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Characterization of RapGAP1 from *Dictyostelium discoideum*

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TABLE OF CONTENTS

ABBREVIATIONS

ATP	Adenosine 5’-trisphosphate
bp	Base-pair(s)
aa	Amino acid
C-	Carboxy-
BSA	Bovine serum albumin
DNA	Desoxyribonuleic acid
dNTP	Desoxiribonucleotide trisphosphate
DMSO	Dimethyl-sulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylene-diamine-tetraacetic acid
et al	And others
Fig.	Figure
h	Hour(s)
His-tag	Histidine-tag
ITPG	Isopropyl-ß-thiogalactopyranoside
kb	kilo-base(s)
kDa	Kilodalton
l	Liter(s)
µ	Micro
M	Moles/l, molar
min	Minute(s)
ml	Milliliters

µm	Micrometer
mM	Millimolar
N-	Amino-
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Negative decadic logarithm of proton concentration
PH	Pleckstrin homology
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
TBE	Tris/borate/EDTA
TBS	Tris buffered saline
TEMED	N,N,N’,N’-tetramethylenediamine
Tris	Tris-hydroxylmethyl-ammoniumethane
Tween 20	Polyoxyethylene-sorbitanmonolaurate
U	Units
V	Volt
v/v	Volume per volume
w/v	Weight per volume

SUMMARY

Rap1 is a ubiquitous Ras-like guanine-nucleotide-binding protein that is involved in a variety of signal-transduction processes especially during cytoskeletal rearrangements. Rap1 is regulated by guanine-nucleotide- exchange factors (GEFs) and GTPase-activating proteins (GAPs) which increase the slow intrinsic GTPase activity by many orders of magnitude and allow tight regulation of signaling.

In this study a new *Dictyostelium* RapGAP1 gene was cloned and characterized. RapGAP1 was discovered by screening the sequences of the *D. discoideum* database. The *Dictyostelium* RapGAP1 gene encodes a protein with 1212 amino acids protein which shows at the C-terminal region 53% sequence similarity to human RapGAP. RapGAP1 mRNA was present during all stages of *D. discoideum* development with a strong upregulation at 9 hours of development. Furthermore, to investigate the role of RapGAP1 in cellular processes RapGAP1 null cells where generated by inserting a gene replacement construct into the endogenous gene. RapGAP1 minus mutants did not show any significant phenotypic abnormalities except that there was a slight delay in development. This delay by about three hours was confirmed by testing the expression of developmentally regulated genes like csA, a cell adhesion protein, and MUD1, a prespore-specific cell surface antigen. However, the mutant was able to complete normal developmental and to form fruiting bodies containing mature spores. Studies on cell motility showed that RapGAP1 null cells moved faster than AX2 wild type cells. This finding suggests that RapGAP1 belongs to a signal transduction chain which ultimately leads to changes in cytoskeletal dynamics.

ZUSAMMENFASSUNG

Rap1 ist ein weit verbreitetes Ras-ähnliches Guaninnukleotid-bindendes Protein, das in einer Vielzahl von Signaltransduktionsketten und besonders bei Restrukturierungen des Zytoskeletts beteiligt ist. Rap1 seinerseits wird durch Guaninnukleotid-Austauschfaktoren (GEFs) und GTPase-aktivierende Proteine (GAPs) reguliert. GAPs erhöhen die langsame intrinsische GTPase Aktivität über mehrere Größenordnungen.

In der vorliegenden Arbeit wurde das erste *Dictyostelium* RapGAP Gen kloniert und charakterisiert. Das RapGAP1 Gen wurde durch eine gezielte Suche in der *D. discoideum* Genom-Datenbank entdeckt und kodiert für ein Protein mit 1212 Aminosäuren. Die GAP-Domäne am C-Terminus ist zu 53% der entsprechenden Domäne im menschlichen RapGAP ähnlich. RapGAP mRNA konnte in allen Stadien der Entwicklung von *D. discoideum* nachgewiesen werden, allerdings wurde bei 9h nach Beginn des Entwicklungszyklus eine starke Hochregulierung der mRNA Mengen festgestellt. Um die Funktion von RapGAP1 in zellulären Abläufen genauer zu bestimmen, wurden RapGAP1 Null-Mutanten mit Hilfe eines ‚gene replacement' Konstrukts isoliert. Die Null-Mutanten zeigten außer einer leichten Verzögerung in der Entwicklung keinerlei Veränderung im Phänotyp. Diese Verzögerung um ungefähr drei Stunden konnte durch die Analyse der Entwicklungsmarker csA, einem Zelladhäsionsmolekül, und MUD1, einem Präsporen-spezifischen Antigen, bestätigt werden. Von der Verzögerung abgesehen waren die Mutanten in der Lage, die Entwicklung mit der Bildung von normalen Fruchtkörpern mit ausgereiften Sporen abzuschließen. Untersuchungen der Zellmotilität zeigten, dass sich RapGAP1 Null-Mutanten schneller bewegen als der AX2 Wildtyp. Dieser Befund lässt vermuten,dass auch in *D. discoideum* RapGAP1 zu einer

Signaltransduktionskette gehört, die letzlich Veränderungen in der Zytoskelett-Dynamik bewirkt.

CHAPTER 1

INTRODUCTION

The regulated reaction of an individual cell to environmental changes requires a finely tuned and coordinated system of extracellular and intracellular signal transduction. Cell surface receptors enable the cell to monitor its environment for the presence of signals like hormones or growth factors, which function usually as ligands for specific receptors. Ligand binding results in receptor activation that propagates the message to the interior side of the cell membrane and initiates the intracellular signaling machinery towards cellular processes like growth, proliferation, differentiation and appropriate gene expression profiles. Among the numerous signaling proteins small GTPases play an important role in cellular signaling. They act as molecular switches by introducing or terminating signals and thus regulating functions of other proteins.

1.1 Ras family members: molecular characteristics

The behavior of Ras family members at the molecular level is described by the GTPase cycle, a characteristic that allows them to function as molecular switches. Conversion to the active, GTP-bound state is enhanced by guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP from the nucleotide-binding site of the GTPase. As soon as GDP is released, GTP enters the binding site, as a consequence of the relatively high affinity of Ras proteins for guanine nucleotides in combination with the 10-fold excess of GTP (1-10 mM) over GDP in the cytoplasm (Boriack *et al.*, 1998). 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 signaling cascades (Pai *et al.*, 1989; Marshall, 1996). Each Ras-like protein is supplied with an intrinsic GTP hydrolyzing activity. The rate at which different GTPases hydrolyze bound GTP varies, but in most cases incompatible with the rapid changes that are required for proper signaling. However, the hydrolyzing activity can be strongly enhanced by GTPase activating proteins (GAPs) (Scheffzek *et al.,* 1998), which are able to bind to the GTPase and thereby stabilize the GTP-GDP transition state (Scheffzek *et al.,* 1997). 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. Over-expression of dominant active or negative versions of GTPases has been widely used as an approach to study small GTPases (Downward, 1990).

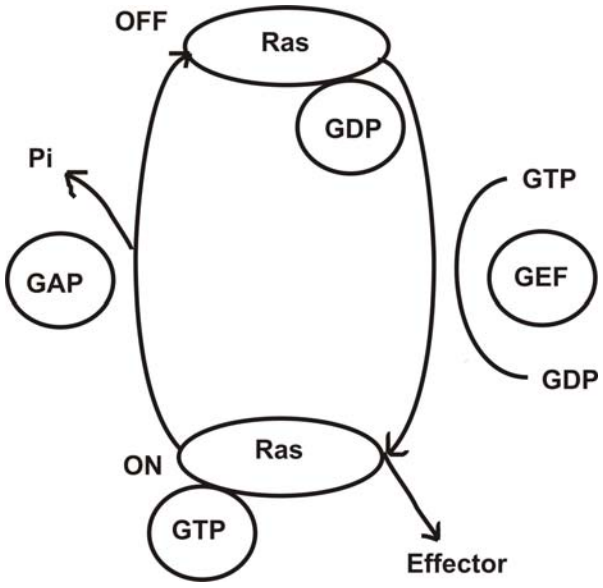

Fig. 1 The Ras GTPase cycle.

The Ras cycle between an inactive GDP-bound form and an active GTP bound form. This is catalyzed by a guanine nucleotide exchange factor (GEF) that displaces the GDP, allowing GTP to bind. Inactivation is regulated by a GAP that stimulates hydrolysis of GTP on the GTPase. Active GTP-bound GTPase is competent to bind directly to effector proteins that propagate the response.

1.2 Small GTPases: switches in signaling cascades

The best-known small GTPase is the Ras protein that was first discovered in rat sarcoma and became the founding member of a superfamily of homologous proteins. At present nine families of small GTPases of the Ras superfamily are known. Ras family proteins are involved in pathways that regulate cell growth, differentiation and apoptosis (Zwartkruis *et al.,* 1995). The Rho proteins (Ras homology) in turn have been implicated in numerous cellular processes, involving cytoskeletal dynamics, cell migration, transcriptional regulation as well as vesicle trafficking (Ridley 2001). Rab GTPases are key regulators of vesicular transport and vesicular fusion (Seabra *et al.*, 2002). Arfs (ADP ribosylation factor) are important regulators of membrane dynamics and protein transport (Catherine *et al.,* 2000). Ran (Ras-related nuclear proteins) is mainly involved in nuclear transport, mitotic spindle assembly, regulation of cell cycle progression and nuclear assembly. Rad (Ras-associated with diabetes) was discovered first in muscle cells of diabetes patients, its biochemical functions are mostly unknown (Bilan *et al.,* 1998). Rheb (Ras homolog enriched in brain) is involved in cell growth and cell cycle progression. Rheb also antagonizes the transforming activity of Ras. The exact function of Rheb is, however, still a mystery (Urano *et al.*, 2000; Clark *et al.*, 1994). In PC6 cells the Rit GTPase represents a distinct regulatory protein, capable of mediating cell survival and differentiation (Spencer *et al.*, 2002). Human RagA and RagB are functional homologues to *S. cerevisiae* Gtrlp, and are both localized within the cytoplasm (Sekiguchi *et al.*, 2001; Hirose *et al.,* 1998; Quinn *et al.,* 1992).

1.3 Rap proteins

In mammalians the Rap subgroup consists two pairs of quasi-identical GTP-binding proteins, (Rap1A and Rap1B; and Rap2A and Rap2B) which differ only in a few residues 90% identical at the amino acid level, respectively. By contrast, Rap1 and Rap2 proteins are only 60% identical to each other (Caron, 2003). Like many small GTPases also Rap1A and B are posttranslationally modified at their C-terminus by a geranylgeranyl moiety, which mediates membrane attachment of the proteins (Bokoch, 1993; Torti, *et al.*, 1994). Rap1 isoforms have been independently isolated by three laboratories by different methods: they have been isolated as homologs of Ras proteins by hybridization (Pizon *et al*, 1988), they have been purified as small G proteins (smg p21) by column chromatography (Kawata *et al.*, 1988) and they have been identified as K-Rev1 in a screen for cDNA that revert the morphology of Ki-Ras-transformed cells (Kitayama *et al.*, 1989). Ras and Rap1 are extremely similar at the amino acid level, which at first glance suggests that they have the same biochemical properties and related cellular function. The region of highest sequence similarity corresponds to the switch one and switch two regions, that is the two regions that adopt different conformations when bound to GDP or GTP, which suggests that Ras and Rap1 share the same effectors. Although qualitative studies (e.g. yeast two-hybrid analysis) have seemed to validate this hypothesis, the in vitro affinities for Rap1 and Ras for a given target (e.g. Raf-1 or RalGDS) differ dramatically (Vetter *et al.*, 1999). Another evidence for this difference is a growth-factor-induced activation of Rap1 versus a distinct sub cellular location in the case of Ras. Whereas Ras is converted to the GTP-bound form at the plasma membrane, Rap1 activation takes place in the perinuclear region both in the PC12 and COS-1 cells

(Mochizuki *et al.,* 2001). Therefore, despite the strong homology between Ras and Rap1, both their different affinities for downstream effectors and sub cellular distributions argue for distinct biological functions.

1.4 GEFs and GAPs regulating Rap1

A remarkable variety of GEFs and GAPs contribute to the regulation of the Rap1 GTPase cycle. Among these GEFs, different families have recently been discovered and characterized.

The first Rap specific GEF to be studied was C3G, which was first identified from human spleen and placenta. It was originally isolated as one of the two major proteins bound to the SH3 domain of the Crk adaptor protein. Crk is an adaptor protein which promots assembly of signal-generating complexes upon a variety of extracellular stimuli. C3G consists largely of three regions. The carboxyl-terminal region, which shares a homologous sequence with CDC25, catalyzes the guanine-nucleotide exchange reaction for Rap1. The central part contains a proline-rich domain, which interacts with the SH3 domain of members of the Crk adaptor proteins, Crk1, Crk11 and Crk L. Although the function of the amino-terminal region of C3G remains unknown, recently $p130^{cas}$, a major Crk SH2-binding protein, has been shown to bind in this region in vitro (Ichiba *et al.,* 1999).

A second exchange factor for Rap1 is CalDAG-GEF1 (calcium- and diacylglycerol-regulated GEF1), a protein from rat brain, which is structurally related to RasGRP but acts on Rap1 instead of Ras (Zwartkruis *et al.,* 1999). It has enriched expression in brain basal ganglia pathways and their axone-terminal regions, and has binding domains for calcium and diacylglycerol.

Rap1 activation by an increase in intracellular cAMP is mediated by Rap1-specific GEFs named Epac (for exchange protein activated by cAMP). Epac is ubiquitously expressed but is particularly abundant in kidney and heart (Kawasaki *et al*., 1998**).** The cAMP-binding site of Epac shares significant sequence identity with the slow and fast cAMP-binding site of the two regulatory subunits of the cAMP-dependent protein kinase (PKA). The GEF-homology domain shares significant homology with C3G. The Ras exchange motif (REM) region, may be important for the stabilization of the GEF structure, and the DEP (Dishevelled, Egl-10, Pleckstrin) domain (important for mediating intracellular protein targeting and regulation of protein stability in the cell) may be involved in membrane attachment (Rooij *et al.,* 1998).

The latest GEF for Rap is PDZ-GEFs. It contains multiple functional domains including a PDZ domain, which is involved in protein-protein interactions, in particular with C-terminal tails of membrane proteins. This autoinhibitory domain is related to the cAMP-binding domain of Epac, but does not interact with cAMP. It also contains a Ras/Rap1 association domain (RA), as well as a GEF catalytic domain. Studies in *Drosophila* show that PDZ-GEF is highly involved in the MAPK signaling pathway, specifically in photoreceptor cell development (Lee *et al.*, 2002).

1.5 Rap-specific GTPase-activating proteins

The RapGaps represent a group of heterogeneous proteins, which exhibit GAP activity specifically for Rap and not for Rho and Rac. Rap specific GAPs have no sequence homology with Ras-GAP and other GAPs such as RanGAP or RhoGAP (Brinkmann *et al.,* 2002). RapGAP was first identified in bovine brain and characterized biochemically as GAP for Rap (Rubinfeld *et al.*, 1991). Biochemical and genetical studies have so far

identified the following GAPs for Rap. Rap1GAP (Rubinfeld *et al.*, 1991) and its isoform Rap1GAP11 (Mochizuki *et al.*, 1999), Tuberin (Wienecke *et al.*, 1995), Spa-1 (Kurachi *et al*., 1997), $GAP1^{IP4BP}$ (Cullen *et al.*, 1995) and E6TP1 (Gao *et al.*, 1999).

The first family of GAPs for Rap proteins contains the members Rap1GAP and the amino terminally extended version named RapGAP11. Rap1GAP was first purified from bovine brain membranes as an 88 Kd protein, which showed high specificity to Rap1A and B and not for Rap2. RapIGAP was abundant in fetal tissue and undifferentiated cells, suggesting a role in growth promoting signals. It was also present in brain and certain tumor cell lines, particularly the Wilms' kidney tumor, SK-NEP-1, and the melanoma, SK-MEL-3, cell lines (Rubinfeld *et al.*, 1991). Progressive deletions at the carboxy and amino termini of human Rap1GAP showed that of the 663 amino acids, only residues from 75 to 416 were sufficient for a catalytically active Rap1GAP. Removal of an additional 27 amino acid beyond residue 75 resulted in complete loss of catalytic activity suggesting that active domain of Rap1GAP resides at the amino terminal portion of the protein while the carboxy-terminal portion is the major site of phosphorylation by cAMP dependent kinase and the cell cycle $p34^{cdc2}$ kinase. (Rubinfeld *et al.*, 1992; Brinkmann *et al.*, 2002; Polakis *et al.*, 1992). Deletion studies have also shown that the NH2 terminal domain of Rap1GAP can bind to activated $G\alpha_z$ subunit of trimeric G proteins revealing Rap1GAP as a possible signal integrator between $G\alpha_z$ signaling and Rap1 signaling.

Rap1GAP11 cDNA was originally isolated from Jurkat T cells and is expressed mainly in heart, liver, kidney, cerebrum. Rap1GAP11 differs only in an additional 31 amino acids before the first Met of the prototype Rap1GAP. Rap1GAP11 is present mainly in the cytosol fraction of the cell and it can bind specifically to the α-subunits of the G_i family

of heterotrimeric G-proteins. Stimulation of the G_i coupled m2- muscarinic receptor (muscarinic receptors are membrane-bound proteins whose extracellular domain contains a recognition site for acetylcholine) translocates Rap1GAP11 from the cytosol to the membrane and decreases the amount of GTP-bound Rap1. This decrease in GTP bound Rap1 activates ERK/MAPK. Thus the α-subunits of G_i activate the Ras-ERK/MAPK mitogenic pathway by membrane recruitment of Rap1GAP11 and reduction of GTP-bound Rap1 (Mochizuki *et al.*, 1999; Stork, 2003).

A second family of GAPs for RAP consists of signal-induced proliferation-associated gene 1 (SPA-1). Sub-cellular fractionation analysis reveals that SPA-1 was present mostly in the insoluble membrane fraction and immunostaining studies indicate that it is localized in the perinuclear ER and Golgi region, probably colocalized with Rap1 and Rap2. SPA-1 mRNA was expressed quite abundantly in lymphoid tissues such as thymus, spleen and PBL (Kurachi *et al.*, 1997). In HeLa cells SPA-1 was found in the cortical area mostly associated with the cortical cytoskeleton. Overexpression of SPA-1 resulted in the rounding up and detachment of the cell, which suggests that Rap1GTP is required to maintain cell adhesion (Tsukamoto *et al.*, 1999).

E6TP1 is another RapGAP, which can interact with high-risk human papilloma virus E6 oncoproteins (HPV E6) and can undergo E6AP dependent ubiquitination and degradation. It is ubiquitously expressed and has a GAP domain, a leucine zipper region and a PDZ domain (Gao *et al.*, 1999). Tuberous sclerosis (TCS) is a human genetic syndrome characterized by the development of benign tumors in a variety of tissues, as well as rare malignancies. Two different genetic loci have been implicated in TSC; one of these loci, the tuberous sclerosis-2 gene (TCS2), contains a region of limited homology to

the catalytic domain of Rap1GAP. Tuberin is expressed in many different cell types and was found mainly in the small blood vessels and neurons (Wienecke *et al.,* 1995; 1997). It is not clear whether the GAP function of tuberin orthologs is required for cell size regulation in *Drosophila* or for polycyctin-1 trafficking in rat kidney cells. However, 11 human disease-associated TSC2 missense mutations occur in the RapGAP related segment (Bernards, 2003). $GAP1^{IP4BP}$ has GAP activity towards both Ras and RAP1 (Cullen *et al.*, 1995). It is solely found at the plasma membrane, most likely due to binding of its PH/BTK domain to inositol 1,3,4,5- tetrakisphosphate (Lockyer *et al.*, 1997).

The mechanism of the GTP hydrolysis of RAP, stimulated by RapGAP, is still unknown. In the case of Ras, glutamine-61 is essential for Gap mediated GTP hydrolysis, whereas Rap1 contains a threonine at this position and Rap is the only small GTP-binding protein which harbors a threonine instead of a glutamine residue at position 61. Replacement of threonine by glutamine does not affect Rap1GAP mediated GTP hydrolysis, indicating that this residue is not critical for this reaction (Rubinfeld *et al.,* 1991). All GAPs characterized so far, with the exception of RanGAP, introduce an arginine residue, the so-called arginine finger, into the active site of their corresponding G protein (Scheffzek *et al.,* 1998). This arginine provides a positive charge, which is thought to stabilize the transition state of GTP hydrolysis. However, this was not the case with Rap1GAP. Understanding of this mechanism came from the mutational studies conducted by Brinkmann *et al.,* (2002) on different residues of Rap1GAP. From the mutational studies he was not able to identify a conserved arginine that when mutated to alanine had any reduction in GAP activity. However, the mutation of two conserved lysines (Lys^{194} and

Lys^{285}) to alanine dramatically reduced the activity of Rap1GAP (by 25-fold and 100-fold, respectively). From this it was clear that Rap1GAP stimulated GTPase reaction does not employ an arginine finger like in Ras, Rho and Rab (Brinkmann *et al.,* 2002).

1.6 Multiple roles of Rap1

Studies have shown that Rap1 is involved in signaling pathways distinct from Ras, while showing similar or identical effector pathways. Ohtsuka *et al.,* (1996) showed that Rap1 can induce the activation of the B-Raf dependent Map kinase cascade and does not antagonize the Ras-induced activation of the B-Raf dependent MAP kinase cascade. It is now well established that interfering with the Rap1 GTP-cycle has profound effects on a wide range of adhesive processes, morphogenesis, phagocytosis, cell-cell adhesion, cell migration and spreading. In HeLa cells overexpression of membrane-tagged RapGEF, exhibited enlarged and flattened cells with extensive cytoplasmic protrusions whereas those transfected with Spa-1 (RapGAP) showed small and round cells (Tsukamoto *et al.*, 1999). Similarly in case of *Dictyostelium* the activated mutant (V12Rap1) showed flattened spread morphology (Rebstein *et al.*, 1997). Rap1 functions as a regulator of morphogenesis in vivo. Rap1 mutations disrupt normal cell shape and morphogenesis in the eyes, ovary and wing of *Drosophila* embryos (Asha *et al.,* 1999). Rap1 controls several specialized types of cell cell adhesion in immune cells. In *Dictyostelium,* Rap1 regulates phagocytosis of bacteria and latex beads (Seastone *et al.,* 1999) and in mammalian cells, Rap1 is found associated with maturing phagosomes (Garin *et al.*, 2001). The ability of Rap1 to regulate integrin-mediated cell adhesion and cell motility has been demonstrated in many cell systems. 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 it blocked granulocyte colony stimulating factor to induce adhesion to tissue culture dishes (Tsukamoto *et al.*, 1999). Mouse embryo fibroblasts that are deficient in C3G show increased cell motility, decreased integrin dependent cell attachment and spreading (Stork, 2003; Caron 2003).

1.7 Role of Rap1 in *Dictyostelium discoideum*

According to current searches, *Dictyosteluim* has three Rap genes, of which there is only one published member of the *Dictyostelium* Rap family (Rap1). Rap1 is expressed at all stages during development but its expression peaks during the aggregation and culmination periods (Robbins *et al.*, 1990). Like human Rap1, DdRap1 also contains a threonine residue instead of the more characteristic glutamine in one of the conserved guanine-nucleotide-binding domains. The *Dictyostelium* protein differs from the human Rap proteins at the extreme amino terminus in that it is two amino acids longer and three of the next six amino acids are different (Robbins *et al.*, 1990). Even though attempts where made to disrupt the Rap1 gene by standard procedures it was not successful, suggesting that Rap1 might be an essential gene in *Dictyostelium.* Thus, most Rap1 studies were based on overexpression and transcriptional regulation by folate induced gene expression. Rebstein *et al.*, (1993, 1997) have described many cytoskeletal defects in cells overexpressing Rap1. The cells are (i) flattened and have a spread morphology, (ii) there is an uniform distribution of F-actin around the cell periphery as opposed to the characteristic punctate distribution found in the parental AX2 wild-type strain, and (iii) the rounding response that occurs when nutrient media is added to starving *Dictyostelium* cells is inhibited.

Studies have also shown that Rap1 acts as a part of the signal transduction pathway that regulates phagocytosis in *Dictyostelium.* Cell lines overexpressing Rap1WT and constitutively activated [Rap1 G12T(+)] exhibited an increase in phagocytic rate when compared to AX2. On the contrary, cells expressing Rap1WT showed no significant changes in the pinocytic rate when compared with parental AX2 cells. Thus in *Dictyostelium* Rap1 appears to positively regulate phagocytosis whereas it negatively regulates macropinocytosis (Seaston *et al.*, 1999).

There have been reports that Rap expression can antagonize the effects of an activated Ras gene in cultured mammalian cells (Kitayama *et al*., 1989), and in *Dictyostelium*, Louis *et al.*, (1997), for the first time showed that Rap can antagonize the aberrant effect of an activated Ras gene on development. Overexpression of RasD (M1 strains) resulted in the formation of multi-tipped aggregates and did not proceed beyond this multi-tipped aggregation stage. However, an increased expression of Rap1 helped the M1 cells to elongate upward from the aggregate to produce structures very similar to the first finger stage observed for wild-type cells as they progress from aggregate to slug stage. Thus, overexpression of Rap1 during development was capable of partially reversing the developmental defects produced by activated RasD.

Kang *et al.,* (2002) showed that gradual reduction of Rap1 protein levels by inducible expression of an antisense construct results in transformants that exhibit decreased growth and viability, implicating that Rap1 is an essential protein. Osmotic shock-induced accumulation of cGMP is reduced in Rap1 depleted cells, but enhanced in cells expressing activated Rap1. Furthermore, the kinetics of Rap1 activation in response to osmotic shock is similar to those of cGMP accumulation.

1.8 *Dictyostelium discoideum* as a model organism

Dictyostelium offers numerous advantages as an experimental system. Like other microbial genetic organisms, *Dictyostelium* is cultured easily and cheaply and can be frozen and stored almost indefinitely, its genome is relatively small. Most importantly, DNA-mediated transformation and homologous recombination allow the isolation of knock-out and knock-in mutants. Because several selectable markers are available, strains can be constructed with complex genotypes.

Dictyostelium is a free-living amoeba that grows and divides in the soil. When the food supply, usually bacteria, is exhausted, individual cells stop growing and migrate into a quasi multicellular organism made up of as many as 10^5 cells. Differentiation and sorting of spores and stalk cells takes place at the multicellular stage, giving rise to a mature fruiting body. The life cycle of *Dictyostelium* is summarized in Fig. 2.

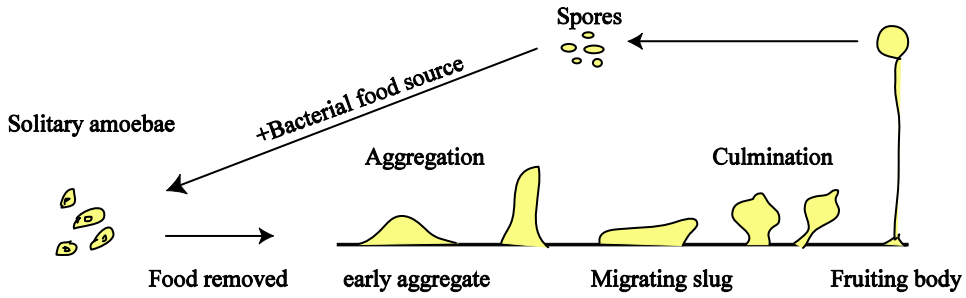

Fig. 2 The life cycle of Dictyostelium discoideum

Spores germinate to give rise to individual amoebas, which feed on bacteria as a food source, and the vegetative cells divide and multiply. Upon depletion of the food source, the cells chemotatically aggregate to form a cell mass that elongates to form the “first finger”, which then either migrates as a slug or directly enters the culmination stage leading to the formation of a fruiting body consisting of the terminally differentiated stalks cells and the spores which can initiate another round of the cycle.

Complex signal transduction networks are activated at the beginning of the growth process and are used throughout the remainder of development, to coordinate the morphogenetic and cellular differentiation events. Signaling in *Dictyostelium* involves the

use of extracellular cues to coordinate changes in cell behavior, cell fate, and cell-cell communication. For example, in vegetative, growing cells, extracellular folate stimulates a chemotactic response, which is thought to be used for the hunting of bacteria. This response is GTP-sensitive and thought to be mediated through heterotrimeric G protein signaling (Parent *et al.*, 1999). Another series of G protein-mediated signals, initiated with cAMP as the extracellular cue is used during the aggregation process, to collect individual cells into the multicellular organism. Study of vegetative growth and early development signaling events has led to the identification of serpentine-type, G protein coupled receptors, heterotrimeric and low molecular weight G proteins, and effectors such as adenylyl cyclase, protein kinase A, and cAMP-dependent phosphodiesterases (Soderbom *et al.*, 1998). Molecular genetic studies of these signaling pathways have also led to the identification of a number of other well-known signaling components, including MAP kinase, phospholipase C, protein kinase B, and STAT proteins (Charles, 1999). This shows that in many cases the functions in *Dictyostelium* are similar to those described in mammalian cells.

In addition, *Dictyostelium* is a major contributor for understanding the amoeboid cell movement. Both *Dictyostelium* cells and leukocytes use G protein mediated signaling to regulate chemotaxis. The second messenger pathways seem to be similar, and Rho family GTPases are important in controlling chemotactic responses (Postma *et al*., 2003). *Dictyostelium* cells have long been a favorite for understanding the changing interactions of actin filaments and actin associated proteins during cell movement and cytokinesis. The ability to generate null mutants in cytoskeletal proteins by means of homologous recombination has allowed cell biologists to evaluate the role of myosin proteins and

other actin binding proteins in actin dynamics (Noegel and Schleicher, 2000). The use of green fluorescent protein (GFP) fusions proved to be a valuable tool for understanding the proteins involved in cytoskeletal activities and for revealing the spatial and temporal dynamics of those contributions.

Dictyostelum harbors an unexpectedly large number of small GTPases. The Ras and Rac proteins together with Rabs and Rans and several Arfs bring the number of small GTPases in *Dictyostelium* to more than in *Drosophila* and *C. elegans.* More importantly, the *Dictyostelium* genome and cDNA sequencing project offers a unique opportunity to explore the advantages of *Dictyostelium* to characterize the signal transduction pathways regulated by small GTPases, especially the Ras-superfamily GTPases. The *Dictyostelium* genome consists of 34 Mb carried on 6 chromosomes, plus a multicopy 90 kb extrachromosomal element that harbours the rRNA genes. The genome of *Dictyostelium* harbours 10,000 to 12,000 genes as estimated from the complete sequence of Chromosome 2 and many of the known genes show a high degree of sequence similarity to homologues in vertebrate species (Eichinger *et al.*, 2003). This information is complemented by sequencing of vegetative, developmental and sexual cDNA libraries, which has yielded a number of non-redundant ESTs (Morio *et al.*, 1998).

1.9 Goals of the project

The goal of the project was to identify and characterize new RapGAP genes in *D. discoideum*. Studies on *Dictyostelium* Rap1 have shown that this gene is important for reactions of the cytoskeleton (Rebstein *et al.*, 1997; Seaston *et al.*, 1999). Since GAP acts as a negative regulator of Rap, we were interested to find out if RapGAP1 has a similar

role in controlling the cytoskeleton and whether it is involved in other signaling cascades?

RapGAP1 was discovered by screening the sequences of the *D. Discoideum* database. The GAP domain exhibits 53% similarity to the corresponding human Rap1GAP domain. The major aim of the work was to characterize the cell biological and biochemical functions of RapGAP1 in detail, and to understand the signaling pathways of RapGAP1. To address these questions, we have undertaken a systematic analysis of the RapGAP1 gene. RapGAP1 null cells were generated by gene disruption. Several independent RapGAP1 null cells were isolated and studied. The cells were able to undergo normal development and to form fruiting body similar to that of wild type, although there was a slight delay of about three hours. Studies on cell motility showed that RapGAP1 null cells were faster than AX2 wild type suggesting that RapGAP1 plays a role in cytoskeletal dynamics.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Enzymes for molecular biology

Calf intestine alkaline phosphatase	Roche
DNA polymerase 1	Roche
Lysozyme	Sigma
Restriction enzymes	Amersham, Boehringer, Eurogentec, Gibco-BRL, New England Biolabs, Promega
RNase A	Sigma
T4 DNA ligase	Gibco-BRL, Promega
Taq polymerase	Amersham
Pfu Turbo polymerase	Stratagene

2.1.2 Antibodies

Anti-Actin (mAb Act1)	(Simpson *et al.*,1984)
MUD1 (mAb)	(Voet *et al.*, 1984)
Contact site Ab (mAb)	(Bertholdt *et al.*, 1985)

Goat anti-mouse IgG antibody (coupled with peroxidase)	Dianova
Goat anti-mouse IgG antibody (Conjugated with Cy3 or FITC)	Dianova

2.1.3 Protein inhibitors

Benzamidine	Sigma
PEFA-block	Roth
Phenylmethylsulfonylfluoride (PMSF)	Serva
Protease inhibitor cocktail (p2714)	Sigma

2.1.4 Antibiotics

Ampicillin	Roth
Blasticidin S	ICN Biomedicals
Kanamycin	Sigma
Penicillin/Streptomycin	Sigma

2.1.5 Chemical reagents

Unless otherwise stated, chemicals were obtained from Fluka, Merck, Pharmacia, Roth, Serva or Sigma and have the purity grade of “p.a”.

Agarose (SeaKem Me)	FMC Bioproducts
Bacto-agar, -peptone, -tryptone	Difco
Chloroform p.a	Riedel de Haen

DE52 (Diethylaminoethyl-cellulose)	Whatman
Hydroxylapatite	Bio-Rad
IPTG (Isopropyl-β-D-thiogalactose-pyranosid)	Gerbu
Oligonucleotides	MWG-biotech
Peptone	Oxoid
Phenol	Appligene
Phosphocellulose (P11)	Whatman
Proteose peptone	Oxoide
Triton X-100	Pierce
Yeast extract	Oxoide

2.1.6 Media

All media and buffers were made with deionized water and sterilized by autoclaving at 120^{o}C for 20 min. Antibiotics were added to media when cooled to about 50^{o}C.

2.1.6.1 Media for *D. discoideum* culture

AX medium (pH 6.7)

14.3 g	peptone
7.15 g	yeast extract
50 mM	glucose
3.5 mM	Na_2HPO_4
3.5 mM	KH_2PO_4

SM agar plates (pH 6.5)

9 g	agar
10 g	peptone
50 mM	glucose
1 g	yeast extract
4 mM	$MgSO_4$
16 mM	KH_2PO_4
5.7 mM	K_2HPO_4

-Both media were filled up to 1 liter with dH_2O.

Soerensen Phosphate buffer (pH 6.0)

14.6 mM KH_2PO_4

2 mM Na_2HPO_4

Salt solution

10 mM NaCl

10 mM KCl

2.7 mM $CaCl_2$

HL-5 medium

10 g yeast extract

50 mM glucose

8.5 mM KH_2PO_4

1.25 mM Na_2HPO_4

-filled up to 2 L with dH_2O

Phosphate agar plates (pH 6.0)

15 g Bacto agar

- filled up to 1 liter with Soerensen buffer

2.1.6.2 Medium for *E. coli* culture

LB-medium (pH 7.4)

10 g Bacto-tryptone

5 g yeast extract

86 mM NaCl

pH adjusted with NaOH and medium filled up to 1 L with dH_2O.

For LB-agar plates, 1.5% (w/v) agar added into the medium and selection was provided by introducing 50 mg/ml ampicillin and/or 25 mg/ml kanamycin.

2.1.7 Buffers and other solutions

Those not shown here will be described in their corresponding sections under Methods.

100x Denhardt's reagent

2%	Ficoll 400
2%	polyvinylpyrrolidon
2%	Bovin serum albumin

10 x NCP buffer (pH 8.0)

100 mM	Tris/HCl
1.5 M	NaCl
5 ml	Tween 20
0.02%	NaN_3

-Filled up to 1 L with dH_2O

PBS (pH 7.2)

70 mM	Na_2HPO_4
150 mM	NaCl
30 mM	KH_2PO_4
2.7 mM	KCl

-Autoclave

TE buffer (pH 8.0)

10 mM	Tris/HCl
1 mM	EDTA

-autoclave

10 x TEB buffer (pH 8.3)

890 mM	Tris
890 mM	Boric acid
20 mM	EDTA

-autoclave

Tris-Phenol (pH 8.0)

-1 vol. melted phenol was equilibrated with 1 vol. 1 M tris/HCl, pH 8.0

2.1.8 Equipment

Axiophot microscope	Zeiss
CCD camera (C5985-10)	Hamamatsu
Conductivity meter (LF 537)	WTW
Confocal laser scanning microscope (LSM 510)	Zeiss

Diavert inverse microcscope (Axiovert 200)	Zeiss
Digital color video CCD camera (TK-C1380)	JVC
Dounce homogenizer	Braun
Eagle Eye II	Stratagene
Electroporation apparatus	BioRad
Fluorescence spectrophotometer (Aminco Bowman)	Sopra
FPLC device (BioLogic)	BioRad
Nuclepore filter	Costar
Parr bomb	Parr Instrument Company
PCR thermal cycler	Biometra
PH meter	Knick
Protein fraction collector	Pharmacia
Rotary shaker	GFL
Semi-dry protein transfer Trans-Blot SD	BioRad
SMART system	Pharmacia
Spectrophotometer	Pharmacia
Speed-Vac concentrator	Bachhofer
Stereomicroscope (MZ12)	Leica
Ultrafiltration centricon	Amicon
Vortex	Bender & Hobein

Water baths	GFL
Balances	Sartorius
X-ray film developing machine (Curix 60)	AGFA

2.1.9 Other materials

2.2 ml sterile tubes for freezing of cells	Nunc
3 MM filter paper	Whatman
4-well borosilicate glass chamber slides	Nunc
24-well plates	Costar
Dialysis membrane	Biomol
Eppendorf tubes (0.1 ml, 0.5 ml, 1.5 ml)	Eppendorf
Falcon centrifuge tubes (15ml, 30ml)	Falcon
Nitrocellulose membranes (BA85)	Schleicher & Schuell
Petri dishes	Greiner
Polaroid film (667)	Polaroid
Polyallomer ultracentrifuge tubes 1.5 ml	Beckman
Quartz cuvettes	Hema
Sterile filters (0.22 μm, 0.45 μm)	Millipore
Tissue culture flasks	Nunc
X-ray films (X-Omat)	Kodak

2.1.10 Centrifuges and rotors

Centrifuges

J2-21 M/E	Beckman

J6-HC	Beckman
G6-SKR	Beckman
Optima LE-80K ultracentrifuge	Beckman
Optima TL 100 ultracentrifuge	Beckman
Table-top centrifuge (5415)	Eppendorf

Rotors

JA 14, JA 20, JS-4.2, Ti45, Ti 70, TLA 100.3	Beckman

2.1.11 Computer programmes

The laboratory was equipped with the latest version of several of routine software from major software vendors. Special software used in this work was

Husar	for sequence analysis
3 Dias	for quantitave analysis of cell migration.

2.2 Vectors and strains

2.2.1 Vectors

The following vectors were used: pMalC_2-vector (New England Biolabs), PQE30 (Qiagen), pLPBLP-vector (Faix *et al.).*

2.2.2 Bacterial strains

The following *Escherichia coli* strains were used: XL-1 Blue for cloning (Sambrook *et al.*, 1989), BL21 and M15 for protein expression.

2.2.3 Cultivation of *E.coli*

Bacteria were cultivated according to standard methods (Sambrook. *et al*., 1989) on agar plates or in liquid culture (240rpm). The cultivation temperature was 37^{o}C. For long-term storage, 930 µl of bacterial culture were mixed with 70 µl DMSO and stored at –80^{o}C.

2.2.4 *D. discoideum* strains

The wild type *D. discoideum* strain AX2, which can be cultivated under axenic conditions (Raper, 1935), was used for this study.

2.2.5 Cultivation of *D. discoideum*

2.2.5.1 Growth in liquid medium (Claviez *et al*., 1982)

From spores or bacterial lawns on SM-agar plates, the wild type AX2 strains were inoculated into AX or HL-5 medium containing the antibiotics streptomycin sulfate (400 µg/ml) in order to be free of the containing *K. aerogenes*. The generation time at 21^{o}C and 150 rpm is about 10 hours. For large-scale generation of cells for protein purification, cells cultivated in 4 x 2.5 l cultures up to a density between 5 x 10^{6} and 1.2 x 10^{7} cells/ml were harvested after centrifugation giving normally a yield of about 100g wet weights. As for cell biological studies, cells were allowed to grow up to a maximum density of 5 x 10^{6} cells/ml to avoid the stationary phase.

2.2.5.2 Growth on agar plates

Isolation of transformant clones was carried out on SM agar plates. 100 µl of a suspension of *K. aerogenes* in salt solution were placed on each agar plate, spread evenly together with the *Dictyostelium* cells, and the plates were kept at 21^{o}C for about 2 days. The doubling time is around 3-4 h and the *Dictyostelium* colonies appeared as round clearings on the bacteria lawn.

2.2.5.3 Development of *D. discoideum*

Development in Dictyostelium is induced by starvation. Cells grown to a density of 2-3 x 10^6 cells/ml were pelleted by centrifugation at 2,000 rpm for 2 min at 4^{o}C and were washed two times in an equal volume of cold Soerensen phosphate buffer in order to remove all the nutrition present in the culture medium. 5 x 10^7 cells/ml were then resuspended in 3ml Soerensen phosphate buffer and evenly distributed onto phosphate-buffer agar plates (90 mm) or water agar plates (90 mm). The plates were air-dried and any excess liquid was carefully aspirated without disturbing the cell layer. The plates were then incubated at 21 ^{0}C.Different stages of development were observed and the images were captured at indicated time points. For development in suspension culture, the cells were resuspended in Sorensen phosphate buffer at a density of 1 x 10^7 cells/ml and were shaken at 160 rpm at 21^{0}C for desired time periods.

2.2.5.4 Preservation of spores

Cells from the axenic culture were harvested by centrifugation at 300 g for 10 min, washed once with cold Sorensen buffer and resuspended at a cell density of 2 x 10^8 cells/ ml. 500 µl of the cell suspension were spread out onto each phosphate agar plate, and the cells were able to develop into fruiting bodies within 2-3 days. The spores were then harvested by knocking onto the lid of the petri dish and taken up in 10 ml of cold Sorensen buffer. 1 ml aliquots were dispensed into Nunc tubes (2.2 ml), shock-frozen in liquid nitrogen and stored at -70^{o}C. For inoculation with spores, an aliquot was thawed at room temperature and cultured in AX medium, whereby after 3 days at 21^{o}C and 150 rpm, a cell density of about 5 x 10^6 cells/ml was reached

2.3 DNA methods

2.3.1 Agarose gel electrophoresis

The separation of DNA fragments according to length was done in gels with 1% agarose I X TBE buffer. For the detection of DNA fragments, 0.05 μg/ml ethidium bromide was added to the liquid agarose. Gels were run with 50-200 V. Probes were incubated with 1/6 (v/v) loading dye before loading. After separation, the fragments were detected with a UV illuminator and documented with the Eagle Eye CCD camera system (Stratagene, Heidelberg).

DNA sample buffer

40%	Sucrose
0.5%	SDS
0.25%	Bromophenol blue

-taken up in TE buffer

2.3.2 DNA extraction from agarose gels

DNA bands were cut out of the gel with a scalpel and transferred into a sterile micro tube. The isolation procedure was performed with the DNA extraction kit (Qiagen) according to the manufacturer's manual.

2.3.3 Determination of DNA concentration

DNA concentration in agarose gels was estimated by comparing the intensity of the band with the bands of the molecular weight marker. Alternatively, the OD_{260} was measured (50 μg/ml of DNA have an OD_{260} of 1, Sambrook *et al*., 1989).

2.3.4 Preparation of plasmid DNA

2.3.4.1 Isolation of plasmid DNA by the method of Homes and Quigley (1981)

An inoculum of bacteria was taken from a streak culture and suspended in 200µl of STET/lysozyme buffer. Alternatively, 1 ml of bacteria from a fresh overnight shaking culture was harvested using a table-top centrifuge (5000 rpm, 1 min, RT), the supernatant discarded and the pellet resuspended in STET buffer. The suspension was boiled in a water bath for 1 min, centrifuged (14,000 rpm, 10 min, RT), and the insoluble cell debris removed using a sterile toothpick. The nucleic acids in the supernatant were precipitated with 200 µl of isopropanol for 5 min at RT and sedimented using a table-top centrifuge (14,000 rpm, 30 min, RT). The DNA pellet was then washed with 70% ethanol, vacuum-dried with a Speed-vac concentrator, and dissolved in 30 µl of TE buffer.

STET buffer (pH 8.0)

50 mM	Tris/HCl
50 mM	EDTA
5%	Triton-X-100
8%	Sucrose
1 mg/ml	lysozyme (added prior to usage)

2.3.4.2 Isolation of plasmid DNA by the method of Qiagen

Large-scale preparation of plasmid DNA from *E. coli* was carried out with the Midi-kit purchased from the Qiagen company. Bacteria were cultivated in 150 ml of LB medium containing 50 µg/ml ampicillin overnight at 37^{o}C with agitation. The cells were harvested by centrifugation (10,000 x g, 5 min, 4^{o}C) and resuspended in 10 ml of buffer P1. For cell

lysis, 10 ml of buffer P2 was introduced and the resultant mixture was gently mixed by inverting the tube several times. After incubation for 5 min at RT, 10 ml of chilled buffer P3 was added, the suspension mixed gently by inverting tube, and then incubated on ice for 15 min. Following centrifugation (35,000 g, 30 min, 4^{o}C), the sediment of proteins and cell debris was discarded, while the supernatant containing the plasmid DNA was centrifuged for a further period of 15 min. The clear supernatant was passed through a Qiagen tip 100 columns previously equilibrated with 5 ml of buffer QBT. After washing twice with 10 ml each of buffer QC, the plasmid DNA was eluted with 5 ml of buffer QF, precipitated with 0.7 vol. of isopropanol, spun down (30,000 g, 30 min, 4^{o}C), and the DNA pellet washed with 70% ice-cold ethanol, air-dried and dissolved in 200 μl of TE buffer.

The DNA concentration was determined using a spectrophotometer whereby an OD^{260} of 1.0 corresponds to 50 μg of DNA. The ratio of OD^{260}/OD^{280} should be between 1.8 and 2.0.

2.3.4.3 Phenol extraction and precipitation of DNA

To separate the DNA from contaminating proteins, the DNA solution was mixed with 50% (v/v) phenol and 50% (v/v) chloroform/isoamylalcohol (24:1). The mixture was heavily vortexed and centrifuged at 13,000 rpm at room temperature for 5 min. The upper phase was removed and the DNA was precipitated with ethanol. For ethanol precipitation, 10% (v/v) of a 3 M Na-acetate solution of pH 5.2 as well as 2.5 volumes of 100% ethanol were added to the DNA solution. It was then incubated at –20^{o}C for 1 hour. After a centrifugation of 30 min at 13,000 rpm and 4^{o}C, the pellet was washed with 70%

ethanol, centrifuged again for 15 min under the previous conditions and dried in a Speed Vac. The dried pellet was resuspended in a suitable volume of H_2O.

2.3.5 DNA cleavage with restriction enzymes

The cleavage was performed in a volume of 50 µl. The buffer suitable for the restriction enzyme(s) was added and the volume adjusted with H_2O. After addition of the enzyme(s), the tube was incubated at the appropriate temperature for at least 1h. The digest was analysed on an agarose gel.

2.3.6 Ligation of DNA into a plasmid vector

To avoid re-ligation and concatamer formation of a cut vector, the vector was dephosphorylated with alkaline phosphatase (CIP, Calf Intestinal Phosphatase). The reaction was performed according to manufacturer's instructions (New England Biolabs). The vector was purified on a gel and incubated with the corresponding insert. The reaction volume was set to 10 µl. 50 ng of vector were ligated with a threefold molar excess of insert. Ligases of various suppliers were used (Gibco, Roch, NEB) together with the supplied buffers. After the ligation, the DNA was extracted with phenol, precipitated with ethanol and resuspended in 5-10 µl of H_20.

2.3.7 Preparation of electroporation competent cells

1 liter LB was inoculated with 10 ml of an *E. coli* overnight culture and grown to an OD_{600} of 0.6 at 37^{o}C under vigorous shaking. All flasks and solutions subsequently used were sterilized and cooled to 4^{o}C. Proper cooling was essential for obtaining competent cells of a good quality. Cells were spun down at 3000x g for 15 min and resuspended in1 l of H_20. After another centrifugation, the cells were resuspended in 500 ml of H_2O,

pelleted again, washed with 20 ml of 10% glycerol and finally resuspended in 3 ml of 10% glycerol. The cells were stored at -80^{o}C in 50 µl aliquots.

2.3.8 Electroporation of *E. coli*

For transformation, electroporation competent *E. coli* cells were thawed on ice. 50 µl of cells were mixed with 1-5 µg of DNA or the ligation mixture resolubilized in dH_2O, and placed in a pre-chilled electroporation cuvatte (Eurogentec; 2 mm gap between electrodes). After a pulse of 2.5 kV, 200 Ω and 25 µF, 1 ml of SOC medium (Sambrook *et al.*, 1989) was immediately added and the cells regenerated at 37^{o}C for 45 min with agitation. 1, 10 and 100 µl of cells were plated out on LB agar plates containing 50 µg/ml of ampicillin or 25 µg/ml of kanamycin and incubated overnight at 37^{o}C.

SOC medium

2%	Bacto-tryptone
0.5%	Yeast extract
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$
20 mM	glucose

2.3.9 Screening for positive *E.coli* transformants

To isolate *E. coli* colonies carrying the desired DNA fragment, colonies were selected and mini preparations of plasmid DNA were performed followed by restriction analysis

to determine the correct orientation of the DNA insert. Finally the authenticity of the DNA sequence was verified by sequencing (Toplab)

2.3.10 *E. coli* permanent cultures

Important transformants were preserved as permanent cultures. An inoculum of bacteria was resuspended in 1 ml of LB medium containing 7% of DMSO. The cells were shock frozen in liquid nitrogen and stored at -70^{o}C.

2.3.11 Transformation of *D. discoideum*

Recombinant gene expression in *D. discoideum* was regulated by a plasmid harbouring the *Dictyostelium* actin 6 promoter (A6P) and actin 6 terminator (A6T) sequences. The presence of an appropriate antibiotic resistance cassette on the expression plasmid allowed for selection of transformants with or blasticidin.

For transformation, *Dictyostelium* cells cultivated to a density of 2-3 x 10^6 cells/ml were harvested by centrifugation (300 g, 5 min, 4^{o}C), washed once with cold Soerensen buffer, followed by washing twice with ice-cold electroporation buffer and the cells finally resuspended in chilled electroporation buffer at a density of 1 x 10^8 cells/ml. 500 µl of the cell suspension were mixed with 15-30 µg of the desired plasmid DNA in a pre-chilled electroporation cuvette (4 mm gap between electrodes). After a pulse at 1.0 kV and 3 µF with the aid of an electroporation device (Gene Pulser, Biorad), the cells were immediately transferred to a petri dish and incubated at RT for 10 min, after which $CaCl_2$ and $MgCl_2$ were added to an end concentration of 1 mM each and the cells incubated for an additional 15 min at RT with gentle agitation. Finally, the cells were diluted with HL-5 medium to a density of 1 x 10^6 cells/ml and allowed to recover at 21^{o}C for 24 h before selection pressure was added.

Electroporation buffer (pH 6.1)

50 mM sucrose

10 mM K_2HPO_4

pH was adjusted with KOH solution, and the buffer sterilized by filtration

2.3.12 Polymerase chain reaction (PCR)

The amplification of DNA fragments was carried out by "polymerase chain reaction". For PCR (colony screening), *Taq* polymerase (Boehringer) and Pfu Turbo polymerase (Boehringer) were used. The reactions contained 2 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM 5'- and 3'- primer and 1/250 vol of Taq-polymerase. cDNA, genomic DNA and plasmid DNA were used as templates. Prior to amplification, the DNA was denaturated for 5 min at 95^{o}C. The denaturation was followed by 25 amplification cycles: 95^{o}C, 1 min; 50-65^{o}C (depending on the annealing temperature of the primers used), 1 min; 72^{o}C 1 min-3 min (depending on the length of the fragment to be amplified). The last elongation step; 72^{o}C, 3 min. Based on the number of guanine and pyrimidine nucleotides, the annealing temperature ™ of a particular oligonucleotide primer could be calculated according to the formula of Suggs *et al*. (1981): 4(G+C) +2(A+T) – 10 = Tm (^{o}C). Preparative PCR was carried out with the Expand high fidelity PCR system from Boehringer Mannheim. The reactions were carried out according to the supplier's instructions.

2.3.13 Purification of PCR products

For cloning purposes, the PCR products were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. Buffers used were provided by the kit and all centrifugation steps were done at 14,000 rpm at RT using a table-top microcentrifuge. 5 vol. PB buffer were added to 1 vol. of the PCR reaction and

mixed. The sample was applied to a QIAquick spin column and centrifuged for 1 min to bind DNA to the column while the flow-through collected in 2 ml collection tube was discarded. DNA was washed with 0.75 ml of PE buffer by centrifuging for 1 min. Residual ethanol was removed by centrifuging for an extra 1 min. The spin column was then placed in a clean 1.5 ml appendorf tube and the DNA eluted by the addition of 50 µl of dH_2O to the column and centrifuging for 1 min. The purified DNA was subsequently used for restriction digest for 2-6 h and finally purified from agarose gel with the aid of the QIAquick gel extraction kit from Qiagen.

2.3.14 Oligonucleotides (Primers)

PH-07F	5'- GGG GGA TCC CAG TCA GAA TTA TTA ATC GG-3'
PH-07R	5'- GGG AAG CTT ATA TGC TTT TTC ATC AGT TG
Rap-07F	5'-GGG GGT ACC GGA CCA TTG GCA TTC AGT TTG -3'
Rap-07R	5'- GGG GCT GCA GTT ATA AAA ATT GAG AAA TAT AAT
PH-07-koF	5'-GGG GTA CCG CAA ACA CAA ATA ATG GAT TAT CAG AT-3'
PH-07-koR	5'-CCCAAGCTTCCAATCCAGTTTATCTTTATTTGAATCGA-3'
Rap-07-koF	5'-AAC TGC AGG CTT CAA ATC AAT CAG AGT TAC AA-3'
Rap-07-koR	5,-CGG GAT CCT AAA GAT TCT CTA GTT CTA GTA AT-3'
KO scr 1	5'-TTG AAA CAC GAC CTT GAG AAG GTA-3'
KO scr 2	5'- GGTCGTGTTTCAA-3'

PH-07F/PH-07R: Used for protein expression, N-terminals of RapGAP1 gene with PH domain.

Rap-07F/Rap-07R: Used for protein expression, C-terminals of RapGAP1 gene with RapGAP domain.

PH-07-KOF/ PH-07-koR : Used for knocking out the RapGAP1 gene.

Rap-07-KOF/ Rap-07-koR: Used for knocking out RapGAP1 gene.

KO scr1: Used for screening RapGAP1 mutant.

KO scr2: Used for screening RapGAP1 mutant.

2.3.15 Southern Blotting

Dictyostelium genomic DNA was digested with various restriction enzymes and separated on 0.7% agarose gel (30 V, for 24 h). DNA was denatured in 0.5 M NaOH for 20 min and gel was rinsed with H_2O briefly. Gel was submerged in neutralization solution (1 M Tris, pH 7.5 for 20 min) and equilibrated with 20 x SSC buffer for 20 min. DNA was blotted from the gel by capillary transfer to the membrane (nitrocellulose filter; Schleicher & Schuell) using 20 x SSC buffer. The blotted membrane was rinsed with 6 x SSC buffer and baked at 80°C for 1.5 h. The membrane was rinsed with hybridization buffer and hybridized with ^{32}P-labelled sample overnight. The membrane was rinsed 3 times with 2 x SSC/ 0.1% SDS- buffer at 37°C for 10 min and rinsed again with wash buffer at 37°C for 1 h.

<u>20 X SSC (pH 7.5)</u>

3 M NaCl

0.4% Na-citrate

2.4 Analysis of RapGAP1 by biochemical methods

2.4.1 SDS-polyacrylamide gel electrophorosis (SDS-PAGE)

Protein mixtures were separated by discontinuous SDS-PAGE (Laemmli, 1970). For this purpose, 10-15% resolving gels with 3% stacking gels were used (7.5 x 10 x 0.05 cm). The stacking gel deposits the polypeptides to the same starting level at the surface of the resolving gel, and subsequently the SDS-polypeptides complexes are separated in the resolving gel according to size under uniform voltage and pH. Prior to SDS-PAGE, 1/3 vol. 3 x SDS gel loading buffer was added to the protein samples to be separated and boiled for 3 min. Electrophoresis was carried out at a constant voltage of 150 V, after which the gel could be stained with Coomassie Blue dye and destained for direct observation of the protein bands, or protein from the gel could be blotted onto a nitrocellulose membranes and detected indirectly via antibodies. As standard, a mixture of proteins of defined molecular masses was electrophoresed.

Stacking gel

125 mM	Tris/glycine (pH6.8)
0.1%	SDS
3.3%	acrylamide:bisacrylamide (30:0.8) per 16 ml:
240µl	20% ammonium persulfate (APS)
8 µl	TEMED

Resolving gel

380 mM	Tris/glycine (pH 8.8)
0.1%	SDS

10-15% acrylamide:bisacrylamide (30:0.8)

per 42 ml :

480 μl 20% APS

12 μl TEMED

10x SDS-PAGE running buffers (pH 8.3)

250 mM Tris

1.9 M Glycine

1% SDS

3xSDS gel loading buffer

150 mM Tris/HCl (pH 6.8)

30% Glycerol

6% SDS

15% β-mercaptoethanol

0.3% bromophenol blue

2.4.2 Coomassie blue staining of proteins

Following SDS-PAGE, gels were stained in Coomassie Blue solution for at least 30 min with agitation, after which the unbound dye was removed by shaking in a destaining solution.

Coomassie Blue solution

0.1% Coomassie Brillant Blue R250

50% methanol

12% glacial acetic acid

Solution filtered via a Whatman filter

Destaining solution

10% ethanol

7% glacial acetic acid

2.4.3 Drying of SDS-PAGE gels

For permanent recording, SDS-polyacrylamide gels after Coomassie Blue staining were washed in water with agitation with a couple of changes of water to remove the destaining solution. The gels were then shaken in a drying solution for 20 min, after which each gel was placed between 2 dialysis membranes pre-wetted in the drying solution and then air-dried overnight.

Drying solution for polyacrylamide gels

24% ethanol

5% glycerol

2.4.4 Western blotting

Following separation of protein by SDS-PAGE, the proteins were transferred from gels onto nitrocellulose membranes (Schleicher & Schuell BA85) according to the modified protocol of Towbin *et al.* (1979) with the aid of a protein transfer apparatus (Trans-Blot SD, BioRad).

In this "semi-dry" method, the gel and its attached nitrocellulose filter were sandwiched between pieces of Whatman 3MM filter paper which had been soaked in transfer buffer and protein transfer was carried out at RT at 12 V for 40 min, after which the nitrocellulose filter was blocked overnight at 4^{o}C in 5% (w/v) milk powder in 1 x NCP buffer (without sodium azide) and incubated with primary antibody (polyclonal antibody

raised against the RapGAP domain of the RapGAP1 gene, monoclonal antibody against contact site A and perspore-specific antibody(mud1) 1:1000,1:100 in NCP buffer) for 4 h. After washing several times with NCP buffer, the membrane was incubated with a secondary antibody (diluted 1:10,000 in NCP buffer) for 1h. For the experimental purposes, goat anti-mouse IgG conjugated with peroxidase (Dianva) was used as the secondary antibody. Finally, the membrane was washed a few times with NCP buffer and protein bands were detected via chemiluminescence, by incubating the membrane in enhanced chemiluminescence reagents (ECL, Amersham) for 1 min, and then exposed to X-ray films (X-omat AR5, Kodak).

Transfer buffer

25 mM	Tris/HCl (pH 8.5)
190 mM	glycine
20%	methanol
0.02%	SDS

20x NCP buffer (pH 7.2)

48.4 g	Tris
348 g	NaCl
20 ml	Tween 20

-Filled up to 2 l with dH_2O.

Chemiluminescence reagents (light sensitive)

200 µl	Luminol (250 mM in DMSO; Luminol: 3-amino-phthalazinedione)
89 µl	p-Coumaric acid (90 mM in DMSO)
2 ml	Tris/HCl (1M, pH 8.5)

-Filled up to 20 ml with dH_2O.

2.4.5 Bradford assay

Protein quantification was performed according to the method of Bradford (1976). In principle, the Coomassie brilliant blue G250 dye interacts primarily with arginine residues of proteins and causes a shift in the absorbance peak from 465 nm to 595 nm. Bovine serum albumin was used to make a standard colour response curve from which the concentration of protein samples could be determined.

2.4.6 Protein purification

2.4.6.1 Purification of histidine-tagged constructs

Proteins with an affinity tag of six consecutive histidine residues (the 6x His-tag protein) were purified using the Ni-NTA system (nickel-nitrilotriacetic acid, Qiagen). The 6x His tag is much smaller (0.84 kDa) than most other affinity tags and is uncharged at physiological pH. Qiagen Ni-NTA products use the four chelating sites of NTA to bind nickel ions more tightly than alternative metal-chelating purification systems that have only three sites available for interaction with metal ions. The presence of an additional chelation site prevents nickel-ion leaching resulting in greater binding capacity, and also minimizes non-specific binding leading to purer protein preparations. The RapGAP domain of the RapGAP1 gene was amplified by PCR using genomic DNA as template and digested with KpnI and PstI in the first construct, and Asp718, Sal 1 was used for the second construct. These were then inserted into the pQE30 vector (Qiagen). The His-tagged fusion protein was expressed in M15 cells. For large prepration, 2 L LB-medium were inoculated with a stationary 10 ml culture of the constructs, the bacteria was grown

for another 2-4 h at 37^{o}C and 240 rpm and induced with 0.1mM IPTG. Expression was carried out for 4 h at 37^{o}C and 240 rpm. Cells were harvested (10 min, 50000 rpm, Sorvall rotor GSA, 4^{o}C). Recombinant proteins were purified according to the Qiagen protocol.

2.4.6.2 Purification of maltose-binding-protein (MBP) fusion constructs

In this protein fusion and purification system, the cloned gene is inserted into a pMAL vector (NEB) downstream from the malE-gene, which encodes maltose-binding protein (MBP). This results in the expression of an MBP-fusion protein. The technique uses the strong tac-promoter and the translation initiation signals of MBP to express large amounts of the fusion protein. The fusion protein is then purified by one-step affinity purification for MBP. pMAL-c2 vector has an exact deletion of the malE signal sequence, resulting in cytoplasmic expression of the fusion protein. Between the malE sequence and the polylinker is a spacer sequence coding for 10 asparagine residues. This spacer insulates MBP from the protein of interest, increasing the chances that a particular fusion will bind tightly to the amylose resin.

The 3’ region of the RapGAP1 gene was amplified using primer PH-07 and cut with BamHI, HindIII inserted into the pMAL-c2 vector (NEB), and expressed in XL1 Blue cells.

2.4.7 Microscopy

2.4.7.1 Microscopy of cells and colonies on agar plates

To determine development of *Dictyostelium* on phosphate agar plates Leica MZFIII stereo microscope were used. Images were processed using Adobe Photoshop.

2.4.7.2 Microscopy for analysis of cell motility

To analyze the cell motility of mutant cells, one-drop of mutant and wild type cells were taken from a 2x 10^6cells/ml stock culture and placed on a clean glass coverslip with 0.5ml of fresh media. Cell migration was recorded at intervals of 10s by using an Axiovert-200 inverted microscope and the axioision software (Carl Zeiss, Jena, Germany). The time laps movies were analyzed with the DIAS program (Solltech, Oakdale, IA; Wessels *et al.,* 1998).

2.4.8 RT-PCR

For performing RT-PCR total RNA was isolated using the Qiagen RNeasy mini kit. Both reverse transcription and PCR where carried out sequentially in the same tube. RT-PCR reactions were performed in 50 µl according to the manufactures instruction (Qiagen one step RT-PCR kit)

Reaction components:

RNA from *Dictyostelium discoideum*	2 µg
5x Qiagen onestep RT-PCR buffer	1x
dNTP mix (containing 10 mM of each dNTP)	400µM of each dNTP
Primer 1 (5'-TCTGATTTGATTCACTTA-3')	1.0 µM
Primer 2 (5'- TCGATTCAAATAAAGATAAACTG-3')	1.0 µM
Qiagen one-step RT-PCR enzyme mix	2.0µl

Reverse transcription was carried out at: 50°C for 30 min followed by an initial PCR activation step: 95°C for 15 min. Amplification was as following: 94°C for 1 min; 54°C for 1 min; and 72°C for 1 min; and the final extension at 72°C for 10 min.

CHAPTER 3

RESULTS

3.1 Molecular characterization of RapGAP1

3.1.1 Sequence analysis

The RapGAP1 gene was discovered by searching the *D. discoideum* databases for proteins which contain PH domains. PH domains are found in many proteins that are involved in intracellular signaling and in anchoring proteins to membranes. It consists usually of about 100 amino acid residues, and is also suggested to play a role in accumulating GTPase activating proteins (GAPs) at specific cellular sites of phosphoinositide production (reviewed in Donovan *et al*, 2002)

The gene coding for RapGAP1 is present on chromosome two and has in the most recent database version (5/2005) the preliminary gene number DDB0166986.

Experimental analysis of the genome structure of RapGAP1 showed that the gene is 3.6 Kb in length, it does not contain introns and encodes a protein of 1212 amino acids with a predicted molecular mass of 132 kDa (Fig. 3). From the sequence analysis of chromosome 2 of *D.discoideum* it was clear that *Dictyostelium* genes are rich in A+T and repetitive elements. This was also the case for the RapGAP1 gene with an A+T content of more than 70%. The RapGAP1 cDNA starts with ATG, preceded by the 4 nucleotides AACC and ends with the characteristic TAA stop codon. Motif searches and structural analysis predicted a PH domain at the N terminus (aa 210-306) of the protein and a RapGAP domain (aa 1029-1208) towards the end of the open reading frame.

```
1    ATGAAAATTAAATTATTTATTAAAAAAAAAAAAAAATTTAATTATAATCATCGACATTAT
     M  K  I  K  L  F  I  K  K  K  K  K  F  N  Y  N  H  R  H  Y
61   ATTATCATCATCAACAACAACAACAACAACAACAACAACAACAAATACAACAACCACAA
     Y  Y  H  H  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  I  Q  Q  P  Q
121  ATACAACAACAACAAATACAACAAAAATTACAGTCAGAATTATTAATCGGTATTAATGGA
     I  Q  Q  Q  Q  I  Q  Q  K  L  Q  S  E  L  L  I  G  I  N  G
181  CATATGAAAAAGATAACGTTAAAATTAAAGAATAATTTAGAAGAGGATCAATATTCAAAT
     H  M  K  K  I  T  L  K  L  K  N  N  L  E  E  D  Q  Y  S  N
241  TGTGTAAAATATGGGTGGATTGAGAAAAGATGTGGTAAAAATCAATCATTTAGTTCATGG
     C  V  K  Y  G  W  I  E  K  R  C  G  K  N  Q  S  F  S  S  W
301  AAAAGAATGTGGTTTATATTAAAAGATAGTAAACTTACATATTATATTAAAGAAAAGAAT
     K  R  M  W  F  I  L  K  D  S  K  L  T  Y  Y  I  K  E  K  N
361  TTAAAGAAAAGATCATCAACAATTGGTAGTACTCAAGAATTTTTTAAACAACAACATGGA
     L  K  K  R  S  S  T  I  G  S  T  Q  E  F  F  K  Q  Q  H  G
421  ATAGATAATAATAATTGTACAAATAGTAATAGTAATAATAATAATAATAACTCTGATTTG
     I  D  N  N  N  C  T  N  S  N  S  N  N  N  N  N  N  S  D  L
481  ATTCACTTATCAGCACCTTCATTATCATCATCAACATCATCAACAATATCACCAATAAGC
     I  H  L  S  A  P  S  L  S  S  S  T  S  S  T  I  S  P  I  S
541  TCATCATCATCATTAACAACTACAACAACTACTACAACAACAACTACAAATGCAAACACA
     S  S  S  S  L  T  T  T  T  T  T  T  T  T  T  T  N  A  N  T
601  AATAATGGATTATCAGATTCAATAAATCATGTATATTTAGAAGGTGAACTTGGTAAAAAG
     N  N  G  L  S  D  S  I  N  H  V  Y  L  E  G  E  L  G  K  K
661  AAAGAAGGTAAAGGATGGAAAGTTAGATGGATGAAACTATTGGAACATTCATTAGTTTAT
     K  E  G  K  G  W  K  V  R  W  M  K  L  L  E  H  S  L  V  Y
721  TATAAATCATCAAAAGATAAAGAACCATTGGGTATAGTTAATTTGAATGAGTGTCAAGAT
     Y  K  S  S  K  D  K  E  P  L  G  I  V  N  L  N  E  C  Q  D
781  TGTGAAGTTAATAAAGAATCATCGAATAAATTCGTTGTTGTACATTCAAGTAATCGATAC
     C  E  V  N  K  E  S  S  N  K  F  V  V  V  H  S  S  N  R  Y
841  TATTTTAAAACCAATACAAAACAAGAACTCACTCAATGGGTTAAATCCGTTAAATCTAGA
     Y  F  K  T  N  T  K  Q  E  L  T  Q  W  V  K  S  V  K  S  R
901  ATACCTTCAATCAATCAAACAAAGATGGATTTAGATCAATTCCAATTAAAAGGTGTCATA
     I  P  S  I  N  Q  T  K  M  D  L  D  Q  F  Q  L  K  G  V  I
961  GAATTTAATAGTATACAACAAATCGCTGAAACTTTCAAATTTAATAGTCAACCAAATTGT
     E  F  N  S  I  Q  Q  I  A  E  T  F  K  F  N  S  Q  P  N  C
1021 CTAACAATTACAACTGATGAAAAAGCATATTATCTATCATTCGATTCAAATAAAGATAAA
     L  T  I  T  T  D  E  K  A  Y  Y  L  S  F  D  S  N  K  D  K
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1081 CTGGATTGGTTAAATCAATTGAATTTAACTATAAATAAATTTAAAGGTGGTTTCAATAGT
     L  D  W  L  N  Q  L  N  L  T  I  N  K  F  K  G  G  F  N  S
1141 GGTGGTGGTTCAAATAATTCTTCACCATCATCTTTACAATCACAACAAGCTAAGGAGAGT
     G  G  G  S  N  N  S  S  P  S  S  L  Q  S  Q  Q  A  K  E  S
1201 GGTAATGGTGGTAGTTTAAGTCCAAATATACTTGGTAACTTTAAATCATCTATTAAACCA
     G  N  G  G  S  L  S  P  N  I  L  G  N  F  K  S  S  I  K  P
1261 TGGAGATTCTCATCTTCACCTTCTCAAGGTCGTGTTTCAATTGGAGGTGGTGGTGATAGA
     W  R  F  S  S  S  P  S  Q  G  R  V  S  I  G  G  G  G  D  R
1321 TCATCAACTCAAGTTAAATTTGTTGATAATCCTTTATCAAAAAGTTCAAATAAAGAGGAG
     S  S  T  Q  V  K  F  V  D  N  P  L  S  K  S  S  N  K  E  E
1381 TTTGATGGTGGTGAATATGGTTCACAAATTTTACCAAGAAAATCAACAATCATTGGTCCA
     F  D  G  G  E  Y  G  S  Q  I  L  P  R  K  S  T  I  I  G  P
1441 AATGGTGGTGTTAAAGTTAGTACAAGTGGTGCTGTTGAATTATCACCAGTTTTAATTTCA
     N  G  G  V  K  V  S  T  S  G  A  V  E  L  S  P  V  L  I  S
1501 AGTAATAATAACAATAATAATACAATAAGTTCAAGTGGTAATTCAATACCAACTTGTTTA
     S  N  N  N  N  N  N  T  I  S  S  S  G  N  S  I  P  T  C  L
1561 AGTGATTTAATTAATGAATCAAGTGATAATGAAGATGGTGATGATTTAAAGATGATGATA
     S  D  L  I  N  E  S  S  D  N  E  D  G  D  D  L  K  M  M  I
1621 CCAGAATCATTAAAGAATGAAATGATGAGAAGATCAGCAATTAGTTCATTGAAAAATTTT
     P  E  S  L  K  N  E  M  M  R  R  S  A  I  S  S  L  K  N  F
1681 AAGTTGGAAATTTTTATTTGGTCAAATCATCAAGAGGTTTTCACATTTCTTTTCTCTGAT
     K  L  E  I  F  I  W  S  N  H  Q  E  V  F  T  F  L  F  S  D
1741 TCAGTGTTGGTAGATCAAGTTAAAGCATTTGCTTTTAAAAAGATACCTTCATTAGCAAAT
     S  V  L  V  D  Q  V  K  A  F  A  F  K  K  I  P  S  L  A  N
1801 TTATCAGTATTGGATTATAGATTAGGTATTGATGAAGATACACTATTGGAAGTGGAATTC
     L  S  V  L  D  Y  R  L  G  I  D  E  D  T  L  L  E  V  E  F
1861 TTAAAGTTTATATATAGTCATACAATGGTTGAATTGGCATTAAAGACTTGTGGTATCGTA
     L  K  F  I  Y  S  H  T  M  V  E  L  A  L  K  T  C  G  I  V
1921 AAGATTGGTATTTTCCATCATCGTAAGGATAGAAGAGTTAAAGAGAAATTATATTCAGAT
     K  I  G  I  F  H  H  R  K  D  R  R  V  K  E  K  L  Y  S  D
1981 AAATTCTATGGTCATCTTTTATCACAATCACCAGATGGTCGTAAAGGTAATGGTGTTGGT
     K  F  Y  G  H  L  L  S  Q  S  P  D  G  R  K  G  N  G  V  G
2041 ATTGGTGGTAATGGTATACCTATTGAGTATTCAAGAAGTGAACCAAATCTTCAATCATGT
     I  G  G  N  G  I  P  I  E  Y  S  R  S  E  P  N  L  Q  S  C
2101 CTATCATCTTCACCTTCAACTAGAGAAACTATGGTACCATCTTCACCATCATCACATCAA
     L  S  S  S  P  S  T  R  E  T  M  V  P  S  S  P  S  S  H  Q
2161 TTAATAACACCACCACCATCATTAAAACAATATGAACAACAATTATCATCATCATCATCA
     L  I  T  P  P  P  S  L  K  Q  Y  E  Q  Q  L  S  S  S  S  S
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2221 TCATCGTCACAACAACTACAACTACAACTACAACAACAAGAACAAGAACAACTATTACAA
     S  S  S  Q  Q  L  Q  L  Q  L  Q  Q  Q  E  Q  E  Q  L  L  Q
2281 GAACAACCAGAAGCTGAACAATCACAACCAGAACCACAACCACAATTAGAACCAGAATCA
     E  Q  P  E  A  E  Q  S  Q  P  E  P  Q  P  Q  L  E  P  E  S
2341 GAAATAGAGATTGAAGAATTAGAACAACCAATTGAAGAACTATTGAATATTTCAACTACA
     E  I  E  I  E  E  L  E  Q  P  I  E  E  L  L  N  I  S  T  T
2401 CCAATATCAATACGTAGTAATTCAATTTTATCAAGTTCAACATCTGCATCATCATCACCA
     P  I  S  I  R  S  N  S  I  L  S  S  S  T  S  A  S  S  S  P
2461 TCTTCAACACCATCATTGACACCAGTAATTGAAAGACAACAAAAAATGTCAGCAGCAGGT
     S  S  T  P  S  L  T  P  V  I  E  R  Q  Q  K  M  S  A  A  G
2521 TGGCATAGTGTTAATCCAAGTGTAACATTGAATCAACGTAGACAATTGATTAGTGGTTGT
     W  H  S  V  N  P  S  V  T  L  N  Q  R  R  Q  L  I  S  G  C
2581 CCAGGTTGGAATATTGAAGTATCGAATCCAACTTCAACATTCACCAGTCAAGGTTTCAAA
     P  G  W  N  I  E  V  S  N  P  T  S  T  F  T  S  Q  G  F  K
2641 CCAAAATTCGATAAACAAGAGCATGGATTCTATAGACGTTATAATTTCGATGGTACAAGT
     P  K  F  D  K  Q  E  H  G  F  Y  R  R  Y  N  F  D  G  T  S
2701 ACGGTTCAAAGCTTTTTAGGTGTAGATATGAAAATGGGACCATTGGCATTCAGTTTGGCA
     T  V  Q  S  F  L  G  V  D  M  K  M  G  P  L  A  F  S  L  A
2761 AAAGATGCCAATGATAACTATCGTGGTGTACTTCATACAAAACATGGCGCAAAGACAATT
     K  D  A  N  D  N  Y  R  G  V  L  H  T  K  H  G  A  K  T  I
2821 AGTGAAGATTCTAAAAATATCGTTGGTATTTTAAATCTCCTTTCATTGAGTAAAAAAGTA
     S  E  D  S  K  N  I  V  G  I  L  N  L  L  S  L  S  K  K  V
2881 AAGACAAAGAAAGTGGTCTCCCATTTAATTGGATTATTGGATCCATCGATCGATGCAAAA
     K  T  K  K  V  V  S  H  L  I  G  L  L  D  P  S  I  D  A  K
2941 CTTTTAAATTTAGCTTCAAATCAATCAGAGTTACAAAAAGAACTCTTATCATTTGAGGAA
     L  L  N  L  A  S  N  Q  S  E  L  Q  K  E  L  L  S  F  E  E
3001 CGTCAAACCACAAGTGGTTTTAAATTTGGTATGGTCTATTGCAGACATGGTCAAGTCACA
     R  Q  T  T  S  G  F  K  F  G  M  V  Y  C  R  H  G  Q  V  T
3061 GACGATGAAATCTTCTCCAATAAACAAGGTAGTCCAGAGTGGGACGAGTTCTTATCATTG
     D  D  E  I  F  S  N  K  Q  G  S  P  E  W  D  E  F  L  S  L
3121 ATTGGTGACAAGATTGAATTGGTCGGTTGGCCACATTATAGTGCTGGTTTGGATGTTAAA
     I  G  D  K  I  E  L  V  G  W  P  H  Y  S  A  G  L  D  V  K
3181 TTCAATTCAACAGGTACTCACTCTCTATACACCGATTATCATGGAAATGAAGTTATGTTC
     F  N  S  T  G  T  H  S  L  Y  T  D  Y  H  G  N  E  V  M  F
3241 CATGTTTCAACAATGTTACCTTTCTCCACTACCGATTATCAACAAATTGAAAGAAAACGT
     H  V  S  T  M  L  P  F  S  T  T  D  Y  Q  Q  I  E  R  K  R
3301 CAAGTTGGCAATGATATTTGTGTTGTCATCTTTAATGATGGTACTCTCTCTTATATGCCA
     Q  V  G  N  D  I  C  V  V  I  F  N  D  G  T  L  S  Y  M  P
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3361 AATACAATCACTTCTCAATTCAATCATGTTATCATTCTTGTTCAATACGATAAACAAAAC
     N  T  I  T  S  Q  F  N  H  V  I  I  L  V  Q  Y  D  K  Q  N
3421 AATGGTTATAAAGTTTCAATGGCTTGTAAAGATGGTGTTAAATCACCTTTCGAACCCCTA
     N  G  Y  K  V  S  M  A  C  K  D  G  V  K  S  P  F  E  P  L
3481 TCTCCAAATAATTTAATTAAAAAATCTGATATTAAAGATTTCATTTTAACTAAATTAATT
     S  P  N  N  L  I  K  K  S  D  I  K  D  F  I  L  T  K  L  I
3541 AATGGTGAATTGGCTTCATTACAAGCTCCTGTATTTGCTTCAAAAATTACTAGAACTAGA
     N  G  E  L  A  S  L  Q  A  P  V  F  A  S  K  I  T  R  T  R
3601 GAATCTTTATTAAATTATTATATTTCTCAATTTTTA***TAA***
     E  S  L  L  N  Y  Y  I  S  Q  F  L  *
```

Fig. 3 Sequence of *D. discoideum* RapGAP1

The complete DNA sequence of the RapGAP1 gene along with the predicted amino acid sequence is shown above. The 5' ATG which is the initiation codon and the stop codon TAA are indicated in bold and italic. The underlined region in the N terminus is the PH domain. The RapGAP domain at the C terminus is also underlined. Numbering of the nucleotides is indicated at the left side.

3.1.2 Sequence comparison with different RapGAPs

Down regulation of small GTP-binding proteins usually requires the interaction with their corresponding GTPase-activating proteins (GAPs), which increase the slow intrinsic GTPase activity by several orders of magnitude. Malfunction of this regulatory step may dramatically interfere with cellular signal transduction pathways (Kraemer *et al*, 2002). The RapGAP domain was aligned with RapGAP domains from other species (Fig. 4). The highest percentage of similarity was found in *Drosophila melanogaster* RapGAP (54.6% similarity and 43.7% identity). An almost similar score was obtained in a comparison with human Rap1GAP (53.2% similarity and 41.3% identity).

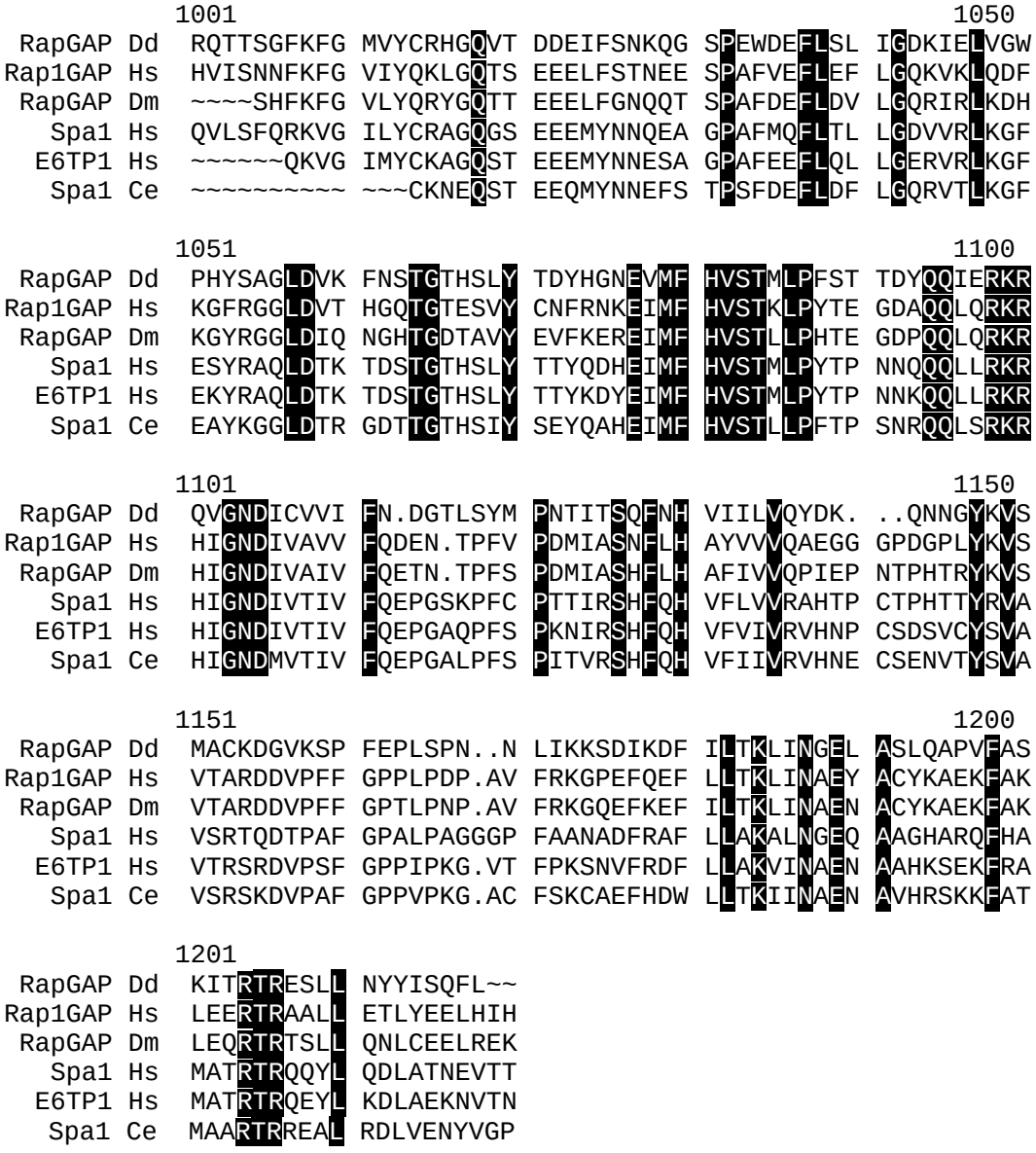


Fig. 4 Alignment of different RapGAP domains

The sequences used in the alignment are from *D. discoideum* RapGAP1, *Homo sapiens* Rap1GAP (Swiss-Prot number P47736), *Drosophila melanogaster* RapGAP (Swiss-Prot number O44090), *H. sapiens* Spa1 (Swiss-Prot number O60618), *H. sapiens* E6TP1 (Swiss-Prot-number Q9UNU4), and *Caenorhabditis elegans* Spa1 (Swiss-Prot number Q20016). The numbering refers to the amino acid position of *D. discoideum* RapGAP1. The alignment was performed using the program Clustal from the UWGCG program package. Invariant residues are boxed.

3.1.3 RapGAP1 transcript accumulation during development

There are two distinct phases in the life cycle of *D. discoideum*, growth and development. Amoebae continue to grow as long as a bacterial substrate is available. The depletion of their food source serves as a signal to trigger the onset of a developmental cycle. Then amoebae aggregate by chemotaxis and pass through various morphogenetic stages from single amoebae to pseudoplasmodia and fruiting bodies.

To determine whether transcription of the RapGAP1 gene is regulated during development, RT-PCR was conducted for all the developmental stages of *D. discoideum* starting from vegetative amoebae up to the formation of mature fruiting bodies. Total RNA was isolated from the different developmental stages and RT-PCR was performed as described in the Materials & Methods section. The expected 660bp product (from base position 2952-3612) was observed throughout all developmental stages, with a strong upregulation at 9 hours of development (Fig. 5). The Ste20-like kinase DST1 was used as a positive control using total RNA from growth phase cells and primers that led to the amplification of a 1Kb PCR product. To be sure that the total RNA was not contaminated with DNA, a control containing RNA from growthphase cells (0 h) was added after the RT step and before the real PCR step began. Lack of the PCR product shows the absence of significant contaminations with genomic DNA.

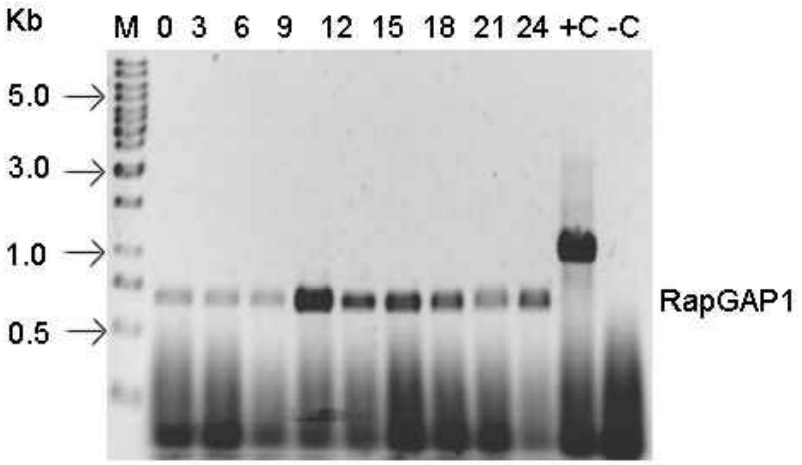


Fig. 5 RT-PCR analysis of RapGAP1 gene expression

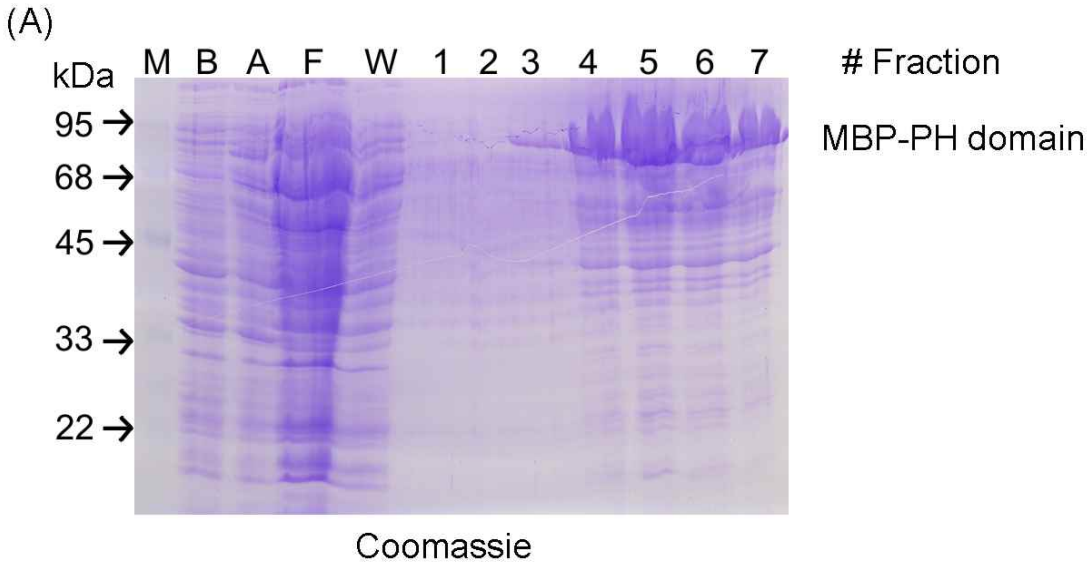
RT-PCR analysis performed on total RNA isolated from all stages of *D. discoideum* development in order to monitor the level of RapGAP1 transcript, hours of development are indicated at the top. DST1 was used as a positive control (lane +C). The RapGAP1 gene does not contain introns, therefore the absence of contaminating genomic DNA was tested in the PCR step only (lane -C).

3.2 Biochemical and cell biological characterization of RapGAP1

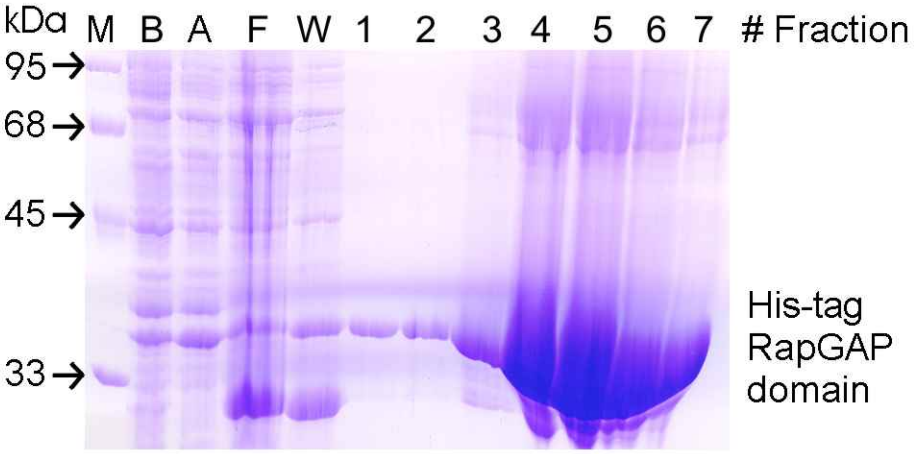
3.2.1 Expression and purification of recombinant RapGAP1

For bacterial expression of selected regions from the RapGAP1 gene, two major domains where cloned independently and expressed. The 5' region, which encodes the PH domain (1-1050 bp) was cloned into the pMALC2 vector and expressed as maltose-binding fusion protein. The detailed cloning strategy is explained under Materials & Methods. Recombinant protein showed a molecular mass of about 76 kDa. Two constructs originated from the 3' region of the gene, both containing the RapGAP domain. The two constructs differed only in a few base pairs. They were cloned into a pQE30 vector and the recombinant proteins had a molecular mass of 33 kDa in the construct made from base position 2736-3639, and 36 kDa in the second construct, which spanned 2622-3639

bp. Since the protein was found in the inclusion bodies, protein from the above constructs had to be purified under denaturing conditions. All the above constructs were used to raise antibodies. Construct (2736-3639 bp) was used for both monoclonal and polyclonal antibodies against the GAP domain. Unfortunately both approaches failed. Only the second construct from base position 2622-3639 (36 kDa protein) was successful in raising polyclonal antibodies against RapGAP1 (Fig. 6C).

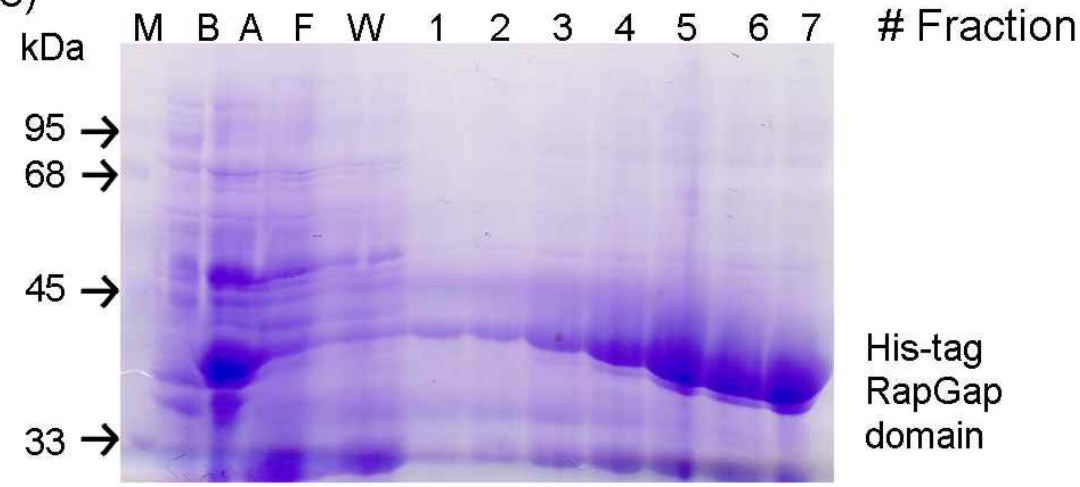


(B)



Coomassie

(C)



Coomassie

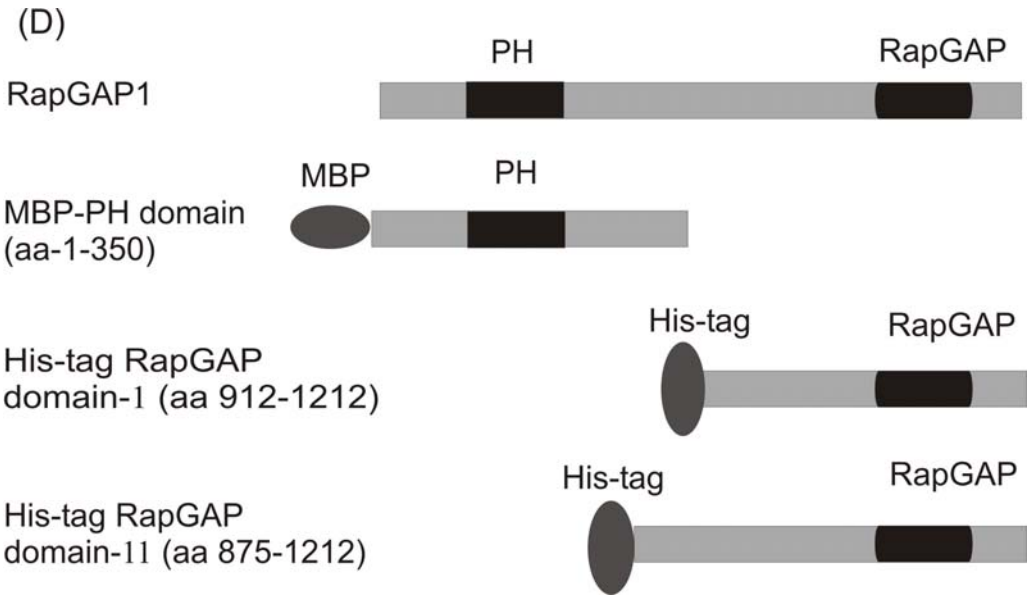


Fig. 6 Purification of recombinant RapGAP1 constructs

A: The 5' region of the RapGAP1 gene, which encoded the PH domain, was expressed as MBP-tagged fusion protein (76 kDa). B&C: The 3' region of the RapGAP1 gene was cloned into a pQE30 vector and expressed as His-tagged fusion protein. In Fig. 6B the recombinant protein has a size of 33 kDa and in Fig. 6C the protein is 36kDa in size which is a consequence of the slightly larger construct. Fig. 6D full length RapGAP1 and other corresponding RapGAP1constructs are schematically depicted. The first five lanes in all three gels correspond to (M) marker, (B) before induction, (A) after induction, (F) flow through of the affinity column and (W) wash.

3.2.2 Western blot analysis of RapGAP1

The expression of RapGAP1 protein in AX2 wild type was analyzed in western blots. The antisera raised against the GAP domain were able to recognise a 133 kDa protein in AX2 wild type homogenate. Later it could be shown that the labelling is specific because the band was absent in RapGAP1 null-cells (see below). Unfortunately despite affinity purification of the serum, the specificity of anti RapGAP1 was not sufficient enough for immunofluorescence and immunoprecipitation approaches.

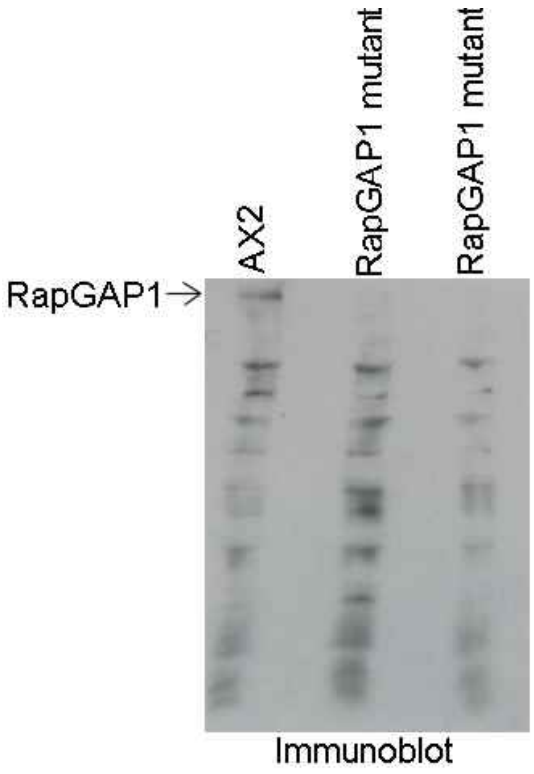


Fig. 7 Identification of cellular RapGAP1 using western blot analysis.

Total protein from AX2 wild type and RapGAP1 mutants (2×10^5 cells/lane) were separated by SDS–PAGE and the protein blot was probed with rabbit antibody raised against the RapGAP domain. In the lane with AX2 homogenate the 133 kDa band represents full length RapGAP1, whereas this band is absent in the knock-out mutants.

3.2.3 Disruption of the RapGAP1 gene by homologous recombination

To investigate in detail the role of RapGAP1, a *D. discoideum* null-mutant was generated with the Cre/LoxP recombination system. Cre-/LoxP recombinant system of bacteriophage P1 has been employed for genetic manipulation of higher eucaryotic cells (Sauer *et al*, 1988). Cre recombinase recognizes a 34 base pair Cre recombination signal sequence called loxP, and can delete genes that are flanked by two loxP sites in the same direction. The pLPBLP vector consists of two head to tail loxP site flanking the combination of an actin 6 promoter, the blasticidin resistance gene and the actin 6 terminator. Two independent fragments of the RapGAP1 gene from base positions 443-

940 using the restriction sites KpnI and HindIII and another one from base positions 2952-3612 using PstI and BamHI were cloned into the multiple cloning site present at either side of the cassette. The plasmid was cut with KpnI and BamHI to avoid ectopic integration. This linearized plasmid was then transformed into AX2 cells and incubated at 21°C with blasticidin as selection marker until colonies were visible. The strategy is summarized in Fig. 8. Screening for the absence of the RapGAP1 gene by PCR showed a shift of a distinct band indicating the disruption of RapGAP1 gene. (Fig. 9)

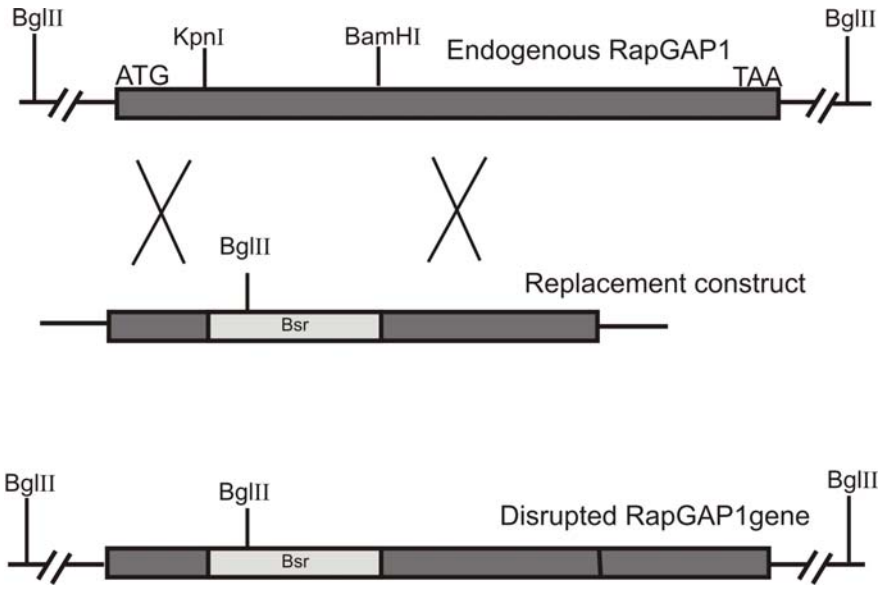


Fig. 8 Sequential steps in the disruption of the RapGAP1 gene

First, 5' and 3' RapGAP1 gene fragments were cloned into the pLPBLP vector with the blasticidin resistance cassette in the middle. Next, the circular knock out construct was linearized and transformed into AX2 cells. Gene disruption was achieved by homologous recombination between the endogenous DNA and the plasmid DNA. A complete disruption would replace genomic DNA with the Bsr cassette thus deleting endogenous HindIII and PstI sites, and introducing a BglII site from the Bsr cassette.

3.2.4 Screening for RapGAP1 Mutants

Screening for RapGAP1 mutants was done by PCR, using three sets of primers. In Fig. 9A the first diagram shows the targeting vector and the second diagram represents the genomic DNA with primer 1 and 2 and the third diagram represents the disrupted RapGAP1 gene with primer 3. Among the three primers, the first one named as (primer 1) was designed from the 5'-region of the RapGAP1 gene which was not included in the gene replacement construct. Primer 1 was used as forward primer for screening both negative and positive clones. Primer 2 was designed from the region of the gene that would be replaced by the blasticidin resistance cassette. A PCR product obtained by combining primers 1 and 2 should give an amplicon of 826 bp and would show that no homologous recombination has occurred. Whereas primer 3 was designed from the end region of the blasticidin resistance cassette in the reverse direction. Combination of these two primers (primer 1 and 3) should give a 1.3 kb fragment if the gene was disrupted.

To screen for RapGAP1 knockout mutants by PCR, blasticidin resistant clones were picked and allowed to grow under selection to a density of 2×10^7 cells/ml. Genomic DNA was isolated using the Roche genomic DNA isolation kit following the manufacturer's instructions. 2 μg of genomic DNA was used as a template for PCR reactions. As shown in Fig. 9D lanes 1,4,5,6,8,9 correspond to RapGAP1 null-cells because a 1.3 kb piece could be amplified; lanes 2,3 show negative clones (826bp product).

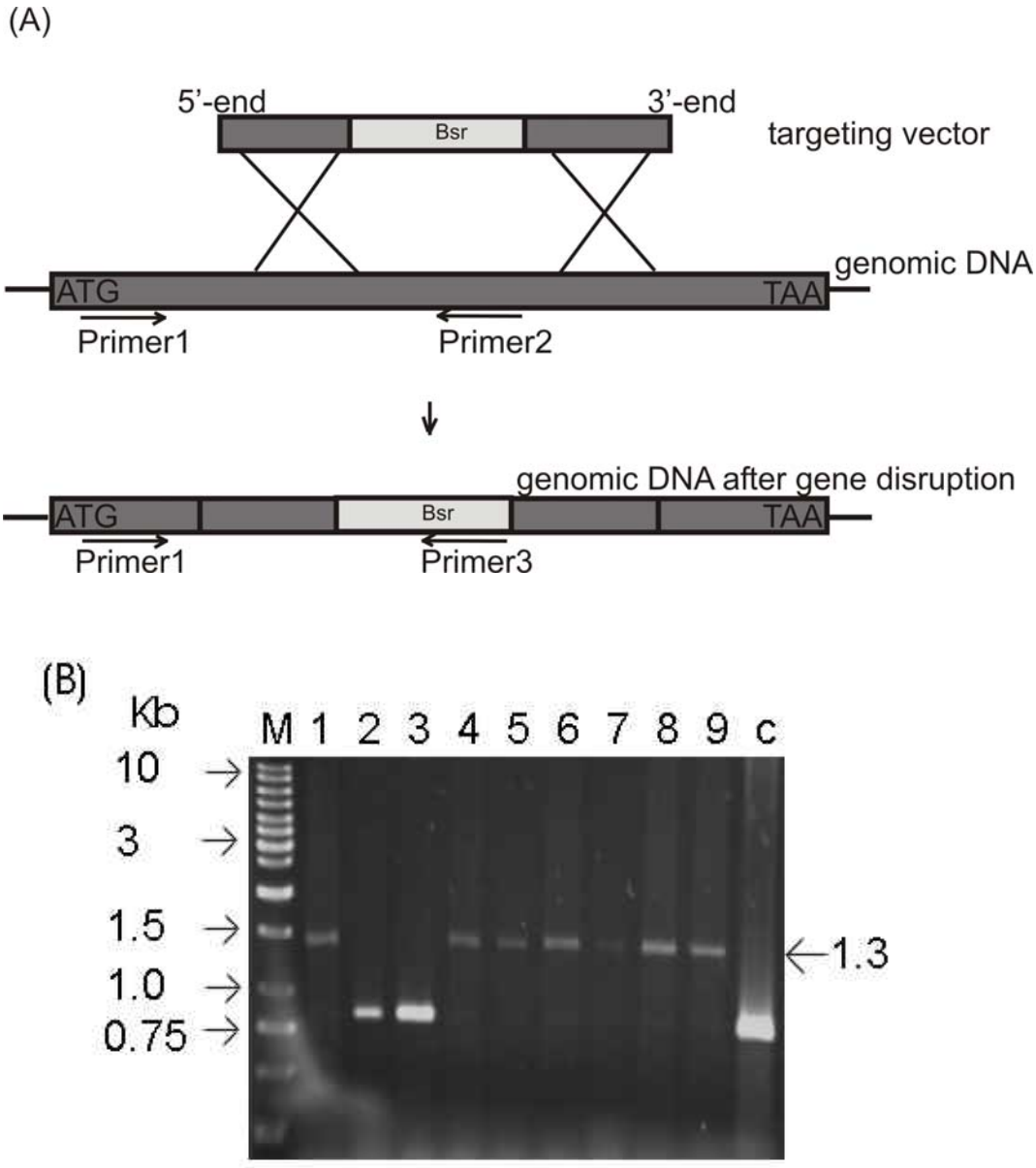


Fig. 9 Screening of RapGAP1 mutants by PCR.

A: Schematic diagram explaining the approach for screening RapGAP1 mutants by PCR. (A) PCR product obtained by the combination of primers 1 and 2 denotes no homologous recombination. Whereas a combination of primer 1 and 3 should give a 1.3kb fragment corresponding to positive clone. B: Genomic DNA isolated from independent clones, was used for screening for RapGAP1 null-cells. Two different sizes of bands were observed when the PCR samples were analyzed on a 0.8% agarose gel. Six independent null-cells (lanes 1,4,5,6,8,9) were obtained out of 12 clones selected for screening. Genomic DNA isolated from AX2 wild type was used as a positive control.

3.3 Analysis and characterization of the RapGAP1 knockout mutant

3.3.1 RapGAP1 mRNA is absent in the knockout mutant

RT-PCR was performed to prove the RapGAP1 gene disruption. Total RNA from vegetative cells of AX2 wild type and RapGAP1 mutants was isolated and 2µg of RNA were used for the reaction. In the wild type the primers amplified a 660bp DNA piece (#2952 - #3612, see Fig. 10), whereas no product could be obtained from mutant RNA.

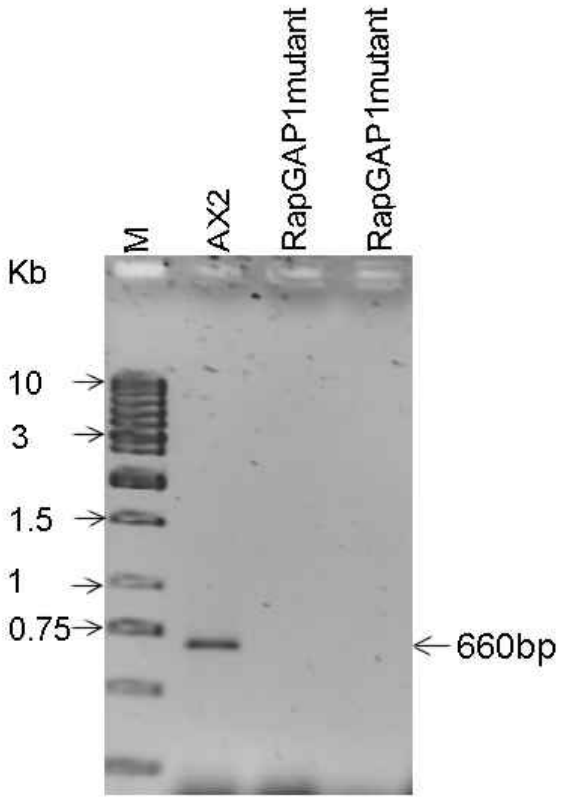


Fig. 10 Analysis of RapGAP1-null cells by RT-PCR

Total RNA was isolated from AX2 wild type and RapGAP1-null cells to perform RT-PCR. AX2 showed a band at 660bp whereas the two independent RapGAP1 mutants failed to show a band confirming that the RapGAP1 gene was disrupted in these two mutants.

3.3.2 Confirmation of the gene disruption by Southern blot analysis

Genomic DNA from AX2 wild type and two independent RapGAP1 transformants was digested with different restriction enzymes (HindIII and EcoRI double digestion, single digestion EcoRI, HindIII) and the digested DNA was hybridised with a radioactively labelled RapGAP1 fragment between bases #443 - #940. We observed with all enzyme combinations a band shift with DNA from the RapGAP1 transformants when compared to AX2 thus confirming the disruption of the RapGAP1 gene.

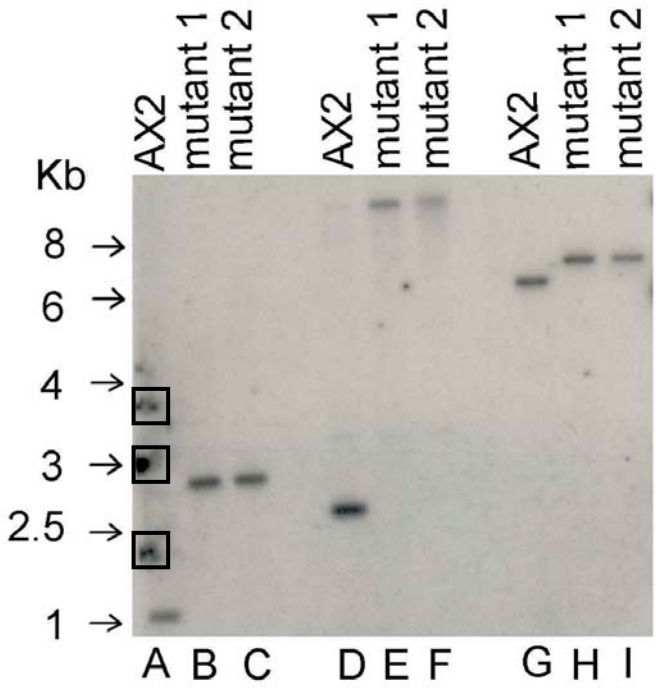


Fig. 11 Southern blot analysis of genomic DNA from wild type AX2 and RapGAP1mutants.

Genomic DNA was isolated from AX2 wild type and RapGAP1 mutants and then digested with different restriction enzymes: A/B/C: double digest with EcoRI and HindIII; D/E/F: single digest with EcoRI, and G/H/I: single digest with HindIII. 5µg of digested DNA were separated in a 1% agarose gel, blotted onto a nitrocellulose membrane, and hybridised with a radioactively labelled RapGAP1 probe. In all cases the different restriction patterns confirm the disruption of the RapGAP1 gene. The frames on the left side indicate spread DNA from the marker lane, the 1 Kb DNA band is specific for the RagGAP1 gene.

3.3.3 Growth of RapGAP1-minus cells in liquid medium

It was reported that in *Dictyostelium* a reduction of Rap1 protein levels resulted in reduced cell growth (Kang *et al.*, 2002). This prompted us to look for growth of RapGAP1 mutants in shaking culture and on solid media. For growth in shaking culture an initial inoculum of 5×10^4 cells/ml was used. Cell density of AX2 wild type and two independent RapGAP1 mutants was determined in triplicate twice a day until the cells reached the stationary phase. As shown in Fig. 12, the mutants did not show any defects under these conditions.

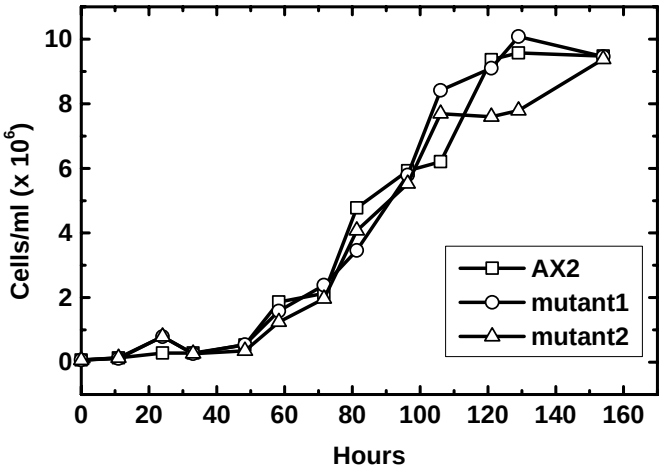


Fig. 12 Growth in shaking culture

AX2 wild type and two independent RapGAP1-null cells were grown at 150 rpm, the results are the mean of three experiments.

3.3.4 Growth of RapGAP1 mutants on bacteria

For growth of mutants on bacteria, *Klebsiella aerogenes* were used as the bacterial strain. 0.2 ml of an overnight culture of *Klebsiella* was plated on SM agar plates and a small

amount of *Dictyostelium* cells was placed at the center of the plate and incubated at 21^{o}C. The diameter of the emerging colonies was measured for mutants and wild type once a day. The data are plotted in Fig. 13. There was no distinguishable difference to be observed between wild type and RapGAP1 mutants.

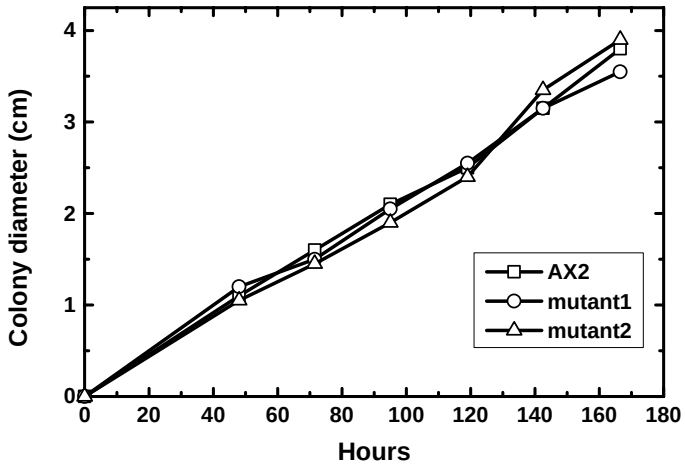


Fig. 13 Growth on Klebsiella aerogenes

Growth of the RapGAP1 mutants and AX2 was determined by measuring colony diameters on a lawn of *K. aerogenes.* The results are the mean of three experiments.

3.3.5 Cell motility analysis of RapGAP1 null cells

Cell motility analysis of RapGAP1 null cells was done using the DIAS computer software. Cells were allowed to settle on the surface of a small petri dish for 5 minutes and positioned on the stage of a Zeiss inverted microscope (see also Materials and Methods). Usually we took at least three times 100 pictures at 10 sec intervals in medium and the same number of pictures after changing to Soerensen buffer. Total path length, directionality, speed etc were determined with the 3D-DIAS computer program, the values from usually more than 150 cells were combined and averaged for the analyses in medium and in Soerensen buffer. Fig. 14 shows the motility of AX2 wild type cells and

of two independent RapGAP1 null mutants. In both cases the mutants showed increased cell motility in medium and in phosphate buffer as compared to the wild type control.

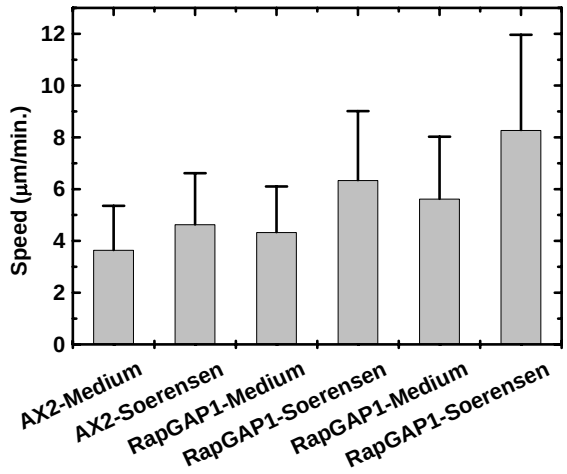


Fig. 14 Analysis of random cell motility exhibited by wild type and RapGAP1 null cells.

Cell motility was monitored in HL-5 medium and in Soerensen phosphate buffer using the DIAS computer-assisted method. In all cases motility increases after replacing full medium by Soerensen buffer. However, the motility of Rap GAP1 null cells was significantly increased under all conditions. The error bars represent standard deviations

3.3.6 Development of RapGAP1 mutants

To study the developmental behaviour of RapGAP1 null mutants, cells were grown in axenic medium to a density of 1×10^8 per plate. The cells were collected and washed 3 times in phosphate buffer, plated on non-nutrient agar plates and incubated for different stages of development starting from t3 to t24. Photographs were taken at different developmental stages.

Compared to wild type cells, RapGAP1 null strains were slightly delayed, especially at the onset of the mound stage. As shown in Fig. 15 after 9 hours of starvation mutants showed loose mounds whereas the AX2 wild type showed already tight mounds. A slight delay in development was observed even after 12 hours of starvation, but during later

stages there was no considerable difference to be observed. This may be due to the existence of other RapGAPs in *Dictyostelium*, which may replace the existence of knocked out RapGAP1, enabling the cell to undergo proper development.

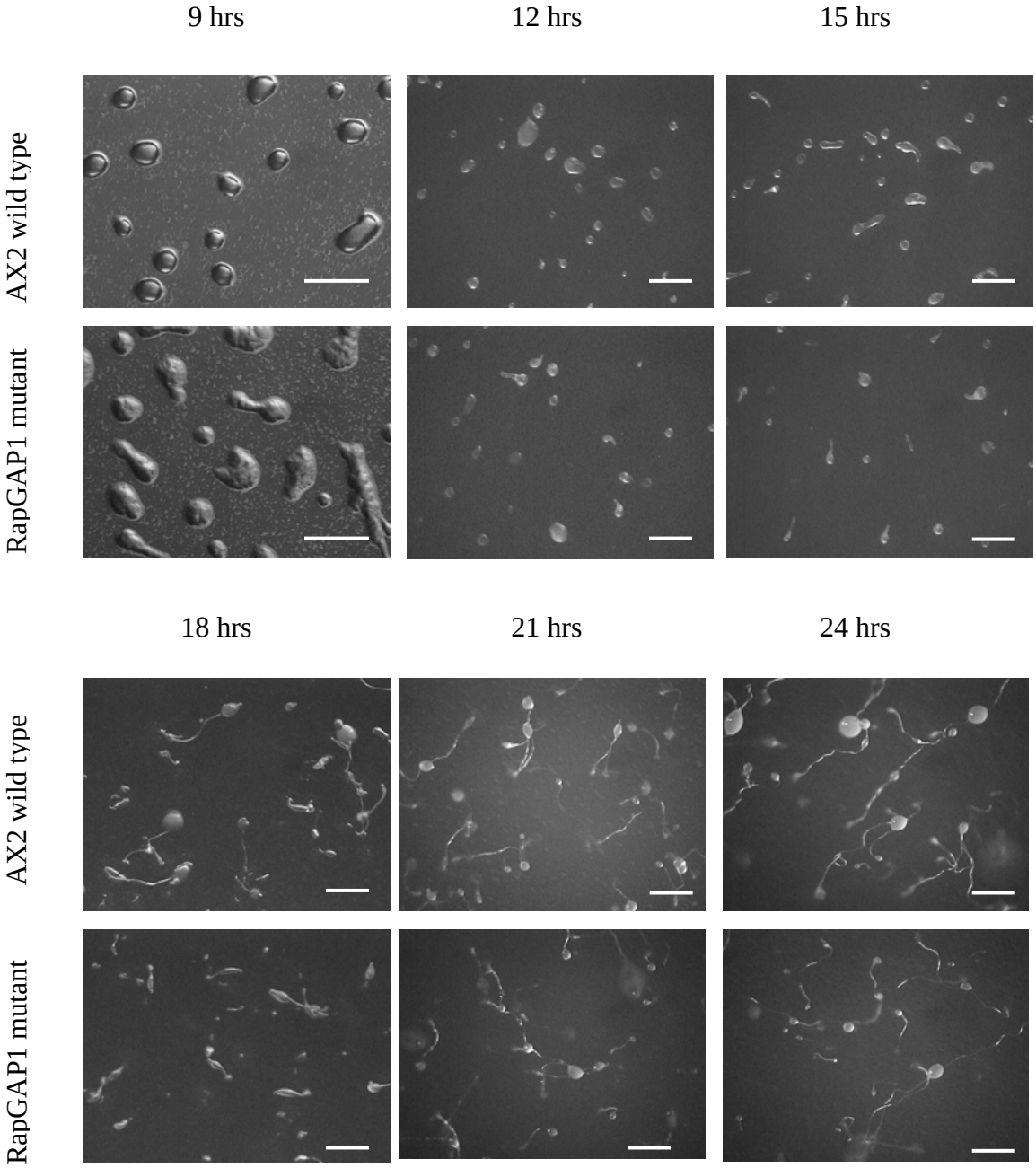


Fig. 15 Development of RapGAP1 null cells and AX2 wild type

Cells were grown in axenic medium, washed in phosphate buffer and plated on non-nutrient agar plates. Photographs were taken at the developmental stages indicated. After 9-12 h of starvation the RapGAP1 mutant developed slower when compared to AX2 wild type. Photographs from t9 in both wild type and mutants had higher magnification when compared to other stages of development. Pictures were taken with a Leica MZFIII stereomicroscopy. Bar, 1 mm (note that T9 has different bar size).

3.3.7 Expression of contact site A protein in the RapGAP1 mutant

To test for a developmental delay at the biochemical level, we performed Western blot analysis for all developmental stages of AX2 wild type and RapGAP1 mutants using an antibody against the cell adhesion molecule contact site A (csA). Cells were lysed by the addition of 1x SDS sample buffer and boiled for 5 minutes. The amounts loaded were equivalent to 2×10^5 cells/lane, equal loading was confirmed by running a parallel gel and staining with Coomaissie Blue. For Western blots the proteins were transferred onto nitrocellulose and probed with mAb 294 (Bertholdt *et al.,* 1985).

Incubation with csA antibody showed a difference between the wild type and the RapGAP1 mutants. In AX2 the contact site could be detected already after 3 hours (t3), it accumulated at t6 and t9, declined at t12, t15 and t18 and came up again between t21 and t24. This observation differs from earlier reports in so far that csA is detectable usually only after 6-9 hours during aggregation and can not be detected during later developmental stages (Noegel *et al*., 1986). The reasons for these differences are not entirely clear and could be due to slight changes in culture conditions. However, in all cases we could detect a delayed accumulation of csA in RapGAP1 null mutants (Fig. 16).

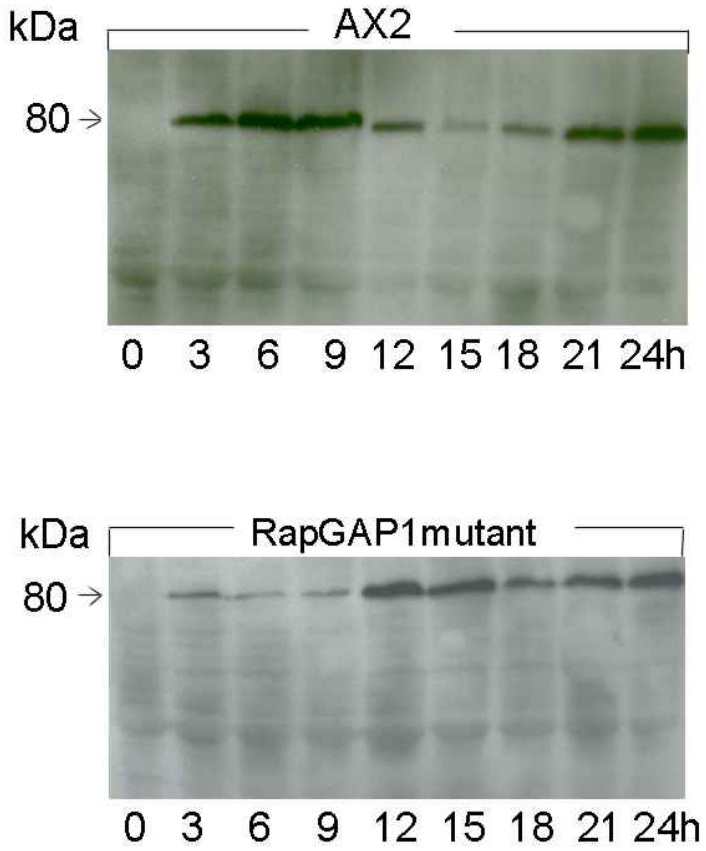


Fig. 16 Western blot analysis of csA expression in AX2 wild type and RapGAP1 null-cells

Cells were harvested from non-nutrient agar plates (0h to 24 h). Total protein of 2×10^5 cells was applied per lane, and the csA protein were labelled with mAb 294. The 80 kDa band shown on the blots denotes the csA glycoprotein. The contact site protein usually reaches highest concentration after 6-9 h of starvation. In the Rap GAP1 minus mutant the accumulation of this developmental marker is clearly delayed by 3-6 h.

3.3.8 Expression of Mud1, a prespore marker

MUD1 is a prespore-specific cell surface antigen of about 30 kDa. Prespore cells first appear in the cell mound stage at the onset of tip formation and are located in the basal region of the tip. (Takeuchi *et al*, 1982). Cells from different developmental stages (0-24 h) were lysed and the extracts loaded onto a 15% SDS polyacrylamide gel (2×10^5 cells/lane) essentially as described for detection of csA protein. A clear difference was observed between AX2 wild type and RapGAP1 null mutant. In the case of AX2 wild

type, a prominent band of 30kDa was observed at 9 hours to 24 hours of starvation, whereas in the mutant extracts the antigen could be detected as a faint band only at t12 and thereafter. This observation is in agreement with previous findings (Fig. 15).

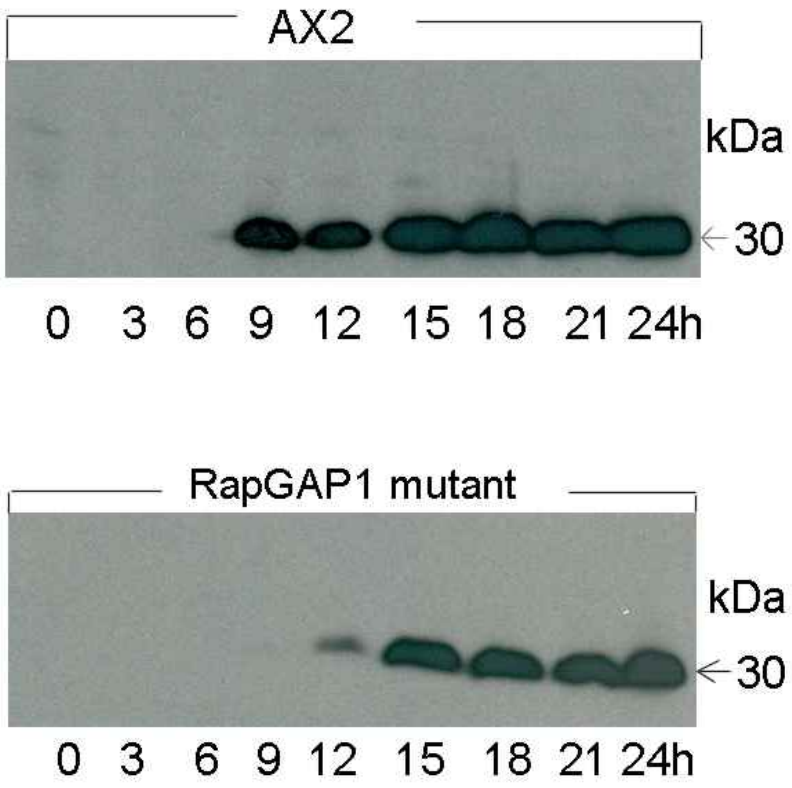


Fig. 17 Analysis of MUD1 as developmental marker in wild type and RapGAP1 null cells

The samples were prepared as described above and the appearance of the prespore cell surface antigen was identified using *Dictyostelium* MUD1 antibody and the ECL reagents. The 30 kDa bands seen in the blots repesents the prespore cell surface antigen. Also in this case development of the mutant seems to be delayed by 3-6 h.

CHAPTER 4

DISCUSSION

We screened the sequences of the *D. discoideum* cDNA project database using PH (pleckstrin homology) domains as query and discovered a cDNA clone, which contained a RapGAP domain and a PH domain. Pending the identification of the physiological function this was designated as RapGAP1. The 3.6 kilobase cDNA insert contains an open reading frame coding for a protein of 1212 amino acids with a predicted molecular mass of 132 kDa. The first RapGAP, Rap1GAP, was purified from bovine brain as a soluble as well as a membrane-associated protein, the latter as a higher molecular weight isoform. Rubinfeld *et al*., (1992) have shown that it is only the catalytic domain, which is sufficient for Rap1GAP activity. This domain was later identified also in the human genes SPA1 and E6TP1 which encodes tuberin. The *Drosophila* genome contains only a single *rap* gene and its loss is lethal at the larval stage which suggests that the Rap protein is performing essential functions (Hariharan *et al*, 1991). In contrast, flies lacking RapGAP1 did not result in obvious phenotypic abnormality. The absence of an obvious phenotype in loss-of function RapGAP1 mutation argues for the existence of functionally similar other GAPs for Rap1 (Chen *et al*, 1997). *Dictyostelium* also harbours many genes that code for RapGAPs and one cannot exclude that a certain functional redundancy masks phenotypic changes under laboratory conditions.

4.1 Why Dictyostelium needs so many RapGAP proteins?

There is only one published member of the *Dictyostelium* Rap family (Rap1), at least two novel putative *rap* genes. Up to eleven RAPGAPs have been identified in the *Dictyostelium* genome (Eichinger *et al.*, 2005). This seems a very large number when compared to the single Rap gene identified from the completed *Drosophila* genome project. In *Drosophila* the genome size, degree of cell specialization or any other evolutionary standards, are large when compared to *Dictyostelium*. Why then does a relatively simple eukaryote like *Dictyostelium* have so many Rap and RapGAP genes? One explanation could be that the RapGAP genes in *Dictyostelium* represent a redundant genetic system, where accidental gene duplication events have created more RapGAP genes than the organism requires. Another explanation might be that though higher eukaryotes have relatively few Rap and RapGAP genes relative to their genome sizes, alternative splicing provides considerable diversity in the primary structure of Rap and its regulators. Since most *Dictyostelium* genes contain no or only a few introns, each protein must be encoded by a distinct gene. This speculation is difficult to discount because very little information is available on the numbers and functions of different RapGAP isoforms in higher eukaryotes.

The evolutionary justification for cell specialization is the compartmentalization of specific biological functions into specific tissues, to allow the biological processes to proceed more efficiently. Cell specialization requires differential gene expression which is likely to affect the availability of effectors and regulators of Rap. This is not the case with *Dictyostelium* as an essentially omnipotent cell. With very little opportunity to alter the network of signaling pathways by cell specialization, their diversity of response

requires an existing diversity of signaling pathways. This may be the reason why *Dictyostelium* has so many different RapGAP genes.

4.2 Developmental regulation of RapGAP1 expression

One of the most prominent features of *Dictyostelium* is that it has a relatively simple and well-defined life cycle. It is the depletion of the food source that enables the cells to start the developmental programme which includes coordinated transcription and sorting of cell populations.

We conducted RT-PCR on the different developmental stages of *Dictyostelium* to study the accumulation of the RapGAP1 message, and found that RapGAP1 mRNA accumulates throughout the developmental cycle of the organism with a maximum at 9 hours after starvation, when cells start to form mounds. This finding was in contrast to the expression pattern exhibited by the human Rap1GAP gene. Rubinfeld *et al*, (1991) showed that human Rap1GAP is not ubiquitously expressed but appears to be most abundant in fetal and undifferentiated cells, and during differentiation, the Rap1GAP immunoreactivity was reduced to an almost undetectable level. In the case of *Drosophila*, the RapGAP1 gene is expressed in specific groups of cells. For instance in the eye-imaginal disc, RapGAP1 is expressed in the photoreceptor clusters posterior to the morphogenetic furrow and is not expressed in the proliferating cells anterior to the morphogenetic furrow (Chen *et al.,* 1997). Our data suggest that in *Dictyostelium* both Rap1 and RapGAP1 are always present. *Dictyostelium* Rap1 mRNA peaks during the aggregation (6-8 hrs) and culmination period (18-22 hrs) (Robbins *et al,* 1990). Since both Rap1 and RapGap1 are expressed throughout development we would expect that

just like Rap1, Rap1GAP also could regulate diverse functions like control of cytoskeletal dynamics, regulation of phagocytosis and development in *Dictyostelium*.

4.3 Role of RapGAP1 during development

Multicellularity and development are important features of *Dictyostelium.* The mechanism for generating multicellularity in *D. discoideum* differs significantly from the early steps of metazoan embryogenesis. Contrary to metazoans, in *D. discoideum* growth and differentiation are well separated. Cells multiply first and then gather chemotactically to form a multicellular organism. However, subsequent processes depend on cell-cell communication on both systems, and basic processes such as differential cell sorting, pattern formation, stimulus-induced gene expression and cell type specific regulation are common to *D. discoideum* and metazoans.

The most obvious defect we noticed in the RapGAP1 null cells was the delay in development. However, the development was not drastically delayed because the cells could overcome this defect and proceed to the formation of normal fruiting bodies after 24h. There was no difference to be observed in the length of the stalks or shapes of the fruiting bodies. Furthermore, we made use of selected marker proteins to confirm the developmental delay. There is an accumulating body of evidence in the literature that Rap and its effectors are involved in cell proliferation and differentiation. Rap1 mutants disrupt normal cell shape and morphogenesis in the eye, ovary and wings of *Drosophila* embryos (Asha *et al.,* 1999). The selective presence of Rap1GAP in undifferentiated HL60 cells suggests that it may be involved in the programming of cell growth and division (Rubinfeld *et al*, 1991). Tuberin, which function as a GAP for Rap1 can trigger

mammalian cell size reduction and a dominant-negative TSC2 mutant induces increased size (Rosner *et al*, 2003).

Upon starvation, individual amoebae of *D. discoideum* start to aggregate which leads often to the formation of migrating slugs. A migratory slug is composed of about 100,000 cells with several cell types and sub-types. Within the slug the main subpopulation of cells are prespore and prestalk cells. The eventual fate of all prestalk cells is identical; they form vacuolated stalk and basal disk cells. Prespore cells occupy the posterior region within the slug, representing about 80% of the slug cells. Prespore specific antigen (PsA) is a developmentally regulated glycoprotein, expressed selectively on the surface of prespore cells (Browne *et al*, 1989) and recognized by the mAb MUD1 (Krefft *et al*, 1983).

In the case of RapGAP1 null cells the absence of MUD1 epitope at the mound stage of *Dictyostelium* development shows that there was a delay in the appearance of prespore cells. Our results suggest that this delay in prespore cell diffentiation might be due to the loss of RapGAP1-dependent stimulation of genes necessary for prespore development. To date the collected knowledge on the role of *Dictyostelium* RapGTPase on development is limited. There is one data published by Louis *et al*, (1997) which states that overexpression of Rap1 during development was able to partially rescue the developmental defects of activated RasD. In the same way RapGAP1 might also be involved in different signaling cascades. However, to resolve this problem single and multiple mutants for all *Dictyostelium* RapGAPs need to be generated

4.4 Control of cell motility

Dictyostelium amoebae are equipped with a complex actin cytoskeleton that endows the cells with chemotactic and motile behavior comparable to that of leukocytes. The role of Rap GTPases in the establishment of cell motility and cell migration has been studied in different cell types. Mouse embryo fibroblasts derived from C3G knock-out animals showed delayed cell spreading and increased motility, both of which are suppressed by overexpression of active Rap1 (Ohba *et al*., 2001). Shimonaka *et al*. (2003) demonstrate that expression of dominant active Rap1V12 in lymphocytes stimulated shear-resistant adhesion, robust cell migration on immobilized intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, and transendothelial migration under flow. Likewise, our studies showed that under normal conditions without any external stimuli RapGAP1 mutant showed defects in cell motility. Most notable is that the RapGAP1 null cells showed increased cell motility when compared to AX2 wild type.

The central roles of the actin cytoskeleton in association with Rap1 for proper polarization of cells have been described already several times. The RapGAP SPA-1 was detected diffusely in the periphery of floating unpolarized T cells, whereas in stimulated cells it was highly concentrated at the actin-rich adhesion region (Harazaki *et al*, 2004). In HeLa cells SPA-1 and RapV12 were co-localized with the cytoskeleton anchoring protein AF-6 at the cell attachment site (Li Su *et al*, 2003). And in platelets, Rap2 directly and specifically interacts with polymerized, but not with the monomeric form of actin (Torti *et al*, 1999).

In *Dictyostelium* Kang *et al*. (2002) suggest that Rap1 may act upstream in a pathway that leads to guanylyl cyclase activation. Since cGMP is an important second messenger that

affects the function of myosin in cellular motility especially during cAMP chemotaxis (Haastert *et al*, 1997) we think that Rap and its regulators may function as key mediators of cellular motility both during growth and during multicellular development.

4.5 RapGAP1 null cells are not defective in axenic growth and growth on *Klebsiella*

Dictyostelium cells lacking RapGAP1 did not show any difference in growth when compared to AX2 wild type, which denotes that RapGAP1 null cells do not have defects in uptake of nutrients and cell growth. From the literature it is clear that one of the putative functions of Rap1 is regulation of cell growth. One suggestive evidence for a role of Rap1 in cancer comes from studies on oncogenic human papilloma viruses (HPV). A cellular Rap1GAP, called E6TP1, is targeted for ubiquitin-mediated degradation by the viral E6 oncoprotein, suggesting that resultant hyperactivation of Rap1 might contribute to cervical and other cancers associated with chronic HPV infection (Sing *et al*., 2003). In mouse, overexpression of an active form of Rap1 promoted the proliferation of normal hematopoietic progenitors, while SPA-1 overexpression markedly suppressed it (Ishida *et al*., 2003). Normal growth of RapGAP1-minus cells in liquid culture or on bacterial lawn suggests again that a functional redundancy of RapGAP isoforms masks the loss of only one gene. This is reminiscent of fairly old data on D. *discoideum* mutants that lacked the highly conserved actin-binding protein α-actinin (Wallraff *et al.,* 1986). In almost all parameters tested, there was no major difference between wild type AX2 cells and the mutant. Only in 1:1 mixed shaking cultures the loss of α-actinin resulted in a mild growth disadvantage between 1-5%. This disadvantage is certainly enough to guarantee a high

evolutionary pressure towards a fully functional α-actinin. However, under laboratory conditions it required the additional knock-out of a second F-actin crosslinking protein to demonstrate a functional redundancy (Witke *et al*., 1992).

CHAPTER 5

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