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Imaging the translocations of CLIC4 and Epac1

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Chapter 3

Direct spatial control of Epac1 by cAMP

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Direct spatial control of Epac1 by cAMP

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Abstract

Epac1 is a GEF for the small G-protein Rap and is directly activated by cAMP. Upon cAMP binding, Epac1 undergoes a conformational change that allows interaction of its GEF domain with Rap, resulting in Rap activation and subsequent downstream effects, including integrin-mediated cell adhesion and cell-cell junction formation. Here, we report that cAMP also induces the translocation of Epac1 towards the plasma membrane. Combining high-resolution confocal fluorescence microscopy with TIRF and FRET assays, we observed that Epac1 translocation is a rapid and reversible process. This dynamic redistribution of Epac1 requires both the cAMP-induced conformational change as well as the DEP domain. In line with its translocation, Epac1 activation induces Rap activation predominantly at the plasma membrane. We further show that the translocation of Epac1 enhances its ability to induce Rap-mediated cell adhesion. Thus, regulation of Epac1-Rap signaling by cAMP includes both the release of Epac1 from auto-inhibition and its recruitment to the plasma membrane.

Introduction

cAMP is an important second messenger that mediates many cellular hormone responses. It has become more and more appreciated that along with the cAMP effector protein kinase A (PKA), Epac proteins also play pivotal roles in many cAMP-controlled processes, including insulin secretion [1,2], cell adhesion [3-7], neurotransmitter release [8-10], heart function [11-13] and circadian rhythm [14]. **Epac1 and Epac2 are cAMP-dependent guanine nucleotide exchange factors (GEFs) for the small G-proteins Rap1 and Rap2 [15,16].** They contain a regulatory region with one (Epac1) or two (Epac2) cAMP-binding domains, a Dishevelled, Egl-10, Pleckstrin (DEP) domain, and a catalytic region for GEF activity [17]. Binding of cAMP is a prerequisite for catalytic activity *in vitro* and *in vivo* [17]. Recently, the structure of both the inactive and active conformation of Epac2 was solved [18,19]. This revealed that in the inactive conformation the regulatory region occludes the Rap binding site, which is relieved by a conformational change induced by cAMP binding.

Like all G-proteins of the Ras superfamily, Rap cycles between an inactive GDP-bound and active GTP-bound state in an equilibrium that is tightly regulated by specific GEFs and GTPase-activating proteins (GAPs). GEF-induced dissociation of GDP results in the binding of the cellular abundant GTP, whereas GAPs enhance the intrinsic GTPase activity of the G-protein, thereby inducing the inactive GDP-bound state. Besides Epac, several other GEFs for Rap have been identified, including C3G, PDZ-GEF and RasGRP, and these act downstream of different signaling pathways [20]. Since Rap localizes to several membrane compartments, including the Golgi network, vesicular membranes and the plasma membrane [21-26], spatial regulation of its activity is expected to be established by the differential distributions of its upstream GEFs, each activating distinct pools of Rap on specific intracellular locations.

Similar to Rap, Epac1 is also observed at many locations in the cell, including the cytosol, the nucleus and the nuclear envelope, endomembranes and the plasma membrane [17,27,28-31]. These various locations may reflect the many different functions assigned to Epac1, such as regulation of cell adhesion, cell junction formation, secretion, regulation of DNA-PK by nuclear Epac1, and regulation of the Na⁺/H⁺ exchanger NHE3 at the brush borders of kidney epithelium [29,32,33]. Apparently, specific anchors are responsible for this spatial regulation of Epac1. Indeed, Epac1 was found to associate with phosphodiesterase 4 (PDE4) in a complex with mAKAP in cardiomyocytes [11], with MAP-LC bound to microtubules [34] and with Ezrin at the brush borders of polarized cells (M. Gloerich, J. Zhao and J.L. Bos, in preparation).

In this study, we report the unexpected observation that, in addition to the temporal control of Epac1 activity, cAMP also induces translocation of Epac1 towards the plasma membrane. Using confocal fluorescence microscopy, Total Internal Reflection Fluorescence (TIRF) microscopy and Fluorescence Resonance Energy Transfer (FRET)-based assays for high spatial and temporal resolution, we observed that translocation of Epac1 is immedi-

ate and that Epac1 approaches the plasma membrane to within ~7 nm. In line with this, Epac1-induced Rap activation was registered predominantly on this compartment. Epac1 translocation results directly from the cAMP-induced conformational change and depends on the integrity of its DEP domain. We further show that Epac1 translocation is a prerequisite for cAMP-induced Rap activation at the plasma membrane and enhances Rap-mediated cell adhesion. Thus, cAMP exerts dual regulation on Epac1 for the activation of Rap, controlling both its GEF activity and targeting to the plasma membrane.

Results

cAMP induces translocation of Epac1 towards the plasma membrane

To study the subcellular localization of Epac1 during activation by cAMP, we monitored GFP-Epac1 using time lapse confocal imaging in HEK293 cells. In accordance with previous reports [17,27,28,30,31], GFP-Epac1 is observed at the nuclear envelope, at the plasma membrane (PM), at endo-membranes and in the cytosol. Upon addition of forskolin, which activates adenylate cyclase to produce cAMP, we observed a pronounced redistribution of GFP-Epac1 towards the periphery of the cell (Fig. 1A and Suppl Movie). Automated image analysis [35] showed that the GFP-Epac1 redistribution is manifest both as a decrease in cytosolic fluorescence and an increase in PM-localized fluorescence, occurring within 2 minutes after stimulation ($\tau_{1/2}$ ~40 s; Fig. 1A). In contrast, fluorescence in the nucleus was constant throughout the experiment (Fig. 1A). We also employed Total Internal Reflection Fluorescence (TIRF) microscopy to monitor GFP-Epac1 selectively at the basal membrane. We observed forskolin-induced accumulation of GFP-Epac1 as an increase of 60 +/- 4 % (mean +/- SEM; n=17) relative to pre-stimulus levels (Fig 1B), suggesting that Epac1 translocation represents a large-scale recruitment to the PM.

Similarly, isoproterenol stimulation, which induces physiological cAMP increases via activation of β -adrenergic receptors, induced rapid Epac1 translocation in A431 cells (Fig. 1C). Epac1 translocation was observed in all cell types tested (HEK293, A431, OVCAR-3, ACHN, RCC10, MDCK, N1E-115, HeLa, Rat-1, GE11, H1299) and over the full range of expression levels (data not shown). Importantly, staining of OVCAR-3 cells with a monoclonal Epac1 antibody showed an increased presence of endogenous Epac1 at the PM after treatment with forskolin, reflecting the PM-translocation of endogenous Epac1 (Fig. 1D). Similar results were obtained with a polyclonal Epac1 antibody (data not shown).

Due to the limited resolution of conventional confocal and TIRF microscopy (~300 nm and ~90 nm, respectively) it is difficult to judge whether GFP-Epac1 indeed translocates to the PM or, alternatively, merely to the cortical actin cytoskeleton just below it. Therefore, we monitored Fluorescence Resonance Energy Transfer (FRET) between YFP-Epac1 and CFP-CAAX, which is membrane-anchored by its prenylated K-Ras CAAX-motif and in these cells con-

Figure 1. Epac1 translocates towards the PM upon elevation of cAMP levels

A) In HEK293 cells stimulation with forskolin (25 μ M) induces translocation of GFP-Epac1 towards the cell periphery. Inset: fluorescence intensity along the red line, showing sharp demarcation of the plasma membrane (width at half-maximum $\sim$ 300 nm). Right: fluorescence intensity at the plasma membrane (red, PM) and in the cytosol (blue, Cyt) as well as the ratio PM/Cyt (green) during the response to forskolin; fluorescence levels in the nucleus (grey, Nucl) were constant. Traces are representative for $n > 15$.

B) Representative TIRF experiment showing the forskolin-induced accumulation of GFP-Epac1 at the basal membrane of HEK293 cells. Mean increase relative to pre-stimulus levels was $60 \pm 4\%$ (mean $\pm$ SEM; $n = 17$).

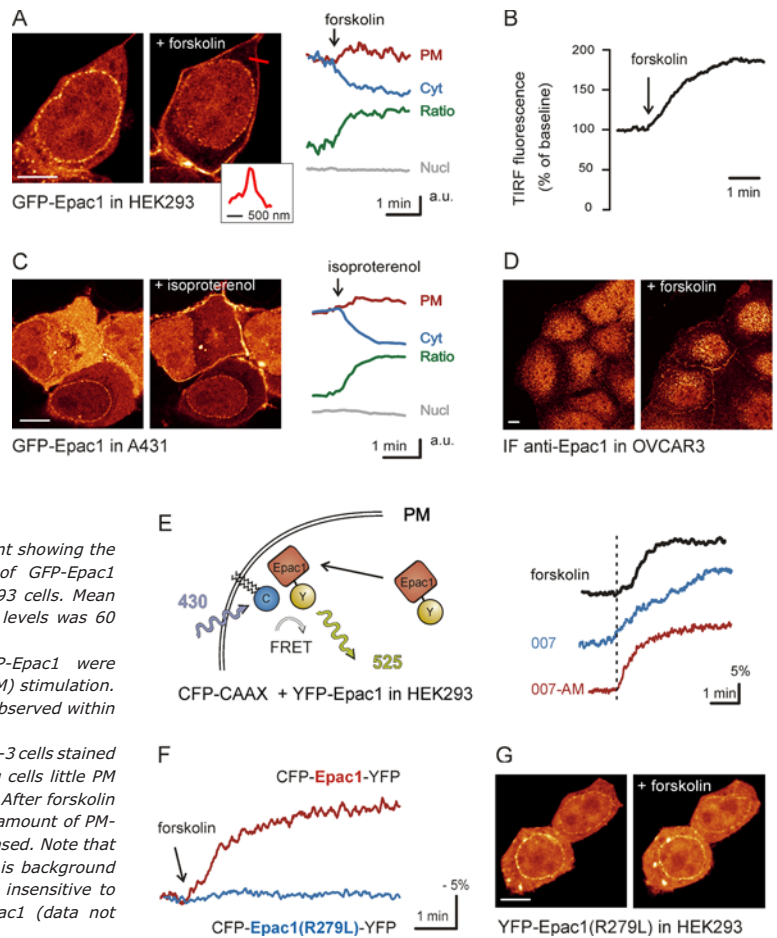
C) A431 cells expressing GFP-Epac1 were imaged during isoproterenol (1 nM) stimulation. Translocation of GFP-Epac1 was observed within 1 minute ($n = 8$).

D) Immunofluorescence of OVCAR-3 cells stained for endogenous Epac1. In resting cells little PM localization of Epac1 is observed. After forskolin stimulation (25 μ M, 10 min) the amount of PM-localized Epac1 is markedly increased. Note that the nuclear immunofluorescence is background staining rather than Epac1, as it is insensitive to siRNA-mediated silencing of Epac1 (data not shown).

E) Measurement of FRET between the PM-marker

CFP-CAAX (see M&M) and translocating YFP-tagged Epac1 (traces are representative for $n > 5$). FRET, expressed as YFP/CFP ratio, increases upon addition of forskolin (25 μ M, black). FRET increases were also induced by the Epac-specific cAMP analogue 8-pCPT-2'-O-Me-cAMP (007, 100 μ M; blue) or the more membrane-permeable analogue 8-pCPT-2'-O-Me-cAMP-AM (007-AM, 1 μ M; red). F) Forskolin (25 μ M) treatment of HEK293 cells transfected with the FRET sensor CFP-Epac1-YFP. The sensor reports the cAMP-induced conformational change as a loss of intramolecular FRET. In contrast to the wild-type sensor (red), the mutant FRET construct CFP-Epac1(R279L)-YFP (blue) lacks the ability to change conformation upon changing cAMP concentrations.

G) Mutagenesis of Arginine279 to Leucine eliminated the ability of YFP-Epac1(R279L) to translocate upon forskolin stimulation (25 μ M). Scale bars: 10 μ m.



stitutes a marker of the PM (see M&M). Forskolin induced immediate increases in the YFP/CFP ratio (10-15%, Fig. 1E), indicating that translocated GFP-Epac1 approaches the PM to within approximately 7 nm. Similar results were obtained using a C-terminally tagged Epac1 (data not shown).

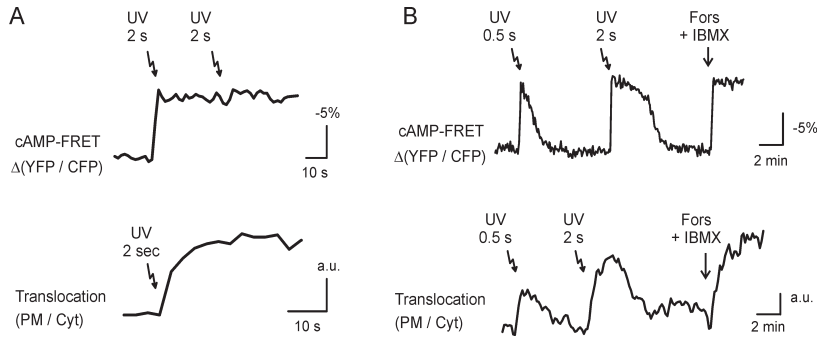
The translocation of Epac1 can also be induced by the Epac-specific cAMP analogues 8-pCPT-2'-O-Me-cAMP (007) [36] and the more cell-permeable 8-pCPT-2'-O-Me-cAMP-AM (007-AM) [37; chapter 9 of this thesis] (Fig. 1E), suggesting that cAMP induces Epac1 translocation through

activation of Epac1 itself rather than via parallel pathways such as PKA. Indeed, Epac1(R279L), which is mutated in the cAMP binding domain and thereby locked in the auto-inhibited, inactive conformation ([31]; see also FRET analysis on CFP-Epac1(R279L)-YFP and the wildtype sensor in Fig. 1F), lacked the ability to translocate (Fig. 1G), indicating that Epac1 must be in its open conformation to translocate. In addition, forskolin-induced translocation of GFP-Epac1 was not affected by inhibition of PKA with H89 (data not shown). Thus, we show that Epac1 translocates towards the PM when it is bound by cAMP.

Figure 2. Epac1 translocation is a highly dynamic and reversible event

A) cAMP-uncaging experiments in HEK293 cells. Upper trace: release of NPE-caged cAMP (arrows; see methods for details) saturated the FRET-based cAMP sensor CFP-Epac(Δ DEP)C.D.-Venus in that a subsequent UV-flash did not induce further FRET changes. Lower trace: ratio between PM and cytosolic fluorescence of GFP-Epac1 (expressed at comparable levels as CFP-Epac1(Δ DEP)C.D.-Venus) showing the immediate translocation upon identical photolysis of caged-cAMP ($\tau_{1/2} < 5$ s).

B) cAMP-uncaging in GE11 cells. Upper trace: due to high speed of cAMP clearing in these cells, dosed release of NPE-caged cAMP evokes transient cAMP rises. Amounts of released cAMP are approximately proportional to the duration of UV flashes. Lower trace: release of identical amounts of caged cAMP in GE11 cells induces transient translocations of GFP-Epac1. The PM/cytosol ratio shows that the degree of translocation correlates with the dose of released cAMP.



Epac1 translocation dynamically follows cAMP levels

High-resolution confocal imaging shows that translocating GFP-Epac1 is recruited from a homogeneous cytosolic pool. Indeed, fast FRAP experiments [35] confirmed that the mobility of GFP-Epac1 approaches that of free GFP (data not shown). Conversely, active transport appears not to be involved, since Epac1 translocation is insensitive to disruption of the actin cytoskeleton (Cytochalasin D, 1 μ g/ml, or Latrunculin A, 1 μ M) or the microtubule network (Nocodazol, 25 ng/ml) (data not shown). Thus, upon cAMP-binding Epac1 finds the PM by passive diffusion.

To examine the dynamics of Epac1 translocation, we loaded HEK293 cells with NPE-caged cAMP and transiently released cAMP by UV-induced photolysis of the NPE-cage. Using CFP-Epac(Δ DEP-CD)-Venus [38], an improved variant of the previously published cAMP sensor [39], we first established experimental conditions to instantly saturate Epac1 with cAMP (Fig. 2A). When applying identical UV pulses to cells expressing comparable levels of GFP-Epac1, we observed very rapid translocation that was halfway within 5 seconds and near-complete in approximately 20 seconds (Fig. 2A).

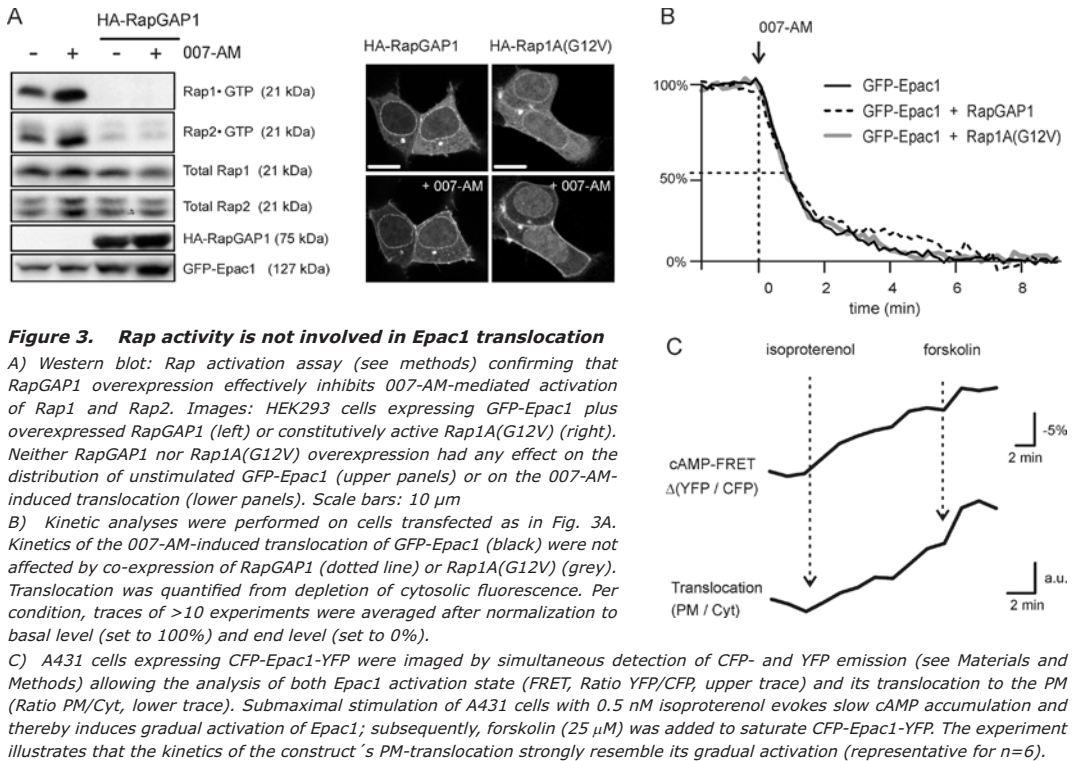
To investigate whether PM recruitment of Epac1 is a reversible event, we photoreleased NPE-caged cAMP in GE11 cells, which rapidly clear cAMP, likely due to high PDE activity [39,40]. This allowed cAMP transients to be evoked repetitively, as detected by the FRET-based cAMP sensor (Fig. 2B). When similar amounts of cAMP were released in GE11 cells expressing GFP-Epac1, we observed rapid translocations to the PM followed by relocation to the cytosol (Fig. 2B). Analyses of the PM/cytosol ratio show that the translocation kinetics closely resemble the dynamic course of the cAMP levels. These experiments indicate that Epac1 translocation is a rapidly reversible event and that the momentaneous cAMP levels dictate the degree of PM localization.

Epac1 conformational change rather than downstream signaling is required for its translocation

As opening up of Epac1, which is essential for cAMP-induced translocation (Fig. 1G), also releases its catalytic activity, we examined whether downstream signals may be required for its recruitment to the PM. As shown in Fig. 3A, overexpression of RapGAP1 inhibits cAMP-induced Rap activation by keeping both Rap1 and Rap2 in the GDP-bound, inactive state. RapGAP1 overexpression did not affect the translocation of GFP-Epac1 (Fig. 3A,B), suggesting that Rap activity is not required. In line with this, co-expression of constitutively active Rap1A(G12V) did not affect GFP-Epac1 localization in unstimulated cells (Fig. 3A), nor did it affect the magnitude or kinetics of the 007-AM-induced translocation (Fig. 3A,B). Furthermore, in OVCAR-3 cells, the cAMP-binding mutant Epac1(R279L) does not translocate when Rap is transiently activated through activation of endogenous Epac1 (data not shown). Taken together, these data indicate that Epac1 translocation results from its conformational change rather than downstream signaling via Rap. This was further supported by using the CFP-Epac1-YFP probe, that allows the simultaneous visualization of localization as well as conformational state via intramolecular FRET. These experiments showed that the kinetics of Epac1 translocation closely follow those of its conformational state (Fig. 3C).

The DEP domain is essential but not sufficient for Epac1 translocation

To determine which domains of Epac1 are involved in the translocation, a series of deletion mutants were analyzed (Fig. 4A). Removal of the DEP domain, which is essential for proper intracellular targeting and functioning of DEP domain-containing proteins such as Dishevelled [39] and numerous RGS proteins [27,41-45], completely abolished Epac1 translocation (Fig. 4B). Mutation of Arginine 82



within the DEP domain of Epac1, which localizes within a proposed interaction surface crucial for the DEP-mediated targeting of Dishevelled-1 and Ste2 [46-49], also abolished the cAMP-induced translocation, further illustrating the critical role of the DEP domain in mediating the cAMP-induced translocation. Nonetheless, GFP-DEP (comprising amino acids 50-148) was not observed at the PM (data not shown). Similarly, the YFP-tagged regulatory region of Epac1 (YFP-Epac1-Reg), comprising the DEP domain and the cAMP binding domain, did not localize at the PM nor did it translocate to the PM upon cAMP elevations (Fig. 4A,B). However, application of 007-AM to cells transfected with both YFP-Epac1-Reg and the CFP-tagged complementary catalytic region of Epac1 (CFP-Epac1-Cat), which are able to reconstitute the structure of the full length protein (data not shown), induced the combined translocation of both fragments (Fig. 4C). This demonstrates that the DEP domain is required for the translocation of Epac1 to the PM, but that it can function only in conjunction with the catalytic region.

Epac1 translocation enhances Rap-dependent cell adhesion

As the main pool of Epac1 redistributes to the PM after its activation, we explored whether the translocation of Epac1 is a prerequisite for activation of Rap at this compartment. For this, HEK293 cells were transfected with the YFP-tagged Ras-binding domain (RBD) of RalGDS

(YFP-RBD(RalGDS)), which recognizes Rap1 specifically in its GTP-bound, activated state [23]. When cells were co-transfected with HA-Epac1, addition of 007-AM resulted in the rapid accumulation of YFP-RBD(RalGDS) at the PM, as visualized both by TIRF (Fig 5A, left panel) and confocal microscopy (right panel). Interestingly, such accumulation was not observed on other subcellular compartments, suggesting that in HEK293 cells cAMP signaling via Epac1 activates Rap predominantly at the PM (Fig 5A). To test for the role of Epac1 translocation in Rap activation at the PM, we compared YFP-RBD(RalGDS) membrane recruitment in cells expressing either CFP-Epac1 or CFP-Epac1(Δ DEP) by TIRF microscopy. 007-AM induced recruitment of the probe to the basal membrane in cells expressing CFP-Epac1 (12 of 14 cells), whereas this was almost absent in cells co-expressing CFP-Epac1(Δ DEP) (1 of 9 cells; $p < 0.01$; Fig. 5B). Thus, translocation of Epac1 is required for Rap activation at the PM.

Epac1-Rap signaling is involved in integrin-mediated cell adhesion by regulating both the affinity and avidity of actin-associated integrin molecules [50]. For Jurkat T-cells, it has been shown that this requires the presence of active Rap at the PM [23]. To study the role of Epac1 translocation in its ability to mediate integrin regulation, we measured adhesion of Jurkat T-cells in response to 007. For this, the cells were transfected with luciferase together with either wild-type Epac1 or the non-translocating Epac1 variants mutated in their DEP domain, and

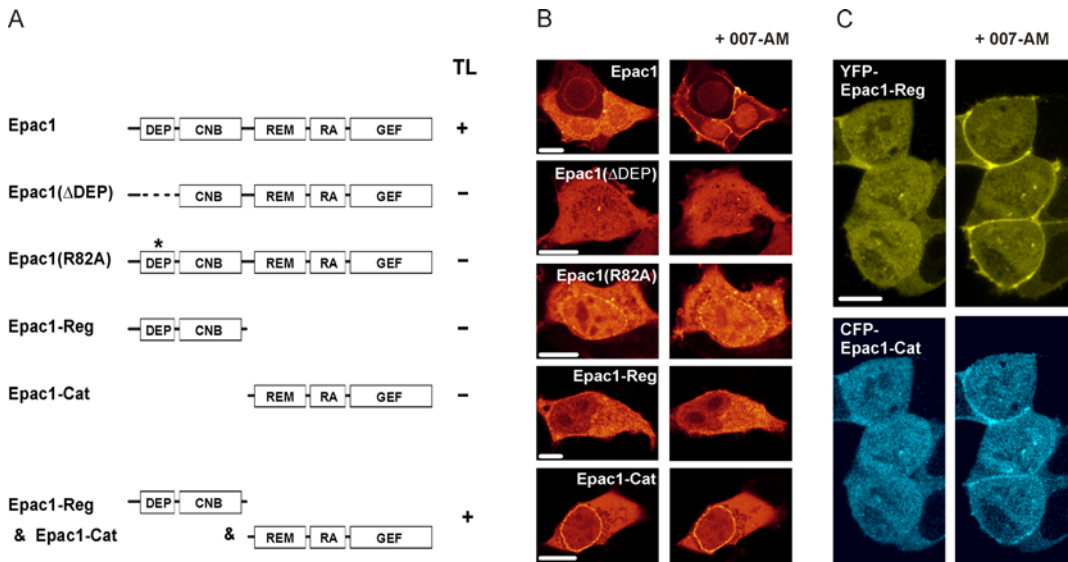


Figure 4. The DEP domain is essential but not sufficient for Epac1 translocation

A,B) Overview of Epac1-mutants and their abilities to translocate (TL) upon addition of 007-AM (1 μ M). Full-length Epac1 consists of a DEP domain (amino acid 50-148), the cyclic nucleotide binding domain (CNB), the Ras exchange motive (REM), a putative Ras association (RA) domain and the catalytic CDC25 homology GEF domain. Removal of the DEP domain (amino acid 50-148) or disruption of the interaction surface within the DEP domain (R82A) abolishes the translocation of Epac1 in response to 007-AM. The separate regulatory region (YFP-Epac1-Reg, amino acid 1-328), containing both the DEP domain and cAMP binding domain (CNB), did not translocate after 007-AM stimulation, indicating that the DEP domain cannot mediate the localization of Epac1 at the PM in the absence of the catalytic region. In accordance with the crucial role of the DEP domain, the complete catalytic region (CFP-Epac1-Cat, amino acid 330-881) was not present at the PM either.

C) In contrast to the separately expressed regulatory and catalytic region, coexpression of YFP-Epac1-Reg (upper panels) + CFP-Epac1-Cat (lower panels) restored the 007-AM-induced translocation, resulting in colocalization of both constructs at the PM.

Scale bars: 10 μ m

adhesion was quantified by detection of luciferase emission. Indeed, 007 induced strong adhesion to fibronectin of wildtype Epac1-transfected cells (Fig. 5C). In contrast, this cAMP-induced effect on cell adhesion was impaired when the translocation-deficient mutants Epac1(Δ DEP) or Epac1(R82A) were expressed (Fig. 5C). Rap1-GTP pull-down experiments in Jurkat T-cells show that expression of these mutants indeed result in less Rap1 activation compared to wildtype Epac1 (Fig. 5D), implying that a significant fraction of Rap1 resides at the PM in these cells. These data indicate that the translocation of Epac1 significantly enhances the signaling cascade towards Rap-mediated cell adhesion.

Discussion

In the current study we have shown that cAMP induces the translocation of cytosolic Epac1 towards the PM. Epac1 translocation is a generally occurring, physiological event, as it was observed in a wide array of cell types, both with overexpressed and endogenous Epac1 (Fig. 1). The translocation depends solely on the cAMP-induced conformational state of Epac1, since it could be induced by the

Epac-selective analogue 007(-AM) and was prevented by a mutation in the cAMP binding pocket (R279L, Fig. 1F,G). Furthermore, since neither activation nor inhibition of Rap could affect Epac1 translocation, involvement of Rap-mediated signaling could be excluded (Fig. 3). Finally, the degree of Epac1 translocation closely followed the levels of free cAMP within the cells and showed similar kinetics as the cAMP-induced conformational change (Fig. 2 and Fig 3C). Thus, in addition to releasing Epac1 from auto-inhibition, direct binding of cAMP also regulates the translocation of Epac1 towards the PM.

Epac1 translocation is based on passive diffusion, since fluorescence distribution in the cytosol is homogeneous throughout the translocation without discernible discrete moving structures that would indicate active transport. In line with the diffusion model, Epac1 translocation shows rapid and reversible kinetics after cAMP-uncaging (Fig. 2) and is not affected by disruption of the actin cytoskeleton or the microtubule network (data not shown). These data imply that upon transition to its opened conformation, Epac1 acquires an affinity for an anchoring factor at the PM, to which it is subsequently targeted via passive diffusion.

Deletion and point mutations have indicated the DEP domain as an essential determinant of translocation. This

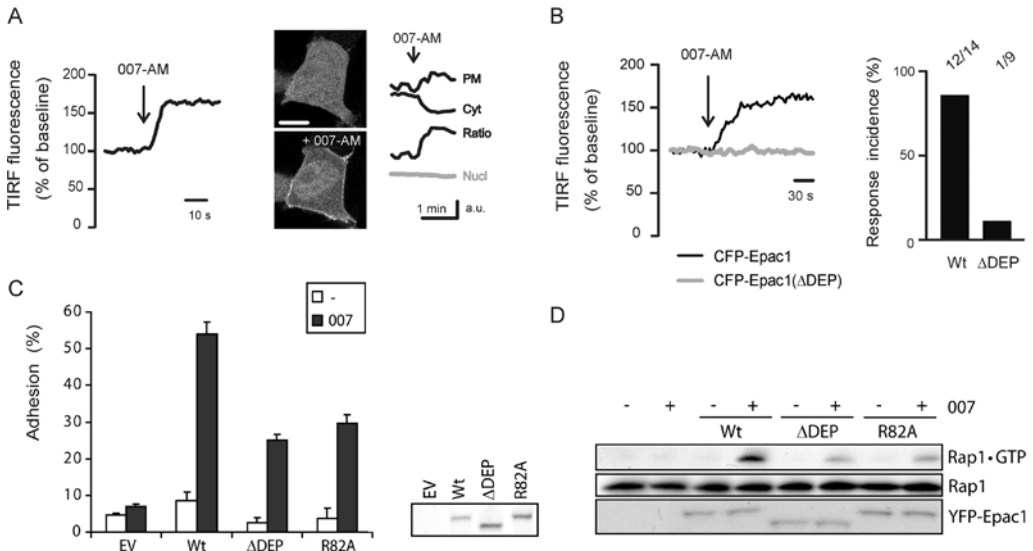


Figure 5. The translocation of Epac1 enhances Rap activation and Rap-mediated adhesion of Jurkat T-cells

A) Left panel, *in vivo* TIRF imaging of Rap1 activation in HEK293 cells using YFP-RalGDS(RBD). When co-expressed with HA-Epac1, YFP-RalGDS(RBD) translocates to the basal membrane within seconds after 007-AM stimulation (1 μ M). The 007-AM-induced increase was 46 $\pm$ 6 % (mean $\pm$ SEM) relative to pre-stimulus levels ($n=9$). Right panel, confocal imaging of YFP-RalGDS(RBD) translocation showing accumulation at the plasma membrane (PM) as well as the simultaneous depletion of cytosol (Cyt); fluorescence in the nucleus was constant (Nucl). Accumulation of YFP-RalGDS(RBD) was not observed on intracellular membranes. Scale bar: 10 μ m.

B) HEK293 cells were transfected with CFP-Epac1 or CFP-Epac1(Δ DEP) and recruitment of co-transfected YFP-RBD(RalGDS) to the basal membrane was measured by TIRF microscopy. Cells expressing low levels of CFP- and YFP-tagged fusion proteins were selected. Traces are representative experiments. Bar graph shows relative occurrence of 007-AM-induced membrane accumulation of YFP-RBD(RalGDS): CFP-Epac1, 12 out of 14 cells; CFP-Epac1(Δ DEP), 1 out of 9 cells.

C) Jurkat T-cells were transfected with either wildtype Epac1 or the non-translocating Epac1 mutants (Epac1(Δ DEP) and Epac1(R82A)) together with a luciferase reporter. Transfected cells were allowed to adhere to fibronectin-coated surface for 45 minutes and adhesion was subsequently detected as luciferase emission. Wildtype-Epac1, when activated by 007 (100 μ M, black bars) greatly enhanced the adhesion as compared to unstimulated conditions (white bars). This effect was impaired when the translocation-deficient mutants Epac1(Δ DEP) or Epac1(R82A) were transfected and it was absent in empty vector-transfected cells (EV). Shown are data from a representative experiment performed in triplo ($n=4$). Total luciferase levels were comparable in all transfections. Right: western blot labeled with the Epac1 antibody (5D3) showing expression levels of the transfected wildtype- and mutant Epac1 used in the adhesion assay.

D) Jurkat T-cells were transfected with similar amounts of either wildtype Epac1 or the translocation-deficient mutants Epac1(Δ DEP) and Epac1(R82A) and GTP-bound Rap1 was pulled down from lysates of cells after stimulation with 007 (100 μ M, 10 min).

is analogous to the targeting function of DEP domains in other proteins such as Dishevelled and RGS [1, 8, 27, 31, 40]. However, crystal structure studies on Epac2 [52] suggest that the DEP domain is solvent exposed regardless of cAMP binding. Therefore, a model wherein the cAMP-induced conformational change renders the DEP domain accessible is unlikely. Indeed, the separate DEP domain or regulatory region of Epac1 did not localize to the PM (Fig. 4). Thus, the DEP domain can only fulfill its function in the context of the structure of the full length protein. The interaction surface for PM anchoring is thus established by combined structural features of the DEP domain and a determinant in the catalytic region, which are dependent on the cAMP-bound conformation. The identification of the membrane anchor would likely help to define the underlying structural mechanism.

Many downstream effects of Epac1 occur at the PM: cell adhesion, cell-cell junction formation and the regulation of NHE3. These effects may require localized activation of Epac1 and, thereby, localized activation of Rap. Indeed, we showed that the translocation of Epac1 is a prerequisite for Rap activation (Fig 5B). Furthermore, translocation strongly enhances Rap-mediated cell adhesion, as the translocation-deficient mutants Epac1(Δ DEP) and Epac1(R82A) were impaired in their ability to induce Rap-dependent adhesion of Jurkat T-cells to fibronectin. It is important to note here, that purified Epac1(Δ 1-148) mediates GDP-dissociation from Rap1 *in vitro* equally well as full-length Epac1 does [51], indicating that the DEP domain is not required for the catalytic activity. The residual effect of the Epac1 mutants on cell adhesion may be due to the relatively high expression of Epac1 in this

system, allowing a fraction of the mutant Epac1 to locate near the PM regardless of the DEP-dependent translocation, thereby activating Rap. Indeed, TIRF experiments showed membrane recruitment of YFP-RBD(RalGDS) in cells expressing CFP-Epac1(Δ DEP) at relatively high levels (data not shown), but not at lower levels (Fig 5B).

Recently, the DEP domain of Epac1 has been shown to be essential for the ability of the regulatory region to disrupt TSH-mediated mitogenesis, further supporting the notion that proper localization of Epac1 via its DEP domain is required for its function [52]. Different anchors may target Epac1 to other cellular compartments and thereby regulate alternative functions of Epac1 that are not linked to (processes at) the PM. Indeed, in cardiomyocytes Epac1 was found in a complex with muscle specific mAKAP, phosphodiesterase 4A and PKA to regulate ERK5 activity [11]. Epac1 is also present at the nuclear membrane (see Fig. 1A) and this binding is maintained during the early time points of cAMP stimulation, indicating that only a subfraction of Epac1 translocates to the PM. Thus, in this respect Epac1 resembles protein kinase A, which is targeted to distinct subcellular compartments through the binding to AKAPs.

PM-localization has also been reported to be essential for signaling via Epac2, which is mediated by binding of its RA domain to activated Ras [30,53]. Originally this was proposed to be regulated by cAMP [30]. However, binding of Epac2 to Ras does not require the open conformation of Epac2 and is not affected by cAMP [53] and Supplemental Fig 1). In addition, expression of an active Ras mutant suffices for targeting Epac2 to the PM [30,53]. Conversely, deletion of the putative RA domain within Epac1 does not affect its cAMP-induced translocation as demonstrated by increased FRET between CFP-CAAX and an YFP-Epac1 mutant lacking the RA domain (see Supplemental Fig 2). These data exclude the possibility that the RA domain is the missing determinant within the catalytic region of Epac1. The different mechanisms of PM targeting distinguish the roles of Epac1 and Epac2, which may add to the understanding of their specific biological functions.

Based on our data, we propose a model where binding of cAMP regulates Epac1 in two manners: it targets Epac1 towards the PM and simultaneously releases the activity of its GEF domain. Such dual regulation imposes signal specificity by guaranteeing that cAMP predominantly affects PM-localized Rap molecules (Fig. 5A), whereas, for example, growth factors such as EGF activate a perinuclear pool of Rap [54]. Analogously, negative regulation of Rap by GAPs may also be spatially confined, as it has been reported that a PM pool of RapGAP restricts Rap activation in COS1 cells [55]. Thus, it appears that the localization of GEFs and GAPs rather than that of the G-protein itself determines the intracellular location of G-protein activity.

We can only speculate why the activation of Epac1 is dynamically regulated by the simultaneous cAMP-dependent translocation rather than by more static confinement via stable association to the PM. The translocation mechanism may serve to dynamically regulate the availability of Epac1 throughout the cell. In addition, the separation between Epac1 and Rap in resting conditions may be an ultimate guarantee against stimulation of Rap by residual

Epac1 activity. Adding a spatial component to the cAMP-mediated regulation of Epac1 undoubtedly renders the transition from unstimulated to stimulated Rap at the PM more pronounced.

Materials and Methods

Reagents and antibodies

Forskolin, IBMX and H89 were from Calbiochem-Novabiochem Corp. (La Jolla, CA); Isoproterenol, EGF, Cytochalasin D, Latrunculin A and Nocodazol from Sigma Chemical Co. (St. Louis, MO); 1-(2-nitrophenyl)ethyl adenosine-3',5'-cyclic monophosphate (NPE-caged cAMP) from Jena Bioscience GmbH (Jena, Germany); 8-pCPT-2'-O-Me-cAMP (007) and 8-pCPT-2'-O-Me-cAMP-AM (007-AM) from Biolog Life Sciences (Bremen, Germany); Fura-Red-AM and BAPTA-AM from Molecular Probes Inc. (Eugene, OR); the Rap1 antibody from SantaCruz Biotechnology (SC-65) and the Rap2 antibody from BD Transduction laboratories (610216). The Epac1 antibody (5D3) has been described [56]. Fibronectin was purified as described [57].

DNA constructs

The following expression vectors were described elsewhere: pcDNA3 CFP-Epac1-YFP and pcDNA3 CFP-Epac1- Δ DEP-C.D.-YFP [39], pcDNA3 CFP-Epac1(Δ DEP-C.D.)-Venus (55), pMT2-SM-HA Rap1A(G12V) and pMT2-SM-HA RapGAP1 [58], pMT2-SM-HA Epac1 [17], pcDNA3 CFP-CAAX [35]. Epac1 (RapGEF3, homo sapiens, GI: 3978530) was cloned C-terminal to YFP in a pcDNA3 vector. Mutations were introduced by site-directed mutagenesis. The separate regulatory (amino acid 1-328) and catalytic (amino acids 330-881) region of Epac1 were cloned into pcDNA3 with an N-terminal CFP and YFP tag, respectively. GFP-RBD(RalGDS) was a kind gift from Mark Philips.

Cell culture

HEK293 (Human Embryonal Kidney) cells and A431 human carcinoma cells were cultured in DMEM; OVCAR-3 and the Jurkat T-cell line JHM1 2.2 were grown in RPMI medium, all supplemented with 10% serum and antibiotics.

Live cell experiments

Cells were seeded in 6-well plates on 25-mm glass cover slips and cultured in 3 ml medium. Constructs were transiently transfected using Eugene 6 Transfection Reagent (Roche Inc.). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 23 mM NaHCO₃, with 10 mM HEPES added), pH 7.2, kept under 5% CO₂, at 37°C. Agonists and inhibitors were added from concentrated stocks.

Dynamic monitoring of YFP/CFP FRET

Cells on coverslips were placed on an inverted NIKON microscope equipped with 63x lens (N.A. 1.30) and excited

at 425 nm. Emission of CFP and YFP was detected simultaneously by two photon multiplier tubes (PMTs) through 470 +/- 20 nm and 530 +/- 25 nm bandpass filters, respectively. Data were digitized by Picolog acquisition software (Picotech) and FRET was expressed as the normalized ratio of YFP to CFP signals. The ratio was adjusted to 1 at the onset of the experiment and changes are expressed as percent deviation from this initial value. For some of these experiments, a CFP-tagged version of K-Ras-CAAX was used as PM marker. Whereas in hippocampal neurons K-Ras-CAAX may translocate to endomembranes under certain conditions [59], under our conditions CFP-CAAX localizes to the plasma membrane as we have extensively documented [35,57]. For the experiment described in Fig. 3C the YFP/CFP FRET ratio was determined in imaging mode, by detecting CFP- and YFP images simultaneously on a Leica fluorescence SP2 microscope equipped with a dual-view attachment and a Coolsnap-HQ CCD-camera (Roper Scientific), using ASMDW acquisition software.

Confocal microscopy

Coverslips with cells expressing various constructs were mounted in a culture chamber and imaged at 37 °C using an inverted TCS-SP5 confocal microscope equipped with 63x immersion oil lens (N.A. 1.4; Leica, Mannheim, Germany). Imaging settings were: CFP, excitation at 442 nm, emission at 465-500 nm; GFP, exc. at 488 nm, em. at 510-560 nm; YFP, exc. at 514 nm, em. at 522-570 nm.

For detection of endogenous Epac1 in OVCAR-3 cells, cells were grown on 12 mm glass cover slips for 72 h and after 10 min of stimulation with 25 μ M Forskolin, fixed with 3.8% formaldehyde, permeabilized using 0.1% Triton X100 and blocked in 2% BSA. Cells were incubated with the Epac1 antibody (5D3) and subsequently with Alexa-conjugated secondary antibodies (Invitrogen). Mounted slides were examined using a Axioskop2 CLSM microscope (Zeiss) (63x magnification lenses, N.A. 1.4).

Digital image analysis

For translocation studies, series of confocal images were taken from a medial plane at 5 or 10 s intervals. To quantitate the translocation of constructs, the ratio of cytosolic to PM fluorescence was calculated by post-acquisition automated assignment of regions of interest (ROIs) using Leica Qwin software (see [35] and <http://research.nki.nl/jalinklab/Homepage%20%Phys&ImgGrp%200.htm>). Note that in the individual traces gain of fluorescence at the PM appears small compared to the loss in the cytosol because of the under-representation of membrane area in medial sections; the majority of membrane is present in the basal membrane and in the strongly curved apical parts of the cell. Post-acquisition brightness and contrast adjustments were performed with ImageJ software (NIH).

TIRF microscopy

Cells expressing GFP-Epac1 or YFP-RBD(RaIGDS) were mounted in a culture chamber and imaged on a Leica TIRF setup, equipped with 488 argon excitation laser and Hamamatsu EM-CCD detector. A 63x, 1.45 NA objective was used and evanescent field penetration depth was set

to 90 nm. TIRF imaging was at ambient temperature and analysis was with LAS-AF software.

Loading and flash photolysis of NPE-caged cAMP

Cells were loaded by incubation with 100 μ M NPE-caged cAMP for 15 min. Uncaging was done with brief pulses of UV light (340-410 nm) from a 100 W HBO lamp using a shutter. To define exposure times and UV-light intensities for desired cAMP-release, cAMP was monitored ratiometrically (CFP/YFP) using the Epac-based sensor CFP-Epac(Δ DEP-C.D.)-Venus [38]. Translocation of GFP-Epac1 was monitored in parallel experiments.

Rap activation assay

Rap activity was assayed as described previously [61]. Briefly, HEK293 cells grown in 9 cm plates were lysed in buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 10% glycerol, 2 mM MgCl₂ and protease and phosphatase inhibitors. Lysates were cleared by centrifugation and active Rap was precipitated with a GST fusion protein of the Ras-binding domain of RaIGDS precoupled to glutathione-sepharose beads.

Adhesion assay

The adhesion of Jurkat T-cells to fibronectin was measured as described previously [62]. In brief, 96-Well Nunc Maxisorp plates were coated with 5 μ g/ml fibronectin and blocked with 1% bovine serum albumin. 1.2×10^7 Jurkat cells were transiently transfected by electroporation (950 μ F, 250 V) with 5 μ g CMV-luciferase plasmid and pcDNA3 YFP-Epac1 plasmid adjusted to get equal expression levels, supplemented with pcDNA3 empty vector to a total of 40 μ g plasmid DNA, using the Gene Pulser II (Bio-rad). Cells were harvested two days after transfection and resuspended in TSM buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂). 2.5×10^4 cells for each well were allowed to adhere for 45 min and non-adherent cells were removed with 0.5% BSA in TSM buffer. Adherent cells were lysed and subjected to a luciferase assay as described previously [63]. For Rap1 activity measurements in Jurkat T-cells, transfected cells were subjected to the Rap activation assay after resuspension in TSM buffer and stimulation with 007 (100 μ M, 10 min).

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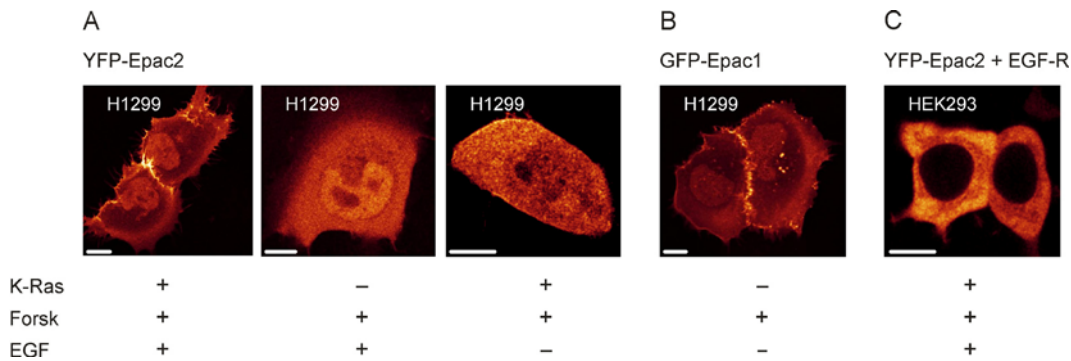
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Supplemental Figures



Suppl Fig 1
The mechanism of Epac1 translocation is distinct from that of Epac2 translocation
A) In accordance with Li et al. [30], YFP-Epac2 (RapGEF4, *mus musculus*, GI:9790086, cloned C-terminal to YFP in a pcDNA3 vector) translocates to the plasma membrane in H1299 cells after both forskolin (25 μ M) stimulation and EGF (25 ng/ml)-mediated activation of overexpressed K-Ras (left), while this translocation was not observed when omitting Ras overexpression (middle) or EGF stimulation (right).
B) In contrast, forskolin alone is sufficient for the translocation of Epac1 in H1299 cells.
C) In any of the other cell types positive for Epac1 translocation, such as HEK293 cells, we could not reproduce the translocation of Epac2, even under conditions of EGF-mediated activation of co-expressed Ras. Scalebars: 10 μ m

