Spatial Regulation of Cyclic AMP-Epac1 Signaling in Cell Adhesion by ERM Proteins^{▽¶}

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Epac1 is a guanine nucleotide exchange factor for the small G protein Rap and is involved in membrane-localized processes such as integrin-mediated cell adhesion and cell-cell junction formation. Cyclic AMP (cAMP) directly activates Epac1 by release of autoinhibition and in addition induces its translocation to the plasma membrane. Here, we show an additional mechanism of Epac1 recruitment, mediated by activated ezrin-radixin-moesin (ERM) proteins. Epac1 directly binds with its N-terminal 49 amino acids to ERM proteins in their open conformation. Receptor-induced activation of ERM proteins results in increased binding of Epac1 and consequently the clustered localization of Epac1 at the plasma membrane. Deletion of the N terminus of Epac1, as well as disruption of the Epac1-ERM interaction by an interfering radixin mutant or small interfering RNA (siRNA)-mediated depletion of the ERM proteins, impairs Epac1-mediated cell adhesion. We conclude that ERM proteins are involved in the spatial regulation of Epac1 and cooperate with cAMP- and Rap-mediated signaling to regulate adhesion to the extracellular matrix.

Cyclic AMP (cAMP) is a second messenger that relays a wide range of hormone responses. The discovery of Epac as a direct effector of cAMP (15, 29) has triggered the elucidation of many cAMP-regulated processes that could not be explained by the previously known effectors protein kinase A (PKA) and cyclic nucleotide-regulated ion channels (21). Both Epac family members, Epac1 and Epac2, act as guanine nucleotide exchange factors (GEFs) for the small G proteins Rap1 and Rap2. Thereby, Epac functions in processes such as exocytosis (28, 48, 59), cell-cell junction formation (13, 20, 30, 53, 64), and cell-extracellular matrix (ECM) adhesion (55). Adhesion to the ECM induced by Epac1 and Rap is mediated by actin-linked integrin molecules and is implicated in diverse biological processes such as homing of endothelial progenitor cells to ischemic tissue (9), remodeling of the vasculature (10, 36), and transendothelial migration of leukocytes (37, 60).

Epac1 and Epac2 are multidomain proteins containing a C-terminal catalytic region, which consists of a CDC25 homology domain responsible for GEF activity, a Ras exchange motif (REM), which stabilizes the CDC25 homology domain, and a

Ras association (RA) domain. In the autoinhibited state, the catalytic site is sterically covered by the N-terminal regulatory region, which harbors a DEP (Dishevelled, Egl-10, and pleckstrin) domain and one or two cyclic nucleotide-binding domains in Epac1 and Epac2, respectively. As demonstrated by the crystal structures of both active and inactive Epac2, autoinhibition is released by a conformational change induced by the binding of cAMP (56, 57).

After its production at the plasma membrane (PM) by adenylyl cyclases, cAMP becomes compartmentalized due to local degradation by spatially restricted phosphodiesterases (1). Further compartmentalization of cAMP signaling is established by the confined targeting of the cAMP effector proteins. Numerous adaptor proteins that target PKA to distinct subcellular locations and mediate the assembly of large signaling complexes have been identified (3). Similarly, cAMP-Epac signaling appears to be spatially regulated by diverse anchoring mechanisms, which may reflect the many different functions assigned to Epac. For instance, the DNA damage-responsive kinase DNA-PK is regulated by nuclear Epac1 (26), whereas membrane recruitment by activated Ras is essential for the role of Epac2 in neurite outgrowth (34, 35). Recently, we reported that Epac1 translocates to the PM upon the binding of cAMP and that this translocation contributes to Rap-mediated cell-ECM adhesion (51). Although the anchor at the PM remains elusive, it has become clear that the cAMP-dependent translocation of Epac1 involves its DEP domain (amino acids 50 to 148) and requires the cAMP-induced conformation.

In this study, we reveal an additional targeting mechanism of Epac1 by showing that its N terminus interacts 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 PM (18, 42, 47).

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Inactive ERM proteins reside in the cytoplasm in an autoinhibited state maintained by an intramolecular interaction between the N-terminal FERM (4.1 protein, ezrin, radixin, moesin) domain and the C-terminal actin binding domain (ABD). This autoinhibition is released by binding to phosphatidylinositol-4,5-bisphosphate (PIP₂) and threonine phosphorylation of the ABD, which induce the open conformation of the protein (reviewed in reference 8). Several kinases have been implicated in phosphorylation of this threonine in the ABD, including protein kinase C α (PKC α), PKC θ , NIK, Mst4, and the Rho effector ROCK (2, 40, 46, 50, 61). Active ERM proteins directly link the actin cytoskeleton to the PM and allow the recruitment of multiple signaling proteins. In this manner, ERM proteins function in numerous processes, such as the formation of microvilli, adherens junction stabilization, and leukocyte polarization (12, 18, 42, 47). Here, we demonstrate that ERM proteins also function as PM anchors for Epac1. The underlying interaction is mediated by the N terminus (residues 1 to 49) of Epac1 and is independent of its conformational state. Instead, the interaction is regulated at the level of the ERM proteins, which bind Epac1 when they are in their active, open conformation. G protein-coupled receptor (GPCR)-mediated signaling that results in activation of ERM proteins increases binding of Epac1 and results in a clustered localization of Epac1 at the PM. Together with DEP domain-mediated PM translocation, ERM proteins control cell adhesion mediated by Epac1. In conclusion, our data show that ERM proteins mediate PM recruitment of Epac1 and couple Epac1 activity to integrin-mediated cell adhesion.

MATERIALS AND METHODS

Reagents and antibodies. 8-pCPT-2'-O-Me-cAMP (007) and the more cell-permeable 8-pCPT-2'-O-Me-cAMP-AM (007-AM) were obtained from Biolog Life Sciences (Bremen, Germany), 8-bromo-cAMP was obtained from Biomol International, and human thrombin and sphingosine 1-phosphate (S1P) were obtained from Sigma-Aldrich. The mouse monoclonal green fluorescent protein (GFP) antibody was obtained from Roche, Flag M2 antibody was from Sigma-Aldrich, monoclonal V5 antibody was from Invitrogen, phospho-ERM (ezrin T567, radixin T564, moesin T558) antibody was from Cell Signaling Technology, monoclonal HA antibody was from Covance (HA11), and monoclonal radixin antibody was from BD Biosciences. The 5D3 anti-Epac1 antibody (Cell Signaling) has been described previously (53). Thrombin receptor-activating peptide (TRP; residues SFLRRN) was synthesized in-house.

DNA constructs. Ezrin (*Homo sapiens*; GI 161702985), radixin (*Homo sapiens*; GI 62244047), moesin (*Homo sapiens*; GI 53729335), Epac1 (RapGEF3; *Homo sapiens*; GI 3978530), Epac2 (RapGEF4; *Mus musculus*; GI 9790086), the radixin actin binding domain (ABD; amino acids 492 to 584), and radixin Δ ABD (amino acids 1 to 492) were cloned N or C terminally to either GFP, citrine yellow fluorescent protein (YFP), red fluorescent protein tandem tomato (TdTom), or a Flag-His tag using a pCDNA3 vector or to a hemagglutinin (HA) tag using a PMT2 vector with the Gateway system (Invitrogen). cDNA for ezrin, radixin, and moesin was obtained from RZPD (Berlin, Germany). Epac1 Δ DEP (amino acids 50 to 148 deleted), Epac1 Δ 1-49, Epac1 Δ 114-148, and point mutants were generated by site-directed mutagenesis. The cyan fluorescent protein (CFP)-Epac1 Δ DEP-YFP fluorescence resonance energy transfer (FRET) was generated by site-directed mutagenesis of the previously described full-length Epac1 FRET probe (52).

Yeast two-hybrid screening. Human full-length Epac1 (RapGEF3; *Homo sapiens*; GI 3978530), cloned using a pB27 vector, was screened with a randomly primed human placenta library by Hybrigenics S.A. (Paris, France), as previously described (54).

Cell culture. HEK293 (human embryonic kidney) cells were cultured in Dulbecco's modified Eagle medium (DMEM); the Jurkat T-cell line JHM1 2.2, the monoclonal JHM1 2.2 cell line stably expressing Epac1 (68), and Ovar3 cells were cultured in RPMI medium; and ACHN cells were cultured in Eagle's minimal essential medium (MEM) containing 2 mM L-glutamine, 1.5 g/liter

sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate. All media were supplemented with 10% fetal bovine serum and antibiotics.

Immunoprecipitation. HEK293 cells cultured in 6-cm dishes were transfected using Eugene 6 transfection reagent (Roche Inc.). For experiments in which Epac was immunoprecipitated, cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 20 mM MgCl₂, 1% NP-40, 10% glycerol, and protease and phosphatase inhibitors. For the reverse experiments, in which ERM proteins were immunoprecipitated, a buffer containing 1% Triton X-100, 0.5% deoxycholate, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, pH 8.0, and protease and phosphatase inhibitors was used. Cell lysates were cleared by centrifugation, and lysates were incubated with protein A-agarose beads (Pharmacia) coupled to the appropriate antibody. After extensive washing with lysis buffer, bound proteins were eluted in Laemmli buffer and analyzed by SDS-PAGE.

For immunoprecipitation of endogenous Epac1 from ACHN cells, a lysis buffer containing 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl, and protease inhibitors and phosphatase inhibitors was used. Immunoprecipitation with the monoclonal Epac1 antibody 5D3 was performed in the presence of 1 mM 8-bromo-cAMP.

Confocal microscopy. Cells were seeded in 6-well plates on 25-mm glass coverslips and transiently transfected using Eugene 6 transfection reagent (Roche Inc.). Coverslips were subsequently mounted in a culture chamber in bicarbonate-buffered saline (containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 23 mM NaHCO₃, and 10 mM HEPES; pH 7.2), kept under 5% CO₂ at 37°C, and imaged using an inverted TCS-SP5 confocal microscope equipped with a 63 $\times$ immersion oil lens (numerical aperture [NA], 1.4) (Leica, Mannheim, Germany). Imaging conditions were as follows: GFP, excitation at 488 nm, emission at 510 to 560 nm; YFP, excitation at 514 nm, emission at 522 to 570 nm; TdTom, excitation at 551 nm, emission at 560 to 600 nm. For detection of Epac1-YFP together with endogenous radixin, cells were grown on 12-mm glass coverslips, stimulated for 10 min with 50 μ M TRP, and subsequently fixed with 3.8% formaldehyde, permeabilized using 0.1% Triton X-100, and blocked in 2% bovine serum albumin (BSA). Cells were incubated with the radixin antibody and subsequently with an Alexa-conjugated secondary antibody (Invitrogen). Mounted slides were examined using an inverted TCS-SP5 confocal microscope equipped with a 63 $\times$ immersion oil lens (NA, 1.4) (Leica, Mannheim, Germany).

Postacquisition image adjustments for brightness and contrast enhancement were performed using ImageJ software (NIH).

Dynamic monitoring of TdTom/YFP FRET. Cells on coverslips were placed on an inverted Nikon microscope equipped with a 63 $\times$ lens (NA, 1.30) and excited at 490 nm. Emission of YFP and TdTom was detected simultaneously by two photon multiplier tubes (PMT) through 555 $\pm$ 20-nm and 610 $\pm$ 25-nm band-pass filters, respectively. Data were digitized by Picolog acquisition software (Picotech), and FRET was expressed as the normalized ratio of TdTom to YFP signals. Changes are expressed as percent deviations from this initial value.

Adhesion assay. The adhesion of Jurkat T cells to fibronectin was measured as described previously (14). In brief, 96-well Nunc Maxisorp plates were coated with 5 μ g/ml fibronectin and blocked with 1% bovine serum albumin. Jurkat cells (1.2×10^7) were transiently transfected by electroporation (950 μ F, 250 V) using the Gene Pulser II (Bio-Rad) with a cytomegalovirus (CMV)-luciferase plasmid, together with a YFP-Epac1 plasmid and/or V5-radixin (ABD), supplemented with pcDNA3 empty vector (EV) to a total of 40 μ g plasmid DNA. Cells were harvested 2 days after transfection and resuspended in buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂, and 0.5% BSA. Cells (2.5×10^4 for each well) were allowed to adhere in the absence or presence of 100 μ M 007 for 45 min, and nonadherent cells were removed by washing with buffer. Adherent cells were lysed and subjected to a luciferase assay as described previously (43).

The adhesion of Ovar3 cells was measured as described previously (38). In brief, 48-well polystyrene cell culture dishes were precoated with 5 μ g/ml fibronectin and subsequently blocked with 1% bovine serum albumin. Sixty hours following transfection with either ezrin (LQ-O17370-00-0005), radixin (LQ-O11762-00-0005), and moesin (LQ-O11732-00-0005) or control Dharmacon ON-TARGETplus small interfering RNAs (siRNAs) using HiPerfect transfection reagent (Qiagen) according to manufacturer's protocol, cells were trypsinized, washed once in RPMI medium containing 10% fetal calf serum (FCS), and allowed to recover surface proteins for 1.5 h in suspension in RPMI medium containing 0.5% FCS, glutamine, and 10 mM HEPES, pH 7.4, at 37°C with gentle rolling. Subsequently, 6.0×10^5 cells were plated per well in the absence or presence of 100 μ M 007. Adhesion was allowed to proceed for 45 min at 37°C, and unbound cells were discarded by washing with phosphate-buffered saline

(PBS). Adhered cells were lysed in buffer containing 0.4% Triton X-100, 50 mM sodium citrate, and 10 mg/ml phosphatase substrate (Sigma-Aldrich). The reaction mixture was incubated at 37°C, and the total amount of cellular protein was determined by measuring absorption at 405 nm.

In vivo Rap activation assay. Rap activity was assayed as described previously (62). Briefly, transfected Jurkat T cells were stimulated for 10 min with 100 μ M 007 and subsequently lysed in buffer containing 1% NP-40, 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 glutathione *S*-transferase (GST) fusion protein of the Ras-binding domain of RalGDS precoupled to glutathione-Sepharose beads. Bound proteins were eluted in Laemmli buffer and analyzed by SDS-PAGE.

RESULTS

The N-terminal 49 amino acids of Epac1 contribute to Epac1-mediated cell adhesion. We have previously shown that the DEP domain-mediated PM translocation of Epac1 contributes to Epac1-regulated adhesion of cells to the ECM (51). In Jurkat T cells, which do not express Epac1 endogenously and are therefore suitable for the comparison of different Epac1 mutants, activation of exogenous Epac1 with the Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP (007) increases adhesion of cells to fibronectin (Fig. 1B). This induction of adhesion is significantly impaired when a DEP domain mutant of Epac1 (Epac1 Δ DEP) is transfected instead of wild-type Epac1, indicating that Epac1-mediated cell adhesion requires activation of Rap1 at the PM (51) (Fig. 1B). Interestingly, deletion of the complete N terminus of Epac1, including both the DEP domain and the 49 amino acids N-terminal to the DEP domain (Epac1 Δ 1-148), completely abrogated Epac1-induced adhesion to fibronectin (Fig. 1B). This suggests that Epac1 contains an additional sequence outside its DEP domain that contributes to its correct localization to control cell adhesion. Indeed, Epac1 lacking its N-terminal 49 amino acids, which we refer to as N49 (Epac1 Δ 1-49) (Fig. 1A), displays a partial reduction in 007-induced cell adhesion (Fig. 1B). Pull-down of Rap1-GTP from these cells showed that 007-induced activation of Epac1 Δ DEP as well as Epac1 Δ 1-49 results in reduced activation of Rap1 compared to activation by wild-type Epac1 (Fig. 1C). Moreover, Epac1 Δ 1-148 was completely unable to induce activation of Rap1 in these cells, in accordance with its incapability to induce cell adhesion (Fig. 1C). Importantly, purified Epac1 Δ 1-148 mediates GDP dissociation from Rap1 *in vitro* as well as full-length Epac1 does (31), implying that the decrease in Rap1 activation in Jurkat T cells is caused by the incorrect targeting of these mutants. Taken together, these results indicate that, besides its DEP domain (residues 50 to 148), Epac1 has an additional targeting sequence within its N terminus (residues 1 to 49), which is required for efficient Rap activation and the induction of cell adhesion.

N49 interacts with proteins of the ERM family. To identify binding partners for the N terminus of Epac1, a yeast two-hybrid screen was performed using a human placenta cDNA library and full-length Epac1 as bait. Positive clones containing partial cDNAs encoding the membrane-associated proteins ezrin and radixin were isolated. 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 PM (18, 42, 47). To confirm the interaction between Epac1 and ERM proteins in mammalian cells, HEK293 cells were transfected with HA-Epac1 and Flag-tagged variants of ezrin,

FIG. 1. The N-terminal 49 amino acids of Epac1 contribute to Epac1-mediated cell adhesion. (A) Domain architecture of Epac1. Epac1 contains a CDC25 homology domain (CDC25-HD), which mediates GEF activity, a Ras exchange motif (REM), which stabilizes the CDC25-HD, a cyclic nucleotide binding (CNB) domain, a DEP (Dishvelled, Egl-10, and pleckstrin) domain, and a Ras association (RA) domain. (B) Adhesion of Jurkat T cells transfected with empty vector (EV), wild-type (Wt) Epac1, or different Epac1 mutants together with a luciferase reporter. Transfected cells were allowed to adhere to a fibronectin-coated surface for 45 min, and adhesion was subsequently detected by measurement of luciferase activity. The induction of adhesion by activation of Epac1 with 007 (100 μ M) is partially impaired with the Epac1 mutants lacking the DEP domain (Epac1 Δ DEP) or N49 (Epac1 Δ 1-49), whereas adhesion is completely abrogated when both the DEP domain and N49 are deleted (Epac1 Δ 1-148). Shown are average data with standard deviations from three individual experiments, with adhesion normalized to 100% for 007-activated wild-type Epac1. Statistical analysis was performed using a one-tailed Student *t* test. The Western blot labeled with the Epac1 antibody (5D3) shows expression levels of the transfected Epac1 constructs. (C) Jurkat T cells were transfected with similar amounts of either wild-type Epac1, Epac1 Δ DEP, Epac1 Δ 1-49, or Epac1 Δ 1-148, and GTP-bound Rap1 was pulled down from lysates of cells after stimulation with 007 (100 μ M, 10 min).

radixin, or moesin. Indeed, all ERM proteins were able to coimmunoprecipitate with Epac1 (Fig. 2A). As the ERM proteins share high sequence similarity, we have arbitrarily chosen to generally use radixin as a representative family member to further explore the interaction with Epac1.

To examine whether radixin is indeed a binding partner for the N terminus of Epac1, the interaction of radixin with Epac1 mutants lacking either N49 or the DEP domain was tested. This revealed that the presence of N49 within Epac1 is essential for its binding to radixin, whereas the DEP domain is dispensable (Fig. 2B). Although Epac1 and Epac2 are similar in domain architecture, the amino acids within N49 of Epac1 are not conserved between the two proteins. As expected, Flag-radixin was not able to coimmunoprecipitate with Epac2 (Fig. 2C), demonstrating that the interaction with ERM proteins is specific for the N terminus of Epac1.

Epac1 is targeted to the plasma membrane by activated ERM proteins. In resting ERM proteins, binding to the PM

FIG. 2. Epac1 directly interacts with ERM proteins. (A) HA-Epac1 coimmunoprecipitation (IP) with Flag-ezrin, Flag-radixin, and Flag-moesin in HEK293 cells. TL, total lysate. (B) Coimmunoprecipitation of Flag-radixin with YFP-tagged wild-type Epac1, Epac1 Δ 1-49, and Epac1 Δ DEP in HEK293 cells. The 49 N-terminal amino acids of Epac1 are required for the interaction with radixin. (C) Coimmunoprecipitation of Flag-radixin with HA-tagged versions of Epac1 or Epac2 in HEK293 cells. In contrast to HA-Epac1, HA-Epac2 is unable to coimmunoprecipitate Flag-radixin.

and actin cytoskeleton is prevented by an intramolecular interaction between the N-terminal FERM domain and C-terminal actin binding domain (ABD) (Fig. 3A). This autoinhibition can be relieved by PIP₂ binding and threonine phosphorylation within the ABD (T564 within radixin) (8). Coimmunoprecipitation experiments using a truncated mutant of radixin lacking the ABD (radixin Δ ABD, amino acids 1 to 492) showed that Epac1 binding is mediated by the N-terminal part of radixin (Fig. 3B). Moreover, the interaction of Epac1 with this truncated mutant is greatly increased compared to that with wild-type radixin, suggesting that the presence of the ABD suppresses Epac1 binding. To test whether Epac1 indeed has increased affinity for ERM proteins in the open conformation, the binding of Epac1 to two constitutively open radixin mutants was examined. First, radixin containing a phosphomimicking mutation at the above-mentioned phosphorylation site [radixin(T654D)] was tested. Second, a novel radixin mutant, in which residues within the ABD that form a hydrophobic interaction surface with the FERM domain were replaced by negatively charged aspartic acid residues [radixin(I577D, F580D)] was designed based on the crystal structure of moesin (49). Indeed, both mutations result in a loss of interaction between the N- and C-terminal halves of radixin (data not shown) and thus enforce the constitutively open conformation of the protein. Both of these radixin mutants showed a dramatic increase in interaction with Epac1 compared to wild-type radixin (Fig. 3C), indicating that Epac1 preferably interacts with ERM proteins that are in their open conformation.

When in their closed conformation, ERM proteins reside in the cytosol, whereas activated ERM proteins are targeted to the PM (19) (Fig. 3D). To test whether activated ERM proteins function as anchors for Epac1 at the PM, we cotransfected YFP-tagged Epac1 with the constitutively open and thus PM-localized radixin mutant Flag-radixin(T564D). Whereas Epac1-YFP, when expressed alone, localized mainly to the cytosol and the nuclear envelope (Fig. 3E), it showed complete accumulation at the PM when radixin(T564D) was coexpressed (Fig. 3E). This was not observed with Epac1 Δ 1-49-YFP, while the isolated N terminus of Epac1 (GFP-N49-Epac1) was sufficient for PM targeting by radixin(T564D) (Fig. 3E). This indicates that this PM accumulation of Epac1 is indeed mediated by the interaction with ERM proteins. As expected, this relocalization of Epac1 was independent of

the presence of its DEP domain (Fig. 3E). Furthermore, radixin(T564D) expression similarly induced PM accumulation of Epac1(R279L)-YFP (Fig. 3E), which is mutated in its cAMP binding domain and thereby locked in the autoinhibited conformation (51). These data indicate that recruitment by ERM proteins is independent of the conformational state of Epac1 and instead is regulated by activation of the ERM proteins. Although Epac1 interacts with ERM proteins independently of its conformational state, likely it still requires cAMP binding to acquire its open, active conformation. To test this, we employed fluorescence resonance energy transfer (FRET) to monitor the conformational state of Epac1 (52) during 007-AM stimulation in cells overexpressing radixin(T564D). To avoid potential changes in FRET because of the previously described cAMP-induced redistribution of Epac1 (51), an Epac1 FRET probe lacking the DEP domain was used (CFP-Epac1 Δ DEP-YFP). In cells showing complete accumulation of the probe at the PM (Fig. 3F, top), 007-AM could induce strong loss of FRET (Fig. 3F, bottom). This indicates that cAMP binding is still required for ERM-targeted Epac1 to acquire its active, open conformation.

GPCR signaling-induced ERM activation results in a clustered localization of Epac1 at the PM. We next investigated the recruitment of Epac1 to ERM proteins when their conformational opening is evoked by physiological stimuli. In HEK293 cells, activated thrombin receptors induce threonine phosphorylation and thereby the opening of ERM proteins (45) (Fig. 4A). Indeed, coimmunoprecipitation experiments demonstrated that the interaction between Epac1 and wild-type radixin is enhanced upon thrombin stimulation (Fig. 4A; quantification in Fig. 4B). This could also be visualized in living cells by detecting FRET between YFP-radixin and Epac1 tagged with the red fluorescent protein TdTom (Epac1-TdTom). Addition of thrombin receptor-activating peptide (TRP) induced an increase in FRET ratio (Fig. 4C), reflecting the interaction between Epac1 and radixin. This experiment also revealed the rapid kinetics of this interaction, as maximal FRET levels were usually reached within $\sim$ 30 s. Importantly, TRP stimulation did not result in an increased FRET ratio between YFP-radixin and Epac1- Δ 1-49-TdTom (Fig. 4C). Taken together, these findings show that the binding of Epac1 to radixin is not restricted to heterologously expressed mutant proteins but also follows receptor-induced unfolding of wild-type radixin.

FIG. 3. ERM proteins require the open conformation to bind Epac1. (A) Domain architecture of radixin. The FERM (4.1 protein, ezrin, radixin, moesin) domain and actin binding domain (ABD) form an intramolecular interaction and are linked by an α -helical region. Phosphorylation of T564 is required for the open conformation of radixin, and I577 and I580 are part of the hydrophobic interaction surface between the ABD and the FERM domain. (B) Coimmunoprecipitation of HA-Epac1 with Flag-tagged wild-type radixin and radixin lacking the ABD (Flag-radixin Δ ABD, residues 1 to 492) in HEK293 cells. The separate N terminus of radixin shows enhanced binding to Epac1 compared to full-length radixin, implying that the presence of the C-terminal ABD suppresses binding of radixin to Epac1. (C) Coimmunoprecipitation of HA-Epac1 with wild-type Flag-radixin and the constitutively open mutants Flag-radixin(T564D) and Flag-radixin(I577D, F580D) in HEK293 cells. The amount of coimmunoprecipitated HA-Epac1 is significantly increased with these radixin mutants. (D) Live imaging of YFP-tagged wild-type radixin and the constitutively open mutant radixin(T564D) in HEK293 cells, showing that upon conformational opening radixin is redistributed to the PM. (E) Live imaging of Epac1 in HEK293 cells. The indicated constructs were expressed alone or with Flag-radixin(T564D). (F) 007-AM (1 μ M) treatment of HEK293 cells transfected with the FRET sensor CFP-Epac1 Δ DEP-YFP. The sensor reports the cAMP-induced conformational change as a loss of intramolecular FRET; deletion of the DEP domain precludes changes in intermolecular FRET due to cAMP-dependent relocation (51). When this Epac1 FRET construct is recruited to the PM by coexpressed radixin(T564D), its conformational opening upon 007-AM treatment remains unaffected. Scale bars (all images), 10 μ m.

Next, we tested whether GPCR-mediated ERM activation is able to induce the interaction between endogenous Epac1 and ERM proteins. For this, we used ACHN human kidney carcinoma cells, which express relatively high levels of Epac1 and ezrin and, in addition, express the receptor for sphingosine 1-phosphate (S1P), which upon activation induces the open conformation of ezrin (5, 19). In unstimu-

lated cells, a minor fraction of ezrin coprecipitated with Epac1, which indicates that under basal conditions a portion of ezrin is in the open conformation in these cells (Fig. 4D). Upon stimulation with S1P, an increased interaction between both endogenous proteins was observed, confirming that GPCR-induced ERM activation results in the increased binding of Epac1 (Fig. 4D).

FIG. 4. GPCR-mediated activation of ERM proteins induces binding of Epac1. (A) Coimmunoprecipitation of HA-Epac1 with Flag-radixin in HEK293 cells stimulated with thrombin (0.2 U/ml, 2 min). Thrombin stimulation results in increased phosphorylation of the ERM proteins (ezrin T567, radixin T564, and moesin T558) (right) and the enhanced interaction of HA-Epac1 with Flag-radixin. (B) Quantification of the relative binding of Epac1 to radixin from three independent experiments, as performed for panel A. Statistical analysis was performed using a one-tailed Student *t* test. (C) Measurement of FRET between YFP-radixin and Epac1-TdTom or Epac1 Δ 1-49-TdTom in HEK293 cells during stimulation with thrombin receptor-activating peptide (TRP; 50 μ M). FRET between YFP-radixin and Epac1-TdTom, expressed as ratio of TdTom/YFP fluorescence, increases upon TRP addition, confirming that binding to Epac1 is increased when radixin is driven in the open conformation. The traces are representative of three independent measurements. The average increase ($\pm$ standard deviation) in the Δ TdTom/YFP ratio was $6.0\% \pm 1.1\%$. (D) Coimmunoprecipitation of endogenous ezrin with endogenous Epac1 in ACHN cells with or without prestimulation with sphingosine 1-phosphate (S1P; 1 μ M) to induce the conformational opening of ezrin.

The PM recruitment of Epac1 by ERM proteins activated by GPCR signaling was examined by live imaging of Epac1-YFP during thrombin receptor stimulation in HEK293 cells. This revealed the PM accumulation of Epac1-YFP upon activation of the thrombin receptor (Fig. 5A), which was dependent on the presence of N49 (Fig. 5B). Interestingly, after thrombin-induced ERM binding, Epac1 does not localize uniformly at the PM but shows a clustered localization within this compartment (Fig. 5A). Cotransfection of Epac1-TdTom with YFP-radixin (Fig. 5C) or costaining of Epac1-YFP with endogenous radixin (Fig. 5D) showed that activated ERM proteins display a similar clustered localization at the PM in these cells. Thus, the distribution pattern of Epac1 induced by GPCR-induced activation of ERM proteins differs notably from those described for the cAMP-induced translocation of Epac1 (51), which distributes Epac1 uniformly along the PM (51). To demonstrate the distinct localization patterns at the PM more directly, we cotransfected HEK293 cells with CFP-Epac1-N49 and YFP-Epac1 Δ 1-49 and stimulated these cells with TRP and 007-AM to simultaneously trigger both mechanisms of Epac1 recruitment to the PM. Indeed, whereas YFP-Epac1 Δ 1-49 translocated to the PM uniformly, CFP-Epac1-N49 was targeted asymmetrically along this compartment (Fig. 5E). This demonstrates the clustered localization of Epac1 at the PM in response to receptor-induced activation of ERM proteins.

Interestingly, the recruitment of Epac1 by activated ERM proteins downstream of the thrombin receptor can be blocked

by the RhoA inhibitor C3 toxin (Fig. 5F) and mimicked by overexpression of the catalytic region of p190-RhoGEF (Fig. 5G). This implies that activation of ERM proteins downstream of the thrombin receptor depends on signaling via the small G protein RhoA, and thereby ERM proteins further connect the signaling pathways by the small G proteins Rho and Rap. Importantly, the clustered distribution of Epac1 observed in RhoGEF-overexpressing HEK293 cells remains unaltered upon stimulation with 007-AM. This indicates that cAMP-induced translocation is not dominant and Epac1 remains bound to ERM proteins when it is activated by cAMP.

ERM binding is required for efficient Epac1-mediated cell adhesion. The observations that efficient cell adhesion by Epac1 requires N49 (Fig. 1B) and that this region mediates PM recruitment by activated ERM proteins (Fig. 3 and 5) suggest that ERM proteins function in Epac1-mediated cell adhesion. Therefore, we blocked the interaction of Epac1 with ERM proteins by overexpression of the isolated ABD of radixin. This domain is expected to bind the N termini of all three ERM proteins (49), which would disrupt binding of Epac1. Indeed, coexpression of the ABD in HEK293 cells resulted in a decrease in binding between Epac1 and full-length radixin (Fig. 6A). This confirms that the binding site for Epac1 is shielded in the closed conformation of the ERM protein. Accordingly, the TRP-induced recruitment of Epac1 to ERM proteins at the PM was abolished by overexpression of the radixin ABD, whereas its subsequent 007-AM-induced PM translocation was

FIG. 5. GPCR-mediated activation of ERM proteins induces a clustered localization of Epac1 at the PM. (A) Live imaging of Epac1-YFP before and after 50 μ M TRP stimulation to induce phosphorylation and conformational opening of ERM proteins in HEK293 cells. In response to TRP, Epac1-YFP has a clustered localization at the PM. Quantitative analysis reveals the rapid kinetics of TRP-induced recruitment of Epac1 in this experiment. Shown are fluorescence intensities at the right flank of the plasma membrane (PM; red) and in the cytosol (blue) as well as the PM/cytosol ratio (green). (B) Live imaging of Epac1 Δ 1-49-YFP before and after 50 μ M TRP stimulation in HEK293 cells. Epac1 Δ 1-49-YFP is not targeted to the PM in response to TRP. (C) Live imaging of YFP-radixin and Epac1-TdTom after 50 μ M TRP stimulation to induce phosphorylation and conformational opening of radixin in HEK293 cells. In response to TRP, both constructs are similarly clustered at the PM. (D) Subcellular localization of Epac1-YFP and endogenous radixin after 50 μ M TRP stimulation in HEK293 cells. (E) Live imaging of HEK293 cells that were transfected with CFP-N49 and Epac1 Δ 1-49-YFP and stimulated with TRP (50 μ M) and 007-AM (1 μ M). Both constructs accumulate at the PM but show different subcellular distributions. (F) Live imaging of Epac1-YFP in HEK293 cells, showing the inhibition of plasma membrane recruitment by TRP (50 μ M) by preincubation with the exoenzyme C3, an inhibitor of RhoA (16 h, 30 μ g/ml). (G) Live imaging of Epac1-YFP in HEK293 cells, showing the recruitment of Epac1 to the plasma membrane by coexpression of the catalytic domain of p190-RhoGEF. The clustered localization of Epac1 remains upon activation of Epac1 by stimulation with 007-AM (1 μ M). Scale bars (all images), 10 μ m.

unaffected (Fig. 6B). Next, we tested the requirement of the Epac1-ERM interaction for Epac1-mediated adhesion in Jurkat T cells. Importantly, these cells contain significant levels of activated ERM proteins under basal conditions (22). Jurkat T cells that stably express Epac1 (68) were transfected with either empty vector (EV) or the radixin ABD, and adhesion of cells was detected by measurement of cotransfected luciferase. This showed that adhesion of EV-transfected cells to fibronectin was greatly enhanced by activation of Epac1 by 007 and that this enhancement was significantly decreased when the radixin ABD was transfected (Fig. 6C). To confirm that this inhibition by the radixin ABD is a consequence of disruption of the

Epac1-ERM interaction, we examined its effect on adhesion induced by Epac1 Δ 1-49. This mutant cannot be targeted to the PM by ERM proteins (Fig. 3E and 5B) but is still partially able to induce cell adhesion because of its DEP domain-mediated PM anchoring (Fig. 1B) (51). Indeed, 007-induced adhesion of YFP-Epac1 Δ 1-49-expressing cells was not altered by overexpression of V5-radixin ABD (Fig. 6D). Finally, we confirmed the role of ERM proteins in Epac1-mediated cell adhesion by siRNA-mediated depletion of ERM proteins in Ovar3 cells. 007-mediated activation of endogenous Epac1 increased adhesion to fibronectin of Ovar3 cells transfected with control siRNA (Fig. 6E). This induction of adhesion was significantly

FIG. 6. ERM binding is required for efficient Epac1-mediated cell adhesion. (A) Coimmunoprecipitation of HA-Epac1 with Flag-radixin in the presence of the V5-tagged C-terminal ABD of radixin in HEK293 cells. Overexpression of V5-ABD decreases the interaction of HA-Epac1 with Flag-radixin. (B) Live imaging of YFP-Epac1 in HEK293 cells coexpressed with CFP-radixin ABD. In the presence of CFP-radixin ABD, plasma membrane recruitment by TRP (50 μ M) is inhibited, whereas the subsequent 007-AM (1 μ M)-induced translocation of Epac1 remained unaffected. (C) Adhesion of Jurkat T cells that stably express Epac1 transiently transfected with either empty vector (EV) or the ABD of radixin to compete for binding of Epac1 to endogenous ERM proteins. Transfected cells were allowed to adhere to a fibronectin-coated surface for 45 min, and adhesion was subsequently detected by measuring the activity of cotransfected luciferase. Adhesion induced by activation of Epac1 with 007 (100 μ M) is decreased by overexpression of the ABD of radixin. Shown are average data with standard deviations from three individual experiments, with 007-induced adhesion in EV-transfected cells normalized to 100%. Statistical analysis was performed using a one-tailed Student *t* test. The Western blot labeled with the V5 and Epac1 antibody (5D3) shows the expression of Epac1 and the transfected V5-radixin ABD construct. (D) Adhesion of Jurkat T cells transiently transfected with YFP- Δ 1-49-Epac1 and either EV or the ABD of radixin. Adhesion of transfected cells was measured by a method similar to the method used for Fig. 5C. Results show that the radixin ABD does not reduce adhesion induced by YFP- Δ 1-49-Epac1. Shown are average data with standard deviations from three individual experiments, with 007-induced adhesion in EV-transfected cells normalized to 100%. Statistical analysis was performed using a one-tailed Student *t* test. The Western blot labeled with the V5 and Epac1 antibody (5D3) shows the expression of YFP- Δ 1-49-Epac1 and the V5-radixin ABD construct. (E) Adhesion of Ovar3 cells transfected with either control or ezrin, radixin, and moesin SMARTpool siRNAs. Sixty hours after siRNA transfection, cells were allowed to adhere to a fibronectin-coated surface for 45 min in the absence or presence of 100 μ M 007, and adhesion was subsequently detected by measuring endogenous phosphatase activity. Shown are average data with standard deviations from two individual experiments, normalized to adhesion of control siRNA-transfected cells. Statistical analysis was performed using a one-tailed Student *t* test. The Western blot shows the expression of endogenous ezrin, radixin, moesin, and tubulin (as a loading control).

inhibited when ERM protein levels were depleted (Fig. 6E). Together, these data confirm that the interaction with ERM proteins is required for efficient Epac1-mediated cell-ECM adhesion.

DISCUSSION

Guanine nucleotide exchange factors are usually multidomain proteins that function in the temporal and spatial control of G protein signaling networks (7). We have previously shown that Epac1, a GEF for Rap proteins, is regulated both in time and in space by cAMP. cAMP is required for the release of autoinhibition of Epac1 (15) and in addition induces its translocation to the PM (51). This translocation is mediated by the DEP domain in Epac1 in its active conformation and is re-

quired for efficient Epac1-mediated adhesion of cells to the ECM (51). Here, we report on an additional signaling event that regulates the PM recruitment of Epac1. We observed that for efficient Epac1-mediated adhesion of cells to fibronectin the N-terminal 49 amino acids of Epac1 are required. This region was found to interact with the ERM proteins ezrin, radixin, and moesin. More-detailed analysis of this interaction revealed that Epac1 specifically interacts with the ERM proteins in their active, open conformation. Indeed, stimuli that induce the activation of ERM proteins, like sphingosine 1-phosphate and thrombin, increase the interaction between Epac1 and ERM proteins and induce the recruitment of Epac1 to distinct sites of the PM. Finally, disruption of the interaction of Epac1 with ERM proteins inhibited Epac1-mediated cell-ECM adhesion. From these results we conclude that Epac1 has

at least two independent mechanisms for recruitment to the cell periphery for efficient adhesion.

In the regulation of Rap activation and integrin-mediated cell adhesion by Epac1, ERM proteins cooperate with the DEP domain-mediated translocation of Epac1. Deletion of the DEP domain results in a partial decrease in cAMP-induced Rap activation and cell adhesion by Epac1 (Fig. 1B and C), as Epac1 Δ DEP has lost part of its PM targeting mechanism. The residual cell adhesion induced by this Epac1 mutant depends on binding to active ERM proteins, and, when activated by cAMP, these ERM-bound Epac1 molecules represent an additional active pool of Epac1 at the PM. Similarly, deletion of N49 results in partial reduction of cAMP-induced cell adhesion by Epac1 (Fig. 1B and C), as Epac1 loses its PM targeting by ERM proteins. Only when both targeting sequences are deleted are activation of Rap1 and, consequently, cell adhesion downstream of Epac1 completely disrupted (Fig. 1B and C). This implies that these two regulatory mechanisms are not redundant to each other but rather are both required for efficient Epac1-induced cell adhesion. Importantly, purified Epac1 Δ 1-148 mediates GDP dissociation from Rap1 *in vitro* as well as full-length Epac1 does (31), indicating that the decreased Rap1 activation by Epac1 Δ 1-49 and Epac1 Δ DEP results from their cellular mislocalization rather than from a direct effect on their catalytic activity. As both mechanisms recruit Epac1 to the PM, their cooperative effect may be explained by an increase in affinity of Epac1 for the PM by the two independent membrane anchors, and thereby more efficient signaling to PM-localized Rap. However, the difference in the distributions of Epac1 at the PM, with DEP-anchored Epac1 uniformly targeted and ERM-bound Epac1 showing a clustered localization in HEK293 cells (Fig. 5), suggests that ERM-mediated targeting of Epac1 may be required for more specifically localized adhesion. In addition, both anchoring mechanisms may allow distinct signaling downstream of Epac1 and Rap. In other cell systems, the distinct localization of ERM-bound Epac1 may serve different functions of the Epac1-Rap pathway. For instance, in kidney and intestinal epithelial cells, Epac1 has been implicated in the control of the sodium protein exchanger NHE3 (25, 44), a protein that is physically linked to ERM proteins (11, 67).

Depletion of N49 of Epac1 and overexpression of the ABD of radixin, which disrupt the interaction of Epac1 with ERM proteins, both result in reduced Epac1-induced cell adhesion (Fig. 1 and 5). siRNA-mediated depletion of the individual ERM proteins did not have any significant effect on Epac1-mediated adhesion in different cell types tested (data not shown), whereas depletion of all three ERM family members in Ovar3 cells also reduced Epac1-mediated cell adhesion (Fig. 6E). This may be explained by redundancy of the ERM family members in the spatial control of Epac1. All three ERM proteins are widely expressed in vertebrates, albeit with variable expression ratios among different tissues (26). The redundancy of ERM proteins is illustrated by knockout models for the individual ERM genes. Whereas loss of *Drosophila* moesin, the only ERM protein expressed in this organism, results in severe defects in anterior-posterior polarity and leads to lethality (35), mice depleted of moesin do not show any obvious abnormalities (36). Epac1 is able to interact with all three ERM proteins (Fig. 2A), and although we have demonstrated

the regulation of Epac1 recruitment using radixin as the representative ERM protein, similar results were obtained with ezrin (data not shown). Thus, ERM proteins appear to be redundant for PM recruitment of Epac1 and thereby for linking Epac1 activity to cell adhesion.

As Epac1 recruitment and coupling to cell adhesion require ERM proteins in their open conformation, signaling pathways that lead to ERM activation will converge with Epac1-Rap signaling. Recruitment of Epac1 by activated ERM proteins downstream of the thrombin receptor is blocked by C3 toxin and mimicked by overexpression of the catalytic region of the RhoGEF p190 (Fig. 5F and G), indicating that this depends on signaling via the small G protein RhoA. RhoA activity may lead to ERM activation either by direct phosphorylation of the threonine in the ABD by the Rho effector ROCK or by enhanced generation of PIP₂ via phosphatidylinositol 4-phosphate 5-kinase (23, 24, 33, 40, 41, 66). Thereby, the Epac1-ERM interaction further connects the actions of Rho and Rap. Both small G proteins play central roles in actin remodeling and share involvement in processes such as cell adhesion and migration (4, 6, 58). Cross talk between Rap and Rho at several levels has previously been reported. Rho can activate the atypical phospholipase C- ϵ (PLC- ϵ), which acts as a GEF for Rap1 (27, 63), and conversely the Rho-GAPs ARAP3 and RA-RhoGAP have been identified as effectors of Rap1 (32, 65). However, the direct relevance of these interactions for either Rho or Rap1 function is currently largely elusive. Our current data show that Rho signaling confers spatial regulation to Epac1-mediated Rap activation. Similarly, signaling cascades activating alternative kinases for the ERM proteins, such as PKC α (46), PKC θ (50), NIK (2), and Mst4 (61), may link to Epac1-mediated signaling by controlling the cellular distribution of Epac1. Indeed, also the activation of ezrin by Mst4, which can be triggered in LS174T-W4 cells by doxycycline-induced Strad α expression (61), results in the relocalization of Epac1 to ERM proteins at the apical membrane (data not shown). Whether all signals that activate ERM proteins lead to Epac1 translocation remains to be determined, but the GFP-tagged N-terminal 49 amino acids of Epac1 appear to be a useful tool to visualize the activation of ERM proteins in living cells.

Spatial confinement of Epac1 by ERM proteins provides further support for the compartmentalization of Epac1 signaling. ERM proteins function as a direct anchor for Epac1, as evidenced by their binding in a yeast two-hybrid model and the interaction of bacterially purified ezrin with N-terminal peptides of Epac1 (K. Taskén and J. L. Bos, unpublished data). Together with alternative anchors, such as the mAKAP-PDE4D complex (16) and the light chain of MAP1 (39), ERM proteins control specific functions of the GEF by recruitment to distinct subcellular locations. Interestingly, ERM proteins form a platform not only for Epac1 but also for PKA signaling as well (17). This suggests the coordinated regulation of both cAMP targets and may contribute to the interconnectivity between these cAMP-regulated pathways. A similar role has been described for mAKAP (16), and coordinated activation of PKA and Epac1 within one single complex may represent a common feature of cAMP signaling.

ERM proteins are important regulators of the organization of the PM, which underlies their role in diverse cellular pro-

cesses such as polarization, migration, and cell adhesion (12, 18, 42, 47). In addition, ERM proteins serve as scaffolds to assemble large signaling complexes at the cortical membrane. Our results show that ERM proteins also recruit Epac1, a component of the Rap signaling network, and are required for efficient cAMP-induced, Epac1-mediated cell adhesion. This newly identified role of the ERM protein family further aids in the understanding of signaling by the ERM proteins and the diverse biological processes in which they function.

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