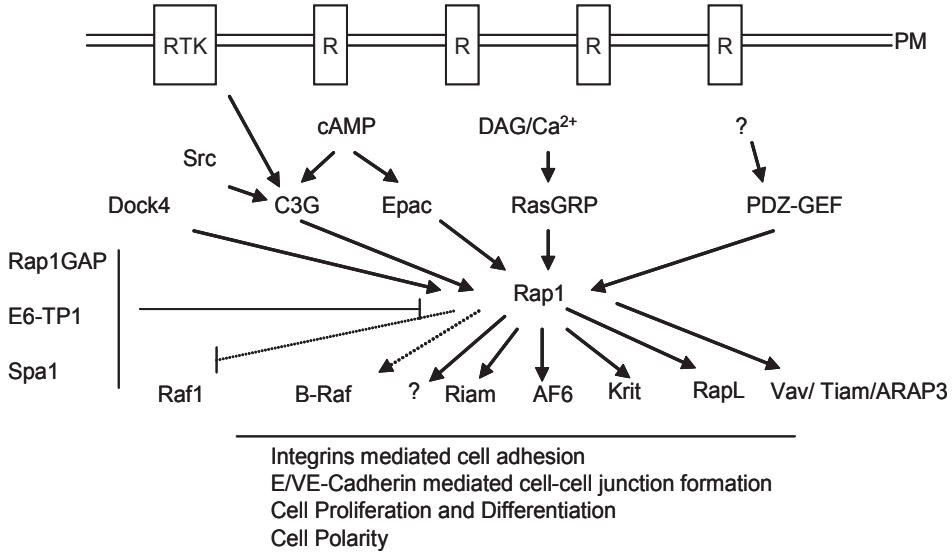

Figure 4. Regulation of Rap GTPase and its downstream signaling pathways
cAMP, calcium (Ca^{2+}) and diacylglycerol (DAG) can activate Rap guanine nucleotide exchange factors, thus, leading to the activation of Rap. GTP bounded Rap can be down-regulated by RapGAP, SPA1 and E6-TP1. Rap may regulate different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