Protein-protein interactions involved in Rap1-mediated signal transduction

Eiwit-eiwit interacties betrokken bij Rap1-gemedieerde
signaal transductie
(met een samenvatting in het Nederlands)

Proefschrift

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George, I think half-pint wants to learn to fly.
-Wiley Post

亦借此書紀念生母羅惠君女士逝世20周年

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Contents

Abbreviations

AF6: Acute lymphoblastic leukemia 1 fusion partner from chromosome 6
BSA: bovine serum albumin
cAMP: cyclic adenosine 3',5' monophosphate
CDC25: cell division cycle 25
CNB: cyclic nucleotide binding domain
8-CPT-2'OMe-cAMP: 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate
C-terminus: caboxy-terminus
DAG: diacylglycerol
DEP: Disheveled, Egl-10, Pleckstrin
EBP50: ERM binding phosphoprotein 50
Epac: Exchange Protein directly activated by cAMP
ERM: Ezrin, Radixin and Moesin
FERM: Four-point one, Ezrin, Radixin, Moesin
FN: fibronectin
ERK: Extracellular signal regulated kinase
GAPs: GTPase activating proteins
GEFs: Guanine nucleotide exchange factors
GFP: Green fluorescene protein
GST: Glutathion S-transferase
HA: Haemagglutinin
HUVEC: human umbilical vascular endothelial cells
ICAM: Intercellular adhesion molecule
ISO: isoproteronol
kDa: kilo Dalton
MAGI: MAGUK with inverted domain structure
mAKAP: muscle-specific A-kinase anchoring protein
mGppNHp: 2'-/3'-O-(N'-Methylanthraniloyl)-guanyl-5'-yl-imidodiphosphate
MAPK: Mitogen activated protein kinase
NHE3: sodium-proton exchanger 3
N-terminus: amino-terminus
PDZ: PSD-95/DlgA/ZO-1
PH: pleckstrin homology domain
PKA: protein kinase A
PKB: protein kinase B
PKC: protein kinase C
PVDF: polyvinyl difluoride
RA: Ras binding domain
RAPL: Regulator of Adhesion and cell Polarization enriched in Lymphoid tissues
RanBP2: RAN binding protein 2
RBD: Ras binding domain
REM: Ras exchange motif
RhoGDI: Rho GDP Dissociation Inhibitor
RIAM: Rap1-Interacting Adaptor Molecule
S1P: Sphingosine-1-phosphate
SD: standard deviation
siRNA: Small interfering RNA
SPA-1: Signal-induced proliferation associated gene-1
TCR: T-cell receptor
TK: thymidine kinase
WT: wide type
WB: western blot

Chapter

1

General introduction

Introduction

The Ras superfamily consists of a large group of monomeric G proteins which cycle between GDP bound inactive and GTP-bound active state. This switch function of Ras-like proteins is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs induce the release of G-protein bound nucleotide, allowing rebinding of the cellular more abundant GTP. GTP-binding changes the conformation of the small G-protein in the switch 1 and switch 2 regions (1-3), which are involved in nucleotide-binding and allows the association with its effectors. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of the G-protein, which results in the hydrolysis of GTP to GDP and thereby terminates signalling of the G-protein.

Rap family

Rap family proteins were first identified in a screen for Ras homologous genes (4) and comprise of Rap1A, Rap1B, Rap2A, Rap2B and Rap2C. Independently, Rap1 was identified as a suppressor of the K-Ras transformation phenotype (5), and this finding invoked attentions to Rap proteins and the signalling pathways they might be involved in. Due to the high similarity between the effector regions of Rap and Ras, Rap was hypothesized to antagonize Ras signalling by trapping Ras effector proteins in an inactive complex. Indeed, Rap binds to Ras effectors with similar affinities (6-8). Moreover, overexpression of active Rap interferes with ERK activation in fibroblasts (9). However, it was shown that Rap might also be able to activate Ras effector proteins and induce oncogenic transformation of Swiss 3T3 fibroblasts (10,11). Although overexpression of Rap may interfere in Ras signalling, endogenous Rap1 is unlikely able to efficiently titrate Ras effector molecules (12,13). This indicates a signalling function of Rap, which is independent of Ras. Indeed, Rap was found to regulate integrin mediated cell adhesion in lymphocyte T cells (14). Subsequent studies demonstrate a crucial role for Rap1 in the regulation of inside-out signalling to integrins, which will be discussed in more detail later. More recently Rap1 has been implicated in E-cadherin (15,16)

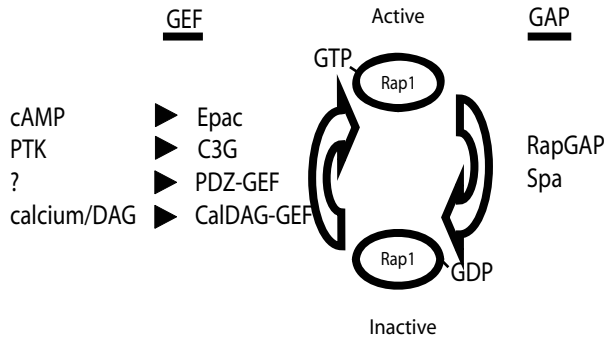

Fig. 1 Regulation of the Rap1 GTPase. Rap activity is upregulated by a number of RapGEFs and downregulated by RapGAPs. Rap1GEFs are controlled by second messengers or protein tyrosine kinases. PTK: protein tyrosine kinase.

and VE-cadherin-mediated junction formation (17-20). In this context Rap was first shown to positively regulate the adhesion of cells to E-cadherin (16). A subsequent report showed that C3G, a guanine nucleotide exchange factor (GEF) for Rap1, regulates the signalling mediated by E-cadherin and nectin in epithelial cells (15,21). In human umbilical vascular endothelial cells (HUVEC), the Epac-Rap1 pathway regulates VE-cadherin-mediated cell-cell contacts (17-20).

Activation of Rap by guanine nucleotide exchange factors

A variety of GEF-proteins are able to catalyse nucleotide exchange of Rap (Fig. 1). Like other GEF proteins for G-proteins of the Ras family, they contain a CDC25-homology domain and a Ras exchanger motif (REM) domain. The CDC25-homology domain is the catalytic domain. Studies revealed that the isolated domain is sufficient to induce nucleotide exchange (22,23). REM domains always occur together with CDC25-holomolgy domains (24) and have a mainly structural function by shielding the hydrophobic surface area of the CDC25-homology domain (25). Except for these common elements, RapGEFs vary in domain composition as will be described in the following sections.

Epac

The observation that cAMP induces Rap activation independent of protein kinase A (PKA) encouraged de Rooij and co-workers to search the sequence database for genes encoding proteins that contain both a cyclic nucleotide binding (CNB) domain and a CDC25 homology domain. This resulted in the identification of the Exchange protein directly activated by cAMP (Epac) and indeed Epac was shown to be directly activated by cAMP *in vivo* and *in vitro* (26). In an independent screen, Epac was identified as a protein differentially expressed in the brain and containing a putative second messenger binding domain (27). The Epac protein family consists of 3 members: Epac1, Epac2 and Repac (Related to Epac) (Fig. 2). Epac1 is

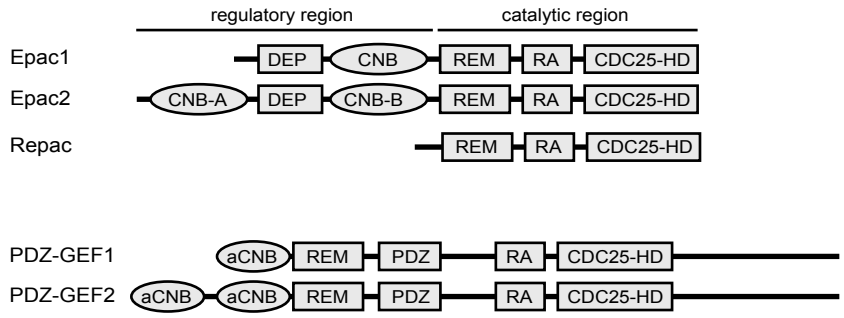

Fig. 2 Domain structure of Epac and PDZ-GEF. Domain organization of Epac1, Epac2, Repac, PDZ-GEF1 and PDZ-GEF2 are shown. DEP, Disheveled, Egl-10 and Pleckstrin domain; CNB, cyclic nucleotide binding domain; REM, Ras exchanger motif; RA, Ras association domain; CDC25-HD, CDC25-homology domain; aCNB, atypical cyclic nucleotide binding domain; PDZ, PSD-95, Dlg, ZO-1 domain.

widely expressed with high expression levels in kidney, ovary, brain and skeletal muscles, whereas Epac2 expression is mainly restricted to the brain, liver, adrenal gland, pituitary and β-cells of the pancreas (27-30). Epac consists of a regulatory and a catalytic region (Fig. 2). The major difference between Epac1 and Epac2 is an additional N-terminal CNB domain in Epac2. This CNB domain has a lower affinity for cAMP than the second CNB domain and is not required to keep Epac2 in the auto-inhibited state (28,31). Both Epac1 and Epac2 contain a Dishevelled, Egl-10 and Pleckstrin (DEP) domain, which is not required for the regulation of Epac by cAMP but mediates its localisation at the plasma membrane (28,32). An ubiquitin-like folded Ras association (RA) domain is inserted between the REM domain and the CDC25-homology domain, which for Epac2 was shown to bind H-Ras ((33), addendum 1 of this thesis).

The analysis of Epac-mediated effects was facilitated by the development of the Epac selective cAMP analogue 8-pCPT-2'-O-Me-cAMP (007), which is not able to bind to or activate PKA. With the use of 007, Epac1 was linked to integrin-mediated and cadherin-mediated adhesion (16,34). Epac2 was shown to fulfil a predominate role in the potentiation of insulin secretion and the regulation of neurotransmitter release (35,36). The effects of Epac on insulin secretion were suggested to be partially Rap independent.

Unlike Epac1 and Epac2, Repac lacks a regulatory region and is therefore constitutively active (28). It is currently unclear whether Repac is regulated by the interaction with other proteins, which could take over the function of the regulatory region.

C3G

C3G is ubiquitously expressed and was originally identified as a protein bound to the SH3 domain of c-Crk(37). C3G can act on Rap-1, Rap-2, and R-Ras via its C-terminal catalytic region (37-40). The Crk-C3G complex is recruited to the plasma membrane upon activation of receptor tyrosine kinases by several growth factors and cytokines. In epithelial cells C3G interacts with the cytoplasmic domain of E-Cadherin, which is responsible for Rap1 activation during cell-cell contact formation (15,41).

PDZ-GEF

Two isoforms of PDZ-GEF, PDZ-GEF1 and 2, exist in mammalians (Fig. 2). Both proteins contain a PSD-95, Dlg, ZO-1 (PDZ) domain, a putative RA domain, a REM-domain, a CDC25-homology domain and an atypical CNB (aCNB) domain, which is not able to bind cAMP (42,43). PDZ-GEFs have been reported to be Rap specific exchange factors (42,43), however, this specificity was challenged by the suggestion that PDZ-GEF1 can also activate Ras (44). It is currently unclear, how PDZ-GEFs are regulated. Recently the function of PDZ-GEF was analysed genetically in *C.elegans* and *Drosophila.* In *C.elegans*, the PDZ-GEF (*pxf-1*)/Rap pathway is required for the maintenance of epithelial integrity (45) and in *Drosophila* PDZ-GEF (*Dizzy*) is involved in Rap-mediated integrin-dependent cell adhesion during cell migration in embryonic development (46).

CalDAG-GEF

The CalDAG-GEFs (or Ras guanine nucleotide releasing proteins, RasGRPs) are regulated by the second messengers diacylglycerol and calcium. They differ in their specificities towards G-proteins. CalDAG-GEF1 acts on Rap proteins (47,48), CalDAG-GEF2 on H-Ras and K-Ras but not on Rap (48-50) and CalDAG-GEF3 on both Rap and Ras proteins (48,51). CalDAG-GEF4 has not been studied extensively, but it shows specificity for Ras (52).

Inactivation of Rap by GTPase activating proteins

The intrinsic GTPase activity of Rap proteins is very low and therefore rapid inactivation of Rap only occurs with the aid of specific GAP proteins (Fig. 1). Members of the SPA-1 (signal-induced proliferation associated gene-1) family contain a catalytic domain called GAP-related domain (GRD), which is responsible for stimulating the intrinsic GTPase activity of Rap proteins by several orders of magnitude.

Originally SPA-1 was found as a mitogen-inducible nuclear protein (41) and was later found to be a principal Rap1-specific GAP in the lymphohematopoietic tissues (53). The structurally related proteins of SPA-1, E6TP1 (E6-targeted protein 1), SPAR (spine-associated RapGAP),

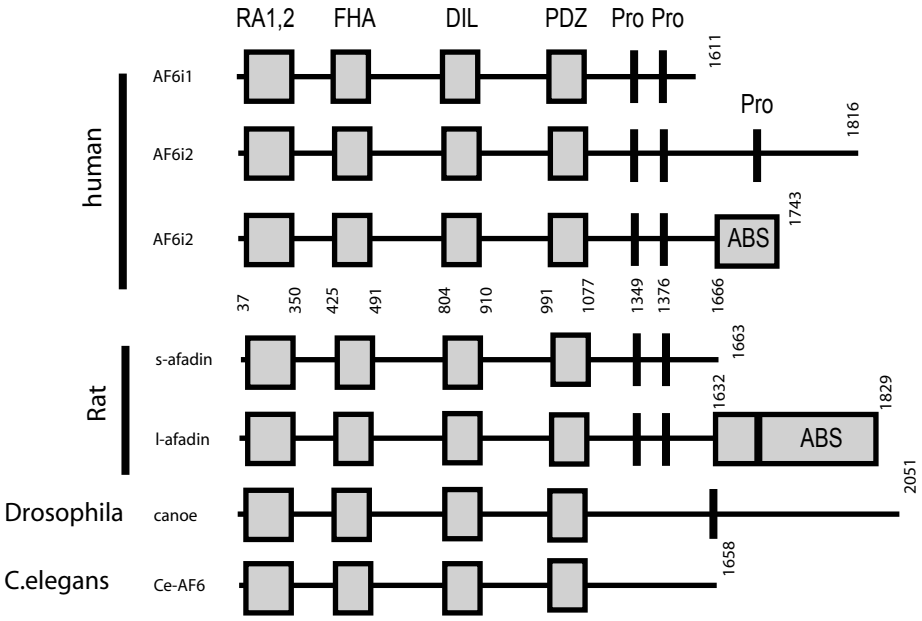

Fig. 3 Domain structure of AF6, afadin, Canoe and Ce-AF6. Domain structure of the three human AF6 isoforms (AF6i1, AF6i1 and AF6i3), the two its rat homologue afadin (long l-afadin and short s-afadin), its Drosophila homologue Canoe and its C.elegans homologue Ce-AF6 are shown. The relative positions of individual domains are shown. RA1, 2, Ras-associating domain 1 and 2; FHA, forkhead-associated domain; DIL, a dilute domain; PDZ, PSD-95, Dlg, ZO-1 domain; pro, proline-rich region, ABS, actin-binding site.

and several SPA-1-like proteins (SPA-Ls) make up the SPA family, but all show a unique cellular distribution in various tissues (53-56). Spa-1 was reported to be regulated by the recruitment of AF6 and subsequent downregulate Rap mediated adhesion (57).
Rap1GAP, a protein with a molecular weight of 73 KDa, was the first identified GAP for Rap (58). It exists as the two splice variants Rap1GAPI and Rap1GAPII, with the latter contains an additional N-terminal region. The α-subunit of the G_i-family proteins binds specifically to Rap1GAPII and recruits it to the membrane, which results in the attenuation of Rap1 activation (59). More recently, Rap1GAP2, which exhibits 50% similarity with Rap1GAPI was identified in human platelet (60).

The Rap binding protein AF6

As a result of chromosome translocation, the ALL-1 (Acute Lymphoblastic Leukemia) gene is fused to other genes on partner chromosomes, which results in chimeric proteins. Originally identified as an ALL-1 fusion partner on chromosome 6 (61), AF6 is a protein conserved from human to nematode and fly, although it is known under various names in different species (Fig. 3). AF6 contains two RA domains, a forkhead-associated (FHA) domain, a dilute domain (DIL), a PDZ domain, and a proline-rich region. The two RA domains mediate the interaction of AF6 with the GTP-bound forms of Rap and Ras (62,63). The PDZ domain was shown to be involved in the association with nectin and Spa-1 (57,64,65). Recently a new human AF6 isoform was cloned (66), namely AF6 isoform 3 (AF6i3). AF6i3 differs from AF6 isoform 1 (AF6i1), which was the only human isoform being studied, by an additional C-terminal F-actin-binding region. This actin-binding region of AF6i3 shares high similarity with the C-terminus from one of its Rat homologs, l-afadin. The actin-binding was suggested to facilitate AF6i3 to positively regulate adhesion (66). Even though AF6 can interact with both Rap and Ras, most of its biological effects were assigned to Rap signalling. *Canoe*, the *Drosophila* ortholog of AF6, functions as an effector of Rap1 during embryonic development and it is required for the proper dorsal closure process (67). In addition, *Canoe* acts downstream of the Epidermal Growth Factor (EGF) receptor and Ras by controlling ommatidal rotation during planar cell polarity (PCP) establishment in the *Drosophila* eye (68).
At subcellular level AF6 is localized at epithelia adherens junction. It binds to several junction proteins, including Zona Occludens 1 (ZO-1) (69), Junctional Adhesion Molecule (JAM) (70), profilin (62) and Nectin (71). Several studies suggest that AF6 is important for the formation and/or stability of cellular adherens junctions. In the context of E-cadherin-mediated adhesion, the PDZ domain of AF6 has been shown to interact with C-terminus of nectin and this interaction facilitates E-cadherin-dependent cell-cell adhesion (64,65). Takai and co-workers have shown that the Rap/AF6 complex promotes the interaction between E-cadherin and p120-catenin in a Rap1-dependent manner and stabilises E-cadherin-mediated adhesion (71). By contrast, recent study shows that AF6i3 is associated with the actin

cytoskeleton and is required for the stabilization of E-cadherin-dependent adhesion in a Ras/Rap independent manner (66). Interestingly, in the context of integrin-mediated adhesion, AF6 plays a role as a negative regulator ((57) and chapter 2). In human fibroblasts AF6 enhances the inhibition of Spa-1 on Rap1 induced cell adhesion (57). The opposite effects of AF6 on E-cadherin- and integrin-mediated adhesion indicate a potential role for AF6 as a balance keeper between the cell-cell adhesion and cell-matrix adhesion. It seems possible that AF6 plays a significant role in cadherin-mediated cell-cell adhesion by keeping Rap1 away from integrin mediated cell-matrix adhesion.

Rap in T cells

Lymphocytes are blood cells that are responsible for the adaptive immune response. In mammals two classes of lymphocytes, the B cells and T cells account for the antibody response and cell mediated immune responses, respectively. T cells derive their names because they develop in the thymus. In cell-mediated immune responses, activated T cells recognise foreign antigens that are presented by the Major Histocompatibility Complex (MHC) proteins on the cell surfaces. T cells are divided into two main classes: the cytotoxic T cells and helper T cells. Once activated by binding to the MHC complex of an antigen presenting target cell, cytotoxic T cells induce apoptosis of the target cell. Instead of killing the target cells directly, helper T cells activate macrophages, B cells and cytotoxic T cells. Inactive lymphocytes circulate continuously between the lymph and blood. In response to stimuli, lymphocytes switch rapidly from a non-adherent state to an adherent state and this process requires inside-out signalling mediated by integrins.

Rap in T-cell adhesion

Stimulation of cell adhesion is one of the best-studied effector pathways of Rap1 (14) (Fig. 4). In lymphocytes, stimulation of the T cell receptor (TCR) is followed by rapid Rap activation (72). Work from different groups suggested that Rap is crucial for the control of integrin-mediated cell adhesion (14,73,74). “Inside-out” signalling is the regulated integrin-mediated adhesion by intracellular signals. It has been proposed that Rap1 couples diverse stimuli such as TCR (75), phorbol ester (76), CD31 (14), CD98 ligation (77) and SDF-1 (78,79) to integrin activation through “inside-out” signalling. On the other hand Rap is a target of signals generated in response to the binding of integrins to their extracellular ligands, termed “outside-in” signalling (80). This suggests a potential positive feedback loop that can further enhance integrin function. In agreement with the studies in tissue culture cells, lymphocytes from Rap-/- mice show impaired cell adhesion (81).

Rap in T-cell migration

The lymphocyte transmigration from vessels into surrounding tissues is controlled by adhesive interaction with the vascular endothelium. Chemokines and adhesion molecules, such as integrins, mediate the adhesive interactions and subsequently influence the rolling,

firm adhesion and transmigration processes. During lymphocyte emigration Rap is activated upon chemokines stimulation within seconds and triggers rapid integrin activation (78). An active Rap mutant can transform lymphocytes into polarized cells and stimulate cell migration over the adhesion molecules ICAM-1 and VCAM-1. Rap activation was shown to be critical for firm attachment of lymphocytes and the subsequent transmigration through the vascular endothelium. A pivotal role of Rap1 was suggested in regulation of chemokine-induced lymphocyte extravasation (82).

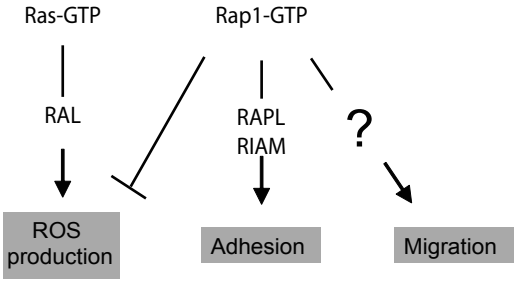

Fig. 4 Rap1 downstream signaling effects in T cells. RIAM and RAPL have been reported to interact with Rap and function as effectors on adhesion. Requirement for Rap1 activity in several cellular processes has been reported such as Reactive Oxygen Species (ROS) suppression, cell adhesion and cell migration.

ROS suppression

Reactive Oxygen Species (ROS) represent a heterogeneous group that includes oxygen anions and radicals or hydrogen peroxide (83). Diverse stimuli increase the intracellular oxygen radicals and promote cellular events, such as proliferation, gene activation, cell cycle arrest and apoptosis (84). In T lymphocytes, transient production of ROS modulates transcriptional and proliferative responses to TCR signalling (85). In contrast, chronic oxidative stress results in a reduced proliferative response and an enhanced transcription of inflammatory gene products. Ras/Ral signalling pathway has been shown to be required and sufficient to mediate phorbol ester-induced ROS production (85). Rap1 is also activated in T lymphocytes upon TCR stimulation. In a phosphatidylinositol 3'-kinase (PI3K)-dependent manner, Rap1 suppresses agonist- and Ras/Ral-induced ROS production, whereas it has no effect on basal ROS production (85). Although the PI3K-dependent ROS suppression was demonstrated by using a PI3K inhibitor (LY294002), no direct association between Rap1 and PI3K in T cells has been observed. This suggests an involvement of a PI3K-dependent unknown Rap effector for ROS suppression in T lymphocytes.

Rap1 effectors in T-cells

RAPL

Regulator of Adhesion and cell Polarization enriched in Lymphoid tissues (RAPL) was isolated in a yeast two-hybrid screen from a human leukocyte cDNA library using Rap1A-G12V, a constitutively active form of Rap, as a bait (86). RAPL encodes a 256 amino acids protein that consists of a Ras binding domain (RBD) and a C-terminal coiled-coil domain. RAPL was first identified as an alternative splice product of the RassF5 gene that is predominantly

expressed in lymphoid tissues (87). In T cells, upon Rap1 activation RAPL forms a complex with LFA-1 and re-localises together with its effector Mst-1 to the leading edge of polarized T cells (88). Plasma membrane localised Mst-1 increases the adhesiveness of the T cell integrin LFA-1 to ICAM-1. A RAPL mutant that is deficient in Rap1-binding blocks Rap1-, TCR-, and chemokine-dependent adhesion. In agreement with the results obtained from studies based on overexpression of RAPL, lymphocytes derived from RAPL knock-out mice show reduced adhesion, demonstrating the essential role for RAPL in integrin-mediated adhesion (89). In addition to integrin activation, RAPL was also reported to be capable of inducing cell polarization of lymphocytes and enhancing Rap-dependent migration of vascular endothelial cells (90). Interestingly, the restricted expression of RAPL indicates that it is unlikely responsible for Rap1 induced adhesion in non-haematopoietic cells.

RIAM

Rap1-Interacting Adaptor Molecule (RIAM) was identified in a yeast two-hybrid screen using another constitutively activate form of Rap, Rap1A-Q63E, as a bait to screen a Jurkat cDNA library (91). RIAM contains an RA domain, a pleckstrin homology (PH) domain and several proline-rich sequences. In Jurkat cells, over-expressed RIAM induces Rap-induced integrin-mediated adhesion. This adhesion was abolished by the knockdown of RIAM with siRNA. Interestingly RIAM also interacts with profilin and Ena/VASP proteins, which are modulators of actin dynamics. In RIAM knockdown cells the content of polymerized actin is reduced and Rap1 is not recruited to the actin cytoskeleton at the sites of adhesion anymore, indicating a role for RIAM in actin dynamics.

Insights from mouse models

To study the Rap effects on lymphocytes in living organisms, Rap1 knockout mice or transgenic mice overexpressing constitutively active Rap1 mutants were generated. Studies using Rap1A-G12V transgenic or Rap1A knockout mice demonstrated that Rap augments T cell activation via the enhancement of integrin-dependent adhesion (74,81). T lymphocytes from mice that constitutively express Rap1A-G12V show an enhanced TCR response (74). In Rap1A deficient mice no significant defects in lymphoid cells differentiation or maturation were observed (81). Intriguingly, studies on tissue culture T cells from either SPA-1-deficient or Rap1A-Q63E transgenetic mice models suggested that Rap1 is involved in T cells unresponsiveness and anergy, a state in which T cells become refractory to stimulation (92,93). SPA-1 deficient mice developed an age-dependent T cell dysfunction preceding the myeloid disorders (92). Helper T cells from mice that express Rap1A-Q63E show an increase of $CD4^+CD103^+$ regulatory T cells fraction. These T cells exhibit a potent inhibition of T cells proliferation and IL-2 production (93-95). Together, these evidences suggested that Rap1 plays a role as a negative regulator of T cells function. The discrepancy from different mice models could be due to different T cells used (96), effects from different Rap mutants (93) or different transgenetic systems used in each study (96).

ERM proteins

The ERM family consists of three closely related proteins, Ezrin, Radixin and Moesin. Radixin was originally isolated as a constituent of adherence junctions in rat liver (97); Ezrin was originally identified as a component of intestinal microvilli (98,99); and Moesin was identified as a heparin-binding protein in bovine uterus smooth muscle cells (100)**.** ERM proteins belong to the erythrocyte protein 4.1 super-family, which are characterized by a conserved N-terminal Four-point one, Ezrin, Radixin, Moesin (FERM) domain. The globular FERM domain, which has been reported to bind proteins and lipids (101), contains three sub-domains (F1, F2 and F3) (Fig. 5). F1 adopts an ubiquitin-like fold, F2 is structurally similar to the acyl-CoA binding protein and F3 shows structural homology to PH domains. In ERM proteins, the FERM domain is followed by an alpha-helical and a charged C-terminal region with a filamentous actin (F-actin) binding site.

Biophysical and structural analysis have shown that the C-terminal region of ERM proteins can interact with the N-terminal FERM domain (102-105). This internal interaction results in a closed conformation referred to as "inactive". Disruption of this interaction switchs the ERM proteins to an open or "active" state. The open conformation allows ERM proteins to interact with other proteins and to function as scaffolds (Fig. 6). The equilibrium between the open and the closed conformation is influenced by several regulatory signals.

Regulation of ERM proteins

Phosphorylation of threonine 558 of Moesin was first noted during thrombin activation of platelets (106). The phosphorylation of the corresponding residues, T564 for Radixin and T567 for Ezrin, were found to reduce the interaction between the FERM-domain and the C-terminal region. Indeed T558 is localised within the interaction surface between the two halves of the protein. Overexpression of phosphorylation-mimicking mutants induces the formation of cell surface structures (107). Rho kinase (ROCK) (108,109), the two isoforms of protein kinase C (PKC), PKCα (110) and PKCθ (111), and Protein kinase B (PKB) (112), have been shown to phosphorylate this conserved threonine and stabilize ERM in the open form.

The FERM domain binds phosphatidylinositol 4, 5-bisphosphoate (PIP_2), which is involved in the activation of ERM proteins (113,114). It is currently unclear whether this "activation"

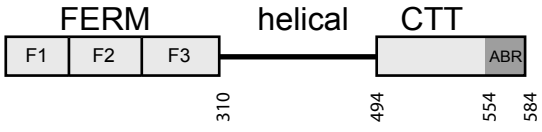

Fig. 5 Domain structure of ERM proteins. ERM proteins contain a FERM domain on the N-terminus followed by an α-helix and a C-terminus tail domain (CTT), which can associate with the FERM domain. The last ~30 residues in the CTT are involved in actin-binding. FERM, Four-point one, Ezrin, Radixin, Moesin. F1, F2 and F3 are sub-domains of the FERM domain and they are structurally similar to an ubiquitin-like fold, acyl-CoA binding protein and PH domain, respectively.

is due to the induction of the open conformation or due to a PIP_2 induced translocation of ERM proteins to PIP_2 enriched membrane compartments. For example, evidence exists that the FERM domain can bind PIP_2 even though it is bound to the C-terminal actin binding domain. (113).

Compared to the activation of ERM proteins, little is known about their inactivation. Myosin light chain phosphatase has been shown to bind to Moesin and dephosphorylate the critical carboxy-terminal threonine (115). In addition, apoptosis induces dephosphorylation of Ezrin (116). Besides dephosphorylation it has been shown that Ezrin is sensitive to calpain, a protease regulated by intracellular calcium (117). More recent studies have shown that in T cells stimulation by chemokines such as stromal derived factor 1 alpha (SDFα1) or secondary lymphoid tissue cytokine result in the dephosphorylation of ERM and a loss of microvilli in a Rac1 dependent manner (118,119).

Biological function of ERM proteins

The localization of ERM proteins has been analyzed in various tissues and cultured cell lines. In most cells all three family members of ERM proteins are co-localised at actin-rich surface structures such as microvilli, lopodia and membrane ruffles (120,121). It is interesting to notice that Radixin is also localised to adherent junction and focal contact sites, where Ezrin and Moesin are absent (122). The expression pattern of ERM proteins varies among tissues. Ezrin is found primarily in epithelial and mesothelial cells in intestine, stomach, lung and kidney. Moesin is enriched in endothelial and homatopoietic cells and found on high levels in lung and spleen. Radixin is highly expressed in liver and intestine (122).

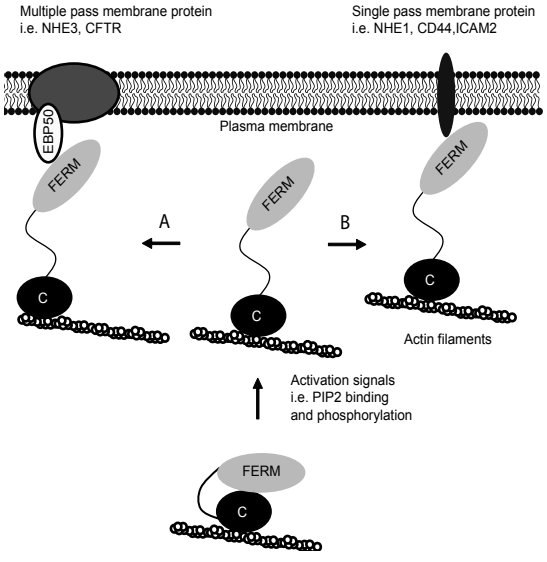

Fig. 6 A working model for the activation and function of ERM proteins. Following activation to unmask binding sites, FERM domain and the C-terminus region are available to bind to membrane proteins or actin, respectively. A, Through scaffold molecules such as EBP50 and E3KARP, ERM proteins link membrane to actin cytoskeleton. B, Activated ERM proteins can also direct provide a linkage between actin and transmembrane proteins. NHE, Na^+/H^+ exchanger; PIP2, Phosphatidylinositol 4, 5-bisphosphoate; CFTR, cystic fibrosis transmembrane conductance regulator; C, carboxy-terminal domain. Modified from (138).

Insight from genomic and genetics

Knock down of the individual ERM proteins in cultured cells induces no significant phenotypic changes on microvilli, cell-cell contacts or cell-substrate contacts. Furthermore, mice that are deficient in one of the ERM proteins do not have any discernable phenotype (123). These results indicate similar or redundant functions between the ERM family members. ERM proteins are conserved during evolution. Human Ezrin, Radixin and Moesin share a high degree of amino acid identity to the single form of ERM proteins in *Drosophila* (Dmoesin) and *C.elegans* (ERM-1) (124,125). Without the influence from functional redundancy, *C.elegans* and *Drosophila* have been used as alternative systems for the genetic study of ERM proteins. In *C.elegans*, ERM-1 is required for apical junction remodelling and tubulogenesis in the intestine (125). In *Drosophila,* Dmoesin is involved in developmental processes such as polarity control, cytoskeletal organization and adhesion (124).

Cytoskeleton-membrane linking

ERM proteins were originally proposed to be cytoskeleton-membrane linking proteins because of their enrichment in cell-surface structures and actin-binding ability. The interaction between ERM proteins and the actin cytoskeleton has been originally studied with Ezrin (117,126,127). Subsequently, the F-actin binding site within the carboxy-terminal 30 residues was identified to be a common feature of ERM proteins (108,128-130). On the amino-terminus of ERM proteins, FERM domain is responsible for the membrane binding. Both direct association of FERM domain with the cytoplasmic tails of transmembrane proteins and indirect association with transmembrane proteins through adaptor proteins have been reported. Many proteins that interact with ERM proteins are adhesion receptors. For example, Ezrin interacts with the Intercellular Adhesion Molecule-2 (ICAM-2) and this interaction facilitates the recruitment of ICAM-2 into uropod, the cell rear of migrating leukocytes (131,132). In addition to the direct association with the cytoplasmic tails of membrane proteins, ERM proteins can also interact with membrane proteins indirectly via with EBP50/NHE and E3KARP, which will be discussed later.

ERM proteins participate in Rho signalling

ERM proteins play a crucial role in the cellular cytoskeletal response to Rho-pathway activation. It was first observed that in response to Rho activation, the soluble pool of cytoplasmic ERM proteins redistribute to the plasma membrane and induce microvillus formation (133-135). The activation of ERM proteins can be achieved by a rise of PIP_2 concentration. PIP_2-producing phosphatidylinositol 4-phosphate 5 kinase (PI4P5K) is a direct Rho effector (136). It has been suggested that Rho activation activates PI4P5K and increases the PIP_2 concentration. Moreover, the interaction between Rho GDP Dissociation Inhibitor (RhoGDI), a potent sequestering factor of Rho, and ERM proteins was shown (105). This interaction suppresses the GDI activity and releases inactive Rho from RhoGDI, allowing Rho to become activated by its exchange factors. In addition, ERM proteins promote Rho

activation through interaction with the tumour suppressor hamartin (137). Together, these findings suggested that ERM proteins participate in the activation of Rho, which again activates ERM proteins as a positive feedback system. Therefore, ERM proteins are both downstream and upstream of Rho.

Involvement of ERM in Na^+/H^+ exchanger function and membrane trafficking

Activated Ezrin, Radixin and Moesin have been shown to connect actin filaments to membrane channels and receptors (138,139). Na^+/H^+ exchangers (NHEs) are Na^+ channels that play a crucial role in Na^+ absorption, acid-base homeostasis and cell volume regulation (140). NHE type 3 kinase A regulatory protein (E3KARP), Ezrin binding phosphoprotein (EBP50) and its rabbit homolog Na^+/H^+ exchanger regulatory factor (NHE-RF) are PDZ domain containing proteins, which bind through the PDZ domain directly to the FERM domain of ERM proteins (104,138). They function thereby as scaffold proteins and provide a link between NHE3 and Ezrin. Ezrin can also interact with PKA (141). It was suggested that Ezrin recruits PKA in the vicinity of the cytoplasmic domain of NHE3 where PKA phosphorylates and inhibits NHE3 actively (142). Recently, another mechanism was suggested, in which P38 triggers the phosphorylation of PKB through MAPK-Activated Protein Kinase-2 (MAPKAPK-2). This results in a PKB-dependent phosphorylation of Ezrin on T567 and the activated Ezrin facilitates its membrane translocation and the activation of NHE3 (112,143). Interestingly, independent of its function as an ion exchanger, NHE1 has been reported to directly interact with ERM proteins and regulate cortical cytoskeleton and PKB-dependent cell survival (144,145).

Besides the involvement in the regulation of the Na^+/H^+ exchanger, Ezrin was recently connected to adrenergic receptor recycling (146). The direct interaction between Ezrin and adrenergic receptor contributes to receptor recycling to the plasma membrane.

Adhesion

The function of ERM proteins on adhesion was first noted by the adhesion deficiency of epithelial cells that were depleted of ERM proteins (147). Moreover, overexpression of ERM proteins enhances cell adhesion (148). ERM proteins can regulate cell adhesion through different mechanisms. As mentioned before, ERM proteins are involved in the regulation of Rho signalling, which controls the actin cytoskeleton remodelling. The phosphorylation of Ezrin by ROCK is required for Rho-dependent focal adhesion assembly (109). Furthermore, the binding between the TSC1 tumour suppressor hamartin and activated ERM proteins is required for focal adhesion formation upon Rho activation signal (137). ERM proteins can also interact with the cytoplasmic tails of adhesion molecules such as CD44, ICAM-1 and ICAM-2 (131,149) and recruit them to the cell membrane.

ERM proteins and human disease

Radixin knockout mice display a breakdown of hepatocyte apical microvilli, which leads to a mild liver injury (150). Hamartin has been linked to ERM proteins in cell adhesion regulation

and hamartoma development (137). Recently Ezrin was identified as a crucial molecule in the dissemination of two pediatric cancer metastasis (151,152). Ezrin expression level is higher in metastatic cells compared to non-metastatic control cells (153,154). Overexpression of Ezrin in low-metastatic cells significantly enhanced the metastatic capacity. Moreover, knockdown of Ezrin by RNAi demonstrates that a high level of Ezrin expression is sufficient for metastatic progression (151,152). Although the importance for Ezrin in tumour metastasis is unambiguously demonstrated, the underlying mechanisms are unknown. Furthermore, this feature is not shared by the other two ERM family members. Radixin and Moesin were found to be down-regulated in lung adenocarcinomas, suggesting a tumour suppressor function of these two molecules (155).

Scope of this thesis

The small G-protein Rap centrally regulates integrin mediated cell adhesion and the formation of E-cadherin based cell-cell junctions. To this end, Rap integrates the input of a variety of signals, which activate Rap via Rap specific guanine nucleotide exchange factors. These GEFs are in part regulated by second messengers and are part of a protein-protein network, which regulates their temporal and spatial activities. Several downstream effectors of Rap mediate the cellular function of this small G-protein. The work described in this thesis deals with the analysis of the Rap signalling network.

Chapter 2 and chapter 3 focus on the downstream effects of Rap. In chapter 2 the relation between AF6 and Rap is analysed. AF6 was reported to be an effector of Rap due to its ability to bind specifically to the active GTP-bound form of Rap. Here we demonstrate that AF6 functions to buffer GTP-Rap in resting cells, maintaining it in a non-productive complex and thereby negatively regulates Rap function in T-cells.

In chapter 3 the analysis of three Rap-like pseudogenes (mRap1A-retro1, mRap1A-retro2 and hRap1B-retro) in mouse and human genome is described. All three retrogenes are expressed and encode functional proteins. These proteins have an increased GTP/GDP binding ratio compared to wild type Rap1. More interestingly they exhibit clear differences in their ability to induce cell adhesion and spreading.

To gain more insight in the protein interaction network, which controls the spatial and temporal organisation of Rap specific GEFs we performed yeast two-hybrid screens using Epac and PDZ-GEF as baits. Addendum 1 gives a general overview of the results obtained from this approach.

Radixin and Ezrin belong to the proteins that were identified to interact with Epac1 in the two-hybrid. This interaction is characterised in detail in chapter 4, which focuses on the interaction between Epac and Ezrin. A short Radixin/Ezrin binding region was identified in the N-terminus of Epac1 and it was shown that Epac can only interact with the open, active conformation of Ezrin. Furthermore, we show that Ezrin couples the activation of the β-

adrenergic receptor to Rap1 signalling via the recruitment of Epac1.

Chapter 5 focuses on the interaction between Epac and Radixin. A novel Radixin mutant is characterized. This Radixin mutant fulfils the classical biophysical criteria of being in an active state.

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Chapter

2

AF6 negatively regulates Rap1-induced cell adhesion

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AF6 negatively regulates Rap1-induced cell adhesion

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Abstract
AF6 is involved in the connection of membrane-associated proteins to the actin cytoskeleton. It binds to Ras-like small GTPases and is suggested to be an effector of both Ras and Rap. Here we show that knockdown of AF6 in T cells by RNAi enhances Rap1-induced integrin-mediated cell adhesion, whereas overexpression of AF6 has the opposite effect. Interestingly, AF6-induced inhibition of cell adhesion correlated with an increase in RapGTP levels. Like AF6, protein KIAA1849 contains a Ras association domain and interacts with Rap1. However, KIAA1849 does not inhibit Rap1-induced cell adhesion. We conclude that AF6 is a negative regulator of Rap-induced cell adhesion. We propose that AF6 inhibits Rap-mediated cell adhesion by sequestering RapGTP in an unproductive complex and thus prevents the interaction of Rap1 not only with effectors which mediate adhesion but also with Rap GAPs. Thus, AF6 may buffer RapGTP in resting T cells and maintain them in a non-adherent state.

Introduction
Rap proteins (Rap1a, 1b, 2a and 2b) are small GTPases closely related to Ras. They are activated by a variety of extracellular signals through the regulation of specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (1). Rap1 is involved in various cellular processes, most notably, the regulation of integrin-mediated cell adhesion and cadherin-mediated cell junction formation (2-4). A variety of effectors have been identified that mediate Rap1 function (5). These effectors include RapL and Riam (6-8). Both proteins interact with Rap1 through a Ras association domain (RA) and mediate Rap-induced integrin-dependent cell adhesion. RapL may function by direct binding to both Rap1 and integrins, whereas Riam may function through an interaction with the actin-regulatory proteins profilin and Ena/Vasp. Arap3 is another RA domain containing protein that interacts with Rap1. This protein is an Arf- and RhoGAP and mediates Rap1-induced inactivation of Rho (9). However, not all effectors of Rap1 have a RA domain. For instance, Vav2, a GEF for the small GTPase Rac, binds to Rap1 through its PH domain and mediates Rap-induced cell spreading (10).
AF6 (also called afadin) has a N-terminal region containing two RAs, one of which interacts with Ras-like small GTPases, including Ras and Rap (11-13). This protein was first identified as the fusion partner of ALL-1 protein in human acute myeloid leukaemia (14). AF6 is a multidomain actin-binding protein that serves as a scaffold protein between cell membrane-associated proteins and the actin cytoskeleton (11). Among the proteins that interact with AF6 are the tight junction protein ZO-1, the cell-cell adherence junction molecule nectin, various Eph receptors and the actin-regulatory protein profilin (11,15-17). AF6 was found to be an effector for Ras in the control of cell junction formation via direct interaction with ZO-1 (15). In addition, in Drosophila, the AF-6 homolog Canoe is an effector of Rap in the

regulation of dorsal closure (18). Recently it was shown that AF6 can interact with Rap GAPs, such as Rap1GAP and Spa1 through its PDZ domain (19). This interaction is mediated by a conserved internal β-turn in the Rap GAPs. AF6 recruits Rap GAPs to negatively regulate the level of Rap1GTP and consequently Rap1-induced integrin-mediated cell adhesion. Since these functional studies were largely performed in overexpression systems, we addressed the question whether AF6 is involved in the control of cell adhesion induced by activation of endogenous Rap and depletion of endogenous AF6. Specific activation of endogenous Rap can be achieved using the cAMP analogue 8-pCPT-2'-O-Me-cAMP (007) that specifically activates Epac (exchange protein directly activated by cAMP) (20). Indeed we observe that activation of Rap1 induced adhesion is negatively regulated by AF6. Importantly, knockdown of AF6 in T cells results in an enhancement of Rap-mediated cell adhesion. Surprisingly, inhibition of Rap1-induced cell adhesion by AF6 correlated with an increase, rather than a decrease in RapGTP. We propose a mechanism of AF6-induced inhibition of Rap1-mediated cell adhesion that includes the sequestration of Rap1GTP in an unproductive complex with AF6 preventing the interaction of Rap1 with both Rap GAPs and effectors of Rap-induced cell adhesion. Thus, AF6 may buffer RapGTP in resting T cells and maintain them in a non-adherent state.

Material and methods

Plasmids and Constructs—Haemaglutamin (HA)-tagged Rap1, Rap1V12 (HA-RapV12), Rap1GAP (HA-RapGAP I) have previously been described (21). PCR fragments containing AF6 or AF6-ΔRA (residues 347 to1612) flanked by a KpnI site at the 5' and a NotI site at the 3' were amplified from AF6 cDNA, provided by Dr. Eli Canaani, (Weizmann Institute of sience, Rehovot, Israel) and subcloned into KpnI/NotI sites of a pGEM-T vector (Promega). Subsequently, these fragments were subcloned into KpnI/NotI digested pcDNA3-HA, and integrities of the constructs were confirmed by DNA sequencing. Myc-AF6-RA (residues 25 to 353) was generated by PCR amplification of a fragment flanked by a EcoRI site at the 5' and a NotI site at the 3' of AF6 from AF6 cDNA. This fragment was subcloned into EcoRI/NotI-digested pcDNA3-Myc vector. Full length Myc tagged AF6 was provided by Dr. Kaibuchi Kozo (Division of Signal Transduction, Nara Institute of Science and Technology, Ikoma, Japan). The AF6L (KIAA1849) cDNA containing the coding sequence was kindly provided by the Kazusa DNA Research Institute (22). Polymerase chain reaction fragments containing AF6L or AF6L-RA (residues 25 to 213) flanked by a SalI site at the 5' and a NotI site at the 3' were subcloned into the pGEM-T vector. These clones were subsequently used to generate HA-AF6L or HA-AF6L-RA by introducing the SalI/NotI fragment containing AF6L or AF6L-RA into SalI/NotI-digested pMT2-SM-HA.

Rap1 Activation Assays and Immunoblotting—Rap1 activation was assayed as described previously (23). Briefly, cells were washed with cold PBS and lysed with buffer containing 1% Nonidet P-40. Lysates were cleared by centrifugation, and active Rap was precipitated with glutathione-Sepharose beads precoupled to a GST fusion protein of the Ras association domain of RalGDS. Precipitates were washed three times with lysis buffer and solubilized in SDS sample buffer. A portion of the cell lysate was reserved for analysis of total Rap content. HA-Rap1 was detected following Western blotting with anti-HA antibodies.

GST pull-down assays—For HA-AF6 and HA-AF6L pull-down assays, glutathione-Agarose beads were loaded with GST-Rap1. HB6 cells were transfected with HA-AF6 or HA-AF6L, followed by cell lysis in lysis buffer containing: 50mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% piridinium betain, 5 mM EDTA, 10 mM NaF, 1μg/ml aprotinin, 1μg/ml

leupeptin. Cell extracts were incubated with GST-immobilized proteins for 1 hour at 4°C. After 4 times washing in lysis buffer, bound proteins were resuspended in Laemmli sample buffer (Bio-Rad Laboratories, CA, USA), resolved by SDS-PAGE, and HA-AF6 or HA-AF6L were detected by Western blotting using anti-HA antibody.

Western Blotting—Western blotting of all protein samples was carried out using polyvinylidene difluoride membranes. The antibodies used for protein detection are the monoclonal anti-HA (12CA5), monoclonal anti-Myc (9E10), monoclonal anti-AF6 (Transduction Laboratories, Lexington, USA), monoclonal anti-Tubulin (Oncogene Science, Cambridge, USA).

Cell Culture, Cell Line, and Transfection—The Epac I monoclonal Jurkat T cell line (HB6) was generated by retroviral transduction of Jurkat cells with amphotropic virus encoding Epac-IRES-GFP (L.S.P. and J.L.B. in preparation). HB6 cells were grown at 37 °C in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated (30 min at 56 °C) fetal bovine serum and 0.05% glutamine in the presence of penicillin and streptomycin. Cells were transiently transfected by electroporation using 35 μg of plasmid DNA in total. Cells ($1.2x10^7$ cells/ml in 0.4 ml of complete medium) were pulsed at 250 V and 960 μF with 5 μg of TK-luciferase plasmid DNA, construct plasmid as indicated in the figure legends, and added vector plasmid to keep DNA amounts constant. Subsequently, 24 h after transfection, cells were transferred to serum-free medium and used 42 h after transfection. For RNAi experiments, cells were transferred to serum-free medium 48 h after transfection and used 72 h after transfection.

Adhesion Assay—For adhesion assays, transiently transfected Jurkat cells, serum starved overnight, were harvested, washed, and resuspended in TSM buffer (20mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) at a concentration of 5×10^5 cells/ml. 24-Well Nunc Maxisorp plates (Corning, NY, USA) were coated with fibronectin (5 μg/ml) overnight at 4 °C, washed, and blocked for 1 hour at 37 °C with 1% bovine serum albumin (BSA), TSM. After washing, 200 μl of TSM was added per well with or without the indicated stimuli. 007 was used at 100 μM and Mn^{2+} was used at 4 mM. Subsequently, 200 μl cell suspension was added per well. Cells were allowed to adhere for 1 hour at 37 °C and non-adherent cells were removed with warmed 0.5% BSA, TSM. Adherent cells were lysed and subjected to a luciferase assay as described previously (24). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Adherent cells were calculated, and the cell numbers were corrected for transfection efficiency and non-specific effects of constructs by measuring luciferase activity of total input cells ((counts in cells bound/counts in total input cells) ×100%).

Small Interfering RNA preparation—To make pTER–AF6, gene specific oligonucleotides were ligated into pTER vector that had been digested with BglII and HindIII according to described protocol (25). The oligonucleotides used are: for AF6 shRNA1, 5'- gatcccgatgatcgggaaggcagatttcaagagaatctgccttcccgatcatctttttggaaa-3' and 5'- agcttttccaaaaagatgatcgggaaggcagattctcttgaaatctgccttcccgatcatcgg'; for AF6 shRNA2, 5'- gatcccggagagagctgacgggtctttcaagagaagacccgtcagctctctccttttttggaaa -3' and 5'- agcttttccaaaaaggagagagctgacgggtcttctcttgaaagacccgtcagctctctcc -3'.

GDI-measurements – Experiments were carried out as described previously (13). In brief, 100 nM Rap loaded with the non hydrolyseable, fluorescent GTP analog 2'-/3'-O-(N-methylanthraniloyl)-guanyl-5'-yl- imidodiphosphate (mGppNHp) were incubated in the presence of 10 μM non labeled nucleotide and various concentrations of the RA-domain AF6L. Fluorescence was measured with a Cary Eclipse (Varian, Australia) equipped with a temperature controlled multi-cuvette holder.

Results

To determine whether AF6 is involved in Rap1-induced cell adhesion, a Jurkat cell line stably expressing Epac (HB6 cells) was used. Cells were transfected with AF6 together with or without constitutive active Rap1V12 and analysed for their ability to adhere to fibronectin. As shown in Figure 1A, AF6 completely inhibited Rap1V12-induced cell adhesion. However AF6 did not inhibit Mn^{2+}-induced cell adhesion, where integrins are activated directly by divalent cations (26). Also, the stimulating effect of Rap1V12 on Mn^{2+}-induced cell adhesion was completely inhibited by AF6. To investigate whether cell adhesion mediated by endogenous Rap1 is also inhibited by AF6, we treated HB6 cells with the Epac-specific analogue 007 to activate Epac and consequently Rap (20). Like Rap1V12-induced adhesion, 007-induced adhesion was inhibited by the overexpression of AF6 (Fig. 1B). AF6 interacts with Rap via RA domains in its N-terminal region (11,13). To investigate whether the interaction of AF6 with Rap is required for its ability to inhibit adhesion, an N-terminal deletion mutant lacking the RA domains was made, namely AF6-ΔRA (deletion of residues 1-346). Pull down experiments with immobilised Rap1 demonstrated the inability of AF6-ΔRA to interact with Rap1 (Fig. 2A). In contrast to full length AF6, AF6-ΔRA was not able to inhibit 007-induced adhesion in HB6 cells (Fig 2B). Conversely, the isolated RA domain of AF6 (residue 1-420) was able to inhibit 007-induced adhesion (Fig. 2C). Surprisingly, full length AF6 was much more efficient in inhibiting 007-induced cell adhesion than the isolated RA domain. From these results we conclude that in HB6 cells, expression of AF6 inhibits Rap-induced cell adhesion. Furthermore, we conclude that although the RA domains are required for this inhibition, additional domains are required for efficient inhibition.

Previously, it was shown that AF6 binds to the RapGAP Spa1, and that AF6 enhanced the Spa1-induced decrease in Rap1GTP levels (19). To investigate the effect of AF6 expression on Rap1GTP levels, HA-Rap1 and HA-AF6 were cotransfected in HB6 cells and the levels of Rap1GTP was determined by a pull-down assay. Surprisingly, AF6 strongly increased the level of HA-Rap-GTP (Fig. 2D). In contrast, AF6-ΔRA did not influence Rap1GTP levels. We further investigated the effect of AF6 expression on the change of endogenous Rap1GTP level in cells. Expression AF6 resulted in an increase in endogenous Rap1GTP level (Fig. 2E). From these results we conclude that AF6 binds to Rap1 through its RA domains and stabilizes Rap1 in the GTP-bound state. This result further suggests that the observed inhibition of cell adhesion by AF6 may be due to sequestration of Rap1 in an unproductive complex.

To investigate whether the negative effect of AF6 on cell adhesion is specific, we used shRNA to knock-down AF6. For that purpose HB6 cells were transiently transfected with two different shRNA constructs. Both resulted in a significant decrease of the endogenous AF6 levels (Fig. 3A). In these experiments the transfection efficiency was between 50 and 70% as measured by GFP cotransfection, indicating that the two shRNA constructs function rather efficiently in knocking down the endogenous AF6. Importantly, both AF6 shRNAs resulted in an increased integrin-mediated adhesion (Fig.3B). To investigate whether AF6 shRNA-induced cell adhesion is Rap dependent, we introduced Rap1GAP to inhibit endogenous Rap (27). Indeed, Rap1GAP completely inhibited AF6 shRNA-induced cell adhesion (Fig.3C). Moreover, knockdown of AF6 further enhanced 007-induced adhesion (Fig.3D). Although over-expression of AF6 resulted in an increased level of Rap1GTP, it could well be that the increased adhesion by AF6 knockdown is caused by increased levels of Rap1GTP. We therefore tested the level of Rap1GTP in the presence of AF6 shRNA. HB6 cells were transiently transfected with AF6 shRNA and Rap1, stimulated with or without 007 and the level of RapGTP was determined by the pull down assay. Knockdown of AF-6

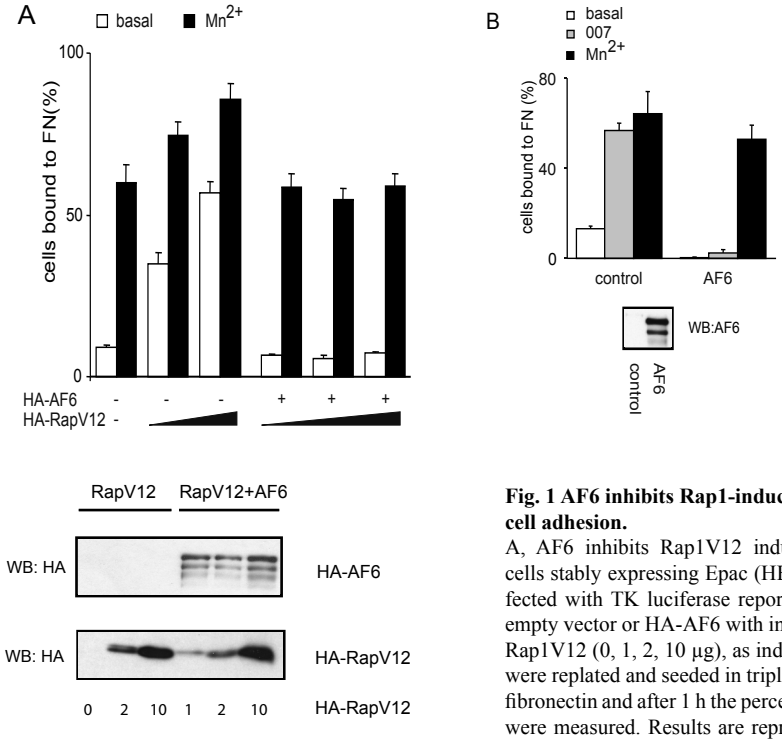

Fig. 1 AF6 inhibits Rap1-induced integrin-mediated cell adhesion.
A, AF6 inhibits Rap1V12 induced adhesion. Jurkat cells stably expressing Epac (HB6 cells) were cotransfected with TK luciferase reporter plasmid and either empty vector or HA-AF6 with increasing amounts HA-Rap1V12 (0, 1, 2, 10 µg), as indicated. After 42 h cells were replated and seeded in triplicate wells coated with fibronectin and after 1 h the percentage of adherent cells were measured. Results are representative of three independent experiments. The bottom panels indicate the expression of HA-AF6 and HA-RapV12 as determined by Westernblotting using anti-HA as a probe. B, AF6 inhibits 007 induced adhesion. HB6 cells were cotransfected with TK luciferase reporter plasmid and either empty vector or HA-AF6 and adhesion was measured as indicated above. After replating, the cells were incubated with either 007 or Mn^{2+} as indicated. Results are representative of three independent experiments. The bottom panels indicate the expression of HA-AF6 as determined by Westernblotting using anti-AF6 as a probe.

did not increase the level of Rap1GTP, but slightly decreased particularly the basal level of Rap1GTP (Fig. 3E). From these results we conclude that endogenous AF6 is a negative regulator of Rap-induced cell adhesion.
In search for effectors of the Rap family we identified a protein (KIAA1849) in the Kazusa DNA database with similarities to AF6 (Fig. 4A). KIAA1849 is characterized by an N-terminal RA domain and a C-terminal PDZ-domain. Due to its similarity to AF6 on domain structure, we called this protein AF6-like (AF6L) (Fig. 4A). We then investigated whether the AF6L can interact with Rap1. Indeed, like AF6, AF6L can be recovered from cell lysate using GST-Rap1 (Fig. 4B). This interaction was further characterised with recombinant AF6L-RA (residues 25 to 213). Binding of effector proteins to small G-protein often results in a reduced exchange of nucleotide bound to the G-protein (GDI-effect) (28). The dissociation of a fluorescently-labelled nucleotide bound to Rap can be detected as a decrease of fluorescence intensity in the presence of an excess unlabelled nucleotide. Indeed, increasing concentration of AF6L-RA reduced the exchange rate of nucleotide bound to Rap1 (Fig. 4C). From this measurement an affinity for the interaction of AF6L-RA and Rap1 of 0.9 µM was determined. This is similar to the affinity of AF6-RA for Rap1 (0.25 µM) (13). From these results we conclude that AF6L can interact with Rap1.

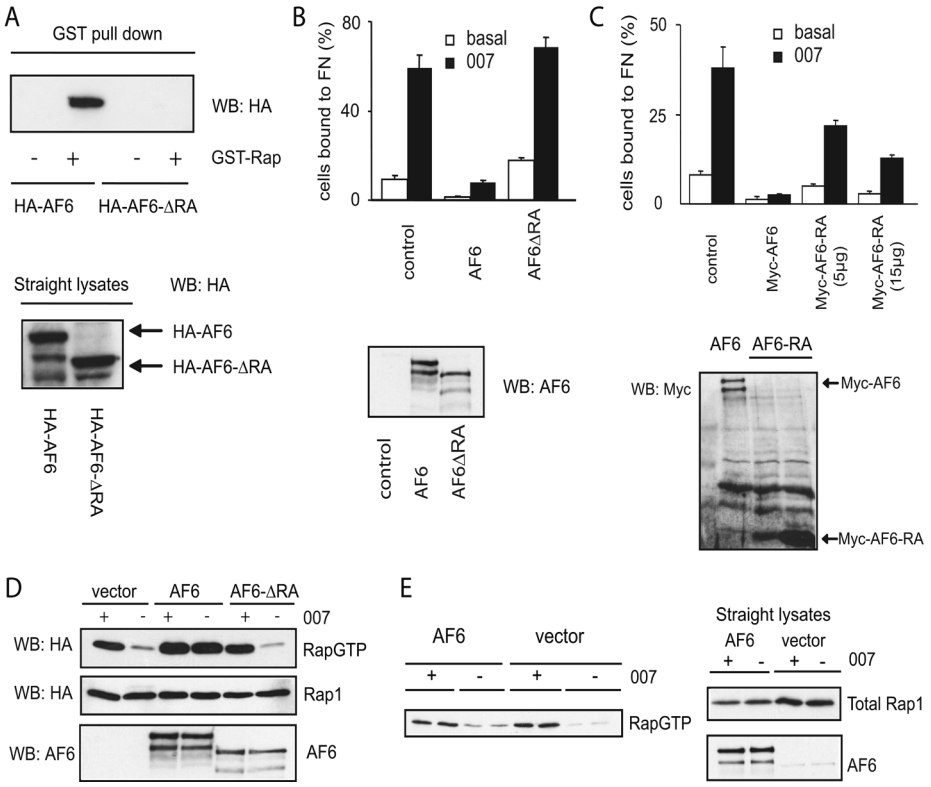

Fig. 2 Inhibition of cell adhesion by AF6 requires the RA domains of AF6
A, AF6 and Rap interaction requires the AF6 RA domains. Purified GST-Rap1 was used to precipitate HA-AF6 and HA-AF6-ΔRA from HB6 cells transiently transfected with either HA-AF6 or HA-AF6-ΔRA. The blot was probed with anti-HA antibody. Expression of each protein was determined by immunoblotting of the straight lysates. B, Deletion of RA domain abolishes AF6-induced inhibition of cell adhesion. HB6 cells were cotransfected with TK luciferase reporter plasmid and either empty vector, AF6 or AF6-ΔRA. Adhesion was measured as indicated in legends figure 1. In the bottom panels expression levels of transfected proteins are shown. Results are representative of three independent experiments. C, The AF6-RA domain blocks Rap-induced adhesion less efficient than full-length AF6. HB6 cells were cotransfected with TK luciferase reporter plasmid and either AF6 or indicated amounts of AF6-RA domain and adhesion was measured as indicated in legend figure 1. Results are representative of three independent experiments. Bottom panel: expression levels of transfected proteins. D, AF6 efficiently increases GTP level of overexpressed Rap1. HB6 cells were cotransfected with empty vector, HA-AF6 or HA-AF6-ΔRA in the combination with HA-Rap1. The upper panel shows the GTP levels of the HA-Rap proteins determined using the pull down-assay (Materials and Methods), the middle panel shows the level of HA-Rap1 in the total lysates and the lower panel shows the expression of HA-AF6 and HA-AF6-ΔRA in the total lysates. The experiment was repeated three times with reproducible results. E, AF6 increases GTP level of endogenous Rap1. HB6 cells were cotransfected with empty vector or HA-AF6. The left panels shows the GTP levels of the Rap proteins determined using the pull down-assay (Materials and Methods), the right panels show the level of endogenous Rap1 in the total lysates and the overexpression of AF6 in the total lysates. The experiment was repeated three times with reproducible results.

We next investigated whether AF6L inhibits cell adhesion. AF6L did not inhibit 007-induced adhesion to fibronectin, even though it was expressed to a much higher level than AF6 (Fig. 4D). Also the level of Rap1GTP was not affected by AF6L (data not shown). Furthermore, AF6L did not rescue the inhibitory effect of AF6 (data not shown). This failure of AF6L to inhibit Rap induced cell adhesion was not due to a failure of AF6L-RA to interact with Rap, since this domain, like AF6-RA domain, inhibited 007-induced cell adhesion (Fig. 4E). Although the domain structures of AF6 and AF6L are quite similar, there are differences between the proteins in amino acid composition. From these results we conclude that the difference between AF6 and AF6L in the inhibition of Rap1-induced cell adhesion is an intrinsic property of the proteins.

Discussion

In this paper we show that AF6 negatively regulates Rap dependent adhesion in T cells. Expression of AF6 results in the inhibition of Rap-induced cell adhesion to fibronectin. This inhibition is observed both when cell adhesion is induced by expression of constitutively active Rap1V12, and when endogenous Rap is activated. This inhibition requires the RA domain containing region of AF6 that interacts with the GTP-bound form of Rap1 (13). AF6-induced inhibition correlates with an increase in the levels of the GTP-bound form of Rap suggesting that AF6 binds to Rap and protects it from hydrolysis by Rap GAPs. One simple explanation for this result would be that AF6 inhibits Rap artificially due to overexpression as was previously shown for the RA-domain of RalGDS (29). However, several observations indicate that the inhibitory effect of AF6 on cell adhesion is specific. First, the isolated region of AF6 that efficiently interacts with Rap (13) was much less efficient in inhibiting Rap-induced cell adhesion than full length AF6. Secondly, knock-down of AF6 by using two independent shRNA constructs resulted in enhanced adhesion. Furthermore, the enhancing effect of AF6 shRNA on cell adhesion was still Rap dependent, which is compatible with the notion that endogenous AF6 inhibits endogenous Rap function. Thus, cell adhesion induced by activation of endogenous Rap1 is enhanced by knockdown of endogenous AF6. Thirdly, while the RA domain of the related AF6L protein can inhibit Rap-induced cell adhesion, the full length AF6L protein fails to do so. These combined observations suggested to us that the inhibitory effect of AF6 on Rap-induced cell adhesion is specific. Apparently, AF6 forms a complex with RapGTP, and thereby competes for effectors of Rap that regulate Rap-induced cell adhesion. A number of these effectors have been identified, most notably RapL and Riam. Both proteins contain RA domains involved in the binding to Rap and both proteins are directly implicated in Rap1-mediated cell adhesion (6,8). Previously, it was shown that AF6 through distinct domains binds to both active Rap1 and Rap GAPs (19). These authors did not observe a significant effect of AF6 alone on the levels of Rap1GTP and Rap-induced cell adhesion. However, both Spa1-induced inhibition of Rap1GTP and inhibition of Rap1-induced adhesion were augmented by AF6. This led to the proposal that AF6 is a negative regulator of Rap-induced cell adhesion by recruiting Rap in a complex with RapGAP. As a consequence RapGTP levels are decreased resulting in the inhibition of Rap effects. These results are at variance with our observations in that AF6 by itself inhibits Rap1-induced cell adhesion and in that AF6 increases the levels of Rap1GTP. Some of the differences may be due to difference in experimental set up, i.e. the level of expression of the various proteins, and the cell lines used. However, it may also point to the intriguing possibility of a dual negative control of Rap1-induced cell adhesion by AF6. First, as shown by our results, AF6 is recruited by RapGTP and prevents RapGTP from RapGAP-induced hydrolysis as well as

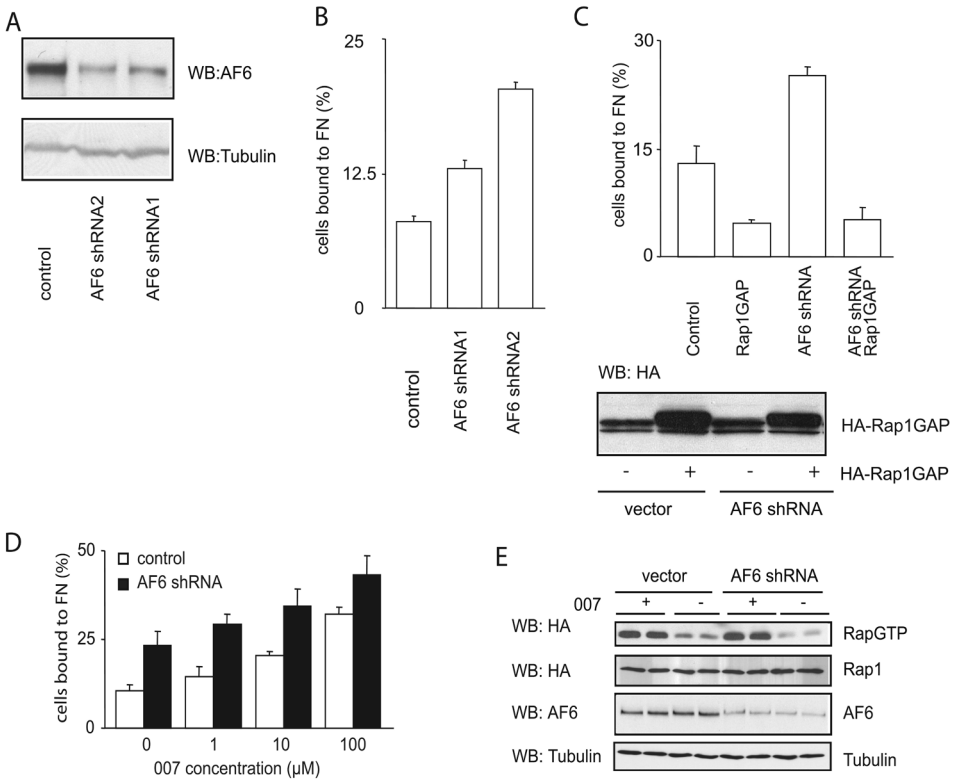

Fig. 3 AF6 depletion increases adhesion.
A, AF6 shRNA decreases protein expression. HB6 cells were transfected with 30 μg of indicated shRNA constructs and grown for 72 hours. In control experiments using GFP we observed more than 50% transfection efficiency using this protocol. Cell lysates were analyzed by Western blotting with anti-AF6 antibody. Anti-tubulin was used to demonstrate equal loading. B, AF6 shRNA induces cell adhesion. HB6 cells were cotransfected with TK luciferase reporter plasmid and the indicated shRNA constructs. After 72 h cells were replated and seeded in triplicate wells coated with fibronectin and after 1 h the percentage of adherent cells were measured. Results are representative of three independent experiments. C, AF6 shRNA-induced cell adhesion is Rap-dependent. HB6 cells were transfected with TK luciferase reporter plasmid, together with either empty vector, Rap1GAP, AF6 shRNA2 or Rap1GAP and AF6 shRNA2. Adhesion was measured as indicated above. Results are representative of three independent experiments. In the bottom panel expression of transfected Rap1GAP is shown. D, AF6 shRNA enhances 007 induced cell adhesion. HB6 cells were transfected with TK luciferase reporter plasmid, together with either empty vector or AF6 shRNA2 and adhesion was measured in the presence of 007 (concentration of 007 as indicated in the figure) as indicated above. Results are representative of three independent experiments. E, AF6 depletion does not affect the Rap1GTP levels. HB6 cells were transfected with HA-Rap1 and either empty vector or AF6 shRNA2. After 72 h the level of Rap1GTP was measured by the pull-down assays. The experiment was repeated three times with reproducible results.

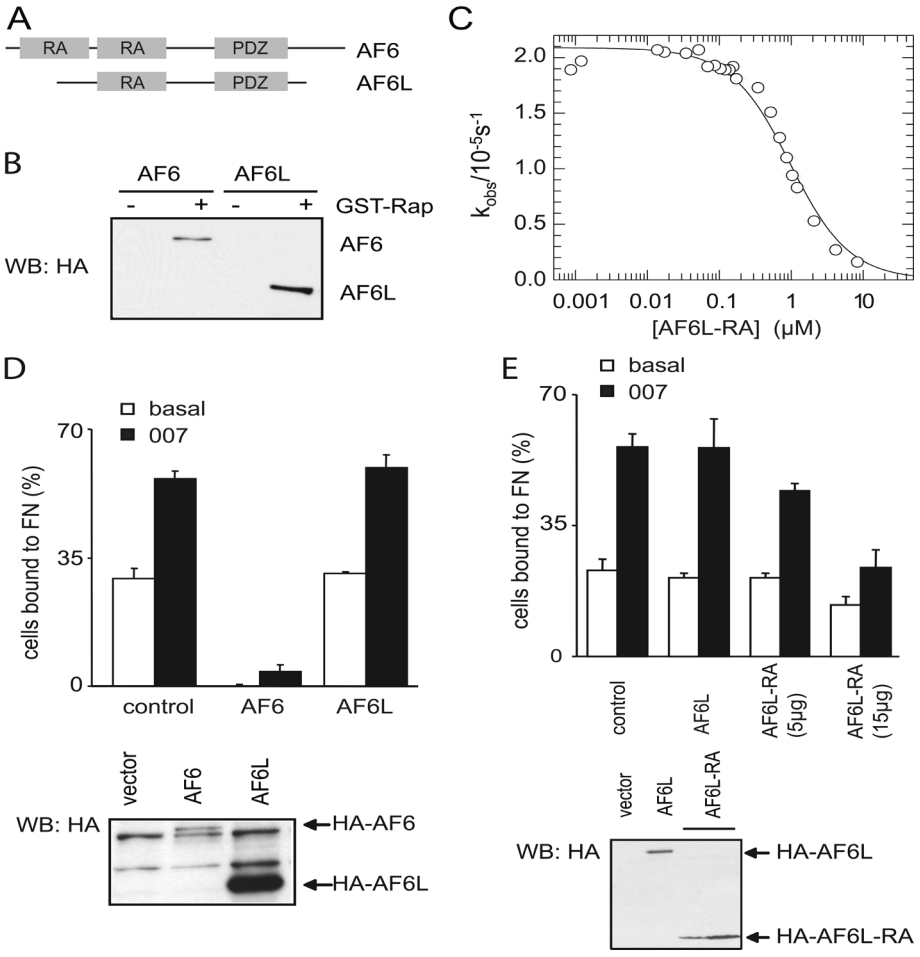

Fig. 4 AF6L is not a negative regulator of Rap1 in cell adhesion
A, Schematic comparison of AF6 and AF6L. AF6L consists of a RA domain in the N terminus and a PDZ domain in the C terminus. B, AF6L binds to Rap1 in a pull down assay. HA-AF6 and HA-AF6L were expressed in HB6 cells and recovered by GST-Rap1. Blot was probed with anti-HA (12CA5) antibody. C, Dissociation rate constant of mG-ppNHp from Rap1A in dependence of AF6L-RA concentration. The rate constant were measured at 37°C and plotted against the concentration of the RA-domain. From this data an affinity of 0,9 μM was calculated. D, AF6L does not block Rap1-induced adhesion. HB6 cells were cotransfected with TK luciferase reporter plasmid and either AF6 or AF6L and adhesion was measured as indicated in legend figure 1. Results are representative of three independent experiments. Bottom panel: expression of AF6 and AF6L proteins. E, AF6L-RA domain inhibits Rap-induced adhesion. HB6 cells were cotransfected with TK luciferase reporter plasmid and either AF6L or indicated amounts of AF6L-RA domain and adhesion was measured as indicated in legends figure 1. Results are representative of three independent experiments. Bottom panel: expression levels of transfected proteins.

interaction with effectors. Secondly, Rap GAPs are recruited in the complex and inhibit free Rap1. In this way Rap is efficiently inhibited. Indeed, full-length AF6 is much more efficient in inhibiting Rap1-induced cell adhesion than the isolated AF6 RA domain, supporting the notion that additional domains of AF6 are required for the efficient inhibition of Rap1. Effectors may compete with AF6 for binding to Rap1. Interestingly, when we knocked down AF6 we observed a slight decrease in Rap1GTP levels, but an increase in Rap1 function. We interpreted this results that in the absence of AF6, RapGTP is released and therefore exposed to GAP activity but also free to interact with effectors.

In the absence of an immune challenge, T cells circulate the body in a non-adhesive state. The maintainance of this non-adhesive condition prevents inappropriate immune responses from occuring. An increase in Rap activation is sufficient to rapidly upregulate T cell adhesiveness, demonstrating that Rap signalling must be tightly controlled in unstimulated T cells (30,31). We observed that reduction of endogenous AF6 expression by RNAi was sufficient to induce T cell adhesion in the absence of stimulation. This suggests that endogenous AF6 may function to buffer GTP-Rap in resting cells, maintaining it in a non-productive complex. Loss of this function of AF6 may therefore result in immunological disorders.

In Drosophila the AF6 homolog Canoe is an effector of Rap1 in the regulation of dorsal closure, presumably by regulating adherens junctions formation (18). This implies that AF6 may be a mediator of Rap1 functions other than integrin-mediated cell adhesion. Moreover, in the regulation of cell junction formation AF6 was shown to be an effector of Ras, rather than Rap (15). This suggests a possible model in which AF6 is an effector of Ras but a negative regulator of Rap1, which is consistent with the opposing functions of these proteins in cell adhesion.

Finally our results show that a protein closely related to AF6, AF6L, is able to interact with Rap1. However, this protein may have a different function than AF6 in that it does not function as a negative regulator of Rap1 in integrin-mediated cell adhesion. Further research is required to establish the real function of this protein and the possible connection with small GTPases.

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Chapter

3

Rap1 retrogenes encode activated isoforms with differential signalling capacity.

To be submitted

Rap1 retrogenes encode activated isoforms with differential signalling capacity.

M. Duchniewicz, Z. Zhang, T. Paluch, T. Zemojtel and F.J.T. Zwartkruis

ABSTRACT

Point mutations in Ras, which cause amino acid substitutions e.g. at position 12 or 61 result in proteins with a high transforming capacity and occur frequently in human tumors. These constitutively active proteins are highly GTP-bound. Here we describe three transcribed processed pseudogenes/retrogenes of Rap1A and B, all of which have limited amino acid substitutions resulting in an increased GTP/GDP binding ratio. Like active Rap1, human Rap1B-retro (G12R, K42E) and mouse Rap1A-retro1 (A59V) bind to the Ras-binding domain of RalGDS. In contrast, mouse Rap1A-retro2 (I9L, T35M, L96V) does not interact, most likely as a consequence of an amino acid substitution in the effector domain. Strikingly, upon over-expression only human Rap1B-retro increases adhesion to fibronectin. Cell spreading can be induced by both hRap1B-retro and mRap1A-retro1. Together, these data indicate that generation of Rap1 retrogenes, encoding activated isoforms is commonly used means to create differential gene function for Rap1-like GTPases, which may be independent of external stimuli.

INTRODUCTION

Ras-like GTPases function as molecular switches, which are active when bound to GTP. Guanine nucleotide exchange factors (GEFs) can activate Ras-like GTPases by facilitating the exchange of GDP for GTP. GTPase activating proteins (GAPs), which catalyze the hydrolysis of GTP, mediate negative regulation of Ras-like GTPases. Due to their prominent role as regulators of proliferation and differentiation in various organisms, many activating mutations in prototypic Ras proteins have been discovered. For example, oncogenic point mutations in Ras were discovered in about thirty percent of human tumors. Residues 12, 13 and 61 are most frequently mutated ((1) http://www.sanger.ac.uk/perl/genetics/CGP), but oncogenic Ras proteins with a change at position 59 have also been identified (2). These Ras proteins are insensitive to RasGAPs and consequently are more GTP-bound than wild type Ras.

Rap1A and Rap1B are members of the family of Ras-like GTPases and differ only by nine amino acids, which are mostly located at the very C-terminal part (3). Human Rap1A is identical to mouse Rap1A and the same holds true for the Rap1B protein (4,5). Targeted disruption of either Rap1A (6) or Rap1B (7) in the mouse results in specific defects. It is at present unclear whether this is caused by differential gene function or tissue-specific

expression. Loss of Rap1B impairs platelet aggregation due to a diminished activation of $\alpha_{IIb}\beta_3$ integrins. In addition, defects in endothelial cells may be causing bleedings, resulting in a high percentage of embryonic lethality. Rap1A mice are fully viable and fertile, but haematopoietic cells from the spleen and thymus adhere less efficiently to fibronectin or collagen as compared to wild type cells. Also the polarizing capacity of T cells following CD3 stimulation is attenuated. These phenotypes were predicted on the basis of biochemical studies in studies in primary and tissue culture cells.

In the course of our analysis of Rap1A-deficient mice, we cloned two different cDNAs encoding Rap1A-like proteins. At the genomic level corresponding, intron-less sequences were found, suggesting that the cDNAs were derived from transcribed processed pseudogenes/ retrogenes. Strikingly, we also identified a human Rap1B retrogene, which on the basis of BLAST searches appeared to be expressed (8,9). Here we present a first characterization of all three retrogenes and demonstrate that despite the fact that all are more GTP-bound than wild type Rap1, they differ in their signaling capacity.

RESULTS

We recently generated a mouse strain, in which the Rap1A locus was inactivated by homologous recombination. Because Northern analysis still showed the presence of Rap1A mRNA of about the wild type length, Rap1A cDNAs were cloned to see if the allele had been targeted correctly (6). Surprisingly, sequencing a number of cDNAs revealed that transcripts were derived from the (targeted) Rap1A locus, while others showed high homology to Rap1A, but encoded proteins with either a single or triple amino acid substitution. We named these genes Rap1A-retro1 (A59V) and Rap1A-retro2 (I9L, T35M, L96V). An alignment of these genes is shown figure 1. Based on mutational analysis of Ras, it seemed likely that Rap1A-retro1 would encode for an activated version of Rap1A. This is reminiscent of the situation in human, where an activated retrogene for Rap1B has been described (9). Database searches revealed the presence of two intronless, genomic sequences homologous to Rap1A-retro1 Rap1A-retro2 at chromosome 7. However, no ESTs were found, which matched the cloned cDNAs. To further establish that the cloned Rap1A-retro1 and -2 were not derived

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mRap1A          MREYKLVVLGSGGVGKSALTVQFVQGIFVEKYDPTIEDSYRKQVEVDCQQCMLEILDTAG
mRap1A-retro1   MREYKLVVLGSGGVGKSALTVQFVQGIFVEKYDPTIEDSYRKQVEVDCQQCMLEILDTVG
mRap1A-retro2   MREYKLVVIGSGGVGKSALTVQFVQGIFVEKYDPMIEDSYRKQVEVDCQQCMLEILDTAG
                ********:*********************:*********************:*

mRap1A          TEQFTAMRDLYMKNGQGFALVYSITAQSTFNDLQDLREQILRVKDTEDVPMILVGNKCDL
mRap1A-retro1   TEQFTAMRDLYMKNGQGFALVYSITAQSTFNDLQDLREQILRVKDTEDVPMILVGNKCDL
mRap1A-retro2   TEQFTAMRDLYMKNGQGFALVYSITAQSTFNDLQDVREQILRVKDTEDVPMILVGNKCDL
                **********************************:*********************

mRap1A          EDERVVGKEQGQNLARQWCNCAFLESSAKSKINVNEIFYDLVRQINRKTPVEKKKPKKKS
mRap1A-retro1   EDERVVGKEQGQNLARQWCNCAFLESSAKSKINVNEIFYDLVRQINRKTPVEKKKPKKKS
mRap1A-retro2   EDERVVGKEQGQNLARQWCNCAFLESSAKSKINVNEIFYDLVRQINRKTPVEKKKPKKKS
                **********************************************************

mRap1A          CLLL
mRap1A-retro1   CLLL
mRap1A-retro2   CLLL
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Figure 1, Alignment of the retro-Rap genes. Comparison of the two mouse retro-Rap with wild type mouse Rap1A. High identities between the Rap genes are indicated by stars and amino acid substitution are indicated by arrows.

from amplification of genomic DNA, two control experiments were done. First, reverse transcriptase was left out the RT-PCR reaction, which abolished amplification of the cDNAs. Secondly, the cDNAs were amplified using the Superscript™ II Reverse Transcripatase kit, which relies on removal of the CAP from mRNA and subsequent ligation of a PCR primer to the now available phosphate group at this end of the mRNA. Also with this approach Rap1A-retro1 and -2 sequences could readily be amplified.

In order to functionally characterize the Rap1 retrogene encoded proteins, we first tested for their ability to bind guanine nucleotides. Cos-7 cells were transfected with HA-tagged versions of wild type Rap1A, human Rap1B-retro, mouse Rap1A-retro1, mouse Rap1A-retro2 or Rap1A-V12 and labeled with [^{32}P]. HA-tagged proteins were isolated by immuno-precipitation and their GTP/GDP content determined by thin layer chromatography (Fig. 2A). As we and others reported previously, the percentage of GTP bound to wild type Rap1

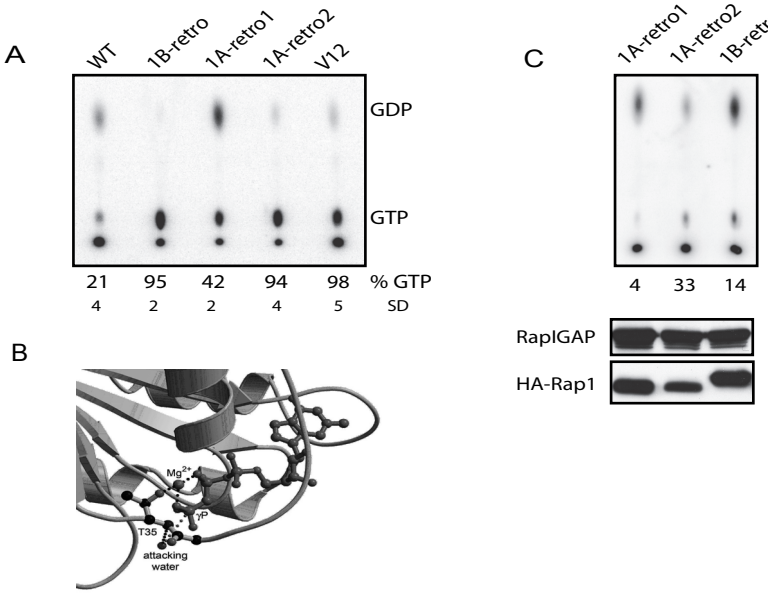

Figure 2, Determination of the GTP/GDP ratio bound to Rap retro-genes. A, Comparison of GTP binding between Rap retro-genes and Rap1V12. Cos-7 cells were transfected with 2 µg different Rap1 constructs as indicated, starved overnight, labelled with [32P] orthophosphate for 5 h. HA-Rap1 was immunoprecipitated with the 12CA5 anti-HA monoclonal antibody, and bound nucleotides were separated using thin layer chromatography, followed by PhosphorImager detection. SD, standard deviation. B, Structural representation of the region around T35 in Ras showing its function in coordinating the attacking of H_2O. Structural representation of the region around T35 in Ras showing its functions in positioning the attacking water and co-ordinating the magnesium ion. T35 and the nucleotide are shown in ball-and-stick presentation. Dotted lines are representing hydrogen bounds. The figure was generated using MolScript (20) and RasterR3D (21) based on the PDB entry 5P21 (22). C, Rap retro-genes are sensitive to RapGap. Cos7 cells were transfected with HA-RapGap in combination with mouse Rap1A-retro1, mouse Rap1A-retro2 and human Rap1B-retro. GDP and GTP, bound to transfected Rap1, was measured as indicated above. The bottom panels indicate the expression of HA-RapGap and HA-Rap as determined by Westernblotting using anti-HA as a probe.

is around twenty percent, while Rap1-V12 is highly GTP-bound. Since mutations at position 12 and 59 are transforming mutations in Ras, we expected human rap1B-retro and mouse Rap1A-retro1 to be more GTP-bound than wild type Rap1. Indeed, more than ninety percent of human rap1B-retro and about forty percent of Rap1a-retro1 was GTP-bound. mRap1A-retro2 was also almost exclusively GTP-bound. Although we were initially surprised by this result, inspection of the crystal structure of Ras, shows that threonine 35 helps in coordinating the gamma-phosphate of GTP and the attacking water molecule required for hydrolysis (Fig. 2B). Previously, it has been reported that V12 mutations in Rap1 are still sensitive to Rap1GAP. As expected, co-transfection of Rap1GAP with any of the retrogenes caused a strong increase in the GDP/GTP ratio. However, this decrease was clearly less pronounced for mRap1A-retro2 (Fig. 2C), underlining the importance of T35 for GTP hydrolysis.

The result above indicates that the conformation of the retrogene isoforms resembles that of active GTP-bound Rap1. GTP-bound Rap1 and Rap1-V12 are known to strongly interact with the Ras-binding domain (RBD) of RalGDS. Therefore, we compared the binding of the retrogene isoforms to GST-RBD with that of Rap1A-V12 using a pull down assay (Fig. 3). The binding of hRap1B-retro and mRap1A-retro1 to RalGDS RBD was comparable to that of Rap1A-V12. In contrast, mouse Rap1A-retro2 did not significantly bind to this RBD. This is most likely caused by the T35M mutation in the effector domain region, since the other two amino acid changes are highly conservative.

One of the best-documented functions of Rap1 is to enhance integrin-dependent adhesion to extra-cellular matrices (10). We assayed the ability of retrogene isoforms to increase adhesion of HB6 cells (Jurkat T cells expressing Epac1) to fibronectin-coated surfaces. Both human Rap1A-V12 and human Rap1B-retro clearly increased adhesion (Fig. 4). As reported previously, expression of the RBD of RalGDS decreased adhesion by preventing endogenous Rap1 effectors to interact with Rap1. Strikingly, expression of mouse retrogenes did not affect adhesion positively or negatively. From this result we conclude that whereas human Rap1B-retro has dominant-active properties in terms of regulation of integrin-mediated adhesion, the mouse retrogenes are inactive in this respect.

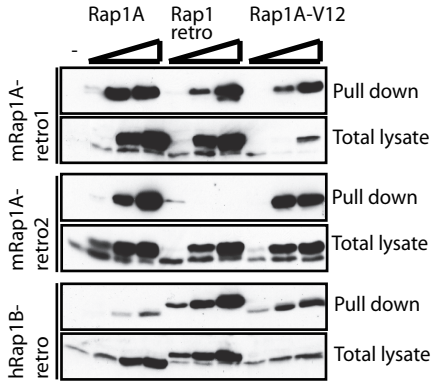

Figure 3, Analysis of activation of Rap retro-genes. Cos7 cells were transfected with empty vector, 0.1, 0.5 or 2 µg wide type Rap1A, 0.1, 0.5 or 2 µg Rap retro-genes or 0.1, 0.5 or 2 µg Rap1A-V12. After 48 hours cells were lysed, and Rap-GTP was isolated using GST-RalGDS-RBD and detected by using Western blotting with an anti-HA antibody, indicated as "pull down". The expression of HA-Rap was determined by Westernblotting using anti-HA as a probe, indicated as "total lysate".

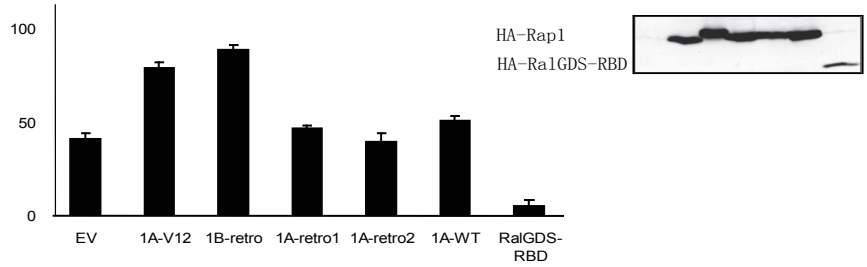

Figure 4, Analysis of the effect of Rap retro-genes on cell adhesion. Jurkat cells stably expressing Epac (HB6 cells) were cotransfected with TK luciferase reporter plasmid and either empty vector or Rap constructs as indicated. After 42 h cells were replated and seeded in triplicate wells coated with fibronectin and after 1 h the percentage of adherent cells were measured. Results are representative of three independent experiments. The bottom panels indicate the expression of HA-Rap and HA-RalGDS-RBD as determined by Westernblotting using anti-HA as a probe.

Cell spreading is a second function of Rap1, which is independent of the control of integrin-mediated adhesion (11). To test for this function, retrogenes were co-transfected with histon2B-GFP into A431 cells, grown on glass coverslips, followed by staining with the anti-HA antibody 12CA5 or phalloidin. First we confirmed that virtually all cells expressing GFP also co-expressed HA-tagged Rap1. Next, we used the phalloidin staining to measure the surface area of GFP-positive cells. Untransfected and empty vector-transfected cells mostly have a round morphology under these conditions. Expression of HA-Rap1V12, HA-hRap1B-retro and HA-mRap1A-retro1 induced a clear increase in the fraction of spread cells (Fig. 5A and B). In contrast, HA-Rap1A-retro2 did not significantly alter cell spreading. Together these results demonstrate that the proteins encoded by the different retrogenes differ in their biological activities.

DISCUSSION

Reverse transcription of mRNAs followed by insertion into the genome is an event, which in germ cells can yield additional copies of genes for future generations. In most cases they will develop into transcriptionally silent pseudogenes, which harbor mutations in their coding regions, preventing the production of functional proteins. However, if these processed pseudogenes have a distinct tempero-spatial expression pattern and/or have acquired neomorphic functions as a consequence of mutation, their presence may be advantageous to the organism and expression may be maintained. Indeed, a number of reports have recently appeared, suggesting that expression of processed pseudogenes (termed retrogenes) might be more common than previously anticipated. For example, the DPPA3 gene, which has been used as a marker for pluripotency, was found to have a number of related retrogenes, of which at least one was found to be expressed in adult tissues (12). Expressed retrogenes are not simple evolutionary relics, but can obtain essential functions as was shown for the Utp14B protein. Mutation of this autosomal gene results in juvenile spermatogonial depletion (13).

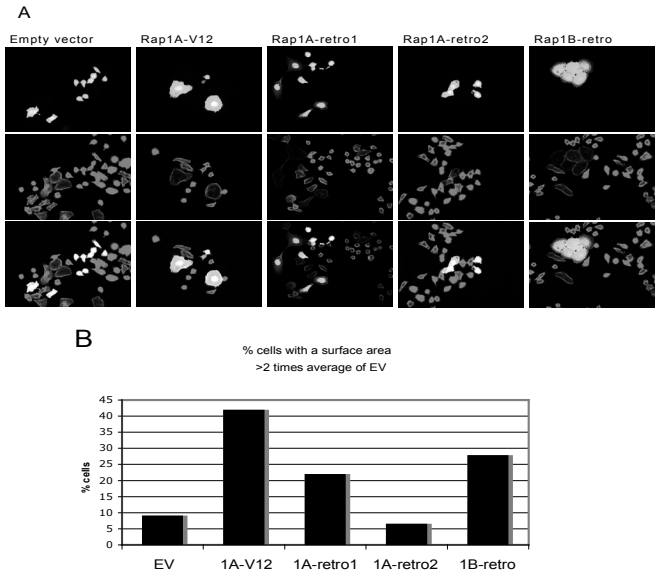

Figure 5, Analysis of the effect of Rap retro-genes on cell spreading. A, A431 cells were co-transfected with histon2B-GFP and Rap constructs as indicated. Cells were grown overnight in tissue culture wells and then photographed. GFP-positive cells were visualized by phalloidin staining. B, To quantitate the cell spreading, two independent fields of ~100 transfected cells were counted and cell bodies that had a flattened morphology were scored as spread. The data are expressed as the percent of cells that were spread and represent the means for two independent experiments.

In the course of analysis of our Rap1A knock out mice, we isolated cDNAs, highly homologous to Rap1A from various embryonic tissues. Although corresponding cDNAs were not identified in EST databases, control experiments confirmed that they did not represent inadvertently amplified genomic sequences. More specifically, they were not amplified if reverse transcriptase was left out of the PCR reaction and they could be amplified in a system, which relies on 5' ligation of a PCR primer to mRNA, from which the 5' cap structure has been removed. Furthermore, the existence of Rap1-like retrogenes appears not to be unique to mice. Recently, we and others reported the existence of ESTs from the human hRap1b-retro retrogene, whose expression might be linked to development of myelodysplastic syndromes (8,9).

Among the detected retrogenes, hRap1B-retro and mRap1A-retro1 were of special interest since they had amino acid substitutions, which suggested that the encoded proteins were constitutively active. Indeed, both retrogene-encoded isoforms had an increased GTP/GDP binding ratio and interacted strongly with the Ras-binding domain of RalGDS in pull-down assays. Strikingly, only hRap1B-retro increased integrin mediated cell adhesion, whereas mRap1A-retro1 was practically inactive in this assay. Cell spreading on the other hand was increased upon over-expression of mRap1-retro1. These results are reminiscent of those obtained with various Rap1 mutants, obtained via site directed mutagenesis (14). In that study, Rap1A G12V appeared to be more powerful in suppressing growth of tumorigenic HT1080 cells than wild type Rap1, but its capacity to induce a flat revertant phenotype of in Ras-transformed DT cells was not strongly increased. In contrast, a Rap1A A59T mutant showed opposite characteristics with a very mild effect on HT1080 proliferation but a clearly enhanced effect in the flat revertant assay. Together, these results indicate that the Rap1

retrogenes have different biological activities, suggesting that they may activate distinct (sets of) Rap1 effectors, with different biological activities. Indeed, Rap1 effectors have been described, which preferentially increase cell spreading like VAV (11) or both cell spreading and cell-matrix interaction like RIAM. It will be interesting to see if the Rap1 retrogene products indeed selectively interact with such effectors.

The fact that Rap1 retrogene encoded proteins are in a GTP-bound state suggest that retrogene expression would allow for activation of Rap1-dependent processes in a stimulus independent way. This phenomenon is well known in the case of Ras, where somatic mutations result in oncogenic proteins, which stimulate the ERK and PI-3K pathways in the absence of growth factors. Eras represents an example, in which a constitutively active GTPase is expressed in a more controlled fashion of expression (15). This retrogene-derived protein is about 50% identical to Ras, and is GTP-bound due to amino acid changes at positions corresponding to position 12, 59 and 63 of Ras. Although Eras can transform cells and is tumorigenic, its expression is confined to embryonic stem cells, where it contributes to proliferation. A second example of controlled expression of an activated GTPase is found in the case of Rac1B, where inclusion of an alternatively spliced exon splicing underlies production of a GTP-bound protein (16). The fact that this extra exon is found in various mammalian species like macaque and mouse, strongly suggests it has a physiological function. Although we still have to detect a function for Rap1A-retro2, mouse Rap1A-retro1 and human Rap1B-retro share their cell spreading capacity. Future studies should further establish their physiological significance and address whether these genes represent a recent example of convergent evolution.

MATERIALS AND METHODS

RT- PCR reaction and cloning of retrogenes

Taq PCR core kit from Qiagene was used. 2-4µl of RT reaction was mixed with PCR buffer, dNTPs, water, Taq polymerase and 10 pmol of 5' and 3' specific primers. The following PCR conditions were used: 1 cycle: 96°C, 2 min. 25-35 cycles: 96°C-20 sec, Tm-50°C - 20 sec, 72°C-1min/1kb / 1 cycle: 72°C, 10 min/4°C ∞. The following primers were used to amplify a 1186 bp fragment: 5'-GCGGGATTGTCAATATTTAAAC-3' and 5'-GCCATAGAAATCAGTTATCCC-3'. PCR products were cloned into vector from TOPO TA cloning kit (Invitrogen) and then sequenced using standard primers recommended by manufacturer. To specifically amplify retrogenes the following primers were used: 5'-GCCTGCTGTA AGAGACACTC-3' and 5'GGCACAGTTACACCACTG TCTTG-3'.

For the expression and activity studies both retrogenes were amplified using high proof readable Pfu polymerase with primers compatible for cloning full length proteins into pMT2HA.

DNAse I treatment of RNA

RNA was deprived of contaminating DNA using DNAseI kit from Promega following manufacturer's recommendations. The DNAaseI reaction was performed at 37°C for 30 min. RNA was purified by phenol/chloroform extraction, following DNA precipitation with isopropanol for 15 min at RT. After the RNA pellet was washed with cold 70% ethanol. The pellet was diluted in 20-50 µl of RNAse-free water and stored at -80°C.

Reverse Transcription

Superscript™ II Reverse Transcripatase kit from Invitrogen was applied. First 4 µl of DNAseI treated RNA was mixed with oligos (Invitrogen), and incubated for 10 minutes at 70°C, cooled on ice and then RT reaction mixture was added containing reaction buffer, DTT, dNTPs and RT polymerase. The sample was incubated at 42°C for 50 min and after 15 min at 70°C for deactivation. Simultaneously, a control reaction was prepared for the same RNA sample but w/o RT polymerase to check whether DNAseI digestion had been successful.

Digest with NLAIV

20 µl of each PCR reaction was use for digest in a total volume of 50 µl. The digest was performed with an excess of enzyme for 3 h. The samples were purified with PCR purification kit (Invitrogen) and separated on 1% gel.

Gene Racer reaction - full length RNA ligase-mediated rapid amplification of 5' cDNA ends (RLM-RACE PCR)

The Gene Racer kit was purchased from Invitrogen and procedure was performed according to manufacturer's recommendation. Briefly, RNA was dephosphorylated to eliminate truncated mRNA and non-mRNA from subsequent ligation with the GeneRacer RNA Oligo. Next, Cap Structure from intact, full-length mRNA was removed using Tap enzyme. This treatment generates a 5'phosphate required for ligation to the "GeneRacer RNA Oligo". Subsequently, ligation of RNA Oligo to decapped mRNA was performed utilizing T4 RNA ligase. Then, cDNA was produced on this RNA using random primers. Hereafter, PCR was performed in order to obtain 5'ends. For this purpose, the first-strand cDNA was amplified using "GeneRacer 5' Primer" (homologous to the GeneRacer™RNA Oligo): 5'-CGACTGGAGCACGAGGACACTGA-3' and a reverse gene-specific primer: 5'-CTTCCCCACGCCTCCTGAACCAA-3'**.** Only mRNA that ligated to "GeneRacer RNA Oligo'' and is completely reverse transcribed will be amplified. This PCR was prepared the standard way using Taq polymerase from Quiagene. Subsequently, this PCR reaction was further used for nested PCR. Here, "5'GeneRacer Nest primer": 5'-GGACACTGACATGGACTGAAGGAGTA-3' and reverse nested gene specific primer: 5'-CTTCCCCACGCCTCCTGAACCAA-3' were used. The PCR reaction was performed in the standard way using Taq polymerase from Qiagene. This PCR product containing 5' UTR of Rap1Aretro-1 was cloned into TOPO TA cloning kit (Invitrogen) and sequenced.

Sequencing of Cosmid DNA harbouring Rap1A-retro2

Cosmid MPMGc121C10270Q2, obtained from RZPD was identified during the screen for

the Rap1A gene. In order to sequence Rap1A-retro2 the following oligos were used: 5'-GCGGGATTGTCAATATTTAAAC-3'; 5'-CTTCCCCACGCCTCCTGAACCAA-3'and 5'-GGCACAGTTACACCACTGTCTTG-3'

Cloning of hRap1B-retro into pMT2HA vector

The fragment containing the ORF of hRap1Bretro was amplified from human genomic DNA using primers: 5'-ATGTCGACCATGCGTGAGTATAAGCTAGTCGT-3' and 5'- ATGCGGCCGCAGACCTGGCTCAGAGCTACA-3'. We used Advantage polymerase from Clontech with PCR conditions: 1 cycle: 95°C 1min/ 35 cycles: 95°C 15sec, 68°C 1min/ 1cycle:68°C 10 min. The PCR product was cloned into vector from TOPO TA cloning kit (Invitrogen) and then re-cloned into SalI and NotI of pMT2HA vector.

Activity measurements for Rap1 and related proteins

Determination of the GDP/GTP ratio using [^{32}P]-labeling of cells or using a non-radio active pull-down assay were done as described in Zwartkruis et al. 1998 (17).

Cell spreading assay

To measure cell spreading, A431 p120 RNAi cells (18) were plated on glass cover slips. The next day cells were transfected with either empty vector or pMT2HA vectors expressing the various Rap1 cDNAs and histon2B-GFP using Fugene. After 24 hours, cells were fixed and stained either for the HA-tag to confirm that GFP positive cells also expressed Rap1 protein (which was the case; data not shown) or with TRITC phalloidin. Cell surface area was measured using the ImageJ program version 1.36b (NIH).

Adhesion Assay

For adhesion assays, transiently transfected Jurkat cells, serum starved overnight, were harvested, washed, and resuspended in TSM buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) at a concentration of 5×10^5 cells/ml. 24-Well Nunc Maxisorp plates (Corning, NY, USA) were coated with fibronectin (5 µg/ml) overnight at 4°C, washed, and blocked for 1 hour at 37°C with 1% bovine serum albumin (BSA), TSM. After washing, 200 µl of TSM was added per well with or without the indicated stimuli. Subsequently, 200 µl cell suspension was added per well. Cells were allowed to adhere for 1 hour at 37 °C and non-adherent cells were removed with warmed 0.5% BSA, TSM. Adherent cells were lysed and subjected to a luciferase assay as described previously (19). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Adherent cells were calculated, and the cell numbers were corrected for transfection efficiency and non-specific effects of constructs by measuring luciferase activity of total input cells ((counts in cells bound/counts in total input cells) ×100%).

Plasmids

Haemaglutamin (HA)-tagged Rap1, Rap1V12 (HA-RapV12), Rap1GAP (HA-RapGAP I) have previously been described (17).

Cell culture

Cos-7 cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS) and serum-starved in DMEM at 1.5% FCS. A431 cells expressing human p120 short interfering RNA (siRNA) was described previously (18). Cells were cultured in DMEM containing L-glutamine (Hyclone), 10% fetal bovine serum (FBS) (Hyclone), and 1% penicillin/streptomycin (Gibco/Invitrogen).

ACKNOWLEDGMENT

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Chapter

4

Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling.

submitted

Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling.

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Abstract

Epac is a cAMP-regulated exchange factor for the small GTPase Rap, which is involved in various cAMP-dependent processes including cell adhesion and secretion. Here we show that Epac1 is particularly abundant at the apical surface of epithelial cells. In search for an apical anchor protein for Epac1 we identified the membrane-cytoskeleton linker protein Ezrin as an Epac1-interacting protein. Indeed Epac1 colocalised with Ezrin at the apical membrane of polarised epithelial cells and at the apical, lumenal surface of kidney tubule epithelial cysts grown in collagen gels. The interaction between Ezrin and Epac1 was dependent on the Ezrin FERM domain and was promoted by receptor activation, suggesting that Epac1 interacts with the open conformation of Ezrin. For Epac1, the N-terminal 49 amino acids are both necessary and sufficient for apical localisation. Importantly, either deletion of Ezrin by RNA interference or disruption of the Epac-Ezrin interaction with the N-terminal 49 amino acids displaced Epac1 from the apical membrane and inhibited Rap1 activation following β-adrenergic receptor stimulation. From these results we conclude that Ezrin is an anchor protein for Epac1 that regulates the apical localisation of Epac1 and couples β-adrenergic receptor stimulation to Rap1 signalling.

Introduction

Epac1 (exchange protein directly activated by cAMP) is a widely expressed guanine nucleotide exchange factor (GEF) for the small GTPase Rap1 and Rap2[1-4]. The N-terminal regulatory region of the protein consists of a DEP (Dishevelled, Pleckstrin, Egl-10) domain, involved in membrane localization and a cAMP-binding domain. The cAMP-binding domain maintains Epac in an auto-inhibited state in the absence of cAMP[5]. The cAMP analogue 8-pCPT-2'-O-Me-cAMP (007) that specifically activates Epac but not protein kinase A[6], has allowed a number of biological processes to be assigned to Epac1. Most notably, Epac1 has been implicated in integrin-mediated cell adhesion[7], E-cadherin[8,9] and VE-cadherin-mediated cell junction formation[10-13], regulation of sodium proton exchange activity[14] and regulation of

secretion[15,16]. The presence of several protein or lipid interaction domains in Epac1 indicates that Epac1 is recruited to specific regions in the cell. Indeed Epac1 is reported to be located in mitochondria[17], in the perinuclear region[18], in cell-cell junctions[8] and in the brush border of kidney tubules[14].

Ezrin is a member of the ERM (Ezrin Radixin Moesin) family of proteins, which link integral plasma membrane proteins with the actin cytoskeleton[19]. ERM proteins possess an N-terminal FERM (band 4.1 ERM) domain and a C-terminal domain which includes an actin binding domain and residues that mediate an intra-molecular interaction with the FERM domain which prevents interaction with the membrane and F-actin[20]. Ezrin is regulated by phosphorylation and by phospholipid binding, which contribute to release of auto-inhibition and thus promote its crosslinking function[21-23]. Ezrin is expressed principally at the apical domain of epithelial cells, where it enhances microvilli formation[24-26]. Ezrin is also involved in the regulation of adherens junctions and focal adhesions and membrane ruffles and consequently plays a role in adhesion and migration[27,28]. In addition to stabilising membrane-actin interactions, Ezrin also functions as a scaffold for cell signalling, associating with the β-adrenergic receptor and PKA (protein kinase A) and thus bringing together a source of cAMP with its target kinase[29,30]. By linking up with PKA-regulated membrane channels, such as NHE3, Ezrin targets this signalling complex to its effector proteins[31]. A recent report showed that Epac also regulates NHE3 function[14].

We show here that Ezrin binds to Epac1 and is responsible for the targeting of Epac1 to the apical membrane. Binding of Epac1 requires the active conformation of Ezrin. Indeed, receptor stimulation induces this association. Furthermore, the Epac-Ezrin interaction is required to mediate β-adrenergic receptor-dependent activation of Rap. From these results we conclude that Ezrin is an anchor protein for Epac1, that regulates the apical localisation of Epac1 and couples β-adrenegic receptor stimulation to Rap1 signalling.

Results

Apical localisation of Epac1

We showed previously that Rap1 is activated by the Epac1-specific cAMP-analogue 007 in the ovarian carcinoma cell line OvCar3[6]. We therefore examined the expression of Epac1 in these cells. We observed that the monoclonal antibody 5D3, which was raised against Epac1, recognized a double band of the predicted size of Epac1. These bands were absent in cells treated with siRNA against Epac1 (Fig. 1A). Two additional Epac1 siRNAs also suppressed Epac1 expression (not shown). Moreover, a polyclonal antibody raised against Epac1, α-Epac1-1, recognized the same two bands (not shown). Confocal analysis of OvCar3 cells showed that Epac1 was located predominantly in punctate structures and at sites of cell-cell contact, a staining that was abolished after treating the cells with Epac1 siRNA (Fig. 1B upper panel, and data not shown). Interestingly, Z-stack analysis of OvCar3 cells demonstrated a predominantly apical plasma membrane localisation of Epac1, suggesting that the punctate structures represented microvilli (Fig 1B lower panel).

To study the apical localisation of Epac1 further, we used Madin Darby Canine Kidney cells (MDCK), which develop a polarised 'cobblestone' morphology with distinct apical and basal-lateral domains. Since MDCK cells do not express detectable levels of Epac1, we stably expressed human Epac1 in these cells by retroviral transduction (Price et al 2004). The localisation of Epac1 in these cells was similar to that observed in OvCar3 cells (Fig. 2A). Z-stack analysis of cells co-stained for Epac1 and the tight junction marker ZO-1 showed

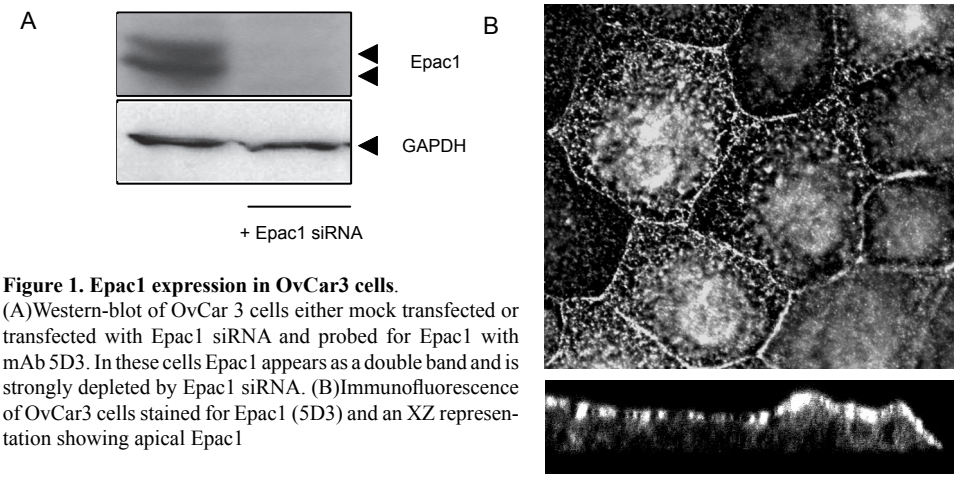

Figure 1. Epac1 expression in OvCar3 cells.
(A)Western-blot of OvCar 3 cells either mock transfected or transfected with Epac1 siRNA and probed for Epac1 with mAb 5D3. In these cells Epac1 appears as a double band and is strongly depleted by Epac1 siRNA. (B)Immunofluorescence of OvCar3 cells stained for Epac1 (5D3) and an XZ representation showing apical Epac1

that Epac1 is located predominantly at the apical surface. This result was corroborated by immunoelectron microscopy (EM) showing that the majority of Epac1 is located in microvilli at the apical surface of the cell. In addition to the apical localisation, Epac1 was found in the perinuclear region, including the Golgi (Fig. 2B), particularly in less polarized cells. We conclude that in polarized epithelial cells, the majority of Epac1 is present in microvilli at the apical plasma membrane.

Epac1 interacts with the apical membrane-cytoskeleton linker protein, Ezrin

To identify possible apical anchor proteins for Epac1 we performed a yeast 2-hybrid screen of a human placenta cDNA library using full length Epac1 as bait. This screen identified Ezrin and Radixin as Epac1-interacting proteins (9 and 5 colonies respectively). Ezrin and Radixin are members of the Ezrin-Radixin-Moesin (ERM) family of proteins, that exist either in a closed, auto-inhibited state whereby a C-terminal region interacts with the FERM domain, or in an open conformation which permits association with interacting proteins (see introduction). Importantly, Ezrin is predominantly localized in apical membranes[32] and thus is a prime candidate for being the apical anchor protein for Epac1. Co-immunoprecipitation experiments were performed to test whether Epac1 interacts with Ezrin *in vivo*. HA-tagged Epac1 was transfected into 293T cells together with flag-tagged forms of either full-length Ezrin or with the N-terminal 492 amino acids of Ezrin (Ezrin-N3) lacking the auto-inhibitory C-terminus (Fig. 3A). Immunoprecipitation of Ezrin-N3, resulted in co-precipitation of Epac1 (Fig. 3B). In contrast, full-length Ezrin only weakly associates with Epac1 suggesting that Epac1 interacts preferentially with the FERM domain-containing N-terminal region of Ezrin and not with auto-inhibited Ezrin. Similarly, Epac1 also interacted with Radixin-N3 but not full-length Radixin (not shown). To confirm that the endogenous proteins interact as well, we examined the interaction of Epac1 and Ezrin in ACHN human kidney carcinoma cells. Indeed, Ezrin co-immunopreciptates with Epac1 (Fig. 3C). S1P induces activation of the small GTPase Rho, and the subsequent synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2)[33], which promotes Ezrin association with the plasma membrane and acquisition of an open conformation[23,34]. We therefore tested whether stimulation of ACNH cells with S1P would result in an increased association between Epac1 and Ezrin. Indeed, co-immunoprecipitation

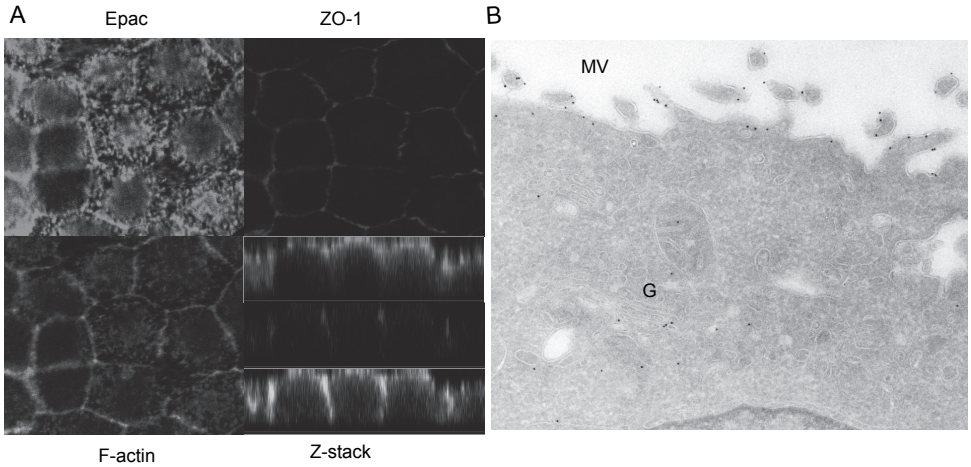

Figure 2. Apical Epac1 expression in polarised MDCK cells.
(A) Immunofluorescent micrographs of confluent MDCK cells stably expressing Epac1 stained with anti-Epac1 monoclonal antibody (5D3), showing a predominantly apical localisation of Epac1. Tight junctions are labelled with anti-ZO-1 polyclonal antibodies and Alexa-488 phalloidin to label filamentous actin. XZ representations are shown in the lower right panel. (B) Transmission electron microscopy image of MDCK-Epac1 cells labelled with a-Epac1-1. The microvilli (MV) and the Golgi aparatus (G), which show most Epac1 labelling are indicated.

of Ezrin by Epac1 was enhanced when ACNH cells were stimulated with sphingosine-1-phosphate (S1P) (Fig. 3C). Co-immunoprecipitation of endogenous Epac1 and Ezrin was also observed in OvCar3 cells following S1P stimulation (not shown). We conclude from these results that Epac1 interacts with the open conformation of Ezrin *in vivo*. These results further imply that the interaction is regulated by stimuli that activate Ezrin.
Epac1 contains a number of domains that mediate protein-protein interactions, nucleotide exchange and membrane localisation (Fig. 3D). We therefore examined a series of deletion mutants of Epac1 to identify the region of interaction with Ezrin. We observed that deletion of the N-terminal 49 amino acids (Epac1-Δ49) strongly reduced co-immunoprecipitation with Ezrin-N3 (Fig. 3E), suggesting that Ezrin interacts with the N-terminal 49 amino acids of Epac1.

The N-terminal tail of Epac1 is required for co-localization with ezrin at the apical plasma membrane.

We next investigated whether Epac1 colocalizes with Ezrin in the apical membrane. We observed that transiently expressed Myc-tagged Epac1 colocalises with Ezrin at punctate structures (Fig. 4A, upper panels). We also examined co-localisation of these proteins in cells grown in 3-dimensional collagen gels, which allows a more developed level of apical-basolateral polarisation. Under these conditions, stably expressed Epac1 shared an almost identical distribution with Ezrin at the apical lumenal surface of MDCK epithelial cell cysts (Fig. 4B), supporting the notion that these proteins interact. Interestingly, non polarised clusters of cells showed a disordered distribution of both Ezrin and Epac1. In agreement with the failure of Epac1-Δ49 to interact with Ezrin, this mutant did not show the punctate microvillar distribution and did not co-localise with Ezrin. Instead, Epac1-Δ49 showed a cytoplasmic and nuclear distribution, while the distribution of Ezrin remained punctate (Fig. 4A, middle panel). Interestingly, a fusion protein of CFP with the isolated N-terminal 49

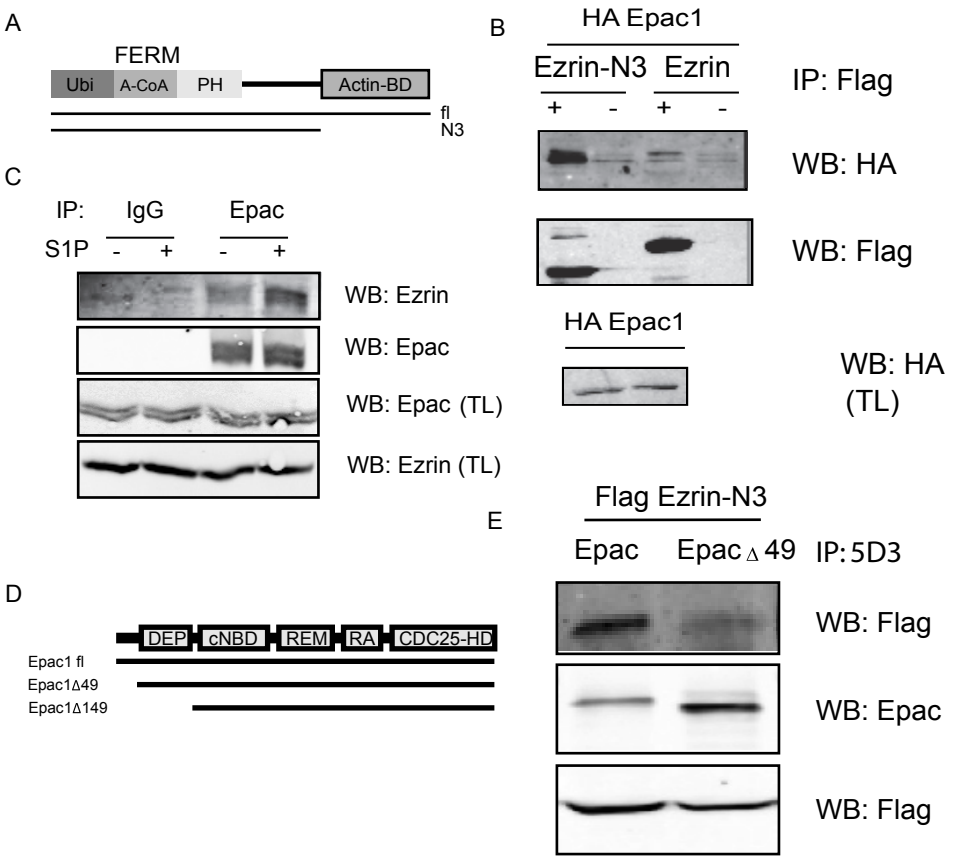

Figure 3. Epac1 interacts with ezrin in vitro.
(A) Domain structure of Ezrin, showing the N-terminal FERM domain, which consists of a ubiquitin-like fold, acyl Co-A binding region and a pleckstrin homology (PH) domain. The C-terminal contains an actin-binding domain and residues required for interaction with the N-terminal. (B) Anti-HA western blot (top panel) shows co-immunoprecipitation of flag-Ezrin with HA-Epac1 from lysates of 293T cells. Flag-tagged Ezrin-N3 but not flag-tagged full length Ezrin precipitates HA-Epac1 using beads coupled to anti-flag antibodies (+) but not beads coupled to anti-myc control antibody (-). Western blots also show that equal amounts of flag-tagged Ezrin were precipitated (middle panel) and HA-Epac1 was equally present in the total lysates, which were subsequently divided for the flag and control IP's (lower panel). (C) Co-immunoprecipitation of endogenous Ezrin with anti-Epac1 antibodies (5D3) from lysates of ACHN cells stimulated with sphingosine 1-phosphate (S1P), but not from unstimulated cells (upper panel). Epac1 was equally precipitated by 5D3 antibodies, but was not precipitated by control anti myc antibodies (IgG). Ezrin and Epac1 were present equally in the cell lysates (lower two panels). (D) Domain structure of Epac1: DEP domain also contributes to membrane targetting, nucleotide binding domain (NBD), Ras Exchange Motif (REM), Ras association domain (RA) and CDC25 homology catalytic domain. The deletion mutants used in this study are also shown. (E) Anti-flag blot showing co-immunoprecipitation of flag-Ezrin-N3 by myc-tagged full length Epac1 (Epac), but less by myc-tagged Epac1Δ49 (EpacΔ49). The lower panel shows approximately equal expression of Ezrin-N3 in original lysates.

amino acids of Epac1 (CFP-Epac1-N49) did colocalise with Ezrin at microvilli (Fig. 4A, lower panel). Similarly, GFP-Epac1-N49 also localised to the lumen of epithelial cysts (Fig. 4C). The microscopy findings were confirmed by cell fractionation experiments; deletion of N49 resulted in an increase in Epac1 present in the cytosolic fraction. Deletion of an extended region of the N terminus of Epac1 which includes the DEP domain (Epac1Δ148) resulted in a further increase in cytosolic Epac1, confirming a previous report that the DEP domain contributes to the membrane localisation of Epac1 (Fig. 4D and[2]). Together, these results show that the majority of Epac1 colocalizes with Ezrin and that the N-terminal region

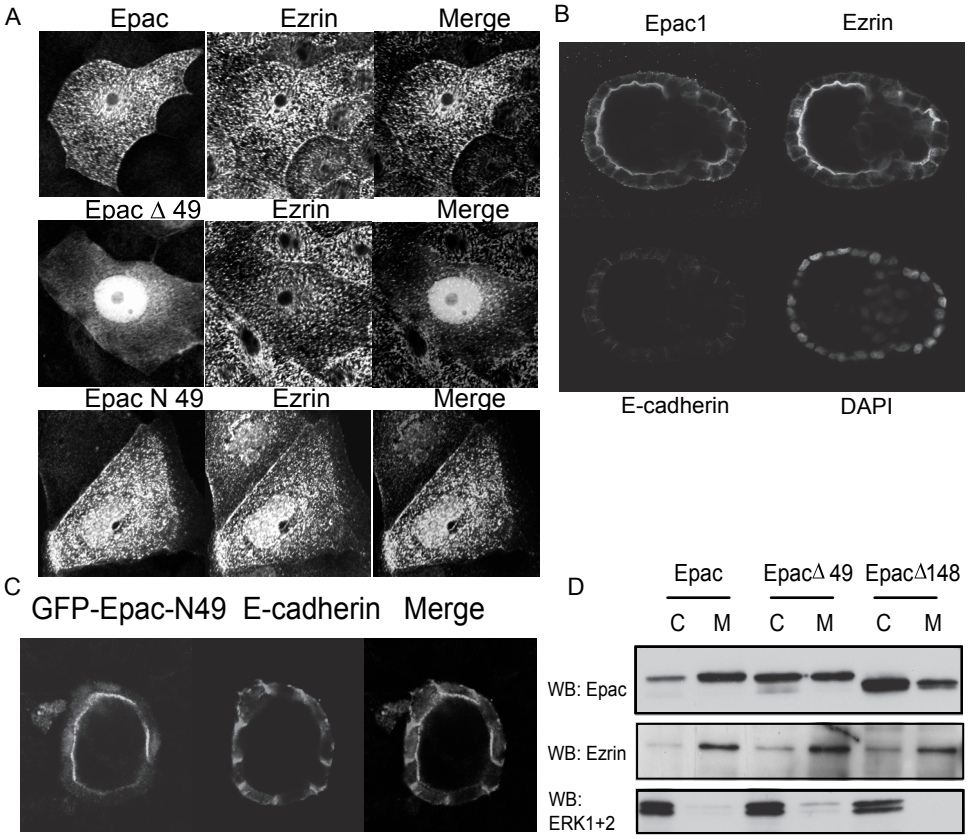

Figure 4. Ezrin colocalisation with Epac1.
(A) Confocal micrographs of MDCK cells transiently transfected with myc-tagged full length Epac1, myc-tagged Epac1Δ49 (lacking the N terminal 49 residues) and CFP fused to the N-terminal 49 amino acids of Epac1 (CFP-Epac1-N49). Cells were labelled with anti-Ezrin antibodies. Myc-tagged Epac1 proteins are labelled with anti-Epac1-1 polyclonal antibodies. CFP fluorescence reveals CFP-Epac1-N49 localisation. Note, cells were fixed 40 hours after transfection and seeding on coverslips and are therefore quite flat. (B) Epac1 expressing MDCK cells grown in a 3-dimensional collagen gel and stained with anti-Epac1 polyclonal antibody, anti-Ezrin monoclonal antibody, anti E-cadherin rat monoclonal antibody (DECMA) and DAPI to label nuclei. (C) Confocal micrographs of MDCK cells stably expressing GFP fused to the N-terminal 49 residues of Epac1 (GFP-Epac1-N49), showing an apical distribution. E-cadherin staining (DECMA) is also shown. (D) Fractionation of MCF-7 cells expressing full length or deletion mutants of Epac1 showing that both the N49 tail and DEP domain contribute to membrane localisation of Epac1. C: cytosol, P: particulate fraction. Erk1/2 and Ezrin are localised predominantly in the cytosol and particulate fractions respectively.

of Epac1 is responsible for the colocalization.

Ezrin targets Epac1 to the apical membrane.

The above findings indicate that Ezrin targets Epac1 to the apical plasma membrane. To test this further, we treated OvCar3 cells with siRNA to deplete Ezrin and examined the localisation of Ezrin and Epac1 by immunofluorescence. The efficiency of Ezrin knockdown varied between cells producing a mosaic of Ezrin expression. We observed that in cells where Ezrin was strongly depleted, Epac1 is localised to the nucleus (Fig. 5 upper panels). We also examined the effect of simultaneous depletion of Ezrin, Radixin and Moesin on Epac1 distribution. Western blot analysis confirmed that all three proteins were depleted by at least 50% (not shown). We observed a similar effect on knockdown of Ezrin and mislocalisation of Epac1 as when Ezrin alone was depleted (Fig. 5 lower panels). From these results we conclude that indeed Ezrin is required for the apical localisation of Epac1 and suggest that in OvCar3 cells, Radixin (and Moesin) do not contribute significantly to this localisation.

Apical targeting of Epac1 is required for efficient Rap1 activation.

To determine whether the apical localisation of Epac1 was important for its function in activating Rap, we expressed deletion mutants of Epac1 in A14 NIH 3T3 fibroblasts and examined Rap1 activation using 'pull-down' assays. Overexpression of full length Epac1 was sufficient to induce HA-Rap1 activation in the absence of additional cAMP-inducing stimulation. We observed that deletion of N49 from Epac1 strongly reduced its capacity to activate Rap1. Deletion of N148, which also includes the DEP domain, completely abolished the capacity of Epac1 to activate Rap1 (Fig. 6A). Thus, the ability of Epac1 to activate Rap1 correlated with its localisation to the plasma membrane (compare with Fig. 4D). Previous studies showed that deletion of the N-terminal 148 amino acids of Epac1 does not affect the regulation of Epac1 activity *in vitro*[2]. Taken together, these findings suggest that localisation of Epac1 via its N-terminal 49 amino acids are required for efficient activation of Rap1 in cells.

Ezrin is required for efficient activation of Rap1 by Epac.

Ezrin is reported to associate both directly and indirectly with the β-adrenergic receptor (β-AR), one of the stimuli that activate Rap1 through Epac[7]. We therefore hypothesized

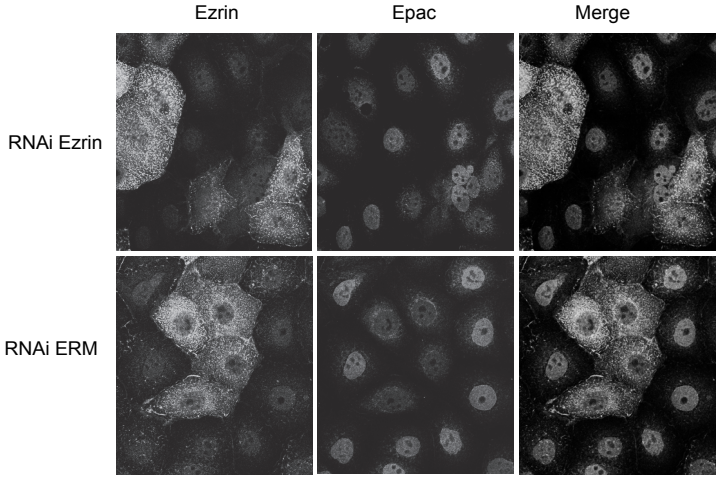

Figure 5. Ezrin is required for Epac1 localisation and function.
Confocal microscopy of OvCar3 cells labelled with anti-Epac1 and anti-Ezrin antibodies following treatment with siRNAi oligos for Ezrin (upper panels) and Ezrin, Radixin and Moesin combined (lower panels). Note that in Ezrin depleted cells, the corresponding localisation of Epac1 is disrupted, resulting in strong nuclear localisation.

that Ezrin brings Epac1 into the proximity of β-AR-induced local increases in cAMP concentrations and is thus required for efficient coupling of β-AR signalling to Rap1. To test this hypothesis, we examined the effect of RNAi-mediated depletion of Ezrin on the activation of Rap1 by β-AR stimulation. Stimulation of OvCar3 cells with isoproteronol to activate β-AR results in activation of Rap1 in OvCar3 cells and also induced Rap1 activation in cells which had been treated with scrambled siRNA. However, Rap1 activation induced by isoproteronol treatment was inhibited in cells depleted of Ezrin (Fig. 6B). Rap1 activation induced by the Epac-specific analog 007 was also inhibited by Ezrin RNAi, although to a lesser extent. Simultaneous depletion of Ezrin, Radixin and Moesin had a similar effect on Rap1 activation as depletion of Ezrin alone. These findings suggest that Ezrin couples adrenergic receptor signalling to Epac-Rap1 activation. Since the effect of Ezrin depletion on Rap1 activation could also be due to more general disruption of microvilli, we examined the effect of inhibiting the Ezrin-Epac1 interaction using the N-terminal 49 amino acids of Epac1, which we predicted would function as a competitive inhibitor of the interaction. Indeed, when overexpressed, GFP-Epac1-N49 results in the release of Epac1 from the microvilli (Fig. 6C). In addition, GFP-Epac1-N49 potently inhibited isoproteronol-induced Rap1 activation and also induced moderate inhibition of 007-induced Rap1 activation (Fig. 6D). We conclude from these experiments that Ezrin-dependent targeting of Epac1 to the apical plasma membrane is required for efficient coupling of β-AR stimulation to Epac-Rap1 signalling.

Discussion

We show here that Epac1 is localised predominantly on microvilli at the apical plasma membrane of various epithelial cell lines. Furthermore, we identified the apical membrane-cytoskeleton cross-linking protein, Ezrin, as a binding partner of Epac1. Ezrin is a member of the Ezrin-Radixin-Moesin (ERM) family of proteins that contain a C-terminal actin binding domain and an N-terminal FERM domain, which interacts with integral membrane proteins. ERM proteins exist either in a closed, auto-inhibited state whereby a C-terminal region interacts with the FERM domain, or in an open conformation which permits association with interacting proteins. The conclusion that Epac1 interacts with Ezrin is based on a number of observations. Firstly, Ezrin binds to Epac1 in a yeast two hybrid screen. Secondly, endogenous Epac1 co-immunoprecipates with endogenous Ezrin. Thirdly, Epac1 and Ezrin co-localize at the apical site of cells. Fourthly, the N-terminal 49 amino acids of Epac1 are required both for apical localisation and for interaction with Ezrin. Finally, knock-down of Ezrin results in a release of Epac1 from the apical surface. We therefore concluded that indeed Ezrin is a *bona fide* interaction partner of Epac1 that targets Epac1 to the apical plasma membrane. An apical localisation of Epac1 was previously described in proximal tubes of the kidney[14], a result confirmed by us using our antibodies (data not shown). This implies that the apical localisation is physiologically relevant.

We mapped the region required for Epac1 binding to the first 492 amino acids of Ezrin, which contains the FERM domain. This region is also required for interaction with other Ezrin binding partners, such as EBP50/NHERF, ICAM-2, CD44 and FAK[35-37]. Further deletions of either the N or C termini disrupted interaction with Epac1 – presumably due to disruption of the tertiary structure of the FERM domain. Epac1 did not bind to full length Ezrin, suggesting that the interaction was suppressed by the C-terminal auto-inhibitory region. Indeed, addition of the C-terminal region of Radixin inhibits Epac1 association

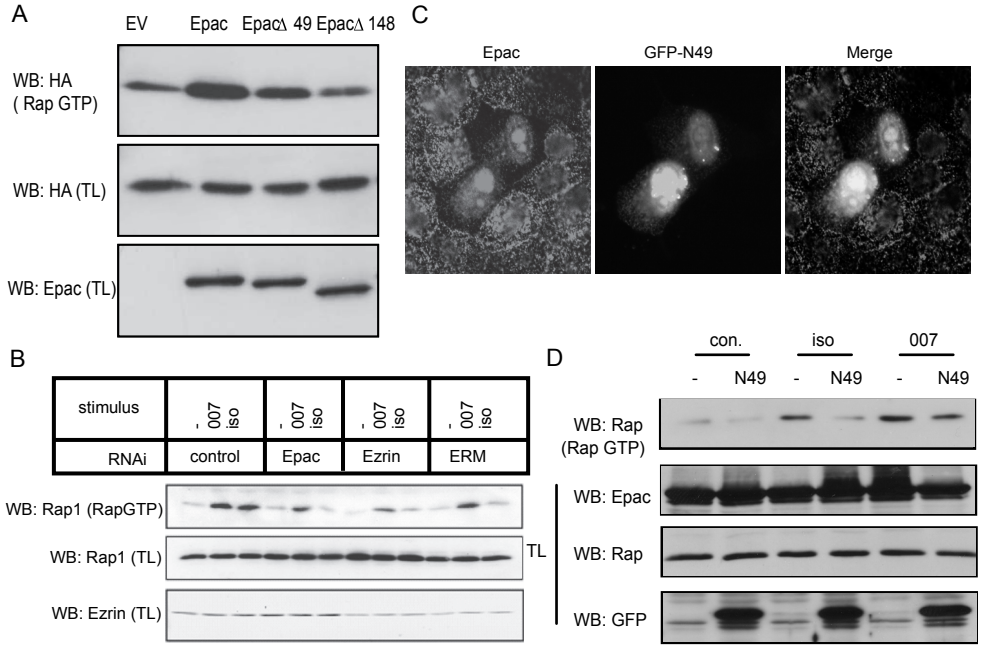

Figure 6. Ezrin-Epac1 interaction is required for Rap1 activation.
(A) Deletion of the N-terminal 49 amino acids and DEP domain of Epac1 impair its capacity to activate Rap. Empty vector, full length myc-Epac1, myc-Epac1-Δ49 and myc-Epac1-Δ148 were transfected into A14 NIH 3T3 cells together with HA-Rap1. Precipitated active HA-Rap1, and expression of HA-Rap1 and myc-Epac1 constructs in total cell lysates are shown. (B) Pull-down assay of active Rap1 from OvCar3 cells treated with siRNA duplexes for Epac1, Ezrin and Ezrin, Radixin and Moesin combined or control oligos. Cells were treated with 007 (100 uM) for 15 minutes, isoproterenol (100 uM) for 2 minutes or buffer control. The upper panel shows the amount of active Rap1 precipitated from cells. The middle panel shows that equivalent levels of Rap1 were present in cell lysates. Ezrin levels werereduced both by Ezrin and by pooled ERM siRNA oligos (Lower panel). (C) Confocal microscopy showing the displacement of Epac1 in cells transiently expressing GFP-Epac1-N49.
(D) Pull down assay of activate Rap from MDCK-Epac1 cells with or without stable expression of GFP-Epac1-N49 and stimulated with 007 (100 uM, 15 minutes) or isoproteronol ('iso', 100 uM, 2 minutes). Rap, Epac1 and GFP-Epac1-N49 expression in total lysates are also shown.

with the N-terminal part of Radixin (not shown). Consistent with a requirement for an open Ezrin conformation, the interaction between endogenous Epac1 and Ezrin was induced upon stimulation with sphingosine-1-phosphate (S1P), which via Rho activation induces PIP2 synthesis, which has been shown to promote Ezrin conformational activation. This indicates that Epac1 activity is subject to dual regulation – firstly by agonists that induce increases in intracellular cAMP and thus Epac1 activation and secondly, by agonists that regulate Ezrin conformational activation and thus Epac1 targetting.
We showed previously that stimulation of the β-AR results in Epac-mediated activation of Rap1[7]. Interestingly, Ezrin also associates both directly and indirectly via EBP50 with the β-AR[29,38], which after stimulation induces cAMP through activation of adenylate cyclase. We therefore tested whether association of Epac1 with Ezrin is required for an efficient coupling of the β-AR to Epac-Rap1 signalling. We observed that depletion of Ezrin by siRNA inhibited Rap1 activation induced by isoproteronol and to a lesser extent by the Epac1

specific analogous 007. This was unlikely to be a consequence of disruption of the apical membrane due to Ezrin loss, since expression of Epac1-N49, which functions as an interfering peptide and releases Epac1 from the apical site, also inhibited isoproteronol-induced Rap1 activation. Importantly, we observed only a partial inhibition of Rap1 activation when the cells were incubated with 007. Also, when we expressed Epac1 lacking the first 49 amino acids, we observed a residual Rap1 activation, indicating that Epac1 that is not linked to the apical membrane can still activate Rap1 although less efficiently. This decreased efficiency is not due to an intrinsic defect of the Epac1 protein, since deletion of the first 148 amino acids has no apparent effect on the ability of Epac1 to activate Rap1 in vitro[2]. We conclude from these results that the targeting of Epac1 to the apical plasma membrane by Ezrin is required for β-AR-mediated activation of Epac1 and subsequent activation of Rap1.

The localisation of Epac1 at the lumenal surface is consistent with recent reports describing a role for Epac1 in the regulation of cytoplasmic pH via the Na^+/H^+ exchanger (NHE3) at the brush border of proximal tubules[14] and exocytosis in the collecting ducts in the kidney[16]. NHE3 also binds directly to Ezrin[39], suggesting that the Ezrin-Epac1 interaction that we describe here is important for NHE3 regulation. Unfortunately, we were thus far unable to make this connection.

Not all functions of Epac can be attributed to a localisation at the apical site of cells. For instance, we have previously reported that Epac-Rap1 signalling enhances integrin-mediated cell adhesion[7] and the formation of E-cadherin-mediated cell-cell contacts[8,9,40]. Interestingly, Ezrin also localises to and regulates cell-cell contacts in epithelial cells[41] and via binding to FAK, mediates focal adhesion formation[27]. Whether the interaction of Epac with Ezrin plays a role in these processes remains to be investigated. It should be noted however, that Epac1 is found at other locations as well, including the plasma membrane and the perinuclear region. We did not observe significant co-staining with the mitochondrial marker mitotracker (not shown) a result which is at variance with a previous report[17]. Epac1 has been observed at the nuclear envelope in other cell types cells[42]. These differences may reflect cell type variation, but more specifically, may be a consequence of differences in apical-basolateral polarity and the lack of competition for Epac1 by apically localised or conformationally active Ezrin. Interestingly, deletion of the N-terminus of Epac1, depletion of Ezrin by RNA interference or overexpression of the isolated N-terminus of Epac1 all resulted in the nuclear localisation of Epac1. The factors regulating the nuclear localisation of Epac1 and the intriguing possibility that Epac1 performs a signalling function in the nucleus require further investigation.

The interaction of Epac1 with Ezrin is also interesting with respect to the observation that Ezrin binds PKA[30]. In addition, Epac1 was found to interact with phosphodiesterase 4D3[18]. This intriguing network of interactions suggests that Ezrin may play host to an entire cAMP-dependent signalling pathway, including the activating β-AR. A similar role was recently described for mAKAP, a muscle specific adaptor protein which coordinates a complex between PKA, Epac1 and PDE4D3[18]. Coordinated activation of PKA and Epac1 on AKAPs may turn out to be a common feature in cAMP-dependent signalling.

We have shown here that Ezrin targets Epac1 to the apical plasma membrane of polarised epithelial cells, coupling adrenergic receptor activation with the Rap signalling pathway. This gives insight into the mechanism of Epac1 control. It was shown previously that Epac, through the cAMP binding domain, interacts with the light chain of microtubule associating protein[43]. Further proteins domains that may mediate interactions are the DEP domain and the RA-domain. Although the interaction partners of these domains are currently unclear, one

can envision complexity in the regulation of Epac1, a feature that seems to be common for GEFs of small GTPases.

Methods

Constructs and antibodies

To generate vectors containing Epac1 full length (aa 2-881), Epac1Δ148 (aa 149-881), Epac1Δ49 (aa 50-881) and Epac1-N49 (aa 1-49), the corresponding coding sequences of human Epac1 (GI:3978530) were introduced into a donor vector, pDONR201 (Invitrogen) to allow sub-cloning by the "Gateway"-procedure (Invitrogen). The constructs were then recombined in pcDNA3-Flag-His, pcDNA3-Myc-, pcDNA-meGFP-, pcDNA-meCFP- and pcDNA3-meYFP-destination vectors according to standard protocols (Invitrogen). Ezrin was obtained as clone IRALp962A182.1 from the Deutsches Ressourcenzentrum für Genomforschung (RZPD) (Berlin, Germany). Using Gateway adapted PCR, we amplified Ezrin and Ezrin-N3 (residues 1 to 492) and cloned these into pDONR201 and then into pcDNA3-flag as above. pcDNA-meCFP and pcDNA-meYFP were kindly provided by O. Rocks and P. Bastiaens (EMBL, Heidelberg, Germany). Ezrin-GFP was kindly provided by Erik Sahai (Cancer Research UK, London).

The mouse Epac1 monoclonal antibody (mAb) 5D3 and the rabbit Epac1 polyclonal antibody (pAb) (α −Epac1-1) were raised against recombinant Epac1Δ148. Rabbit pAb against ZO-1 was from Zymed. Anti-Rat-E-cadherin mAb (DECMA) was from Sigma. Ezrin was from Becton Dickinson. Anti-flag was from Kodak. Anti-HA monoclonal antibody 12CA5 was purified by our laboratory. Anti-ERK1/2 rabbit pAb was generated by our laboratory. Goat-anti-mouse/rabbit/rat Alexa 405,488,568 and 647 secondary antibodies were from Molecular Probes. Fluorescein and Rhodamine phalloidin was from Sigma.

Cell culture:

MDCK-II, MCF7, 293T and A14 cells were routinely cultured in DMEM provided with 10% FCS, 0.5% glutamine, penicillin and streptomycin (all from BioWhittaker, Belgium) at 37 °C in 6.0 % CO2. OvCar3 cells were from ATCC and were cultured as above in RPMI. ACHN cells were cultured in Eagle's MEM contained 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The MDCK Epac1 cell line was generated and cultured as described before8.

Yeast 2-hybrid screening

A human placenta random primed library was custom-screened with Epac1 full length and Epac1 residues 1-328 (Epac1: RapGEF 3, homo sapiens, GI: 3978530) by Hybrigenics S.A. (Paris, France) as previously described[44].

Transfection.

MCF7 were transfected with the indicated constructs using FuGENE 6 (Roche, Diagnostics). MDCK cells were washed with PBS containing 1 mM EGTA, detached by trypsinisation (PBS + 0.2% trypsin, 1 mM EDTA), resuspended in complete medium at a density of $1X10^7$ cells/ml and transfected with 20ug of the indicated constructs by electroporation (1000F, 260v). A14 cells were transfected with using the calcium phosphate method. Cells were lysed or fixed 40 h after transfection.

For si-RNA transfection of OvCar3 cells, siRNA duplexes were diluted in Optimem (Gibco).

Oligofectamine (Invitrogen) was added and the mix added to cells according to manufacturers instructions. siRNA oligos were as follows: Epac1 (No.1 GACCGGAAGTACCACCTTA; No.2 CCATCATCCTGCGAGAAGA; No.3:GCACCTACGTCTGCAACAA) were from Dharmacon, siRNA's against Ezrin (No.1GAACAGACCTTTGGCTTGGAGTTGA; No.2 TGGCCTCCACTATGTGGATAATAAA; No.3 CCTCAAAGAGTGATGGACCAGCACA), against Radixin (GCCAGGCTACCTGGCTAATGATAGA), and Moesin (TGGCCTCGTATGCTGTCCAGTCTAA) were from Invitrogen. Ezrin siRNA is the mixture of No.1, 2 and 3; ERM siRNAi is the mixture of Ezrin No.1, Radixin and Moesin oligos).

Co-immunoprecipitation
293T cells were transiently transfected with 10 μg of plasmid DNA as indicated in the figure legends. After culturing for 40 h, cells were lysed in 1 ml of 1% Triton X-100 buffer containing additionally 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, aprotinin (1 μg/ml), and pepstatin (1 μg/ml). Cell lysates containing equal amounts of total protein were incubated for 2 h at 4°C with 10 μl of antibody pre-coupled protein A-Sepharose beads (Pharmacia). The beads were washed extensively with lysis buffer and bound proteins were analysed by SDS-PAGE and Western blotting. For immunoprecipitation of Epac1 from ACHN cells, cells were lysed in a buffer containing 20 mM Tris pH 8.0; 1% Triton X-100, 0.5% Na-DOC; 10 mM EDTA; 150 mM NaCl and protease inhibitors. After clearing the lysate, lysates were incubated in the presence of 1 mM 8-Br-cAMP (MP biochemicals) with anti-Epac1 monoclonal antibody 5D3 and protein A beads for 2 h. 8-Br-cAMP is included in the IP since binding of Epac1 5D3 is dependent on an open (cAMP-bound) conformation (JZ unpublished findings). Precipitates were washed and subjected to SDS-PAGE and Western blotting.

Subcellular fractionation
MCF7 cells were treated with hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT and 1 mM $NaVO_3$, 1 μM leupeptin, 1 μM aprotinin) and homogenized through a 23G 11/4 microlance**.** Trypan blue staining of the cell lysate showed that more than 90% of the cells were broken. The lysate was centrifuged directly at 100,000g at 4°C for 90 minutes and the cytosol-containing supernatant was removed and SDS-sample buffer added (C). The pellet, containing all non-cytosolic material, was washed in lysis buffer and dissolved in sample buffer (P).

Immunofluorescence of cells cultured in 2-dimensions and in 3-dimensional collagen gels.
Cells were cultured on glass coverslips. Before fixation, medium was removed and cells were washed three times with ice-cold PBS. Fixation was performed with ice-cold methanol for 2 min for OvCar3 cells or with 3.8% formaldehyde for 20 min for MDCK cells, followed by permeabilisation with 0.2% TritonX-100 for 10 min. The samples were incubated with blocking buffer containing 4% goat serum and 0.2% bovine serum albumin (BSA) in PBS for 1 h. Cells were labelled with primary antibody for 2 h followed by washing 3 times with PBS. Alexa-conjugated secondary antibodies were applied for 1h. MDCK cells were cultured in collagen I gels as previously described[45]. Briefly, MDCK-II cells stably expressing Epac1 were suspended in a solution containing in 1% collagen and 3.7g/l $NaHCO_3$ in complete medium and added to 24-well plates and allowed to polymerise at 37°C for 20 min. Complete medium was added and replaced every 3 days. For tubulogenesis experiments, to prepare

gels for microscopy, gels were incubated with collagenase (Sigma), (5ug/ml), then fixed for 30 minutes in a solution containing 3.7% formaldehyde, 10 mM PIPES pH 6.8, 0.3 M sucrose, 100 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.1% Triton X-100. Gels were washed, permeabilised further with 0.2% Triton X-100 for 5 min , blocked for 1 h with 10% FBS with 0.01 M glycine in PBS. Gels were incubated overnight in primary antibody, washed 3 times in PBS and then incubated for at least 3 h with secondary antibody. Gels were washed extensively with PBS and mounted between glass slide and cover slip in Immu-mount (Shandon). Images were recorded using a LSM510 laser scanning confocal microscope (Zeiss Microimaging). All pictures were obtained by confocal microscopy.

Electron Microscopy
MDCK Cells were fixed by adding 4% freshly prepared formaldehyde and 0.4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 to an equal volume of culture medium for 10 min, followed by post-fixation in 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 without medium. Cells were stored until further processing in 1% formaldehyde at 4ºC. Processing of cells for ultrathin cryosectioning and immuno-labeling according to the protein A-gold method was done as described[46]. In brief, fixed cells were washed with 0.05 M glycine in PBS, scraped gently from the dish in PBS containing 1% gelatin and pelleted in 12% gelatin in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose and afterwards mounted on aluminum pins and frozen in liquid nitrogen. To pick up ultra thin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used[47].

Rap1 pull down and Western blotting
OvCar3 cells were seeded in 10 cm dishes at 40% confluence the day before treatment with siRNA duplexes as described above. A14 cells were transiently transfected with the constructs as indicated. Active Rap1 was determined by 'pull-down' assay as described previously[48]. In brief, cells were stimulated as described, washed with ice-cold PBS and lysed in a buffer containing 1%NP40, 150 mM NaCl, 50 mM Tris-Cl pH 7.4, 10% glycerol, 2 mM $MgCl_2$ with leupeptin and aprotinin. After clearing by centrifugation, active Rap1 was precipitated with the recombinant Rap1 binding domain (RBD) of RalGDS fused to GST immobilized on glutathione beads. Active Rap1 precipitates and samples of total cell lysate were resolved by SDS-PAGE, transferred to PVDF membrane and probed with the appropriate primary and secondary antibodies.

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Addendum

Identification of Epac and PDZ-GEF interacting proteins

Zhongchun Zhang, Holger Rehmann, John de Koning and Johannes L. Bos

Introduction

Proteins of the Epac- and PDZ-GEF-family are Rap specific guanine nucleotide exchange factors (GEFs). Rap proteins are small G-proteins belonging to the Ras family, which cycle between a GTP bound active and a GDP bound inactive state. The exchange of GDP to GTP is catalysed by GEFs. Only in its GTP bound state Rap can interact with a couple of effector proteins, such are Rassf3 or Riam, and thereby control a variety of cellular effects. The most prominent understanding of Rap1 function is its role in integrin-mediated cell adhesion and E-cadherin mediated cell junction formation (1-4).

Epac1 is a multi-domain protein that consists of a regulatory and a catalytic region. The regulatory region contains a Disheveled, Egl-10, Pleckstrin (DEP) domain and a cyclic nucleotide binding (CNB) domain (5); the catalytic region contains a Ras exchanger motif (REM) domain, an ubiquitin-like Ras association (RA) domain, and a CDC25-homolog domain (CDC25-HD) (Fig. 1). Epac2 has an additional N-terminal CNB domain (CNB-A). The catalytic activity towards Rap is mediated by the CDC25-HD, which is found together with a REM-domain in all GEFs for members of the Ras family. The REM domain shields hydrophobic surface areas of the CDC25-HD against the solvent and has thus a mainly structural function by stabilising the fold of the CDC25-HD. Epac is inactive in the absence of cAMP and activated upon binding of cAMP to the CNB domain. The second CNB domain of Epac2 is sufficient to keep the protein in the auto-inhibited state in the absence of cAMP and thus the function of the first CNB domain, which has a lower affinity for cAMP than that of the second CNB domain, is still elusive. Membrane localisation of Epac is mediated by the DEP domain, as demonstrated by immuno-fluorescence studies. The RA-domain of Epac2 interacts with RasGTP and this interaction contributes to the determination of the cellular localisation of Epac2.

Both PDZ-GEF1 and PDZ-GEF2 are ubiquitously expressed with highest expression levels in brain and placenta (6,7). Both proteins contain an atypical cyclic nucleotide binding (aCNB) domain, a REM domain, a PDZ domain, RA domain and a CDC25-HD. PDZ domains are typically found in scaffold proteins, where they are mediating protein-protein interactions. PDZ-domains specifically bind to the C-termini of proteins, which are characterised by the consensus sequence X-S/T-X-V/L or X-Φ-X-Φ (8) (Φ, hydrophobic amino acid; X, unspecified amino acid). It is currently unclear, how PDZ-GEF is regulated. The aCNB domains are neither able to bind cAMP nor cGMP, which is in agreement with the observation that these

domains are lacking the typical consensus motives for cyclic nucleotide binding. It might be that a yet unidentified second messenger or protein is binding to the aCNB domain of PDZ-GEF and thereby activating the protein in a similar manner as in the case of cAMP and Epac.

Signalling by Epac and PDZ-GEF takes place in the context of a protein-protein interaction network. This network controls the temporal and spatial localisation of the GEFs in the cell and thus adding an important level of regulation to the biological system. Epac1, for example, interacts with phosphodiesterase 4D (PDE). PDEs catalyse the breakdown of cAMP and cGMP to AMP and GMP, respectively. Together with PDE4D, Epac is found in a complex together with A-kinase anchoring protein (AKAP) and protein kinase A (PKA), allowing a tightly controlled cross-talk between the PKA and the Epac pathway as well as a negative feedback control of cAMP signalling (9). The identification of novel Epac or PDZ-GEF interacting proteins is therefore of high interest and fundamental to a comprehensive understanding of the biological function of these proteins. To do so we initiated yeast-two-hybrid screens using both Epac and PDZ-GEF as a bait. In the screens we identified known interaction partners of Epac and PDZ-GEF, which validate this method. More notably, the novel interactions identified give a new light on Epac and PDZ-GEF functions.

Material and Methods

Baits encoding both full-length proteins and protein fragments were constructed, namely the regulatory domain of Epac1 (aa 1-328), full-length Epac1 (aa 2-881), full-length *Drosophila* Epac (aa 1-993), the regulatory domain of *Drosophila* Epac (aa 1-335), the catalytic domain of *Drosophila* Epac (aa 335-993), full-length PDZ-GEF1 (aa 1-1499) and full-length PDZ-GEF2 (aa 1-1601). Yeast two-hybrid screens were carried out by Hybrigenics S.A. (Paris, France) as previously described (10,11).

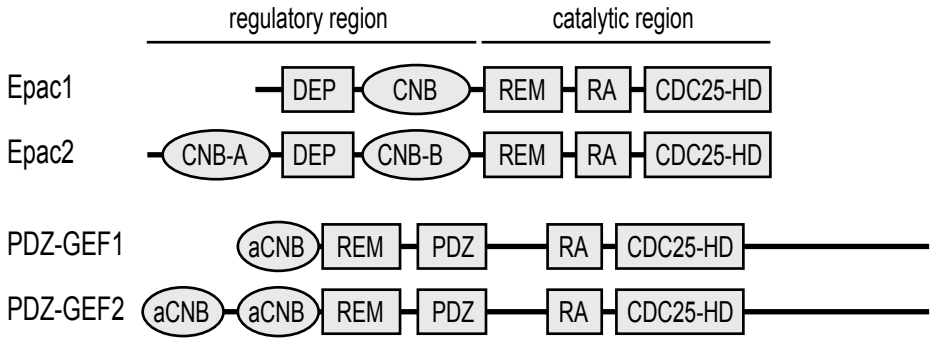

Fig. 1 Domain organization of Epac and PDZ-GEF. cAMP, cAMP-binding domain; DEP, Disheveled, Egl-10, Pleckstrin; CNB, cyclic nucleotide binding; REM, Ras exchanger motif; RA, Ras association; CDC25-HD, CDC25-homolog domain; aCNB, atypical cyclic nucleotide binding domain; PDZ, PSD-95, Dlg, ZO-1 domain.

Results

The crystal structure of Epac2 in its auto-inhibited conformation has demonstrated that in the absence of cAMP the CNB-domains are positioned in such a way that the access of Rap to the catalytic site is blocked. Thus relative large surface areas in the regulatory and the catalytic region are masked by an internal interaction between the regulatory and the catalytic regions. Therefore we generated several bait constructs of Epac and PDZ-GEF (see Fig.1 and Material and Methods) and subjected not only the full length proteins to the yeast-screen, but also vectors containing the regulatory and the catalytic regions separately. In the latter more surface areas are accessible for protein-protein interactions and the screen might result in candidates, which can interact with the "active"conformation of Epac.

	Gene name	Cat	GI number	description/function
Bait	**Drosophila Epac**			
	Ras	A	563359	oncogene, signal transduction
	Akt	A	24647358	serine-threonine protein kinase
	Rm62	A	24644479	DEAD box protein, RNAi machiner
	eIF3-S9	A	24654452	translation initiation factor
	mitochondrial ribosomal protein S31	A	17977676	protein synthesis in mitochondrial
	CG3304	B	45550116	unknown
Bait	**human Epac1-FL**			
	Ran binding protein 2	A	6382079	nucleocytoplasmic transport
	radixin	A	33990951	membrane cytoskeleton crosslinker
Bait	**human Epac1-RD**			
	CHD4	A	24047226	DEAD box protein, transcription rep
	bone morphogenetic protein 1	B	75517262	cell differentiation
	Radixin	B	33990951	membrane cytoskeleton crosslinker
	zinc finger protein 406	B	33878189	transcription regulation
	DLG5	B	19070635	scaffold protein
	U2 small nuclear RNA auxiliary factor 2	B	33873977	RNA splicing
	FACTP140	B	5499741	transcription initiation
Bait	**human Epac2**			
	heterogeneous nuclear ribonucleoprotein M	A	40555782	RNA splicing
	Epac1	A	46250001	cAMP target protein
	Kiaa 0663	A	40788320	unknown
	DDX23	A	41327771	DEAD box protein, RNA splicing
	FLJ10074	A	61354688	serine/threonine kinase
	clathrin	A	30353925	endocytosis
	synaptojanin2	B	27695876	endocytosis
	CDC25B	B	56204123	cell cycle control
	RNA binding motif protein 21	C	12383073	Unknown
	HT010	C	74214647	unknown
	utrophin	C	57162444	cytoskeletal protein

Table 1 Overview of yeast two-hybrid results from Epac. Columns depict putative interaction picked up in yeast two-hybrid screens, reliability of the interaction and description or function of the gene. The scores are listed whereby A stands for high reliability and E for low reliability.

Yeast two-hybrid screens were carried out by Hybrigenics S.A. (Paris, France). The obtained results are listed in Table 1 and 2. All hits were scored for reliability based on an algorism developed by Hybrigenics. The algorism takes into account not only how many independent fragments were picked up in a screen, but also how frequently each fragment was picked up in the same library with different baits. A fragment that is picked up more frequently and/or more independently of the nature of the bait is likely to represent a more unspecific interaction. The scores are listed in table 1 and 2 and are given by five categories A to E, whereby A stands for a high reliability and E for a low reliability. For human Epac and PDZ-GEF a human placenta library was screened, and we also used the *Drosophila* Epac to screen a *Drosophila* library. The yeast two-hybrid screens resulted in approximately 30 candidate proteins (Table 1 and Table 2), which will be discussed in more detail below.

Epac interaction proteins

Vesicle associated proteins

Screens using Epac2 as bait identified Clathrin light chain and Synaptojanin2 as interacting proteins. Both proteins are involved in endocytosis, a process which is mainly mediated by clathrin coated vesicle. Endocytosis is initiated by the enrichment of clathrin at a certain place of the plasma membrane. The heavy and the light chains of clathrin associate with each other and form a basket-like structure that divides the vesicle from the membrane and surrounds it from the outside. Synaptojanin2 is a ubiquitously expressed polyphosphoinositide phosphatase. Depletion of Synaptojanin2 results in a strong defect in clathrin-mediated receptor internalization due to a decrease in clathrin-coated pits and vesicles.

Epac2 was shown to potentate insulin secretion in pancreatic β-cells (12) and a function of Epac1 in Ca^{2+} mediated acrosomal exocytosis was suggested (13). The maintenance of a constant cell volume requires a well controlled balance between endocytosis and exocytosis. In this context the putative involvement of Epac in both processes is highly interesting.

Interestingly, endocytic component has also been reported to negatively regulate Ras signalling independent of its endocytic function (14). Therefore, it will be also interesting to investigate the function of Epac2 on endocytosis as well as the function of the endocytic proteins on Epac/Rap signal transduction pathway.

ERM proteins

Ezrin and Radixin were obtained from screens using full-length Epac1 and the regulatory region of Epac1 as baits. Ezrin and Radixin are members of the ERM protein family (chapter 1). The interaction between Epac and ERM proteins were confirmed and characterised in detail in chapter 4 and 5 of this thesis.

CDC25B

CDC25B was picked up using the regulatory region of Epac2 as bait. CDC25-proteins are phosphatases that specifically dephosphorylate tyrosine and threonine. Three isoforms,

CDC25A, B and C, exist in mammalian cells, which play a key role in cell cycle control and checkpoint response. CDC25B was traditionally assumed to be active only during the late G2-phase and mitosis. However, recent evidences suggest that all three isoforms regulate cell division in a cooperation manner (15). Interestingly, CDC25B shuttles between the nucleus and cytoplasm, which is mediated by the interaction of CDC25B with 14-3-3 proteins and by its nuclear export signal (NES) sequences and nuclear localisation sequences (NLS). The interaction between Epac2 and CDC25B may link Epac and Rap signalling to cell cycle control and cell growth. In addition, this interaction may explain the nuclear localisation of Epac (J.Zhao private communication).

RanBP2

In the yeast two-hybrid screen using the regulatory region of Epac1 as bait, a multitude of fragments belonging to RanBP2 were isolated (Fig. 2). RanBP2 is a protein of 358 kDa in size that comprises eight zinc-finger motifs and four Ran binding domains (RanBDs) (16,17). All fragments identified by the yeast screen are coding for parts of the zinc-finger region, but there is no sequence in common between all of the fragments. This implies either the existence of two independent binding sites for Epac1 in RanBP2 or an artefact in the yeast reporter system caused by the zinc finger domains. Thus the putative interaction between RanBP2 and Epac1 requires further conformation by techniques independent of the yeast system, such as co-immunoprecipitation assay and GST-pulldown assay.

Studies by Qiao et al. have shown a cell cycle dependent localisation of Epac1 (18). During interphase Epac is localized to nuclear membrane and mitochondria and is localized to mitotic spindle and centrosomes in metaphase. RanBP2 is part of the nuclear pore complex and is involved in the Ran mediated nuclear import and export processes. Recent studies in our laboratory have shown that Epac1 is partially localised in the nucleus and the nuclear localisation of Epac1 is enhanced upon disruption of its membrane localisation e.g. loss of the anchoring protein (chapter 4 and J. Zhao private communication). Therefore this interaction is of putative interest in the light of these biological findings. It might generate valuable insight into the regulation of Epac1 localisation.

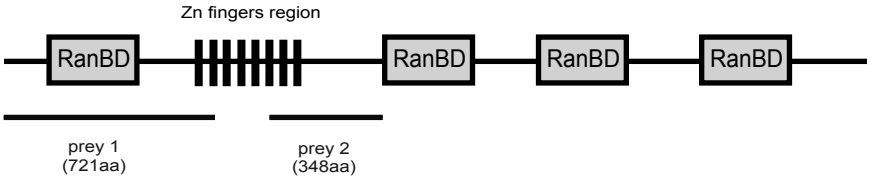

Fig. 2 Domain organization of RanBP2. RanBP2 contains eight zinc-finger motifs and four Ran binding domains (RanBDs). The fragments isolated from the yeast two-hybrid screen are located in different regions on the protein.

DLG-5

DLG-5 was found to interact with the regulatory region of Epac1. DLG-5 protein belongs to the membrane-associated guanylate kinase (MAGUK) family and contains a NH_2-terminal coiled-coil-like domain, four PDZ domains, a Src homology 3 (SH3) domain and a prototypical guanylate kinase (GuK) domain. As a scaffold protein, DLG-5 recruits junction proteins such as Vinexin-β and β-Catenin at cell-cell contact sites (19). Previous study showed that Epac is also localised at cell-cell contact sites (20), whereas, the mechanism of this localization is unknown.

DEAD-box proteins

Several DEAD-box proteins were identified as interacting proteins for both Epac1 and Epac2 (Fig. 3). DEAD box polypeptide 17 (DDX17) and CHD4 were found as interacting protein with the regulatory region of Epac1. DEAD box polypeptide 23 (DDX23) was found to interact with the regulatory region of Epac2 and Rm62 was identified in the screen by using the catalytic region of *Drosophila* Epac.

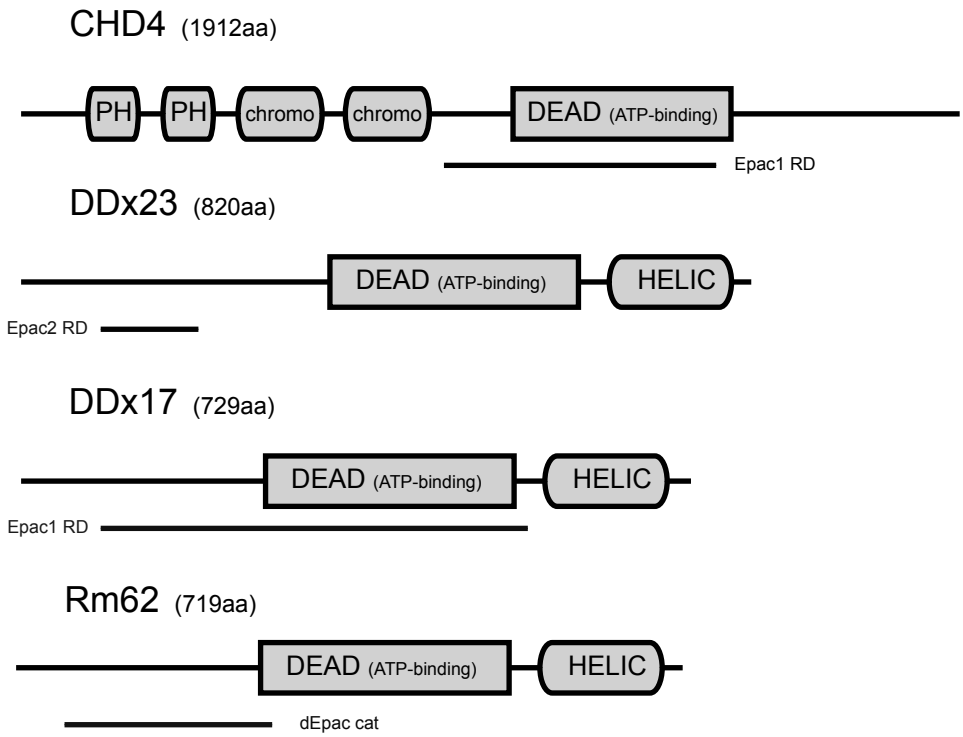

Fig. 3 Domain organization of DEAD-box proteins. Overview of DEAD-box containing proteins isolated in yeast two-hybrid screens with Epac is shown. Both the fragments isolated from the yeast two-hybrid screens (preys) and the baits used in the screens were indicated. HELIC stands for helicase superfamily c-terminal domain; PH stands for pleckstrin homology domain; Chromo stands for Chromatin organization modifier domain; Epac1 RD stands for regulatory domain of Epac1; Epac2 RD stands for regulatory domain of Epac2; dEpac cat stands for catalytic region of *Drosophila* Epac.

The DEAD-box protein family of RNA helicases forms a widely dispersed family of proteins found in all organisms. They are named according to the conserved amino-acid sequence Asp-Glu-Ala-Asp. DEAD-box proteins are required in many processes where RNA is involved, including transcription and translation, RNA splicing, RNA export from the nucleus, RNA degradation, nonsense-mediated decay (NMD), ribosome biogenesis and RNA interference (21). DDX17 is involved in U1snRNP (small nuclear ribonucleoprotein) mediated pre-mRNA splicing (22). DDX23 is a homolog of Prp28p, a component of the U5 snRNP complex. Both biochemical and genetic studies showed that Prp28 facilitates the exchange of U1 at the 5' splice site (23,24). Chromodomain helicase DNA-binding protein 4 (CHD4) is an ATP-dependent, nucleosome remodelling subunit of the Nucleosome Remodeling and Histone Deacetylation (NURD) complex. It is required for the transcription repression function of transcriptional compressor NGF1-A/EGR1-Binding protein (NAB2) (25). Rm62 unwinds dsRNA in an ATP-dependent manner and is necessary for efficient RNAi function (26,27). Recent study showed that Rm62 negatively regulates the insulator activity and nuclear organisation, suggesting a regulatory function of the RNAi machinery on nuclear architecture organization (28).

The interaction between DEAD box proteins and Epac that are described here, might link Epac signalling to RNA-mediated processes. However, different regions of Epac appear to interact with different regions of DEAD box proteins (Fig.3) and it is thus not possible to define a common interaction motif between Epac and DEAD box proteins.

Ras

Both full length *Drosophila* Epac and the catalytic region of *Drosophila* Epac identified Ras as an Epac interacting protein. This is in agreement with the presence of a RA-domain in the catalytic region of Epac. To confirm the interaction between an Epac protein and Ras, we analysed this interaction *in vitro*. Binding of effector proteins to small G-protein often results in a reduced exchange of nucleotide bound to the G-protein (GDI-effect) (29). The

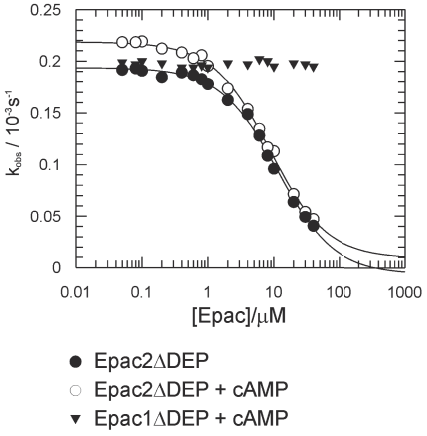

Fig. 4 Epac2 interacts with Ras
Dissociation rate constant of mant-GppNHp from Ras in dependence of Epac concentration at 37°C. The rate constant was measured. 100 nM H-Ras loaded with the non hydrolyseable, fluorescent GTP analog 2'-/3'-O-(N-methylanthraniloyl)-guanyl-5'-yl- imidodiphosphate (mGppNHp) were incubated in the presence of 10 μM non labeled nucleotide and various concentrations of the different Epac protein in the presence or absence of cAMP. Fluorescence was measured with a Cary Eclipse (Varian, Australia) equipped with a temperature controlled multi-cuvette holder.

dissociation of a fluorescently-labelled nucleotide bound to Ras can be detected as a decrease of fluorescence intensity in the presence of an excess unlabelled nucleotide. Indeed, increasing concentration of Epac2ΔDEP, which still contains the RA-domain, reduced the exchange rate of nucleotide bound to H-Ras and an affinity for 10μM was found (Fig. 4). The interaction was shown to be independent of the presence of cyclic nucleotide. Opposite to Epac2, Epac1 is not able to interact with Ras. These results are in agreement with data recently published by the group of Quilliam, who showed that the interaction of Ras and Epac2 contributes to proper localisation of Epac2. The interaction between Epac2 and H-Ras was demonstrated here for the first time *in vitro*, and validates the yeast two-hybrid approach.

PDZ-GEF interaction proteins

RalGDS and ARRB1 (arrestin-beta 1)

For both PDZ-GEF1 and PDZ-GEF2, an interaction with RalGDS was identified. β-arrestin was found to interact with PDZ-GEF2. RalGDS and β-arrestin are both components of the Ral pathway (30,31). RalGDS is a GEF for RalA and RalB (32). It contains a RA domain, which interacts with RasGTP. Binding of RalGDS to RasGTP leads to the trans-location of RalGDS to the plasma membrane, where it activates Ral. Ral is thus a downstream effector of Ras signalling and an important regulator of the exocyst complex (33).

β-arrestin is an ubiquitously expressed multifunctional adaptor protein. It was originally identified due to its function in the regulation of GPCR (G protein coupled receptor) (34). β-arrestin associates with agonist-evoked GPCRs, which have been phosphorylated by G protein

	Gene name	Cat	GI number	description/function
Bait	**PDZ-GEF1**			
	WWP1	A	2072501	E3 ligase
	Rap2A	A	57160784	small GTPase, signal transduction
	PDZ-GEF1	A	63990428	guanine nucleotide exchange factor
	Ras	A	47117697	small GTPase, signal transduction
	RalGDS	B	37589539	guanine nucleotide exchange factor
	Alex3	B	7576448	tumor suppression
	Rap1B	C	47115315	small GTPase, signal transduction
	TGM2	C	6706797	tissue transglutaminase
Bait	**PDZ-GEF2**			
	MAGI-3	A	12003994	scaffold protein
	RalGDS	D	24307909	guanine nucleotide exchange factor
	PDZK3	D	87196343	cell proliferation
	ARRB1	D	13177715	endocytosis

Table 2 Overview of yeast two-hybrid results from PDZ-GEF. Columns depict putative interaction picked up in yeast two-hybrid screens, reliability of the interaction and description or function of the gene. The scores are listed whereby A stands for highly reliability and E for low reliability.

receptor kinases (GRKs) and mediates homologous receptor desensitization. In addition, β-arrestin functions as an adaptor in the clathrin-dependent endocytosis (35). Recently β-arrestin was implied in the regulation of small G-proteins. β–arrestin2 interacts directly with RalGDS, resulting in cytoskeletal rearrangement (31). Upon agonist stimulation of the receptor, β–arrestin1 serves as a scaffold for the exchange factor ARNO (ARF nucleotide exchange factor) and ARF6 (36). Finally, β–arrestin1 was shown to be criticaly involved in Gαq/11 mediated RhoA activation and subsequent stress fiber formation (37).

The results presented here, suggest a link between β–arrestin and Rap signalling. The interaction between RalGDS and PDZ-GEF might mediate a spatial connection between Ral and Rap signalling.

WWP1

WWP1 is a ubiquitous expressed E3 ligase that contains four tandem WW domains and a HECT (homologous to the E6-associated protein carboxyl terminus) domain (38). The WW domain consists of approximately 40 amino acids that bind to the PY motif, a proline-rich sequence followed by a tyrosine residue. Like other E3 ubiquitin ligases, WWP1 is critical in determining substrates specificity and subsequent degradation. WWP1 was shown to target Smad, a gene regulatory protein, for degradation and regulate Smad-dependent signalling pathway activated by TGF-β (39,40). WWP1 was also reported to mediate ubiquitination of human Krupper-like factor (KLF5), an important transcription factor in human carcinogenesis (41). Interestingly, the recognition motif of the first and third WW domains of WWP1 (42), namely PPXY (P: proline; X: any residue; Y: tyrosine), is present in both PDZ-GEF1 and PDZ-GEF2. Therefore, the interaction between PDZ-GEF and WWP1 may suggest a mechanism of how PDZ-GEF activity is regulated in a post-translational level.

MAGI-3

MAGI (membrane-associated guanylate kinase with inverted orientation) proteins are one of the five subgroups of MAGUKs, which are suggested to function as molecular scaffolds in cells. The MAGI protein family consists of three members MAGI-1, MAGI-2 and MAGI-3 (43). As a common structure feature all MAGI proteins contain six PDZ domains, a guanylate kinase domain and two WW domains. The name of MAGI proteins comes from the inverted ordering of their protein-protein interaction domains, which is different from the other MAGUK proteins. It was shown previously that MAGI-1 associates with PDZ-GEF1 in vascular endothelial cells and it is important for Rap1 activation upon cell-cell contact (44). PDZ-GEF1 was also shown to interact with MAGI-2, which is a synaptic scaffolding molecule (45). The localization of MAGI-2 on the synaptic structure implies that PDZ-GEF1 could be localized there as well. In spite of the studies on PDZ-GEF1 and MAGI family proteins, little is know about the relation between PDZ-GEF2 and MAGI. Previous studies suggested that MAGI-3 plays important roles as a scaffold protein in different signaling pathways and recruits signalling molecules to cell surface (46,47). The interaction between

PDZ-GEF2 and MAGI-3 may gain insight into the function and localization of PDZ-GEF2 in a MAGI-3 dependent manner.

PDZD2

PDZD2 (PDZ-domain-containing 2; also known as PAPIN, AIPC, PDZK3 and PIN1) is a multi-PDZ protein expressed in pancreas and many other tissues (48). The PDZD2 C-terminus shares high amino acids similarity with pro-interleukin-16 (pro-IL-16), a precursor of T lymphocyte chemoattractant factor IL-16 (49). PDZD2 is localised mainly to the endoplasmic reticulum (ER). Like pro-IL-16, PDZD2 is proteolytically cleaved at its C-terminus to generate a secreted peptide. sPDZD2 (secreted PDZD2) contains two PDZ domains and is highly expressed in pancreatic islet β-cells. PDZD2 stimulates the proliferation of the insulinoma cell line INS-1E and suppresses slightly the insulin promoter activity at a high concentration (50).

Conclusion

In the study presented here we aimed for the identification of novel Epac and PDZ-GEF interacting proteins. For this we have chosen a yeast two-hybrid approach and indeed we identified a couple of putative interacting proteins. The identification of some proteins like MAGI for PDZ-GEF or Ras for Epac, which were already known to interact with the bait proteins, proves the validity of this approach.

In general, only a few proteins were identified to interact with both PDZ-GEF1 and PDZ-GEF2 or Epac1 and Epac2, indicating a certain specificity of the GEFs. It is thus likely that the different isoforms of the GEFs function both in common and distinguish pathways. The identified interacting proteins are ranging from scaffolding proteins to enzymes with their own catalytic activities. The scaffolding proteins might contribute to the temporal and spatial organisation of Rap signalling. It requires clearly further experiments to validate the interactions found here by independent methods. However, the detailed analysis of the interaction between Epac and Ezrin and Radixin, which is described in Chapter 4 and 5 of this thesis, is the first step towards a more comprehensive evaluation of all interactions described here and an example for the fruitfulness of the yeast hybrid approach.

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Chapter

5

Active mutant of Radixin binds Epac1

To be submitted

Active mutant of Radixin binds Epac1

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Abstract

The Ezrin-Radixin-Moesin (ERM) proteins function as cross-linkers between cell membrane and cytoskeleton by binding to membrane proteins via their N-terminal FERM-domain and F-actin via their C-terminal tail domain. Previous studies from our laboratory have shown that Ezrin targets Epac1 to the apical surface and couples receptor activation to Rap1 signalling. In the present study, we characterised a novel Radixin mutant, which results in a constitutive active Radixin by abolishing the intramolecular interaction of the FERM-domain with the C-terminal tail domain. Using this mutant we show that Epac1 interacts specifically with the active conformation of Radixin. Furthermore, we demonstrate that Radixin interacts with Epac1 but not Epac2 and the isolated C-terminal tail domain is sufficient to inhibit the Epac1-Radixin interaction.

Introduction

Radixin belongs to the ERM (Ezrin, Radixin and Moesin) family. ERM proteins are essential in a variety of cellular processes, such as cell shape maintance, microvilli formation, cell-cell adhesion, cell migration and cell polarity (1-4). ERM proteins consist of an N-terminal Four-point one, Ezrin, Radixin, Moesin (FERM) domain of ~300 amino acids followed by an α-helical region and a C-terminal tail (CTT) domain of ~90 amino acids. The C-terminal ~30 amino acids of ERM proteins consist an actin binding region (ABR), which is required for the binding to F-actin (5). ERM proteins exist in equilibrium between two conformations: a closed "inactive" state in which the CTT domain associates with the FERM domain and an open "active" state, in which this interaction is disrupted (6). The CTT-domain interacts with actin and in the open conformation the FERM domain interacts with membrane proteins, thereby ERM proteins link the actin cytoskeleton to the plasma membrane. In the closed conformation the surface areas involved in these interactions are masked (6,7).

The equilibrium between the open and the closed state of Radixin is directly regulated by the phosphorylation of Thr564 (8,9). This residue is conserved in Radixin, Ezrin and Moesin suggesting a conserved mode of regulation. Thr564 is located in the ABR and pointing

inside the interaction surface of the CTT-domain and the FERM domain. Phosphorylation of Thr564 destabilises this interaction as was shown with phosphorylation mimicking mutations *in vitro*. Thr564 was identified as a substrate for Rho kinase (ROCK) (8,10), PKCα (11), PKCθ (12), and Protein kinase B (PKB) (13). Activated ERM proteins have been shown to function as a crosslinker and join actin filaments to membrane proteins (14,15). They also participate in the activation of Rho, which again activates ERM proteins as a positive feedback system (14).

Structurally the FERM domain consists of three sub-folds. It contains an N-terminal ubiquitin-like fold, followed by a region resembling the fold of Acetyl-CoA-binding domains and a C-terminal PH-domain-like fold. A grove is formed between the ubiquitin-like fold and the PH domain-like fold, which functions as a PIP_2 binding site (16). PIP_2 binding to the FERM domain is assumed to contribute to the membrane localisation of ERM proteins (17). Thereby it enhances the "activity" of ERM proteins.

Epac1 is a Rap-specific nucleotide exchange factor that can be directly activated by cAMP *in vivo* and *in vitro* (18). The cAMP-Epac signalling regulates integrin-mediated and cadherin-mediated cell adhesion via Rap (19-21). The first 49 amino acids of Epac1, which are not conserved in Epac2, have been shown to bind Ezrin (chapter 4). This interaction facilitates Epac to localize on the apical membrane of polarised epithelial cells and couple receptor activation to Rap1 signalling.

In this study we biochemically characterise the novel Radixin mutant I577D/F580D. This mutation favours the open conformation of Radixin, as was shown by the inability of the mutated CTT domain to interact with the FERM domain. Moreover, we found that Epac1 interacts with Radixin in the activation conformation.

Results

Radixin-I577D/F580D is trapped in the open conformation.

To explore the function of the open conformation of Radixin, the Radixin double mutant I577D/F580D was generated. We designed this mutant based on the crystal structure of the highly related protein Moesin (7). I577 and F580 are localised in the ABR of the Moesin CTT domain (Fig. 1A). The corresponding residues in Moesin form a hydrophobic interaction surface with the FERM domain and thus we hypothesised that the replacement of these residues by negative charged asparagines might destroy this interaction (Fig. 1B). The isolated CTT domain is able to interact with the FERM domain and thus the interaction can be monitored directly e.g. by co-immunoprecipitation of Radixin-N with Radixin-C (6,7,22,23). As predicted, Radixin-C-I577D/F580D does not co-precipitate with Radixin-N, opposite to the non-mutated CCT domain (Fig. 1C).

Phosphorylation of Thr564 is known to induce the open conformation of Radixin and the related

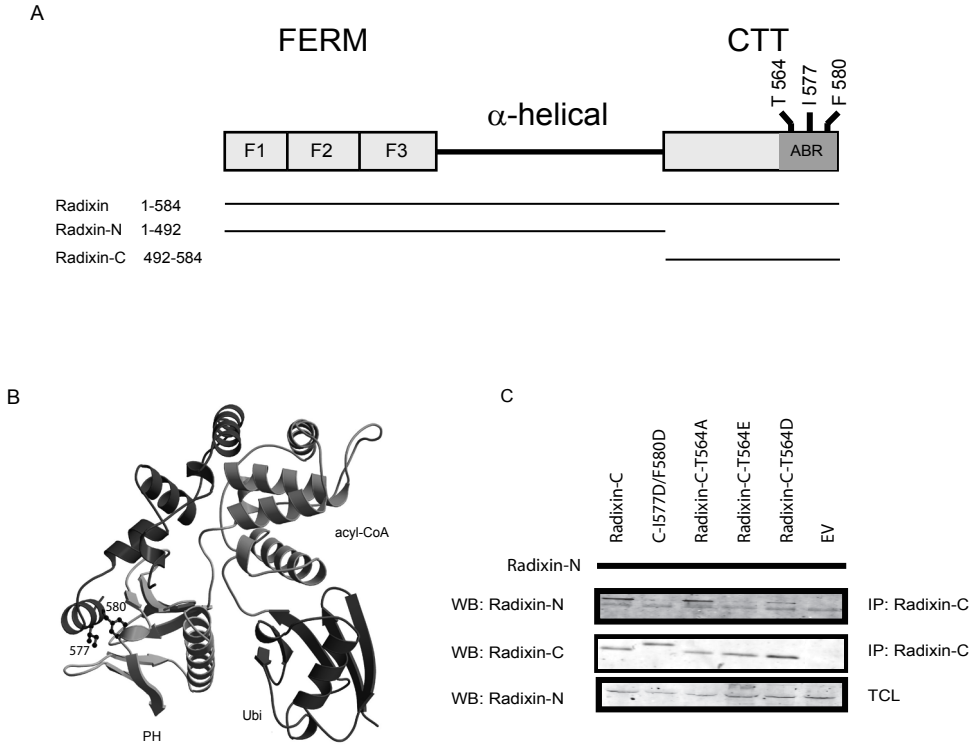

Fig.1 Radixin I577D/F580D is trapped in the open conformation. A, Domain organisation of Radixin. FERM, (Four-point one, Ezrin, Radixin, Moesin); CCT, C-terminal tail; ABR, actin binding region. T564, I577 and F580 are shown. The borders of the used constructs are indicated. B, Interaction between the FERM domain (green and yellow) and the C-terminal tail domain (red) of Moesin (7) is shown. Three sub-domains from FERM domain are indicated. Ubi, ubiquitin-like fold; acyl-coA, Acetyl-CoA-binding domain; PH, PH-domain-like fold. I577 and F580 are shown in ball-and-stick representation. The figure was generated using MolScript (38) and RasterR3D (39). C, 293T cells were transfected with Flag-tagged Radixin-N with the indicated V5-tagged Radixin-C mutant. V5-tagged Radixin-C was precipitated and the co-immunoprecipitated Radixin-N was probed with anti-Flag antibody (Top panel). The precipitated V5-tagged Radixin-C was detected by V5 antibody (middle panel) and the expression of Radixin-N was determined by immunoblotting of the straight lysates (lower panel).

Moesin and Ezrin proteins (14,24). Several studies have therefore used the phosphorylation mimicking mutations T564D and T564E and the non-phosphorylatable mutant T564A (25). Here these mutants are compared with the I577D/F580D mutant. Radixin-N specifically co-precipitated with Radixin-C as well as Radixin-C-T564A. The mutation T564D only reduced the interaction, whereas the interaction is abolished in case of T564E (Fig. 1C).

Radixin-I577D/F580D represents the active conformation

I577 and F580 are localised in the ABR and thus, even though Radixin-I577D/F580D is in the open conformation, binding of Radixin-I577D/F580D to actin might be affected. To exclude

this, an actin-binding assay was performed. Purified actin was polymerised to generate F-actin and incubated in the presence or absence of recombinant Radixin proteins. F-actin and actin-bound proteins were separated by ultracentrifugation from the soluble fraction. The obtained fractions were subjected to SDS-PAGE gel and stained with Coomassie blue (Fig. 2A). A significant fraction of both wild type Radixin and Radixin-I577D/F580D were found in the sediment together with F-actin, indicating that Radixin-I577D/F580D is able to bind to actin.

Since the open confirmation of ERM proteins links F-actin to the plasma membrane proteins (24,26,27), Radixin-I577D/F580D is expected to show a corresponding cellular localisation. The cellular distribution of Radixin in MDCK cells was investigated using confocal microscopy. Indeed Radixin-I577D/F580D showed a more prominent localisation at the plasma membrane in contrast to wild type Radixin, which was localized in the cytosol (Fig. 2B). This finding is in line with the previous described localisation of Radixin T564D in neuronal cells (28). Notably, Radixin-I577D/F580D is highly colocalized with actin as demonstrated by phalloidin co-staining (Fig. 2B).

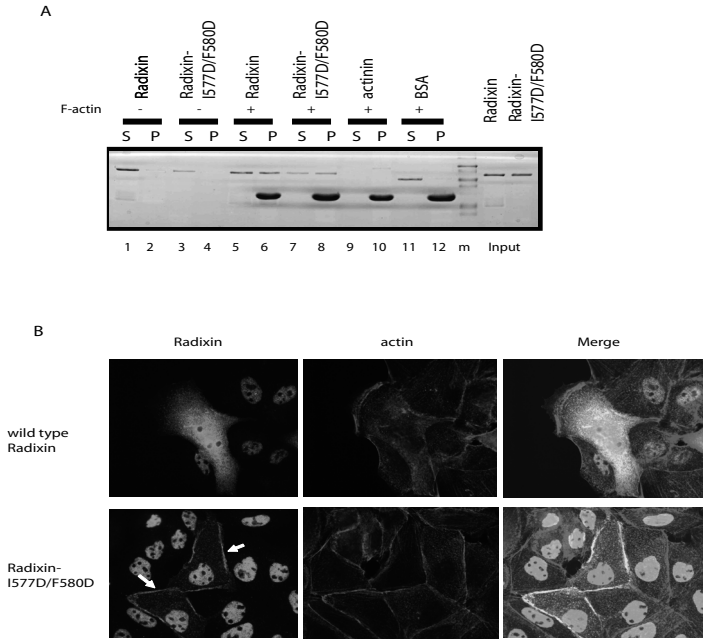

Fig.2 Characterization of Radixin-I577D/F580D. A, Characterization of F-actin-binding capacity of Radixin-I577D/F580D in a cosedimentation assay. Recombinant proteins were prepared, and F-actin cosedimentation assays were performed as described in Materials and Methods. Plus and minus indicates the presence or absence of F-actin in the experiment. S: supernatant, P: pellet, m: protein marker. B**,** Radixin-I577D/F580D colocalizes with actin on the plasma membrane. Confocal micrographs of MDCK cells transiently transfected with Flag-Radixin-I577D/F580D or Flag-WT-Radixin. Cells were labelled with anti-Flag antibodies. Actin was detected by alexa-labeled Phalloidin. Arrows depict prominent membrane localization.

Epac1 interacts specifically with the open conformation of Radixin

Epac1 interacts with Radixin and its close relative Ezrin in a yeast two-hybrid screen. Furthermore we showed that Ezrin targets Epac to the apical site of polarized cells. We therefore tested whether Epac1 interact with Radixin in a co-immunoprecipitation assay and whether this is affected by the active conformation of Radixin. We observed that Epac1 co-immunoprecipitated with Radixin-I577D/F580D and Radixin T564E but not with wild type Radixin, Radixin T564D or T564A (Fig. 3A). This result indicates that Epac1 specifically binds to the active conformation. We next showed that the interaction between Radixin-I577D/F580D and Epac1 can be blocked completely by overexpressing Radixin-C (Fig. 3B).

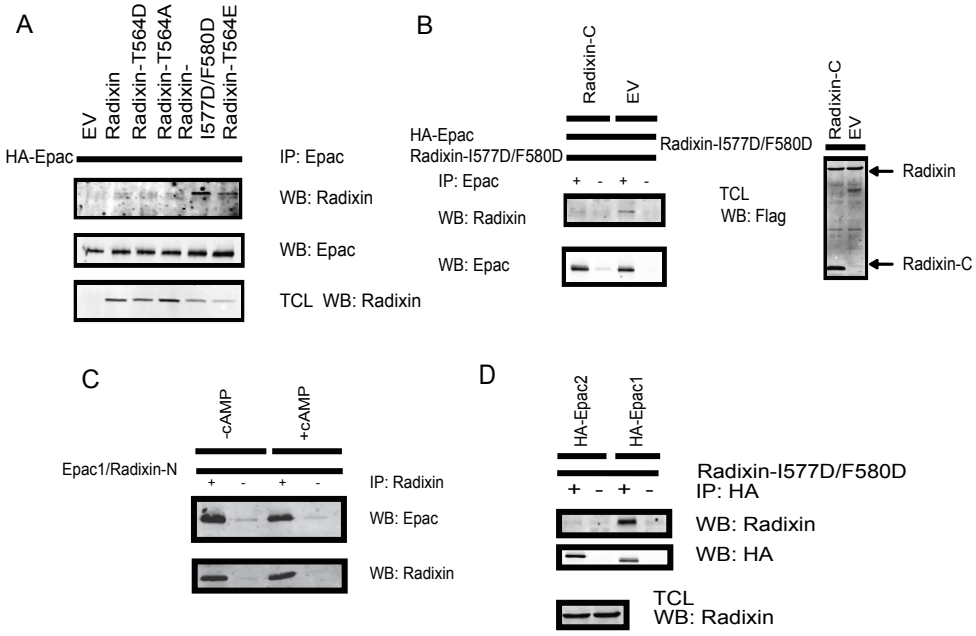

Fig.3 Epac1 interaction with Radixin. A, Radixin-I577D/F580D augmented the interaction with Epac1. HA-tagged Epac was precipitated from 293T cells transiently transfected with HA-tagged Epac1 and indicated Flag-tagged Radixin constructs. The co-immunoprecipitated Radixin was probed with anti-Flag antibody (Top panel). The precipitated Epac was detected by HA antibody (middle panel) and the expression of Radixin was determined by immunoblotting of the straight lysates (lower panel). B, 293T cells transiently transfected with HA-tagged Epac1 and Flag-tagged Radixin-I577D/F580D together with either Flag-tagged Radixin-C or empty vector control. The co-immunoprecipitated Radixin was probed with anti-Flag antibody. The precipitated Epac was detected by HA antibody and the expression of both Flag-Radixin-N and Flag-Radixin-C were determined by Flag antibody. C, cAMP does not influence the interaction between Epac1 and Radixin. 293T cells were co-transfected with Radixin-N and Epac1. After 42h, the cells were incubated with either 8-Br-cAMP for 15 minutes followed by co-immunoprecipitation assay. D, Radixin interacts with Epac1 but not Epac2. 293T cells were co-transfected with Radixin and either Epac1 or Epac2 and cell lysates were either directly probed or immunoprecipitated as indicated. The bottom panel indicates the expression of Radixin as determined by Western blotting.

The activity of Epac1 is regulated by direct binding of cAMP (18,29). Thus it might be possible that the binding of cAMP to Epac in addition influences the ability of Epac to interact with Radixin. However, no differences in the efficiency of co-immunoprecipitation of Epac with Radixin were observed in the presence or the absence of additional cAMP (Fig. 3C).
To demonstrate the specificity we also tested whether Epac2 might interact with Radixin. However, the interaction between Epac2 and Radixin was not observed (Fig. 3D).

Discussion

Radixin exists either in a closed "inactive" state or in an open "active" state. The closed state is mediated by an internal interaction between the N-terminal FERM domain and the CTT domain. Here we have described a novel, functional active mutant of Radixin, which is trapped in the open conformation. The I577D/F580D mutation was designed with the intention to abolish the intramolecular interaction of Radixin. This concept was confirmed experimentally, since the mutated CTT domain was shown to be unable to interact with the FERM domain. Traditionally, Thr564 (or the corresponding residues in Ezrin and Moesin) is mutated to aspartate or glutamate to induce the open conformation of ERM proteins (8,9). Thr564 is phosphorylated by Rho-associated kinase, PKB, PKCα, PKCθ and PKB thus the mutations are mimicking the natural activation mechanism of ERM-proteins. Here we have analysed the efficiency of these mutations by biochemical methods and compared them to the novel mutation I577D/F580D. In case of T564E and I577D/F580D the interaction between the FERM domain and CTT domain was completely abolished, whereas Radixin-C-T564D showed only a reduced ability to interact. This observation is in agreement with structural considerations. Thr564 of the CTT domain (Thr558 in Moesin) is localised at the border of the interaction area between the FERM- and the CTT-domain, but pointing towards the FERM domain. The introduction of the negative charged, bulky phosphate group is causing repulsive forces and thus destabilising the interaction of the CTT domain with the FERM domain. Previous studies using phosphorylation mimicking mutants revealed that the phosphorylation of Moesin on T558 weakens the intramolecular interaction due to both electrostatic and steric effects (7). Aspartate, as well as glutamate, is mimicking the negative charge of the phosphate group. However, in addition to be negatively charged, glutamate is sterically more demanding than aspartate. It is thus expected that in RadixinI577D/F580D and RadixinT564E a much higher fraction adopts the open conformation than in RadixinT564D. This is also reflected by the ability of the mutants to interact with Epac. Neither wild type Radixin nor RadixinT564D co-immunoprecipitate with Epac, whereas RadixinI577D/F580D and RadixinT564E do. This result is in agreement with the observations of Chamers et al. who reported only a small effect of EzrinT567D on the biochemical activation of Ezrin (30). Wild type Radixin-CCT is able to bind to the FERM-domain of RadixinI577D/F580D and

RadixinT564E. This interaction blocks the ability of Epac to bind to the constitutive open mutant of Radixin.

Together, these results demonstrate that the majority of RadixinI577D/F580D is trapped in the open conformation. However, the biological activity of the open form of Radixin depends on its ability to cross-link the plasma membrane to actin. In this context it is important to notice that both Ile577 and Phe580 are localised in the ABR and thus the possibility that the ability to bind actin is lost in RadixinI577D/F580D needs to be excluded. Previous studies have shown that wide type ERM proteins are able to bind to actin (31,32). Here it was demonstrated, that RadixinI577D/F580D co-sediments with F-actin as well as wild type Radixin. Furthermore RadixinI577D/F580D co-localises with the actin cytoskeleton and the plasma membrane.

Radixin is highly related to Ezrin. The interaction of Ezrin and Epac was described in our previous study and was shown to be responsible for the localisation of Epac at microvilli structures. It is thus expected that the interaction of Epac and Radixin has a similar function in the spatial regulation of Epac signalling. The localisation of Epac is thus coupled to the activation state of ERM-proteins. Even though Radixin and Ezrin are highly homologues, there are slight differences in their biological function. Both Ezrin and Radixin are co-localised at actin-rich surface structures such as microvilli, lopodia and membrane ruffles. However, Radixin is in addition found in adherent junction and focal contact sites (33,34). The expression of Ezrin and Radixin differs in individual tissues. Ezrin is found primarily in epithelial and mesothelial cells in intestine, stomach, lung and kidney while Radixin is enriched in liver, brain and skeletal muscle (33,34). Finally, during tumour development the expression of Ezrin is up-regulated while Radixin was found to be down-regulated (35). These evidences indicate different functions between the family members. It was shown that Epac1 localises predominantly at microvilli at the apical plasma membrane. However, taken the important function of Rap in cell-matrix adhesion and cell-cell adhesion (20,21,36,37) it is an intriguing possibility that Radixin is targeting Epac to cell-cell contact sites. Whether Radixin has influences on Epac/Rap signalling pathway is currently under investigation.

Materials and methods

Antibodies

Mouse monoclonal anti-Flag (clone M2) is from Sigma. Mouse monoclonal anti-Myc (9E10) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal anti-NV5 was from Invitrogen, goat-polyclonal anti-α-actinine was from Santa Cruz biotechnology. Mouse monoclonal anti-HA (12CA5) was purified by our laboratory. Odyssey secondary Alexa Fluor 680-labeled monoclonal- or polyclonal- antibodies were from Li-Cor's biosciences. After the blots were washed with TPBS and analyzed using the Odyssey infrared imaging

system (Li-Cor, USA).

Plasmids

Human Radixin cDNA (IRAKp961G1091Q) was obtained from the Deutsches Ressourcenzentrum für Genomforschung (RZPD) (Berlin, Germany). To generate vectors containing Radixin full length (aa 1-584), Radixin N (aa 1-492) and Radixin C (aa 492-584), the corresponding coding sequences were introduced by PCR into a donor vector, pDONR201 (Invitrogen, USA) to allow sub-cloning by the "Gateway"-procedure (Invitrogen, USA). T564E, T564A, T564D, I577D/F580D mutations in WT-Radixin or Radixin-C were introduced according to the QuikChange procedure (Stratagene, USA).

Recombinant protein purification

Radixin-WT and Radixin-DM were expressed as GST-fusion proteins (pGEX4T2) in the E. coli strain CK600K. The cells were grown in Standard I media (Merck, USA) containing 100 mg/l ampicillin and 50 mg/l kanamycin to an A600 of 0.8, induced with 100 µM IPTG and incubated overnight at 25°C. Cells were re-suspended in 100 mM Tris-HCl pH7.5, 50 mM NaCl, 5 mM EDTA, 5mM DTE and 5% glyclerol and lysed by sonification. After centrifugation the supernatant was loaded onto a 20 ml GSH column (Pharmacia, USA). The column was washed five volumes of 50 mM Tris-HCl (pH 7.6), 400 mM NaCl, 5% glycerol and 5mM DTE, with 350 ml of 50 mM Tris-HCl (pH 7.6) 100 mM KCl, 10 mM MgCl2, 5 % glycerol, 5 mM DTE, 2 mM ATP and with 2 column volumes of 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM $CaCl_2$ 5 % glycerol and 5 mM DTE (buffer T). Thrombin cleavage (thrombin from bovine plasma, Serva, Heidelberg) was performed with 150 units over night in buffer T at 4°C (1 unit clots a standard fibrinogen solution in 15 seconds at 37°C) and the same buffer. The obtained protein was further purified by gel filtration on a Superdex 75 equilibrated with 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM DTE and 2.5% glycerol.

Actin binding assay

An actin binding protein spin-down assay kit from Cytoskeleton, Inc's was used. F-actin was prepared by polymerize G-actin for 1 hour at RT. 60 µM test protein was used and incubated with polymerized F-Actin for 1 hour at RT. Samples, including a positive (α-actinin, 5 µg) and negative (Bovine Serum Albumin, BSA, 1 µg) control, were then centrifuged at 55,000 RPM for 3 hours at 24˚C. Sample supernatant and pellet were analyzed on a SDS-PAGE gel stained with simply blue (Invitrogen, USA).

Cell Culture, Transfection and Immunoprecipitation

293T cells were transiently transfected by the calcium phosphate method. For immunoprecipitation, anti-Flag, anti-V5 or anti-HA (12CA5) antibody were incubated with protein A-Sepharose beads (Santa Cruz Biotechnology, USA) for 1 h. Cells were then harvested, lysed in 1 ml of 1% Triton X-100 buffer containing additionally 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride,

0.5% Nonidet P-40, aprotinin (1 µg/ml), pepstatin (1 µg/ml) and incubated with beads for 2 h. Beads were then washed 3 times. Precipitates and samples of total cell lysate were resolved by SDS-PAGE, transferred to PVDF membrane and probed with the appropriate primary and secondary antibodies. The amount of cell extract loaded on the gel was 5% that used for the immunoprecipitation.

Immunofluorescence of MDCK cells

Cells were cultured on glass cover slips. Before fixation, medium was removed and cells were washed three times with ice-cold PBS. Fixation was performed with ice-cold 3.8% formaldehyde for 20 min, followed by permeabilization with 0.2% TritonX-100 for 10 min. The samples were incubated with blocking buffer containing 4% goat serum and 0.2% bovine serum albumin (BSA) in PBS for 1 h. Cells were labelled with primary antibody for 2 h followed by washing 3 times with PBS. Alexa-conjugated secondary antibodies were applied for 1h. Actin was detected by alexa labelled Phallodin. All pictures were obtained by confocal microscopy.

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Chapter

6

Discussion

General discussion

Signal transduction pathways play important roles in cell differentiation, proliferation, and survival. Specific protein-protein interactions mediate the assemblies of protein complexes in response to different signals therefore regulate the proper transmission of cellular signals. Inappropriate protein-protein interactions within signalling pathways can lead to many diseases, including cancer. Proteomics-based approaches, which enable the quantitative investigation of protein-protein interactions involved in signalling networks, provide us with techniques to define the molecules controlling various signalling pathways.

Ras-like small G-proteins, such as Rap, function in signal transduction by cycling between an inactive GDP-bound state and an active GTP-bound state. Exchange of GDP to GTP and thus activation is facilitated by guanine-nucleotide exchange factors (GEFs). In the GTP bound conformation, Rap interacts with and thus transmits the signal to effector proteins. GTPase activating proteins (GAPs) stimulate the hydrolysis of GTP bound Rap and convert Rap to GDP-bound inactive state (see chapter 1). This implies a rather simple two-state switch function of Rap. However, the switching takes place in a rather complicated network of protein-protein interaction, which allows Rap to function as a sophisticated control unit in signal transduction.

The main goal of this thesis was to elucidate molecular mechanisms in the Rap1 signalling network. Initially, we studied the role of the known Rap-binding protein AF6 and showed that AF6 acts as a negative regulator of Rap-mediated cell adhesion (Chapter 2). Three Rap-like pseudogenes (mRap1A-retro1, mRap1A-retro2 and hRap1B-retro) in mouse and human genome were analyzed (Chapter 3). We show that these retrogenes encode functional proteins and exhibit clear differences between each other in their ability to induce cell adhesion and spreading. To obtain a better understanding of how RapGEFs exert their function, a number of yeast two-hybrid screens were performed, utilizing the RapGEFs Epac and PDZ-GEF (Chapter 4 addendum). A number of interacting proteins were identified including the ERM (Ezrin-Radixin-Moesin) proteins Ezrin and radixin. Based on these results we investigated the effect of this interaction on Epac localisation and on Epac- and Rap1-induced effects (Chapter 4). Furthermore, we developed a potent active mutant of Radixin (Chapter 5).

The involvement of AF6 in Rap-mediated adhesion

Rap has a crucial role in the inside-out regulation of integrin-mediated cell adhesion (1-3) and cadherin-mediated cell-cell adhesion (4-7). Overexpression of active Rap1 mutant increases cell adhesion whereas RapGAP inhibits stimulus-induced adhesion. Several lines of evidence suggest that AF6 is involved in Rap1 signalling. In *Drosophila* AF6 functions as an effector of Rap1 during embryonic development and it is required for the proper dorsal closure process (8). During E-cadherin-mediated junction formation, AF6 interacts with nectin and facilitates cell-cell adhesion (9). Furthermore, the interaction between E-

cadherin and P120-catenin can be stabilised by the AF6/Rap complex, which results in an increase on adhesion (10). Interestingly, Su and co-workers have shown that AF6 recruits RapGAPs to negatively regulate Rap1-induced integrin-mediated adhesion in fibroblast (11). Consistent with this notion, we show that over-expression of AF6 inhibits 8-pCPT-2'-OMe-cAMP induced adhesion in T cells (Chapter 2). Importantly, knockdown of AF6 enhances Rap1-induced adhesion. Since AF6 interacts with Rap in a RapGTP dependent manner (12), we propose that in the adhesion process AF6 traps Rap1 in an inactive complex rather than functioning as an effector. This does not exclude the possibility that AF6 does function as a Rap effector in another Rap-mediated process such as the formation of cell-cell junctions. Indeed, as expected from an effector that binds specific to the GTP-bound form of Rap1, AF6 prevents Rap1GTP from RapGAP-induced hydrolysis. In Chapter 2 we also described the identification of KIAA1849 as a novel Rap1 binding protein. Due to the similarity to AF6, we named this protein AF6-like (AF6L). AF6L interacts with Rap1 but does not influence integrin-mediated adhesion. Compared to AF6, several domains are missing in AF6L, including an N-terminal RA domain and a C-terminal proline-rich region, which are both involved in protein-protein interaction and signalling. However, whether AF6L is a true Rap1 effector or whether it might interact with other Ras-like small GTPases as well needs further investigations.

Epac and PDZ-GEF interacting proteins.

To gain insight into how the activity of RapGEFs is modulated, we investigated which proteins are able interact with Epac and PDZ-GEF. Yeast two-hybrid is a fast and straightforward approach to identify proteins that interact directly with the protein under study. Since both hybrid proteins have a relative high expression weak or transient interactions can be detected. Proteins found to interact with Epac and PDZ-GEF range from scaffold proteins to enzymes. All hits were scored based on an algorism for reliability (Addendum 1). The scaffold proteins might contribute to the temporal and spatial organisation of Rap signalling. In the Epac yeast two-hybrid screens, Rap that is known to be directly activated by Epac (13,14) was not picked up, indicating that the results described in Chapter 4 do not provide a complete list of Epac and PDZ-GEF interaction partners. There are several reasons for false positive or false negative results. Firstly, over-expression of both hybrid proteins increases the chance of unspecific binding. Secondly, to be detected the interaction has to take place in the nucleus of the yeast cell and thus strong localisation signals of human proteins might prevent the fusion protein from entering the yeast nucleus. Thirdly, the regulation of the reporter genes by the fused protein could influence the result. Fourthly, post-translational modification of human protein might not be achieved in yeast. Therefore, although yeast two-hybrid shows real direct interactions, other evidence is required to prove that the interaction id biological significant, other methods to verify these interactions are required, such as co-immunoprecipitation assay and GST-pulldown assay. Furthermore, mass spectroscopy of the

RapGEF complex is a valuable method to achieve better understanding, which allows us to identify modifications of RapGEFs. It will be interesting to compare results from cells grown under different physiological conditions, such as different levels of second messengers, in order to find alterations in modifications.

The involvement of ERM proteins in Epac/Rap signalling

Whereas the activity of Epac is directly regulated by cAMP, the interaction of Epac with scaffold proteins mediates the spatial control of Epac activity. The results presented in chapter 4 and 5 demonstrate that Epac1 interacts with the ERM protein Ezrin and Radixin and that this interaction contributes to the regulation of Epac. The family of ERM proteins were originally proposed to function as cytoskeleton-membrane linker proteins (15-17). More recently, a growing body of evidence suggests that ERM proteins are also involved in signal transduction (18,19). ERM proteins exist in two conformations, an inactive closed conformation due to an intramolecular interaction and an active open conformation (Chapter 1). In the active state the N-terminal FERM domain and the C-terminal actin-binding site are available for binding to membrane-protein and F-actin, respectively (18). In this way, ERM proteins directly link the actin cytoskeleton to the plasma membrane. In addition, they serve as scaffold proteins and recruit protein complexes to actin-rich surface structures such as microvilli, lopodia and membrane ruffles. In chapter 4 the interaction between Epac1 and Ezrin was described. This interaction was confirmed by both co-immunoprecipitation assay and subcellular localization studies. We found that the first 49 amino acids of Epac1 are required for this interaction. Ezrin also binds to beta adrenergic receptor (β-AR), which induces synthesis of cAMP upon stimulation (20). We observed that knockdown Ezrin inhibits Rap1 activation induced by the β-AR agonist isoproteronol. Overexpression of the first 49 amino acids releases Epac1 from its apical localisation and inhibits 8-pCPT-2'-OMe-cAMP-induced Rap1 activation. Apparently Ezrin recruits Epac1 to the apical surface for efficient coupling it to the β-AR. It is currently unclear which process is benefited the most from the apical localisation of Epac.1. A good candidate would be the reported regulation of the sodium, proton exchanger 3 (NHE3), which also forms a complex with erzin through EBP50. However, we have thus far not be able to confirm this effect. Alternatively it mediates the effect of Epac to junction formation or to cell adhesion.

It was reported, that muscle-specific A kinase anchoring protein (mAKAP) function as a scaffold protein and co-ordinates a complex between Epac1, PKA and phosphodiesterase 4D3 (PDE4D3) (21). Interestingly, Ezrin also binds PKA and since Epac1 interacts directly with PDE4D3 (21,22), Ezrin may form a complex and synchronise the activation of PKA and Epac1. Interestingly, an Epac1 mutant that does not bind Ezrin, is located predominat in the nucleus (chapter 4). Since Epac binds specifically to the active form of Ezrin, this might indicate that nuclear localisation of Epac is regulated by Ezrin. In addition it suggests that Epac might have a nuclear function. Perhaps the interaction we observe with ranBP2 in the

yeast two hybrid screen may play a role in this process (Addendum 1). However, we have currently no clue about this function.

Due to the differences between Ezrin and Radixin with respect to their expression profiles (23-25); the question was raised whether Radixin also contributes to the control of Rap1 pathways. We demonstrated that Epac1 only interacts with the open conformation of Radixin (Chapter 5). Based on the structure of ERM proteins (26) we generated a novel open mutant of Radixin, Radixin-I577D/F580D, which was shown to adopt the functional active conformation. This mutant exhibits less intramolecular interaction compared to the known phosphorylation mimic mutants. More interestingly, it interacts with Epac1 with higher efficiency as determined by co-immunoprecipitation assay. Together, these findings illustrate that ERM proteins are anchor proteins (18,19) that can regulate Rap1 signalling by localising Epac1.

Activation of the ERM proteins require relief of the intramolecular association, and this is believed to involve phosphorylation of threonine 564 (27,28). Studies have therefore employed the T564D phosphomimetic mutant to explore the consequences of Radixin activation (29,30). By characterization of Radixin-I577D/F580D mutant we show that the inhibition of the intramolecular association results in unmasking both membrane-binding site and actin-binding site. More interestingly, compared to Radixin-I577D/F580D, the Radixin-T564D mutation has a small effect on the biochemical activation of Radixin, indicating that Radixin-T564D is not fully activated as generally considered. Another Radixin phosphomimetic mutant, Radixin-T564E, exhibit an inhibition of the intramolecular interaction. Aspartate, as well as glutamate, is mimicking the negative charge of the phosphate group. However, in addition to be negatively charged, glutamate is sterically more demanding than aspartate; therefore blocks the internal interaction. This observation is in agreement with a previous report (31). We show that the interaction between Epac and Radixin is dependent on the activation of Radixin. Furthermore, Epac1 interacts with Radixin-I577D/F580D and Radixin-T564E mutant but not with Radixin-T564D mutant. We conclude that the Radixin-I577D/F580D mutant is a powerful tool to study the consequences of activated Radixin.

Concluding remarks

Other results shows that AF6, an established effector of Rap1 in cell-cell junction formation may have an additional function, being an inhibitor of Rap1 signalling in integrin mediated cell adhesion. In addition, we show that specific anchor proteins for Rap1GEF exist that might be responsible for the special control of Rap1 signalling. Most notably, Ezrin and Radixin localize Epac1. Interestingly, Epac binds specifically to the active form of these proteins indicating that the localisation of Epac can be regulated by stimuli that modulate Ezrin/Radixin activity. Indeed, Epac-mutants that can not bind to these proteins are "mislocalized" and appear in the nucleus, pointing to perhaps a novel function of Epac at this location. Clearly we are just at the beginning of our journey to understand the molecular mechanism of Rap1 signalling, but it is a good starting point for further adventures.

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Summary

The small GTPase Rap1 belongs to the Ras family and shares high similarity with Ras protein. Rap1 cycles between an inactive GDP-bound state and an active GTP-bound state. This cycle is regulated by guanine nucleotide exchange factors (GEFs), such as Epac and GTPase activating proteins (GAPs), such as Rap1GAP. The most well-established processes where Rap1 is involved in include integrin-mediated adhesion and cadherin-mediated cell-cell adhesion. The GEFs are regulated by second messengers and are part of a protein-protein network, which regulates their temporal and spatial activities. The objective of the work described in this thesis was to investigate how Rap1 signaling pathway is regulated and to identify and characterize signaling proteins that direct the Rap1 pathway.

In chapter 2, we studied the relation between AF6 and Rap1. We show that the overexpression of AF6 inhibits Rap1 induced adhesion. In addition, knocking-down the endogenous AF6 increases adhesion. These results show that in Jurkat T cells, AF6 functions to buffer GTP-Rap in resting cells and negatively regulates Rap1 function. In chapter 3 we analyze three Rap-like pseudogenes (mRap1A-retro1, mRap1A-retro2 and hRap1B-retro) in mouse and human genome. We show that all three retrogenes are expressed and encode functional proteins. These proteins appeared to stay more in a GTP-bound state compared to wild type Rap1. More interestingly, they exhibit clear differences in their ability to induce cell adhesion and spreading. To gain more insight in the protein interaction network, which controls the spatial and temporal organisation of Rap specific GEFs we performed yeast two-hybrid screens using Epac and PDZ-GEF as baits. This is described in chapter 4 addendum. A general summary of the results is given and candidate proteins were discussed. In chapter 4 and chapter 5 we characterized in detail the interaction between ERM (Ezrin-Radixin-Moesin) proteins and Epac1. We show that both Ezrin and Radixin interact with Epac1 in an activation-dependent manner. The Ezrin/Radixin binding region was identified in the N-terminus of Epac1. Furthermore, we demonstrate that this region is also required for the localization of Epac1 at microvilli in fully polarized cells. We show that Ezrin couples the activation of the β-adrenergic receptor to Rap1 signalling via the recruitment of Epac1. In chapter 5 a novel Radixin mutant was characterized. This mutant Radixin in which both I577 and F580 are substituted for aspartic acids fulfils the classical criteria of being in an active state.

Samenvatting in het Nederlands

Het kleine GTPase Rap1 behoort tot de Ras-familie van kleine G-eiwitten, welke voorkomen in een inactieve GDP-gebonden en actieve GTP-gebonden vorm. De activering van Rap1 wordt gereguleerd door ´guanine-nucleotide exchange factoren´ (GEFs), welke ervoor zorgen dat Rap-gebonden GDP vrijkomt waardoor Rap GTP kan binden. GTPase activerende eiwitten (GAPs) daarentegen stimuleren de hydrolyse van GTP naar GDP en daarmee de inactivering van Rap1. Rap1 is betrokken bij de regulatie van integrine-afhankelijke adhesie van cellen aan de extracellulaire matrix en cadherin-gemedieerde adhesie van cellen onderling. Het doel van het werk beschreven in deze thesis is te achterhalen hoe de Rap1 signaalcascade gereguleerd wordt en de eiwitten betrokken in dit signaalpad te identificeren en karakteriseren.

In hoofdstuk 2 wordt de relatie tussen AF6 en Rap1 bestudeerd. In de literatuur wordt AF6 beschreven als een effector van Rap, doordat AF6 specifiek kan binden aan GTP-gebonden Rap. Echter, wij laten zien dat over-expressie van AF6 de Rap1 geïnduceerde adhesie van cellen remt en dat omgekeerd een verlaging van endogeen AF6 adhesie stimuleert. Dit suggereert dat AF6 GTP-gebonden Rap kan wegvangen en dus een negatief effect heeft op de Rap1 signaalcascade. Dit is een interessant nieuw concept voor een eiwit dat specifiek bindt aan de GTP-gebonden vorm van Rap, maar niet werkt als effector maar competeert met andere effectoren voor de binding.

In hoofdstuk 3 worden drie Rap-achtige pseudogenen (mRap1A-retro1, mRap1A-retro2 and hRap1B-retro) in het muizen en humane genoom geanalyseerd. We laten zien dat al deze drie retrogenen tot expressie komen en coderen voor functionele eiwitten. In vergelijking tot klassiek Rap1 komt een hoog percentage van deze eiwitten voor in de GTP-gebonden vorm. Interessant is dat de pseudogenen een verschillende potentie hebben om cel adhesie en cel spreiding te induceren; zowel hRap1B-retro en mRap1A-retro1 stimuleren integrine-afhankelijke cel spreiding terwijl alleen hRap1B-retro cel adhesie medieert.

Om meer inzicht te krijgen in het eiwit-eiwit interactie netwerk dat Rap-specifieke GEFs controleert is een yeast two-hybrid screen uitgevoerd met Epac en PDZ-GEF (addendum 1). In hoofdstuk 4 en hoofdstuk 5 wordt de interactie tussen Epac1 en ERM (Ezrin-Radixin-Moesin) eiwitten in detail bestudeerd. We laten zien dat de N-terminale regio van Epac1 alleen bindt aan de actieve vorm van Ezrin en Radixin. Deze regio is nodig voor de localizatie van Epac1 in de microvilli van volledig gepolarizeerde cellen. Ezrin koppelt de activatie van de β-adrenerge receptor aan het Rap1 signaalpad via rekrutering van Epac1. In hoofdstuk 5 wordt een nieuwe Radixin mutant beschreven die de actieve vorm van Radixin nabootst.

Curriculum Vitae

Zhongchun Zhang was born on the 29th of January 1979 in NanJing, China. He obtained his secondary school diploma in July 1997. In September the same year he entered the China Pharmaceutical University in NanJing to study Biopharmaceuticals. In September 2001 he started the Master program in Wageningen University, the Netherlands. From July 2002 to January 2003 he did his master research project under the supervision of Professor Hans Bos in Utrecht University. On 29th of January 2003 he obtained his MSc degree in Biotechnology. From February 2003 till February 2007 he worked as a graduate student under the supervision of Professor Hans Bos in the Department of Physiological Chemistry at the University Medical Center in Utrecht. Research performed during that period was focused on the regulation of Rap signaling pathway and is described in this PhD thesis. In May 2007 he will begin as a postdoctoral fellow in the laboratory of Intracellular Signaling headed by Professor Marc Therrien at the Institute for Research in Immunology and Cancer in Montreal, Canada.

Publication List

Zhongchun Zhang, Holger Rehmann, Leo S. Price, Jurgen Riedl, and Johannes L. Bos
AF6 negatively regulates Rap1-induced cell adhesion. (2005) *J. Biol. Chem. 280, 33200-5*

Jun Zhao*, **Zhongchun Zhang***, Matthijs R.H. Kooistra, Sander Basten, Viola Oorschot, John de Koning, Judith Klumperman, Fried J. Zwartkruis, Holger Rehmann, Johannes L. Bos and Leo S. Price
Ezrin is an Epac1-anchoring protein that couples receptor activation to Rap1 signalling.
*These authors contributed equally to this work. *Submitted*

M. Duchniewicz, **Zhongchun Zhang**, T. Paluch, T Zemojtel and F.J.T. Zwartkruis
Rap1 retrogenes encode activated isoforms with differential signalling capacity.
To be submitted

Judith H. Raaijmakers1, Laurence Deneubourg, Holger Rehmann, John de Koning, **Zhongchun Zhang**, Sonja Krugmann, Christophe Erneux and Johannes L. Bos
The PI3K effector Arap3 forms a SAM domain-mediated interaction with the PIP3 phosphatase SHIP2. *Submitted*

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Notes