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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