ERM proteins recruit Epac1 to the plasma membrane and facilitate Epac1-induced cell adhesion

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Abstract

Epac1 is a GEF for the small G-protein Rap and is directly activated by cAMP. Epac1-Rap signaling is involved in plasma membrane-localized processes such as integrin-mediated cell adhesion and cell-cell junction formation. We previously showed that cAMP induces the translocation of Epac1 to the plasma membrane, thereby enhancing Rap-mediated cell adhesion. We here report an additional mechanism of Epac1 recruitment to the plasma membrane via an interaction with members of the Ezrin/Radixin/Moesin (ERM) family. In contrast to cAMP-dependent Epac1 translocation, this recruitment depends neither on the DEP domain nor on the conformational state of Epac1. Instead, it is regulated by conformational opening of the ERM proteins. Furthermore, whereas cAMP binding targets Epac1 uniformly along the plasma membrane, ERM proteins recruit Epac1 to polarized subcompartments. Finally, we show that the ERM-interaction contributes to Epac1-mediated cell adhesion. Taken together, our data suggest that ERM proteins spatially confine Epac1 to subcompartments of the plasma membrane, thereby contributing to the efficiency of localized Rap signaling.
**Chapter 4**

**Introduction**

cAMP is a second messenger in a wide variety of hormone responses. cAMP is produced at the plasma membrane by adenylate cyclases, and subsequently becomes compartmentalized due to degradation by spatially restricted phosphodiesterases (PDEs) [1]. Further compartmentalization of cAMP signaling is established by the confined targeting of cAMP effector proteins. For the classical cAMP target Protein Kinase A (PKA), more than 50 A-kinase anchoring proteins (AKAPs) have been identified. AKAPs differentially target PKA to subcellular compartments and are thereby implicated in distinct biological functions of PKA [2].

The discovery of Epac as a direct effector of cAMP [3,4] has triggered the elucidation of many cAMP-regulated processes that could not be explained by the known effectors PKA and cyclic nucleotide-regulated ion channels. Epac1 and Epac2 act as guanine nucleotide exchange factors (GEFs) for the small G proteins Rap1 and Rap2, and thereby function in processes such as cell adhesion [5-9], insulin secretion [10,11] and cell polarity [12,13]. Their catalytic region contains the enzymatically active CDC25 domain, a Ras Association (RA) domain and the Ras Exchange Motif (REM). In auto-inhibited Epac, the catalytic site is sterically covered by the regulatory region, which harbors the cAMP-binding domain(s) and the DEP domain [14]. CAMP binding releases Epac from auto-inhibition by inducing a conformational change, as demonstrated by the crystal structures of active and inactive Epac2 [15,16].

Similar to the compartmentalization of PKA, cAMP-Epac signaling is spatially regulated by several anchoring proteins. For instance, both Epac1 and Epac2 are targeted to microtubules by interacting with Microtubule Associated Protein-Light Chain (MAP-LC) [17]. Other anchors bind specifically to either Epac1 or Epac2, thereby contributing to their distinct functions. For instance, only Epac1 is targeted to the mAKAP complex at the nuclear envelope [18] and, conversely, Epac2 is part of the Rim2-Piccolo complex involved in cAMP-dependent exocytosis [19,20]. In addition, more dynamic targeting mechanisms allow the regulation by intracellular signalling events. For example, Epac2 is recruited to the plasma membrane by the activated GTPase Ras [21] and this was shown to be crucial for its involvement in neurite outgrowth [22]. Recently, we reported that Epac1 translocates to the plasma membrane upon binding of cAMP and we showed that this translocation enhances Rap-mediated cell adhesion [23]. Although the anchor at the plasma membrane remains elusive, it has become clear that cAMP-dependent Epac1 translocation involves the DEP domain (residues 50-148) and depends on the cAMP-induced conformational opening.

In an attempt to identify new binding partners for Epac1, we performed a yeast-two-hybrid screen, which revealed interactions with members of the Ezrin-Radixin-Moesin (ERM) family. ERM proteins show high sequence similarity and function as scaffolding proteins that link the actin cytoskeleton to the plasma membrane [24]. Inactive ERM proteins reside in the cytoplasm in an auto-inhibited state maintained by an intramolecular interaction between the N-terminal FERM domain and the C-terminal Actin Binding Domain (ABD). For their transition to scaffolds at the plasma membrane, ERM proteins require PI(4,5)P_2 binding and acquisition of the open conformation [25]. The latter requires phosphorylation of the ABD, for which several kinases have been implicated, including PKCα and the Rho-effector Rock [26-30]. ERM proteins directly link the actin cytoskeleton to the PM and recruit of multiple signalling proteins. In this manner, ERM proteins function in numerous processes that involve actin dynamics, such as the formation of microvilli [31], adherens junctions stabilization [24] and leukocyte polarization [32].

In the present study, we show that ERM proteins serve as membrane anchors for Epac1. The underlying interaction is mediated by the Epac1 N-terminus (residues 1-49) and is independent of its conformational state. These properties imply that ERM proteins do not constitute the anchor for the previously described cAMP-induced translocation, which depends on the DEP domain and conformational opening [23]. Instead, the interaction is regulated on the level of the ERM proteins, which selectively bind Epac1 when they are in their active, open conformation. In line with this, ERM activation via thrombin stimulation triggers the immediate recruitment of Epac1 to cortical ERM proteins. This recruitment targets Epac1 to polarized compartments along the plasma membrane, adding a component of spatial regulation to Epac1 functioning. Importantly, we find that the ERM-interaction enhances the ability of Epac1 to induce Rap-mediated adhesion of Jurkat T-cells. Taken together, our data suggest that spatial regulation by ERM proteins regulates the coupling of cAMP-dependent Epac1 activity towards Rap.

**Results**

**Epac1 interacts via its N-terminus to proteins of the ERM family**

In an attempt to identify new binding partners for Epac1, a yeast two-hybrid screen was performed using a human placenta cDNA library and full length Epac1 as bait. Positive clones were isolated encoding partial cDNA for the membrane-associated proteins Ezrin and Radixin. Together with Moesin, these proteins belong to the Ezrin/Radixin/Moesin (ERM) family and function as adaptor proteins that link the actin cytoskeleton to the plasma membrane [33].

To confirm the interaction between Epac1 and the ERM family proteins in mammalian cells, HEK293 cells were transfected with HA-Epac1 and Flag-tagged variants of Ezrin, Radixin or Moesin. Indeed, all ERM proteins co-precipitated with Epac1 (Fig 1A). As the ERM proteins share high sequence similarity and bind Epac1 to a similar extent, Radixin was used as a representative family member to further explore the interaction between Epac and ERM proteins. Epac1 and Epac2 are similar in domain architecture, except that Epac2 contains an additional cAMP binding domain [14]. However, Flag-Radixin was not able to co-immunoprecipitate with Epac2 (Fig 1B). Thus, binding to ERM proteins is specific for Epac1, suggesting that the interaction is mediated by a region that
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is not conserved in Epac2. Mutational analyses revealed that the interaction with Radixin requires the N-terminal 49 amino acids of Epac1 (Fig 1C), which are indeed absent in Epac2. To further establish this, we tested binding of bacterially purified Ezrin to a series of 20-mer peptides from the Epac1 N-terminus. This assay demonstrated that the Epac1-ERM interaction is direct and further revealed that the interaction surface resides between amino acid 19 and 48 of Epac1 (Supplementary Fig S1). Thus, the Epac1 N-terminal N49 is not only essential but also sufficient to bind ERM proteins.

Importantly, these data exclude the possibility that the interaction with ERM proteins underlies the recently described cAMP-induced PM translocation of Epac1 [23], which is mediated by its DEP domain. In line with this, deletion of the DEP domain (residues 50-148) did not affect the interaction with Radixin (Fig 1C). Thus, our data indicate that Epac1 binds ERM proteins via its N-terminal 49 residues, providing a targeting mechanism that is distinct from the cAMP-induced PM translocation.

Epac1 selectively binds to ERM proteins that are in the open conformation

In resting ERM proteins, binding to the plasma membrane and the actin cytoskeleton is prevented by the intramolecular interaction between its N-terminal FERM domain and C-terminal actin binding domain (ABD) (see scheme in Fig 1A). This auto-inhibition can be relieved by threonine phosphorylation within the ABD (in Radixin: Thr564) [26,34]. Co-immunoprecipitation experiments using either the truncation mutant containing the N-terminal FERM domain and α-helical region (residues 1-492) or the separate C-terminal ABD (residues 493-584) showed that Epac1 binding is mediated by the N-terminal half of Radixin (Fig 2B). Since this region is partially buried in the globular, inactive ERM conformation, we tested whether Epac1 specifically binds ERM proteins that are in the open conformation. For this, two Radixin mutants were tested (see residues in Fig 2A). The first, Radixin(T564D), contains a phospho-mimicking mutation at the threonine position [35]. The second, Radixin(1577D/F580D) is a novel mutant based on the crystal structure of Moesin [36]. In this mutant, residues of the ABD that form a hydrophobic interaction with the FERM domain were replaced by negatively charged asparagines. Both mutations prevented the interaction between the N- and C-terminal truncation constructs of Radixin (data not shown), and thus result in the constitutively open conformation of full length Radixin. Indeed, both Radixin mutants showed dramatically increased interaction with Epac1 compared to wildtype Radixin (Fig 2C), indicating that Epac1 displays an increased affinity for the open conformation of the ERM binding partners.

To test this further, we stimulated HEK293 cells with thrombin, which induces threonine phosphorylation and thus conformational opening of ERM proteins ([37] and Fig 2D). As shown in Fig 2D, the interaction between Epac1 and wild-type Radixin was enhanced upon thrombin treatment. This could also be measured in vivo by measurement of Fluorescence Resonance Energy Transfer (FRET) between YFP-Radixin and Epac1-TdTom. Addition of Trombin Receptor-activating Peptide (TRP) induced an increase in FRET (Fig 2E; ΔTdTom/YFP = 6.0 +/- 1.08 % (average +/- st-dev), n=3), reflecting the instant interaction between Epac1 and Radixin. These data confirm that Epac1 preferably binds to the open conformation of ERM proteins.

In a converse experiment, we examined whether the open conformation of Radixin is truly required for binding to Epac1. For this, the separate C-terminal ABD of Radixin was overexpressed to bind the N-terminus of full-length Epac1.
This mimics the intramolecular interaction and thus the closed conformation of FL-Radixin. Indeed, co-expression of the Radixin C-terminus resulted in a dose-dependent decrease in binding between Radixin and Epac1 (Fig 1F). All in all, our data show that the binding of Epac1 to ERM proteins is regulated on the level of ERM conformation.

Activated ERM proteins rapidly recruit Epac1 to the plasma membrane

Epac proteins are spatially regulated by interactions with diverse anchoring proteins, and the inducible Epac1–ERM interaction may also represent such a regulatory mechanism. In the inactive, closed conformation, ERM proteins reside in the cytosol [34,38], whereas activated ERM proteins localize to the plasma membrane. To study the potential role of ERM proteins in regulating the subcellular localization of Epac1, we used the constitutively open and thus PM-localized Ezrin(T567D). Whereas Epac1-YFP, when expressed alone, localized mainly to the cytosol and the nuclear envelope, it showed marked accumulation at the PM when Ezrin(T567D) was overexpressed. This was not observed with CFP-Epac1(Δ1-49), while the isolated N-terminus of Epac1 (GFP-N49) was sufficient for PM targeting (Fig 3A), indicating that the PM accumulation of Epac1 is indeed mediated by the interaction with ERM proteins.

Strikingly, Ezrin(T567D) expression similarly induced PM accumulation of Epac1(R279L)-YFP, which is mutated in its cAMP binding domain and thereby locked in the auto-inhibited conformation [23,39] (Fig 3A). The latter implies that recruitment by ERM proteins is independent of Epac1 conformational state.

Activated ERM proteins are tethered to the PM via their FERM domains [40], implying that they may recruit Epac1 closely to the PM. To test this, we drove Epac1-YFP to the constitutively open mutant Ezrin(T567D) in cells co-expressing CFP-CAAX, which is membrane-anchored by its prenylated K-Ras CAAX-motif. We observed a prominent loss of FRET upon addition of Ionomycin (Fig 3B; average +/- s.e.m. 23 +/- 3% decrease in ratio YFP/CFP), which
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This implies, that ERM-bound Epac1-YFP resides within 10 nm from the PM. This was not observed when Epac1(Δ49)-YFP was used (average +/- sem 0.5 +/- 0.1 %, data not shown). Without overexpressed Ezrin(T567D), Ionomycin-induced loss of FRET was significantly smaller (average +/- s.e.m. 5 +/- 1%; Fig 3B). The residual effect suggests membrane-targeting of Epac1 by endogenous ERM proteins.

Next, we used confocal microscopy to simultaneously monitor the subcellular localization of YFP-Radixin and Epac1-TdTom during TRP stimulation. Thrombin receptor activation drives ERM proteins into their open conformation [37] and induces their interaction with Epac1 (Fig 2D). We observed PM accumulation of YFP-Radixin and the simultaneous recruitment of Epac1-TdTom to these areas of accumulated YFP-Radixin (Fig 3C; movie can be on the web-site), in agreement with the TRP-induced FRET increase shown in Fig 2E. Also without Radixin overexpression, TRP elicited the recruitment of Epac1-YFP to the PM (Fig 3D). This was disabled by deletion of N49 (Epac1(Δ49)-YFP, Fig 3E), implying that ERM proteins, when activated by thrombin receptor signaling, recruit Epac1 to the PM. TRP-induced recruitment was also observed for the cAMP-binding mutant Epac1(R279L)-YFP (Fig 3F) and GFP-N49 (data not shown). Furthermore, it was sensitive to the RhoA-inhibitor C3 toxin (Fig 3G), establishing that TRP induces ERM proteins activation through Rho signaling [28,29].

Thus, from our data we conclude that Rho-dependent activation of ERM proteins causes their translocation to the PM and releases their ability to recruit Epac1.
Activated ERM proteins recruit Epac1 to polarized areas of the PM

Interestingly, the distribution patterns induced by ERM-activating differ notably from those described for cAMP-induced translocation of Epac1 [23]. Activated ERM proteins, which localize asymmetrically themselves (Fig 4A, left panel), recruit Epac1 to polarized areas of the PM, whereas cAMP-bound Epac1 distributes more uniformly along the PM (Fig 4A right panel; see also Fig 3C,D,F). This becomes even more apparent when Rho signaling is promoted by co-expression of the separate DH-PH domain of p190-RhoGEF (p190(DHPH)) [41], resulting in heavily polarized distributions of Epac1-YFP (Fig 4C) and Epac1(R279L)-YFP (data not shown). Colocalization in polarized PM areas was also observed with endogenous radixin, as visualized by immunofluorescence stainings in HEK293 cells stimulated with TRP or transfected with p190(DHPH) (Supplemental Fig 2).

Thus, cAMP-induced translocation and ERM-mediated recruitment induce differential localization patterns of Epac1. To demonstrate this more directly, we transfected HEK293 cells with CFP-N49 and YFP-Epac1(∆49) and stimulated these cells with both TRP and 007-AM. PM targeting was observed for both constructs, however, leading to different localizations at the PM. YFP-Epac1(∆49), which can translocate upon binding of 007-AM but lacks the ERM interaction domain, distributes uniformly along the PM. On the other hand, CFP-N49, which can interact with ERM proteins but is insensitive to 007-AM, accumulated asymmetrically along the PM (Fig 4B). Thus, in contrast to cAMP-induced Epac1 translocation, activated ERM proteins have the potential to recruit Epac1 to polarized areas of the PM.

Epac1 recruitment by ERM proteins is independent of Epac1 activation state (Fig 3A, 3F), in marked contrast with its cAMP-dependent translocation. Thus, in the absence of cAMP-raising stimuli, ERM proteins recruit auto-inhibited, inactive Epac1 to polarized areas of the PM. We wondered whether this spatial confinement is affected when cAMP binding subsequently elicits the DEP-dependent affinity of Epac1 for the PM. Therefore, we added 007-AM to cells expressing Epac1-YFP and p190-(DHPH). As shown in Fig 4C, 007-AM did not induce lateral redistribution of the asymmetrically localizing Epac1. Thus, although cAMP induces homogeneous PM translocation in the absence of ERM interactions (YFP-Epac1(∆49); Fig 4B and data not shown), active ERM proteins can restrict the localization of cAMP-activated Epac1 to polarized areas along the PM. Taken together, our data imply that ERM proteins can target the cAMP-dependent GEF activity of Epac1 to specialized areas of the PM.

The Epac1-ERM interaction facilitates 007-induced cell adhesion

Since active ERM proteins are capable of restricting activated Epac1 to a subcompartment of the PM, we wondered whether this interaction facilitates Epac1-mediated Rap signalling. In Jurkat T-cells, activation of Epac1-Rap signalling induces adhesion by increasing the affinity of integrins for their extracellular matrix substrates [42]. To assess whether the interaction of Epac1 with ERM proteins contributes to its ability to induce adhesion, we transfected Jurkat T-cells with wildtype Epac1 or deletion mutants lacking the DEP domain (Δ50-148) or both domains (Δ1-148). In all cases, a luciferase construct was cotransfected so that adhesion to fibronectin could be quantified by measurement of luciferase activity. As previously described [23], the 007-induced increase in adhesion was greatly impaired, albeit not completely abolished, upon deletion of the DEP domain (Δ1-148). In all cases, a luciferase construct was cotransfected so that adhesion to fibronectin could be quantified by measurement of luciferase activity. As previously described [23], the 007-induced increase in adhesion was greatly impaired, albeit not completely abolished, upon deletion of the DEP domain (Δ1-148). Interestingly, a similar intermediate effect was observed upon deletion of N49 (Fig 5). This implies that besides the cAMP-induced, DEP-dependent translocation, also the ERM interaction is involved in Rap signalling by Epac1. Indeed, in Jurkat T-cells expressing an Epac1 mutant deficient in both recruitment mechanisms (Epac1(Δ1-148)), the 007-induced adhesion was entirely lost. These data strongly suggest that the DEP and the N49 domains cooperate to convey Epac1 GEF activity to PM-localized Rap, thereby inducing cell adhesion.
ERM-mediated Epac1 recruitment

In the current study we reveal the direct interaction between Epac1 and members of the Ezrin/Radixin/Moesin (ERM) family (Fig 1). We determined that the Epac1 N-terminus (residues 1-49, or N49) interact with the N-terminal half of ERM proteins, which also harbors the FERM domain (Fig 2). Importantly, the Epac1-ERM interaction is independent of the conformational state of Epac1, since it also applies to the cAMP binding mutant Epac1(R279L), which is locked in the inactive, auto-inhibited conformation (Fig 3). Instead, we show that the interaction is regulated on the level of the ERM proteins. To bind Epac1, ERM proteins require the open, activated conformation that is triggered by threonine phosphorylation in the ABD (Thr564 in Radixin) (Fig 2).

Recently, we reported that conformational opening upon cAMP binding also triggers the translocation of Epac1 to the PM. To date, the identity of the involved membrane anchor has remained unknown. Although the ERM proteins, which can directly bind the PM, appear interesting candidates, their role as anchors in the cAMP-dependent translocation is excluded by their ability to recruit the auto-inhibited Epac1(R279L). Furthermore, cAMP-dependent translocation is mediated by the DEP domain (residues 50-148) and does not require the N-terminal N49 (Fig 4B), which is the determinant for ERM-interaction.

Instead, we show that ERM proteins are anchors for an alternative mechanism of Epac1 recruitment. To localize to the PM, ERM proteins require the activated, open conformation [26], which is also a prerequisite for Epac1 binding (Fig 2). Taken together, recruitment of Epac1 to the PM directly follows from targeting of ERM proteins themselves. This was clearly illustrated by the simultaneous PM recruitment of YFP-Radixin and Epac1-TdTom (Fig 3C) when we activated ERM proteins via thrombin receptor stimulation [37]. Using FRET sensors for cAMP [43] and for PKA-activity [44] we further established that cAMP levels are not elevated upon TRP stimulation (data not shown), supporting the notion that ERM-mediated Epac1 recruitment is independent of Epac1 activation state.

The recruitment to ERM proteins contributes to the ability of Epac1 to induce Rap-dependent [8] adhesion of Jurkat T-cells. The 007-induced adhesion of cells expressing Epac1(Δ1-49) was significantly reduced as compared to cells expressing wildtype Epac1 (Fig 5). Interestingly, we previously reported that also the DEP-dependent translocation of cAMP-bound Epac1 enhances Jurkat cell adhesion [23]. Indeed, while deletions of DEP or N49 alone resulted in partial decreases in adhesion, their combined deletion (Epac1(Δ1-148)) could suppress 007-induced adhesion completely. These data suggest that the two targeting mechanisms involving DEP and N49 cooperate to establish Rap activation. This implies that the interaction with ERM proteins facilitates the coupling between cAMP-activated Epac1 and its effector Rap.

In our current model, ERM proteins contribute to the Epac1-mediated activation of Rap by spatial confinement of the RapGEF. First, they position Epac1 in the vicinity of the PM (<10 nm), as demonstrated by FRET experiments using the PM-marker CFP-CAAX (Fig 3). Second, Epac1 accumulates at subdomains of the PM due to the asymmetrical distribution of ERM proteins themselves (Fig 4). Although the ERM proteins provide PM targeting of Epac1, Rap-mediated cell adhesion is still dependent on Epac1 activation by 007. The observation that Epac1 activation does not affect the ERM-induced spatial confinement (Fig 4) implies that ERM proteins can spatially regulate the GEF activity of Epac1.

The observation that spatial regulation of Epac1 activity by ERM proteins facilitates Rap-dependent adhesion may be explained in multiple ways. First, the combination of ERM- and cAMP-dependent targeting is expected...
to increase the avidity for the PM, thereby increasing the frequency of interaction events between Epac1 and Rap molecules. Second, the asymmetrical localizations, to which Epac1 is targeted, may reflect signaling platforms specialized for the coupling between Epac1 and Rap. In support of this, Rap has been shown to display polarized PM distributions in several cell types [12,45-47]. Third, the inhomogeneous targeting may result in the activation of a specific pool of Rap and, thereby, of specific Rap effectors. For example, ERM proteins may confine Rap activity to areas where integrin-mediated adhesion can occur. Finally, ERM-mediated Epac1 targeting may serve a combination of these functions.

Signaling via RhoA has been reported to induce ERM activation [37]. Using the RhoA-inhibitor C3, we established that TRP induces Epac1 recruitment via RhoA activation (Fig 3G). Thereby, the Epac1-ERM interaction further connects the actions of RhoA and Rap. Both GTPases play central roles in actin remodeling and share involvement in processes such as cell morphology, regulation of focal adhesions [48] and migration [49]. Interestingly, cross-talk between Rap and RhoA at several levels has been reported. Two Rho-GAPs, ARAP3 [50] and RA-RhoGAP [51] have been identified as effectors of Rap1. Conversely, RhoA can activate the atypical PLCζ [52], which acts as a GEF for Rap1 [53]. In addition, Schmidt and co-workers found that PLCζ can be activated downstream of Rap2B activity [54]. Our current data add to this interconnectivity by showing that RhoA signaling confers spatial regulation to Epac1-mediated Rap activation. Besides RhoA signaling, ERM proteins can be activated by several other signaling pathways, including phosphorylation by PKCζ [27], PKCθ [55] and NIK [56]. This implies that such pathways also cross-talk to Rap via spatial regulation of Epac1.

Epac1 targeting by ERM proteins may also be of major importance in epithelial cells, where ERM proteins strongly accumulate to the microvilli at the apical membrane. ERM proteins are involved in the dynamic regulation of actin assembly, which underlies the highly curved architecture of the microvilli [57]. Indeed, along with ERM proteins, Epac1 strongly accumulates in the microvilli of epithelial cell types (e.g. MDCK, HeLa, OVCAR; data not shown) and we find that this depends on ERM binding (data not shown). Epac1 has been reported to inhibit the apically localized sodium-proton (H⁺) exchanger 3 (NHE3) in the proximal tube of the kidney [58] and in the intestinal epithelia [59]. Since NHE3 functions in signaling complexes that also contain Ezrin [60,61], it is likely that ERM-mediated Epac1 targeting is of great importance for this function.

Spatial confinement of Epac1 by ERM proteins is another example of compartmentalized Epac1 signaling. The ensemble of Epac anchors, also containing MAP-LC [17], mAKAP [18] and the Rim2-piccolo complex [19], allows the involvement of Epac in several functions at different subcellular locations. In addition, our recruitment studies indicate that the anchoring function of ERM proteins are subject to dynamic regulation. Analogously, signaling of cAMP effector PKA is extensively compartmentalized by >50 AKAPs. It is interesting to note that Ezrin also serves as an AKAP [59,62,63]. Thereby, Ezrin may form a cross-road for two cAMP-dependent signaling routes.

Our current data show that ERM-mediated PM recruitment facilitates Rap-dependent cell adhesion. This strongly suggests that the ERM proteins define a compartment at the PM where Epac1-Rap signaling is efficiently coupled to downstream adhesive events.

## Materials and Methods

### Reagents and antibodies

8-pCPT-2′-O-Me-cAMP (007) was obtained from Biolog Life Sciences (Bremen, Germany), human Thrombin from Sigma-Aldrich (T7572). Thrombin Receptor-activating Peptide (TRP, residues SFLRRN) was synthesized in our institute. The mouse monoclonal GFP antibody was obtained from Roche, the FlagM2 antibody from Sigma-Aldrich, the phospoERM (Erzin T567, Radixin T564 and Moesin T558) antibody from Cell Signaling Technology and the monoclonal anti-HA antibody from Covance (HA11). Ezrin recombiant protein was purified as described previously.

### DNA constructs

Ezrin (ERZ, homo sapiens, GI: 161702985), Radixin (RDX, homo sapiens, GI: 62244047), Moesin (MSN, homo sapiens, GI: 53729335), Epac1 (RapGEF3, homo sapiens, GI: 3978530) and Epac2 (RapGEF4, mus musculus, GI:9790086) were cloned C-terminal to either a Citrine, TomatoRed or Flag–His tag in a pC DNA3 vector or an HA tag in a pMT2-SM vector, using the Gateway system (Invitrogen). cDNA for Ezrin, Radixin and Moesin was obtained from RZPD (Berlin, Germany). ∆DEP-Epac1 (amino acid 50-148 deleted), ∆E9–Epac1 (N-terminal 49 amino acids deleted), N-terminal Radixin (amino acid 1-492), C-terminal Radixin (amino acid 492-584) and the indicated point mutants were generated by site-directed mutagenesis.

### Yeast two-hybrid screening

Human full length Epac1 (RapGEF3, homo sapiens, GI: 3978530) cloned in a pB27 vector was screened with a randomly primed human placenta library by Hybrigenics S.A. (Paris, France), as previously described (ref).

### Cell culture

HEK293 (Human Embryonal Kidney) cells were cultured in DMEM, supplemented with 10% serum and antibiotics.

### Immunoprecipitation

HEK293 cells cultured in 24cm dishes were transfected with FastGene 6 Transfection Reagent (Roche Inc.) with the indicated constructs. For experiments in which Epac1 was immunoprecipitated, cells were lysed in a buffer containing 50mM Tris pH 7.5, 200mM NaCl, 20mM MgCl₂, 1% NP40, 10% glycerol and protease and phosphatase inhibitors. For the reverse experiment in which ERM proteins were immunoprecipitated, a buffer containing 1% Triton X100, 0,5% DOC, 50mM Tris pH 7.5, 150mM NaCl, 2mM EDTA pH 8.0 and protease and phosphatase inhibitors was used. Cell pellets were spun down by centrifugation and lysates were incubated with sepharose A beads (Pharmacia) cou-
plied to the appropriate antibody. After extensive washing with lysis buffer, bound proteins were eluted in Laemmli buffer and analyzed by SDS-PAGE.

**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 Fugene 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 MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM glucose, 23 mM NaHCO\(_3\), with 10 mM HEPES added), pH 7.2, kept under 5% CO\(_2\) at 37°C. Agonists and inhibitors were added from concentrated stocks.

**Dynamic monitoring of TomatoRed/YFP FRET**

Cells on coverslips were placed on an inverted NIKON microscope equipped with 63x lens (N.A. 1.30) and excited at 490 nm. Emission of YFP and TomatoRed was detected simultaneously by two photon multiplier tubes (PMT) through 555 +/- 20 nm and 610 +/- 25 nm bandpass filters, respectively. Data were digitized by Picolog acquisition software (Picotech) and FRET was expressed as the normalized ratio of TomatoRed to YFP signals. Changes are expressed as percent deviation from this initial value.

**References**


Chapter 4


Supplemental Figures

Suppl Fig 1.
Mapping the residues essential for interaction with ERM proteins.
Binding of GST-Ezrin to a peptide array of 20-mer peptides comprising the N-terminal region of Epac1. The region between amino acid 18 to 48 within Epac1 is essential for the interaction with ezrin.

Suppl Fig 2.
Polarized distribution of endogenous radixin in TRP-stimulated HEK cells
A) Colocalization of GFP-N49, the ERM-interaction domain of Epac1, and endogenous Radixin (labeled with Alexa594-conjugated secondary antibody) in fixed HEK293 cells. Upper panels: in the absence of ERM activating stimuli, radixin showed modest peripheral accumulation and so did GFP-N49. Lower panels: in cells stimulated with TRP, endogenous radixin shows polarized accumulation at the PM, to which GFP-N49 colocalizes. Note that GFP-N49 tends to accumulate in the nucleus, likely due to basic residues in the N49-sequence constituting a pseudo-NLS.
B) When cells were transfected with p190-RhoGEF(DHPH) for activation of RhoA, endogenous radixin and Epac1-YFP colocalized to highly polarized areas of the PM.