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Gene Wiki Review

Exchange protein directly activated by cAMP encoded by the mammalian rapgef3 gene: Structure, function and therapeutics

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Abstract

Mammalian exchange protein directly activated by cAMP isofrom 1 (EPAC1), encoded by the RAPGEF3 gene, is one of the two-membered family of cAMP sensors that mediate the intracellular functions of cAMP by acting as guanine nucleotide exchange factors for the Ras-like Rap small GTPases. Extensive studies have revealed that EPAC1-mediated cAMP signaling is highly coordinated spatiotemporally through the formation of dynamic signalosomes by interacting with a diverse array of cellular partners. Recent functional analyses of genetically engineered mouse models further suggest that EPAC1 functions as an important stress response switch and is involved in pathophysiological conditions of cardiac stresses, chronic pain, cancer and infectious diseases. These findings, coupled with the development of EPAC specific small molecule modulators, validate EPAC1 as a promising target for therapeutic interventions.

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Keywords: RAPGEF3, cAMP signaling, Signalosomes, Therapeutic target

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1. Introduction

Second messengers play a critical role in relaying the extracellular cues to trigger various intracellular signaling. Identified more than 50 years back, adenosine 3',5'-cyclic monophosphate (cAMP) represents the first known second messenger (Rall and Sutherland, 1958). The elucidation of the cAMP signaling cascade has led to several ground-breaking discoveries in biology and transformed the practice of modern medicine and pharmacuetics. Intracellular cAMP is produced from adenosine triphosphate (ATP) by the action of a family of enzymes known as adenylyl cyclases (ACs) in response to the activation of guanine nucleotide-binding proteins (G-proteins) initiated by the binding of extracellular ligands to G-protein coupled receptors (GPCRs). cAMP is known to be involved in regulating a myriad of biological functions, including metabolism, gene transcription, cell proliferation, differentiation, migration, and apoptosis (Fig. 1).

Earlier, it was widely accepted that cAMP exerted its action mainly through the ubiquitous cAMP-dependent protein kinase/protein kinase A (PKA) initially discovered in 1968 (Walsh et al., 1968) or tissue-specific cyclic nucleotide-regulated ion channels (CNG and HCN). However, not all cAMP mediated cellular events are dependent on PKA, suggestive of yet unidentified additional mechanisms and molecular targets. In 1998, these discrepancies were better explained following the discovery of a new family of cAMP sensors, namely exchange proteins directly activated by cAMP (EPACs) or cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), cAMP is known to be involved in regulating a myriad of biological functions, including metabolism, gene transcription, cell proliferation, differentiation, migration, and apoptosis (Fig. 1).

2. Origin of EPAC

The formation of EPAC gene, through the fusion of two functional elements, cAB and GEF motifs, is evolutionally more recent than that of PKA. While PKA is present in unicellular eukaryotes like Saccharomyces cerevisiae, EPAC proteins have been only found in multicellular organisms. The cAB domain of EPAC is presumably devised from the two tandem cAB domains (cAB-A and cAB-B) of the regulatory subunit of PKA. In lower eukaryotes, such as Caenorhabditis elegans and Drosophila, only one EPAC gene with both cAB domains is found. The divergence of EPAC1 and EPAC2 isoforms most likely originated from a gene duplication event through evolution, during which EPAC2 physically retains both cAB domains while EPAC1 lacks the cAB-A site. Interestingly, although the cAB-A site is present in EPAC2, its measured cAMP binding affinity (87 μM) is significantly weaker than that of the cAB-B site (1.2 μM), which is similar to that of EPAC1 (4 μM) (De Rooij et al., 2000). Therefore, it appears that the cAB-A site in EPAC2 and EPAC1 sequences with homology to both cAB domain of PKA and GEF domain for Ras/Rap1 (De Rooij et al., 1998). As the name suggests, EPAC/cAMP-GEF is a novel class of protein that contains both cAB and GEF domains within a single polypeptide chain. In mammals, two independent genes known as Rap guanine nucleotide exchange factors 3 and 4 (RAPGEF3 and RAPGEF4) encode for the two EPAC isoforms, EPAC1 and EPAC2; respectively. The discovery of EPACs has opened up additional exciting avenues for exploration of the cAMP signaling pathway. This review focuses on the EPAC1 isoform. Since excellent reviews related to EPAC structure and function have been published earlier (Gloerich and Bos, 2011; Schmidt et al., 2013), significant efforts will be placed on discussion of more recent findings related to in vivo studies of the physiological functions of EPAC1 and the potential of EPAC1 as a therapeutic target for human diseases.

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**Fig. 1.** Schematic representation of cAMP signaling pathways. cAMP signaling pathways are initiated by the extracellular binding of the activating ligand to the G-protein coupled receptor (GPCR), which lead to a cascade of reactions involving the sequential activation of G protein and adenylyl cyclase (AC), and consequently the conversion of ATP to cAMP. Increase in intracellular levels of cAMP results in the activation of cAMP sensors, including the ubiquitously expressed cAMP-dependent protein kinase/protein kinase A (PKA) and exchange protein directly activated by cAMP/cAMP-regulated guanine nucleotide exchange factor (EPAC/cAMP-GEF), as well as tissue-specific cyclic nucleotide-regulated ion channels (CNG and HCN).
has lost its functionality in cAMP binding either partially or completely. The c-terminal GEF domain of EPAC is very closely related to the Ras superfamily GEF proteins. While the EPAC GEF domains contain all three structurally conserved regions (SCRs) found in RasGEFs (Boguski and McCormick, 1993), they have evolved sufficiently to form a distinct sub-cluster.

3. EPAC1 gene and transcripts

Human EPAC1 gene, RAPGEF3, is located on the chromosome 12 (12q13.11: 47,734,367–47,771,041), and contains 28 exons. It is reported to have up to 20 potential transcripts (Ensembl: http://e79.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000079337; r=12:47734367-47771040). Among these predicted transcripts or splice variants, three EPAC1 transcript variants have been validated in the NCBI database. Transcript variant 1 (6239 bp), the longest transcript, encodes the longer EPAC1 isoform i.e. EPAC1a with 923 amino acids. On the other hand, transcript variant 2 (5773 bp) and 3 (6003 bp) differ in the 5′ untranslated regions (UTRs) and contain an alternative 5′ exon when compared to variant 1, which leads to translation initiation at a downstream starting codon. Variants 2 and 3 encode the same EPAC1 isoform, EPAC1b with a shorter N-terminus and a total of 881 amino acids. Similarly, mouse EPAC1 gene, located on the chromosome 15 (15qF1: 97,744,770–97,767,972), also has three validated transcript variants (Ensembl: http://useast.ensembl.org/Mus_musculus/Info/Index?db=core;g=ENSMUSG0000022469;r=15:97744770-97767972). Transcript variant 1 (3825 bp) is the longest transcript and encodes the longest EPAC1 isoform 1 with 926 amino acids. While transcript variant 2 (3801 bp) uses two alternate in-frame exons, transcript variant 3 (3774 bp) lacks an alternate in-frame exon, both in the central coding region. These differences result in shorter isoforms 2 (918 aa) and 3 (909 aa). The functional differences, tissue distribution and significance of these EPAC1 isoforms have not been studied and are not clear.

The promoter region of EPAC1 contains two glucose responsive element (GRE) (CACGTG) sites corresponding to nucleotides -1112 to -1106 (GRE1) and -479 to -473 (GRE2) (Sun et al., 2011), as well as a hypoxia responsive element (HRE) (ACGTG) site located at -1232 to -1228 (Lai et al., 2012).

4. EPAC1 protein structure and mechanism of activation

EPAC1 proteins share considerable sequence and structural similarities with EPAC2. For example, human EPAC1a and EPAC2a share 50% sequence identity and 68% similarity. With the exception of the N-terminus, EPAC1 contains identical structural domains as found in EPAC2 (Fig. 2A). Specifically, the N-terminal regulatory region of EPAC1 consists of a lead sequence followed by Dishevelled, Egl-10, Pleckstrin (DEP) and cAB domains. On the other hand, C-terminal catalytic region is characterized by the presence of a RAS exchange motif (REM), a RAS-association (RA) domain and a CDC25 homology domain, also known as the guanine nucleotide exchange factor for Ras-like small GTPases (RasGEF) domain. While the REM region interacts with the GEF region and stabilizes the CDC25 homology domain, it is not directly involved in interacting with the small GTPase (Boriack-Sjodin et al., 1998).

Functionally, EPAC in its ligand-free form remains in an auto inhibitory mode wherein cAB domain sterically blocks the catalytic site. This is supported by the observation that a deletion mutant EPAC1 protein lacking the regulatory region is constitutively active in vitro (De Rooij et al., 1998). Furthermore, individually isolated EPAC N-terminal regulatory half (EPAC1-RD1–328), as well as the cAB domain alone (EPAC1-CAT324–881), can form a stable complex with the C-terminal catalytic half (EPAC1-CAT324–881) and inhibit its GEF activity. The addition of cAMP obliterates this inhibitory effect as it is in the case of full-length EPAC1 (De Rooij et al., 2000). Interestingly, a cAB domain construct shorter by eleven amino acids (EPAC1-RD1–317) does not inhibit EPAC1-CAT324–881. This observation leads to the identification of a conserved sequence motif 321VLVLE325 required for the auto-inhibition of EPAC. Mutation of the VLVLE motif into AAAAA results in a constitutively active EPAC protein in the absence of cAMP (Rehmann et al., 2003). Crystallographic analysis of the full-length EPAC2 reveals that these residues participate in the formation of a central “switchboard” structure that is critical for maintaining the proper orientation between the
regulatory and catalytic region to keep EPAC in its auto-inhibitory state (Rehmann et al., 2006, 2008).

The crystal structure of a deletion EPAC2 lacking the first 305 amino acids in complex with Sp-cAMPS and Rap1B (EPAC2Δ305: Sp-cAMPS:Rap1B) provides a snapshot of the active conformation of EPAC proteins. It reveals the conformational changes that accompany the EPAC activation in atomic details (Rehmann et al., 2006, 2008). Indeed, structural comparison between EPAC2Δ305: Sp-cAMPS:Rap1B and full length autoinhibitory EPAC2 shows that cAMP binding causes a mostly rigid body movement between the regulatory and catalytic regions pivoting around the hinge. As a consequence, the regulatory half moves to the rear side of the catalytic half, thereby exposing Rap binding site and relieving the autoinhibition (Fig. 2B). Interestingly, the cAMP-induced hinge motion brings the switchboard closer to the cAMP binding pocket. In fact, the VLVLE motif forms part of the lid capping the cAMP binding site (Rehmann et al., 2006, 2008). These results support the notion that while EPAC proteins employ switchboard as a unique structural feature for its functionality, they share a similar activation mechanism, namely hinge motion, with other cyclic nucleotide binding proteins (Rehmann et al., 2007). Indeed, disrupting the hinge helix by replacing the conserved F53 residue with a G in EPAC2 leads to constitutive activation (Tsalkova et al., 2009; White et al., 2012).

In addition to the static conformational changes revealed by X-ray crystallography, enhanced deuterium exchange-mass spectrometry (DXMS), NMR and molecular simulation analyses suggest that protein dynamics also play important roles in EPAC activation (Li et al., 2011; Van Schouwen et al., 2011). cAMP binding causes extensive allosteric perturbation of dynamics in the catalytic region. In particular, reducing the dynamics of the helical hairpin that interacts directly with RAP1 may reduce the entropic penalty and promote the binding of RAP1 substrate (Li et al., 2011; Van Schouwen et al., 2011).

It is important to note that majority of the structural analyses of EPAC proteins are based on EPAC2 as isolated recombinant EPAC1 protein is much more delicate and resistant to crystallographic study. NMR analyses of the cAB domain deletion proteins have been performed and provided important insights into understanding the roles of alloster and dynamics during EPAC1 activation (Das et al., 2008; Harper et al., 2008; Mazhab-Jafari et al., 2007; Selvaratnam et al., 2011, 2012). To date, no full-length active structure for either EPAC1 or EPAC2 is available; therefore, major gaps in our understanding of EPAC activation remain.

5. EPAC specific modulators

5.1. Agonists

Selective EPAC pharmacological modulators have been developed to better understand and discern the independent roles of EPAC or PKA mediated cAMP signaling. Through comparative sequence analyses, it was discovered that a conserved glutamic acid residue of cAB domain of PKA regulatory subunits and cyclic nucleotide regulated ion channels is missing in EPAC protein. This glutamic acid forms a hydrogen bond with the 2′-OH group of the cAMP ribose moiety and is required for high-affinity cAMP binding (Su et al., 1995; Zagotta et al., 2003). The initial agonist discovery efforts explored this critical difference and led to the identification of a cAMP analog, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-pCPT-2′-O-Me-cAMP) that is capable of selectively activating EPAC1 (Christensen et al., 2003; Enserink et al., 2002). Subsequent modifications and optimizations allowed the development of more membrane permeable and PDE-resistant EPAC-specific agonists (Poppe et al., 2008; Vliem et al., 2008). Detailed discussion of cAMP analogs as pan EPAC activators has been reviewed extensively previously (Chen et al., 2014; Holz et al., 2006, 2008).

Achieving selectivity between the two isoforms of EPAC has generally been challenging due to the greater sequence homology between them. Nonetheless, major efforts made towards the discovery of cAMP based selective EPAC1 and EPAC2 modulators have shown initial promise. For instance, 8-pCPT-2′-O-Me-cAMP with 2′-O methyl substitution on the ribose moiety of cAMP structure along with 8 position para-chlorophenylthio (pCPT) substitution was more selective for EPAC1 agonism (AC50 = 1.8 μM; relative kmax = 3.6) than for EPAC2 (AC50 = 3.5 μM; relative kmax = 0.8). Moreover, structural basis for this observed activity has been further elucidated and attributed it to the Gln270 moiety present in EPAC1 in contrast to the Lys405 in EPAC2 (Schouwen et al., 2015). This was further confirmed by site directed mutagenesis and structural studies wherein this unique Gln270 residue has been shown to favorably influence the hinge region conformation upon 2′-O alkylated ligand binding in EPAC1 (Schouwen et al., 2015). On the other hand, Sp-8-Bnt-Me-cAMPS with axial sulfur in the cyclic phosphate along with 8 position benzylthio group (BntT) is an excellent activator of EPAC2 (AC50 = 2.1 μM; relative kmax = 6.6) but a poor one for EPAC1 (AC50 = 13 μM; relative kmax = 0.3). Structural analysis of EPAC2:Sp-8-Bnt-Me-cAMPS complex reveals that the aromatic ring of the BntT of Sp-8-Bnt-Me-cAMPS is sandwiched between Leu379, a part of the core cAB domain, and Lys405, a part of the lid, thereby stabilizing the active conformation. The corresponding Lys405 residue in EPAC1 is Glu315. EPAC2K450E mutant shows a reduced ability to be activated by Sp-8-Bnt-Me-cAMPS, suggesting that difference between Lys405 and Glu315 in EPAC2 and EPAC1, respectively, is responsible for the preference of Sp-8-Bnt-Me-cAMPS for EPAC2 over EPAC1 (Schouwen et al., 2015). To date, no non-cAMP analog EPAC activator has been identified.

5.2. Antagonists

The designer cAMP analog approach has one major limitation for crafting EPAC antagonists because cAMP analogs can bind and inhibit phosphodiesterases (PDEs), which are enzymes critical for metabolizing and desensitizing cyclic nucleotide signaling. Inhibiting PDEs increases cyclic nucleotide concentration in cells and counters the intended antagonistic effects. De novo screening of a diverse drug-like small molecule library with a robust EPAC2-based high throughput screening assay led to the successful identification and validation of several novel EPAC specific inhibitors (ESIs) (Almahairaq et al., 2013; Chen et al., 2012; Chen et al., 2013a; Tsalkova et al., 2012a, 2012b). Surprisingly, two of the ESIs, ESI-05 and ESI-07, act as EPAC2 selective antagonists with minimal activity towards EPAC1 by binding to a previously unidentified allosteric binding site: the interface between the two cAB domains of EPAC2 (Tsalkova et al., 2012b). The discovery of first-in-class EPAC specific antagonists represents a major leap forward in our ability to pharmacologically dissect the roles of EPAC isoforms. In particularly, one of the pan-ESIs, ESI-09 (Chen et al., 2013b), has been shown to have excellent bioavailability with no major toxicity when administrated in vivo to mice. This favorable pharmacological/toxicological profile of ESI-09 makes it a useful pharmacological tool for probing physiological functions of EPAC proteins and for testing therapeutic potential of targeting EPAC in animal disease models (Almahairaq et al., 2015a; Gong et al., 2013; Yan et al., 2013; Zhu et al., 2015). Another EPAC specific antagonist recently reported in literature, CE3F4, is a representative of the tetrahydroquinoline class of compounds (Courilleau et al., 2012). Upon structure activity relationship study, the (R) enantiomer was found to possess improved potency than racemic CE3F4 and (S)-CE3F4. Additionally, it demonstrated 10-fold selectivity for EPAC1 over EPAC2 (Courilleau et al., 2013).

6. EPAC1 tissue and cellular distribution

In contrast to EPAC2 expression patterns, the expression of EPAC1 is relatively ubiquitous in mice. Particularly, the kidney, ovary, skeletal muscle, thyroid and certain areas of the brain express higher levels of EPAC1 mRNA (Kawasaki et al., 1998). In agreement with the mRNA expression level, high EPAC1 protein staining was observed in the apical
brush board membrane of mouse proximal tubules (Honegger et al., 2006; Li et al., 2008). Similar expression pattern of EPAC1 was found in rat and human kidney (Li et al., 2008). According to Human Protein Atlas, EPAC1 RNA is detected in all tissues while EPAC1 protein is detected at high or medium expression levels in 48 of 80 analyzed normal tissue cell types (http://www.proteinatlas.org/ENSG00000079337-RAPGEF3/tissue). The expression of EPAC1 gene is also regulated developmentally. Real time PCR analysis of EPAC1 mRNA in mice brain, heart, kidney and lung revealed that EPAC1 expression increased moderately but significantly after birth, reaching maximal expression levels at age of 3 weeks in all the above mentioned tissues (Ulucan et al., 2007).

At the cellular levels, EPAC1 has been observed in various subcellular locations and during different stages of the cell cycle. EPAC1 is mainly found on the nuclear membrane and mitochondria of interphase COS7 cells but it is localized to the mitotic spindle, centrosome and the contractile ring during mitosis (Qiao et al., 2002). Deletion of the first 148 amino acids of EPAC1 disrupts its mitochondria and membrane cellular localization. While the DEP domain is important for EPAC1 membrane association, it is not important for mitochondrial targeting. Instead, a putative mitochondrial-targeting sequence has been identified at the N-terminal of the EPAC1b protein (Qiao et al., 2002). It was realized very early on that correct cellular targeting, particularly membrane association, is important for the cellular function of EPAC1 as while the DEP domain is not necessary for activation of Rap1 in test tubes, a EPAC1 deletion mutant without DEP domain is incapable of activating Rap1 and PKB in cells (Mei et al., 2002).

It appears that in addition to activating EPAC1, cAMP also regulates its subcellular targeting of EPAC1. Ponsioen et al. show that upon cAMP binding, EPAC1 is targeted to the plasma membrane assisted by the DEP domain (Ponsioen et al., 2009). Subsequent studies revealed that the binding of cAMP to EPAC1, in addition to activating its GEF activity, also induces a conformational change in the DEP domain (Li et al., 2011). This results in the reorientation of a phosphatidic acid (PA) binding motif within the DEP domain, therefore enabling EPAC1 binding to PA at the plasma (Consonni et al., 2012). On the other hand, low intracellular cAMP levels favor the distribution of EPAC1 towards microtubule (MT) cytoskeleton (Qiao et al., 2002). This is likely due to the ability of EPAC1 to directly interact with MT and to promote its polymerization independent of Rap1 (Mei and Cheng, 2005; Sehrawat et al., 2008). In primary rat alveolar macrophage (AM), EPAC1 is detected on punctate and tubular membranes throughout the cell body, predominantly at the perinuclear region with low level on the plasma membrane. In addition, EPAC1 is also co-localized with microtubule organizing centers (MTOCs). Following treatment with prostaglandin E2 (PGE2), EPAC1 redistributes from MTOC to nuclear envelope and accumulates on late phagosomes (Brock et al., 2008). The cellular distribution of EPAC1 allows it to signal at specific subcellular compartments via interaction with its cellular partner to form functional signalosomes as described below in Section 7.

7. EPAC1 interaction partners and signalosomes: compartmentalization and integration of cAMP signaling

7.1. Roles of phosphodiesterases and A-kinase anchoring protein (AKAP)

While cAMP is a small molecule second messenger capable of free diffusion in solution, cellular signaling mediated by cAMP is exquisitely specific and precise both spatially and temporally. This is achieved by a highly coordinated network of two multi-membered families of important signaling molecules with opposing functions, namely the adenylyl cyclases and phosphodiesterase. The regulation of cAMP signaling is further aided by a family of structurally diverse scaffolding proteins, A-kinase anchoring protein (AKAP), which tethers PKA and related signaling molecules to form signalosomes at discrete subcellular locations (Scott et al., 2013). The identification of a putative cAMP-responsive signaling complex containing EPAC1, PDE4D3, PKA and ERK5, held together by mAKAP (a.k.a. AKAP6), at the perinuclear membranes of rat neonatal ventriculocytes provides the first evidence of a direct coupling of EPAC1 and PKA to integrate and coordinate cAMP signaling output for optimal spatial and temporal regulation (Fig. 3) (Dodge-Kafka et al., 2005). Of note, while EPAC1 and PKA, individually, have been shown to be able to form cAMP signaling complexes with PDE3B or PDE4D in both human aortic endothelial cells (HAECs) and human...
microvascular endothelial cells (HMVECs), PKA- and EPAC1-containing signaling complexes have otherwise been found to be non-overlapping. Such complexes contain a matrix of at least 4 distinct populations in which unique combinations of cAMP effector proteins (PKA or EPAC) and CAMP-PDEs (PDE3B or PDE4D) are present (Netherton et al., 2007). Similar conclusion is reached in HEK293T cells where PKA and EPAC function in distinct signaling complexes (Raymond et al., 2007). Therefore, the formation of signalosomes containing both cAMP sensors EPAC1 and PKA appears to be cell-type specific. In prostate cancer cells, it has been proposed that active EPAC1 forms a multiprotein signal complex with PDE3B, PDE4D, AKAP, Raptor and Rictor to facilitate the activation of mTORC1 and mTORC2 (Misra and Pizzo, 2012). Moreover, EPAC1 has been shown to form a complex with AKAP450 (a.k.a. AKAP9) in human umbilical venous endothelial cells. While AKAP9 is essential for EPAC1-mediated microtubule polymerization and enhancement of the endothelial barrier function, it is not required for EPAC1-induced Rap1 activation, reorganization of cortical actin and VE-cadherin adhesion (Sehrawat et al., 2011).

Majority of the aforementioned studies based their conclusions on co-immunoprecipitation and colocalization analyses. Therefore, exact architecture and composition of the signalosome, especially how EPAC1 interacts with other components of the complex, are not clear. Maurice and colleagues demonstrate that PDE3B interacts directly with EPAC1 and one of the regulatory subunit of PK3y, P84. Peptide array analyses lead to the identification of specific regions of interactions: the N terminal of PDE3B (amino acids 1–25) interacts with two sections of the EPAC1, the initial portion of the Cα domain (amino acids 218–246) and part of REM domain (amino acid 398–422); while P84 binds to a hydrophobic section (amino acids 436–460) of PDE3B. This PDE3B-based signalosome, integrating EPAC1 and PK3y signaling, allows dynamic cAMP-dependent regulation of cell adhesion, spreading, and tubule formation in human arterial endothelial cells (HAECs) (Wilson et al., 2011). Similarly, the interaction site between PDE4D and EPAC1 involving residues spanning amino acids 362–397 in EPAC1 has also been mapped (Rampersad et al., 2010).

More recently, a direct interaction between EPAC1 and β-arrestin 2 (β-ar2), an important regulator of GPCR signaling, has also been demonstrated. Differential recruitment of EPAC1-β-ar2 or PDE4D5-β-ar2 complex by activated β1 or β2 adrenergic receptor (βAR), respectively, allows discrete signal output. Specifically, activation of βAR leads to the recruitment of EPAC1-β-ar2, activation of H-Ras via Rap2B-PLC signaling, which induce pro-hypertrophic gene expression during cardiac myocyte remodeling. On the other hand, activation of β2AR results in the recruitment of PDE4D5-β-ar2 complex and leaves EPAC1-β-ar2 in the cytosol where it activates Rap1 non-hypertrophic signaling (Berthouze-Duquesnes et al., 2013).

7.2. Ezrin–radixin–moesin (ERM)

While direct interaction of cAMP with EPAC1 results in conformational changes that allow the latter’s spatial regulation via DEP domain assisted plasma membrane (PM) translocation (Ponsioen et al., 2009), additional membrane targeting mechanisms, involving the Ezrin–radixin–moesin (ERM) family of scaffolding proteins, have also been identified. ERM proteins operate as scaffolds linking PM with actin cytoskeleton by virtue of their N-terminal FERM (4.1 protein, ezrin, radixin, moesin) lipid binding domain, middle helical domain and C-terminal actin binding domain (ABD). Open conformation of ERM proteins is generated on PI(4,5)P2 binding to FERM and threonine phosphorylation of the ABD thereby leading to the release of autoinhibition. Subsequently, these cytoplasmic proteins translocate to the PM and can serve as PM anchors for other proteins. Yeast two-hybrid screen and co-immunoprecipitation experiments were employed to confirm that EPAC1 binds to activated ERM directly via its N-terminal 49 amino acids. These results underscore the importance of less appreciated sites of the EPAC1 and the interesting role such residues also play in the spatial regulation of EPAC1. Another distinguishing aspect of ERM-EPAC1 binding is that the activation state of EPAC1 doesn’t affect the interaction, unlike interaction between PM and EPAC1 via its DEP domain. Overall, increased EPAC1 binding to GPCR-induced ERM proteins leads to clustering and localization of EPAC1 in plasma membrane. Ultimately this influences efficient adhesion to extracellular matrix (Gloerich et al., 2010). A separate study by Altschuler and colleagues in 2011 independently identifies radixin, an ERM family member, as a EPAC1 cellular partner from a yeast two-hybrid screen using the first 200 residues of EPAC1 as the bait. In vitro binding and immunoprecipitation experiments further validate the interaction and allow the identification of a new ERM binding motif spanning residues 1–52 in EPAC1, which binds directly to the N-terminal half (1–318) of radixin. Co-localization and functional analyses further reveal that radixin can act as a scaffold that integrates EPAC1 and PKA, through its FERM and helical domain, respectively, into functional signalosomes at specific cellular loci to mediate thyroid stimulating hormone (TSH) induced cell proliferation in thyroid cells (Hochbaum et al., 2011).

7.3. RanBP2

Using mass spectrometry based proteomics study, small G protein Ran and Ran binding protein 2 (RanBP2), as well as importin β-1, nucleoporins 98 and 205, were identified as purported binding partners for EPAC1 in HEK293 cell line stably transfected with Flag-EPAC1 (Liu et al., 2010). This finding associates EPAC1 with the nuclear pore complex. Through various studies including in vitro GST pulldown assay, staining and imaging of endogenous EPAC1, Ran and RanBP2 were shown to act specifically as nuclear pore anchors for EPAC1. This anchoring was found to be independent of CAMP binding and feasible to occur in non-excited cells as well. Two possible modes of interaction include either direct binding to RanBP2 or engagement with Ran facilitating tethering to RanBP2. Initial reports suggested that the RA site of EPAC1 is critical for mediating the binding interaction with Ran-GTP and RanBP2 proteins. This revelation was particularly intriguing since the RA domain’s role in binding Ras had been exemplified in EPAC2 before, however; this was the first evidence confirming a specific role of the RA domain of EPAC1. Next, Ran-EPAC1 interaction was found to mediate Rap1 activation at the nuclear envelope. These findings led Stork and colleagues to propose that nuclear localization of EPAC1 may facilitate Rap1 activation in response to elevated levels of cAMP. While several earlier reports had established that the EPAC1 localizes to the nuclear envelope (DiPilato et al., 2004; Dodge-Kafka et al., 2005; Magiera et al., 2004; Qiao et al., 2002; Wang et al., 2006) and participate in regulation of DNA-dependent protein kinase nuclear/cytosolic shuttling (Huston et al., 2008), the current findings hold special significance. Previously, the proposed mechanism for this phenomenon was that perinuclear concentration of EPAC1 proceeds in an anchoring protein mAKAP mediated fashion. It is important to note that RanBP2 as binding partner provides a more general mechanism and better explains the perinuclear localization of EPAC1 in cells lacking mAKAP. The results of this study were somewhat contradictory to later findings where the exact mechanism of EPAC1 directly binding to RanBP2 was established to involve the zinc finger (ZNF) domain of RanBP2 (Gloerich et al., 2011). In contrast to the earlier report designating RA domain as the binding site, EPAC1 domain required for RanBP2 binding was found to be the CDC25-HD domain. Replacement of the RA domain failed to impact the nuclear localization of EPAC1 adversely, moreover, Ran was found to be dispensable during the nuclear localization event. Since the CDC25-HD site is responsible for GEF activity as well, contrary to earlier hypothesis, RanBP2 binding to EPAC1 was found to abolish Rap activation. Justification for the discrepancies was given by accounting for the RA mutant employed by Liu and colleagues with an altered CDC25-HD site that prevents binding RanBP2 or phosphorylation of its ZNF sites was capable of reversing the negative regulation RanBP2
exerts on EPAC1 and restore Rap1 activation (Gloeirich et al., 2011). Adding complexity to the matter further, Yawood and colleagues recently identified a putative nuclear pore localization signal within the CDC25-HD domain of EPAC1 (residues 764–838) that is important for the nuclear envelop targeting of EPAC1. However, these authors were not able to demonstrate direct interaction between EPAC1 and RanBP2 (Parnell et al., 2015). Therefore, while all three studies show the localization of EPAC1 to the nuclear pore the molecular mechanism of EPAC1 nuclear targeting remains debatable.

7.4. G-protein-coupled receptor kinase 2 (GRK2)

G-protein-coupled receptor kinases (GRKs) are a family of serine/threonine kinases that phosphorylate the intracellular domain of activated GPCRs. GRK-phosphorylated residues act as docking sites for the recruitment of arrestins, which block the reassociation of the G-proteins and reactivation of GPCRs. GRK2 plays important roles in modulating cAMP signaling by its ability to phosphorylate βARs. GRK2 is also known to exert diverse cellular functions by interacting with a range of non-GPCR substrates (Evron et al., 2012). Heightened Rap1 activation by EPAC agonist has been observed in presence of diminished GRK2 levels. Subsequent co-immunoprecipitation studies indicated that GRK2 directly interacts with transfected HA-tagged EPAC1 especially upon stimulation with 8–pCPT treatments. At endogenous level, GRK2 was found to bind to low–molecular-weight splice variant of EPAC1 in tissue extracts of dorsal root ganglia (DRG) and spinal cord (Eijkelkamp et al., 2010). Subsequent functional analyses have established an important function role of GRK2/EPAC1 signaling balance in pain sensation and the development of chronic pain, which will be discussed in detail in Section 8.2.

7.5. Transforming growth factor-β (TGF-β)

Type 1 TGF-β receptor (TβRI) is the key receptor for propagating TGFβ signaling. A proteomic study reveals that EPAC1 binds to activated TβRI. This interaction requires the intact kinase activity of TβRI and is independent of the cAB domain of EPAC1. Moreover, EPAC1 inhibits TGFβ1-induced Smad2 phosphorylation and antagonizes TGFβ1/TβRI-mediated transcripational activation, inhibition of cell adhesion and stimulation of cell migration (Conrotto et al., 2007). It appears that TGFβ1 and EPAC1 signaling forms a reciprocal negative feedback loop as TGFβ1 decreases transcriptional expression of EPAC1 while overexpression of EPAC1 inhibits TGFβ1-induced collagen synthesis in fibroblasts (Yokoyama et al., 2008). However, it is unclear if this relationship between EPAC and TGFβ signaling is generally applicable in other cell types, particularly in vivo. Recent study using EPAC1 knockout mice shows that deletion of EPAC1 in T cells leads to an increased level of SMAD7, a reduced expression of SMAD4 and a suppressed SMAD2 phosphorylation in response to TGFβ1 treatment (Almahariq et al., 2015b). These results suggest that EPAC1 positively modulates TGFβ1 signaling in T cells.

8. Physiological functions and involvement in diseases

To date, most functional analyses of EPAC1 have been performed under in vitro settings. While these studies are important for mechanistic understanding of EPAC1–mediated cellular signaling, they may or may not directly translate into EPAC1’s physiological functions in vivo. Over the last few years, studies using EPAC1 or EPAC1/2 double knockout mouse models have led to an increasing appreciation of the important roles that EPAC1 plays in normal physiological and disease states. It appears that deletion of EPAC1 or even double knockout of EPAC1 and EPAC2 does not generate major physiological defects in mouse. Considering the fact that CAMP is mainly a stress–response signal not essential for survival, this is not completely surprising. The subtle phenotypic manifestations of these EPAC1 knockout mice under normal basal conditions make the dissection of physiological functions of EPAC1 protein difficult. Therefore, finding the “right” stress conditions and disease model systems to interrogate the EPAC1 deletion animals has been the key to reveal the in vivo functions and disease relevance of EPAC1 proteins. For example, using high fat diet (HFD) feeding as a stress trigger, Yan et al. show that EPAC1 null mice are resistant to HFD-induced obesity, hyperleptinemia and glucose intolerance (Yan et al., 2013). This study uncovers an important EPAC1 function in energy homeostasis and leptin resistance (Almahariq et al., 2014). Recent progresses related to physiological functions of disease implications of EPAC1 are discussed below.

8.1. EPAC1 and cardiac stress

EPAC functions in cardiovascular system have been reviewed extensively (Bisserier et al., 2014). Therefore, we will focus on the most recent updates in the field. Abnormal cardiac adrenergic receptor activation has been associated with cardiac hypertrophy, a leading indicator of arrhythmias, cardiomyopathy and heart failure. The involvement of EPAC1 in cardiac hypertrophy was suggested by the observations that EPAC1 levels were upregulated in isoproterenol-induced left ventricular hypertrophy and in pressure overload-induced hypertrophy (Metrich et al., 2008; Ulucan et al., 2007). Additionally, it was shown that EPAC1 is the main EPAC isoform expressed in human heart and its levels were increased in heart failure tissues (Metrich et al., 2008).

In 2014 Okumura and coworkers using EPAC1 and EPAC2 knockout mouse models show that deletion of EPAC1 protects mice from various cardiac stresses, including arrhythmogenic stress, whereas loss of EPAC2 shows no cardioprotective effects. Specially, while EPAC1 KO mice display a similar degree of aortic banding induced cardiac hypertrophy when compared with wild type controls, EPAC1 deficiency suppresses subsequent cardiac dysfunction by preventing cardiac myocyte apoptosis and fibrosis. Likewise, mice lacking EPAC1 are resistant to isoproterenol- and aging-induced cardiomyopathy and attenuation of arrhythmogenic activity. At the cellular and molecular levels, EPAC1 deletion reduces phospholamban (PLN) phosphorylation at serine-16, a known major PKA phosphorylation site. On the other hand, activation of EPAC in cardiomyocytes promotes PLN serine-16 phosphorylation in a PLC/PKCε dependent and PKA-independent manner. These results suggest that EPAC1 is an important regulator of cardiac stresses and inhibition of EPAC1 may represent an alternative strategy for the treatment arrhythmia and/or heart failure (Okumura et al., 2014). Results from this study are not in agreement with another genetic study based on individual EPAC1, EPAC2 and double KO mice, which reported that EPAC2, instead of EPAC1, mediated β1-adrenergic-dependent sarcoplasmic reticulum Ca2+ leak and arrhythmia (Pereira et al., 2013). The reason for the difference is not clear although the genetic backgrounds on which the KO mice were generated are different between the two studies, which could potentially account the observed phenotypic difference.

A more recent study reports that activation of EPAC1 in neonatal cardiomyocytes promotes autophagy during cardiomyocyte hypertrophy. Deletion of EPAC1 in mice protects animals against β1-AR-induced cardiac remodeling and prevents the induction of autophagy (Laurent et al., 2015).

8.2. EPAC1 and chronic pain

cAMP signaling is one of the first pathways identified in regulating pain sensitivity. Implication of EPAC signaling with pain sensing was initially made by Levine and colleagues. It was revealed that β2-adrenergic receptor/cAMP signaling acts through EPAC, but not PKA, to stimulate protein kinase C isofrom ε (PKCε), a key signaling molecule important for nociceptor sensitization and the transition from acute to chronic pain (Hucho et al., 2005). Moreover, activation of EPAC in IB4(+) DRG neurons stimulates PKCε to induce hyperalgesia via phospholipases C
and D in rat nociceptive mechanical threshold behavioral experiments. It is well-known that PEG2 induces transient hyperalgesia and nociceptor sensitization via a cAMP/PKA-dependent mechanism. Interestingly, PGE2 induced sensitization of purinergic P2X receptor, an important mechanism responsible for abnormal pain response to inflammatory injury, was found to switch from PKA-dependent in normal condition to both PKA- and EPAC/PKC-dependent after complete Freund’s adjuvant (CFA)-treatment in DRG neurons. This transition could be attributed to changes in EPAC signaling as EPAC1 protein level was increased in CFA-treated rat DRG neurons (Wang et al., 2007).

Subsequent study by Eijkellkamp et al. showed that GRK2 protein levels in rat DRG neurons were reduced during peripheral paw inflammation. Using Na\textsubscript{a},1.8\textsuperscript{+} sensory neuron tissue specific GRK2 null and heterozygous mice, it was further demonstrated that GRK2 contributed to PGE2-induced hyperalgesia: down-regulation of GRK2 in SNS-GRK2\textsuperscript{−/−} mice prolonged PGE2-induced hyperalgesia in a PKA-independent but EPAC-dependent manner. Furthermore, coimmunoprecipitation experiments revealed that GRK2 acted as a binding partner for EPAC1, hence reduced GRK2 during inflammation leads to increased activation of EPAC1 signaling. Consequently, aggravated hyperalgesia is observed in vivo during inflammatory response via biased cAMP signaling shifting from PKA to EPAC\textsubscript{1}/Rap1 and ERK/PKC\textsubscript{α} pathways (Eijkellkamp et al., 2010). This association of GRK2 attenuation in nociceptors with enhanced inflammatory hyperalgesia was further confirmed in rat model using intrathecal antisense GRK2 oligonucleotide, which led to a heightened and prolonged hyperalgesia in response to PGE2, epinephrine and carrageenan by activation of the cAMP-dependent pathways down-stream of GPCR (Ferrari et al., 2012).

The connection between GRK2 and EPAC1 and the involvement of EPAC1 in chronic pain were further solidified with the use of EPAC1 knockout mouse models. Consistent with the finding of increased EPAC1, but not EPAC2, expression in DRG innervating the ipsilateral paw after a unilateral L5 nerve transection (SNT) in comparison with DRG innervating the contralateral paw from sham-operated mice, SNT-induced allodynia is greatly reduced in EPAC\textsuperscript{−/−} and EPAC\textsuperscript{+/-} mice when compared with WT counterparts. These results suggest that EPAC1 is required for the development of chronic neuropathic pain in mouse model. Mechanistic analysis further reveals that EPAC1 signaling enhances mechanotransduction induced by Piezo2, a well-studied mechanotransducer involved in the development of allodynia and that EPAC activation leads to a long-lasting mechanical allodynia independent of Na\textsubscript{a},1.8\textsuperscript{+} (Eijkellkamp et al., 2013). Using two mouse models of hyperalgesic priming, Wang et al. showed that induction of GRK2 expression in vivo by viral-based gene transfer or reduction of EPAC1 in mice via EPAC1 antisense oligonucleotide and gene knockout approaches prevented the development PGE2-induced chronic hyperalgesia. In addition, increasing GRK2 or decreasing EPAC1 inhibited chronic pain in the CFA chronic inflammatory pain model. Taken together, these results convincingly demonstrate that the balance between GRK2 and EPAC1 signaling plays an important role in pain sensitivity. Thus this represents a promising therapeutic target for the prevention and treatment of abnormal pain (Wang et al., 2013).

8.3. EPAC1 and cancer

cAMP plays an important role in regulating cell migration in various cancers, especially in melanoma, where EPAC1 enhances tumor migration and metastasis (Bajjinyam et al., 2010, 2011, 2014). EPAC1 is also over-expressed in human pancreatic ductal adenocarcinoma (PDA) tissue compared to the surrounding normal pancreatic tissue (Lorenz et al., 2008). The functional role this EPAC1 overexpression in PDA was probed by using an EPAC-specific inhibitor and EPAC1-based RNAi gene silencing, which led to the finding that EPAC1 plays an important role in pancreatic cancer cell migration and invasion in vitro (Almahariq et al., 2013). Furthermore, by employing an orthotopic metastatic mouse model in which MIA PaCa-2 PDA cells stably expressing luciferase were injected into the pancreas of athymic nude mice, Almahariq et al. showed that genetic suppression of EPAC1 or its pharmacologic inhibition with ESL-09 inhibited invasion and metastasis of PDA cells as monitored by in vivo imaging of their local and distant dissemination, as well as by histological assessment of the number of metastatic foci in the liver. Mechanistically, EPAC1 promotes activation and trafficking of integrin (β1, a key molecule involved in PDA migration and metastasis (Almahariq et al., 2015a). These data validate that EPAC1 promotes metastasis of PDA cells and is a novel therapeutic target for developing anti-metastasis agents for PDA.

The EPAC1/Rap1 signaling pathway can also promote oncogenesis via upregulating glucose uptake and metabolism (Onodera et al., 2014). By applying a physiologically relevant three-dimensional cultures of laminin-rich extracellular matrix (3D IrECM), which restores crucial microenvironmental cues absent from conventional 2D culture and allows phenotypic discrimination between nonmalignant and malignant mammary cells, Onodera and colleagues show that increased glycolytic activation by overexpressing glucose transporter type 3 (GLUT3) in nonmalignant human breast cells results in a malignant transformation phenotype of disorganized, proliferative and nonpolar colonies by activating known oncogenic signaling pathways, including EGRF, β1 integrin, MEK, and AKT. On the other hand, reduction of glucose uptake in malignant cells promoted the formation of organized and growth-arrested acinus-like structures, and suppressed oncogenic pathways. Surprisingly, the canonical metabolic signaling pathways including AMPK, mTOR, and HIF in 2D cultures are not important for the transformation between “normal” and malignant phenotypes in 3D cultures. Instead, loss of epithelial integrity involved activation of EPAC1/Rap1 signaling mediated by pyruvate kinase M2 (PKM2) interaction with soluble adenyl cyclase, as well as O-linked N-acetylgalactosamine modification downstream of the hexosamine biosynthetic pathway (Onodera et al., 2014). These observations based on the ex vivo 3D IrECM system is a major step forward and may have profound implications on our understanding of oncogenesis and cancer treatment if validated in vivo.

8.4. EPAC1 and infections

Numerous microbial pathogens exploit the cAMP signaling pathways of their hosts as a strategy to enhance their infectious virulence (McDonough and Rodriguez, 2012). For example, anthrax toxin edema factor (EF) is a highly active calmodulin-dependent adenylate cyclase that can dramatically increase the intracellular level of cAMP in host cells and cause extensive tissue damages. It was determined that while EF did not block cell proliferation and survival it inhibits chemotaxis and causes changes in cytoskeleton in primary human microvascular endothelial cells (HMVECs) through activating the cAMP/EPAC/Rap1 signaling pathway (Hong et al., 2007). Consistent with these general findings, Peterson and colleagues show that EF significantly suppresses human macrophage phagocytosis and induces cytoskeletal changes, including decreased cell spreading and reduced filopodia and F-actin content. Furthermore, EF treatment modulates both the protein levels and activity of EPAC1 and PKA. These data, combined with the fact that EPAC or PKA activation by selective cAMP analogs recapitulates EF effects, suggest that EF can signal through EPAC and PKA to weaken the host immune response (Yeager et al., 2009).

Rickettsiae are obligatory intracellular bacteria that infect hosts by targeting the endothelial lining of the blood vessels. Considering the important roles of EPAC1/Rap1 signaling play in endothelial functions, it was hypothesized that EPAC1 might be involved in the development of rickettsioses. Indeed, deletion of RapGEF3 gene in mice protects them from an ordinarily lethal dose of rickettsiae by suppressing bacterial adhesion and invasion into the endothelial cells. Consistent with the genetic manipulation, pharmacological inhibition of EPAC1 in vivo using an EPAC specific small molecule inhibitor, ESI-09, recapitulates the
9. Conclusion and perspectives

Since the initial discovery of EPAC proteins in 1998, major advances have been made in understanding the structure and function of this important family of molecular switches, as well as in the development of molecular, cellular and pharmacological tool boxes for probing EPAC signaling in vitro and in vivo. However, major gaps remain. For example, little is known about transcriptional, translational and post-translational regulations of EPAC1 and information on human EPAC1 genetics is sparse. In addition, while studies of genetically engineered mouse models have been effective in elucidating the physiological functions of EPAC proteins, limited in vivo genetic analyses of EPAC knockout mice have already generated conflicting results in the literature, which highlights the complexity of animal studies. Besides extrinsic factors, such as variability in experimental and environmental conditions, "irreproducible results" could arise as an intrinsic property of the knockout approach since it is possible that a particular gene knockout may produce a spectrum of apparent phenotypes depending upon the unique genetic background of the mouse strain on which the knockout is based. Solution to this challenge may require the use of tissue-specific/conditional knockout animal models, as well as functional in vivo EPAC specific pharmacological probes. Despite these issues, we believe that our knowledge of EPAC1 gene will progress at an accelerated rate in the near future, which will greatly facilitate not only our basic understanding of cAMP/EPAC signaling but also the development of potentially new diagnostics and therapeutics specifically targeting EPAC1.

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