Ras-like small GTPases in platelet biology

Ras-achtige kleine GTPasen in de biologie van bloedplaatjes

(met een samenvatting in het Nederlands)

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Abbreviations

AC	adenylyl cyclase
ACD	acid, citrate, dextrose
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BAPTA-AM	1,2-bis (2-aminophenoxy) Ethane-N,N,N',N'-tetraacetic acid-acetoxymethylester
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CaM	calmodulin
CaMK	calcium/calmodulin-dependent kinase
cAMP	cyclic adenosine monophosphate
CREB	cAMP responsine element binding protein
C-terminus	carboxy-terminus
DMSO	dimethylsulfoxide
EGTA	[ethylenebis(oxyethylenenitrilo)]-tetraacetic acid
Epac	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinase
DAG	diacylglycerol
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GDS	guanine nucleotide dissociation stimulator
GEF	guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
GP	glycoprotein
GRF	guanine nucleotide releasing factor
GRP	GRF-related protein
GST	glutathion S-transferase
GTP	guanosine triphosphate
HA	haemagglutinin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICAM-1	intercellular adhesion molecule-1
IP_3	inositol 1,4,5-trisphosphate
IP4BP	inositol 1,3,4,5-tetrakisphosphate binding protein
JAK	Janus kinase
kDa	kilo Dalton
LDL	low density lipoprotein
LFA-1	leukocyte function-associated molecule-1
mantGDP	2',3'-bis(O)-N-methylantharanoloyl guanosine diphosphate
MAPK	mitogen activated protein kinase
mSOS	mammalian Son of Sevenless
N-terminus	amino-terminus
OCS	open canalicular system
PAF	platelet activating factor
PAR	protease activated receptor
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PH	pleckstrin homology
PI(3)K	phosphatidylinositol-3OH-kinase
PIP_2	phosphatidylinositol 3,4-diphosphate
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
P70 S6K	p70 ribosomal S6 kinase
PVDF	polyvinyl difluoride
RalBP1	Ral-binding protein 1
RalGPS	RalGEFs with PH domain and SH3 binding motif
RBD	Ras-binding domain
RIP	Ral interacting protein
Rlf	RalGDS-like factor
RTK	receptor tyrosine kinase
scr	structurally conserved region
SDS-PAGE	sodium dodecyl sulfate-poly-acrylamide gelelectrophoresis
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SH2	Src homology 2
STAT	signal transducers and activators of transcription
TCR	T-cell receptor
TK	thymidine kinase
TPA	2-O-tetradecanoylphorbol-13-acetate
TxA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen 4
vWF	von Willebrand factor
wt	wild-type

CHAPTER

1

General introduction

I. Rap1 and Ral in signal transduction

The Ras family: molecular switches in signalling cascades

The Ras superfamily of monomeric GTPases

Intercellular communication tightly controls the behaviour of each individual cell in a human being by means of signals like hormones, growth factors and cytokines. Cell surface receptors enable the cell to monitor its environment for the presence of these signals, which function as ligands for the receptors. Ligand binding results in receptor activation. Activated receptors propagate the message to the interior side of the cell membrane and initiate activation of intracellular signal transduction pathways. The intracellular signalling machinery regulates cellular processes like growth, proliferation, differentiation and appropriate gene expression profiles. Overactivity or a functional block at the level of one of the signalling proteins is incompatible with the required tight regulation of these cascades. Several diseases are known to result from such molecular alterations that underly dysregulation of cellular processes, like for instance tumour formation or excessive bleeding disorders. A first requirement for development of therapeutic intervention strategies, is to understand the molecular details underlying the regulation and mechanisms in cellular signalling. Here, the focus will be on Ras family members in signal transduction.

The superfamily of Ras-like small GTPases comprises proteins that are divided into subfamilies according to structure, sequence homology and function (1), '*Guidebook to the Small GTPases'*, edited by Marino Zerial and Lukas A. Huber, Oxford University Press). The Sar1/Arf and Rab families are both involved in membrane compartmentalisation and vesicular traffic (2,-6). The Ran proteins are used for transport of proteins and RNAs across nuclear pores, mitotic spindle assembly and nuclear envelope formation (7-9). The Rho proteins mediate organisation of the actin cytoskeleton (10,11). The function of the RGK family, named after the GTPases Rad and GEM/Kir (RGK), is largely unknown, although a connection between some of its members and the cytoskeleton has been proposed (12,13). The Ras family regulates cell proliferation and differentiation (14,15). In comparison with heterotrimeric G-proteins, the monomeric Ras-like GTPases have a low molecular mass (20-35 kDa) and therefore are also referred to as the small (or low molecular weight) GTPases.

Ras family members: molecular characteristics

The behaviour of Ras family members at the molecular level is described by the GTPase cycle. They either bind GDP or GTP, a characteristic that allows them to function as molecular binary switches. Conversion to the active, GTP-bound state is mediated by guanine nucleotide exchange factors (GEFs), which catalyse the release of GDP from the nucleotide-binding site of the GTPase. As soon as GDP is released, GTP enters the binding site, as the consequence of relatively high affinity of Ras proteins for guanine nucleotides (dissociation constant = 10^{-2} pM in case of Ras) in combination with the 10-fold excess of GTP (1-10 mM) over GDP in the cytoplasm (16). GTP binding induces a conformational change after which the effector-binding domain is exposed, allowing the GTPase to associate with downstream target proteins and generate signalling cascades (17-22). Each Ras-like protein is supplied with an intrinsic GTP hydrolysing activity. The rate at which different GTPases hydrolyse bound GTP varies, but is in most cases incompatible with the rapid changes that are required for proper signalling. However, the hydrolysing activity can be strongly enhanced by GTPase-activating proteins (GAPs) (23), which are able to bind to the GTPase and thereby stabilise the GTP-GDP transition state (24). Reversion to the GDP-bound state switches the GTPase off, abolishes effector binding and terminates signal transduction. Thus, GTPase activity represents the coordinated balance between the actions of GEFs and GAPs.

Amino acid changes at positions at or near sites of contact with the β- and γ-phosphates of the guanine nucleotide (residues 12, 13, 59, 61 and 63 in H-Ras) lead to reduced intrinsic GTPase activity. Mutations that generate changes at sites of interaction with the guanine base (residues 116, 117 and 119 in H-Ras) decrease the affinity of the protein for

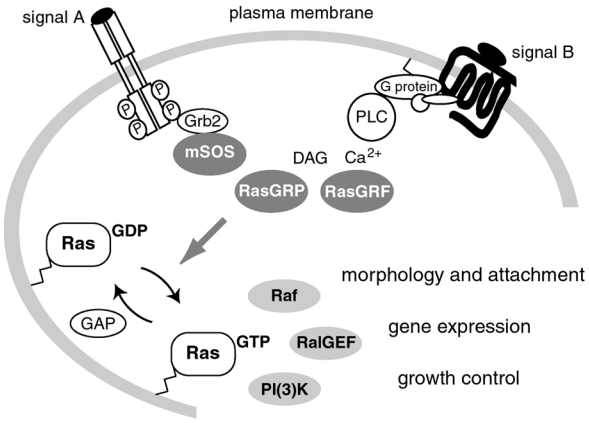

Figure 1. ***Ras, the small GTPase paradigm.***
Communication signals generated by the cell itself (autocrine), neighbouring cells (paracrine), or secreted by other cells in the blood stream (endocrine) arrive at the cell surface where they bind to and activate their corresponding receptors. An activated receptor transduces the signal to the interior side of the cell membrane in a receptor-type-specific way, resulting in activation of a Ras guanine-nucleotide exchange factor (GEF). This either involves the second messengers calcium and diacylglycerol (DAG) or protein complex formation on phosphorylated tyrosine residues. An active RasGEF catalyses the release of GDP out of Ras, that is replaced by GTP. In the active, GTP-bound conformation Ras is able to interact with effector proteins initiating signalling along effector pathways and regulation of downstream cellular processes.

nucleotides and increase the intrinsic exchange rate. These changes all result in a net increase in the GTP-bound, active form of the GTPase. Genetically, these mutants function as dominant active. In addition, mutations that block activation of the protein exist. These cause inability of the protein to change its conformation to the active form. Mutations that change residue 17 in H-Ras for instance result in a dominant negative version. This is thought to result from a constitutive association of the GTPase with the exchange factors. Overexpression of dominant active or negative versions of GTPases has been widely used as approach to study small GTPases (25).

The homology in the core effector region of the Ras family proteins (amino acids 32-44 in H-Ras) is striking and raises concern about target specificity. An interaction results from association between the GTPase effector domain and the Ras-binding domain (RBD) in the effector. RBDs share a ubiquitin-like fold (26-30). The presence of a number of positively charged residues in this tertiary protein structure determines successful interaction with the negatively charged effector-binding region of the small GTPases (31-35). Although Ras, Rap, R-Ras and TC21 can indeed bind the same effectors, the affinities are different (36-41). The amino acid residues flanking the effector region contribute to the electrostatic interaction between GTPase and effector (14,42). For example, changing the residues at position 30 and 31 in Rap1 into E30D and K31E, similar to Ras, results in equal binding affinity between Rap1 and Ras for the RBD of RalGDS, one of the Ras effectors (34,43,44). In general, one GTPase has several effector proteins. Discrimination originates from differential utilisation of amino acids within the effector domain by target proteins. Mutations have been defined whereby certain connections are lost, while others are still functional (45).

Small GTPases contain a membrane localisation domain at their carboxy (C)-terminus. Posttranslational modification of the C-termini is essential for membrane localisation of the Ras proteins and thereby required for proper functioning. Moreover, the differences in the C-termini may give rise to differential membrane localisation and as a consequence contribute to effector specificity (reviewed in (15)).

The Ras signalling pathway

In different human tumours, activating mutations have been identified at high frequency in the founding superfamily member Ras (46). For that reason, Ras has received much attention and has become a paradigm for small GTPase signalling (Figure 1). Extracellular signalling molecules bind to their transmembrane receptors, which propagate the signals in different ways. Either directly or indirectly phosphotyrosine docking sites are created that serve complex formation between the adaptor protein Grb2 and the Ras-specific GEF mSOS. This causes mSOS relocalisation to

the plasma membrane where it can activate Ras (47). Alternatively, activated receptors generate the second messengers calcium and diacylglycerol (DAG) that regulate GEFs like RasGRFs (48) and RasGRPs (49). For Ras, both the exchange reaction and GTPase activation have been visualised in co-crystallisation studies of Ras with the catalytic domain of mSOS (16) and of Ras with the catalytic domain of p120 RasGAP (24). Ras activation, which is (or can experimentally) be mimicked by expression of activated Ras mutants (46,50,51), gives rise to a number of cellular effects. These can be classified as alterations in growth control, morphology and attachment, or gene expression (21,50,52). They all contribute to the oncogenic property of active Ras signalling. In part, the molecular mechanisms underlying these changes overlap. Members of the Raf family (Raf1, A-Raf and B-Raf), phosphatidylinositol-3-OH-kinase (PI(3)K) and RBD-containing RalGEFs (RalGDS, Rlf and Rgl) together with their signalling cascades have been established as genuine Ras downstream effectors pathways (53,54). Obviously, Ras plays an interesting and elaborate function in intracellular signal transduction. From this point of view, it is appealing to speculate on important contributions in cell regulation by the closest relatives of Ras, namely Ral and Rap proteins.

Rap and Ral activity

Rap1 proteins

Several studies have contributed to the discovery and initial characterisation of the Rap (Ras-proximate) proteins (55-60). At protein level, Rap1A and Rap1B (henceforth also collectively referred to as Rap1) are approximately 95% identical (only 9 out of 184 amino acids different), with the major difference at the C-terminus (6 amino acids). Rap1 shares about 50% overall homology with Ras. Strikingly however, their core effector regions are nearly identical (54). Rap1 is ubiquitously expressed and particularly abundant in platelets, neutrophils and brain (61). Both Rap1A and B are posttranslationally modified at their C-terminus by a geranylgeranyl moiety, which mediates membrane attachment of the proteins (62-64). Rap1 localises to intracellular membranes at the perinuclear region (Golgi compartment), endocytic and exocytic vesicles and to the plasma membrane in fibroblasts (65,66), to specific granules in neutrophils (67,68) and to secretory vesicles in parotid gland cells (69,70). The Rap2 proteins share about 70% homology with the Rap1 members at the amino acid level. An interesting difference between the two groups resides in the core effector domain (Bos 98). Rap2A and B are 90% identical (18 amino acids different), with a predominant difference in the C-terminus (61,64,71). As a consequence, Rap2A is farnesylated, while Rap2B is geranylgeranylated (72-74).

Activation of Rap1

Rap1 has been implicated in signal transduction by the finding that cyclic adenosine monophosphate (cAMP) induces an increase in GTP-bound epitope-tagged Rap1 (75). So far, no antibody is able to immunoprecipitate endogenous Rap1. Therefore, activation of endogenous Rap1 could not be studied using the classical GDP/GTP quantification assay (25). This has led to the development of a GTPase pulldown assay to monitor activation of endogenous GTPases (76-78). Using this assay, Rap1 activation has been demonstrated upon treatment with a number of extracellular stimuli, depending on the cell type (54,79). Receptors propagating signals towards Rap1 include receptor tyrosine kinases (RTKs), heterotrimeric G-protein-coupled receptors (GPCRs; reviewed in (80)), immunoreceptors and cytokine receptors (76,81-83). Processes like cell adhesion, cell density and receptor internalisation also play a role in the activation mechanism (84,85). In a diversity of cell types, like for instance platelets, fibroblasts, neutrophils and B-cells, phospholipase C (PLC) and several second messengers appeared pivotal intermediates in Rap1 activation by extracellular ligands (75,76,79,81,82,86). This knowledge has sparked off a successful search for Rap1 activating factors that are differentially regulated by second messengers.

GEFs and GAPs regulating Rap1

A remarkable variety of GEFs and GAPs contribute to the regulation of the Rap1 GTPase cycle (79,87) (Figure 2). Among the GEFs, different families have recently been discovered

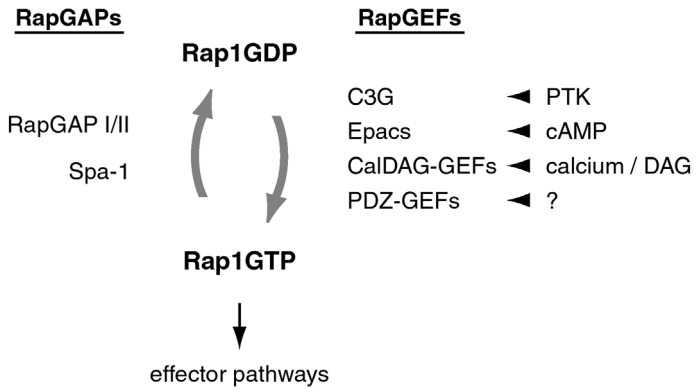

Figure 2. ***Regulation of the Rap1 GTPase cycle.*** Stimulus-induced receptor signalling may either elevate Rap1GTP by promoting the action of guanine-nucleotide exchange factors (GEFs) or by inhibiting (trapping) Rap1 GTPase-activating proteins (RapGAPs). Rap1GEFs are controlled by different mechanisms in which either a second messenger or protein complex formation is involved. (PTK: protein tyrosine kinases).

and characterised, which members contain binding sites for the second messengers calcium, DAG and cAMP. Members of the Epac (exchange protein directly activated by cAMP, also referred to as cAMP-GEF) family contain a cAMP-binding motif that allows direct, allosteric activation of the GEF activity upon cAMP binding (88-90). Elevation of Rap1GTP by calcium and DAG can be explained by the action of members of the CD-GEF (CalDAG-GEF; Cal stands for calcium) family, which contain putative calcium- and DAG-binding sites (91-94). However, consensus about the differential regulation of these GEFs by calcium and DAG remains to be established (79,87). Regulation of the PDZ-GEF family members has not yet been resolved (95-97). The first RapGEF to be studied in detail was C3G (Crk SH3-domain-binding guanine-nucleotide releasing factor; SH3 stands for Src homology region 3 domain). C3G mediates activation of Rap1 induced by a mechanism involving complex formation with the proto-oncogene Crk and membrane localisation (98-104). Since Crk adaptor proteins contain an SH2 domain, tyrosine kinase-linked receptors are able to recruit Crk/C3G complexes by phosphorylating tyrosine residues on membrane-associated proteins like Cas, Cbl and Gab1 (104-108). This is thought to bring C3G in close vicinity of Rap proteins. In addition, tyrosine phosphorylation of C3G has been reported to be involved (109). Other proteins have been implicated as GEFs in Rap regulation, like Nsp2 (110), but these require further characterisation in order to establish *bona fide* RapGEF activity. Strikingly, while the RasN17 mutant tightly associates with RasGEFs, Rap1N17 does not bind to C3G (111). Therefore, RapN17 may not function as a dominant-negative mutant in Rap1 signalling in the way RasN17 does in the Ras pathway.

The first family of GAPs for Rap proteins contains the members RapGAP I and the amino (N)-terminally extended version named RapGAP II (112,113). RapGAP II has been shown to associate with active $G\alpha_i$ -subunits, which results in membrane relocalisation and activation of the GAP (113). Rap1GAP can also bind $G\alpha_o$- and $G\alpha_z$ (114,115). The interaction with $G\alpha_o$ inhibits GAP activity. Serine phosphorylation of RapGAP I by PKA or Cdc2 has been described, which might allow complex formation with other proteins (116-118). A second family consists of signal-induced proliferation-associated gene 1 (Spa-1) and its close homologue E6TP1 (E6 targeted protein 1). These proteins are characterised by the presence of a PDZ domain (119). E6TP1 was identified as a target for degradation by the papilloma virus E6 oncoprotein. E6 expression results in tumour formation, which suggests a link between overactive Rap1 and cell transformation. However, the functionality of the presumed GAP domain needs to be proven. Additional putative RapGAP proteins include tuberin, which contains a GAP domain that may function towards both Rap1 (120) and Rab5 (121). Deletions in the tuberin gene cause familial tuberous sclerosis, a disease characterised by the development of benign tumours (122-126). Finally, GAP^{IP4BP}, which binds the second messenger inositol 1,3,4,5-tetrakisphosphate, may function as RapGAP and RasGAP (127).

The Ral proteins

The GTPases RalA and RalB are 85% identical and predominantly differ in their C-terminus. They have 58% sequence identity with Ras (128-130). Ral and Ras have comparable nucleotide binding characteristics and low intrinsic GTPase activity (131). The Ral proteins are ubiquitously expressed but are particularly abundant in brain, testes and platelets (132-134). Ral is posttranslationally modified by geranylgeranylation (15,135). In subcellular localisation studies it has been found in plasma membrane (136), endocytotic vesicles (130), synaptic vesicles (137) and secretory vesicles (138).

Ral activation mechanisms

An interesting Ras protein connection arose when the first identified RalGEF, Ral gunanine dissociation stimulator (RalGDS, (139)) was found to interact with the GTP-bound form of Ras, R-Ras and Rap1 in a yeast two-hybrid screen (36,140). Subsequently, the RalGEFs RalGDS-like (RGL) and RalGDS-like factor were cloned and shown to possess similar structural and Ras-binding properties (141-143). Together with Rsc these GEFs form the RalGDS family of RalGEFs. Rsc is an oncoprotein that results from a fusion between the yeast Rad23 protein homologue and the C-terminal region of the RalGEF designated Rgr (144). Members of this family are characterised by the presence of a Cdc25-like catalytic region and a C-terminal RBD. Rgr forms an exception, since it lacks this RBD region. While both RalGDS and Rlf are ubiquitously expressed (41,139), Rgl is predominantly present in brain, heart, lung kidney and testis, whereas Rgr is mainly present in lung and skin (142,145). The involvement of Ral in Ras signalling has been firmly established by *in vivo* proof (reviewed in (146)). Upon Ras activation, RalGDS proteins are recruited to the plasma membrane. Activation of these GEFs requires an intact plasma membrane localisation signal in Ras (143,147-149). In agreement, membrane targeting of RalGDS and Rlf renders them constitutively active (143,149).

In addition, Ras-independent mechanisms participate in the regulation of Ral, as for instance in response to elevated levels of intracellular calcium (150-152) (Figure 3). Calcium may regulate Ral by inducing the

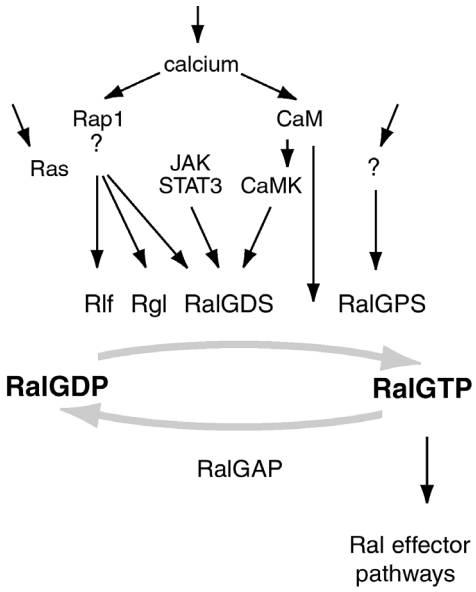

Figure 3. ***Regulation of the Ral GTPase cycle.***
Ral activating mechanisms can be classified as Ras-dependent and -independent. Stimuli that induce RasGTP lead to recruitment and activation of the RalGEFs containing a Ras-binding domain (RalGDS, Rlf and Rgl). Among the Ras-independent pathways are the RalGPS proteins, which may be regulated by protein or lipid interactions with their proline-rich and pleckstrin homology (PH) domains. In addition, a rise in intracellular calcium leads to Ras-independent Ral activation. This may result from direct association between calcium/calmodulin (CaM) and Ral, calcium-induced Rap1 activation or regulation of RalGDS by means of a calcium/calmodulin-dependent kinase (CaMK). Additional pathways have been suggested in the control of Ral, but these are currently less well understood and for that reason not depicted.

binding of calmodulin (CaM) to a CaM-binding domain in the C-terminus of RalA (153,154). Alternatively, an activated calcium/CaM-dependent kinase might contribute by phosphorylating RalGDS and thereby activate the catalytic activity of the GEF (155). Since Rap1 is able to associate with the RalGDS family members, another explanation may be that Rap1 mediates the calcium-induced Ral activation. The family of RalGEFs with pleckstrin homology (PH) domain and SH3 binding motif (RalGPS proteins), which consists of the splice variants 1A, 1B (also named RalGEF2, chapter 2) and 2, also functions independent of Ras. The activity of these members is regulated by the PH domain and a Grb2-binding PXXP motif ((156), chapter2).

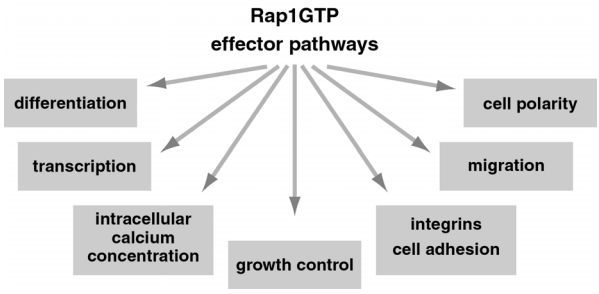

Figure 4. ***Rap1 downstream signalling effects.***
Upon activation, Rap1 is supposed to interact with effector proteins that contain a Ras-binding or Ras-associating domain. Several candidates have been proposed by in vitro and over-expression systems, but the relevance of these interactions in vivo needs to be determined. However, requirement for Rap1 activity in a diversity of cellular processes has been reported, like transcription, differentiation, cell adhesion, cell polarity, migration and the regulation of the intracellular calcium concentration.

Finally, evidence for the involvement of Src-like kinases, PI(3)K and protein kinase C (PKC) has been generated (152) and in addition JAK/STAT3/RalGDS (157) and PI(3)K/PDK-1 (PI 3,4,5-trisphosphate-dependent kinase-1)/RalGDS (158) pathways have been described. Concerning the downregulation of RalGTP, different RalGAP proteins have been postulated but not cloned (159,160). Other RalGEF candidates have been found, namely Rab2L (161), And-34 (110), Rgl-2 (162) and Rgl-3 (also called RPM) (163), but their activity towards Ral needs further proof.

Functions of Rap1 and Ral

Rap1 and the Ras effectors

Rap1 has been identified as the product of a gene that could revert oncogenic Ras-induced transformation of cells (60). As the core effector regions of Ras and Rap1 show striking similarity (87), the hypothesis was raised that Rap1GTP may interfere in Ras signalling by trapping Ras effector proteins out of sight from Ras. Indeed, Rap1 has been shown to bind the Ras effectors PI(3)K, Raf-B and RalGDS *in vitro* and in over-expression studies (36,39,81,143). Many studies have contributed to the amount of conflicting results on the function of Rap1 in the regulation of the MAPK pathway (79,87,164, JM Enserink, submitted). Furthermore, the proposed connection of Rap1 by binding to RalGDS with the upstream regulation of the Ral GTPases (146), as has been discussed, remains intriguing. Moreover, Rap1 has been implicated in cAMP-induced, PI(3)K-mediated activation of protein kinase B (PKB/Akt) in thyroid cells, indicating that Rap1 might in addition be able to regulate PI(3)K *in vivo* (165). However, whether these interactions between Rap1 and the Ras effectors occur between endogenous proteins *in vivo* remains to be determined.

Rap1 in cell adhesion

In search for a protein function, a commonly followed strategy is trying to find phenotypes caused by mutations in that protein in lower eukaryotes, which are used as genetic systems. Investigations in yeast, *Dictyostelium discoideum* and *Drosophila melanogaster* have implicated the Rap relatives in processes ranging from bud site selection to cell migration and morphogenesis (reviewed in (87)) (Figure 4). For instance, distribution of adherens junctions around the lateral circumference of cells within an epithelium was reported to require Rap1 in *Drosophila* (166). In this study, cells mutant for *Rap1* condensed their adherens junctions to one side of the cell, which disrupted normal epithelial cell behaviour. In addition, Rap1 was found enriched at adherens junctions, particularly between divided sister cells. Rap1 may be linked to adherens junctions by its interaction with the multidomain cytoskeletal linker protein canoe, the *Drosophila* orthologue of AF-6 (166). This protein interacts with ZO-1 (167), which in turn associates with the adherens junction component α-catenin (168). Interestingly, these findings suit with the recently discovered exciting link between Rap1 and integrin-mediated cell adhesion.

Integrins are heterodimeric cell surface receptors that are able to attach to extracellular matrix components or adhesion molecules on the surface of other cells (169-172). Upon a variety of signals, inactive integrins are

converted to an active state that has high affinity or avidity for ligand, thereby promoting attachment to extracellular matrix or other cells. One of the first indications for the involvement of Rap1 in this process came from the observations that over-expressing the RapGAP Spa-1 in HeLa cells impaired the attachment to fibronectin-coated plates and in 32D promyelocytic cells blocked granulocyte colony-stimulating factor (G-CSF) to induce adhesion to tissue culture dishes (173). In addition, dominant-negative C3G blocked hepatocyte growth factor (HGF)-induced activation of Rap1 and reattachment of 293 cells to tissue culture dishes (104). Strong arguments have been provided by research using haematopoietic cells. Transient over-expression of activate Rap1 induces $\alpha_L\beta_1$-integrin (leukocyte function-associated antigen 1; LFA-1)-mediated cell adhesion in both human T cell and mouse pre-B cell leukaemia lines (174-178). Similar results have been provided using a mouse model in which Rap1V12 is expressed in the T cell compartment (179). RapN17 expression inhibited T-cell receptor (TCR)-induced adhesion and CD31-induced adhesion could be blocked by overexpression of RapN17, RapGAP and RalGDS-RBD (174). Over-expressing RapGAP II also decreased the ability of WEHI-231 B cells to form phorbol ester-induced LFA-1-mediated homotypic adhesion interactions and decreases adhesion of A20 B cells to immobilised intercellular adhesion molecule-1 (ICAM-1) (79). Integrin regulation by Rap1 does not seem to be restricted to the LFA-1 integrin, as Rap1 is also crucial for CD31- and cytokine-induced haematopoietic cell adhesion depending on the $\alpha_4\beta_1$-integrins very late antigen 4 (VLA-4) and VLA-5 (174,177). The exact mechanism and Rap1 effectors involved in the activation of integrin-mediated cell adhesion remain elusive. However, inhibition of Rap1 function seems to confer a physical restraint on integrins, as induction of LFA-1-mediated cell adhesion by binding of Mn^{2+} to extracellular divalent cation-binding sites or by integrin-activating antibodies (KIM185 and TS2/16) is also blocked by Rap1-inhibitory proteins ((174), chapter 3). The Rap1 family members R-Ras and H-Ras have also been implicated in the regulation of integrins (180,181). However, both the mechanisms of integrin regulation and the subset of integrins regulated may differ. Although RasN17 is also able to block TCR- and CD31-induced adhesion, CD31 stimulation does not induce Ras activation (174). H-Ras requires an intact cytoskeleton and is sensitive to inhibitors of MAPK, PI(3)K and PLC function, while none of these contribute to Rap1-induced adhesion (175,182,183). R-Ras can activate both the $\alpha_m\beta_2$ integrin (Mac-1) (176) and LFA-1 (174), but not VLA-4 (174).

Ral effectors and functions

As the RalGDS proteins are targets of active Ras, it may not be surprising that Ral contributes to Ras-mediated cellular effects. Indeed, Ral has been demonstrated to function in Ras-dependent proliferation (reviewed in (145)). Constitutive active RalGEFs have been demonstrated to induce enhanced growth properties, low serum growth and even contribute to tumour invasion and metastasis (143,144,184,185). Other reported effects of active Ral include anchorage-independent growth (186), DNA synthesis (187), differentiation (188), inhibition of differentiation (189,190) and transcriptional regulation (143,191-196) (Figure 5).

Up to now, several proteins have been described to associate with Ral. The first identified Ral effector was named Ral-binding protein 1 (RalBP1, also called RLIP76 or RIP1) (197-199). The interaction with RalBP1 links Ral to the small GTPases Rac and Cdc42, since RalBP1 harbours GAP activity towards these proteins (198). RalBP1 was found to form a complex with Pob1 (partner of RalBP1, (200) en Reps1 (201)). Ral, complexed with these proteins, has been implicated in binding to active epidermal growth factor (EGF) receptors and, through direct binding to Pob1, the recruitment of Epsin and Eps5. These proteins have been shown to function in the regulation of clathrin-mediated endocytosis of both EGF and transferrin receptors (202,203). By this mechanism, Ral has been implicated in the regulation of ligand-dependent receptor-mediated endocytosis (204). Phospholipase D (PLD) has been described to associate with Ral by binding its N-terminus (134,205-207). This direct association is not dependent on the activation state of Ral and has no effect on PLD activity (134). However, Arf was also found to associate with the Ral-PLD complex and Arf is

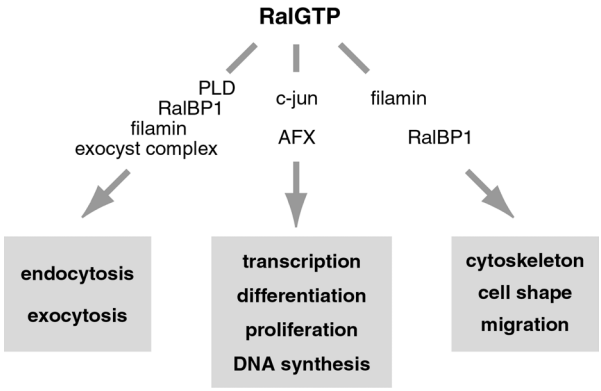

Figure 5. ***Ral downstream signalling effects.***
Several proteins have been reported to interact with Ral and function as effectors. Other proteins have been demonstrated to function more downstream in Ral-regulated signalling pathways (AFX, c-jun). Based on these interactions supplemented with functional studies Ral has been implicated in cellular processes that vary from regulation of transcription, differentiation, proliferation or DNA synthesis, the control of the actin cytoskeleton, cell shape and migration, and finally events in vesicular transport.

able to activate PLD (208,209). Both Arf and PLD are involved in vesicle formation and endocytosis (210), which again points to a role for Ral in the regulation of membrane transport. Further proof for this Ral function is provided by the recently described interaction between RalGTP and Sec5, a subunit of the human exocyst complex (211-213). This multi-protein complex has been implicated in polarised vesicular traffic (214-216) which indeed requires proper Ral activity (212). The Ral-Sec5 interaction may also give rise to Ral-mediated filopodia formation (213). The actin filament crosslinking protein filamin, also called ABP-280, has initially been identified to bind to Ral in a GTP-dependent fashion in platelets (217-219). Interestingly, the dominant-negative version RalN28 was demonstrated to block Cdc42-induced filopodia formation (219). Participation of filamin as downstream target in the filopodia formation was shown by the finding that over-expression of the Ral-binding domain of filamin blocked RalA-induced filopodia formation in 3T3 cells. Moreover, Ral could not induce filopodia formation in filamin-negative human melanoma cells (219). In addition to cross-linking actin filaments, filamin forms a docking site for various cell surface receptors and certain intracellular proteins involved in signal transduction and endocytosis (220,221). In conclusion, the Ral interacting proteins sofar identified implicate Ral in regulating vesicular transport as well as the cytoskeleton. Dual participation of several binding partners to these processes suggests a fine-tuning or integrating function for Ral.

II. Rap1 and Ral in platelet biology

Platelets

Blood platelet as model system to study Rap1 and Ral signalling

Blood platelet forms an attractive model system to study signal transduction by small Ras-like GTPases. First of all, platelets show rapid and massive signalling in an all-or-nothing fashion. Furthermore, platelets enable one to focus on early events, as they lack replication and transcription regulation. The Rho family members RhoA, Rac1, Rac2 and Cdc42 and the Rab family members Rab1, -3B, -4, -6, -8 have been detected in platelets (130,222,223). These GTPases may contribute to the extensive reorganisation of the platelet cytoskeleton and the regulated secretion upon activation. In addition, platelets contain Ras and Ral proteins (130,133,224) and Rap1B expression is even particularly high, as it accounts for 0.1% of total cellular protein in platelets (62). Moreover, Rap and Ral are activated within seconds following stimulation with a variety of agonists (76,86,151,225-227) and have therefore obviously been implicated in the process of platelet activation as regulators of platelet responses.

Platelet function

Blood platelets are small, flat, disc-shaped and anucleated cell fragments that arise from megakaryocytes, a population of cells in the bone marrow (textbook: 'Platelets and their

factors', edited by F. von Bruchhausen and U. Walter, Springer Verlag Heidelberg). Megakaryocyte differentiation is accompanied by the appearance of an extensive membranous network in their cytoplasm, the demarcation membrane. At the time megakaryocytes are fully differentiated, cell fragments are shed off using this mass of membrane structures and enter the blood stream as platelets. They have a diameter of 2 to 4 μm and circulate at a number of 150,000-300,000 per μl. Platelets contain three types of secretory vesicles. The α-granules contain, amongst others, adhesive proteins (e.g. fibrinogen and von Willebrand Factor (vWF)), growth modulators (e.g. platelet-derived growth factor (PDGF)) and coagulation factors (e.g. factor V). Dense granules contain non-metabolic ADP and ATP, calcium and serotonin. Lysosomal granules contain enzymes like cathepsin and β-galactosidase. Peroxisomes and mitochondria are also present. The open canalicular system (OCS) is formed by invaginations of the plasma membrane and functions as an export system for secretory products. The dense tubular system is thought to be a remnant of the megakaryocyte rough endoplasmatic reticulum and is the location for prostaglandin production and calcium storage. A platelet is present in the blood circulation for about 8-14 days. Old platelets are removed from the blood by the liver, spleen and bone marrow.

In case injury occurs to a vessel wall, platelets get in touch with material present underneath the endothelial lining, as for instance vWF or collagen. As a consequence, platelet function is activated which leads to the formation of a haemostatic plug (228). Platelets respond robust and very fast. Their shape changes within seconds to a spherical appearance, which is next converted to an irregular, spinny outline with pseudopodia and lamellipodia stretching in all directions. Regulated secretion and paracrine signalling enhance and sustain the ongoing platelet response. In addition to the shape change, adhesive properties are concomitantly induced (229). As a consequence, platelets bind at the site of damage to adhesive proteins in the injured vessel wall, a platelet clot is built up and finally retraction turns the aggregated platelets into a wound seal. Hereby the platelets cover and close wounds after which bleeding stops (230). During this activation process, plasminogen activator inhibitor-1 (PAI-1) and thromboxane A_2 (TxA_2) are secreted to decrease fibrinolysis and to induce vasoconstriction, respectively. Under pathological conditions, platelets are involved in thrombosis and contribute to atherosclerosis: After adhesion, platelets release PDGF and transforming growth factor β (TGF-β), which induce proliferation and migration of smooth muscle cells.

Platelet adhesion

The platelet plasma membrane contains a diversity of glycoproteins (GPs) that, upon platelet activation function in cell-cell and cell-matrix interactions (229). For instance, GP IIb/IIIa, the major platelet integrin $\alpha_{IIb}\beta_3$, is a calcium-dependent heterodimeric fibrinogen receptor (231). Also other ligands for this integrin have been reported (232). Glanzmann thrombasthenia patients suffer from a severe bleeding tendency due to the absence of functional $\alpha_{IIb}\beta_3$ integrin. In patients with Bernard-Soulier-syndrome, the adhesion receptor for von Willebrand Factor (vWF), the GP Ib-IX-V complex, a member of the leucine-rich glycoprotein gene family (233,234), is non-functional, expressed at low levels or even absent.

Strong evidence suggests that platelets utilise a multi-step adhesion mechanism (235,236). The initial contact of platelets with the vessel wall (platelet adhesion) is a complex process involving multiple adhesive substrates (vWF, collagen, E-selectin) and receptors on the platelet surface (GP Ib-IX-V, integrin $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ and P-selectin) (236,237). Initial platelet adhesion is critically dependent on the binding of the GP Ib-IX-V complex to immobilised vWF, especially under conditions of rapid blood flow and thus high shear stress. This receptor-ligand interaction supports platelet translocation and tethering on the surface of endothelial cells and at sites of vascular injury (238-240). Furthermore, it forms a prerequisite step for subsequent integrin-mediated cell arrest, as it induces signal transduction leading to activation of the platelet and its integrin $\alpha_{IIb}\beta_3$ (235,241). Once this integrin is activated, it is able to bind to vWF through an RGD-containing sequence (242) and fibrinogen, resulting in irreversible platelet adhesion, spreading and thrombus growth (243). This multi-step adhesion mechanism may not only be important for

platelet-vessel wall interactions, but also for platelet aggregation, particularly under high shear rates. Platelet aggregation studies using a cone-plate viscometer, have demonstrated that exposing platelets in suspension to high levels of shear stress, induces platelet aggregation independent of the addition of an exogenous stimulus (reviewed in (238,244)). This shear-induced platelet aggregation is initiated by the binding of soluble vWF to GP Ib-IX-V. This interaction not only tethers platelets to one another but also triggers platelet activation, converting integrin $\alpha_{IIb}\beta_3$ from a low- to a high-affinity receptor which is capable of binding fluid-phase adhesive proteins such as soluble vWF or fibrinogen (243,245). While high shear stress-induced platelet aggregation occurs under pathological circumstances, normally occurring shear stress also contributes to platelet activation and adhesion under physiological circumstances (229,240,244). Studies using mice lacking vWF, fibrinogen or both have demonstrated the central role for vWF in promoting platelet-vessel wall and platelet-platelet adhesive interactions during thrombus growth (246). However, the mechanism linking the vWF/GP Ib-IX-V interactions to activation of $\alpha_{IIb}\beta_3$, particularly under shear conditions, remains poorly understood.

Platelet signal transduction

Under resting conditions, platelets are kept dormant by inhibitors released from the endothelium. Upon vessel wall injury, several factors are released that stimulate platelet activation. The balance between inhibitory and activating signals is maintained by a tightly coordinated signal transduction system. This discussion on platelet signal transduction will be restricted to the major pathways. In general, those can be considered as either activating or inhibiting platelet function.

Inhibitory platelet signals generate the formation of cAMP. Platelets contain five classes of receptors that trigger the production of cAMP (247-249). For example, prostaglandins (PGs) of the I-type such as prostacyclin (PGI_2), which is produced by endothelial cells and is the most potent platelet inhibitor, is recognised by the IP receptor. These receptors act on trimeric G-proteins of the G_s group, which upon activation stimulate adenylyl cyclase (AC) by their α-subunit (250). PKA is thought to play an important role in the inhibitory mechanisms (251-255). The rise in cAMP is counteracted by the action of 3', 5'-cyclic nucleotide phosphodiesterases (256).

Among the signalling pathways that contribute to the rapid platelet action, the GPCRs play again a prominent role (229,250). The major platelet agonists, α-thrombin, platelet-activating factor (PAF), ADP, lysophosphatidic acid (LPA) and TxA_2, all act on serpentine receptors that are coupled to different heterotrimeric G proteins on the platelet surface. Most platelet agonists function through receptors coupled to heterotrimeric G protein G_q, the activated subunit of which ($G\alpha_q$) can induce PLC-β (257). PLC-mediated hydrolysis of phosphatidylinositol 4,5-diphosphate (PIP_2) generates both DAG regulating PKC and inositol 1,4,5- trisphosphate (IP_3). A subsequent rise in cytosolic calcium is sufficient to induce the function of $\alpha_{IIb}\beta_3$ and triggers platelet activation (229,230). Among the different platelet PLC enzymes (258,259), the β-forms are regulated by heterotrimeric G proteins (259-261), while PLC-γ forms are regulated by SH2-mediated protein-protein interactions that depend on the state of tyrosine phosphorylation (260).

Interestingly, the most recent studies point out that platelet aggregation induced by the different agonists results from concomitant signalling through both G_q- and G_i-coupled receptors (226,227). This concept has initially emerged from studies on ADP-induced platelet aggregation. ADP binds to both the P2Y receptor, coupled to G_q (262,263) and $P2Y_{12}$, coupled to G_i (264,265). Activated $P2Y_1$ receptor induces PLC, calcium mobilisation and platelet shape change (262,263), but is unable to trigger aggregation unless G_i-coupled $P2Y_{12}$ receptor is concomitantly activated (266). In addition, mice deficient in either of these receptors fail to aggregate in response to low or moderate concentrations of ADP, indicating that stimulation of both G_q- and G_i-coupled pathways is required for full activation (267-269). Several reports indicate that this dual requirement for full platelet activation is not restricted to ADP, but forms a general feature for many platelet agonists. This can be either in a direct manner, when the agonist functions on both G_q- and G_i-coupled receptors as is the case for ADP and TxA_2 (270), or indirect, as the

consequence of secreted ADP or epinephrine, which activate receptors coupled to G_i (271,272). Inhibition of AC by G_i is an important step in agonist-induced platelet activation. *In vivo* namely, platelets are continuously kept inactive by exposure to prostacyclin, produced by endothelial cells. Suppression of cAMP levels is required to enable full platelet activation. However, a decrease in cAMP alone appears insufficient to account for all the roles played by G_i family members in the support of platelet activation and this is currently under investigation (226). Additional receptor types participate in platelet regulation, like tyrosine kinase receptors, cytokine receptors and glycoproteins (228-230).

Signalling from ligand-bound GP Ib to $\alpha_{IIb}\beta_3$ requires intracellular calcium, PI(3)K and Rho (273-276). Lateral clustering of GP Ib-IX-V complexes may be involved in the up-regulation of the adhesive function of $\alpha_{IIb}\beta_3$ (277). The cytoplasmic domain of GP Ibα is known to interact with both filamin (278,279) and the signalling adaptor protein 14-3-3ζ (278,280,281). The interaction with 14-3-3ζ has been proposed to enable GP Ib to transduce signals necessary for $\alpha_{IIb}\beta_3$ activation (282). The interaction of GP Ib with filamin has a significant influence on various aspects of platelet morphology and GP Ib-IX-V receptor function (279,283). This interaction may be essential for GP Ib-IX-V anchorage at high shear stress (284-286).

In conclusion, on resting platelets, $\alpha_{IIb}\beta_3$ can only bind surface-coated fibrinogen. However, the platelet agonists described above or activation of the GP Ib-IX-V complex initiate platelet signal transduction leading to a conformational change in $\alpha_{IIb}\beta_3$ (inside-out signalling). This enables the integrin to bind soluble fibrinogen and other ligands by increasing its affinity, allowing platelet clot formation. Although the initial signalling events triggered by platelet agonists have been characterised, downstream pathways controlling $\alpha_{IIb}\beta_3$ control are still elusive. Binding of a ligand to $\alpha_{IIb}\beta_3$ and attachment of the integrin to the cytoskeleton upon platelet aggregation initiate so called outside-in signal transduction by the integrin (287,288).

Rap1 and Ral in platelet activation

Rap1 and Ral expression and localisation in platelets

In human platelets, the Rap1B expression level exceeds that of other small GTPases (including Rap1A) by at least ten fold (62,289,290). In platelets, Rap1 has been observed predominantly at the plasma membrane and associated open canicular system, but also at α-granules. Platelet stimulation with α-thrombin was found to relocalise Rap1 to the canalicular system with the secreted α-granules and Rap1 concentrates within the pseudopods formed by the plasma membrane during activation (291). Fractionation studies have described similar distribution patterns (292,293).

RalA and RalB are equally and abundantly expressed in platelets (130,133,294,295). Both isoforms are membrane-associated due to their geranylgeranylation (130). RalA was found associated with dense granules of resting platelets, vesicles that are secreted during platelet activation (296).

Rap1 activity control

Treatment of human platelets with agents like forskolin, PGE_2, PGI_2 and its analogue iloprost, elevate the cAMP level thereby reducing platelet responsiveness to activating agonists (228). In part this may be due to the activation of cAMP-regulated protein kinase A (PKA). Targets of this kinase include actin-binding protein (297), the vasodilator-stimulated phosphoprotein (VASP) (298), the GP Ibβ-chain (299) and thrombolamban (300-302), which was identified as Rap1B. The phosphorylation occurs preferentially at serine 179. Rap1A, also target for PKA, has only the less attractive serine 180 residue (303-305). In addition, cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) is able to phosphorylate the plasma membrane associated pool of Rap1B (292,306,307). The function of the PKA-mediated phosphorylation is unclear (62). However, since the phosphorylation site is close to the membrane attachment site of Rap, it is likely that phosphorylation affects membrane localisation. Indeed, the only effect observed so far *in vivo* caused by phosphorylation is an incomplete

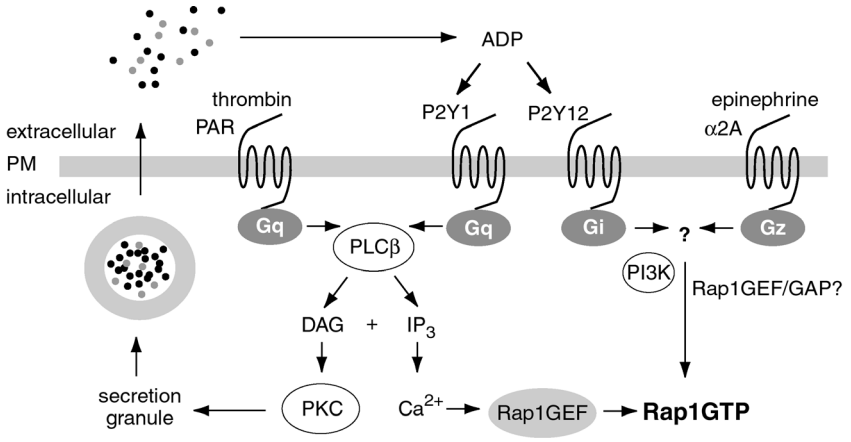

Figure 6. ***Mechanisms of Rap1 activation in platelets.***
In platelets, Rap1GTP can be increased by activating receptors coupled to G_i or G_q family members. Epinephrine binds and activates α_{2A} adrenergic receptors which induce Rap1GTP via a $G\alpha_z$-dependent pathway that at least partially requires PI(3)K. ADP stimulates both G_i- and Gq-coupled receptors, leading to two pathways of Rap1 activation: a calcium-dependent route downstream of G_q and a G_i-dependent cascade which is dependent on PI(3)K. Thrombin binds to PAR1 and PAR3 in human platelets thereby activating G_q. In addition to the PLC pathway this results in release of ADP from dense granules, which in turn stimulates the ADP receptors $P2Y_1$ and $P2Y_{12}$. Thus, thrombin also elevates Rap1GTP through both G_i- and G_q-dependent pathways. Although the exact mechanisms are not yet completely understood, G_q-mediated rise in intracellular calcium likely stimulates a CalDAG-GEF. Rap1 activation by G_i family members appears to be dependent on PI(3)K, but may also involve trapping of some RapGAP isoform.

relocalisation of Rap1B to the cytosol (308-310).

Several upstream pathways have by now been described to contribute to Rap1 activation in platelets. Initially, several platelet agonists elevating Rap1GTP were found to require PLC-mediated mobilisation of calcium (76). In addition, PKC and PI(3)K were demonstrated to play a role in sustained Rap1 activation (86) and LDL has been shown to induce Rap1 activation dependent on p38 MAPK and phospholipase A_2 (PLA_2) activation and consequent TxA_2 formation (225). LDL treatment was in these studies not able to initiate platelet aggregation and secretion, despite the considerable Rap1 activation. This implies that Rap1 activation is insufficient for the initiation of full platelet responses. Recent studies describe that stimulation of a G_i-dependent signalling pathway (for instance by ADP or epinephrine) is necessary and sufficient to induce Rap1 activation and does not require calcium mobilisation (226,227). Instead, G_i-dependent Rap1 activation requires PI(3)K activity (226). Apparently, activation of Rap1 can be accomplished by $G\alpha_i$- and PI(3)K-dependent mechanisms as well as G_q-and calcium-dependent mechanisms. The earlier observed requirement for PKC and PI(3)K in sustained Rap1 activation (86) may rely on the G_i-regulated pathway, induced by PKC-regulated secretion of ADP after initial G_q stimulation. In Figure 6, upstream pathways leading to Rap1 activation in platelets are schematically summarised.

GEFs and GAPs regulating Rap1 in platelets are currently largely unknown. However, the role of calcium and DAG in Rap1 activation (76,86) suggests the involvement of CalDAG-GEF proteins, which is supported by experimental evidence (311). Epac is most likely not expressed or at least not functional (chapter 4). PDZ-GEFs have been were recognised in platelet lysates (B. Kuiperij and J. de Rooij, personal communication). Rap1GAP activity is high in platelets and this does not change upon thrombin stimulation; only epinephrine was found to reduce Rap1GAP activity (312). Two Rap1GAP activities have been partially purified from platelet cytosol (313) and one of these was demonstrated to reside in a 55 kDa protein (64,314).

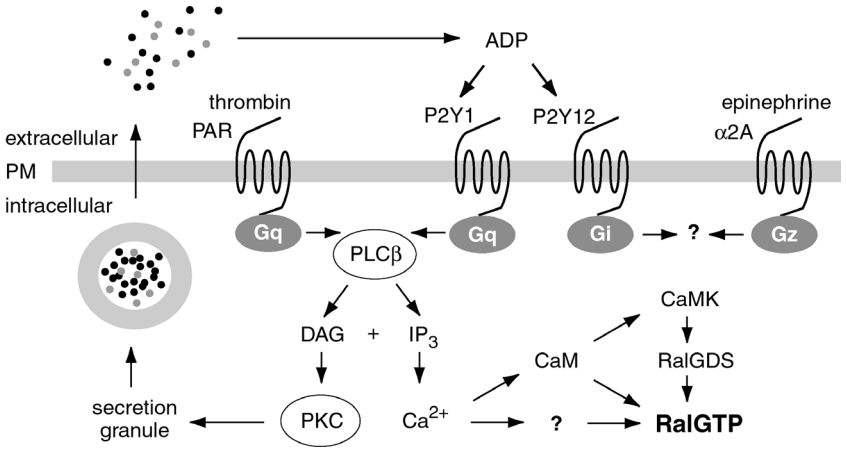

Figure 7. ***Mechanisms of Ral activation in platelets.***
Ral activation in platelets occurs most likely by Ras-independent processes in which G_q protein-coupled receptor-mediated intracellular calcium elevation plays an essential contribution. The exact mechanism linking calcium with Ral activation is not understood, however several options have been proposed, as indicated. Whether a G_i-regulated pathway is involved needs to be investigated. Although RalGEF2 was not found expressed in platelets, the presence of other RalGEFs needs to be examined.

Interestingly, this protein is very similar to the N-terminus of the 88 kDa membrane-bound Rap1GAP I (112,315), which is also expressed in platelets (B. Franke, unpublished results). An interesting aspect of Rap1 concerns its association with the cytoskeleton in activated platelets. Upon platelet activation, rapid and extensive reorganisation of the actin cytoskeleton takes place. An initial breakdown of existing structures is followed by the formation of an elaborate, strongly interconnected F-actin structure (316,317). Upon α-thrombin treatment, Rap1 completely associated with the actin filaments (318,319). In case of other platelet agonists or if aggregation is absent, only 20-40% of Rap1 bound to actin (319,320). Rap1B and Rap2B association with the cytoskeleton induced by vWF involving FcγII receptor-mediated protein tyrosine phosphorylation has also been demonstrated (321). Platelet aggregation was found to downregulate Rap1 activity and the cytoskeleton-associated fraction of Rap1 was in the inactive state (86). Moreover, Rap1GAP I was also observed to associate with the cytoskeleton in thrombin-stimulated platelets (B. Franke, unpublished results). Although these observations are interesting and may form a mechanism for Rap1 activity downregulation, the function of the interaction between these signalling proteins and the cytoskeleton remains obscure.

Ral activity control

An intriguing relation between Rap1 and Ral was found in platelets (146). The proteins seemed to be regulated in a coordinated fashion, as Ral activation correlated with the activation profile of Rap1 instead of Ras. Firstly, this clearly implicated Ral in addition to Rap1 in platelet activation, suggesting also participation of Ral in platelet functions. Interestingly, Ral had been proposed to function in secretion and/or cytoskeletal organisation. Secondly, this formed evidence for Ras-independent ways to regulate Ral activity, attracting attention towards the upstream activation mechanisms of the Ral proteins. Thirdly, the correlation in activities together with the known interaction between Rap1 and RalGDS suggested a function for Rap1 in Ral regulation.

In platelets, RalGTP is rapidly increased upon treatment with α-thrombin, platelet activating factor (PAF) or TxA_2, all ligands for GPCRs (146,225). Activation of Ral by these factors requires an increase in the level of cytosolic calcium, either by the mobilisation from intracellular calcium stores or by the induction of a calcium influx. In Figure 7, a schematic representation of the Ral activation in

platelets is shown. As the RalGDS GEFs can in addition to Ras also bind other Ras family members (36,41,140,141,322-324), Rap1 has been proposed to regulate Ral in platelets (151). Binding studies have revealed that the RBD in RalGDS has a 100-fold higher affinity for Rap1 than for Ras or R-Ras *in vitro* (37). However, in Cos7 cells transfected Ras rather than R-Ras or Rap1 stimulated RalGDS, Rgl and Rlf exchange activity towards Ral (39,142,143). This suggests the calcium/calmodulin-pathway leading to Ral activation might be most prominent in platelets, as also no evidence was found for expression of RalGEF2 (chapter 2). Currently it is unknown whether G_i-regulated signalling, which leads to Rap1 activation, also results in RalGTP accumulation.

Functions of Rap1 and Ral in platelet perspective

Rap and intracellular calcium homeostasis

An interesting direction concerning the role of Rap1 in platelet physiology, originates from its connection with the 97 kDa sarco-/endoplasmic reticulum calcium-ATPase 3b (SERCA 3b). SERCA is associated with IP_3-sensitive calcium stores in intracellular membranes (325-329). Firstly, several lines of research indicate correlated expression of SERCA and Rap1 in platelets and other cell lines (328,330,331). Interestingly, interaction between Rap1 and SERCA 3b in co-immunoprecipitation experiments has been reported, which was abrogated by phosphorylation of Rap1 in the membrane preparations by the catalytic subunit of PKA (327). Rap1, as PKA target, forms the major phosphoprotein in antagonist-inhibited platelets. Therefore, Rap1 has been connected to the PKA-induced removal of calcium from the platelet cytosol due to activation of calcium-ATPases (332-334). Calcium-uptake is stimulated by PKA in platelet microsomes containing both PKA and Rap1B (thrombolamban) (335). In addition, GTPγs, a non-hydrolysable GTP analogue, inhibits both the calcium-ATPase activity and the phosphorylation of Rap1b by the catalytic subunit of PKA in membrane preparations of platelets (336). One problem for this mechanism to occur *in vivo* however, is raised by the finding that PKA-mediated Rap1B phosphorylation is a slow process in intact platelets (303,308) that lags behind inhibition of platelet functions by antagonists, such as removal of cytosolic calcium (76,337). Furthermore, debate is going on whether platelet antagonists induce re-uptake of intracellular cytosolic calcium into stores, or causes inhibition of mobilisation from these stores (338). However, recently also other reports point to a role of Rap proteins in the regulation of intracellular calcium. A cAMP-induced Epac-PLC pathway in which Rap2B may function was found to increase intracellular calcium in HEK293 cells (339). Finally, a cAMP-induced calcium increase in human megakaryoblast has been reported and the involvement of Rap1 was proposed (340).

Rap1 and $\alpha_{IIb}\beta_3$ integrin regulation

As has been described already, Rap1 has been implicated in the control of integrin-mediated cell adhesion. This has raised the question whether Rap1 may be able to regulate the platelet integrin $\alpha_{IIb}\beta_3$ as well. The role of Rap1b in $\alpha_{IIb}\beta_3$ function was investigated by viral transduction of GFP-Rap1 chimeras into murine megakaryocytes, which exhibit inside-out signalling similar to platelets. As a readout for integrin affinity, binding of soluble fibrinogen was measured. Interestingly, in this study solely expression of active Rap1B showed no effect, but it augmented thrombin-induced fibrinogen binding (311). This effect was prevented by inhibition of actin polymerisation. The authors proposed Rap1B might regulate $\alpha_{IIb}\beta_3$ affinity by modulating its interactions with the cytoskeleton. With this respect, it is interesting to note again the reported relocalisation of Rap1 to the actin cytoskeleton upon platelet activation (section *Rap1 activity control*). In addition, Rap1 signalling is also required for TPA-induced adhesion of DAMI human megakaryoblasts to immobilised fibrinogen (chapter 5). Although further research is required to demonstrate this connection in platelets, these studies form initial proof for a function of Rap1 in the control of the platelet integrin $\alpha_{IIb}\beta_3$ and link Rap1 signal transduction to one of the most elementary processes underlying platelet physiology.

Rap1 effector proteins

Also in platelets Rap1 may interact with RBD-containing proteins. The correlation in Rap1 and Ral activity in platelets has raised the idea that Rap1 may regulate RalGDS (146). However, evidence that Rap1 exerts functions *via* on of the Ras effectors *in vivo* has not yet been established. Moreover, expression of RalGDS has not even been demonstrated in platelets and a calcium pathway without contribution of a GEF may be sufficient to regulate Ral activity. Among the (putative) targets for Ras-like GTPases, proteins containg a predicted RBD or Ras-associating (RA) domain, several candidates have additional domains that allow interaction with the cytoskeleton, integrins or adherens junctions, like for instance talin and AF-6. Interaction with these proteins may explain how Rap1 regulates integrins and why Rap1 relocalises to the actin cytoskeleton upon platelet activation. However, in conclusion, no Rap1 effector protein has been established in platelets and this item awaits further research.

Ral binding partners and functions

In contrast to Rap1, for Ral several target proteins and binding partners have been characterised in the past ten years, as described in the section *Ral effectors and functions*. With respect to platelets the nature of these binding partners implicates Ral in the regulation of vesicular traffic and the actin cytoskeleton. Like Rap1, also Ral was observed to associate with the cytoskeleton upon platelet activation (Franke, unpublished results).

III. Conclusions

In conclusion, Rap1 and Ral signal transduction pathways are activated upon a variety of platelet agonists and inhibited by platelet antagonists. This correlation suggests the involvement of both GTPases in platelet function. By the use of several relevant model systems, an augmenting pile of plausible possibilities for cellular functions of Rap1 has been put forward and this is still in progress. Although interaction with Ras effectors remains an option, in terms of platelet physiology connections with vesicular transport, intracellular calcium regulation, cytoskeleton and adhesion, which all have only recently begun to emerge, are more attractive. In addition, Ral has been implicated in the regulation of several types of vesicular transport and the cytoskeleton. If at any time a reason existed to perform functional studies on Rap1 and Ral in platelets, it is definitely most challenging now.

IV. Scope of this thesis

We studied both the upstream regulation of Ral proteins and downstream functions of Rap1 in the light of platelet activation. Beside the advantages of platelets as a model for signal transduction, also a few important disadvantages exist. First of all, platelets cannot be directly used for genetic studies. Transfection experiments are impossible due to the lack of the transcription machinery. Intervention can only take place by using pharmacological inhibitors and activators. Moreover, platelets cannot be cultured. Therefore, blood donation is required, which inevitable increases biological variation due to donor variability. As the consequence of these restrictions, other models (megakaryocytes, lymphocytes and fibroblasts) had to be used as well in order to address our research questions.

We started to investigate the Ras-independent Ral activation mechanism. In chapter 2 the identification and characterisation of RalGEF2 is described. This novel RalGEF belongs to the family of RalGPS proteins. RalGEF2 lacks an RBD domain and its activity is independent of Ras signalling. This GEF possesses a PH domain that plays a role in its membrane localisation and activaty towards Ral. However, regulation of RalGEF2 by second messengers or complex formation has not been identified yet. Next, we focussed on cellular effects regulated by Rap1 signal transduction. In chapter 3 we describe the requirement for Rap1 in integrin-mediated cell adhesion induced by either Mn^{2+} or integrin-activating antibodies. This Rap1 role was demonstrated in a variety of cell types and in different combinations of integrin type and immobilised integrin ligand. We found that active Rap1-induced cell adhesion depends on intracellular calcium and calmodulin. We propose a facilitating function for Rap1 in the

cell adhesion process. In chapter 4 we have studied the putative involvement of Rap1 in a cAMP-induced PKA-independent signalling pathway regulating the intracellular calcium regulation in human megakaryocytes. Using a cAMP analogue that specifically activates the cAMP-regulated RapGEF Epac, we did not find evidence for the involvement of endogenous Rap1 signalling in calcium homeostasis. However, we observed Rap1 activation as the consequence of gentle mixing a sample of megakaryocytes. Further research revealed that in addition to Rap1, several key signal transduction pathways are activated in a variety of human suspension cell lines, solely due to mixing them in a test tube, described in chapter 5. This may well be the consequence of shear or mechanical stress generated by mixing. Furthermore, we show that Rap1 is also required for TPA-induced $\alpha_{IIb}\beta_3$-mediated adhesion of DAMI megakaryoblastic cells to immobilised fibrinogen. This forms an initial indication that Rap1 contributes to the regulation of the major platelet integrin. Finally, in chapter 6 the implications of our research findings are discussed and placed in the context of the latest research developments.

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CHAPTER

2

RalGEF2, a Pleckstrin homology domain containing guanine-nucleotide exchange factor for Ral

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RalGEF2, a Pleckstrin homology domain containing guanine-nucleotide exchange factor for Ral

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Ral is a ubiquitously expressed Ras-like small GTPase. Several guanine-nucleotide exchange factors for Ral have been identified, including members of the RalGDS family, which exhibit a Ras binding domain and are regulated by binding to RasGTP. Here we describe a novel type of RalGEF, RalGEF2. This GEF has a characteristic Cdc25-like catalytic domain at the N-terminus and a pleckstrin homology (PH) domain at the C-terminus. RalGEF2 is able to activate Ral both *in vivo* and *in vitro*. Deletion of the PH domain results in an increased cytoplasmic localisation of the protein and a corresponding reduction in activity *in vivo*, suggesting that the PH domain functions as a membrane anchor necessary for optimal activity *in vivo*.

INTRODUCTION

Ral (RalA and B) is a small GTPase of the Ras family implicated in the control of cell proliferation (1-7), differentiation (8-10), cytoskeletal organisation (11) and vesicular transport (12). Ral is activated by a large variety of extracellular stimuli (13-15) and one of the mechanisms of Ral activation is the direct binding of active Ras to Ral-specific GEFs. These GEFs, RalGDS, Rgl and Rlf, have a C-terminal Ras binding domain (RBD) responsible for the interaction with RasGTP and an N-terminal Cdc25-like catalytic domain (5,16,17). However, other mechanisms of Ral activation occur as well. In platelets and in fibroblasts elevation of intracellular calcium levels induce Ral activation independently of Ras activation (13,18) and in neutrophils phosphatidylinositol-3'-OH-kinase (PI3K) and perhaps Src may mediate Ral activation (15). This suggests that in addition to RalGDS, Rgl and Rlf, other Ral regulatory proteins exist. Indeed, Rsc, a fusion protein isolated from a rabbit squamous cell carcinoma, has RalGEF activity and appears to lack an RBD (19). RalGTP levels are also regulated by GTPase activating proteins (GAPs), including a high molecular mass RalGAP purified from brain and testis and a 43 kDa RalGAP in human platelets (20,21). Finally, Ral A interacts with calmodulin (22,23), which may be involved in calcium-induced regulation of Ral (13,18).

In addition to proteins that regulate its activity, Ral interacts with several other proteins which may function as effectors. The active, GTP-bound form of Ral associates with RalBP (24-26). RalBP is a GAP for Cdc42, a small GTPase involved in actin cytoskeleton organisation and filopodia formation in fibroblasts (27). In addition, RalBP associates with Reps1 and POB1, two proteins implicated in endocytosis (12,28,29). RalGTP also binds to ABP280/filamin1, a cross-linker of actin filaments and a scaffold for several other proteins. This complex may mediate Cdc42-induced filopodia formation (11). Finally, Ral associates with the small GTPase Arf and phospholipase D (PLD) in a nucleotide-independent manner (30-33). Both PLD and Arf have been implicated in many processes, including vesicular transport (34-37). All these

interactions point to a function of Ral in the control of the actin cytoskeleton and processes related to this, such as establishing cell polarity, migration and vesicular transport (see (38).

In this paper we describe the identification of a novel Ral-specific GEF, RalGEF2. This GEF has a characteristic Cdc25-like catalytic domain at the N-terminus and a pleckstrin homology (PH) domain at the C-terminus. This PH domain functions as a membrane anchor necessary for optimal activity of RalGEF2 *in vivo*.

MATERIALS AND METHODS

Plasmids and constructs

The KIAA0351 cDNA containing the complete coding sequence was kindly provided by the Kazusa DNA Research Institute. This cDNA was isolated in a random cloning strategy (39). A PCR fragment containing RalGEF2 flanked by a SalI site at the 5' (primer A: 5'-GTCGACTATGTACA-AGAGGAATGGTCTG-3') and a HpaI site at the 3' (primerB: 3'-CGTTTGGAATACAGTAA-ACTCCAATTG-ATC-5') end, was subcloned into the pGEM-T vector (Promega). This clone was subsequently used to generate HA-RalGEF2, by introducing a SalI fragment containing RalGEF2 into SalI-digested pMT2-SM-HA. Cat-RalGEF2 (amino acids 1-289), flanked by a SalI site at the 5' end (primer A) and a HpaI site at the 3' end (primer C: 3'-CGAGTCTTAGCTTGGTCCTTTACAATTG), and ΔPH-RalGEF2 (amino acids 1-431), flanked by a SalI site at the 5' end (primer A) and a HpaI site at the 3' end (primer D: 3'-CTTGAGGCGTCGACACGGTCAATTG-5'), were also subcloned in pGEM-T. These SalI-HpaI fragments were also cloned into SalI-HpaI digested pBluescript in which SalI-NotI Rlf-ΔRBD-CAAX had been cloned previously to obtain RalGEF2 proteins with the C-terminal polybasic sequence and CAAX box of K-ras (5). Subsequently, SalI-NotI fragments from these clones were isolated and ligated into SalI-NotI-digested pMT2-SM-HA to generate HA-cat-RalGEF2-CAAX (HA-cat-CAAX) and HA-ΔPH-RalGEF2-CAAX (HA-ΔPH-CAAX). In addition, cat-RalGEF2 and ΔPH-RalGEF2 were also isolated as SalI-NotI fragments from the pGEM-T constructs and cloned into SalI-NotI digested pMT2-SM-HA vector, generating HA-cat-RalGEF2 (HA-cat) and HA-ΔPH-RalGEF2 (HA-ΔPH). From HA-cat-RalGEF2 the mutants HA-catRalGEF2 L148A and HA-catRalGEF2 ΔSALQS were generated by site-directed mutagenesis. GST-fusion constructs were made by isolating SalI-NotI fragments from the HA-RalGEF2, HA-cat-RalGEF2-CAAX and HA-ΔPH-RalGEF2-CAAX constructs and cloning these fragments into XhoI-NotI-digested pGEX-4T3 bacterial expression vector, generating respectively GST-RalGEF2, GST-cat and GST-ΔPH.

Homology searches

RalGEF2 was found by searching databases for proteins containing Cdc25 catalytic domain homology. RalGEF2 was analysed for protein domains using the ISREC ProfileScan server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html). The structurally conserved region (scr) sequence was defined from a comparison of various Ras and RalGEFs using the clustalX (40) alignment program, as was done for the alignment of homologous amino acids in the PH domain.

Cell culture, cell lines and transfection

A14 (41) and Cos7 cells were grown at 37 °C in DMEM (Gibco) supplemented with 10% heat-inactivated (30 min. at 56 °C) FCS and 0.05% glutamine. For activation studies cells were serum starved overnight for at least 16 hours. Cells were transfected by the calcium-phosphate method.

Purification of GST-proteins

For purification of GST-fusion constructs, protein expression was induced in DH5α using 100 nM IPTG for 20 h at room temperature. Bacteria were collected and lysed in ice-cold PBS containing 1% TX-100 and protease inhibitors. The lysates were sonicated three times for 20 seconds and centrifuged at 10,000 g for 20 minutes to remove insoluble material. GST-fusion proteins were purified from the cleared lysate by batch-wise incubation with glutathione-agarose beads (Sigma) and after washing the protein was eluted from the beads in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 10 % glycerol and 10 mM glutathione. The eluted protein was dialysed for 20 hours in the same buffer without glutathione. The purification procedure of the small GTPases used in the in vitro experiments is described elsewhere (42-44).

Immunoprecipitation and Western blotting

Western blotting of all protein samples was performed using PVDF-membranes. The antiserum used for detecting endogenous RalGEF2 was raised against a synthetic peptide that consists of the amino acids 543-557 (KSNRPQVPANLMSFE) of RalGEF2. Other antibodies used are anti-HA (12CA5) (45), anti-Ral, anti-Ras and anti-Rap (all three from Transduction Laboratories). Anti-RalGEF2 and 12CA5 were also used to perform immunoprecipitations.

In vivo activation of small GTPases

Cells were transiently transfected with HA-tagged versions of the small GTPases either alone or in combination with RalGEF2, and serum-starved for 20 hours prior to lysis. GTP-bound forms of the different GTPases were isolated using activation specific probes and subsequently quantified, as described (13,46,47). For RalGTP the Ral-binding domain of RLIP was used, for Ras the RDB of Raf1 and for Rap the RBD of RalGDS.

In vitro activation of small GTPases

In vitro GEF activity was measured as described (48). Briefly, 250 nM of purified GTPase, loaded with fluorescently labelled 2',3'-bis(O)-N-methylantharanoloylguanosinediphosphate (mantGDP), was incubated, in the presence of excess unlabelled GDP (5mM), with 50 nM of purified GST-cat in 50 mM Tris pH 7.5, 5 mM Mg^{2+} and 5 mM DTE at 20°C. Release of mantGDP was measured in real-time as a decrease in fluorescence using a Perkin-Elmer fluorometer LS50B (excitation wavelength 366 nm, emission wavelength 450 nm). This decrease in fluorescence is caused by quenching of released mantGDP by water. In the Ral assay, 0.15 mg/ml BSA was added to stabilise the protein. Alternatively, we used a reverse assay (49) in which 150 nM RalGDP was added to a cuvette containing 100 nM mantGDP either alone or in the presence of 50 nM purified GEF protein. Uptake of mantGDP results in an increase in fluorescence.

Subcellular fractionation

Cells were harvested in lysis buffer (20 mM Hepes, pH 7.4, 5 mM EGTA, 1 mM sodium vanadate, 1μM leupeptin, 0.1 μM aprotinin) and subsequently homogenised through a 23 G 1 1/4 Microlance syringe. Intact cells and nuclear components were removed by a sequential two times centrifugation step at 6000 rpm for 1 minute (Eppendorf table centrifuge). Subsequently, the samples were centrifuged at 100,000 g at 4˚C for 90 minutes. The supernatant was collected as the soluble fraction and the particulate fraction was dissolved in buffer containing 1% TX-100, 50 mM Hepes pH 7.5, 50 mM

NaCl, 5 mM EDTA, 1 mM vanadate, 1 μM leupeptin and 0.1 μM aprotinin. Soluble and particulate fractions were analysed by SDS-PAGE and immunoblotting.

RESULTS

A novel Ral guanine-nucleotide exchange factor

In our ongoing search for regulators of Ras-family members, we found a protein in the database from the Kazusa DNA Research Institute (KIAA0351) with similarities to RasGEFs. A schematic representation of the non-coding and coding sequences of KIAA0351 is presented in Figure 1A. As will be shown below, this protein is a GEF for the small GTPase Ral, hence we named it RalGEF2, since this GEF forms a novel subclass, distinct from the RalGEF members which are characterised by the presence of the RBD domain (Figure 1B). RalGEF2 has the characteristic GEF domain present in all RasGEFs and shows considerable homology to previously described RasGEFs, especially in the structurally conserved regions scr1, 2 and 3 (data not shown). In addition, RalGEF2 has a PH domain in its C-terminus that shows highest homology to the PH domain in the Drosophila RhoGEF Still Life (Sif) (50), and the N-terminal PH domains in the RacGEFs Tiam1 and Stef (51,52) (Figure 1C). Surprisingly, no Ras exchange motif (REM) is present in RalGEF2, in contrast to all other Cdc25-like GEFs identified so far.

Expression of RalGEF2

A polyclonal antibody (α-RalGEF2) was raised against a C-terminal peptide of RalGEF2. This antibody recognised the 60 kD HA-RalGEF2 protein immunoprecipitated from Cos7 cell lysate transiently transfected with HA-RalGEF2 using an α-HA-monoclonal antibody (Figure 2). A similar-sized protein was identified in an α-RalGEF2-immunoprecipitate from 293 cells. Both protein bands disappeared when the antibody was preincubated with the immunising peptide. From this result we concluded that α-RalGEF2 recognises RalGEF2 both in immunoprecipitation and blotting experiments. Next, a Western blot containing protein samples from various human tissues was probed with either α-RalGEF2 or α-RalGEF2 blocked with peptide. As shown in Figure 2B, RalGEF2 expression is rather ubiquitous. It is high in brain, heart, kidney, adrenal gland and colon, low in pancreas, skeletal muscle, thymus and liver, and intermediate in lung and spleen. Ubiquitous expression of RalGEF2 was also shown using RT-PCR by the Kazusa DNA Research Institute (http://www.kazusa.or.jp/huge/gfimage/rt-pcr/html/KIAA0351.html).

RalGEF2 activates Ral both in vivo and in vitro

To investigate which Ras-like GTPase is activated by RalGEF2, we incubated GST-cat (Figure 3A) with various Ras-like GTPases loaded with fluorescent mantGDP. Release of guanine-nucleotide bound to Ral was measured in real time as decrease in fluorescence. As shown in Figure 3B, incubation of Ral-mantGDP alone already resulted in a decrease in fluorescence, indicating that a fraction of the protein sample was instable and degraded during the incubation. In the presence of GDP intrinsic exchange activity is measured. We observed a clear increase in exchange when GST-cat was added as well, showing that GST-cat catalyses Ral guanine-nucleotide exchange. GST-cat did not affect the exchange rate of either Ras or Rap2 (Figure 3C). As an alternative assay we used a reverse procedure in which binding of mantGDP to Ral results in an increase in fluorescence. MantGDP either alone, in the presence of GST-cat or in the presence of GST-Rlf was incubated and RalGDP was added. As shown in Figure 3D, both GST-cat and GST-Rlf catalysed guanine-nucleotide exchange. Surprisingly, when a shorter version of Ral lacking an additional 28 residues at the C-terminus was used in the assay, GST-cat failed to induce exchange, in contrast to GST-Rlf. This effect was also observed when corresponding *Drosophila* Ral proteins were used (data not shown). To investigate whether GST-cat may still bind to Ral, we pre-incubated Ral-mantGDP and GST-cat for prolonged period of time followed by the addition of GST-Rlf. GST-cat did not affect the ability of GST-Rlf to induce exchange (Figure 3E), indicating that RalGEF2 fails to interact stably with the C-terminally truncated protein. From these results we conclude that RalGEF2 is indeed a GEF for Ral, and that, in contrast to Rlf, RalGEF2 requires the C-terminal 28 residues of Ral for proper interaction.

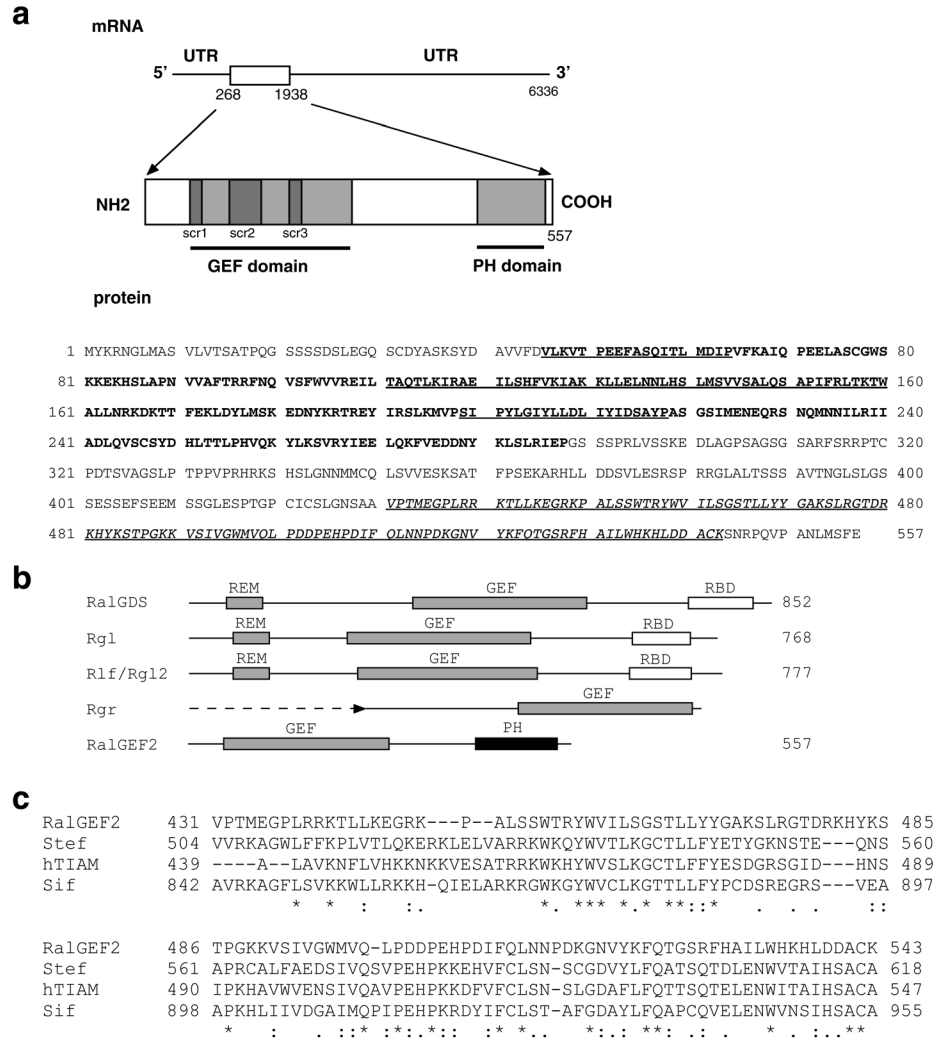

Figure 1. ***RalGEF2 contains a GEF domain and a PH domain.***
A. Schematic representation of the noncoding and coding sequence in KIAA0351 mRNA and the domains present in the predicted protein sequence. UTR, untranslated region; scr 1-3, hyper conserved regions in GEFs for Ras-like proteins. Bold type represents the GEF domain, bold and underlined scr1-3 and underlined the PH domain. B. Schematic comparison of the five RalGEFs currently identified. Both RalGDS, Rgl and Ral contain in addition to the GEF domain, a Ras exchange motif (REM) and a Ras binding domain (RBD). Rgr is a RalGEF that is only found as a partner in a fusion protein isolated from a rabbit squamous cell carcinoma (Rsc). The dashed arrow indicates the other fusion partner. No mouse or human Rgr sequences have thus far been reported. C. Sequence comparison between the PH domain of RalGEF2 and those present in the N-terminal region of the RacGEFs Stef and Tiam-1, and *Drosophila* Still life (Sif).

Subsequently, we analysed whether RalGEF2 also activates Ral *in vivo*. HA-RalGEF2 was cotransfected with HA-tagged RalA in Cos7 cells and Ral activity was measured using activation-specific probes. As shown in Figure 4A, cotransfection with RalGEF2 resulted in a clear activation of Ral. In contrast, RalGEF2 displayed only marginal

activity for HA-Ras and no exchange activity for HA-Rap1 *in vivo* (Figure 4B and C). From this result we conclude that RalGEF2 selectively activates Ral *in vivo*. Next, we addressed the question whether indeed the GEF activity is reponsible for this effect. HA-cat-CAAX, which is described below, activates cotransfected HA-Ral. We made mutants of this protein in a region that is fully conserved in all human RalGEFs, located in the scr2, in order to abolish its catalytic activity. We found that both HA-cat L148A and HA-cat ΔSALQS (residue 146-150) failed to activate HA-Ral (figure 4D). From these data we conclude that activation of Ral *in vivo* is directly mediated by the catalytic activity of RalGEF2.

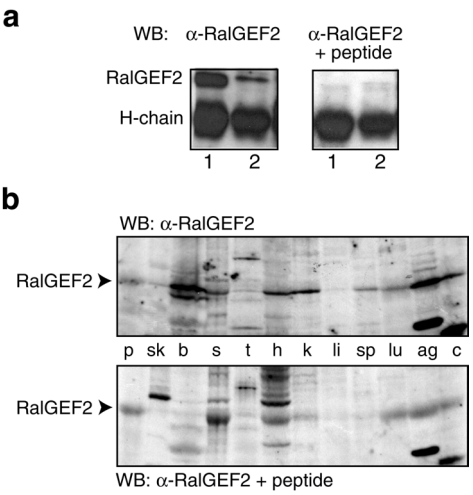

Figure 2. ***Expression of RalGEF2 in human tissue.*** A. HA-RalGEF2 was precipitated from lysate of Cos7 cells transiently transfected with HA-RalGEF2 using anti-HA (12CA5) monoclonal antibody (lane 1), endogenous RalGEF2 was precipitated from lysate of 293 cells using a polyclonal peptide antibody of RalGEF2 (α-RalGEF2; lane 2). Blots were probed with either α-RalGEF2 (left panel) or with α-RalGEF2 preincubated with the peptide used for immunisation (right panel). H-chain: immunoglobulin heavy chain. B. New-born human tissue samples were lysed and equal amounts of protein were separated by gel electrophoresis. Gels were blotted and probed with either α-RalGEF2 (upper panel) or with α-RalGEF2 preincubated with the peptide used for immunisation (lower panel). Lanes represent pancreas (p), skeletal muscle (sk), brain (b), skin (s), thymus (t), heart (h), kidney (k), liver (li), spleen (sp), lung (lu), adrenal gland (ag), colon (c).

Regulatory function of the PH domain

PH domains commonly interact with membrane lipids, in particular phosphorylated phosphatidylinositol (PI) lipids. As such, PH domains function either as membrane anchor or as a regulatory domain that responds to the products of PI3K, PI-3,4-P_2 or PI-3,4,5-P_3. To determine whether RalGEF2 responds to PI3K signalling, we introduced RalGEF2 in A14 cells and stimulated the cells with insulin, a potent inducer of PI3K activity (45). However, we did not observe any increase in RalGEF2 activity by insulin treatment (data not shown). Also other stimuli tested, including EGF, endothelin, forskolin, ionomycin, LPA and serum failed to activate RalGEF2. We therefore investigated whether the PH domain is involved in membrane localisation of RalGEF2. Cells were transfected with HA-RalGEF2 or mutants lacking the PH domain (HA-ΔPH and HA-cat) and the presence of these proteins in the cytosol and the membrane-enriched particulate fraction was determined. Deletion of the PH domain resulted in a clear reduction of the level of RalGEF2 in the membrane fraction (Figure 5A), indicating that the PH domain is involved in membrane localisation of RalGEF2. This reduced association to membranes could be restored by adding the C-terminal polybasic domain and CAAX sequence of K-ras to HA-ΔPH and HA-cat (HA-ΔPH-CAAX and HA-cat-CAAX). This region directs the addition of a C-terminal isoprenyl group and as a consequnce membrane attachment (Figure 5A).

Next, we investigated whether deletion of the PH domain also affects GEF activity *in vivo*. Cells were cotransfected with the various RalGEF2 constructs and HA-Ral, and the level of RalGTP was determined. Even at lower levels of expression (upper panel Figure 5B) full length RalGEF2 is much more efficient in activating Ral than the mutant lacking the PH domain (Figure 5B), showing that the PH domain is required for efficient RalGEF2 activity. The reduced GEF activity of HA-ΔPH can be restored by the addition of a membrane anchor (HA-ΔPH-CAAX; Figure 5 C). Similarly, addition of the CAAX domain to HA-cat increased the efficiency of Ral guanine-nucleotide exchange activity (Figure 5D). From these results we conclude that the PH domain of RalGEF2 is a membrane targetting sequence responsible for efficient RalGEF activity.

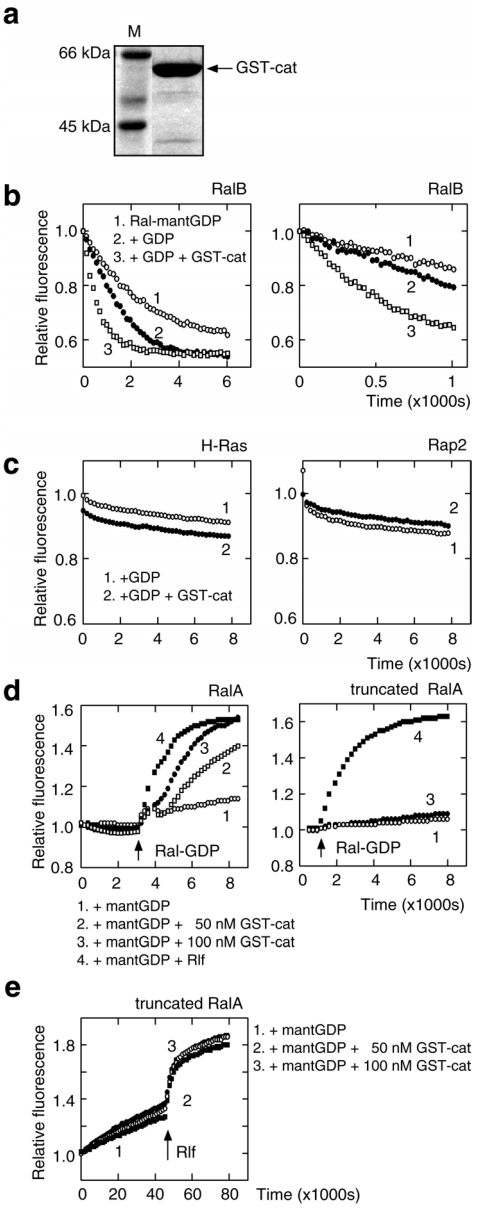

Figure 3. ***RalGEF2 specifically activates Ral in vitro.*** A. Coomassie Blue-stained gel, which demonstrates the purification result of a fusion protein containing GST and the catalytic domain of RalGEF2 (GST-cat; residue 1-289). M stands for marker. B. 250nM human RalB loaded with fluorescent mantGDP was incubated either alone to measure stability of the protein (1, open circles), with 5 mM GDP to measure the intrinsic release (2, closed circles) or with 5 mM GDP and 50 nM GST-cat (3, open squares). The right panel is a magnification of the initial part of the reaction in the left panel, to clearly show the rate differences. C. 250 nM H-Ras.mantGDP (left panel) or 250 nM Rap2.mantGDP (right panel) was incubated with either 5 mM GDP (1, open circles) or 5 mM GDP and 50 nM GST-cat (2, closed circles). D. 100 nM mantGDP was incubated either alone (1, open circles), in the presence of 50 (2, closed circles) and 100 (3, open squares) nM GST-cat, or Rlf (4, closed squares). At the time indicated (arrow) 150 nM simian Ral loaded with GDP was added. Two different RalA proteins were used, one containing residue 1-206 (left panel) and one containing residue 1-178 (right panel). E. 150 nM simian RalGDP (residue 1-178) was incubated with 100 nM mantGDP either alone (1, open circles), or in the presence of 50 nM GST-cat (2, closed circles) or 100 nM GST-cat (3, open squares) for a prolonged period of time. Subsequently, 50 nM Rlf was added (arrow) and incubation continued.

DISCUSSION

We have identified a novel GEF for the small GTPase Ral, RalGEF2. This is a ubiquitously expressed protein that is particularly abundant in brain, heart, kidney, adrenal gland and colon. Previously, four other RalGEFs have been identified, including RalGDS, Rgl and Rlf. These GEFs have in addition to the catalytic region a C-terminal Ras binding domain. The most striking characteristic of RalGEF2 is its PH domain. This domain is most similar to PH domains present in the Rho/RacGEFs Tiam1, SIF and *Drosophila* Still life. PH domains may bind to PI lipids, which are either constitutively present or which are induced by certain stimuli. For instance, the product of PI3K, PI-3,4-P_2, recruits target proteins to the membrane by binding to their PH domains (53,54) Via this relocalisation a protein is for instance either brought into the vicinity of its target or of its activators. However, the fact that insulin, a very strong inducer of PI3K, fails to activate RalGEF2, suggests that RalGEF2 activity is not induced by these lipids. Thus, the PH domain may serve to constitutively anchor RalGEF2 to membranes. Indeed deletion of the PH domain results in an increased cytoplasmic localisation of the protein and a corresponding reduction in the efficiency of Ral activation *in vivo*. Both membrane localisation and efficient GEF activity could be restored by the addition of the C-terminal polybasic region and CAAX motif of K-ras, which targets proteins to the membranes. From these results we conclude that the PH domain is predominantly responsible for membrane localisation of RalGEF2.

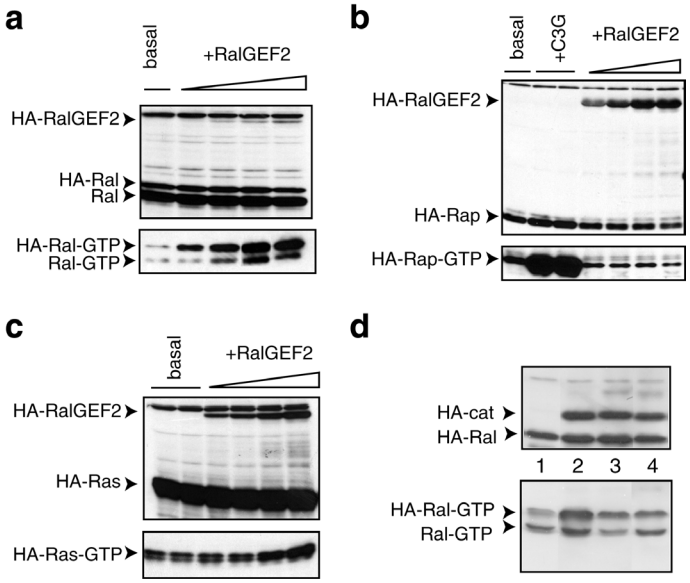

Figure 4. ***RalGEF2 specifically activates Ral in vivo.*** HA-RalA (A), HA-Rap1 (B) or HA-Ras (C) was cotransfected in Cos7 cells either alone or in the presence of HA-RalGEF2 (0.5, 1.0, 2.0 and 5.0 μg). A. Upper panel shows a Western blot of cell lysate incubated with anti-HA and anti-Ral antisera, lower panel shows HA-RalGTP and endogenous RalGTP precipitated with RalBD of RLIP and identified with anti-Ral antiserum. B. Upper panel shows a Western blot of cell lysates incubated with anti-HA, lower panel shows HA-Rap1-GTP precipitated with RalGDS-RBD and identified with anti-HA antiserum. In the second and third lane the Rap1-specific GEF C3G was cotransfected. C. Upper panel shows a Western blot of cell lysates incubated with anti-HA, lower panel shows (HA-)RasGTP precipitated with Raf-RDB and identified with anti-HA antisurum. D. Cos7 cells were transiently transfected with HA-RalA either alone (lane 1), HA-cat (lane 2), HA-cat L148A (lane 3) and HA-cat ΔSALQS (residue 146-150; lane 4). Upper panel shows a Western blot of cell lysates incubated with anti-HA, lower panel shows HA-RalGTP and endogenous RalGTP precipitated with RalBD of RLIP and identified with anti-Ral antiserum.

Our failure to induce the activity of RalGEF2 by external stimuli may indicate that RalGEF2 is a constitutively active GEF, or that we have not yet identified the proper stimulus. Whereas Ral clearly serves as a downstream target of Ras signalling, through the direct binding of members of RalGDS, Rgl and/or Rlf to activated Ras, it is clear from several studies that other pathways are also mediating Ral activation. For instance, calcium can activate Ral independently of Ras (13,18), while in neutrophils as yet unidentified pathways exist (15). RalGEF2 may mediate one of these pathways.

RalGEF2 is distinct from the other RalGEFs in that it does not have a REM domain. This REM domain is thought to play a role in stabilising the catalytic domain of Ras-like GEFs. Perhaps the PH domain or another region serves a similar function. An additional striking difference between RalGEF2 and other RalGEFs, in particular Rlf, is that RalGEF2 does not exchange nucleotide *in vitro* from a truncated version of Ral. Apparantly the C-terminal region of Ral contains amino acids essential for the proper binding of the catalytic domain of RalGEF2. Whether RalGEF2 directly interacts with this region, or if the absence of the REM domain is responsible for this difference awaits further investigation. However, this result clearly indicates that RalGEF2 uses a different molecular mechanism to release GDP from Ral than Rlf does.

Ral has been implicated in a variety of cellular processes. Most notable is the role of Ral in coupling signals from Ras to the induction of transcription, such as transcription from serum-response elements (5,55) and the inhibition of the transcription factor AFX (56). These effects may, at least in part, explain the effects of Ral-mediated signalling on cell proliferation (1-7) and differentiation (8-10). However, the mechanism by which Ral regulates transcription is unclear. Another function of Ral is the regulation of the cytoskeleton. This is indicated by the association of the active form of Ral with RalBP, a GAP for the small GTPase Cdc42, and to filamin/ABP280, a protein involved in the cross-linking of actin filaments. Indeed, Ral was found to mediate Cdc42-induce filopodia formation. Finally, Ral has been implicated in

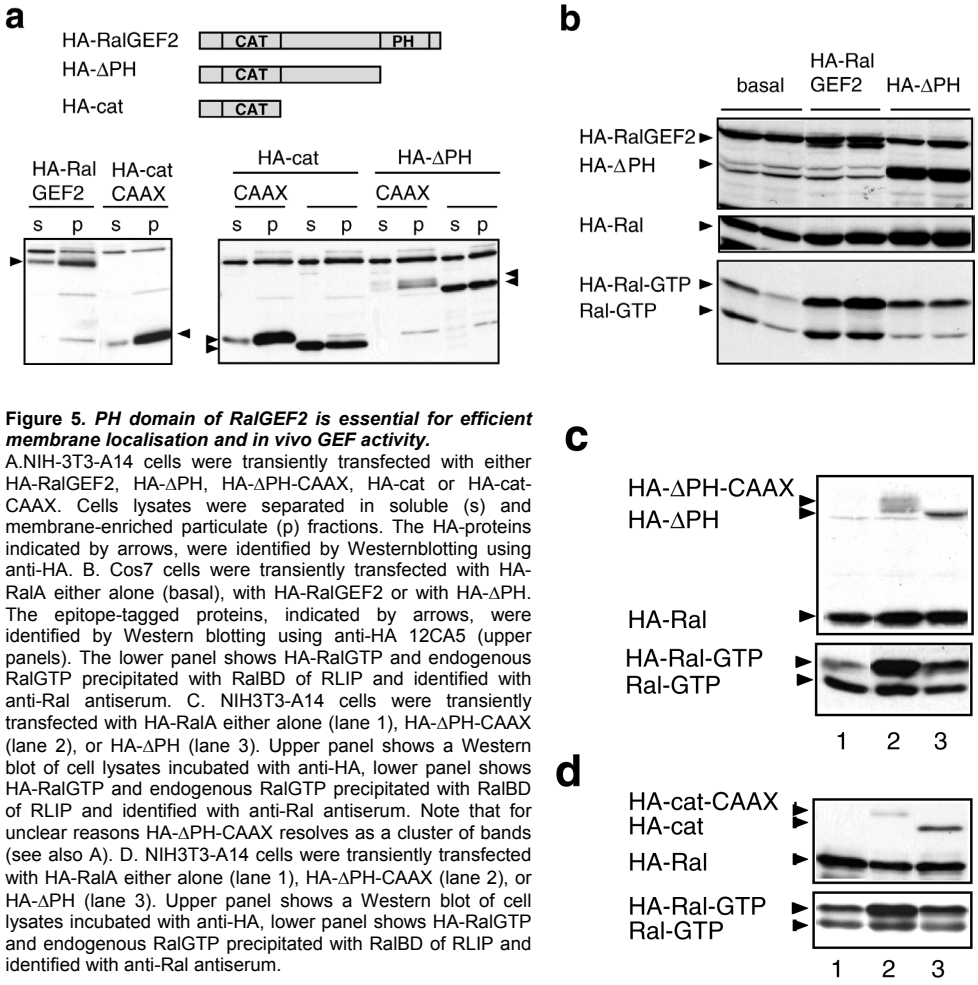

Figure 5. ***PH domain of RalGEF2 is essential for efficient membrane localisation and in vivo GEF activity.***
A.NIH-3T3-A14 cells were transiently transfected with either HA-RalGEF2, HA-ΔPH, HA-ΔPH-CAAX, HA-cat or HA-cat-CAAX. Cells lysates were separated in soluble (s) and membrane-enriched particulate (p) fractions. The HA-proteins indicated by arrows, were identified by Westernblotting using anti-HA. B. Cos7 cells were transiently transfected with HA-RalA either alone (basal), with HA-RalGEF2 or with HA-ΔPH. The epitope-tagged proteins, indicated by arrows, were identified by Western blotting using anti-HA 12CA5 (upper panels). The lower panel shows HA-RalGTP and endogenous RalGTP precipitated with RalBD of RLIP and identified with anti-Ral antiserum. C. NIH3T3-A14 cells were transiently transfected with HA-RalA either alone (lane 1), HA-ΔPH-CAAX (lane 2), or HA-ΔPH (lane 3). Upper panel shows a Western blot of cell lysates incubated with anti-HA, lower panel shows HA-RalGTP and endogenous RalGTP precipitated with RalBD of RLIP and identified with anti-Ral antiserum. Note that for unclear reasons HA-ΔPH-CAAX resolves as a cluster of bands (see also A). D. NIH3T3-A14 cells were transiently transfected with HA-RalA either alone (lane 1), HA-ΔPH-CAAX (lane 2), or HA-ΔPH (lane 3). Upper panel shows a Western blot of cell lysates incubated with anti-HA, lower panel shows HA-RalGTP and endogenous RalGTP precipitated with RalBD of RLIP and identified with anti-Ral antiserum.

vesicular trafficking. Ral localises in part in vesicular membranes and, recently, it was shown that Ral, through RalBP and the RalBP-associated proteins Reps1 and Pob1, is involved in the regulation of receptor endocytosis. In addition, Ral mediates FcεRI-induced histamine secretion (15). It may be that all these effects are pleiotropic, resulting from the activation of a single pool of active Ral. As a consequence, RalGEF2 may be involved in each of these processes. Alternatively, the distinct regulatory mechanism of RalGEF2 exchange activity, PH domain dependent membrane localisation, suggests the possibility that compartmentalised activation of Ral may regulate distinct functions of Ral.

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CHAPTER

3

The small GTPase Rap1 is required for Mn^{2+}- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion

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The small GTPase Rap1 is required for Mn^{2+}- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion

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In T lymphocytes, the Ras-like small GTPase Rap1 plays an essential role in stimulus-induced inside-out activation of integrin LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$). Here we show that Rap1 is also involved in the direct activation of these integrins by divalent cations or activating antibodies. Inhibition of Rap1, either by RapGAP or the Rap1 binding domain of RalGDS, abolished both Mn^{2+}- and KIM185 (anti-LFA-1)-induced LFA-1-mediated cell adhesion to ICAM-1. Mn^{2+}- and TS2/16 (anti-VLA-4)-induced VLA-4-mediated adhesion were inhibited as well. Interestingly, both Mn^{2+}, KIM185 and TS2/16 failed to induce elevated levels of Rap1GTP. These findings indicate that available levels of GTP-bound Rap1 are required for the direct activation of LFA-1 and VLA-4. Pharmacological inhibition studies demonstrated that both Mn^{2+}- and KIM185-induced adhesion, as well as Rap1-induced adhesion, require intracellular calcium, but not signalling activity of the MEK-ERK pathway. Moreover, functional calmodulin signalling was shown to be a prerequisite for Rap1-induced adhesion. From these results we conclude that in addition to stimulus-induced inside-out activation of integrins, active Rap1 is required for cell adhesion induced by direct activation of integrins LFA-1 and VLA-4. We suggest that Rap1 determines the functional availability of integrins for productive binding to integrin ligands.

INTRODUCTION

Circulating lymphocytes require a dynamic and flexible regulation of their integrin-dependent adhesive properties. While these cells circulate in blood and lymph or migrate through tissues, rapid transitions between adherent and non-adherent states are required. The leukocyte function-associated antigen 1 (LFA-1) (integrin $\alpha_L\beta_2$) is a transmembrane heterodimer composed of a unique α subunit (α_L or CD11a) and a β_2 subunit (CD18) that is common to a subset of leukocyte integrins. Expressed at the cell surface of resting leukocytes, LFA-1 is inactive. However, rapid conversion into an active state allows the integrin to bind to its ligands, intercellular adhesion molecule 1, 2, and 3 (ICAM-1, -2 and -3) (1-3). Relevant examples of the LFA-1/ICAM-1-mediated adhesion include leukocyte-endothelial cell interaction to direct lymphocyte homing or extravasation (4) and the establishment and strengthening of contacts between T cells and antigen-presenting cells (APC) (5,6). In addition, lymphocytes express VLA-4 (integrin $\alpha_4\beta_1$) which binds to vascular cell adhesion molecule 1 (VCAM-1) (7).

The binding of ligand by LFA-1 and VLA-4 is activated through inside-out signalling (8,9). Cytokines, chemokines or other T cell surface receptors, such as the antigen-specific T cell receptor (TCR) or CD2, lead to the production of second messengers and subsequent regulation of these integrins. For LFA-1 these signals are also generated by stimuli that increase the intracellular Ca^{2+} concentration or by protein kinase C (PKC) upon phorbol ester treatment. Models explaining the regulation of LFA-1

activity incorporate two distinct mechanisms. The first postulates modulation of the intrinsic affinity of the integrin for its ligand, while the second suggests a critical role for increased integrin receptor clustering or integrin redistribution at the cell surface (avidity regulation) (8). In addition to inside-out signalling, integrins can be activated directly from the outside by divalent cations, like Mn^{2+} or Mg^{2+} (in the presence of EGTA), which bind to the ectodomain (10,11) or after stimulation with activating monoclonal antibodies like the β_2-specific antibody KIM185 (12) and the β_1-specific antibody TS2/16 (13).

Recently, the small GTPase Rap1 has been demonstrated to play a role in TCR-, CD31- and cytokine-induced adhesion mediated by LFA-1, VLA-4 and VLA-5 (14-16). Overexpression of the active mutant RapV12 induced adhesion to immobilised ICAM-1 and VCAM-1. TCR- and CD31-induced activation of integrins were abolished by the introduction of constructs that inhibit Rap1 signalling, i.e. Rap GTPase activating protein (RapGAP), which lowers the level of Rap1GTP, the Rap1 binding domain (RBD) of RalGDS, which presumably inactivates Rap1 by binding to it and the putative dominant negative mutant RapN17 (15). Interestingly, transgenic mice constitutively expressing Rap1A V12 in their T cell lineage have been generated (17). Expression of active Rap1A in primary T cells from these mice is sufficient to induce inside-out signalling leading to β_1 and β_2 integrin-mediated adhesion. In these cells, active Rap1A did not modulate integrin affinity, as was measured by soluble ICAM-1 binding. However, thymocytes expressing Rap1A V12 showed integrin clustering at the surface, pointing to a role for Rap1 in integrin avidity modulation.

In the present study we show that overexpression of Rap1 inhibitory signalling proteins, RapGAPs and RBD, interferes with the ability of Mn^{2+} and KIM185 to induce LFA-1-mediated adhesion to ICAM-1, and Mn^{2+} and TS2/16 to induce VLA-4-mediated adhesion to VCAM-1. Both divalent cations and the activating antibodies do not induce accumulation of active Rap1GTP, consistent with the notion that they are thought to bypass the requirement for intracellular signalling events (18,19). This indicates that direct activation of integrins does not depend on further Rap1 activation. Although TPA-induced adhesion (inside-out integrin activation) may similarly not require activation of Rap1 (14), we demonstrate that it is blocked by inhibition of Rap1 signalling. Apparently, both stimulus-induced inside-out regulation of integrins and activation of integrin-mediated cell adhesion by cations and integrin-activating antibodies require Rap1. Use of pharmacological inhibitors reveals that Mn^{2+}-, KIM185-, but also RapV12-induced LFA-1-mediated adhesion, is critically dependent upon intracellular Ca^{2+} levels, but independent of PKC or MEK-ERK signalling. Furthermore, RapV12-induced LFA-1-mediated adhesion requires calmodulin. We propose that Rap1 activity, either basal or induced, regulates the functional availability of integrins for the adhesion process by or in combination with a calcium/calmodulin-dependent mechanism.

MATERIALS AND METHODS

Plasmids and Constructs

Haemagglutinin (HA)-tagged Rap1, Rap1V12 (HA-RapV12), Rap1GAP (HA-RapGAP I), RalGDS-RBD and PDZ-GEF, as well as pCAGGS-C3G and pSR-His-tagged Spa1 have previously been described (15,20,21). HA-RapGAP II was generated by PCR amplification of a 450 bp SalI/XhoI fragment including the 30 additional N-terminal amino acids of RapGAP II from pCA-RapGAP II (provided by Dr. Michiyuki Matsuda, Department of Tumor virology, Research Institute for Microbial Diseases, Osaka University, Japan). Subsequently, this fragment was subcloned into SalI/XhoI-digested pMT2-SM-HA-RapGAP I to generate pMT2-SM-HA-RapGAP II and integrity of the construct was confirmed by DNA sequencing.

Cell Culture, Cell Line and Transfection

The Jurkat T cell line JHM1 2.2 was provided by Dr. D. Cantrell (Imperial Cancer Research Fund, London, UK) with kind permission of Dr. A. Weiss (University of California at San Francisco, San Francisco, CA). Jurkat T cells were grown at 37°C in RPMI 1640 (Gibco Life Technologies, Paisly, UK) supplemented with 10% heat-inactivated (30 min. at 56°C) foetal bovine serum and 0.05% glutamine, in the presence of penicillin and streptomycin. The erythroleukemic K562 cells, either wild-type or stably expressing LFA-1, were grown in 75% RPMI 1640 and 25% IMDM (Gibco Life Technologies) supplemented with 10% heat-inactivated foetal bovine serum and 0.05% glutamine, in the presence of penicillin and streptomycin. The stable transfectants were cultured in the presence of 2 mg/ml geneticin, as has been described (22). Cells were transiently transfected by electroporation using 35 μg plasmid DNA. Cells (1.2×10^7 cells/ml in 0.4 ml complete medium) were pulsed at 250 V and 960 μF with 5 μg TK-luciferase plasmid DNA, construct plasmid as indicated in the figure legends and added vector plasmid to keep DNA amounts constant. Subsequently, 24 hr. after transfection, cells were transferred to serum-free medium and used 42-48 hr. after transfection.

Adhesion Assay

For adhesion assays, transiently transfected Jurkat cells 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. 96-Well Nunc Maxisorp plates were coated overnight at 4°C with goat anti-human IgG antibodies (Jackson ImmunoResearch, West Grove, PA; 4 μg/ml) in sodium bicarbonate buffer (Sigma, St. Louis, MO), washed, blocked for 30 min. at 37°C with 1% BSA/TSM, followed by incubation for 1 hr. at 37°C with 50 ng/ml recombinant ICAM-1 or 2 μg/ml VCAM-1 human IgG Fc fusion proteins, depending on the integrin studied. For studies on cell adhesion to fibronectin (Sigma, St. Louis, MO), 96-Well Nunc Maxisorp plates were coated overnight at 4°C with 1-5 μg/ml fibronectin in sodium bicarbonate buffer, washed with TSM and blocked for 30 min. at 37°C with 2% BSA/TSM. Poly-L-Lysine (Sigma, St. Louis, MO) was coated on plates for 1h. at room temperature as 0.1% w/v solution in water. After washing, 50 μl TSM was added per well with or without indicated stimuli. Subsequently, 50 μl cell suspension was added per well, after which the cells were spun down for 1 min. at 200 rpm in a Heraeus Sepatech Megafuge 1.0. Cells were allowed to adhere for 30 min. 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 (23). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Cells bound were calculated and numbers were corrected for transfection efficiency and nonspecific effects of constructs by measuring luciferase activity of total input cells ([counts in cells bound/counts in total input cells]×100%). Activating anti-integrin β_1 and β_2 antibodies TS2/16 and KIM185 respectively, have been described previously (12,13) and were used at 10 μg/ml. Mn^{2+} was used at 4 mM, TPA (Sigma) at 100 ng/ml. Preincubations with the following pharmacological inhibitors were performed for 30 min. at 37 °C: Roche 31-8220 (Calbiochem, La Jolla, CA, 5 μM), GF 109203X (Biomol, Plymouth, PA, 5 μM), BAPTA-AM (Molecular Probes, Eugene, Oregon, USA, 20 μM), PD 98059 (Sigma, 10 μM), U0126 (Promega, Madison, WI, 10 μM), LY294002 (Biomol, 10 μM), wortmannin (Sigma, 100 nM), U73122 (1 μM), SK&F96365 (concentration described in legend), calmidazolium chloride (10 μM), E6 Berbamine (10 μM), W-7 (100 μM) (all five from Biomol), calpeptin (Sigma, 100 μM), KN-93 (Calbiochem, 10 μM) and cyclosporin A (Biomol, 200 ng/ml).

Analysis of Rap1 activation in vivo

Jurkat cells were serum-starved overnight and resuspended at 25×10^6 cells/ml in RPMI without serum. 200 μl of this suspension was used per sample. After transfer to Eppendorf tubes, cells were left untreated for 15 min. at 37°C. Next, Mn^{2+} (4 mM), KIM185 (10 μg/ml), TS2/16 (10 μg/ml) or TPA (100 ng/ml) was added for indicated periods of time. Subsequently, cells were lysed for 15 min. at 4°C by the addition of ice-cold lysis buffer (10% glycerol, 1% Nonidet P40, 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 μM leupeptin, 0.1 μM aprotinin) and lysates were cleared by centrifugation at maximal speed in an Eppendorf centrifuge for 15 min. at 4°C. The GTP-bound form of Rap1 was isolated using RalGDS-RBD as an activation specific probe and subsequently quantified by Western blotting using anti-Rap1 antibody, as has been previously described (24,25).

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), polyclonal anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-Rap1 (Transduction Laboratories, Lexington, KY), polyclonal anti-Rap1 (Santa Cruz Biotechnology) and anti-phospho-MAPK (Cell Signaling Technology, New England Biolabs, Inc., Beverly, MA).

RESULTS

Inhibition of Rap1 abolishes divalent cation-induced adhesion to ICAM-1 and VCAM-1

To determine whether Rap1 is involved in Mn^{2+}-dependent activation of LFA-1 we measured the effect of Rap1 inhibitory proteins on Mn^{2+}-induced adhesion of Jurkat cells to ICAM-1, the counter receptor of LFA-1. Cells were transfected with RapGAP I, RapGAP II (which both lower the level of Rap1GTP) or RalGDS-RBD (which can form an inactive complex with Rap1GTP) together with a luciferase construct to detect transfected cells. These cells were subsequently plated on ICAM-1 in the presence or absence of Mn^{2+} and after 30 minutes nonadherant cells were washed away, and the percentage of luciferase- positive cells attached was measured. In control cells transfected with empty vector Mn^{2+} treatment strongly induced adhesion to ICAM-1 (Figure 1A). In contrast, adhesion induced by Mn^{2+} was blocked in cells transfected with RapGAP I, RapGAP II (Figure 1A) or RalGDS-RBD (Figure 1B). The ability of Rap1 inhibitory proteins to block divalent cation- induced integrin adhesion was not limited to LFA-1, as RapGAP I and RalGDS-RBD also blocked Mn^{2+}-induced adhesion of VLA-4 to VCAM-1 (Figure 1C). Furthermore, Mg^{2+}/EGTA-induced adhesion to ICAM-1 was also blocked by RapGAP and RalGDS-RBD (data not shown).

RapGAP proteins act on active Rap1-induced adhesion

To confirm the specificity of RapGAP proteins in blocking Rap1-dependent adhesion, Jurkat cells were transfected with active RapV12, or the Rap1 guanine-nucleotide exchange factors (GEFs) C3G or PDZ-GEF, to activate endogenous Rap1, either alone or in combination with RapGAP II (Figure 2A). Like in our previous studies, RapV12 strongly induced adhesion to ICAM-1, while overexpression of C3G or PDZ-GEF resulted in a more modest but significant induction of adhesion. Cotransfection of cells with RapGAP II however, completely blocked adhesion

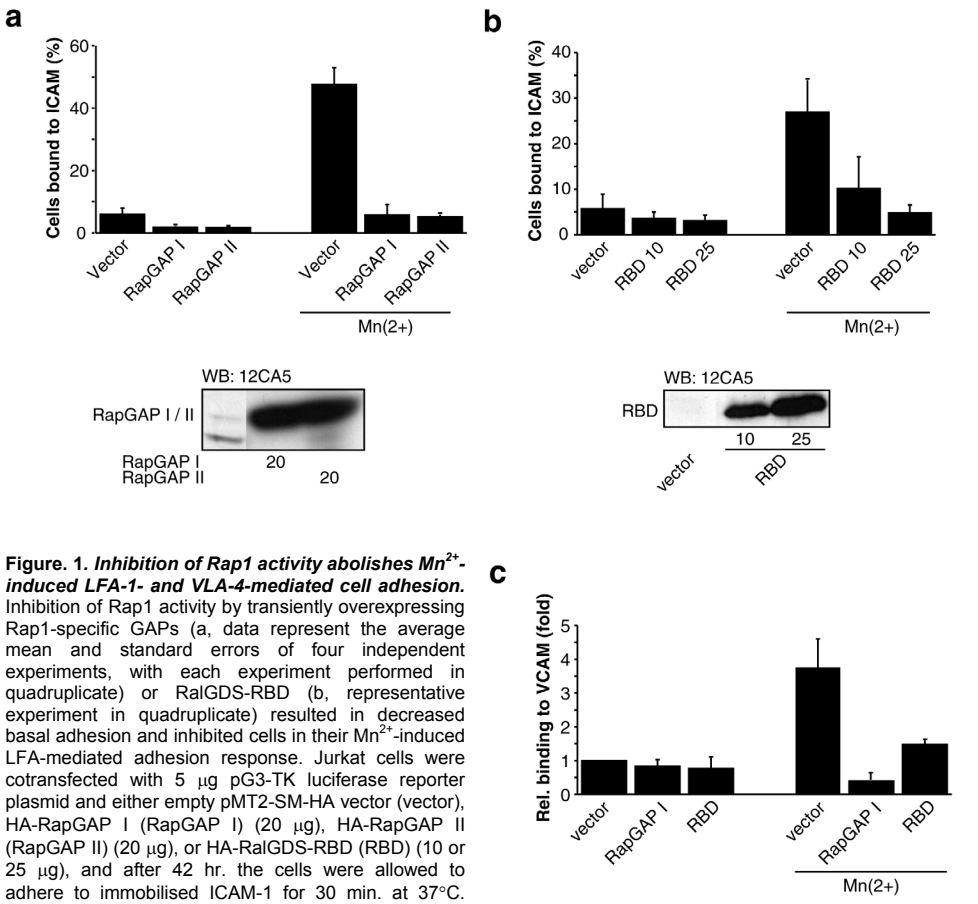

Figure. 1. ***Inhibition of Rap1 activity abolishes Mn^{2+}-induced LFA-1- and VLA-4-mediated cell adhesion.*** Inhibition of Rap1 activity by transiently overexpressing Rap1-specific GAPs (a, data represent the average mean and standard errors of four independent experiments, with each experiment performed in quadruplicate) or RalGDS-RBD (b, representative experiment in quadruplicate) resulted in decreased basal adhesion and inhibited cells in their Mn^{2+}-induced LFA-mediated adhesion response. Jurkat cells were cotransfected with 5 μg pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (vector), HA-RapGAP I (RapGAP I) (20 μg), HA-RapGAP II (RapGAP II) (20 μg), or HA-RalGDS-RBD (RBD) (10 or 25 μg), and after 42 hr. the cells were allowed to adhere to immobilised ICAM-1 for 30 min. at 37°C. Fraction of cells bound was determined by fraction of luciferase activity bound. In the bottom panels expression levels of the transfected proteins are shown. c, Inhibition of Rap1 activity abolished Mn^{2+}-induced VLA-4-mediated adhesion. Jurkat cells were cotransfected with constructs as indicated in a, and the cells were allowed to adhere to immobilised VCAM-1. Results are the average from two independent experiments in quadruplicate and indicated as the relative binding to VCAM-1 as compared to vector control.

induced by RapV12, C3G or PDZ-GEF, indicating that RapGAP II inhibited Rap1-dependent signalling. Precipitation of GTP-bound transfected Rap1 with GST-RalGDS-RBD fusion protein (Figure 2B) confirmed that transfected RapGAP II catalysed hydrolysis of GTP of both wild-type Rap1 and RapV12. Together these results show that RapGAP overexpression blocks Rap1-induced adhesion by decreasing the Rap1GTP amount. We conclude that Rap1GTP is required for induction of LFA-1 and VLA-4 integrin-dependent adhesion induced by divalent cations.

Inhibition of Rap1 blocks adhesion induced by β_1 and β_2 integrin-activating antibodies

Integrin-mediated cell adhesion induced by Mn^{2+} is proposed to occur by direct interactions of these cations with the extracellular domains of integrins rather than via intracellular signalling pathways. However, Mn^{2+} might also influence other cell surface or intracellular signalling proteins. Therefore, we tested the effect of Rap1 on cell adhesion induced by the monoclonal antibody KIM185. This antibody directly and specifically activates LFA-1 integrin in its ligand-binding capacity (12).

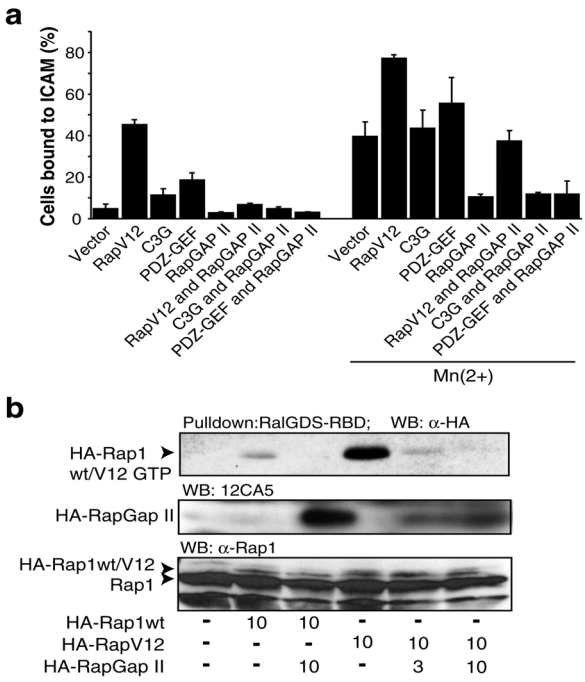

Figure 2. ***Inhibition of active Rap1-induced adhesion and decrease of RapGTP levels by RapGap proteins.*** a, Rap1-specific GEFs induced LFA-mediated adhesion. Both RapV12- and Rap1GEF-induced LFA-1-mediated adhesion could be blocked by cotransfection of RapGAP II (representative experiment in quadruplicate). Jurkat cells were cotransfected with 5 μg pG3-TK luciferase reporter plasmid and either empty vector, HA-RapV12 (10 μg), C3G (10 μg), HA-PDZ-GEF (10 μg), HA-RapGAP II (10 μg) or indicated combinations of them (10 μg of each construct). b, RapGAP II efficiently decreased GTP levels of both cotransfected Rap1wt and RapV12. Jurkat cells were transfected with empty vector, HA-Rap1 or HA-RapV12 alone or in combination with HA-RapGAP II (amounts of DNA [μg] as indicated in the figure). The upper panel shows the GTP levels of the Rap proteins determined using the pulldown-assay (Materials and Methods), the middle panel demonstrates the expression of HA-RapGAP II in the total lysates and the lower panel shows the presence of HA-Rap1wt and HA-RapV12 in the total lysates.

Treatment of Jurkat cells with KIM185 strongly induced adhesion to ICAM-1 (Figure 3A). Similar to Mn^{2+}, cell adhesion induced by KIM185 was completely blocked by transfection of either RapGAP I or RalGDS-RBD. VLA-4-mediated adhesion to VCAM-1 induced by the activating anti-β_1 integrin antibody TS2/16 was also completely blocked by transfection of cells with either of two RapGAPs, RapGAP I or Spa1 (Figure 3B). The inhibitory effect of RapGAP on Mn^{2+}- and KIM185-induced adhesion to ICAM-1 was also observed in the erythroleukemic cell line K562 stably expressing LFA-1 (22) (Figure 3C). In wild-type K562 cells, which only express VLA-5 ($\alpha_5\beta_1$) as β_1 class of integrin (26), RapGAP II abolished TS2/16-induced adhesion to fibronectin (Figure 3D). Our findings demonstrate that Rap1 is required for adhesion induced by integrin-activating antibodies.

Blocking Rap1 specifically affects integrin/ligand-mediated adhesion

To show that the Rap1 requirement is specific for integrin-ligand binding we tested cell adhesion to poly-L-Lysine. No difference in cell attachment was observed upon expression of Rap1V12 or RapGAP, either in the absence or presence of TS2/16 (Figure 4A). We also used another approach to demonstrate that RapGAP inhibits integrin-ligand-based cell binding. Increasing the amount of immobilised fibronectin concomitantly increased basal cell binding, which could be abolished by expression of RapGAP (Figure 4B). Interestingly, we observed that higher fibronectin densities rescued the inhibitory effect of RapGAP on TS2/16-induced adhesion, both with K562 and Jurkat cells. Mn^{2+}-induced adhesion could still be inhibited by RapGAP II at the highest fibronectin concentration used (5 μg/ml; data not shown). From these results we conclude that blocking Rap1 specifically inhibits adhesion mediated by integrin-ligand binding, but that integrins and processes required for adhesion are still functional.

Mn^{2+}-/KIM185-treatment does not activate Rap1

The requirement for Rap1 in Mn^{2+}- and antibody-induced integrin-mediated cell adhesion suggested the possibility that both

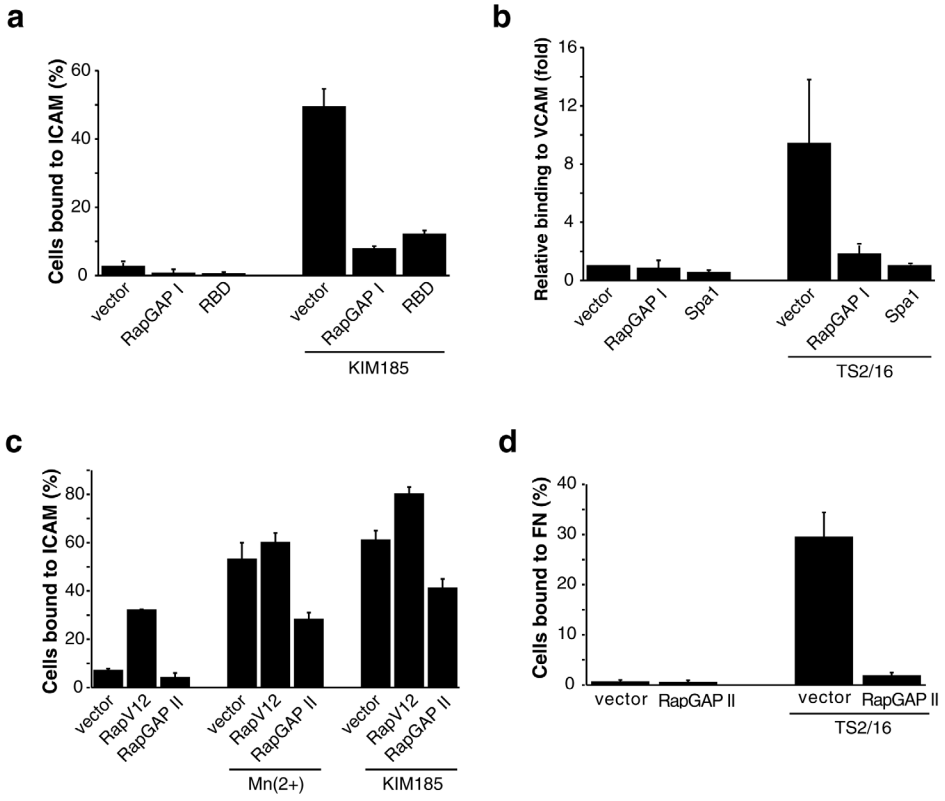

Figure 3. ***Inhibition of Rap1 activity abolishes anti-integrin antibody-induced LFA-1- and VLA-4-mediated cell adhesion.*** a, Inhibition of Rap1 activity by transient expression of either HA-RapGAP I or HA-RalGDS-RBD inhibited KIM185-induced LFA-mediated adhesion (representative experiment performed in quadruplicate). b, Transient expression of either HA-RapGAP I or Spa1 inhibited TS2/16-induced VLA-4-mediated adhesion (average of two independent experiments performed in quadruplicate, normalised to 1 for untreated vector control). c, Transient expression of RapGAP II inhibited Mn^{2+}- or KIM185-induced adhesion to ICAM-1 of K562 cells stably expressing LFA-1. d, RapGAP abolished TS2/16-induced adhesion of wild-type K562 cells to fibronectin (coated with 1 μg/ml concentration). Jurkat and K562 cells with and without stably expressed LFA-1were transfected with 5 μg pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector (vector), HA-RapV12 (RapV12) (10 μg), HA-RapGAP I (RapGAP I) (10 μg), HA-RalGDS-RBD (RBD) (10 μg) or Spa1 (10 μg). After 42 hr. cells were allowed to bind to immobilised ICAM-1 (a and c), VCAM-1 (b) or fibronectin (c and d) in the presence and absence of anti-integrin antibody KIM185 (a and c), TS2/16 (b and d) or Mn^{2+} (c).

stimuli mediated adhesion via activation of Rap1. We therefore tested whether Mn^{2+}, KIM185 or TS2/16 were able to induce Rap1 activation (Figure 5). Cells were stimulated for 30 minutes, corresponding to the duration of the adhesion assay and Rap1GTP was analysed using the RalGDS-RBD as activation specific probe. No activation of Rap1 was observed with Mn^{2+} (Figure 5A). In contrast, strong activation of Rap1 was observed following treatment with TPA. Surprisingly, Mn^{2+} treatment did induce a modest and sustained activation of the MEK/ERK signalling pathway, as indicated by phosphorylation of ERK1 (p44) and ERK2 (p42) (Figure 5A). Treatment of cells with KIM185 (Figure 5B) or TS2/16 (Figure 5C) neither increased the Rap1GTP level nor induced ERK activation. Thus, although both divalent cations and activating anti-integrin antibodies stimulate adhesion in a Rap1-dependent manner, this is not a result of acute activation of Rap1.

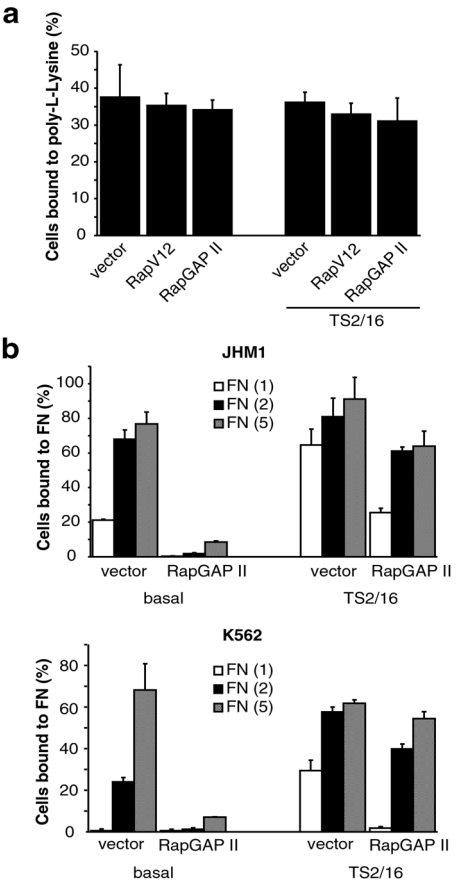

Figure 4. ***Inhibition of Rap1 activity abolishes specifically integrin-ligand-mediated cell adhesion.*** a, Transient overexpression of RapV12 or RapGAP in K562 cells in the presence or absence of TS2/16, did not affect cell binding to immobilised poly-L-Lysine (representative experiment performed in quadruplicate). b, Inhibition of cell adhesion induced by increased ligand density by transient expression of RapGAP. High ligand densities in combination with TS2/16 treatment overcame cell adhesion inhibition by RapGAP overexpression. The upper panel shows adhesion of K562 cells with and without transient expression of RapGAP to fibronectin (FN, with indicated concentrations in μg/ml used for coating), in the presence and absence of TS2/16. In the lower panel a similar situation is shown for Jurkat cells (representative experiments performed in triplicate). Jurkat cells and K562 cells were transfected with 5 μg pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector (vector), HA-RapV12 (RapV12) (10 μg) or HA-RapGAP II (RapGAP II) (20 μg). After 42 hr. cells were allowed to bind to immobilised poly-L-Lysine (a) or fibronectin (plates coated with indicated dilutions of ligand) (b), in the presence and absence of anti-integrin antibody TS2/16.

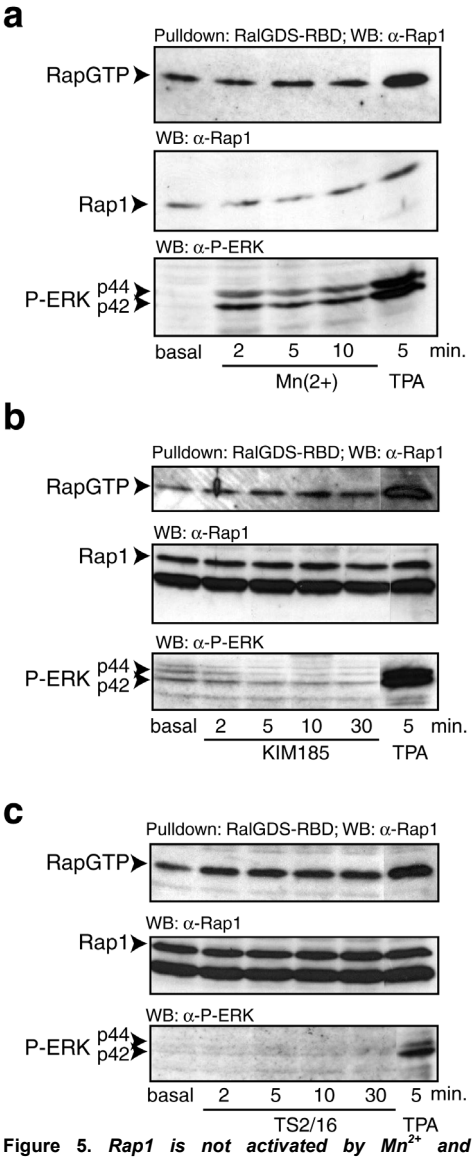

Figure 5. ***Rap1 is not activated by Mn^{2+} and activating anti-integrin antibodies.*** Jurkat cells were unstimulated (basal) or treated for the indicated periods of time with either 4 mM Mn^{2+} (a), the LFA-1 activating antibody KIM185 (b) or the VLA-4 activating antibody TS2/16 (c). Cells were lysed and RapGTP was determined using the RBD pulldown assay. Upper panels: RapGTP levels determined by the pulldown assay. Middle panels: Total Rap1 in the lysates to demonstrate equal input per sample. Lower panels: Lysates were analysed for the presence of phosphorylated ERK. Treatment for 5 min. with TPA (100 ng/ml) was used as a positive control for Rap1 activation.

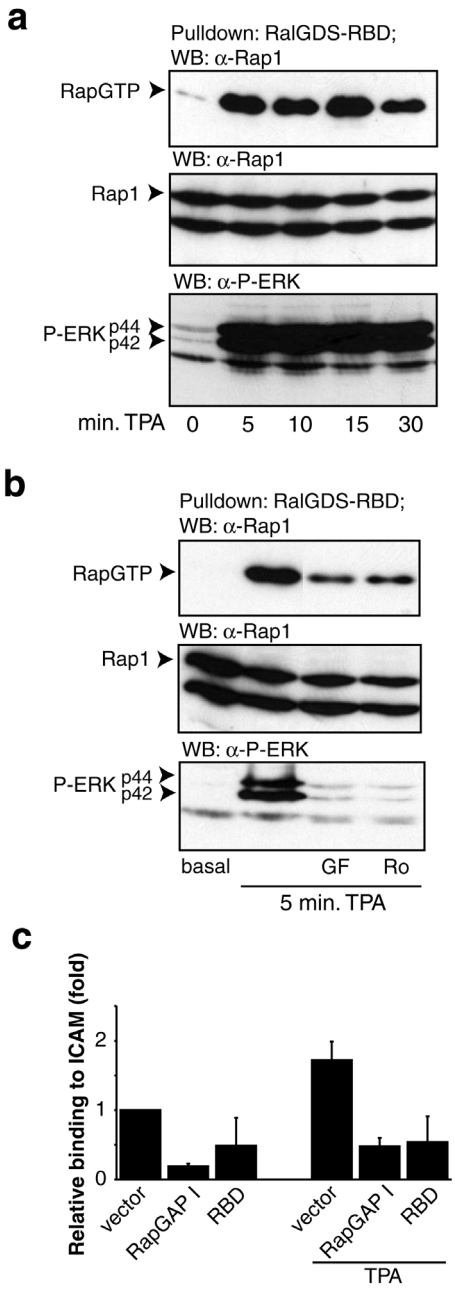

Figure 6. ***Effect of inhibiting basal Rap1 signalling on TPA-induced inside-out activation of LFA-1-mediated adhesion.*** a, Timecourse demonstrating RapGTP levels upon TPA treatment (100 ng/ml) for indicated periods of time. The upper panel shows RapGTP levels. The middle panel shows total Rap1 in the lysates to demonstrate equal input per sample. The bottom panel demonstrates ERK phosphorylation during the timecourse. b, TPA-induced Rap1 and MAPK activity in the presence of pharmacological PKC inhibitors. After preincubation for 30 min. with either GF 109203X or Ro 31-8220 (5 μM) cells were treated with TPA (100 ng/ml) for 5 min. The upper panel demonstrates Rap1GTP levels, the middle panel shows the presence of equal amounts Rap1 in each sample, and in the bottom panel ERK phosphorylation is shown. c, TPA-induced LFA-mediated adhesion to immobilised ICAM-1 is dependent on basal Rap1 activity. Jurkat cells were transfected with 5 μg pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (vector), HA-RapGAP I (20 μg) or HA-RalGDS-RBD (20 μg) after which binding to immobilised ICAM-1 in the presence and absence of TPA (100 ng/ml) was analysed (average of 2-3 independent experiments).

Inhibition of TPA-induced adhesion by Rap1 interfering proteins

The phorbol ester TPA is generally used to induce integrin-mediated cell adhesion by a still elusive pathway. Therefore, we investigated the possible requirement for Rap1 in TPA-induced integrin-mediated cell adhesion as well. Treatment of Jurkat cells with TPA resulted in a rapid and prolonged activation of Rap1 as well as induction of ERK phosphorylation (Figure 6A). Pretreatment of Jurkat cells with pharmacological inhibitors of PKC (Roche 31-8220 and GF 109203X) inhibited TPA-induced activation of both Rap1 and ERK (Figure 6B). Although previous studies have provided evidence that excludes the requirement for Rap1 activation in TPA-induced adhesion (14), overexpression of RapGAP I or RalGDS-RBD blocked TPA-induced adhesion to ICAM-1 (Figure 6C). Pharmacological inhibition of PKC selectively blocked TPA-, but not Mn^{2+}- or KIM185- induced adhesion to ICAM-1 (Figure 7A). These findings show that TPA-induced inside-out activation of adhesion requires Rap1 activity, either basal or induced.

Rap1–induced adhesion requires intracellular calcium and calmodulin

To study the mechanism by which Rap1 regulates integrins, we tried to identify common elements between RapV12- and Mn^{2+}- or activating antibody-induced cell adhesion. Several reports have provided strong evidence that agonist-induced adhesion in a number of heamatopoietic cell types requires influx of calcium from extracellular sources (19,27,28). Indeed, the calcium chelator BAPTA-AM strongly inhibited basal, Mn^{2+}-, TPA- and

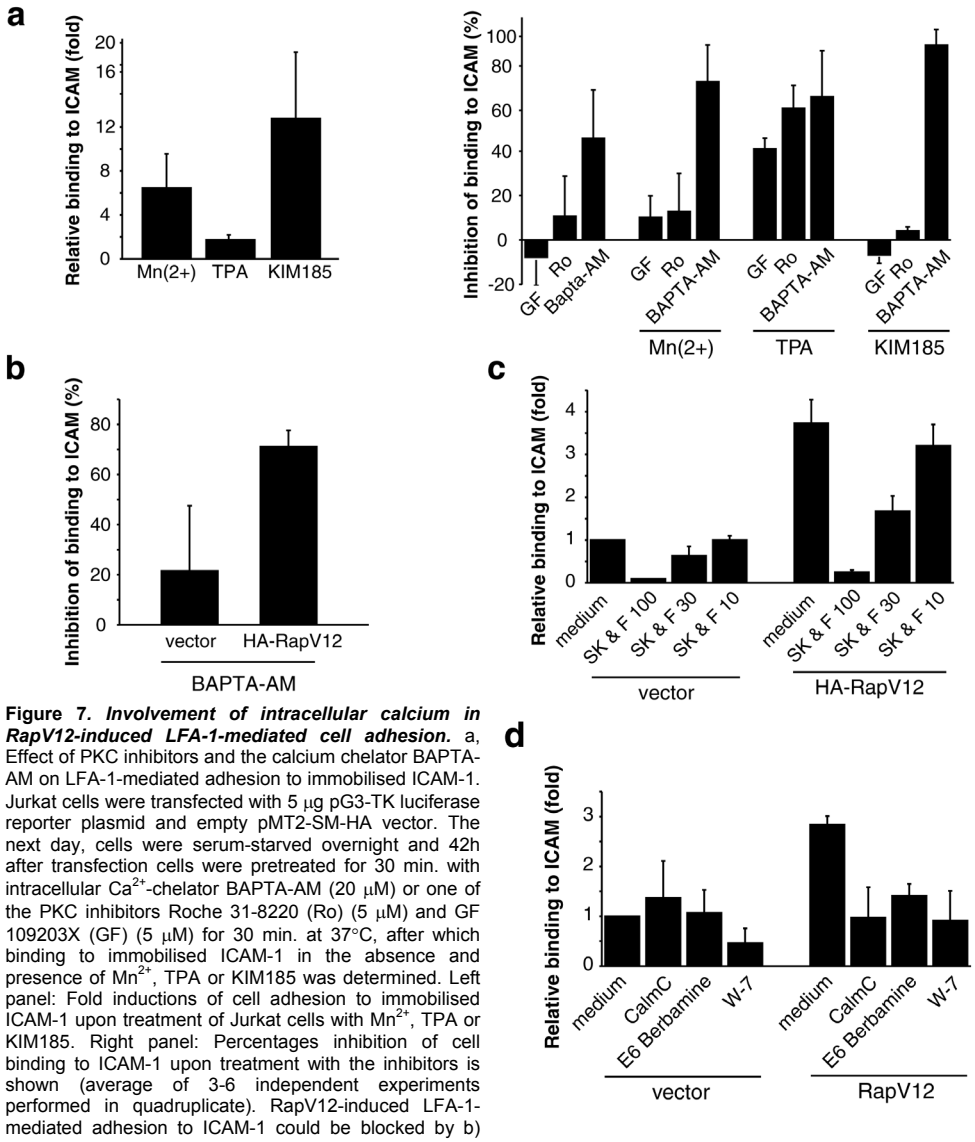

Figure 7. ***Involvement of intracellular calcium in RapV12-induced LFA-1-mediated cell adhesion.*** a, Effect of PKC inhibitors and the calcium chelator BAPTA-AM on LFA-1-mediated adhesion to immobilised ICAM-1. Jurkat cells were transfected with 5 μg pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector. The next day, cells were serum-starved overnight and 42h after transfection cells were pretreated for 30 min. with intracellular Ca^{2+}-chelator BAPTA-AM (20 μM) or one of the PKC inhibitors Roche 31-8220 (Ro) (5 μM) and GF 109203X (GF) (5 μM) for 30 min. at 37°C, after which binding to immobilised ICAM-1 in the absence and presence of Mn^{2+}, TPA or KIM185 was determined. Left panel: Fold inductions of cell adhesion to immobilised ICAM-1 upon treatment of Jurkat cells with Mn^{2+}, TPA or KIM185. Right panel: Percentages inhibition of cell binding to ICAM-1 upon treatment with the inhibitors is shown (average of 3-6 independent experiments performed in quadruplicate). RapV12-induced LFA-1-mediated adhesion to ICAM-1 could be blocked by b) chelation of intracellular calcium with BAPTA-AM (20 μM), c) inhibition of calcium channels with the imidazole compound SK&F 96365 (SK&F) (100, 30 and 10 μM) and d) inhibition of calmodulin with calmidazolium chloride (CalmC; 10 μM), E6 berbamine (10 μM) and W-7 (100 μM). Jurkat cells were transfected with 5 μg pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (vector) or HA-RapV12 (10 μg). After 42 hr., they were left untreated or preincubated for 30 min. with the inhibitors and subsequently cells were incubated for 30 min. at 37°C to allow binding to immobilised ICAM-1. Data represent the average of 3 independent experiments performed in quadruplicate.

Kim185-induced LFA-mediated adhesion (Figure 7A). We therefore examined next whether RapV12-induced adhesion also displayed a similar requirement. Chelation of intracellular calcium with BAPTA-AM strongly inhibited both basal and RapV12- induced adhesion (Figure 7B). Preincubation of RapV12-transfected Jurkat cells with the imidazole compound SK&F 96365, which inhibits calcium channels in leukocytes and thus

lowers intracellular calcium, efficiently blocked basal and RapV12-induced adhesion in a dose-dependent manner (Figure 7C). The calmodulin inhibitors W-7, E6 berbamine, and calmidolium chloride each blocked RapV12-induced adhesion (Figure 7D). From these results we conclude that RapV12-induced adhesion, like adhesion induced by other stimuli, requires calcium signalling.

The MEK-ERK pathway is not involved in Rap1-mediated cell adhesion

We were particularly interested in a possible role of the MEK-ERK pathway in Rap1-mediated cell adhesion, since positive and negative regulation of this pathway by Rap1 is a predominant effect of Rap1 reported in T lymphocytes and other cell types (29-31). To measure whether the MEK-ERK pathway mediates Rap1-induced adhesion, we used the MEK inhibitors PD 98059 and U0126. Although both MEK inhibitors completely inhibited TPA-induced ERK activation (data not shown) they did not affect RapV12- or Mn^{2+}-induced adhesion (Figure 8). Alternatively, Rap1 may mediate cell adhesion by inhibiting ERK activation, suggesting that RapGAP might inhibit cell adhesion by preventing the inactivation of ERK. However, in the presence of the two MEK inhibitors, RapGAP II still inhibited cell adhesion. From these results we conclude that MEK and therefore most likely ERK is not involved in Rap1-mediated cell adhesion. This is confirmed by recent findings that active Rap1 did not interfere with the Ras signalling pathway (17). By utilising the PI(3)-kinase (PI(3)K) inhibitors wortmannin and LY294002 and the phospholipase C (PLC) inhibitor U73122, we did not find a role for PI(3)K or PLC in either RapV12- or Mn^{2+}-induced adhesion (data not shown).

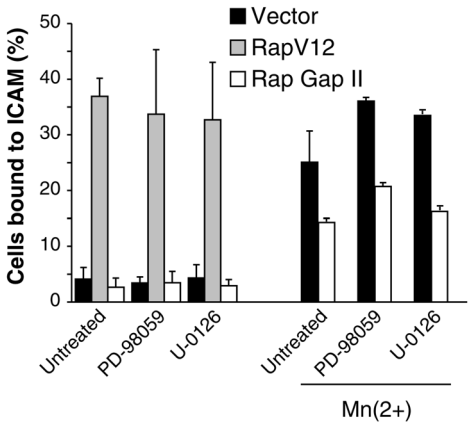

Figure 8. ***MEK-ERK pathway is not involved in Rap1-mediated cell adhesion to ICAM-1.*** Jurkat cells were transfected with 5 μg pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector (vector), HA-RapV12 (10 μg) or RapGAP I (20 μg). After 42 hr., cells were either left untreated or preincubated with the MEK inhibitors PD 98059 (10 μM), or U0126 (10 μM) for 30 min. at 37°C, after which the cells were allowed to bind to immobilised ICAM-1 for 30 min. in the presence and absence of Mn^{2+}. Data represent the mean and standard errors of two independent experiments performed in quadruplicate.

DISCUSSION

Previously, we and others have shown that the small GTPase Rap1 is required for cytokine-induced inside-out activation of various integrins, including LFA-1, VLA-4 and $\alpha_M\beta_2$ (14,15,32,33). In this manuscript we now demonstrate that also direct activation of LFA-1-, VLA-4- and VLA-5-mediated adhesion by Mn^{2+} or integrin-activating antibodies (monoclonal KIM185 for LFA-1 and TS2/16 for VLA-4 and VLA-5) is sensitive to inhibition of Rap1 signalling . This is shown by overexpression of either Rap-specific GAPs, which interfere with Rap1 signalling by reducing levels of endogenous GTP-bound Rap1, or overexpression of RalGDS-RBD, which inhibits Rap1 signalling by binding to GTP-bound Rap1 and sequestering it from potential effector proteins, in Jurkat and K562 (Figure 1 and 3). As an assay to monitor enhanced integrin function we measured binding to immobilised integrin ligands, i.e. ICAM-1 for LFA-1, VCAM-1 for VLA-4 and fibronectin for VLA-4 and VLA-5. This unexpected finding raised the question whether the inhibition is due to an integrin-specific effect or whether Rap1 affects a general requirement for integrin-mediated cell adhesion, like cytoskeletal rearrangements and cell spreading. However, inhibition of Rap1 signalling did not affect integrin-independent adhesion to poly-L-Lysine (Figure 4A). In

addition, inhibition of TS2/16-induced adhesion by blocking Rap1 signalling could be rescued by increasing the fibronectin concentration (Figure 4B). Moreover, inhibitors of actin cytoskeleton dynamics, cytochalasin D (14) and jasplakinolide (K. de B. unpublished results), did not influence RapV12-induced adhesion.

Basal Rap1 signalling may be sufficient to play a role in integrin-mediated adhesion, since we have not observed an increase in Rap1GTP upon treatment with either Mn^{2+} or activating antibodies (Figure 5). This may imply that Rap1 only provides a favourable setting for enhanced integrin function. In agreement with a facilitating function for Rap1 are the recent findings that overexpressing Rap1B V12 in megakaryocytes itself had no effect, but augmented fibrinogen binding to $\alpha_{IIb}\beta_3$ induced by a PAR4 thrombin receptor agonist (34). In addition, we have not observed increased adhesion of human megakaryoblasts overexpressing active Rap1 to immobilised fibrinogen, while we have found inhibition of TPA-induced adhesion by RapGAP (unplubished results). However, RapV12, as well as Rap1-specific GEFs that activate endogenous Rap1, are able to induce increased integrin-mediated cell adhesion (Figure 2), showing that activation of Rap1 is sufficient to enhance the function of LFA-1.

We have also investigated the involvement of putative Rap1 effectors in the regulation of integrins. Although regulation of the MEK-ERK pathway is reported to be a predominant effect of Rap1 in T lymphocytes and other cell types (29-31), MEK inhibitors neither blocked RapV12- nor Mn^{2+}-induced adhesion (Figure 8). Interestingly, this issue was recently analysed in an *in vivo* model system (17). In agreement with our results, transgenic mice constitutively expressing Rap1A V12 in their T cell lineage did not demonstrate a modulating role for Rap1 in the MEK-ERK pathway. Furthermore, inhibitors of the proposed Rap1 targets PLC and PI(3)K did not interfere with RapV12- or Mn^{2+}-induced adhesion. However, pretreatment of cells with the intracellular calcium chelator BAPTA-AM inhibited both basal adhesion and RapV12-, Mn^{2+}- or integrin activating antibody-induced adhesion to a similar extent (Figure 7). Utilisation of the SK&F 96365 inhibitor, which blocks extracellular calcium channels required for leukocyte adhesion, also blocked RapV12-induced adhesion. This result is compatible with recent evidence that extracellular stimuli inducing T lymphocyte adhesion via LFA-1, such as TCR stimulation and phorbol esters, do so by inducing an influx of extracellular calcium (28). Although in several studies Rap1 has been implicated in the regulation of cellular calcium levels (35-37), clear evidence is still lacking. A recent study demonstrated that Rap2b via interaction with PLCε is able to regulate intracellular calcium signalling in HEK293 cells (38). A large number of calcium responsive signalling proteins have been shown to regulate integrin-dependent adhesion in various cell types, like calpain (28,39,40), calmodulin (41), and calcium/calmodulin-dependent kinase II (42-44). In the case of RapV12-induced adhesion, calmodulin is required (Figure 7D). However, we could not demonstrate a role for calpain, calcium/calmodulin-dependent kinase II or calcineurin in RapV12-induced integrin function (Figure 7). The function of this calmodulin requirement is therefore still unclear.

Our results indicate that all stimuli that enhance the function of integrins tested, including inside-out signalling and direct activation, require Rap1. Previously, it was reported that the phorbol ester TPA may induce integrin activation independent of Rap1 activation (14). However, we have found that overexpression of RapGAPs or RalGDS-RBD efficiently blocked TPA-induced adhesion, indicating that Rap1-GTP is also required for TPA-induced integrin activation. How Rap1 enhances integrin function is still elusive. Cell surface expression of integrins is not affected by Rap1 signalling interference (14,15). This indicates that Rap1 is involved in the increase in integrin function, either by inducing a conformational change or by inducing clustering of integrins, two nonexclusive modes of integrin activation (8,9,45). Importantly, the effect of Rap1 on integrin function occurs with both β_1 and β_2 integrins. The most plausible explanation for the role of Rap1 is regulation of auxillary factors for integrin function. These factors may be signalling or structural proteins that associate with integrin cytoplasmic tails (46). Matsuda and co-workers have utilised fluorescent resonance energy transfer (FRET) analysis to visualise Rap1 activation *in vivo*, and

have noted that growth factor-induced Rap1 activation initiates in the perinuclear region (47). Perhaps Rap1 influences integrin processing, the repertoire of integrin-associated proteins that is delivered to surface, or modulates membrane microdomain organisation (lipid rafts) (48,49) influencing integrin function (50).

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CHAPTER

4

Cyclic AMP-induced PKA-independent regulation of intracellular calcium concentration in human megakaryocytes does not involve the Epac-Rap1 pathway

Cyclic AMP-induced PKA-independent regulation of intracellular calcium concentration in human megakaryocytes does not involve the Epac-Rap1 pathway

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Recently, several studies have provided evidence for a function of the small GTPase Rap1 in the regulation of the intracellular calcium homeostasis. Moreover, in human megakaryocytes, cAMP-induced increase in intracellular calcium was found to be independent of protein kinase A (PKA). We therefore tested the hypothesis that the cAMP target Epac, a guanine nucleotide exchange factor (GEF) for Rap1, mediates this increase in calcium. For these analyses the synthetic cAMP analogue E-cAMP was used. This compound specifically activates Epac both *in vitro* and *in vivo*. Here we show that in platelets E-cAMP is indeed specific and did not induce typical cAMP-PKA mediated effects like inhibition of thrombin-induced increase in intracellular calcium concentration and inhibition of thrombin-induced Rap1 activation. In megakaryocytes, we were unable to demonstrate activation of Rap1 by cAMP or E-cAMP, indicating that a functional Epac-Rap1 pathway may not exist in these cells. In addition, in contrast to forskolin, E-cAMP did not induce an increase in the intracellular calcium level. From these results we conclude that the cAMP-induced PKA-independent increase in the intracellular calcium concentration in megakaryocytes is not mediated by the cAMP-Epac-Rap1 pathway.

INTRODUCTION

The small Ras-like GTPase Rap1 (Ras-proximate) was identified both in a screen for proteins related to the small GTPase Ras (1,2) and as the cDNA product able to induce flat revertants of K-*ras*-transformed cells (Krev-1) (3). Remarkably, in addition to reversion of oncogenic transformation Rap1 has been implicated in the induction of oncogenic transformation in other cell types (4,5). The similarity in the effector domains between Ras and Rap1 has raised the attractive hypothesis that Rap1 may interfere in Ras signalling, possibly by interaction with Ras effectors Raf1, B-Raf and guanine-nucleotide exchange factors (GEFs) for Ral (6-9). Although Rap1 has been shown to be able to regulate the extracellular signal-regulated kinases (ERKs) both positively (10,11) and negatively (12,13), debate is still going on about the control of the ERK pathway by Rap1 *in vivo* (14,15, JM Enserink *et al.*, submitted). However, compiling evidence suggests Rap1 functions in other cellular processes. Rap1 has been implicated in platelet activation (16), exocytosis (17), differentiation (18,19) and regulation of integrin-mediated cell adhesion (15,20-24). Moreover, in lower eukaryotes, Rap1 orthologues contribute to processes related to the establishment of cell polarity (reviewed in (14)) and the regulation of adherens junction positioning and cell adhesion (25).

Rap1 can be activated in various cell types with a plethora of stimuli (16,26-30). Different signalling pathways contribute to Rap1 activation, depending on the receptor and the cell type. In general, the second messengers calcium, diacylglycerol (DAG) and cyclic adenosine monophosphate (cAMP) are used in the upstream control. Four families of guanine-nucleotide exchange factors (GEFs) have been

characterised that regulate the activation of Rap1. The first, C3G, may regulate tyrosine kinase-induced Rap1 activation through the adapter proteins Crk and Cbl (29,31,32). Second, the Epacs, which are directly regulated by cAMP (33,34). Members of the third, the CD-GEF family, contain calcium- and DAG-binding domains and show different GTPase specificities (35-37). Evidence exists which indicates differential regulation of these members by calcium and DAG (J. de Rooij, unpublished results). Finally, the PDZ-GEFs, which have so far not been connected to upstream activators ((38), H.B. Kuiperij *et al*. submitted).

Rap1 has been connected to signal transduction initiated by intracellular calcium (Ca^{2+}_i). An increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) was demonstrated to be necessary and sufficient to activate Rap1 in platelets (16). Interestingly however, Ca^{2+}_i might not only function upstream from Rap1, as independent studies suggest a role for Rap1 in the regulation of $[Ca^{2+}]_i$. First, evidence has been provided for a cAMP-regulated Phospholipase-C-ε (PLC-ε)- calcium signalling pathway that is mediated by Rap2B (39). Secondly, Rap1 has been suggested to function in Ca^{2+}_i homeostasis in platelets. Rap1 and the 97 kDa sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), that is associated with inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+}-stores in intracellular membranes linked to the plasma membrane, interacted in co-immunoprecipitation experiments. This interaction dissociated upon phosphorylation of Rap1 in the membrane preparations by the catalytic subunit of PKA (40). GTPγS, a non-hydrolysable GTP analogue, was found to inhibit both SERCA activity and the phosphorylation of Rap1b by the catalytic subunit of PKA in membrane preparations of platelets (41). Moreover, the expression of SERCA and Rap1 in platelets and various other cell lines seem to correlate (42,43). Finally, immature human megakaryocytes have been demonstrated to respond upon iloprost- or forskolin-induced cAMP production with an increase in $[Ca^{2+}]_i$, which is insensitive to the PKA inhibitor H-89 (44). In this latter study, Rap1 activity was reported to parallel the $[Ca^{2+}]_i$ increase induced by cAMP-elevating treatments, suggesting the involvement of the Epac-Rap1 pathway.

We have addressed the question whether the cAMP-regulated Epac-Rap1 signalling pathway controls $[Ca^{2+}]_i$ in megakaryoblasts. We therefore used E-cAMP, a cAMP analogue that activates Epac with a 30-100 times higher affinity as compared to PKA (JM Enserink *et al.*, submitted). This tool enabled us to focus specifically on the downstream effects of cAMP-initiated signalling mediated by the Epac-Rap1 cascade. In the present study we show that treatment of MEG-01 cells with E-cAMP did not result in increased $[Ca^{2+}]_i$. Moreover, we did not find evidence for a functional cAMP-Epac-Rap1 signalling pathway to exist in MEG-01 cells. From these results we conclude that the cAMP-Epac-Rap1 pathway in not involved in cAMP-mediated regulation of intracellular calcium.

MATERIALS AND METHODS

Materials

Fura-2-acetomethylester (Fura-2/AM) and α-thrombin were from Sigma (St. Louis, MO, USA. Forskolin was purchased from Calbiochem (San Diego, Ca, USA) while 8-Bromo-cAMP and E-cAMP were from Biolog Life Science Institute.

Cell Culture and Cell Line

The MEG-01 cell line was grown at 37°C in RPMI 1640 (Gibco Life Technologies, Paisly, UK) supplemented with 20% heat-inactivated (30 minutes at 56°C) foetal bovine serum. OVCAR cells were cultured in RPMI 1640 supplemented with heat-inactivated10% foetal bovine serum. Both cell lines were grown in the presence of 0.05% glutamine, penicillin and streptomycin, in a humidified atmosphere with 5% CO_2. Prior to stimulation, cells were serum-starved overnight.

Platelet Isolation

Donors claimed not to have taken any medication during the preceding 10 days. After informed consent was obtained, freshly drawn venous blood from healthy volunteers was collected into 0.1 volume of 130 mM trisodium citrate. Citrated blood was centrifuged (150×g, 15 minutes at 20°C) and subsequently the platelet-rich plasma was supplemented with prostaglandin I_2 (10 ng/ml) and ACD (2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 ml dH_2O) for acidification to pH 6.5 and centrifuged again (330×g, 15 minutes at 20°C).

Measurement of Calcium Responses

Platelet-rich plasma was supplemented with 3 μM Fura-2/AM and incubated for 45 minutes at 37°C in the dark. Next, the platelet-rich plasma was supplemented with ACD for acidification to pH 6.5 and centrifuged (330×g, 15 minutes at 20°C). Platelets were resuspended in Hepes-Tyrode (HT) buffer (145 mM NaCl, 5 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM Hepes and 0.1% (w/v) glucose) pH 6.5, to a concentration of 2×10^9 and stored at room temperature in the dark. Immediately before $[Ca^{2+}]_i$ measurement, the platelet suspension was diluted 10 times in prewarmed (37°C) HT

buffer (pH 7.4) and GRGDS peptide (100 μM) was added to prevent platelet aggregation. MEG-01 cells were pelleted (5 minutes, 125×g, 20°C) and resuspended in HT buffer pH 7.4 supplemented with 0.1% (w/v) BSA, at a concentration of 5×10^5 cells/ml. Next, Fura-2/AM was added to a final concentration of 3 μM and cells were loaded for 1 hour at 37°C in the dark. Subsequently, cells were spun down, resuspended in HT-buffer without BSA to a final concentration of 1.6×10^6 cells/ml and stored at room temperature in the dark. Immediately before analysis of $[Ca^{2+}]_i$, the cells were resuspended in prewarmed (37°C) HT-buffer at 2×10^5 cells/ml. Measurements were performed at 37°C under mild stirring (50 rpm) on a Hitachi F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). Fura-2 fluorescence was measured at 340 nm (F1) and 380 nm (F2) excitation and 510 nm emission wavelength. Measurements, calibrations and calculations were performed as described previously (44).

Analysis of Rap1 activation using an activation-specific probe

MEG-01 cells were resuspended at 5×10^6 cells/ml in RPMI without serum. 200 μl of this suspension was used per sample. After transfer to eppendorf tubes, cells were left untreated or preincubated with an inhibitor as indicated in the Results section for 30 minutes at 37°C. Next, a stimulating agent was added after which samples were mixed gently and incubated for indicated periods of time. Subsequently, cells were lysed for 15 minutes at 4°C by the addition of ice-cold lysis buffer (10% glycerol, 1% Nonidet P40, 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 μM leupeptin, 0.1 μM aprotinin) and lysates were cleared by centrifugation at maximal speed in an Eppendorf centrifuge for 15 minutes at 4°C. The GTP-bound form of Rap1 was isolated using RalGDS-RBD as an activation specific probe and subsequently quantified by Western blotting using anti-Rap1 antibody, as has been described previously (16,45,46).

Determination of PKA Activity

Analysis of PKA activity in platelets and MEG-01 cells upon indicated treatments was carried out by using the mobility-shift of VASP on Western blot as a readout (47). This is caused by PKA-mediated phosphorylation on Ser 157. Alternatively, phosphorylation of CREB (cAMP responsive element binding protein) was used as read-out for PKA activity.

Western Blotting

Western blotting of all protein samples was carried out using polyvinylidene difluoride membranes. The antibodies used for protein detection are the rabbit polyclonal anti-Rap1 (Santa Cruz Biotechnology, CA, USA) and goat polyclonal anti-VASP (Santa Cruz Biotechnology). The secondary goat-anti-rabbit-HRP antibody was from Santa Cruz Biotechnology and rabbit-anti-goat-peroxidase was purchased from DAKO (Glostrup, Denmark).

RESULTS

E-cAMP does not inhibit thrombin-induced increase in $[Ca^{2+}]_i$ in platelets

Previous studies have demonstrated that treatment with the stable derivative of prostacyclin, iloprost, or forskolin results in a simultaneous accumulation of cAMP and $[Ca^{2+}]_i$ in the immature megakaryoblastic cell line MEG-01. These signalling events were accompanied by an increase in the Rap1GTP level (44). Surprisingly, the rise in $[Ca^{2+}]_i$ was insensitive to the PKA inhibitor H-89, pointing to a possible role for the cAMP-regulated Rap1-specific GEF Epac. To investigate whether indeed Epac mediates cAMP-induced calcium increase, we made use of a recently developed cAMP analogue, E-cAMP, that activates Epac efficiently while it has a strongly reduced effect on PKA (JM Enserink *et al*., submitted). To first test the specificity of this compound we used platelets, which are extremely sensitive to cAMP-PKA-mediated inhibition, including inhibition of thrombin-induced calcium mobilisation (see Figure 1A and B). Pre-treatment with 0.1 mM E-cAMP for a similar period of time did not affect the thrombin-induced calcium response (Figure 1C), showing that PKA is not activated. Only at higher concentrations (0.3 mM) E-cAMP resulted in a partial inhibition of thrombin-induced $[Ca^{2+}]_i$ (Figure 1D). From these results we conclude that E-cAMP at concentrations up to 0.1 mM does not induce PKA-mediated signalling to inhibit thrombin-induced increase in $[Ca^{2+}]_i$.

E-cAMP does not affect Rap1 activation in platelets

We next investigated the effect of E-cAMP on Rap1 activation in platelets. As shown previously (16), Rap1 is very rapidly activated by thrombin and this activation is completely abolished by forskolin-induced cAMP formation (Figure 2A). Again, E-cAMP was unable to inhibit thrombin-induced Rap1 activation, stressing our conclusion that E-cAMP does not activate PKA. Whereas cAMP-PKA clearly inhibits Rap1 activation, it may still be possible that cAMP-Epac activates Rap1. However, treatment of platelets with E-cAMP did not induce Rap1 activation (Figure 2B). To show that indeed E-cAMP is able to induce Rap1 activation, we treated OVCAR cells with E-cAMP as a control. As shown in Figure 2C, treatment with E-cAMP resulted in a clear Rap1GTP accumulation. Treatment with 8-Br-cAMP activated both Rap1 and induced phosphorylation of CREB, an indirect read-out for PKA activity. Together, these findings indicate that E-cAMP used at concentrations up to 0.1 mM neither activates PKA nor Rap1 in platelets.

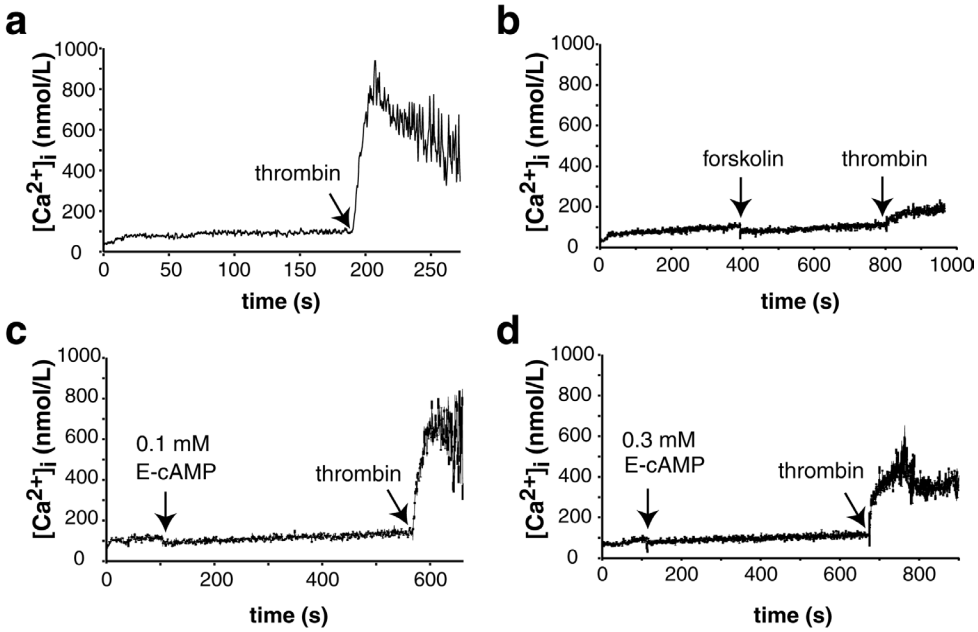

Figure 1. ***Effect of E-cAMP on platelet $[Ca^{2+}]_i$ responses.*** A, Thrombin-induced elevation of $[Ca^{2+}]_i$ in platelets. B, Inhibition of thrombin-induced $[Ca^{2+}]_i$ elevation in platelets by a forskolin pre-treatment. C, Platelets neither increased $[Ca^{2+}]_i$ upon 0.1 mM E-cAMP nor was thrombin-induced $[Ca^{2+}]_i$ elevation inhibited by a pre-treatment with 0.1 mM E-cAMP. D, Used at 0.3 mM, E-cAMP pre-treatment resulted in a partial inhibition of the thrombin-induced rise in $[Ca^{2+}]_i$. Platelets were freshly isolated after which they were loaded with Fura-2-AM. Subsequently, Ca^{2+} responses induced by thrombin (1 U/ml), either in the absence (A) or presence (B) of forskolin (40 μM) or E-cAMP (C, 0.1 mM and D, 0.3 mM) were measured in the presence of 1 mM extracellularly added Ca^{2+}.

E-cAMP neither induces Rap1 activation nor Ca^{2+} mobilisation in megakaryocytes

In addressing the question whether E-cAMP can induce Rap1 activation in the megakaryoblastic MEG-01 cell line as previously reported for cAMP (44), we noticed that gentle mixing the MEG-01 cells already induced Rap1 activation (see Chapter 5). In case we controlled for this effect and gently mixed the untreated samples as well, we did not observe an increase in Rap1 activation after stimulation with either forskolin (Figure 4) or with E-cAMP (data not shown). Despite many attempts we were unable to find conditions to avoid mix-induced Rap1 activation, and therefore we could not measure whether cAMP can induce Rap1 activation in these cells. Forskolin was still able to signal and to induce phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a read-out for PKA activity (47), in MEG-01 cells (Fig. 3B). As expected, E-cAMP did not induce phosphorylation of VASP.

Next, we measured the effect of forskolin and E-cAMP on $[Ca^{2+}]_i$ in MEG-01 cells. Stimulation with forskolin resulted in a small increase in $[Ca^{2+}]_i$ (Figure 3A) as previously reported (44). However, addition of E-cAMP did not generate a calcium response (Figure 3B), while $[Ca^{2+}]_i$ could be elevated by thrombin. From these results we conclude that the Epac-Rap1 pathway is unlikely to mediate cAMP-induced calcium increase.

DISCUSSION

Previously, treatment with iloprost or forskolin was shown to concomitantly elevate $[Ca^{2+}]_i$ and the RapGTP level in MEG-01 cells, both in a H-89-insensitive manner (44). These observations suggested the involvement of the Epac-Rap1 signalling pathway in the regulation of $[Ca^{2+}]_i$. In the present study, we have investigated whether indeed cAMP-Epac-Rap1 signalling plays a role in cAMP-induced rise in $[Ca^{2+}]_i$ in

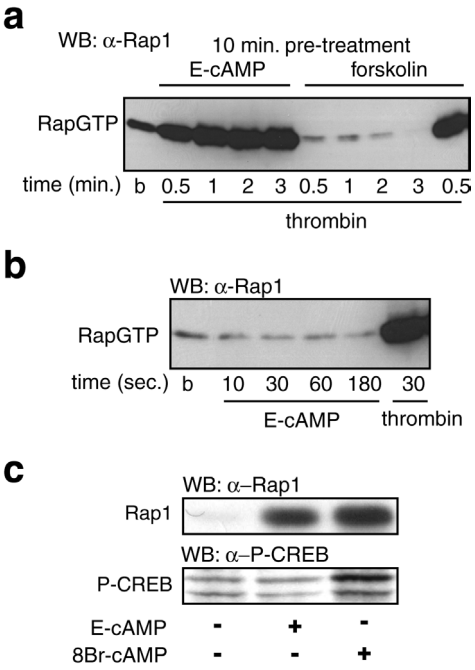

Figure 2. ***Rap1 activity and VASP phosphorylation are not affected by E-cAMP in platelets.*** A, Pre-treatment of platelets with E-cAMP does not block thrombin-induced accumulation of Rap1GTP. Platelets were left untreated or preincubated (10 minutes, 37°C) with either E-cAMP (0.1 mM) or forskolin (40 μM), which was followed by thrombin stimulation (1 U/ml) for the indicated periods of time. Next, platelets were lysed and RapGTP was determined using the RBD pulldown assay. B. E-cAMP does not activate Rap1 in platelets. Isolated platelets were left untreated (basal) or stimulated with 0.1 mM E-cAMP for the indicated periods of time, after which Rap1 activation was measured. thrombin (1 U/ml) was used as a positive control for Rap1 activation. C, Treatment with E-cAMP activates Rap1 but not PKA. OVCAR cells were treated for 15 minutes with 0.1 mM E-cAMP. Cells were lysed after which Rap1 activity was analysed using GST-RalGDS-RBD coupled to glutathion-agarose beads to pulldown Rap1GTP. Phosphorylation of CREB in corresponding cell lysates was determined as a read-out for PKA activity using a phosphospecific CREB antibody. As a control, cells were treated with 0.1 mM 8-Bromo-cAMP for 15 minutes, followed by a similar analysis.

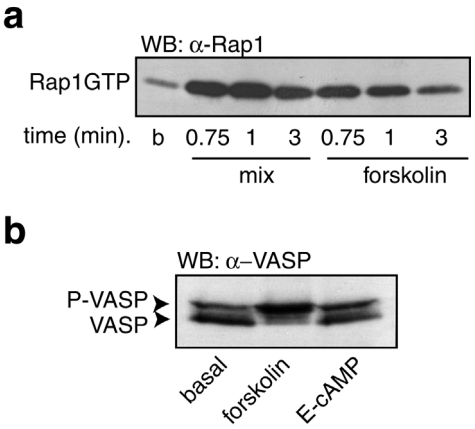

Figure. 3. ***Effect of E-cAMP treatment on Rap1 activation and VASP phosphorylation in MEG-01 cells.*** A, Mixing a sample of resting MEG-01 cells induces accumulation of Rap1GTP. Stimulation with forskolin (40 μM) did not have additional effect on the Rap1 activation. MEG-01 cells were serum-starved overnight and the next day resuspended in fresh, serum-free medium. Samples (1×10^6 cells in 200 μl) were distributed in reaction tubes, after which these were placed in a 37°C waterbath in order to let the cells rest for 30 minutes. Next, samples were left untreated (basal), gently mixed (5 times upside-down) or stimulated with forskolin followed by gentle mixing, after which they were incubated for indicated periods of time. Subsequently, cells were lysed and Rap1GTP was determined using the activation-specific probe assay. B, Treatment of platelets with E-cAMP does not induce VASP phosphorylation. Platelets were left untreated or were stimulated with either forskolin (40 μM) or E-cAMP (0.1 mM) for 1 minute. Next, platelets were lysed using Laemmli sample buffer and phosphorylation of VASP was determined by a mobility shift assay.

immature megakaryocytes. We used a cAMP derivative, indicated as E-cAMP, which when used between 10 and 100 μM, binds and activates the cAMP-regulated Rap1-specific GEF Epac and does not affect PKA (JM Enserink *et al.*, submitted). Indeed, in platelets E-cAMP did not inhibit thrombin-induced $[Ca^{2+}]_i$ elevation or Rap1 activation, effects strongly inhibited by cAMP (Figure 1 and 2). E-cAMP treatment also did not elevate $[Ca^{2+}]_i$ or the Rap1GTP level. Similarly, in MEG-01 cells E-cAMP treatment neither induced a rise in $[Ca^{2+}]_i$ nor Rap1 activation (Figure 3 and 4). From these results we conclude that that the cAMP-Epac-Rap1 is not involved in the regulation of $[Ca^{2+}]_i$.

Our finding that cAMP does not induce Rap1 activation in MEG-01 cells is in contrast with previous results (44). However, we noted that mild disturbance of MEG-01 already results in a considerable Rap1 activation. This could be the explanation for the controversy, but it also implies that cAMP does induce Rap1 activation, but that due to the effect of mixing on Rap1 activation a small increase is undetectable.

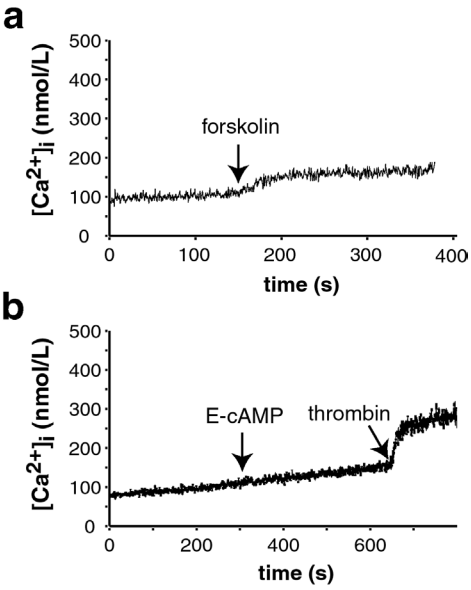

Figure. 4. ***Effect of E-cAMP treatment on $[Ca^{2+}]_i$ in MEG-01 cells.*** A, MEG-01 cells respond upon forskolin with an increase in $[Ca^{2+}]_i$. B, E-cAMP did not generate a Ca^{2+} response although a clear rise in $[Ca^{2+}]_i$ could be evoked, demonstrated here by a thrombin treatment. MEG-01 cells were loaded with Fura-2-AM after which $[Ca^{2+}]_i$ was monitored in the presence of 1 mM extracellular Ca^{2+}. Responses upon (A) forskolin (40 μM) or (B) E-cAMP (0.1 mM) followed by thrombin (1U/ml) were determined. In the case of E-cAMP treatment a representative of duplicate observations with similar results is shown.

Unfortunately, at this moment no antibodies are available that are able to detect endogenously expressed Epac (J.R. de Rooij, personal communication). However, using PCR we were able to demonstrate Epac mRNA (data not shown). Irrespective whether the cAMP-Epac-Rap1 pathway does operate in MEG-01 cells, in contrast to forskolin, E-cAMP did not induce an increase in $[Ca^{2+}]_i$. We therefore conclude that the cAMP-Epac-Rap1 pathway does not mediate the cAMP-induced PKA-independent increase in $[Ca^{2+}]_i$. Interestingly, the cAMP derivative Sp-5,6-DCL-cBIMP, which is able to induce both PKA and Epac activation (J.R. de Rooij, personal communication), did not elevate $[Ca^{2+}]_i$ in MEG-01 cells (44). This points to the involvement of another cAMP target in the regulation of cAMP-induced $[Ca^{2+}]_i$ increase. This pathway, including the cAMP-target is still elusive, but cAMP-regulated ion channels may be good candidates (48).

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CHAPTER

5

The small GTPase Rap1 is activated by shear stress and involved in integrin $\alpha_{IIb}\beta_3$-mediated cell adhesion in human megakaryocytes

The small GTPase Rap1 is activated by shear stress and involved in integrin $\alpha_{IIb}\beta_3$-mediated cell adhesion in human megakaryocytes.

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The small GTPase Rap1 is activated by a large variety of stimuli and functions in the control of integrin-mediated cell adhesion. Here we show that in human megakaryocytes and several other commonly used haematopoietic cell lines, like K562, Jurkat and THP-1, shear stress induced by gently tumbling of the samples induces Rap1 activation rapidly and strongly. Inhibitors that have previously been shown to affect Rap1 activation in human platelets, like the intracellular calcium chelator BAPTA-AM and various PKC inhibitors, could not block this shear stress-induced activation. Also inhibition of actin cytoskeleton dynamics did not influence this activation of Rap1, suggesting that this activation is mediated by cell surface receptors. Human platelets, however, were refractory to this shear stress-induced activation of Rap1. To determine the consequences of Rap1 activation we measured adhesion of megakaryocytes to fibrinogen, which is mediated by the integrin $\alpha_{IIb}\beta_3$, in the presence of inhibitors of Rap1 signalling. Introduction of both Rap1GAP and RalGDS-RBD in the megakaryoblastic cell line DAMI strongly reduced basal adhesion to immobilised fibrinogen. This inhibition was partially rescued by the phorbol ester TPA, but not by α-thrombin. From these results we conclude that in megakaryocytes mix stress induces Rap1 activation that controls $\alpha_{IIb}\beta_3$-mediated cell adhesion.

INTRODUCTION

The small GTPase Rap1 is a molecular switch that cycles between an inactive GDP and active GTP-bound conformation. The protein can be activated by a plethora of stimuli (1-6), indicating that Rap1 activation is a common event in signalling. In general Rap1GTP accumulation is induced by second messengers, like cAMP, calcium ions and diacylglycerol and a number of guanine nucleotide exchange factors (GEFs) have been identified to mediate this activation (7). In human platelets Rap1 is abundantly expressed and is rapidly activated by a large variety of agonists, including a thrombin, thromboxane A_2, epinephrine, platelet activating factor, ADP and the phorbol ester TPA (1). At least two different signalling pathways are involved in this activation, one mediated by G_q, phospholipase C (PLC) and calcium and one mediated by protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI(3)K) (1,8-10). In platelets, Rap1 activation is rapidly inhibited by agents that increase the intracellular cAMP concentration, like for instance prostaglandin I_2 (1). Activation of Rap1 is prior to platelet activation, suggesting a key role of Rap1 in this process. After platelet activation, Rap1 relocalises to the actin cytoskeleton (8,11,12). In the control of Rap1 in platelets, RapGAP, which can interact with active alpha subunit of the heterotrimeric G-protein G_z, plays a role as well (13).

Rap1 has been implicated in a variety of cellular functions including the control of the Raf-MEK-ERK pathway, although this result is questioned (7,14). More recently, in a variety of haematopoietic cell lines Rap1 was found to be involved in the regulation of integrin-mediated cell adhesion. Rap1 controls T-cell receptor-,

CD31- and CD98-induced activation of $\alpha_L\beta_2$ (15-19), but is also required for Mn^{2+}- or activating antibody-induced $\alpha_L\beta_2$-mediated adhesion (20). In macrophages Rap1 regulates $\alpha_M\beta_2$-mediated phagocytosis (19,21) and in a variety of cell types Rap1 was found to regulate β_1-integrins as well (20,22). The mechanism of this activation is still unclear and may involve an increase in both integrin clustering and integrin affinity. In *Drosophila*, Rap1 was found to control cell polarity (23) and to regulate the even distribution of adherence junctions (24).

The role of Rap1 in the regulation of integrins with β_1 or β_2 subunits raised the question whether Rap1 may regulate the activation of integrin $\alpha_{IIb}\beta_3$. Proper control of this integrin forms a key step in platelet adhesion and aggregation. Since platelets are not suitable for genetic interference studies, we have chosen human megakaryocytes, platelet precursor cells expressing $\alpha_{IIb}\beta_3$. In these studies we noted that these cells are highly sensitive to mild disturbances, like tumbling tubes to mix added growth factors. This stress-induced effect was observed in several other haematopoietic cell lines as well. In addition we observed that inhibition of Rap1 strongly reduced cell adhesion to fibrinogen, the counter receptor of $\alpha_{IIb}\beta_3$. This inhibition was partially rescued by treatment with the phorbol ester TPA, but not by α-thrombin. From these results we conclude that in megakaryocytes shear stress is a mechanism to activate Rap1 and that Rap1 is involved in the control of $\alpha_{IIb}\beta_3$-mediated cell adhesion to fibrinogen.

MATERIALS AND METHODS

Materials

The following stimuli and inhibitors were used at concentrations indicated, unless stated otherwise: 8-Bromo-cAMP (Biolog Life Science Institute, 1 mM), forskolin (ICN, 20 μM) BAPTA-AM (Molecular Probes, Eugene, Oregon, USA, 25 μM), Roche 31-8220 (Biomol, Plymouth, PA, USA, 5 μM), GF 109203X (Biomol, Plymouth, PA, 5 μM), staurosporin (Sigma, 200 nM), jasplakinolide (Molecular Probes, 5 μM), cytochalasin D (Biomol, 5 μM), latrunculin A (Molecular probes, 5 μM), latrunculin B (Molecular Probes 5 μM), LY294002 (Biomol, 10 μM), wortmannin (Sigma, 100 nM), H-89 (Biomol, 10 μM), PP1 (Biomol, 50 μM), genistein (Biomol, 100 μM), SB 203580 (Biomol, 10 μM), rapamycin (Biomol, 10 nM), thrombin (Sigma, 1U/ml), TPA (Sigma, 100 ng/ml).

Cell culture, cell lines and transfection

MEG-01 cells (25) were grown at 37°C in RPMI 1640 (Gibco Life Technologies, Paisly, UK) supplemented with 20% heat-inactivated (30 min. at 56°C) fetal bovine serum. DAMI cells (26) were grown in IMDM supplemented with 10% heat-inactivated horse serum. CHRF-288-11 cells (27) were cultured in Fischer's medium enriched with 20% heat-inactivated horse serum. The Jurkat T cell line JHM1 2.2 was provided by Dr. D. Cantrell (Imperial Cancer Research Fund, London, UK) with kind permission of Dr. A. Weiss (University of California at San Francisco, San Francisco, CA). These Jurkat cells were grown as has been described earlier (15). AZU II cells were kindly provided by Dr. M. van de Wetering, Department of Immunology, University Medical Center Utrecht, The Netherlands). They were grown in RPMI 1640 supplemented with 10% foetal calf serum. The erythroleukemic K562 cells, a gift from Dr. C.G. Figdor, were grown in 75% RPMI 1640 and 25% IMDM (Gibco Life Technologies) supplemented with 10% heat-inactivated foetal bovine serum. THP-1 cells were kindly provided by Dr C.J. Heijnen (Department of Immunology, University Medical Centre Utrecht, The Netherlands) and were maintained like the Jurkat cells, but in the presence of 4×10^{-4} % mercapto-ethanol. All cell types were cultured in the presence of 0.05% glutamine, penicillin and streptomycin, in a humidified atmosphere with 5% CO_2. For electroporation, DAMI cells (1.2×10^7 cells/ml in 0.4 ml complete medium) were pulsed at 250 V and 960 μF with 5 μg TK-luciferase plasmid DNA, construct plasmid as indicated in the figure legends and added vector plasmid to keep DNA amounts constant. Subsequently, 24 hrs after transfection, cells were transferred to serum-free medium and used 42-48 hrs after transfection. The constructs used for electroporation have been described previously (20).

Platelet Isolation

Donors claimed not to have taken any medication during the preceding 10 days. After informed consent was obtained, freshly drawn venous blood from healthy volunteers was collected into 0.1 volume of 130 mM trisodium citrate. Citrated blood was centrifuged (150×g, 15 minutes at 20°C) and subsequently the platelet-rich plasma (PRP) was supplemented with prostaglandin I_2 (10 ng/ml) and ACD (2.5 g tri-sodium citrate, 1.5 g citric acid and 2.0 g D-glucose in 100 ml dH_2O) for acidification to pH 6.5 thereby preventing platelet activation during further isolation. Next, platelets were purified from PRP by centrifugation (330×g, 15 min at 20°C). The platelet pellet was resuspended in HEPES-tyrode buffer (10 mM HEPES, 137 mM NaCl, 2.68 mM KCL, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_2$, pH 7.4) containing 5 mM D-glucose at 2×10^8 platelets/ml.

Mixing-induced shear stress

Cells were incubated overnight in serum-free medium. Next, cells were resuspended at 5×10^6 cells/ml (MEG-01) 10×10^6 cells/ml (DAMI, CHRF-288-11) or 25×10^6 cells/ml (Jurkat, AZU II, K562, THP-1) in medium. 200 μl of cell suspension or 500 μl of platelet suspension (2×10^8 platelets/ml) in eppendorf tubes was incubated in a 37°C waterbath for 30 minutes. A Sample was mixed gently, i.e. five times up and down and incubated at 37°C for the indicated periods of time. Subsequently, cells were lysed for 15 min. at 4°C by the addition of ice-cold lysis buffer (10% glycerol, 1% Nonidet P40, 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 μM leupeptin, 0.1 μM aprotinin) and lysates were cleared by centrifugation at maximal speed in an Eppendorf centrifuge for 10 minutes at 4°C.

Measurement of Rap1, Ras and Ral activation

The GTP-bound form of Rap1 was isolated using RalGDS-RBD as an activation-specific probe and quantified by

Western blotting using anti-Rap1 antibody, as has been described previously (1). Similarly, RasGTP and RalGTP were determined using Raf1RBD and RalBP as activation-specific probes (28,29).

Adhesion assay

Adhesion assays were performed and analysed as has been extensively described (15). Briefly, cells were transiently transfected with a luciferase expression contruct in the presence or absence of additional expression constructs as indicated. Cells were harvested, washed and resuspended in TSM buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) containing 0.5% BSA at a concentration of 5×10^5 cells/ml. Coating of 96 well plates was performed as described previously (20) with some minor modifications. Greiner Microlon 96 well plates that had been coated overnight at 4°C with different concentrations of fibrinogen (Chromogenix AB, Mölndal, Sweden) in sodium bicarbonate buffer (50 µl per well) were washed with TSM buffer and blocked for 30 minutes at 37°C with 5% BSA/TSM. After washing the plate again with TSM, 50 µl 0.5% BSA/TSM was added to each well containing a stimulating agent or not. Subsequently, 50 µl cell suspension was added per well, after which the cells were spun down for 1 minute at 200 rpm in a Heraeus Sepatech Megafuge 1.0. Cells were allowed to adhere for 30 minutes 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 (30). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Cells bound were calculated and numbers were corrected for transfection efficiency and nonspecific effects of constructs by measuring luciferase activity of total input cells ([counts in cells bound/counts in total input cells]×100%).

Western blotting

Western blotting of all protein samples was carried out using polyvinylidene difluoride membranes. The antibodies used for protein detection are the rabbit polyclonal anti-Rap1 (Santa Cruz Biotechnology, CA), anti-Ras (Transduction Laboratories). Phosphospecific antibodies against ERK1 and 2, PKB/Akt substrate and S6 were all purchased from Cell Signalling.

RESULTS

Shear stress-induced Rap1 activation

In our analysis of Rap1 activation in MEG-01 cells, we noted that elevated Rap1GTP levels already occurred in the absence of the stimulating agents, solely due to the gentle mixing procedure. This effect was rapid as it occurred within 30 seconds after tumbling the tube and was sustained for at least 5 min (Figure 1A). Addition of the phorbol ester TPA, a strong inductor of Rap1 in platelets, did not or hardly induce a further activation of Rap1 (data not shown). Also cAMP-elevating stimuli, like forskolin, which was previously shown to activate Rap1 in MEG-01 cells, did not further increase the Rap1GTP level (data not shown). A similar mix stress-induced activation was observed in a number of other haematopoietic cell lines, like K562 (erythroleukemia), Jurkat (T lymphocytes), AZU II (B lymphocytes) and THP-1 (monocytes) (Figure 1C). Also in two other megakaryocytic cell lines, DAMI and CHRF-288-11, mix stress induced Rap1 activation (Figure 1D). This activation was neither enhanced nor inhibited by forskolin, an activator of adenylate cyclase resulting in elevation of the intracellular cAMP level. However, TPA has a slight additional effect on DAMI cell, and a more pronounced additional effect on CHRF-288-11 cells. Importantly, mixing-induced shear stress did not induce Rap1 activation in platelets (Figure 1D). From these results we conclude that in megakaryocytes and in a number of other haematopoietic cell lines, but not in platelets, mild mixing is sufficient to strongly induce Rap1 activation.

To investigate the activation pathway responsible for mix stress-induced Rap1 activation, we investigated the effect of several pharmacological inhibitors. Most of the used inhibitors were shown in the past to affect activation of Rap. In certain cell types, an increase in $[Ca^{2+}]_i$ was demonstrated to be necessary or even sufficient to induce Rap1 activation and could be blocked by BAPTA-AM (1,4). Pre-treatment of MEG-01 cells with a combination of BAPTA-AM and EGTA did not inhibit shear stress-induced Rap1GTP increase (Figure 2A). PKC inhibitors (Roche 31-8220, GF 109203X, staurosporine) were described to block Rap1 activation in various cell types (4,8) (6), however they also did not inhibit shear-induced elevation of active Rap1 (Figure 2B). Also PI(3)K and PKA were implicated in the Rap1 activation processes (8,9,14). However, inhibitors of PI(3)K activity (LY294002 or wortmannin) or PKA (H-89) did not interfere with the shear-induced activation (Figure 2C). Contribution of src and p38 MAPK was proposed (4,14,31), but inhibitors of these proteins (PP1/genistein and SB 203580, respectively) showed no effect on this stress-induced Rap1 activation (Figure 2D). Apparently, mix stress-induced Rap1 activation is not mediated by one of these previously described pathways. Finally, we tested whether the effect is direct or mediated by changes in the actin cytoskeleton. However, no role was found for actin cytoskeleton dynamics, as neither actin polymer stabilising (jasplakinolide) nor dissociating (cytochalasin D, latrunculin A and

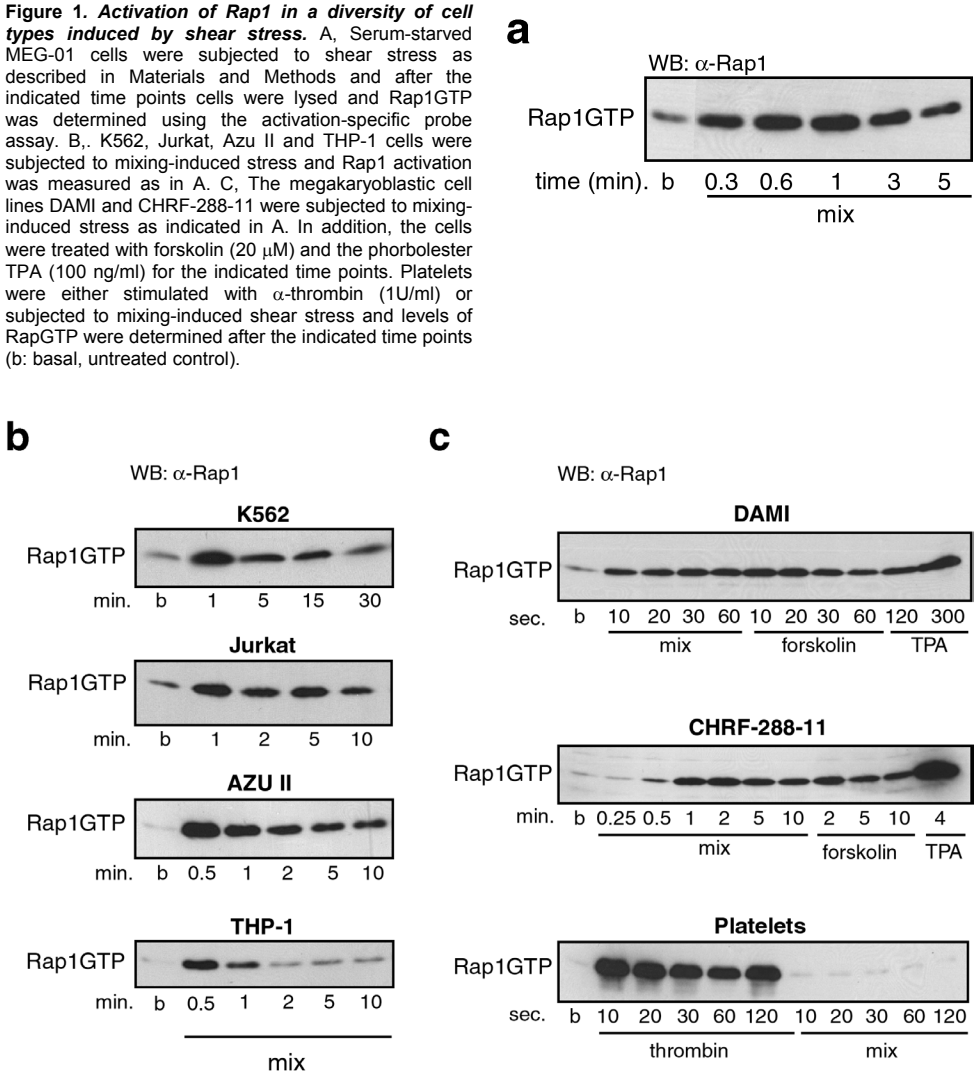

Figure 1. ***Activation of Rap1 in a diversity of cell types induced by shear stress.*** A, Serum-starved MEG-01 cells were subjected to shear stress as described in Materials and Methods and after the indicated time points cells were lysed and Rap1GTP was determined using the activation-specific probe assay. B,. K562, Jurkat, Azu II and THP-1 cells were subjected to mixing-induced stress and Rap1 activation was measured as in A. C, The megakaryoblastic cell lines DAMI and CHRF-288-11 were subjected to mixing-induced stress as indicated in A. In addition, the cells were treated with forskolin (20 μM) and the phorbolester TPA (100 ng/ml) for the indicated time points. Platelets were either stimulated with α-thrombin (1U/ml) or subjected to mixing-induced shear stress and levels of RapGTP were determined after the indicated time points (b: basal, untreated control).

B) agents seemed to affect this Rap1 activation (Figure 2E). The effectiveness of the used pharmacological inhibitors was tested in parallel experiments (data not shown). From these results we conclude that mix stress rather directly, presumably through a cell surface receptor, activates Rap1.

Shear stress-induced activation of Ras and phosphorylation of ribosomal protein S6

To study whether shear stress induces additional signal transduction pathways we determined the activation of both the small GTPases Ras and Ral, and of ERK in MEG-01 cells (Figure 3A). Interestingly, Ras was strongly activated, but at a later time point than Rap1. Two downstream targets of Ras, Ral and ERK were not activated. Using a phosphospecific antibody recognising phosphorylated targets of protein kinase B (PKB) we observed phosphorylation of several proteins in lysates of mixed MEG-01 cells (Figure 3B). However, we did not find activation of PKB itself using phosphospecific antibodies (data not shown), but did observe phoshorylation of the ribosomal protein S6, which is sensitive to the mTOR inhibitor

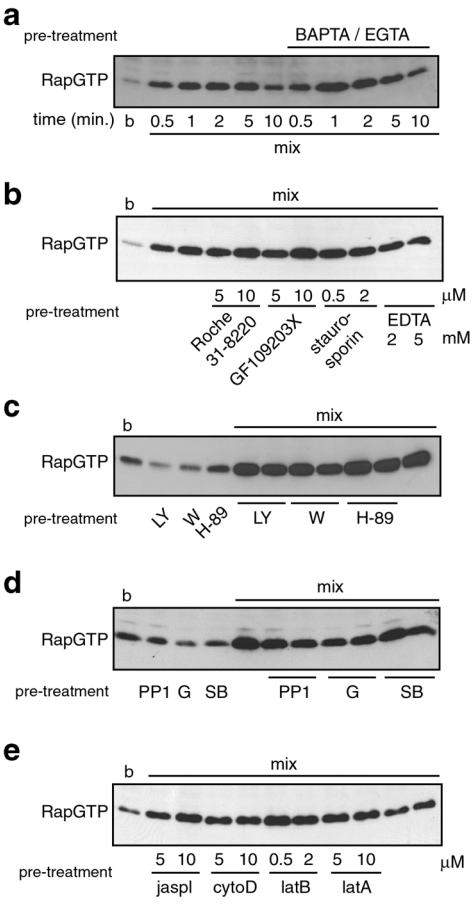

Figure 2. ***Effect of pharmacological inhibitors on the shear stress-induced Rap1 activation.*** MEG-01 cells were serum-starved overnight after which inhibitors were added at the indicated concentration 30 min prior to the induction of mixing-induced stress. In this period cells were kept at 37 °C. Cell were subjected to mixing-induced stress followed by incubation for indicated periods of time (A) or for 1 min (B-E) and Rap1GTP was determined. A, Cells were pre-incubated with 25 μM BAPTA-AM and 5 mM EGTA. B, Cells were incubated with the PKC inhibitors Roche 31-8220, GF 109202X, staurosporin or EDTA**,** used at concentrations as indicated. C, Cells were incubated with the PI(3)K inhibitors LY294002 (10μM, LY) and wortmannin (100nM, W) and the PKA inhibitor H-89 (10 μM). The effect of the inhibitors on mixed-induced stress is in duplicate. D, Cells were incubated with the src inhibitor PP1 (50 μM), the tyrosine kinase inhibitor genistein (100 μM, G) and the p38MAPK inhibitor SB 203580 (10 μM, SB). The effect of the inhibitors on mixed-induced stress is in duplicate. E, Cells were incubated with indicated concentrations of inhibitors of actin dynamics, i.e. jasplakinolide (jaspl), cytochalasin D (cyto D), latrunculin A and B (lat A and lat B) (b: basal, untreated control).

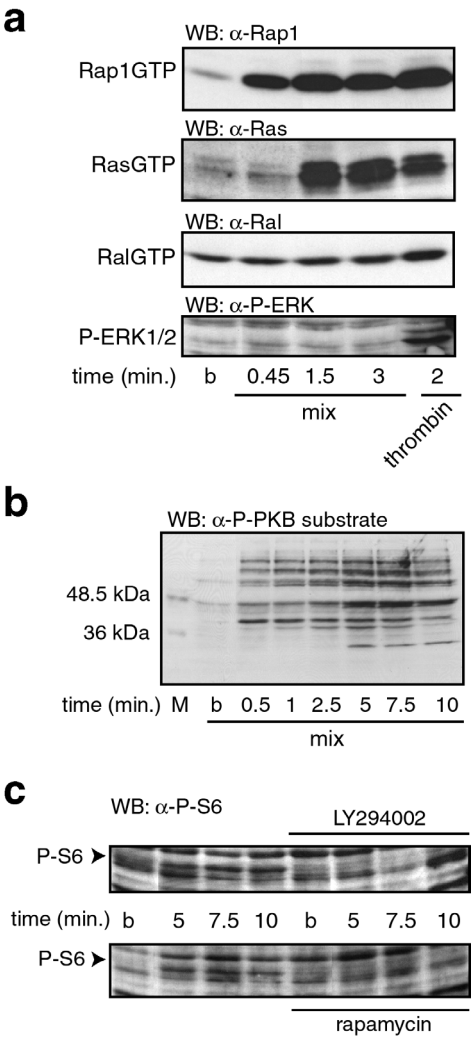

Figure 3. ***Shear stress-induced activation of Ras.*** MEG-01 cells were subjected to mixing-induced shear stress. A, Effect of mix stress on the activation of Ras, Ral and ERK. Activation of Ras and Ral was measured using activation specific probes. Activation of ERK was measured using a phosphospecific antibody that recognises the active form of both ERK1 and ERK2. B, Phosphorylated proteins recognised by a phospho-PKB substrate-specific antibody. C, Ribosomal S6 protein recognised by antibody specific for phosphorylated S6. The PI3K inhibitor LY294002 (25 μM) and mTOR inhibitor rapamycin (10 nM) were added 30 min prior to mixing-induced shear stress as described in the legend of Figure 2. (b: basal, untreated control).

rapamycine and the PI(3)K inhibitor LY294002 (Figure 3C). This suggests that p70 S6 kinase is responsible for the phosphorylation of S6.

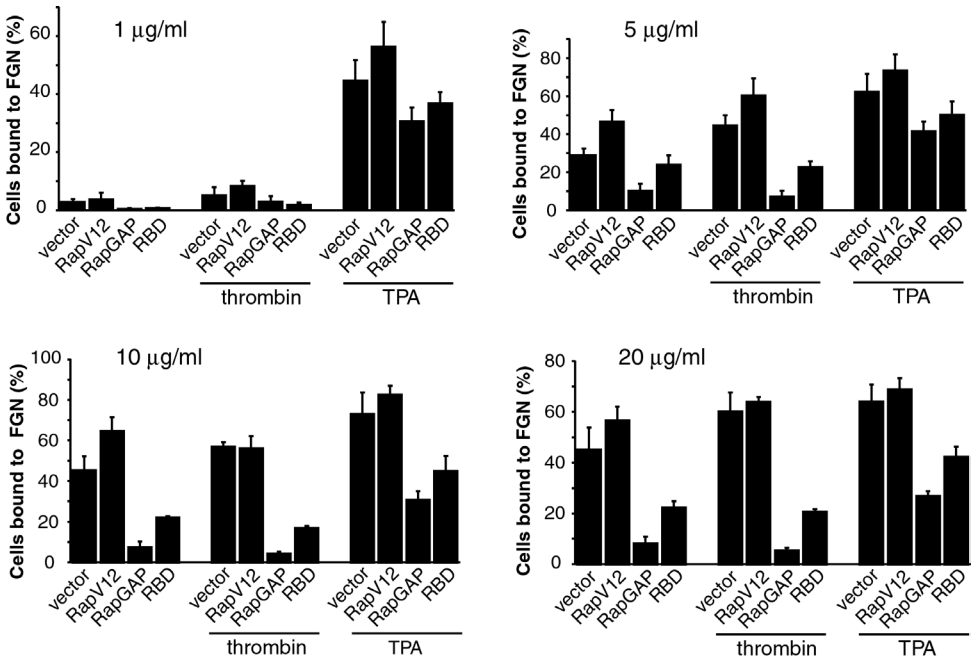

Figure 4. ***Rap1 involved in $\alpha_{IIb}\beta_3$-mediated cell adhesion.*** DAMI cells were cotransfected with 5 μg pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (vector), HA-RapGAP I (RapGAP I) (20 μg) or HA-RalGDS-RBD (RBD) (20 μg). After 42 hrs, the cells were allowed to adhere to immobilised fibrinogen for 30 minutes at 37°C. The percentage of cells bound was determined by fraction of luciferase activity bound, as described in the Materials and Method section. 96-well plates were coated with the indicated concentration of fibrinogen. Where indicated, the cells were treated with α-thrombin (1 U/ml) or TPA (100 ng/ml).

Interestingly, shear stress-induced activation of p70 S6 kinase was previously demonstrated (32). From these results we conclude that mix stress induces activation of several signalling pathways one of which is the activation of Rap1. However, activation of Rap1 appears to be a particularly fast response.

Inhibition of Rap1 abolishes $\alpha_{IIb}\beta_3$-mediated cell adhesion

In contrast to platelets, which do not bind to fibrinogen in the resting state, we observe that DAMI cells adhere spontaneously to fibrinogen (Figure 4), indicating that the integrin $\alpha_{IIb}\beta_3$ is (partially) active. To investigate whether Rap1 is involved in this process, we transfected DAMI cells with two different inhibitors of Rap1 signalling, i.e. Rap1GAP, which lowers the level of Rap1GTP and RalGDS-RBD, which binds with high affinity to Rap1GTP. Cells were allowed to adhere to 96 well plates coated with different concentrations of fibrinogen, the counter receptor of $\alpha_{IIb}\beta_3$. We observed that the concentration dependent adhesion is strongly inhibited by Rap1GAP and significantly inhibited by RalGDS-RBD, indicating that active Rap1 is required for $\alpha_{IIb}\beta_3$-mediated cell adhesion. Introduction of Rap1V12 had little effect on basal level of adhesion, suggesting that the endogenous level of Rap1GTP is already sufficiently high to support cell adhesion. Interestingly, whereas α-thrombin only marginally affects basal level of adhesion, TPA is a strong inductor of cell adhesion. Introduction of Rap1V12 did not significantly increase cell adhesion under these conditions. The reduction in basal adhesion after expression of Rap1GAP and RalGDS-RBD was also observed when cells were stimulated with α-thrombin. However, a much weaker effect of Rap1GAP and RalGDS-RBD was observed after TPA-induced cell adhesion, showing that TPA-induced adhesion is partially independent of Rap1.

DISCUSSION

In this paper we show that in a variety of haematopoietic suspension cells mixing-induced shear stress results in a clear Rap1 activation. The presence of additional stimuli, like forskolin, TPA or thrombin, did not accomplish a clear additional increase in the Rap1GTP level. The effect was rather dramatic and even observed when cell suspensions were either tumbled once up and down or gently stirred with a pipet tip. The mechanism by which shear stress activates Rap1 is still unclear, despite our attemps to find a clue about the initial signalling events. We have tried to interfere using a variety of inhibitors previously described to inhibit Rap1 activation, like inhibitors for PLC, PKC and PI(3)K, and chelators of calcium ions. We have also tested inhibitors of the cytoskeleton or integrin-based cell-cell contacts. However, neither of the inhibitors tested so far showed an inhibitory effect on the shear stress-induced Rap1 activation.

Signalling induced by mixing is probably most similar to signalling induced by mechanical and shear stress, the first induced by changes in cell shape, the second by a fluid flow. For both stimuli the receptors that trigger the events are still elusive, although cell surface receptors and alterations in the cytoskeleton are most likely involved (33-37). Both stimuli trigger a variety of signalling pathways, including the activation of Ras and p70 S6 kinase, like we observe for mix stress, resulting in changes in cell structure and function. Previously, activation of Rap1 was observed after stretching adherent fibroblastic L 929 cells (38). This implies that activation of Rap1 by external stress is not restricted to haematopoietic suspension cells.

One of the most prominent effects of active Rap1 in mammalian cells is the regulation of integrin-mediated cell adhesion. Adhesion mediated by integrins containing either $\beta_1$and β_2 subunits has been shown to be regulated by Rap1. This regulation results in an increased binding of integrins to soluble ligands or increased cell adhesion to immobilised ligands. Megakaryocytes, like platelets, express $\alpha_{IIb}\beta_3$ that allows binding to fibrinogen. Interestingly, megakaryocytes already adhere to fibrinogen in the absence of a stimulus and treatment with α-thrombin, a very potent activator of $\alpha_{IIb}\beta_3$ on platelets, did only marginally increase the adhesion. This adhesion was dependent on fibrinogen and increasing concentration of fibrinogen also increased basal and α-thrombin-induced cell adhesion. Importantly, both basal and thrombin-induced cell adhesion was inhibited by the introduction of Rap1GAP or RalGDS-RBD, inhibitors of Rap1 signalling. This shows that Rap1 is required for $\alpha_{IIb}\beta_3$-mediated cell adhesion and suggests that Rap1 regulates $\alpha_{IIb}\beta_3$ activity. The inhibitory effect of Rap1GAP and RalGDS-RBD on basal adhesion indicates that in resting cells a basal level of Rap1 is sufficient to sustain cell adhesion. However, considering the stress induced by plating cells, it is likely that the basal level of Rap1 is elevated by mixing-induced stress and thus contributes to $\alpha_{IIb}\beta_3$-mediated cell adhesion. While this manuscript was in preparation, Bertoni *et al*. reported that Rap1 regulates the affinity of $\alpha_{IIb}\beta_3$ in primary mouse megakaryocytes (39). In this study basal adhesion was low and insensitive to expression of Rap1GAP, whereas α-thrombin strongly stimulated cell adhesion, which was further augmented by expression of RapV12 and inhibited by RapGAP. However, they did not measure the level of Rap1GTP in these cells. Apparently, these mature megakaryocytes are less sensitive to mix stress-induced Rap1 activation than the DAMI cells, which represents a more immature megakaryoblast.

What could be the reason for cells to activate Rap1 in response to extracellular applied stress? The most likely explanation would be that when cells are subject to forces, they respond by increased binding to their support. Alternatively, Rap1 regulates integrin dynamics to allow cells to respond to shape changes without cell rupture. Interestingly, Rap1 activation is induced by a large variety of stimuli and is thus a very common event. In addition, Rap1 appears to modulate all integrins thus far tested, indicating that it regulates a process required for the activation of all integrins. Elucidation of this process is currently one of the great challenges in cell biology.

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CHAPTER

6

Summarising discussion

Ral and Rap1 in the light of platelet function

A human being consists of billions of cells that work together in organs and tissues. For a proper organisation, communication between these cells is indispensable. Cells send messages to each other by means of communication signals, for instance hormones. A cell is able to receive these signals by specific receptor molecules. Once a signal is received, the activated receptor passes the message on to proteins inside the cell. These proteins in turn propagate the signal to other proteins and so on, generating protein cascades that transmit the signal. Finally, proteins are reached that regulate processes underlying cell growth, proliferation, differentiation or death, like for example the control of which genes are on and off. Normally, each individual cell receives multiple signal inputs at once and a complex network of proteins is needed in order to process the messages and to translate them into the correct cellular responses that enable the desired adaptation in the cellular behaviour. This process of receiving, transmitting and integrating signals is indicated as signal transduction. Small mistakes in these pathways may give rise to inappropriate cell reactions and thereby underlie several diseases, for example uncontrolled cell growth (cancer) or bleeding disorders.

Members of the Ras-like small GTPase protein family play an important role in signal transduction. These proteins participate in signal transduction as molecular switches. This results from their characteristic association with either the guanine-nucleotide GDP, a conformation in which these proteins are inactive, or the guanine-nucleotide GTP, the active state. An activating signal is propagated to a guanine-nucleotide exchange factor (GEF) which releases GDP from the GTPase, leading to the replacement by GTP. In the GTP-bound conformation, the GTPase is active and transmits the signal further on. Due to the action of a GTPase activating protein the GTP is hydrolysed, the Ras protein returns to the GDP-bound, inactive state and signalling is terminated. The Ras protein family is introduced in chapter 1 of this thesis. The studies described in this thesis focus on Ral and Rap1, both of which are close relatives of the Ras protein. Ras has become notorious, because aberrant, persistent Ras signalling due to small mutations was found to play a role in several types of human cancer (1), demonstrating the importance of Ras GTPase signalling.

The main goal of this thesis was to elucidate mechanisms of Rap1 and Ral signalling as they occur in platelets. Platelets are anucleate cell fragments in the blood circulation and their function is to stop bleeding. The behaviour and regulation of platelets is described in chapter 1. Platelets form an attractive model system to study Rap1 and Ral signalling. Ral and Rap1 are both abundant and rapidly activated upon a variety of agonists in human platelets (2-7), suggesting regulatory contribution of these GTPases to platelet function. Furthermore, due to the absence of a nucleus platelets are less complex as compared to for instance a fibroblast. Elucidating signalling pathways that control platelet function enlarges the understanding of defects that underlie diseases caused by improper platelet behaviour.

Initially, the Ral activation mechanism in platelets was addressed. This research has resulted in the characterisation RalGEF2, an exchange factor for Ral that is not regulated by Ras (chapter 2). In chapter 3, 4 and 5 investigations on Rap1 signalling and functions related to platelet physiology form the central theme.

Ras-independent Ral activation: Identification of RalGEF2

Previous studies on Ral activation have established that active, GTP-bound Ras is able to recrute and activate Ras-binding domain (RBD)-containg RalGEFs and thereby induces Ral activation (reviewed in (8)). In platelets however, RalGTP elevation occurs upon treatment with a diversity of different stimuli in a Ras-independent manner. Instead, RalGTP accumulation was found to depend on intracellular calcium and correlated with Rap1 activity (3). Initially, a mechanism whereby Rap1 regulates the RBD-containing RalGEF RalGDS was proposed, but this seemed unlikely to occur *in vivo* (3,9). Alternatively, intracellular calcium might fulfil a second messenger function in upstream Ral regulation

by direct binding to and activation of a Ral-specific GEF. In search for a novel GEF that could account for Ras-independent calcium-regulated Ral activation in platelets we have identified and characterised RalGEF2 (chapter 2). At the same time, different splice variants of RalGEF2 were reported to exist (10). Although we clearly have identified a Ras-independent factor in the regulation of Ral activity, it is unlikely to play a role in platelets. First of all, we could not detect protein expression of RalGEF2 in blood platelets. In addition, no indications exist for regulation of RalGEF2 by intracellular calcium. This implies that other Ras-independent factors that activate Ral should exist. We have performed additional database searches, but have not found other putative RalGEFs. Alternatively, calcium regulates known components in the upstream pathways leading to RalGTP accumulation. Indeed, direct binding of calcium/calmodulin to Ral thereby regulating its activity has been proposed (11,12). In addition, calcium/calmodulin-dependent kinase might regulate RalGDS (13).

New insights on Rap1 signalling and functions

Rap1 and integrin-mediated cell adhesion

Recently, Rap1 was found to play a crucial role in inside-out regulation of integrin-mediated cell adhesion (14-18). Expression of an activated Rap1 mutant results in increased integrin-mediated cell adhesion, while expressing proteins that block Rap1 signalling (RapGAPs and RalGDS-RBD) inhibit stimulus-induced cell adhesion, as described in chapter 1. Integrins can also directly be activated at the outside of the cell. Divalent cations bind to defined binding pockets thereby generating the high affinity state of the integrin (19). In addition, antibodies have been generated that associate with the extracellular part of the integrin in such a way that they induce the conformation in which the integrin displays high affinity for its ligand. For example, the monoclonal antibody KIM185 activates integrin $\alpha_L\beta_2$ (LFA-1) (20), while TS2/16 increases the affinity of $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5) (21). Consistent with the notion that divalent cations (for instance Mn^{2+}) or these integrin-activating antibodies induce this high affinity state without the requirement for intracellular signalling (22,23), treatment with Mn^{2+} or the integrin-activating antibodies does not result in activation of Rap1 (chapter 3). This implies that Rap1 is not in a linear pathway between divalent cations or integrin-activating antibodies and the integrin. However, we have shown that a block of Rap1 activity inhibits the integrin-mediated cell response upon treatment with these agents (chapter 3). Interestingly, in this study, we further demonstrate that induction of cell adhesion induced by expression of active Rap1 requires in addition to intracellular calcium functional calmodulin signalling. Adhesion induced by divalent cations or activating antibodies also required intracellular calcium. This finding suggests that common mechanisms underlie both types of integrin regulation. In addition we describe that stimulus-induced Rap1 activation not always enhances integrin-dependent cell adhesion. TPA induces a strong and prolonged Rap1 activity in Jurkat JHM1 cells, however the effect on cell adhesion is only marginally. The data presented in this study support a facilitating function for Rap1 in the process of integrin-mediated cell adhesion. Expression of active Rap1 stimulates a mechanism allowing increased adhesion at relatively low amounts of immobilised ligand. In case Rap1 signalling is severely impaired, integrin-mediated adhesion can be established by increasing the amounts of immobilised ligand in the presence of integrin-activating antibody (chapter 3). The Rap1GTP level may determine the fraction of a constant integrin pool present at the cell surface that is available for the adhesion process. At least Rap1 facilitates the proces of integrin activation.

Knowing the regulatory Rap1 function in integrin-mediated cell adhesion, the question raised whether Rap1 also contributes to the control of the major platelet integrin $\alpha_{IIb}\beta_3$. As described in chapter 1, upon platelet activation the proper control of $\alpha_{IIb}\beta_3$ integrin activity by signal transduction mechanisms forms an essential aspect for platelet function. We and others have demonstrated that, in addition to the regulation of integrins $\alpha_L\beta_2$ (LFA-1), $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5), Rap1 is required in the regulation of the platelet integrin $\alpha_{IIb}\beta_3$ in megakaryocytes, the cells that develop into platelets ((24), chapter 5). Expression of active Rap1 in megakaryocytes did not promote $\alpha_{IIb}\beta_3$

functionality, as measured on soluble fibrinogen binding. However, thrombin-induced binding of soluble fibrinogen increased as the consequence of active Rap1 expression in these cells and was inhibited by RapGAP expression (24). We observed that megakaryoblastic DAMI cells adhere spontaneously to immobilised fibrinogen, indicating that in these cells integrin $\alpha_{IIb}\beta_3$ is at least partially active (chapter 5). This adhesion, which was dependent on the concentration of fibrinogen coated, could be completely abolished by inhibition of Rap1 signalling, while introduction of Rap1V12 had little effect. The block of $\alpha_{IIb}\beta_3$-mediated adhesion by inhibition of Rap1 was partially rescued by a TPA treatment, indicating that TPA-induced adhesion is partially independent of Rap1 in these cells. Together, these findings again illustrate that Rap1 not directly induces increased integrin functionality but fulfils a facilitating function.

Mechanism of integrin regulation by Rap1

The mechanism by which integrins bind to their ligands is complex (reviewed in (25,26)). In the currently held view, integrins can be regulated by increasing the ligand-binding affinity, by clustering at the cell surface impeding their lateral diffusion in the plasma membrane, by relieve of a restraint and by induced association with the cytoskeleton. Receptor-induced signalling that gives rise to an increase in the intrinsic affinity of an integrin heterodimer for its ligand is denoted inside-out activation. In this situation, the cytoplasmic integrin domain may be modified in such a way that a conformational change in the extracellular integrin domain is brought about. For instance, this could be either phosphorylation or association with cytoskeletal proteins. However, receptor-induced cell adhesion often results from increased integrin avidity. Increased avidity is the result of multiple simultaneous interactions that cooperatively increase the binding strength, as is the situation for antibodies. Multiple integrin-mediated interactions may allow integrin clustering and binding of a cell to extracellular matrix components or to another cell. However, total adhesion strength is limited due to dissociation from ligands and lateral diffusion of the integrins involved. Immobilising the integrins may give rise to stabilisation of these interactions. One could think of recruitment of the integrins to specialised membrane areas, like for instance lipid rafts (27), or anchoring the integrins to the cytoskeleton. Several proteins have been described that associate with cytoplasmic regions in the integrin and consequently couple the integrins to the actin cytoskeleton (28-31). Once attached to the cytoskeleton, additional signals may regulate actin polymerisation, actin-myosin contraction, or actin-membrane interactions that could promote clustering of the integrins thereby increasing their avidity. Rho family GTPases have been demonstrated to promote linkage of the cytoskeleton to integrins by ezrin, radixin, moesin (ERM) proteins and in addition mediate actin polymerisation and reorganisation of the cytoskeleton (32).

In an attempt to explain how Rap1 contributes to integrin regulation, some general considerations deserve attention since they clearly put some restrictions on the oppurtunities. For instance, one needs to take into account that it is not clear whether Rap1GTP brings about changes in integrin clustering or integrin affinity. In mouse T cells, expression of active Rap1 induced clustering of LFA-1 and did not increase affinity of this integrin as measured by soluble ligand binding (18). In contrast, other studies report affinity modulation of LFA-1 by Rap1 in T lymphocytes (14), while a combination of both affinity and avidity modulation was found in B lymphocytes (15). In megakaryocytes, active Rap1 did not alter $\alpha_{IIb}\beta_3$ affinity, but only enhanced the thrombin-induced affinity change (24). These findings imply that integrin regulation by Rap1 could well be dependent on the cell type and the nature of the heterodimer, suggesting that it is not one uniform mechanism in between Rap1 and the integrins. In that case the question remains whether Rap1 couples to so many different effector pathways to be able to differentially regulate each integrin type. Accepting that Rap1 increases the functionality of a variety of integrins, a direct mechanism like phosphorylation of the integrin or an associated protein seems unlikely since a variety of factors that induce integrin function require Rap1, but do not activate it. For instance, Mn^{2+}-induced integrin-mediated adhesion does require Rap1 but Mn^{2+} does not induce Rap1 activity (chapter 3). Furthermore, the presence of Rap1V12 in

megakaryocytes hardly or not induced (soluble) fibrinogen binding to $\alpha_{IIb}\beta_3$ by itself (24) (chapter 5). In summary, these findings suggest that Rap1GTP is not sufficient to directly activate integrins and at least requires the involvement of additional factors.

The results presented in chapter 3 suggest that Rap1 determines somehow whether integrins can participate in the adhesion process or not, which is supported by the finding that active Rap1V12 does not alter the affinity of αIIbβ3 for fibrinogen by itself, but increases thrombin-induced fibrinogen binding in megakaryocytes. Most likely, Rap1 facilitates integrin function by optimising conditions allowing a more active integrin conformation or enabling stimuli to induce integrin activation. One could think of the recruitment of an auxiliary factor involved in proper integrin function or fine-tuning of the integrin connection with the cytoskeleton. For example, Rap1GTP was demonstrated to interact with Krit1 (33). This protein was recently reported to associate with ICAP-1 (34,35), a protein that binds to the cytosolic tail of the β_1 integrin subunit thereby regulating the function of β_1 integrins (36-38). In case Krit1 is bound to ICAP-1, it cannot associate with the integrin tail. Rap1GTP may titrate away Krit1 allowing ICAP-1 to bind the integrin and facilitate its function. Of course this would only explain β_1 integrin regulation, while Rap1 also controls additional integrin types that do not interact with ICAP-1. With respect to the cytoskeletal interactions, Rap1 could disrupt an imposed cytoskeletal restraint, allowing other factors to induce a more active conformation or allowing participation of an increased fraction of the integrins expressed at the cell surface in the adhesion process. Alternatively, Rap1 might contribute to the integrin connection with the cytoskeleton in such a way that the affinity changes or clustering can be brought about. Interestingly, several putative Rap1 effector proteins exist based on the presence of a Ras-associating domain (RA domain) that possess additional protein domains which allow interaction with either integrins, associated adhesion molecules or the cytoskeleton. For instance, talin possesses a band 4.1 domain containing a predicted hidden RA domain. Talin associates with several β integrin cytosolic tails coupling them to the cytoskeleton (39,40). Moreover, new insights point to a role for Rap1 in cell polarisation by regulation of the actin cytoskeleton. In *Dictyostelium discoideum*, Rap1 has been implicated in regulation of cortical actin-based morphological changes by membrane ruffling and the formation of lamellipodia (41,42). Furthermore, the yeast Rap1 orthologue bud1 recruits polarity-establishment factors (including the small GTPase Cdc42, its GEF Cdc24 and the scaffold protein Bem1), controlling thye assembly of the actin cytoskeleton for bud formation. The GEF and GAP required for Bud1 regulation, Bud5 and Bud2, respectively, also contribute to this process (43,44). A role for Rap1 in localised actin cytoskeleton regulation underlying cell adhesion is further supported by protein complex formation between between Rap1 and actin-binding protein AF-6/afadin (45). Interestingly, AF-6 localises to tight junctions in adherens-based adhesion complexes and is thought to connect cell surface adhesion molecules to the cortical actin cytoskeleton. In addition, AF-6 associates with profilin (45), which regulates actin polymerisation (46). In *Drosophila melanogaster*, Rap1 was recently demonstrated to regulate the proper localisation of adherens junctions (47).

Finally, one should consider the intracellular localisation of Rap1. Rap1 is membrane-associated and recently stimulus-induced Rap1 activation was shown to uccur in the perinuclear region (48). Localisation in membranes other than the plasma membrane rules out the opportunity of a direct interaction between Rap1 and integrin complexes. In such a situation Rap1 could only indirectly facilitate integrin activation, for example by modifying the plasma membrane lipid composition, cell shape, localisation of the integrins in the plasma membrane or by regulating the transport of an integrin auxiliary factor to the plasma membrane.

In conclusion, although several likely possibilities exist to explain the involvement of Rap1 in adhesion, the exact mechanisms can only be unravelled by the elucidation of the Rap1 effector pathways leading to the integrins.

Rap1 in other integrin-mediated cellular processes

New insights with respect to a function of Rap1 in the regulation of integrins have paved

the way to explore involvement of Rap1 in the complete scala of integrin-dependent processes, such as morphogenesis, heamotopoiesis, migration and tumour invasion. Indeed, Rap1 functions in agonist-induced, integrin-dependent phagocytosis of opsonised particles in macrophages (16,49). Interference with Rap1 activation by overexpression of wild-type (wt) or inhibitory Crk and C3G proteins correlates with alterations in cellular migratory responses to HGF and stromal-cell-derived factor 1 (SDF-1) (50,51). Genetic analysis in Drosophila melanogaster shows a role for Rap1 in both morphogenesis and cell migration (52). In agreement with these data showing a role for Rap1 in migration, overexpressing RapGAP II to inhibit Rap1 signalling blocked stromal cell-derived factor-1 (SDF-1)-induced migration in two B cell lines (53).

Rap1 regulating calcium homeostasis

We have implicated intracellular calcium in Rap1-induced adhesion (chapter 3). Interestingly, PKA-independent cAMP-induced intracellular calcium rise has been described in human megakaryocytes (54), suggesting a role for the Epac-Rap1 pathway. As described in chapter 1, Epac is a RapGEF that is controlled by the second messenger cAMP. Furthermore, in HEK293 cells a regulating role in the control of the intracellular calcium concentration has been assigned to Rap2B (55). Moreover, in platelets Rap1B associates with the calcium transporter SERCA 3B, as described in chapter 1. Rap1-regulated control of the intracellular calcium homeostasis may not only explain why active Rap1-induced integrin-mediated adhesion is calcium-dependent, but might in addition form a possible pathway for the control of Ral activity in platelets. As discussed already, Ral activation in platelets is Ras-independent, requires calcium and correlates with Rap1 activity. In order to examine a role for the Epac-Rap1 pathway in the control of intracellular calcium in megakaryocyte we made use of a synthetic cAMP derivative. This compound was recently developed to be able to specifically induce Epac-mediated Rap1 signal transduction (J.M. Enserink *et al*., submitted). However, in the MEG-01 megakaryoblast cell system we have not been able to find evidence in favour of cAMP-induced Rap1-mediated intracellular calcium elevation (chapter 4).

Rap1 in mechanical or shear stress-induced signalling

In the blood stream, in addition to several agonists, shear or mechanical stress induces a variety of biochemical and physical changes in cell structure and function. For instance, mechanical stress may alter growth and differentiation (56-60). Haemodynamic shear stress influences regulation of vascular tone and diameter, inflammatory responses, haemostasis and vessel wall remodelling function (61-64), including a time- and position-dependent increase in endothelial cell membrane fluidity (65) and aligment (60,66,67). Interestingly, shear forces have recently been demonstrated to promote lymphocyte migration across vascular endothelium (68). Circulating lymphocytes need to anchor to the luminal side of blood vessels at sites of inflammation. In this process, selectins play an important role by mediating leukocyte tethering and rolling. Subsequent contribution of integrins is indispensable for stable cell arrest (69). As described in chapter 1, also in platelet activation, adhesion and aggregation shear stress is considered to play a pivotal role. Interestingly, we have found Rap1 activation induced by gently mixing MEG-01 human megakaryoblast cell suspensions in reaction tubes, in the absence of a stimulating agent. Surprisingly, we have observed this intriguing effect in a variety of additional human suspension cells representing different blood cell types, like monocytes, B and T lymphocytes (chapter 5). We have not been able to get a clue about the mechanism generating this sensitivity of Rap1 towards mixing, as commonly used inhibitors that affect Rap1 activation in other systems, like PKC and PLC inhibitors or calcium chelators (2,5,9,70,71), did not affect mixing-induced Rap1 activation. However, additional signal transduction events are induced as the consequence of mixing (chapter 5). Among these, we have identified phosphorylation of the ribosomal protein S6, which is sensitive to wortmannin and rapamycin, suggesting involvement of PI(3)K and mTOR pathways. As fluid shear stress has been reported to account for p70 S6 kinase activation in a PI(3)K-dependent manner (72,73), we propose that the mixing-induced activation of Rap1 and other signaling proteins measured takes place as the result of mechanical or shear stress.

The consequence of the mixing-induced Rap1 activation is not only that Rap1GTP elevation in these cell types upon different stimuli is easily disturbed my gently mixing and therefore stimulus-induced Rap1 activation is difficult to measure. In addition, it may implicate Rap1 in physiological processes regulated by shear or mechanical stress. As mentioned already, shear plays a role in lymphocyte transendothelial migration. It is tempting to speculate on the involvement of active Rap1 in this integrin-mediated process. After initial interaction of lymphocytes with the vessel wall, they experience shear or mechanical stress from the blood flow. As a consequence Rap1 might be activated facilitating integrin activation and stable arrest at the endothelium. Additional shear-induced signalling may regulate cell spreading, migration or control of transcription and translation in order to prepare the lymphocyte for its functions after extravasation has occurred. In part, Rap1 could contribute to such processes. Several model systems exist to explore cellular effects induced by shear or mechanical stress in a controlled and quantitative fashion, which may be fruitful implementing in further research on this phenomenon.

The mixing-induced Rap1 activation measured in different human blood cell types was under similar experimental settings not observed in blood platelets. One explanation may be that platelets are only activated at high shear stress levels (74,75). High shear stress-induced activation of the GP Ib-IX-V complex triggers activation of platelet integrin $\alpha_{IIb}\beta_3$ by signalling in which intracellular calcium, PI(3)K and Rho have been implicated. As described in chapter 1, this integrin fulfils an essential function in platelet adhesion and aggregation. Upon treatment of platelets with certain agonists, a rise in intracellular calcium is required and sufficient to induce elevation of Rap1GTP (2). It is not unlikely that shear-induced activation of GP Ib-IX-V induces Rap1GTP accumulation that in turn contributes to the control of αIIbβ3. In order to examine this exciting possibility, it will be of great importance to study Rap1 activation in platelets in an established, quantitative shear stress system.

Concluding remarks

The small GTPases Rap1 and Ral are both rapidly activated in a diversity of cell types upon a variety of stimuli, including platelets, which clearly demonstrates a role for these proteins in signal transduction. The characterisation of a novel Ras-independent pathway contributing to the control of Ral activity presented in this thesis forms further evidence for Ral functions independent of Ras effects. Several recent findings have implicated Ral in the control of the actin cytoskeleton and vesicular transport. Other studies described in this thesis contribute to understanding the Rap1 function in the regulation of integrin-mediated cell adhesion in general. With respect to platelets, the studies link Rap1 to the control of platelet integrin $\alpha_{IIb}\beta_3$. This implies Rap1 is connected with one of the most elementary processes that occur during platelet activation that underlies platelet adhesion and aggregation. In addition, the results implicate Rap1 in mechanical or fluid shear stress-induced signalling in different blood cell types. Whether Rap1 plays a role in shear stress-induced platelet activation requires further examination. In conclusion, signal transduction by the Ras family members Rap1 and Ral is clearly involved in platelet physiology. How these GTPases fulfil their functions in platelets needs further exploration to enlarge the understanding about platelet diseases like bleeding disorders and thrombosis.

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Samenvatting

Achtergrond

Een cel is de kleinste eenheid van leven. Het menselijk lichaam is opgebouwd uit miljarden cellen. Georganiseerd in complexen geven ze vorm aan weefsels en organen. Voor het goed functioneren daarvan is communicatie tussen deze cellen essentieel. Ze beïnvloeden elkaars gedrag door het uitzenden van signaalstoffen, bijvoorbeeld hormonen of groeifactoren. Via de bloedbaan kan een dergelijke boodschap terecht komen bij cellen die met behulp van een specifieke receptor het signaal ontvangen. Het receptor eiwit geeft het bericht door aan een volgend eiwitmolecule in de cel. Dit kan vervolgens op zijn beurt de boodschap weer doorgeven aan andere eiwitten, waardoor een hele cascade van eiwitten ontstaat die het opgevangen signaal overdragen. Uiteindelijk worden eiwitten bereikt die direct verantwoordelijk zijn voor het sturen van cellulaire processen die ten grondslag liggen aan de groei, deling, differentiatie of dood van de cel. Een cel kan meerdere signalen tegelijkertijd ontvangen en beschikt over een netwerk van eiwitten om deze te verwerken, integreren en vertalen in de juiste aanpassing in het gedrag. Het geheel van biochemische reacties dat optreedt in de cel als gevolg van het ontvangen van een boodschap wordt signaaltransductie genoemd. Functioneert een bepaald eiwit uit zo'n netwerk niet naar behoren, dan kan dit afwijkende reacties veroorzaken en leiden tot ziektes.

Leden van de Ras-achtige kleine GTPase eiwitfamilie spelen een belangrijke rol in de signaaltransductie. Ze werken als een soort moleculaire schakelaar. Het ontvangen van een signaalstof kan via een signaaltransductie cascade een Ras eiwit tijdelijk actief maken waardoor diverse processen in de cel in werking treden. Deze eiwitfamilie is vernoemd naar het Ras eiwit, dat als eerste werd ontdekt. Ras kreeg bekendheid toen gevonden werd dat kleine mutaties in de genetische informatie (het gen) die codeert voor dit eiwit tot gevolg hebben dat het Ras eiwit voortdurend actief is. Dit kan resulteren in het ontstaan van kanker (ongecontroleerde celdeling).

De Ras familieleden Rap1 en Ral worden onder andere aangeschakeld in bloedplaatjes op het moment dat die een signaal krijgen om hun functie te vervullen. Dit suggereert dat Rap1 en Ral deelnemen aan de signaaltransductie die de daarvoor benodigde processen stuurt. Bloedplaatjes komen in grote getale voor in de bloedbaan. Ontstaat er ergens in het lichaam een beschadiging aan een bloedvat, dan komen er signaalmoleculen vrij die ter plaatse aanwezige bloedplaatjes activeren. De signaaltransductie in die plaatjes heeft als gevolg dat ze van vorm veranderen en aan elkaar gaan plakken (adhesie) door het actief maken van 'plakmoleculen' (integrines) op hun oppervlak. Dit resulteert in de vorming van een bloedstolsel en het stelpen van de bloeding. Plaatjesactivatie mag alleen plaatsvinden als er een wond is. Onder de verkeerde omstandigheden kan het leiden tot de vorming van een bloedstolsel in een bloedvat met in sommige gevallen een infarct als gevolg. Kennis en inzicht met betrekking tot de signaaltransductie is dus vereist om het functioneren van bloedplaatjes (en cellen in het algemeen) onder gezonde en zieke omstandigheden te begrijpen. De vraagstelling voor het hier beschreven promotie-onderzoek was op welke manier Ras-achtige kleine GTPasen betrokken zijn in signaaloverdracht zoals die in bloedplaatjes plaatsvindt. Een bloedplaatje heeft echter eigenschappen waardoor het voor bepaalde onderzoeksstappen niet bruikbaar is. Om die reden zijn diverse deelprocessen bestudeerd in andere doch relevante celsystemen.

Samenvatting

In het eerste hoofdstuk worden de Ras eiwitten en hun rol in de signaaltransductie belicht. Daarbij gaat de aandacht in het bijzonder uit naar hun gedrag in bloedplaatjes en de consequenties daarvan voor de plaatjesfysiologie.

Voor het onderzoek beschreven in het tweede hoofdstuk heeft het activatie-

mechanisme van Ral in bloedplaatjes centraal gestaan. Uit voorafgaand onderzoek was gebleken dat dit niet Ras- maar calcium-afhankelijk verloopt. Een poging deze calcium-gestuurde signaleringsroute naar Ral nader te definiëren heeft geleid tot de ontdekking van een nieuwe Ras-onafhankelijke Ral activator die RalGEF2 genoemd is. Dit eiwit bezit een PH domein dat zowel een rol speelt in de membraanlocalisatie van RalGEF2 als het vermogen om Ral te activeren.

Naast Ral is ook Rap1 een Ras-achtig eiwit dat deelneemt aan het activatieproces in bloedplaatjes. Hoofdstuk drie biedt nieuw inzicht in de functie van Rap1 met betrekking tot integrine-gemediëerde celadhesie. Rap1 activiteit blijkt een voorwaarde te zijn voor dit proces wanneer het door divalente cationen of integrine-activerende antilichamen aan de buitenkant van de cel geïnduceerd wordt. Voorheen werd gedacht dat het op deze manieren aanzetten tot adhesie onafhankelijk was van de situatie in de cel. Het merkwaardige is dat behandeling van de cellen met deze agentia op zichzelf geen Rap1 activatie tot gevolg heeft. Rap1 activatie immers is reeds voldoende om integrine-gemediëerde celadhesie te bewerkstelligen. Wellicht vervult Rap1 een faciliterende taak in deze vorm van integrine-activatie.

In hoofdstuk vier wordt de mogelijke rol van Rap1 in cAMP-geïnduceerde PKA-onafhankelijke verhoging van de intracellulaire calciumconcentatie onderzocht in humane megakaryocyten. Ook in bloedplaatjes is Rap1 in verband gebracht met de regulatie van de intracellulaire calcium homeostase. Met behulp van een cAMP analoog die selectief aangrijpt op een activatieroute naar het endogeen Rap1 is echter vooralsnog geen aanwijzing gevonden voor de betrokkenheid van Rap1 in dat proces in de megakaryocyten.

De experimenten in hoofdstuk vijf tonen Rap1 activatie aan in diverse bloedcellijnen als gevolg van shear stress. Dit effect is gemeten in cellijnen die representatief zijn voor T en B lymphocyten, megakaryocyten en monocyten. Bovendien blijkt Rap1 activiteit nodig te zijn voor integrine $\alpha_{IIb}\beta_3$-gemediëerde adhesie van humane megakaryocyten. Deze bevindingen suggereren allereerst een mogelijk verband tussen shear stress, Rap1 en de regulatie van integrines. Daarnaast koppelen ze Rap1 aan de regulatie van het belangrijkste plaatjes-integrine. De juiste controle over de activiteit van integrine $\alpha_{IIb}\beta_3$ is onontbeerlijk voor het correct functioneren van bloedplaatjes. Daarmee is een mogelijke connectie gelegd tussen Rap1 en een van de meest elementaire processsen van de plaatjesactivatie.

Doel van dit onderzoek is geweest om een stap verder te komen in het begrijpen van de rol van Ras GTPases in het proces van plaatjesactivatie. Kennis en inzicht omtrent de signaaltransductieroutes die ten grondslag liggen aan de plaatjesregulatie kan leiden tot een beter begrip van afwijkend gedrag van bloedplaatjes zoals dat optreedt bij ziektes als thrombose en atherosclerose. Mogelijkerwijs zouden deze moleculaire reacties in zulke gevallen bijgesteld kunnen worden middels medicinale interventie.

Curriculum vitae

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1987-1993	Gymnasium-β, Collegium Marianum in Venlo
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List of publications

De Bruyn KMT, de Rooij J and Bos JL. The small GTPase Rap1 is activated by shear stress and involved in integrin $\alpha_{IIb}\beta_3$-mediated cell adhesion in human megakaryocytes. *To be submitted.*

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