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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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)

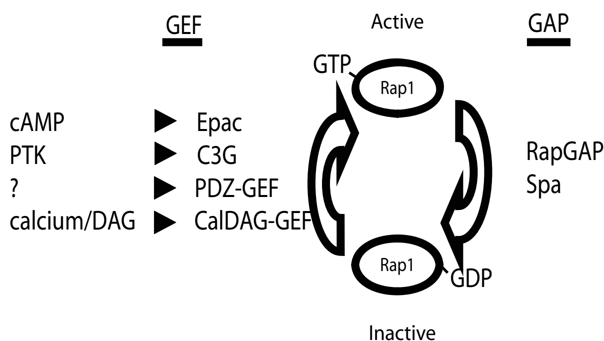

Fig. 1 Regulation of the Rap1 GTPase. Rap activity is upregulated by a number of RapGEFs and downregulated by RapGAPs. Rap1GEFs are controlled by second messengers or protein tyrosine kinases. PTK: protein tyrosine kinase.

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

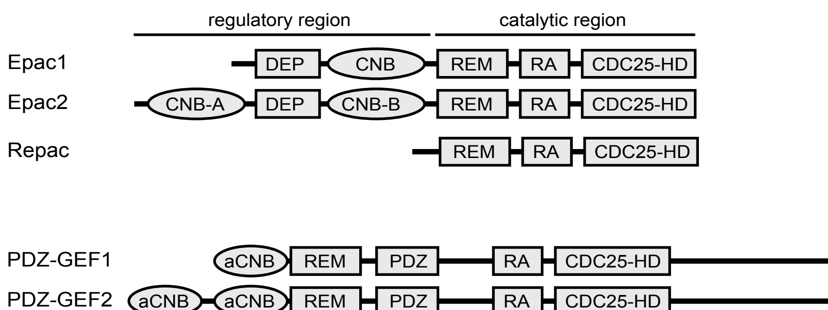

Fig. 2 Domain structure of Epac and PDZ-GEF. Domain organization of Epac1, Epac2, Repac, PDZ-GEF1 and PDZ-GEF2 are shown. DEP, Disheveled, Egl-10 and Pleckstrin domain; CNB, cyclic nucleotide binding domain; REM, Ras exchanger motif; RA, Ras association domain; CDC25-HD, CDC25-homology domain; aCNB,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

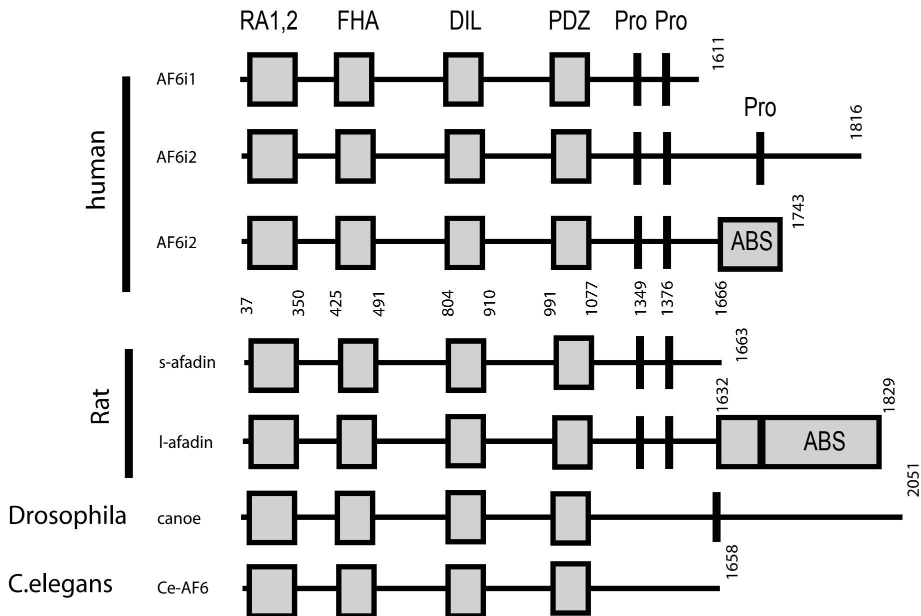

Fig. 3 Domain structure of AF6, afadin, Canoe and Ce-AF6. Domain structure of the three human AF6 isoforms (AF6i1, AF6i1 and AF6i3), the two its rat homologue afadin (long l-afadin and short s-afadin), its Drosophila homologue Canoe and its C.elegans homologue Ce-AF6 are shown. The relative positions 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).

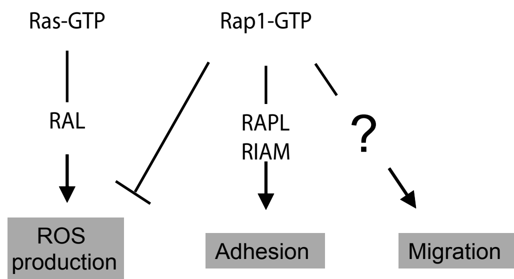

Fig. 4 Rap1 downstream signaling effects in T cells. RIAM and RAPL have been reported to interact with Rap and function as effectors on adhesion. Requirement for Rap1 activity in several cellular processes has been reported such as Reactive Oxygen Species (ROS) suppression, cell adhesion and cell migration.

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”

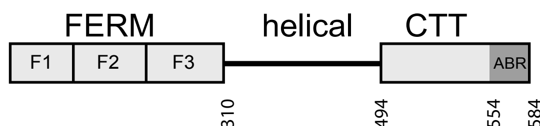

Fig. 5 Domain structure of ERM proteins. ERM proteins contain a FERM domain on the N-terminus followed by an α-helix and a C-terminus tail domain (CTT), which can associate with the FERM domain. The last ~30 residues in the CTT are involved in actin-binding. FERM, Four-point one, Ezrin, Radixin, Moesin. 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).

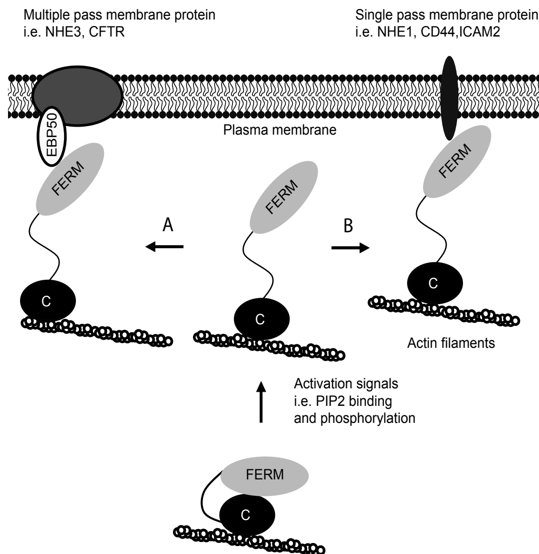

Fig. 6 A working model for the activation and function of ERM proteins. Following activation to unmask binding sites, FERM domain and the C-terminus region are available to bind to membrane proteins or actin, respectively. A, Through scaffold molecules such as EBP50 and E3KARP, ERM proteins link membrane 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 (1.2×10^7 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’- gatcccggagagagctgacgggtctttcaagagaagacccgtcagctctctcctttttggaaa -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

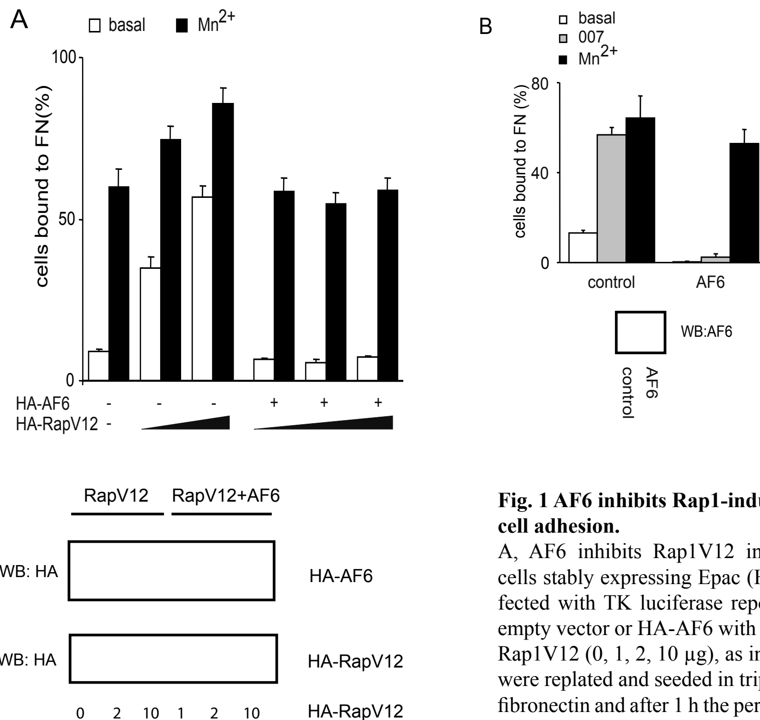

Fig. 1 AF6 inhibits Rap1-induced integrin-mediated cell adhesion.
A, AF6 inhibits Rap1V12 induced adhesion. Jurkat cells stably expressing Epac (HB6 cells) were cotransfected with TK luciferase reporter plasmid and either empty vector or HA-AF6 with increasing amounts HA-Rap1V12 (0, 1, 2, 10 µg), as 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 panel 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 mGppNHp 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.

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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

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.