The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*.

by

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Preface

The work presented in this thesis was carried out at UNIGEN Center for Molecular Biology at the Norwegian University of Science and Technology (NTNU) and the Department of Biology NTNU. Financial support has been provided by The Research Council of Norway and of Norsk Hydro ASA, and is gratefully acknowledged.

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Abstract.

Small GTP binding proteins function as molecular switches which cycles between GTP-bound ON and GDP-bound OFF states, and regulate a wide variety of cellular processes as biological timers. The first characterized member of the small GTPase family, the mutated oncogene p21 src, later known as Harvey-Ras, was identified in the early 1980s (Shih, T. Y. et al. 1980). In the following years small Ras-like GTPases were found in several organisms and it was soon discovered that they took part in processes, such as signal transduction, gene expression, cytoskeleton reorganisation, microtubule organisation, and vesicular and nuclear transport. The first *Rho* (Ras homology) gene was cloned in 1985 from the sea slug Aplysia (Madaule, P. et al. 1985) and because of their homology to *Ras* it was first suspected that they could act as oncogenes. Later studies have shown that even though they participate in processes such as cell migration and motility they are not mutated in cancers.

The first indications that Rho was a signaling protein regulating the actin cytoskeleton, came from experiments where activated forms of human RhoA was microinjected into 3T3 cells (Paterson, H. F. et al. 1990). Another Rho-like GTPase Rac1 (named after Ras-related C3 botulinum toxin substrate) was later shown to regulate actin cytoskeletal dynamics as well, suggesting that Rho-family members cooperate in controlling these processes (Ridley, A. J. et al. 1992). The Rac GTPase was also implicated in regulating the phagocytic NADPH oxidase, which produce superoxide for killing phagocytized microorganisms (Abo, A. et al. 1991). Thus, it soon became clear that Rac/Rho and the related GTPase Cdc42 (cell division cycle 42) had central functions in many important cellular processes.

There are at least three types of regulators for Rho-like proteins. The GDP/GTP exchange factors (GEFs) which stimulates conversion from the GDP-bound form to the GTP-bound form. GDP dissociation inhibitors (GDIs) decrease the nucleotide dissociation from the GTPase and retrieve them from membranes to the cytosol. GTPase activating proteins (GAPs) stimulates the intrinsic GTPase activity and GTP hydrolysis. In addition there are probably regulators that dissociate GDI from the GTPase leaving it open for activation by the RhoGEFs.

Ras and Rho-family proteins participate in a coordinated regulation of cellular processes such as cell motility, cell growth and division. The Ral GTPase is closely related to Ras and recent studies have shown that this GTPase is involved in cross-talk between both Ras and Rho proteins (Feig, L. A. et al. 1996; Oshiro, T. et al. 2002). Ral proteins are not found in plants and they appear to be restricted to animalia and probably yeast. During a screen for small GTPases in *Drosophila melanogaster* I

discovered in 1993 several new members of the Ras-family, such as *Drosophila* Ral (DRal), Ric1 and Rap2. The functions of Ral GTPases in *Drosophila* have until recently been poorly known, but in paper 2 we present some of the new findings.

Rho-like GTPases have been identified in several eukaryotic organisms such as, yeast (Bender, A. et al. 1989), *Dictyostelium discoideum* (Bush, J. et al. 1993), plants (Yang, Z. et al. 1993), *Entamoeba histolytica* (Lohia, A. et al. 1993) and *Trypanosoma cruzi* (Nepomuceno-Silva, J. L. et al. 2001). In our first publication, (Winge, P. et al. 1997), we describe the cloning of cDNAs from *RAC*-like GTPases in *Arabidopsis thaliana* and show mRNA expressions pattern for five of the genes. The five genes analyzed were expressed in most plant tissues with the exception of *AtRAC2* (named *Arac2* in the paper), which has an expression restricted to vascular tissues. We also discuss the evolution and development of *RAC* genes in plants. The third publication, (Winge, P. et al. 2000), describe the genetic structure and the genomic sequence of 11 *RAC* genes from *Arabidopsis thaliana*. As most genomic sequences of the *AtRACs* we analyzed came from the *Landsberg erecta* ecotype and the *Arabidopsis thaliana* genome was sequenced from the *Columbia* ecotype, it was possible to compare the sequences and identify new polymorphisms. The genomic location of the *AtRAC* genes plus the revelation of large genomic duplications provided additional information regarding the evolution of the gene family in plants. A summary and discussion of these new findings are presented together with a general study of small Ras-like GTPases and their evolution in cellular organisms. This study suggests that the small GTPases in eukaryots evolved from two bacterial ancestors, a Rab-like and a MglA/Arp-like (Arf-like) protein. The MglA proteins (after the *mgl* locus in *Myxococcus xanthus*) are required for gliding motility, which is a type of movement that take place without help of flagella.

The second publication describes experiments done with the *Drosophila melanogaster* DRal gene and its effects on cell shape and development. Ectopic expression of dominant negative forms of DRal reveals developmental defects in eye facets and hairs, while constitutive activated forms affects dorsal closure, leaving embryos with an open dorsal phenotype. Results presented in this publication suggest that DRal act through the Jun N-terminal kinase (JNK) pathway to regulate dorsal closure, but recent findings may point to additional explanations as well. The results also indicate a close association between processes regulated by Rac/Rho and Ral proteins in *Drosophila*.

List of papers

1. Cloning and characterization of *rac*-like cDNAs from *Arabidopsis thaliana*. Winge P., Brembu T and Bones A. M. *Plant Molecular Biology*, **35**, 483-495, 1997.

2. The *Drosophila* Ral GTPase regulates developmental cell shape changes through the Jun NH_2-terminal kinase pathway. Sawamoto K, Winge P, Koyama S, Hirota Y, Yamada C, Miyao S, Yoshikawa S, Jin MH, Kikuchi A, Okano H, *Journal of Cell Biology*, **146** (2), 361-372, 1999.

3. Genetic structure and evolution of Rac-GTPases in *Arabidopsis thaliana*. Winge P., Brembu T., Kristensen R. and Bones A. M. *Genetics*, **156** (4), 1959-1971, 2000.

Introduction

The discovery of Rac proteins and their functions

The history behind the discovery of the Rac protein goes back to the mid 80's when researchers were studying the botulinum toxin from the bacterium *Clostridium botulinum*. These studies revealed that the light chain of botulinum toxin was able to ADP-ribosylate certain proteins (Simpson, L. L.1984). One of these proteins was later found to be actin (Aktories, K. et al. 1986; Reuner, K. H. et al. 1987; Vandekerckhove, J. et al. 1988). It was also noticed that in addition to the 40-43 kDa actin proteins, a smaller set of proteins in the 21-23 kDa size range were ADP-ribosylated by the botulinum toxin (Ohashi, Y. et al. 1987; Banga, H. S. et al. 1988; Morii, N. et al. 1988). Morii and co-workers then co-purified a guanosine 5'-(3-O-thio) triphosphate (GTP gamma S) binding activity with the ADP-ribosylation substrate and concluded that it was due to small GTP binding proteins. They also suggested a name for these proteins, Gb (b for botulinum), but the name was never widely used. Later it was shown that this protein fraction did in fact contain two types of small GTP-binding proteins: Rac and Rho. At the same time other researches studying GTP binding proteins in human neutrophils found small GTP binding proteins that could serve as substrate for ADP-ribosylation by botulinum toxin (Bokoch, G. M. et al. 1988). The nature of these proteins were further clarified when it was discovered that they cross-reacted with an anti-ras monoclonal antibody, and it was therefore assumed that the 22 kDa GTP binding proteins were Ras-like (Quilliam, L. A. et al. 1988).

In 1989 the first two human Rac genes was cloned and it was shown that they were good substrates for ADP-ribosylation by the C3 component of botulinum toxin (Didsbury, J. et al. 1989). Didsbury and co-workers were the first to use the name Rac for these proteins, after Ras-related C3 botulinum toxin substrate. Because of their relationship with the Ras proteins they were soon suspected to be regulators of signals from the cell surface and to regulate transport in the cell. Both of the human *rac* genes known at that time, *rac1* and *rac2*, were shown to be expressed in myeloid tissue, and in the coming years extensive studies of Rac proteins were done in myeloid cell lines (Abo, A. et al. 1991).

Evolution and origin of RAS-superfamily GTPases.

Nucleotide binding proteins, so called P-loop NTPases (ATP and GTPases) have one of the most common protein folds found in cellular organisms (Wolf, Y. I. et al. 1999;

Hegyi, H. et al. 2002). Based on the wide distribution and the conserved nature of the family members it has been suggested that P-loop NTPases were present in the last universal common ancestor (LUCA). The cellular functions of the P-loop NTPases are diverse and many are involved in processes such as; protein localization, chromosome partition, membrane transport, protein translation and signaling. A proposed division of the GTPases was recently put forward, where they were divided into two major classes, TRAFAC (after translation factors), and SIMBI (after signal recognition particle MinD and BioD) (Leipe, D. D. et al. 2002). These classes can be further subdivided into 20 distinct monophyletic sub-families. Among the TRAFAC class families are the Ras-like superfamily, the translation factor superfamily, the myosin-kinesin superfamily, the OBG-HflX superfamily and the TrmE-Era-EngA-YihA-Septin-like superfamily. Most proteins of the TRAFAC class are characterized by a conserved threonine or serine residue in loop 2 (AtRAC1 Thr^{38}), which make a hydrogen bond to the Mg^{2+} cation, and a serine residue in loop 10 (AtRAC1 Ser^{159}) involved in guanine base binding, but there are several exceptions. The phyletic distribution of the GTPases show that plants have representative genes in most subfamilies however plants lacks septins and the novel Rag GTPases. When compared to other GTPases the Ras-like superfamily is closest related to translation factors, (especial translation initiation factor IF-2), and may have evolved from them.

The extended Ras-like GTPase family

Several expressed sequence tag (EST) projects, which also include a number of protists, have deposited their sequence data to GenBank at NCBI. Although the sampling of organisms still is a little biased, these sequences gives us some indication of which Ras-like GTPases exist in cellular organisms and how they have evolved. Searching the available cDNA and genomic sequence in GenBank shows that the extended Ras-like GTPase family are found in all three superkingdoms and are greatly expanded in eukaryots. Table 1 shows the phyletic distribution in eukaryots of 20 distinct families from the Ras-like GTPases. From this table it is evident that a major expansion of this superfamily has taken place in Metazoa (vertebrates and invertebrates), Fungi and Mycetozoa. In particular the closely related families Ras, R-

Ras, Rap, Rheb, Ral, Rin/Rit and Gem/Rad have evolved and diversified within these organisms.

	Ver	Inv	Fun	Myc	Ent	Vir	Rhd	Eug	Alv	Dip	Str	Cry
Ras	X	X	X	X	X							
Rap	X	X	X	X	X							
Rheb/Rhes	X	X	X	X			X	X				
R-ras	X	X										
Ral	X	X										
Ric/Rit/Rin	X	X										
Gem/Rad	X											
Rac	X	X	X	X	X	X		X	X	X		
Cdc42	X	X	X									
Rho	X	X	X									
RhoBTB	X	X		X								
TC10/TCL	X											
Rab	X	X	X	X	X	X	X	X	X	X	X	
Tem1/Sid3			X			X				X		
Ran	X	X	X	X	X	X	X	X	X	X	X	X
Sar	X	X	X	X	X	X	X		X	X	X	
Arf/Arl/Arp	X	X	X	X	X	X	X	X	X	X	X	
Rag/Gtr	X	X	X	X				X			X	
"Dual GTPase"	X	X	X			X	X					
AP-GTPase	X	X		X		X						

Cellular organisms	Abb.	# Seq.	Representative Phylum/Sub-Phylum/Classes
Vertebrates	Ver	12260214	Mammalia, Actinopterygii
Invertebrates	Inv	1158659	Pseudocoelomata, Mollusca, Arthropoda,
Fungi	Fun	279724	Ascomycota, Basidiomycota
Mycetozoa	Myc	155884	Dictyostelium, Physarum
Entamoebidae	Ent	80794	Entamoeba
Viridiplantae	Vir	3230864	Embryophyta, Chlorophyta
Rhodophyta	Rhd	13801	Porphyra
Euglenozoa	Eug	159734	Leishmania, Trypanosoma
Alveolata	Alv	135387	Paramecium, Tetrahymena, Theileria, Plasmodium, Toxoplasma
Diplomonadida	Dip	2988	Giardia
Stramenopiles	Str	11328	Phytophthora, Phaeodactylum
Cryptophyta	Cry	615	Guillardia

Table 1. Proteins from each of the 20 families from the extended Ras-like GTPase family were used in BlastP and TBlastX searches against EST and genomic databases at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Homologues genes were identified and phylogenetic relationship assigned. Positive identification of a GTPase homologue is marked by a X. The number of sequences available for each kingdom / phylum is taken from the NCBI Taxonomy Database (September 2002), and the taxomomic division is largely based on the same database (Wheeler, D. L. et al. 2002). In addition genomic sequences from the *Giardia lamblia* genome at Woods Hole, Massachusetts, U.S.A, http://jbpc.mbl.edu/Giardia-HTML/index2.html, was included.

The table also shows that the Rheb family has the widest distribution in eukaryota. Interestingly Rheb-like genes are not found in Viridiplantae, but clear homologs are present in Rhodophyta, Euglenozoa, Mycetozoa, Fungi and Metazoa. This suggests

that Rheb is the founding member of this family group and consequently Ras, Rap, Ral and related gene families were created at a later stage through gene duplications. The functions of Rheb proteins are still largely unknown, but experiments with the yeast homologs have shown that they may regulate uptake of arginine and lysine (Urano, J. et al. 2000) and directly or indirectly affect cell cycle progression (Yang, W. et al. 2001). Prenylated human Rheb is also reported to bind the delta sub-unit of human cGMP phosphodiesterase (PDEδ), a protein structurally similar to RhoGDI (Hanzal-Bayer, M. et al. 2002). The lack of Rheb and Ras-like proteins in Viridiplantae suggests that the Rheb genes were lost in this clade and that "true" Ras proteins evolved just in Entamoebidae, Mycetozoa, Fungi and Metazoa. Alternatively both Rheb and the "true" Ras proteins were lost in Viridiplantae.

The Rab, Ran, Sar and Arf families, which regulate vesicle transport, nuclear transport, mitotic spindle assembly and cell signaling, are highly conserved and probably exist in all eukaryots. Similarly Rac-like proteins appear to be universal in eukaryots and the fact that they have not been detected in Stramenopiles, Rhodophyta and Cryptophyta is probably due to fewer genomic/EST sequences from these organisms. In addition the data indicate that the Rac-like genes have undergone more genetic drift and are less conserved than Rab, Ran and Arf genes.

The novel Rag/Gtr, Tem1/Spg1 and "Dual" GTPase families

Three novel GTPase families, the Rag/Gtr, Tem1/Spg1 and "Dual" GTPases (e. g. *Arabidopsis* At3g63150) all have a distinct phyletic distribution. Apart from the Tem1/Spg1 proteins, which have been thoroughly studied in yeast, the other GTPase family members are in general poorly characterized. The "Dual" GTPases (a temporary name) were discovered from sequencing projects and genome comparison studies (Leipe, D. D. et al. 2002) and has not been given an official name. As the temporary name implies these proteins are composed of two GTPase domains which are separated with a linker region with two calcium binding EF-hand motifs. The N-terminal GTPase domain is probably derived from a Rac-like ancestor while the C-terminal GTPase domain are distantly related to Rab. Database searches shows that Dual GTPases exist in Metazoa, Fungi, Rhodophyta and Viridiplantae, but is apparently missing in Mycetozoa, Entamoebidae and other protists.

Proteins within the Rag/Gtr family are highly conserved and are found in several eukaryots. However they have apparently been lost in Viridiplantae and are also missing in the genome of *Giardia lamblia* (Diplomonadida) (McArthur, A. G. et

al. 2000). The function of these GTPases is mainly known from experiments in yeast and the *Saccharomyces cerevisiae* Gtr1 gene was first identified as a suppressor of a temperature-sensitive mutant of RCC1, a Ran guanine nucleotide exchange factor (Nakashima, N. et al. 1996). This and later experiments indicate that Rag/Gtr proteins may be linked to processes regulated by the Ran GTPase such as, import and export of proteins or mRNA through the nuclear pore complex, or regulation of microtubule aster formation and spindle assembly (Ohba, T. et al. 1999; Bamba, C. et al. 2002). Even though Rag/Gtr proteins are highly conserved in eukaryots their absence in plants and *Giardia lamblia* suggest that their functions are redundant in some organisms.

The Tem1 (Termination of M-phase GTPase)/Spg1 (Septum-promoting GTPase) family has a more restricted distribution within eukaryots and so far they have just been identified in Fungi, Viridiplantae and Diplomonadida. This suggest that Opisthokonts (Metazoa and Fungi) and Amoebozoa (Mycetozoa and Entamoebidae) have lost this GTPase or that there has been a horizontal transfer of Tem1 from bikonts (ancestrally bicillate eukaryots) to yeast. The *S. cerevisiae* Tem1 protein was first identified as a suppressor of the cold-sensitive phenotype of lte1 mutant, a Ras-guanine nucleotide exchange factor, and were shown to be essential during exit from M-phase (Shirayama, M. et al. 1994; Lee, S. E. et al. 2001). Later studies have shown that the yeast Tem1 protein also interacts with Cdc15, a protein kinase regulating mitotic exit (Asakawa, K. et al. 2001; Visintin, R. et al. 2001). The Tem1 protein is regulated by the Bfa1/Bub2 GTPase activating proteins (Byr4/Cdc16 in *Schizosaccharomyces pombe*) and the Ras nucleotide exchange factor Lte1. Neither Bfa1/Bub2 nor Lte1 homologs are found in *Arabidopsis* and suggest that plant Tem1 homologs are regulated by other proteins. There are also indications that Tem1 proteins in yeast regulate different processes than their plant homologues. The yeast Tem1 proteins have for instance been linked to processes that regulate the actomyosin and septin dynamics during cell division (Lippincott, J. et al. 2001). There is a possibility that the plant Tem1 homologues regulate the actomyosin transport network, but as plants do not have septins and cell division in plants is not dependent on F-actin ring formation their function may be somewhat different. However, it is not unlikely that plant Tem1 homologues may regulate the assembly of the phragmoplast, after all, cytokinesis in plants probably have more in common with cell division in yeast and animals than previously thought (Molchan, T. M. et al. 2002; Guertin, D. A. et al. 2002). Another putative function is that Tem1 may regulate the actomyosin transport network during pollen tube growth and other processes that require polarized growth (Vidali, L. et al. 2001). Further indications that Tem1 may be linked to polarized growth, comes from its interaction with the two Kelch domain

proteins Kel1 and Kel2, which are localized to regions of polarized growth (Philips, J. et al. 1998; Hofken, T. et al. 2002). Hofken and co-authors have also suggested that Tem1 is dependent on the cell polarity proteins Cdc42, Cdc24 and Cla4 for targeting its exchange factor Lte1 to the bud cortex.

The AP-GTPase family

The AP-GTPase domain is distant related to the Rab and Rac GTPases and are found in multidomain proteins such as cyclic GMP-binding protein C from *Dictyostelium discoideum* and DAP kinase from humans (Aravind, L. et al. 2001; Goldberg, J. M. et al. 2002). In eukaryots the AP-GTPases are found in Metazoa, Mycetozoa and Viridiplantae, but due to the high diversity of this gene family its distribution is probably wider and may include other Kingdoms/Phylums. Little is known of the AP-GTPase domain but based on the domain architecture of the proteins it is found such as; protein kinases, cGMP-binding proteins and leucine rich repeat (LRR) proteins, indicate that it is part of various signaling networks. The function of the large *Arabidopsis* AP-GTPase is unknown, but its N-terminal part is distantly related to the tropomodulin domain. In mammalia tropomodulins are proteins that bind to the pointed end of actin filaments and modulate thin filament dynamics (Gregorio, C. C. et al. 1995; Weber, A. et al. 1999). This may indicate that the *Arabidopsis* AP-GTPase is another key factor involved in regulating the actin cytoskeleton.

Prokaryotic Rab and Arf-like GTPases

Recent years genome sequencing has shown that the evolution of eukaryotic Ras-like GTPases can be traced back to prokaryots (Leipe, D. D. et al. 2002). Analysis of the genome sequences shows that the prokaryotic GTPases closest related to the extended Ras-like proteins can be divided into three separate groups with representative members from both prokaryote super kingdoms (Archaea and Bacteria) see table 2. Prokaryotic Ras-like GTPases have so far not been found in Fibrobacter/Acidobacteria, Fusobacteria or Spirochaetes. The fact that many prokaryots are missing Ras-like GTPases show that these proteins are not part of the core of “nontransferable prokaryotic genes” (Kyrpides, N. et al. 1999; Nesbo, C. L. et al. 2001).

GTPase	Cr	Eu	Aq	De/Th	Pr	Ac	Fi	Cy	Ch	Ba/Ch
Rab-like					x			x	x	
MglA/Arp-like	x	x	x	x	x	x	x	x	x	
AP-GTPase		x						x		x

Table 2. Phyletic distribution of Ras-like GTPases in prokaryots. Identified GTPases are marked with x. Abbreviations: Cr (Crenarchaeota), Eu (Euryarchaeota), Aq (Aquificae), De/Th (Deinococcus/Thermus), Pr (Proteobacteria), Ac (Actinobacteria), Fi (Firmicutes / gram positive bacteria), Cy (Cyanobacteria), Ch (Chloroflexi), Ba/Ch (Bacteroidetes / Chlorobi group).

Prokaryotic MglA/Arp-like GTPases

The MglA/Arp-like (Arf-like proteins) proteins have the most extensive distribution among prokaryots and are probably the ancestors of the eukaryotic Sar/Arf/Arl/Arp families. Phylogenetic studies show that the Bacterial MglA/Arp proteins can be sub-divided into at least two groups the MglA-like and the Arp-like proteins. See phylogenetic tree figure 1. A clear division exists between Bacterial and Archaeal Arp-like GTPases suggesting that these genes have not undergone substantial horizontal gene transfer. Little is known about these proteins, but the *Myxococcus xanthus* MglA protein are known to regulate a special form of cell movement termed gliding motility, which is important during the development of fruiting bodies and surface colonization in the environment (Stephens, K. et al. 1989; Hartzell, P. et al. 1991). Bacterial gliding motility is defined as smooth translocation of cells over a surface by an active energy dependent process, which is achieved without help of flagella. Instead the bacteria rely on pilus extension and retraction for cell movement (Sun, H. et al. 2000). The gliding mobility regulated by MglA is therefore remarkably similar to processes regulated by Rho/Rac GTPases in eukaryotic cells. However, MglA is closer related to Arf/Sar proteins than Rac/Rho, and complementation experiments have shown that the eukaryotic Sar1 GTPase is able to rescue a sporulation defect *Myxococcus* MglA strain (Hartzell, P. L.1997). The MglA-like gliding mobility proteins (GMPs) are found in three major Bacterial phylums; Proteobacteria, Aquificae and Deinococcus-Thermus. MglB, the chromosomal neighbor to *Myxococcus xanthus* MglA, is related to the *Drosophila* Roadblock/*Chlamydomonas* LC7 proteins, which have been identified as one of the light chains of cytoplasmic dynein (Koonin, E. V. et al. 2000; Wilson, M. J. et al. 2001). It has also been suggested that MglB is a modulator or a putative release factor for MglA.

So far the function of proteins within the Arp group is unknown. However, one interesting observation is that the Arp group GTPases are found in several Actinobacteria and Cyanobacteria, which are known to posses sliding/gliding motility

and it is tempting to suggest that these proteins regulate similar processes as MglA (Martinez, A. et al. 1999; McBride, M. J.2001). The Arp-like GTPases may therefore be important during surface colonization in the environment or in hosts and biofilm development. Whether the MglA/Arp-like proteins regulate the assembly of filaments composed of the bacterial actin homolog MreB is still unknown, but type IV pilus biogenesis and motility in *Vibrio cholerae* may involve a MreB homolog (Marsh, J. W. et al. 1999; van den Ent, F. et al. 2001).

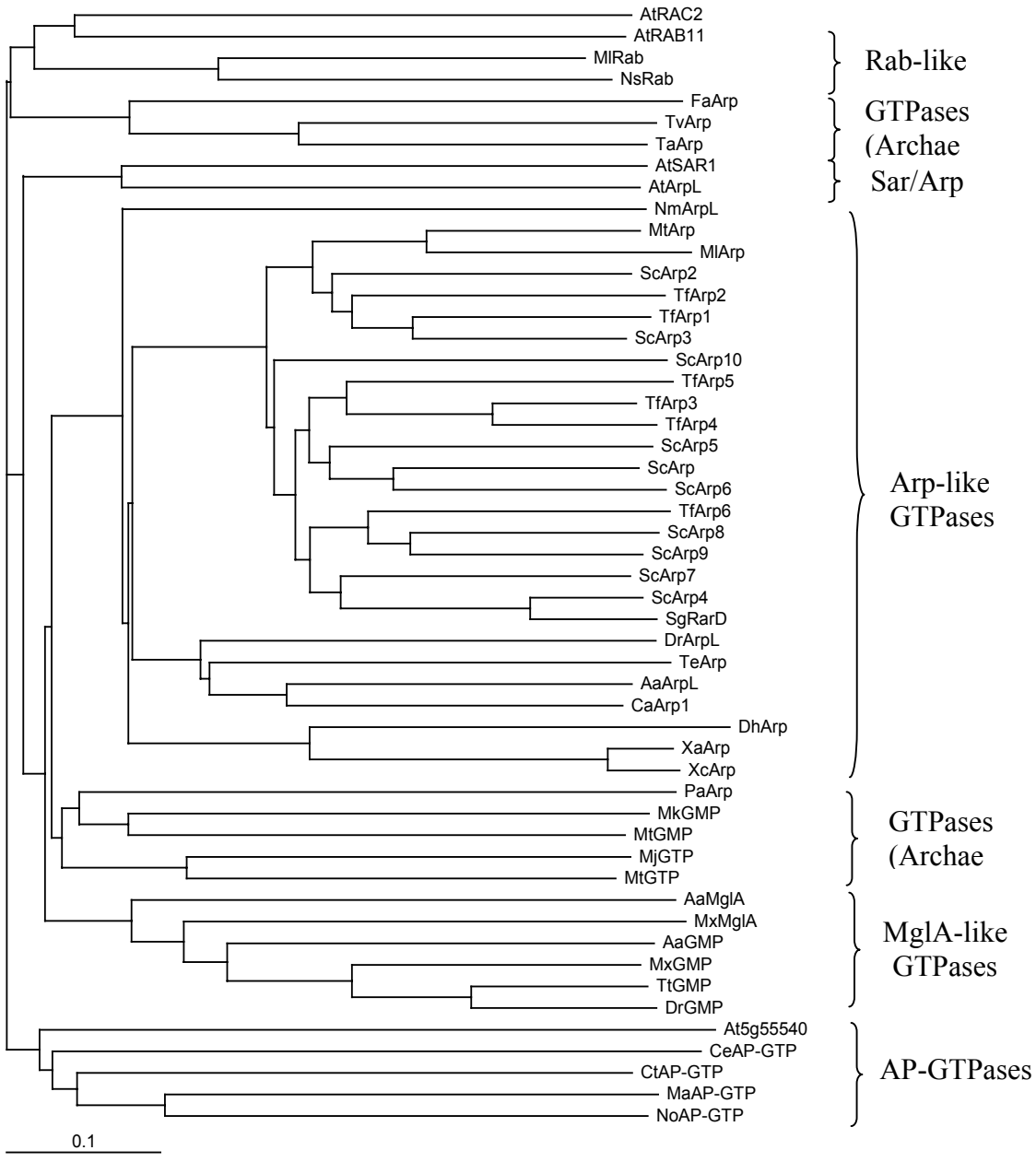

Figure 1. A protein alignment of small GTPases from prokaryots was made using the GeneDoc program (http://www.psc.edu/biomed/genedoc/), and a neighbor joining tree was produced with the ClustalX program. The scale bar indicates the number of amino acid substitutions pr. site. To show their relationship to eukaryotic

GTPases, Rac, Rab, Arf and Sar GTPases from plants and animals were included. Several unique groups of prokaryoyic GTPases can be observed from the figure. Abbreviations: Aa (*Aquifex aeolicus*), At (*Arabidopsis thaliana*), Ca (*Chloroflexus aurantiacus*), Ce (*Caenorhabditis elegans*), Ct (*Chlorobium tepidum*), Dh (*Desulfitobacterium hafniense*), Dr (*Deinococcus radiodurans*), Fa (*Ferroplasma acidarmanus*), Ma (*Methanosarcina acetivorans*), Mb (*Methanothermobacter thermautotrophicus*), Me (*Methanobacterium thermoautotrophicum*), Mj (*Methanococcus jannaschii*), Ml (*Mesorhizobium loti*), Mk (*Methanopyrus kandleri*), Mt (*Mycobacterium tuberculosis*), My (*Mycobacterium leprae*), Mx (*Myxococcus xanthus*), Nm (*Neisseria meningitidis*), Ns (Nostoc sp. PCC 7120), Pa (*Pyrococcus abyssi*), Sc (*Streptomyces coelicolor*), Sg (*Streptomyces griseus*), Ta (*Thermoplasma acidophilum*), Te (*Thermosynechococcus elongatus*), Tf (*Thermobifida fusca*), Tv (*Thermoplasma volcanium*), Xa (*Xanthomonas axonopodis*), Xc (*Xanthomonas campestris*).

Prokaryotic AP-GTPases

The prokaryotic AP-GTPases are distant related to Rab GTPases and are found in Cyanobacteria, Chlorobia (photosynthetic anaerobic green-sulfur bacteria) and Euryarchaeota. AP-GTPases in prokaryots have a conserved domain structure with N-terminal LRR motifs and a C-terminal AP-GTPase domain. A similar domain organization is also found in eukaryotic AP-GTPases, i. e. *C. elegans* LRR and ankyrin repeat protein T27C10.6, but their function are unknown. The possibility of horizontal gene transfer between eukaryots and prokaryots can not be ruled out, but the phyletic distribution of AP-GTPases suggest that they are an old gene family which have evolved independently in both prokaryots and eukaryots. Whether the prokaryotic LRR AP-GTPases has functions related to the bacterial internalins, LRR motif proteins involved in host cell invasions, are not known at present (Marino, M. et al. 2000).

Prokaryotic Rab-GTPases

While prokaryotic AP-GTPases share some similarities to Rab-GTPases, a novel group of Rab GTPases has just emerged as the most likely ancestors of eukaryotic Rab proteins (Winge unpublished). However, just four members of this protein family have been detected in prokaryots so the possibility of gene transfer from eukaryots to prokaryots can not be completely excluded. The existence of these GTPases in three different bacterial phylums, Cyanobacteria (acc. NP_487070.1, *Nostoc sp*. PCC 7120, acc. ZP_00109479.1, *Nostoc punctiforme*), Proteobacteria (acc. BAB50180.1, *Mesorhizobium loti*) and Chloroflexi (acc. ZP_00017677.1, *Chloroflexus aurantiacus*) suggest otherwise. Non of the prokaryotic Rab GTPases have been described and their function remains unknown. Analysis of the *Mesorhizobium loti* “Rab” operon

suggests that the MlRab protein is a regulator of bacterial motility, in this case as a regulator of flagellar motors (Winge unpublished). The MlRab operon encodes proteins with homology to flagellar motor proteins, a putative Rab interacting protein, ion transport proteins and adenylate/guanylate cyclases. In bacteria the flagellar motors are powered by the transmembrane gradient of protons or sodium ions and are dependent on a cAMP-catabolite activator protein (Lloyd, S. A. et al. 1999).

Like the MglA/Arp proteins the prokaryotic Rab proteins are among the smallest GTP binding proteins known with just 165-168 amino acids. In contrast to eukaryotic Rab proteins, which have a variable C-terminal part for membrane attachment, the 18 kDa bacterial Rab proteins lack this region.

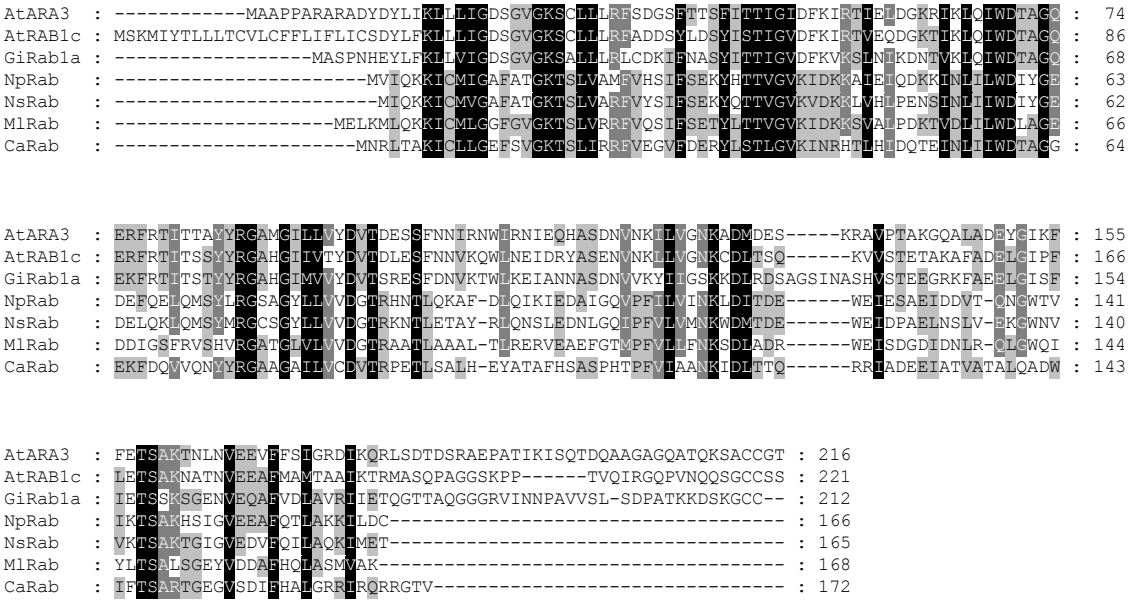

Figure 2. Rab GTPases from bacteria aligned with eukaryotic Rab homologues. Alignment was produced with the GeneDoc program. Eukaryotic Rab GTPases has a C-terminal extension that is post translationally modified to provide membrane association. From the figure it can be seen that prokaryotic Rab GTPases lack this variable part. AtARA3 (*Arabidopsis thaliana*, acc # AAK68735), AtRAB1c (acc # CAB10533), GiRab1a (*Giardia lamblia*, acc # AF183936), NpRab (*Nostoc punctiforme*, acc # ZP_00109479), NsRab (*Nostoc sp*. PCC 7120, acc # NP_487070), MlRab (*Mesorhizobium loti*, acc # BAB50180), CaRab (*Chloroflexus aurantiacus*, acc # ZP_00017677).

Sar and Arf GTPases also have a similar truncated C-terminal part, but in eukaryots both Sar and Arf proteins have instead evolved an N-terminal extension for membrane recruitment and activation (Huang, M. et al. 2001). Thus, prokaryotic Rab proteins have no domains indicative of membrane association and have a very compact globular structure.

Putative MglA/Arp interacting proteins

Prokaryots not only have Ras-like GTPases, like their eukaryotic counterparts they also have regulators of these proteins. The only regulators identified so far is putative nucleotide exchange factors, and interestingly they are found in prokaryotic phylums that are known to harbor MglA/Arp-like GTPases. For instance, the RalF gene from the alpha-proteobacterium *Legionella pneumophila* encodes an Arf guanine nucleotide exchange factor, Arf-GEF, that is required for the localization of ARF on phagosomes containing *L. pneumophila* (Nagai, H. et al. 2002). *L. pneumophilia*, best known as the agent of Legionnaires' disease, is an intracellular parasite that interferes with vesicle traffic in eukaryotic host cells to create a vacuole that supports its own replication. Even though large parts of the *L. pneumophilia* genome has been sequenced a MglA/Arp GTPase has not been identified. If found these GTPases may have important functions during surface colonization and could for instance be involved in biogenesis of type IV pili (Stone, B. J. et al. 1998; Mattick, J. S.2000).

Arf-GEF/Sec7-like proteins are also found in other alpha-proteobacteria such as *Rickettsia prowazekii*, an obligate bacterial parasite responsible for epidemic typhus. The function of the *Rickettsia* Arf-GEF is still unknown. Phylogenetic analysis of the bacterial Sec7/Arf-GEF proteins show that they constitute a distinct sub-group and suggest that eukaryotic Arf-GEFs have evolved from these proteins. Arf-GAP proteins on the other hand do not exist in prokaryotes and suggest that they have a high intrinsic GTPase activity or are regulated by other GAP-like proteins.

The evolution of Rac, Rho and Cdc42 proteins.

Despite the existence of nearly 100 complete prokaryotic genomes, no traces after the ancestors to Ran, Rheb/Ras, Tem1/Sid3, Rag/Gtr, Rac/Rho have been found. If Eukaryots arose through a symbiosis between an Archaea and Bacteria (Golding, G. B. et al. 1995; Lopez-Garcia, P. et al. 2001; Horiike, T. et al. 2001), these gene families must have evolved later through gene duplications of the ancestral prokaryotic GTPases. An alternative view proposed by (Hartman, H. et al. 2002) is that a third type of cell, the chronocyte, existed in parallel with Archaea and Bacteria. This “hypotetical” organism had a cytoskeleton, a complex internal signaling system and fed upon or lived in symbiosis with Archaea or Bacteria. The chronocyte

"hypothesis" is based mainly on the existence of a set of proteins, the so-called eukaryotic signature proteins (ESPs), which is unique for eukaryots. Among the 347 ESP proteins Hartman and colleagues identified are also the GTP-binding proteins, Ras, Rho/Rac, Arf, Ran and Rab {see table 2 (Hartman, H. et al. 2002)}. However, this hypothesis has several shortcomings and can not be verified until additional protist genomes are completed. As both Rab and Arf-like proteins exist in both Archaea and Bacteria they can for sure be removed from the ESP list.

If the symbiosis hypothesis for the origin of eukaryotes is correct, the Ran, Rheb/Ras and Rac/Rho families most likely evolved from a Rab-like ancestor through gene duplications. The origin of the Rag/Gtr and Tem1/Sid3 genes is more uncertain, but they are probably derived from the MglA/Arp or AP-GTPases. Phylogenetic analyses of the eukaryotic Rab proteins show that they had already developed distinct families such as Rab1, Rab2 and Rab11, before the major radiation of protists 1.5 – 2.0 billion years ago (Doolittle, R. F. et al. 1996; Pereira-Leal, J. B. et al. 2001). Even though Rac/Rho proteins have not been identified in Stramenophiles, Rhodophyta and Cryptophyta, the phyletic distribution of Rac/Rho proteins suggest that they exist in most eukaryots and were established as a distinct protein family before the division between bikonts and opisthokonts. In contrast to the Rab and Arf proteins, which diversified into distinct families before the bikont-opisthokont division, the expansion of the Rac/Rho family occurred later. The evolution of Rac/Rho proteins in eukaryots is therefore unique and just a few major sub-families such as Rac and Cdc42 have a broad phyletic distribution.

Rac-like proteins have been found in several unicellular organisms such as, Alveolates (*Paramecium tetraurelia*), Euglenozoa (*Trypanosoma cruzi*), Diplomonadida (*Giardia lamblia*), Entamoebidae (*Entamoeba histolytica*), Mycetozoa (*Dictyostelium discoideum*) and Fungi (*Ustilago maydis*), which suggests that Rac-like proteins are present in most eukaryotic organisms and that their functions are indispensable. However this may not be the case as Rac-like genes have not been identified in the Alveolates *Plasmodium falciparum* or *Plasmodium yoelii yoelii* genomes or in large collections of expressed sequence tags from these organisms (Gardner, M. J. et al. 2002; Cowman, A. F. et al. 2002). The loss of Rac-genes in the genus Plasmodium is also reflected by the absence of Rac interacting proteins / Rac-regulators and indicate that they were lost at an early stage in eukaryotic evolution, see Table 3. It must be noted Plasmodium, which has the capacity to move on solid substrate by gliding motility, does so without changing their shape (Menard, R.2001). Thus, in unicellular organisms with rigid cell walls Rac-like proteins may be dispensable and can even be completely lost in certain phylums/orders throughout evolution, such as in Plasmodium. An example of partial

loss of Rac/Rho genes comes from the Ascomycota phylum where certain yeasts such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have lost the Rac genes, but retained Rho and Cdc42 genes. In contrast, the rice blast fungus (*Pyricularia grisea*) and the dimorphic yeast *Yarrowia lipolytica*, which both belong to the same phylum as *S. pombe* and *S. cerevisiae*, the ascomycota phylum, still have Rac genes. Due to the recent discovery of Rac genes in fungi little is known of their functions in this kingdom, but results indicate that they may be tied to processes such as regulating polarized cell growth (Hurtado, C. A. et al. 2000).

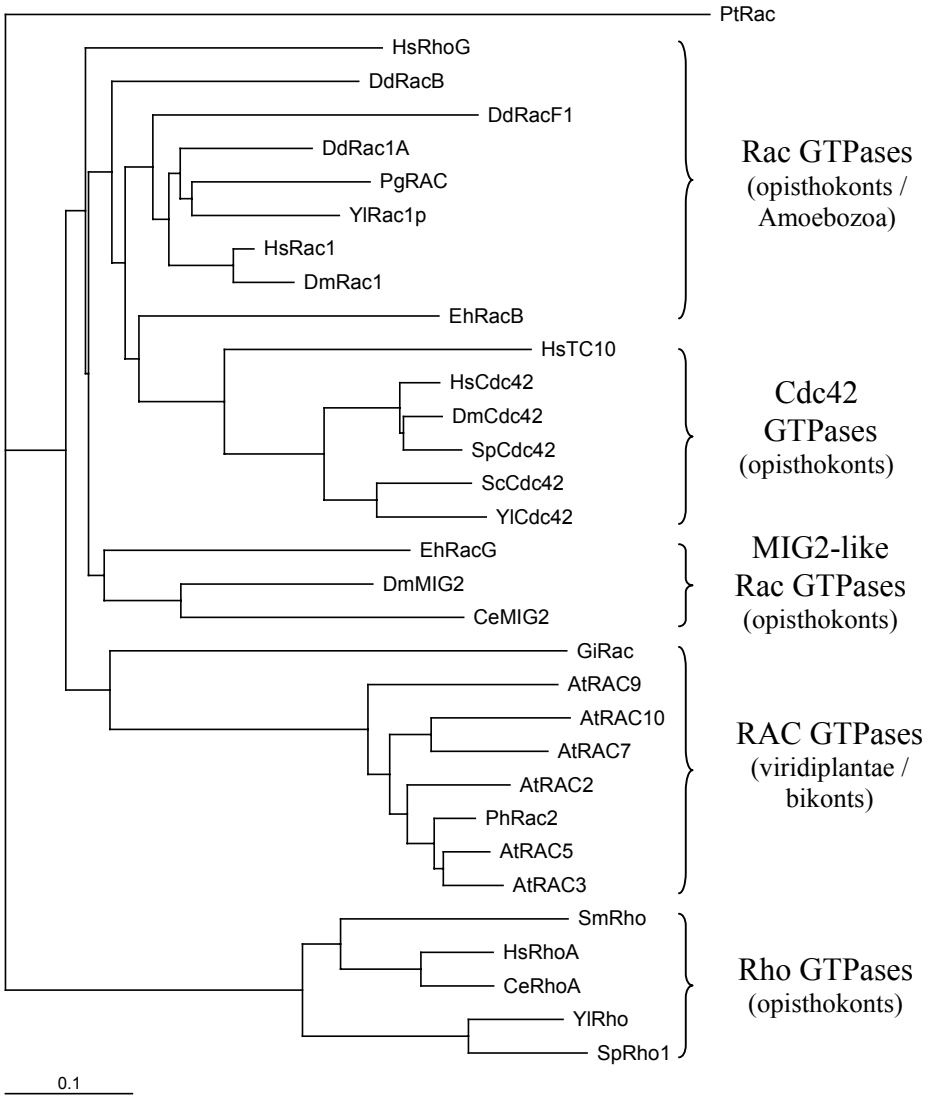

Figure 3. A protein sequence alignment of a selection of Rho family GTPase members was made with the GeneDoc program. The distance matrix was made with the Protdist program, using maximum likelihood estimates based on the Dayhoff PAM 120 matrix. An unrooted tree was constructed with the Fitch program from the Phylip program package, using Fitch-Margoliash and Least-Squares Distance Methods (Fitch, W. M. et al. 1967). Abbreviations: At (*Arabidopsis thaliana*), Ce (*Caenorhabditis elegans*), Dd (*Dictyostelium discoideum*), Dm (*Drosophila melanogaster*), Eh (*Entamoeba histolytica*), Gi (*Giardia lamblia*), Hs (*Homo sapiens*), Pg (*Pyricularia grisea*), Ph (*Physcomitrella patens*), Pt (*Paramecium tetraurelia*), Sc (*Saccharomyces cerevisiae*), Sm (*Schistosoma mansoni*), Sp (*Schizosaccharomyces pombe*), Yl (*Yarrowia lipolytica*).

Despite the fact that Rac-like proteins have evolved and diversified independently in various Kingdoms a phylogenetic analysis suggest that the ancestral "Rho-protein" in fact had more in common with present day Rac proteins, see phylogenetic tree, figure 3. The Rac genes found in Giardiinae, Viridiplantae, Dictyosteliida, Fungi and Animalia are well conserved and have undergone few changes, suggesting that a Rac-like gene was the founder of the "Rho family". The chances that Rho or Cdc42 could be the ancestral genes are remote, as they have so far only been identified in opisthokonts, in contrast Rac genes exist in Mycetozoa, Entamoebidae, bikonts and opisthokonts. Data therefore suggest that Rho and Cdc42 genes evolved from a Rac-like ancestor through gene duplications occurring after the bikont-opisthokont division and consequently they have a more narrow phyletic distribution. Several very divergent Rac/Rho-like proteins exist, but they appear to be fast evolving proteins restricted to distinct phylums/orders and can be excluded as founders of the "Rho-family".

The evolution and diversification of the Rac/Rho family have progressed in multicellular organisms and probably reflects the need for complex regulation of polarized cell growth, motility and other essential functions. As an example, *Drosophila melanogaster*, which has one of the smallest animal genomes sequenced so far, has 7 Rac/Rho-like proteins, while in humans the Rho GTPase family has expanded to at least 21 members. Interestingly, the Rac genes in some amoebae/slime molds have also undergone extensive duplications and at least 12 Rac genes are found in *Entamoeba histolytica* and 11 Rac genes have been identified in *Dictyostelium discoideum* (Rivero, F. et al. 2001). Both *Entamoeba* and *Dictyostelium* on the other hand are mobile organisms with the capacity to make filopodia and phagocytize particulate matter (Voigt, H. et al. 1999; Wilkins, A. et al. 2001). It is therefore not surprising that these organisms also have several Rac regulators and Rac binding proteins, see table 3. Other protists like *Giardia lamblia* (Diplomonadida) have only one Rac-like gene and few known Rac regulators.

	Ver	Inv	Fun	Myc	Ent	Vir	Rhd	Eug	Alv	Dip	Str	Cry
RhoGEF	X	X	X	X								
RhoGAP	X	X	X	X	X	X		X		X		
RhoGDI	X	X	X	X	X	X				X		
Crib	X	X	X	X		X						
Dock180	X	X	X	X		X						
ELMO	X	X		X		X		X				
Arfaptin	X	X										
Phox67	X											

Table 3. Phyletic distribution of Rac/Rho interacting proteins. The RhoGEF proteins are of the Dbl type. A positive identification of protein is marked with a x. Abbreviations: Ver (Vertebrates), Inv (Invertebrates), Fun (Fungi), Myc (Mycetozoa), Ent (Entamoebidae), Vir (Viridiplantae), Rhd

(Rhodophyta), Eug (Euglenozoa), Alv (Alveolata), Dip (Diplomonadida), Str (Stramenopiles), Cry (Cryptophyta).

The evolution RAC/ROP GTPases in Viridiplantae

Little or no information are available about the development of RAC-like proteins in Chlorophyta (algae) and no RAC-like genes have been identified among the 145,000 or so ESTs available from Volvocales, indicating that they either have a low expression or are missing altogether from this order. In addition, all RAC-interacting proteins, which are found in higher plants, are missing in *Chlamydomonas reinhardtii* (Volvocales). The loss of RAC-like genes and RAC-interacting proteins from some unicellular algae may not be an unusual situation. Processes such as polarized growth or cell movement by a dynamic actin cytoskeleton, which typically are regulated by Rac/Rho proteins, are less developed in algae, and living in an aqueous environment, algae rely instead on cilia and flagella for motility (Silflow, C. D. et al. 2001). Flagellar assembly is lost in most higher plants, the exception being Bryophyta which produce flagellated sperm cells for reproduction. We should however expect to find RAC-like proteins in some algae, in particular among the prasinophytes (Lemieux, C. et al. 2000; Turmel, M. et al. 2002). Likewise, the chances of finding RAC-like proteins or RAC-interacting proteins in Charophyta (giant algae) is high, due to their close relationship with land plants (Karol, K. G. et al. 2001).

Like many other multicellular organisms higher the RAC proteins in plants have diversified and produced distinct sub-families (Winge, P. et al. 1997; Li, H. et al. 2001). The diversification of the RAC/ROP family however is a relatively recent event and probably occurred during the emergence of vascular plants. Analysis of *RAC* genes in Bryophyta (*Physcomitrella patens*) show that the genes are almost identical, indicating that no distinct families of *RAC* genes had developed before the split between Bryophyta and Tracheophyta (Winge, P. et al. 2000). The bryophyte RAC proteins are closest related to AtRAC2, AtRAC3, AtRAC4 and AtRAC5, and suggest that this type of RAC protein more closely resemble the ancestral plant RACs (see phylogenetic tree Kitsch). Additional *RAC* sequence information from the Filicophyta (ferns), Anthocerotophyta (hornworts) and Marchantiophyta (liverworts) may resolve these questions.

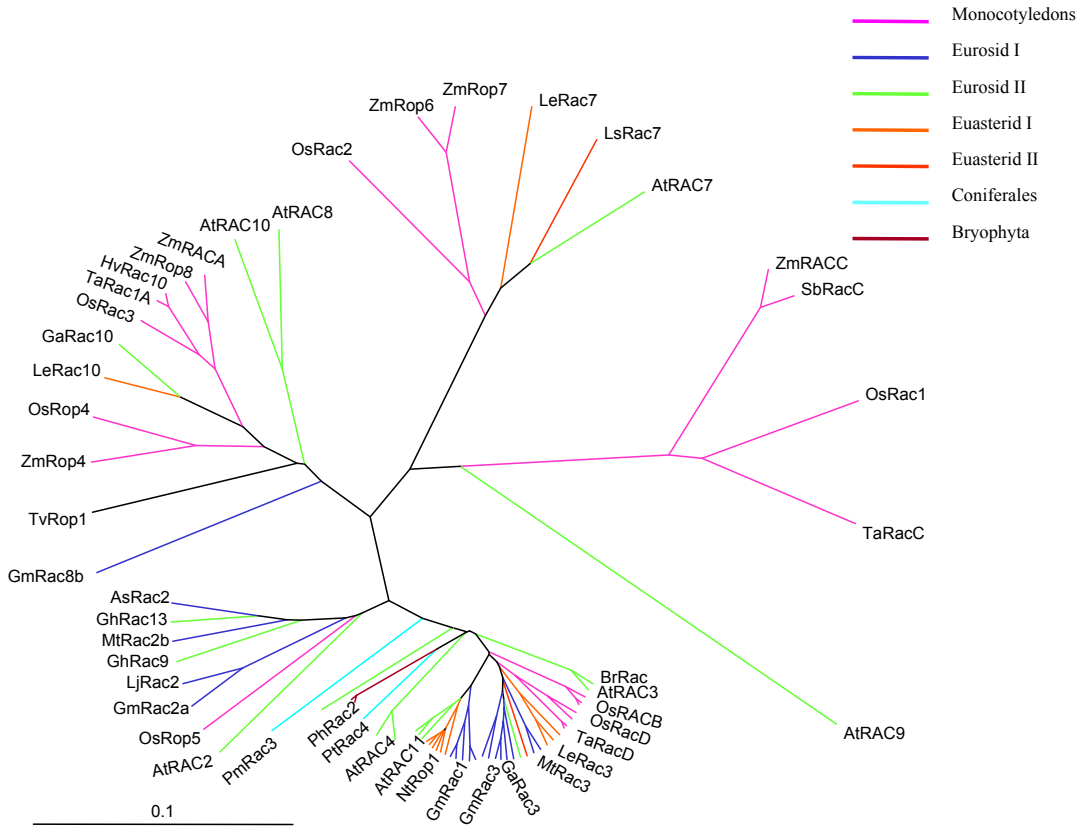

Figure 4. A protein sequence alignment of RAC GTPases from plants was made with the GeneDoc program. A distance matrix was made with the Protdist program, using maximum likelihood estimates based on the Dayhoff PAM 120 matrix. The unrooted tree was constructed with the Kitsch program from the Phylip program package, using Fitch-Margoliash and Least Squares Methods (Fitch, W. M. et al. 1967). Abbreviations: As (*Populus tremula* "Aspen"), At (*Arabidopsis thaliana*), Br (Brassica rapa), Ga (*Gossypium arboreum*), Gh (*Gossypium hirsutum*), Gm (*Glycine max*), Hv (*Hordeum vulgare*), Le (*Lycopersicon esculentum*), Lj (*Lotus japonicus*), Ls (*Lactuca sativa*), Mt (*Medicago truncatula*), Nt (*Nicotiana tabacum*), Os (*Oryza sativa*), Ph (*Physcomitrella patens*), Pm (*Picea mariana*), Pt (*Pinus tadea*), Sb (*Sorghum bicolor*), Ta (*Triticum aestivum*), Tv (*Tradescantia virginiana*), Zm (*Zea mays*).

Evolution of plant RAC genes through duplications.

In contrast to many other plant gene families where genes are duplicated in tandem, *RAC* genes in plants have expanded due to several large duplications, possibly even genome duplications (Winge, P. et al. 2000; Vision, T. J. et al. 2000; Blanc, G. et al. 2000). A phylogenetic analysis of *RAC* genes show that a major division occurred between 150-300 million years ago, resulting in the creation of new type of RAC protein, named by us as group II (or type II) RAC proteins. This event was caused by the addition of a new exon at the C-terminal of the protein and in this process destroying the existing prenylation motif CAAX. As RAC proteins are dependent on proper membrane association the loss of the prenylation motif was later somehow

compensated by one or two Cys residues encoded by the last exon, which can be modified with palmitate or other saturated fatty acids and provide membrane attachment (Lavy, M. et al. 2002). The new exon was caused by a mutation(s) that created a splice site donor in the 3rd base in the last codon before the stop codon and a splice acceptor (NAG) located further downstream, see figure 2 publication 3 (Winge, P. et al. 2000). This is a rare example where an exon has been added to the 3' of a GTPase without "deactivating" the protein. Genes belonging to *RAC* group II have not been found outside the Magnoliophyta (flowering plants), but it can not be excluded that they also exist in Gymnosperms (seed-bearing plants). Before the emergence of magnolids the type II RAC genes underwent an additional duplication, thereby creating the ancestors to AtRAC7 and AtRAC8/AtRAC10. Thus, both mono and dicotyledons have distinct proteins in both these groups, see phylogenetic tree with ESTs (color).

By analyzing the genomic regions which flanks *AtRAC8* and *AtRAC10* it is evident that they were created by a large chromosomal duplication, which now spans several hundred Kbp of *Arabidopsis* chromosome III and V, and consequently these regions share a significant co-linearity (macro-synteny), see figure 5. This recent duplication is probably restricted to the Brassicales order and a *AtRAC10* homologue has also been identified in *Brassica oleracea* (acc. # BZ027674). There is almost a complete lack of synteny between the chromosomal regions occupied by *AtRAC7* and *AtRAC8*/*AtRAC10*, but a close scrutiny reveals some anchor genes and micro-synteny see figure 5. Micro-synteny is also observed when regions flanking *AtRAC7* and *OsRac1*, a rice homologue, is compared. In fact co-linearity is better between *OsRac1* and *AtRAC7* than between *AtRAC7* and *AtRAC8*/*AtRAC10*, providing additional support for a division of type II RAC genes before the development of the magnolids.

Later in evolution another distinct RAC sub-family evolved in dicotyledons that now includes the *Arabidopsis* proteins AtRAC1/AtROP3, AtRAC6/AtROP5 and AtRAC11/AtROP1. Homologues belonging to this sub-group can be found in both Asterids and Eurosids but not in monocotyledons. Due to these relative recent duplications significant co-linearity is observed between the duplicated regions, see figure 7 (Winge, P. et al. 2000).

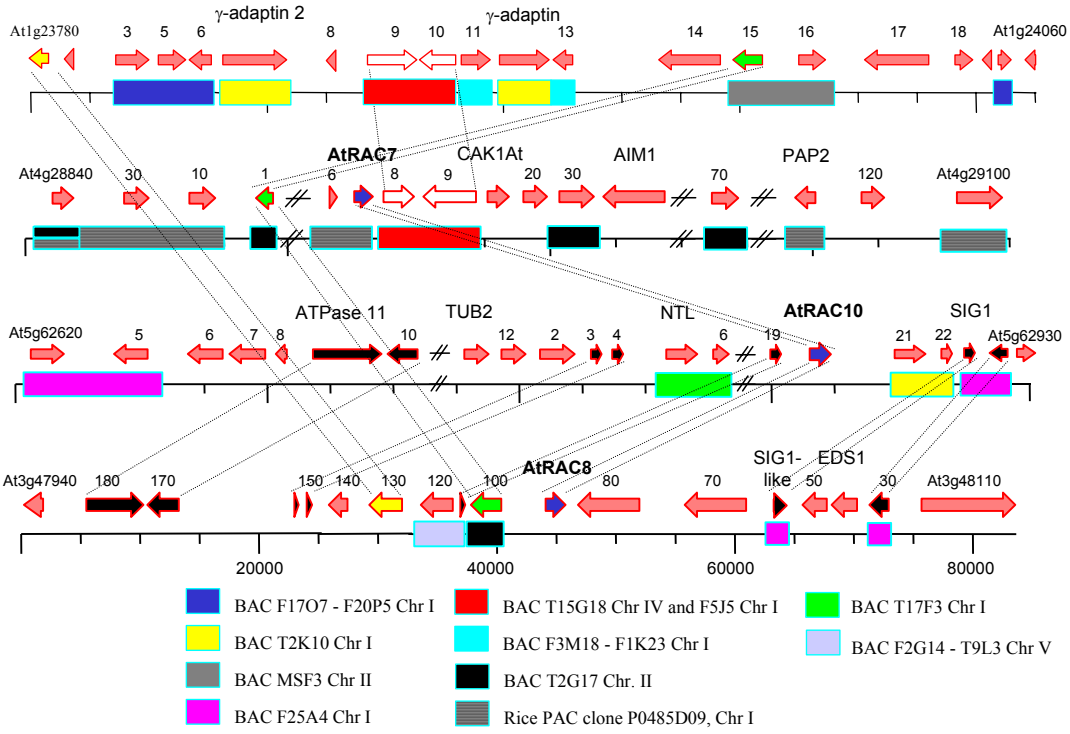

Figure 5. The mosaic structure of the *Arabidopsis* genome is revealed when duplicated genes are analyzed. The regions flanking *AtRAC7* (Chr IV), *AtRAC8* (Chr III), *AtRAC10* (Chr V) and a locus on Chr I were analyzed for orthologous/paralogous genes using BlastN, BlastP and TBlastN programs from NCBI. Homologous genes were identified and Bacterial Artificial Chromosomes (BAC) clones with positive hits identified. Colored boxes indicate other regions in the genome that have homology to genes localized next to the *RAC* genes. Several genes flanking *OsRac1* is also found next to *AtRAC7*.

Large chromosomal duplications or genome duplications are normally followed by massive rearrangement of the genome with additional deletions and duplications (Bancroft, I.2001; Simillion, C. et al. 2002). As a consequence it is often hard to find large genomic duplications in plants that are 200 million years or older. Analysis of synteny and gene homology between rice and *Arabidopsis*, which diverged some 150-200 million years ago, has clearly shown how co-linearity is gradually lost due to chromosomal rearrangements and transposon activity (Goff, S. A. et al. 2002; Salse, J. et al. 2002). Similar observations were reported when genomic regions of *Arabidopsis* and tomato, which diverged approximately 100 million years ago, was analyzed (Ku, H. M. et al. 2000). Thus, the duplications reported in paper 3 figure 7, involving *AtRAC1*, *AtRAC6*, *AtRAC11*, *AtRAC4* and *AtRAC5*, are easily recognized as they probably occurred less than 150 million years or so. An analysis of the *Arabidopsis* genome also reveals several chromosomal locations where *RAC* genes may have been deleted. For instance, a type II *RAC* gene

has probably been deleted from chromosome I (located between gene At1g23780 and At1g24060, see figure 5) and a homologue of *AtRAC11*/*AtROP1* has most likely been deleted from chromosome V (located between gene At5g66080 and At5g66140). An overview of possible regions where *RAC* genes may have been deleted are shown in figure 6. With more complete plant genomes available they can be used to follow the evolution of gene families such as RAC, and eventually they may be used to reconstruct ancestral plant genomes.

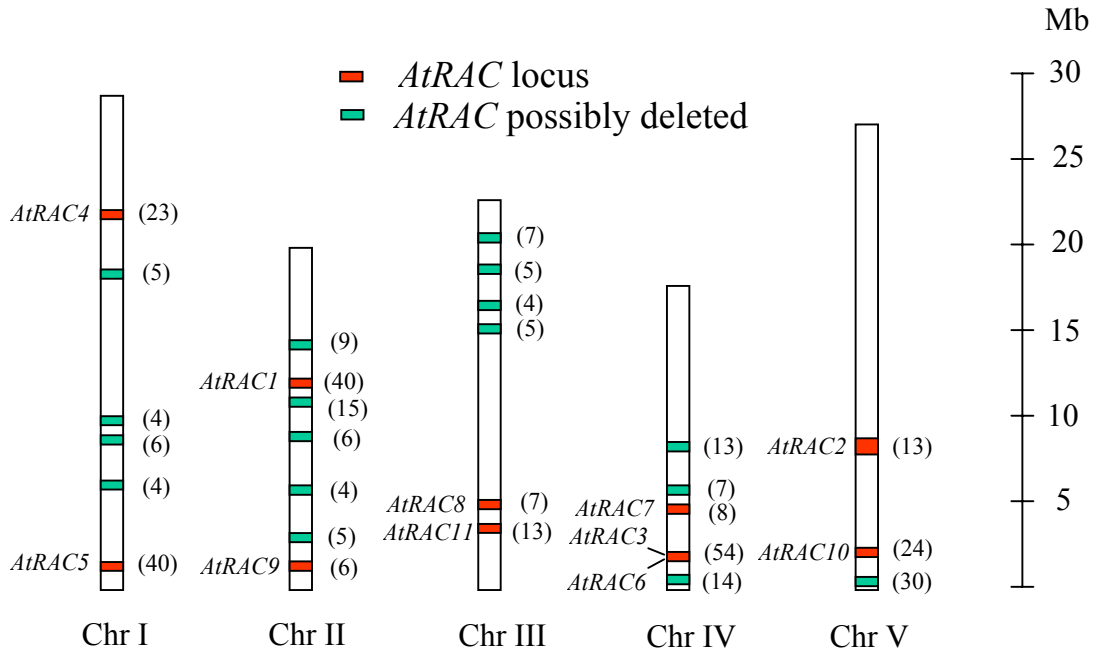

Figure 6. The figure shows the chromosomal localization of *AtRAC* genes and regions with co-linearity to *AtRAC* loci. Open reading frames found in approximately 200-300 Kb of sequence flanking the *AtRAC* genes were analysed with BlastN and TBlastN (NCBI), and used to identify other homologous regions in the genome. Genes with clear homology to *AtRAC* gene neighbours were scored and their genomic location determined. The numbers in parentheses indicate the number of genes with homology to genes flanking various *AtRAC* loci. As an example, the *AtRAC5* locus has at least 40 genes closely related to genes found next to other *AtRAC* genes, most of them due to an internal duplication of Chr I. High co-linearity and homology to AtRAC loci at the end of Chr IV and V suggest that a *RAC* gene may have been deleted there.

Ral GTPases.

Ral GTPases are members of the Ras superfamily and closely related to Ras and Rap proteins. The Ral gene was first discovered in 1986 in a screen for GTP binding proteins in lymphocytes and initial reports suggested that this protein was involved in processes related to vesicle transport (Chardin, P. et al. 1986; Ngsee, J. K. et al. 1990). A later study of GTP binding proteins in the electrode lobe of the marine ray

Discopyge ommata showed that Ral, Rho and the Rab protein Sec4 co-purified with cholinergic synaptic vesicles (Ngsee, J. K. et al. 1991; Volknandt, W. et al. 1993). A similar localization of Ral to synaptic vesicles was observed in mammalian axonal cells and point to related functions for Ral in animals (Bielinski, D. F. et al. 1993). Like most members of the Ras-superfamily the Ral protein is activated by guanine nucleotide dissociation stimulators, RalGDS/RalGEF, some of which is also active against p21 Ras (Albright, C. F. et al. 1993; Hofer, F. et al. 1994). As a consequence of this, it has been suggested that pathways regulated by Ral and Ras are tightly interconnected (Feig, L. A. et al. 1996). In addition, new results show that Ral proteins are directly linked to processes partly regulated by Rac and Rho proteins such as cell motility (Suzuki, J. et al. 2000; Oshiro, T. et al. 2002).

The Ral protein as a regulator of the exocyst

For many years the elusive functions of the Ral protein in vesicle transport were unknown, but recently a flurry of new papers have shed some new light on this subject. It now appears that Ral protein play an important role in the regulation of the exocyst (Sugihara, K. et al. 2002; Moskalenko, S. et al. 2002). The exocyst (Sec6/8 complex) is an octameric protein complex, which is localized to sites of polarized exocytosis and regions of active cell surface expansion. Vesicular transport to the plasma membrane is regulated at several steps and the exocyst has important functions in tethering and directing vesicles to the plasma membrane, for a review see (Novick, P. et al. 2002; Lipschutz, J. H. et al. 2002). This evolutionary conserved protein complex composed of the proteins SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84 was first discovered in *S. cerevisiae* (TerBush, D. R. et al. 1996) and later many of the exocyst proteins have been found in animals and plants. Recent results have shown that the GTPases Rho, Cdc42, Sec4 and Ral physically interact and regulate several members of the exocyst complex and there is also experimental evidence for cross talk between processes/pathways regulated by these GTPases.

The first indications that Ral proteins interacted with the exocyst complex came from protein interaction experiments with GTP-bound Ral (Brymora, A. et al. 2001; Polzin, A. et al. 2002). These experiments identified that GTP-bound Ral was associated with a protein complex containing Sec6 and Sec8 proteins. Further protein interaction studies and results from a yeast two-hybrid screen, where a double mutant of Ral was used as a bait, identified the N-terminal part of Sec5 as the real target for Ral (Sugihara, K. et al. 2002; Moskalenko, S. et al. 2002). Loss of Ral function

appears to destabilize the exocyst complex, inhibit regulated exocytosis and impede filopodia formation. The Ral protein may also regulate filopodia formation independent of the exocyst complex, and the activated Ral protein interact with the actin binding protein filamin (Ohta, Y. et al. 1999). Interestingly, Pak1, a Rac interacting kinase also binds filamin and influence the dynamics of actin cytoskeletal structures, suggesting that processes regulated by Rac and Ral are closely linked in animals (Vadlamudi, R. K. et al. 2002).

Other results that connect the exocyst with the processes regulated by Rho and Cdc42 comes from studies in yeast. During polarized growth the exocysts in yeast cells are located in the bud tip and are co-localized with Rho and Cdc42. These studies have shown that both Cdc42 and Rho1 in GTP-bound form interact with Sec3p and regulate the polarized location of the exocyst (Guo, W. et al. 2001; Zhang, X. et al. 2001). In addition the Sec15 protein interact with the Rab protein Sec4 (Guo, W. et al. 1999). Thus, four different types of GTPases have been implicated in the regulation of the exocyst. However there are some "uncertainties". Ral proteins do not exist in *S. cerevisiae*, the Sec3 protein is poorly conserved between yeast, animals and plants, and it is not confirmed that animal Rac, Rho or Cdc42 proteins bind Sec3-homologues. Indeed it is proposed that another of the exocyst components, Exo70, may serve the recruiting function of Sec3 in mammalian cells (Matern, H. T. et al. 2001). Genes encoding all the exocyst proteins are found in *Arabidopsis* and provide compelling evidence for the existence of an exocyst complex in plants as well. It is therefore not unlikely that cell polarity proteins like RAC/ROP in plants play essential roles in exocyst localization, like their cellular counterparts Rho and Cdc42 in yeast. In plants it is expected that processes that require polarized cell expansion, such as pollen tube and root hair growth, depend on the exocyst.

Ral function has also indirectly been linked to processes regulated by Rac and Cdc42. One of the first proteins which was shown to interact with activated Ral was RalA binding protein 1, RalBP1/RLIP76, which in fact is a RhoGAP protein (Cantor, S. B. et al. 1995; Park, S. H. et al. 1995). In addition one of the Ral nucleotide exchange factors, RalGEF2, has a C-terminal pleckstrin homology (PH) domain that suggest a co-localization with Rac, Cdc42 and Rho proteins (de Bruyn, K. M. et al. 2000; Lemmon, M. A. et al. 2002). PH domains are found on several cytoskeletal proteins, regulators of Rac, Cdc42 and Rho proteins and regulators of vesicle transport. The PH domain probably serves as a tag recruiting proteins to specific membrane regions where their interacting partners or substrates are located. Another binding partner of RalBP1, the Eps homology (EH) domain protein POB1, suggest that RalBP1 may have a regulatory function during endocytosis as well (Yamaguchi, A. et al. 1997; Kariya, K. et al. 2000).

The Ral protein have also been linked to vesicle transport through its interaction with a protein complex that includes Phospholipase D1 (PLD1) and the GTPase Arf (Jiang, H. et al. 1995; Luo, J. Q. et al. 1998). PLD1 is largely associated with secretory granules and co-localize at sites of exocytosis. Recent reports indicate that PLD1 has important functions related to vesicle fusion at the plasma membrane (Humeau, Y. et al. 2001; Choi, W. S. et al. 2002). Interestingly PLD1, which has a N-terminal PH domain, has been reported to interacts with Cdc42 (Walker, S. J. et al. 2000). This may indicate that the protein complex identified in vesicles composed of Ral, PLD1 and Arf (Arf6) physically interact with the exocyst or "prepare" the vesicle before docking at the plasma membrane.

The *Drosophila melanogaster* Ral GTPase.

The Ral GTPases probably evolved from a Ras or Rap-like ancestor through gene duplications and they are most likely restricted to animalia, yeast and/or amoebozoa, which developed Ras and Rap gene families at an early stage in evolution, see table 1. However, Ral proteins have so far only been reported from animalia. In the invertebrates, *C. elegans* and *Drosophila*, Ral exist as a single copy gene, while vertebrates have evolved two Ral genes, RalA and RalB. *Drosophila* has evolved two splice forms of Ral, DRal (acc. # U23800) and Dral2 (acc. # AY024366) that only differ in the membrane binding C-terminal part. Little is known of the function of Ral in Drosophila, but recent work show that it is coupled to processes that regulate cell polarity and probably work together with or in parallel with Rac and Rho GTPases.

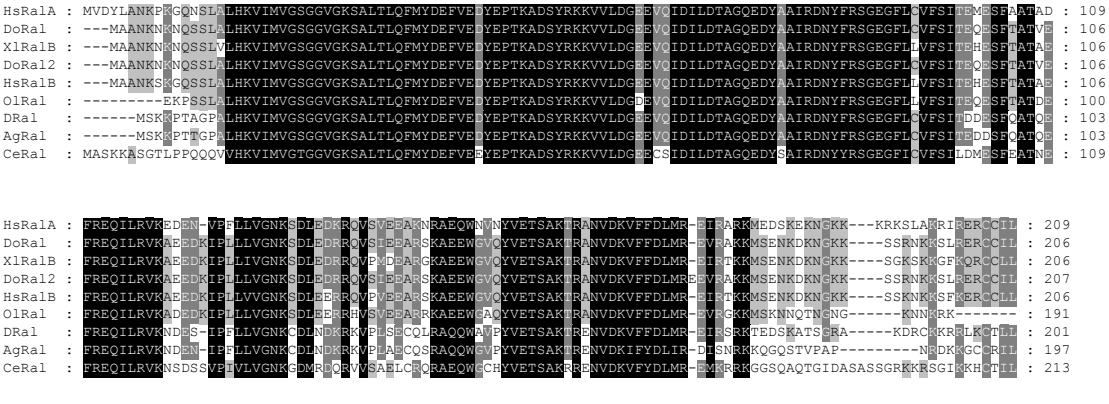

Figure 7. A protein alignment of Ral proteins from animalia produced with the GeneDoc program. Abbreviations; Hs (Homo sapiens), Do (*Discopyge ommata*), Xl (*Xenopus laevis*), Ol (*Oryzias latipes*, based on an EST), D (*Drosophila melanogaster*), Ag (*Anopheles gambiae*), Ce (*Caenorhabditis elegans*).

Functions of Ral during *Drosophila* eye facet development

The compound eye of *Drosophila melanogaster* is composed of about 800 similar facets or ommatidia and has been used as a model system for studying cell polarity and morphogenesis (Reifegerste, R. et al. 1999; Adler, P. N.2002). In wild type *Drosophila* each ommatidia contain eight photoreceptors, which are arranged in a distinct pattern. Several ommatidia mutants have been described and proteins such as the receptor Frizzled (Fz), its ligand Wingless (Wnt) and the signal transducer Dishevelled (Dsh) are known to regulate a pathway that affect the development of the eye facet. Recently Rac and Rho proteins have been implicated in establishing the polarity of ommatidia and are probably downstream of Dsh in the Fz pathway (Fanto, M. et al. 2000). Transient expression of a dominant negative Rac1 resulted in ommatidia with planar-polarity defects and an activated Rac1 produced many misoriented ommatidia.

To study the processes regulated by Ral constitutive active and dominant negative forms of Ral have been expressed in *Drosophila*. Ectopic expression of an activated form of human RalA, which is 71% identical with DRal, results in disruption of the ommatidial organization and the development of a rough eye phenotype (Sawamoto, K. et al. 1999b). The observed changes with constitutive activated Ral, such as irregular orientation of ommatidia, are reminiscent of activated Rac1 and suggest that Ral may co-operate with Rac1 during establishment of cell polarity (Fanto, M. et al. 2000). Furthermore, Sawamoto and co-workers reported that activated human RalA affects the organization of the actin cytoskeleton, and constitutively activated RhoA acts synergistically with activated Ral producing flies with a severe rough eye phenotype.

Expression of mutated forms of DRal affects not only the ommatidia but also influence the development of various tissues in *Drosophila*. For instance, expression of activated DRal in embryonic epidermis results in lethality and some embryos have a severe dorsal-open phenotype (Sawamoto, K. et al. 1999a). Dorsal closure involves coordinated changes in the shape of epidermal cells stretching along the dorsal-ventral axis such that the two lateral epidermal cell sheets slide over the underlying epithelial layer and eventually meet at the dorsal midline to close the dorsal side of the embryo. Dorsal closure does not require new cell divisions and is largely driven by cytoskeletal changes regulated by proteins such as Rac and Rho. Thus, RhoA mutants and downstream effectors of Rac such as the c-Jun N-terminal kinases (JNKs) are known to induce various defects during dorsal closure (Strutt, D. I. et al. 1997; Lu, Y. et al. 1999; Harden, N.2002). Defects during dorsal closure are also produced by *canoe*^{3}, an embryonic lethal allele of *cno*. The *cno* gene encode a PSD-

95/Dlg/ZO-1 (PDZ) domain protein, which is involved in the JNK pathway, and it is reported that some *cno* alleles produces flies with rough eyes (Takahashi, K. et al. 1998). Another gene connected to the Rac pathway in *Drosophila*, myoblast city (*mbc*), cause similar defects in dorsal closure when inactivated (Erickson, M. R. et al. 1997). Later studies have shown that MBC, a homologue of C. elegans CED-5 and human DOCK180, is a unique guanine exchange factor with specificity for Rac GTPases (Brugnera, E. et al. 2002), see "chapter": Rho guanine nucleotide exchange factors, RhoGEFs page X.

Regulation of bristle and hair development by DRal

Drosophila hair and bristle development provides a unique model system to study polarized cell growth and the actin cytoskeleton (Adler, P. N.2002). The 5000 bristles that protrude from the cuticle of an adult *Drosophila* function as either mechanosensors or chemosensors, and are organized in distinct patterns. Sense organ precursors (SOPs)", where the first asymmetric division depends on Frizzled function, differentiate into the ciliated neuron and supporting cells of bristles. Each sense organ consists of four cells, the neuron, the sheet, the tormogen (socket forming cell) and the trichogen (shaft forming cell). Structural studies have shown that bristles have an actin cytoskeleton where actin filaments possess unidirectional polarity and are cross-linked by multiple bridging proteins (Tilney, L. G. et al. 1995; Tilney, L. G. et al. 2000). It is therefore not surprising that *Drosophila* mutants affecting actin binding proteins and proteins that regulate actin cytoskeletal dynamics often produce defects during bristle development (Verheyen, E. M. et al. 1994; Hassan, B. A. et al. 1998; Wahlstrom, G. et al. 2001). For instance, studies of mutant forms of Rac and Cdc42 have shown that they affect the development of wing hairs in *Drosophila* (Eaton, S. et al. 1996). Although the function of DRal was unknown it was assumed that expression of mutated versions of DRal in *Drosophila* produced various bristle and hair phenotypes, due to connections between Ral and Rac pathways in other organisms.

Results show that over expression of wild-type DRal in *Drosophila* does not cause any visible phenotype, whereas a dominant negative DRal mutant cause defects in the development of various tissues such as wing hairs and sensory bristles (Sawamoto, K. et al. 1999a). Dominant negative DRal proteins expressed in developing wings produced duplicated and forked hairs, which often were shorter than normal. On the other hand, expression of dominant negative DRal resulted in loss of bristles on the nota, probably by interfering with shaft initiation, see figure 4 in

paper 2. This phenotype could be rescued by co-expressing wild type DRal protein, see figure 7 paper 2. Further experiments showed that the loss of bristle phenotype could be suppressed when DRal dominant negative flies were crossed with flies having alleles of *basket* (*bsk*) or *hemipterous* (*hep*), which encodes a JNK and JNKK respectively, see figure 8 publication 2. These two protein kinases DJNK and DJNKK are part of a mitogen activated protein kinase, MAPK, pathway in *Drosophila* that are required for epithelial cell shape changes during dorsal closure (Martin-Blanco, E.1997; Agnes, F. et al. 1999).

How the *Drosophila* JNK pathway is able to interfere and suppress the bristle phenotype caused by a dominant negative DRal protein is unknown, but the JNK pathway is known to be regulated by Rac and Cdc42 proteins (Coso, O. A. et al. 1995; Noselli, S.1998; Fanto, M. et al. 2000). It is also conceivable that some of the phenotypes induced by mutated forms of DRal are mediated through its functions as a regulator of the exocyst. One possibility is that loss of Ral function can destabilize the exocyst complex and thereby prevent directed exocytosis, which in turn interfere with shaft initiation and cell expansion.

The development of bristles and hair in *Drosophila* probably has some common characteristics with trichome and hair development in plants. Both plants and flies rely on the actin cytoskeleton and its regulating proteins for developing normal hair, bristles and trichomes (Dickinson, W. J. et al. 1997; Wasteneys, G. O.2000; Bouyer, D. et al. 2001). Furthermore, processes regulated by the Rac pathway have been implicated in development of hair cells and trichomes in both *Drosophila* and *Arabidopsis* (Eaton, S. et al. 1996; Qiu, J. L. et al. 2002). For instance, the phenotypes of the *Arabidopsis* trichome mutant SPIKE1 is caused by an inactivation of a gene encoding a homologue of human Rac/Cdc42 activator Zizimin1, which is related to the RacGEFs CED-5 and DOCK180. Another *Arabidopsis* trichome mutant, *ZWICHEL*, may in similar ways be tied to the Rac pathway through its FERM domain, which may function as a RhoGDI dissociation factor (Bretscher, A.1999; Hamada, K. et al. 2001; Edwards, S. D. et al. 2001). Most studies so far have indicated that *ZWICHEL*, which encode a kinesin like protein, is a microtubule motor protein and therefore just involved in microtubule linked processes, however this may not be its sole function (Mathur, J. et al. 1999).

Results from studies of trichome mutants in *Arabidopsis* as well as bristle and hair mutants in *Drosophila* suggests that these model systems will be valuable tools for studies of the Rac/Ral pathways and cytoskeletal dynamics in general. However, the lack of Ral proteins and many known Ral interacting in plants suggest that there will also be many differences to be identified in these two model organisms.

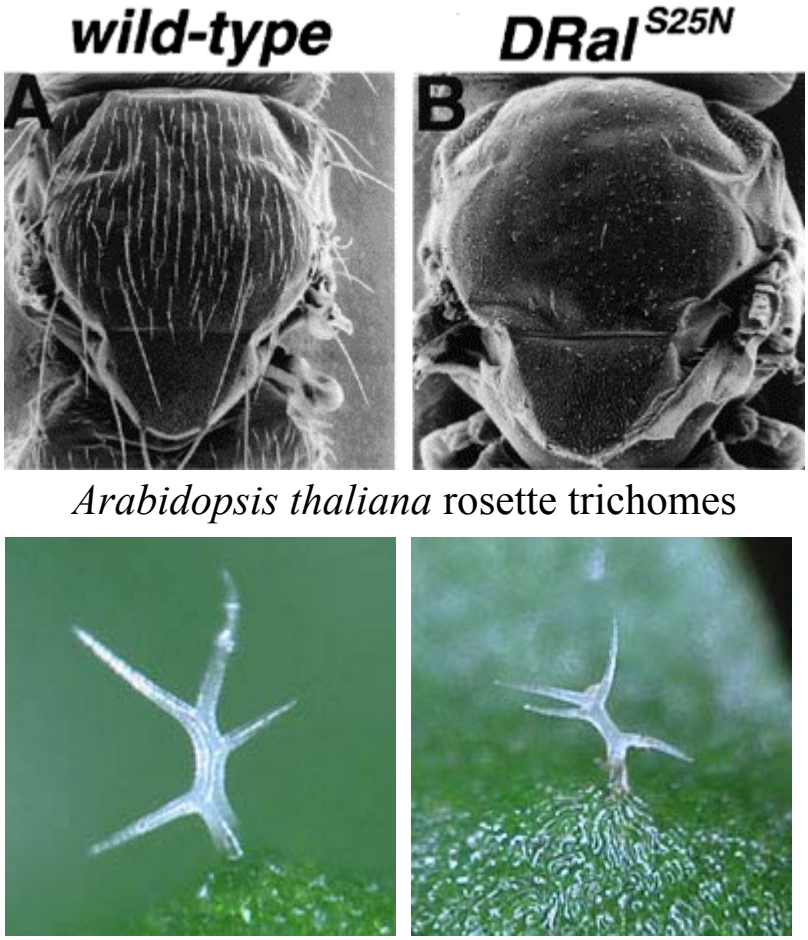

Figure 8. The top figures, A and B shows the effect of expressing the dominant negative DRal mutant on the development of adult nota in *Drosophila*, (taken from figure 4 in publication 2). The two bottom figures show unusual trichomes from an *Arabidopsis* rosette leaf expressing an antisense *AtRAC* gene.

Rac proteins and the oxidative burst.

In animal cells the Rac1 and Rac2 proteins have proved to be an obligatory participant in the activation of the NADPH oxidase that generate superoxide. In animals this multi-component complex is composed of two membrane bound proteins containing cytochrome b_{559}, ($p22^{phox}$) and $p91^{phox}$ in addition to the cytosolic components, Rac, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ that is necessary to activate the oxidase (Segal, A. W. et al. 1993). In resting cells the enzyme complex is latent, but upon activation of various stimuli the cytosolic components translocate to the plasma membrane and activate the NADPH oxidase complex. In phagozytes this process is often described as an oxidative burst (Knaus, U. G. et al. 1991). This activation is in

part regulated by the Rac protein and both in vitro and in vivo studies indicates that $p67^{phox}$ is the principal target of activated Rac protein (Prigmore, E. et al. 1995). One function of Rac may therefore be to recruit the cytosolic $p67^{phox}$ to the plasma membrane. A similar mechanism for membrane targeting has been reported for the protein kinase Raf, which is translocated to cell membrane by the Ras protein (Leevers, S. J. et al. 1994). Another possibility is that Rac regulate polarized exocytosis and thereby regulate transport and localization of the membrane bound NADPH oxidase or components regulating its activity.

Of the cytosolic components known to regulate the NADPH oxidase in mammals just RAC/Rop proteins are found in plants. However, ten NADPH oxidase related proteins have been identified in *Arabidopsis thaliana* (Sagi, M. et al. 2001). The production of reactive oxygen species (ROS) in plants may have various functions, during plant defense against microorganisms and during development (Levine, A. et al. 1994; Kurek, I. et al. 2002; Potikha, T. S. et al. 1999; Yang, Z.2002).

The 3D structure of G-proteins.

All members of the G-proteins share a common structural core consisting of a central six-stranded β-sheet surrounded by α-helixes (Jurnak, F.1985).(Sprang, S. R.1997a) This three dimensional structure, also called the G protein fold, is a variation of the more general nucleotide binding fold found in proteins such as myosin, ATPases and others. From X-ray crystallographic studies the three-dimensional structures of several small GTPases have been solved. They include the proteins: Ha-Ras (Pai, E. F. et al. 1989), Rac1 (Hirshberg, M. et al. 1997), Cdc42 (Rudolph, M. G. et al. 1999), RhoA (Wei, Y. et al. 1997; Rittinger, K. et al. 1997b), Ran (Scheffzek, K. et al. 1995), Rap1a (Nassar, N. et al. 1995), ARF-1 (Goldberg, J.1998), Rab3A (Dumas, J. J. et al. 1999), Sar1 (Huang, M. et al. 2001) and the 3D structures a number of other GTP binding proteins such as EF-Tu and G_i are known. The nucleotide-binding site is defined by five polypeptide loops, G-1 to G-5, which are highly conserved among all G-proteins. The G-1 loop (also called the P-loop) is the diphosphate-binding loop that interact with the α- and β-phosphates of the GDP/GTP molecule while the guanine ring is recognized by residues in the G-4 loop, figure 9. A conserved threonine residue in the G-2 loop coordinate the interaction with a Mg^{2+} ion that is essential for GTPase activity and conformational stability while an Asp residue in the G-3 loop forms the GTP γ-phosphate binding site.

The conformational changes that occur when the GTPase switches between GDP and GTP binding forms have been studied in detail for the Ras protein and have shown that they primarily affect two parts of the protein termed switch I and switch II (Milburn, M. V. et al. 1990). The switch I region which include the G-2 loop, also known as the effector region, are a flexible part of the protein that mediate interaction with other proteins. The switch II region, which includes the G-3 loop plus the α2 helix is flipped out and is more exposed when the protein is in the GTP binding form.

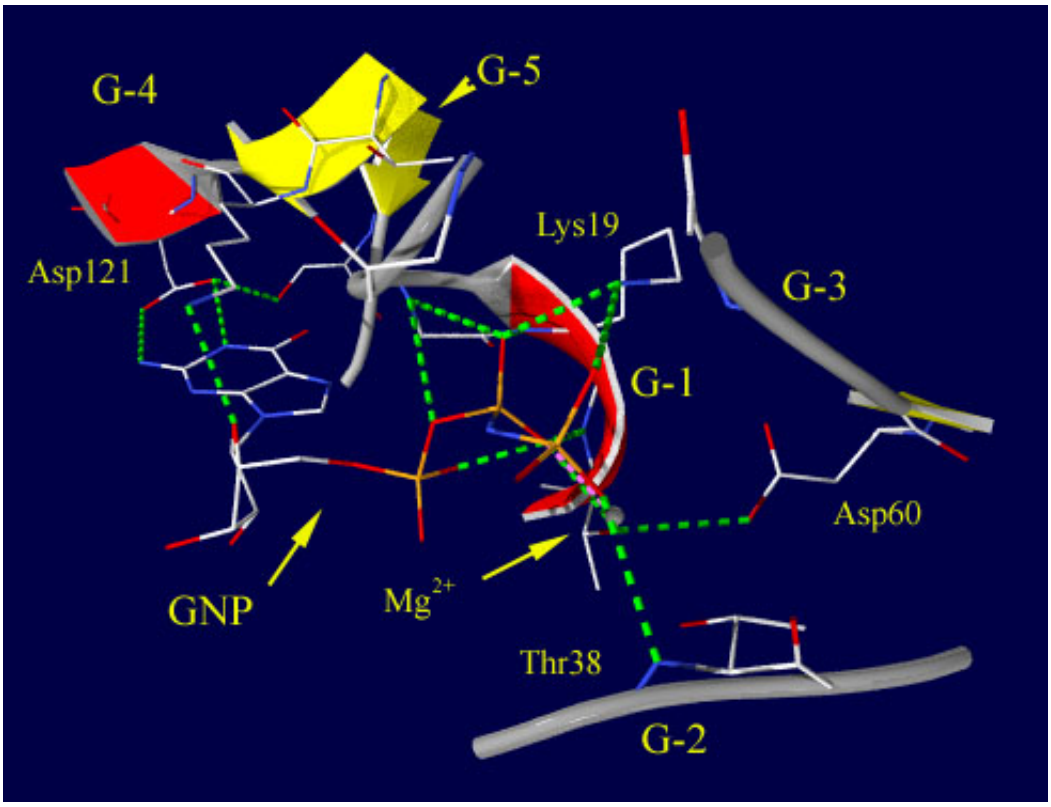

Figure 9. An overview of the nucleotide binding pocket from a Rac GTP binding protein. Green lines indicate hydrogen bonds between the GNP analogue and key residues in the protein. The figure was made with the GlaxoSmithKline Swiss-PdbViewer v3.7 (Guex, N. et al. 1997), and visualized with the raytracer program, Pow-Ray™ version 3.1g (http://www.povray.org/).

Thus, the conformational changes induced by GTP binding enable the GTPase to adapt a conformation, which enables interaction with other proteins such as GAPs, GEFs and various effector proteins. It appears that for most protein interactions both switch I and II regions are necessary for correct complex formation as shown for the Ras : Ras-GAPs complex (Scheffzek, K. et al. 1996). NMR studies of the nucleotide binding region of Ras have shown that switch I and II are dynamic regions capable of undergoing rapid conformational changes thereby confirming the crystallographic data (Kraulis, P. J. et al. 1994).

In the GTP-bound form the G-proteins adopt a confirmation that facilitate GTP hydrolysis. The mechanism of GTP hydrolysis by Ras-like GTPases has been analyzed in detail and shows that a Gln residue in the G-3 loop together with a Thr in the G-2 loop, both highly conserved in most GTPases, activate a water molecule for nucleophilic attack on the γ-phosphate in the GTP molecule (Maegley, K. A. et al. 1996; Sprang, S. R.1997b). Except for the heterotrimeric G_α sub units, the intrinsic GTPase activity of most Ras-like GTPases are low. Most Ras-like GTPases therefore bind GAP proteins which increase the GTPase activity apparently by stabilizing a conformation mimicking the transition state of the GTPase reaction (Scheffzek, K. et al. 1996). In crystal structure studies of Ras:RasGAP1 it has been shown that the enhanced GTPase activity is due to an conserved arginine of GAP, (part of the so called arginine-finger motif), which is placed close to the active site of Ras (Ahmadian, M. R. et al. 1997; Scheffzek, K. et al. 1997). Comparisons of RasGAP and RacGAP/RhoGAP proteins show that this Arg residue is universally conserved (Barrett, T. et al. 1997) also among the plant RacGAP proteins (personal observations). This indicates that GAP proteins enhance the GTPase activity in GTPases through a common mechanism.

Structure of Rac, Cdc42 and Rho GTPases.

The three-dimensional structures of the human Rac1, Cdc42 and RhoA GTPases have recently been solved through X-ray crystal analysis, (Hirshberg, M. et al. 1997; Rudolph, M. G. et al. 1999) (Wei, Y. et al. 1997) (Ihara, K. et al. 1998) and have revealed that their structures are highly similar to Ras, figure 10. All these proteins consist of a central six-stranded β-sheet that is surrounded by loops and helices, which form most of the surface of the protein. The Rac and Rho proteins also have regions that correspond to switch I and switch II in Ras, but from the crystal structures the switch I region appear less exposed than in the Ras proteins (Hirshberg, M. et al. 1997). The conformational changes that take place when the Rho-family proteins cycle between GDP and GTP bound forms are also less extreme than what is seen for the Ras proteins (Hoffman, G. R. et al. 2000b). Like most proteins belonging to the Ras-superfamily, the Rac and Rho proteins have a flexible C-terminal part that tether them to the membrane through a prenylated C-terminal Cys residue. When these GTPases are not complexed with interacting partners this C-terminal part is highly flexible and most crystal structures of Ras and Rho-like GTPases are deduced from truncated proteins lacking the C-terminal part. When complexed to the various

interacting proteins there is however indications that the C-terminal part adopts a well defined conformation (Hoffman, G. R. et al. 2000b).

When the structures of the Rac and Rho proteins are compared they are highly similar and the observed amino acid differences results mainly in different charge distribution across the surface of the proteins. The exposed C-terminal hinge part of the proteins is most divergent, but also the insert and switch regions have distinct differences. The two regions that are most diverging are the insert region and the C-terminal part.

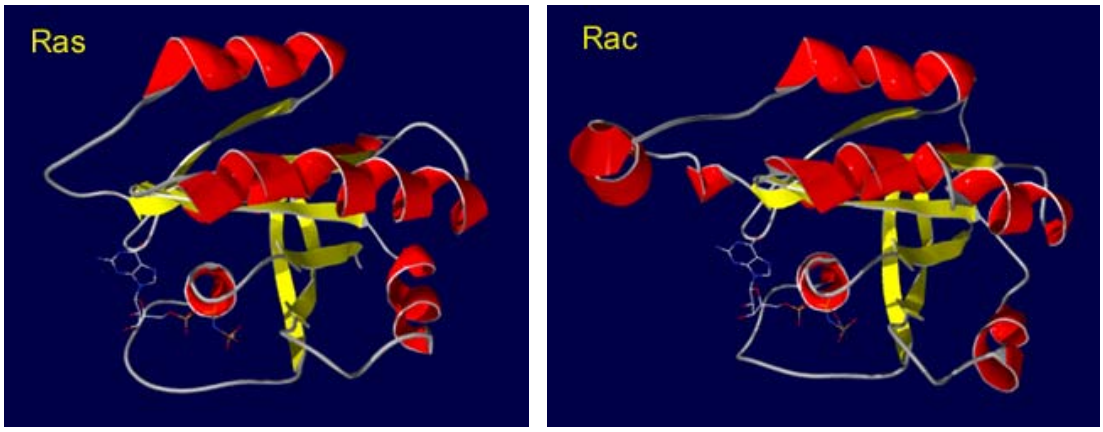

Figure 10. A structural comparison showing differences and similarities between Rac and Ras proteins from humans (HsRac1 pdb acc. # 1MH1, Ras pdb acc. # 5P21). The figures were made with the GlaxoSmithKline Swiss-PdbViewer v3.7 (Guex, N. et al. 1997), and visualized with the raytracer program, Pow-Ray™ version 3.1g (http://www.povray.org/).

The insert region of Rho family proteins.

The major structural difference between Ras and Rho family proteins is the presence of the insert region, which is found in most members of the Rho family. This 11-13 residue long insert is located between β-sheet 5 and α-helix 4 and consists of a loop plus a protruding amphipathic α-helix, see figure 11. The amino acid composition of the insert helix varies between Rho and Rac/Cdc42 proteins. One result of these amino acid changes is that the proteins display differences in charge distribution across the insert helix. The human RhoA protein has for instance three basic residues located in the insert helix while in human Rac and Cdc42 proteins the insert helix contain more exposed acidic residues. These small amino acid variations in the insert region may play an important role in helping to differentiate between the various Rho-family members and their interacting proteins. It has also been proposed that the positive charges of the insert region could mediate interactions during the membrane

attachment / detachment (Wei, Y. et al. 1997), but this has never been experimentally confirmed.

It was also suspected that the insert region was sensitive to the GDP/GTP binding state of the protein, but the crystal structures of human RhoA in GTP and GDP bound form have shown that its conformation is not influenced by the nucleotide binding state of the protein (Ihara, K. et al. 1998). The lack of GDP/GTP induced switching of the insert region was also observed in the solution structure of the human Cdc42 protein (Feltham, J. L. et al. 1997). Whether this is also applies to the insert region of Rac proteins remains unknown. Thus, currently available data indicate that the insert region is both structurally and functionally different from the switch I and II regions and appear not to be influenced by nucleotide binding.

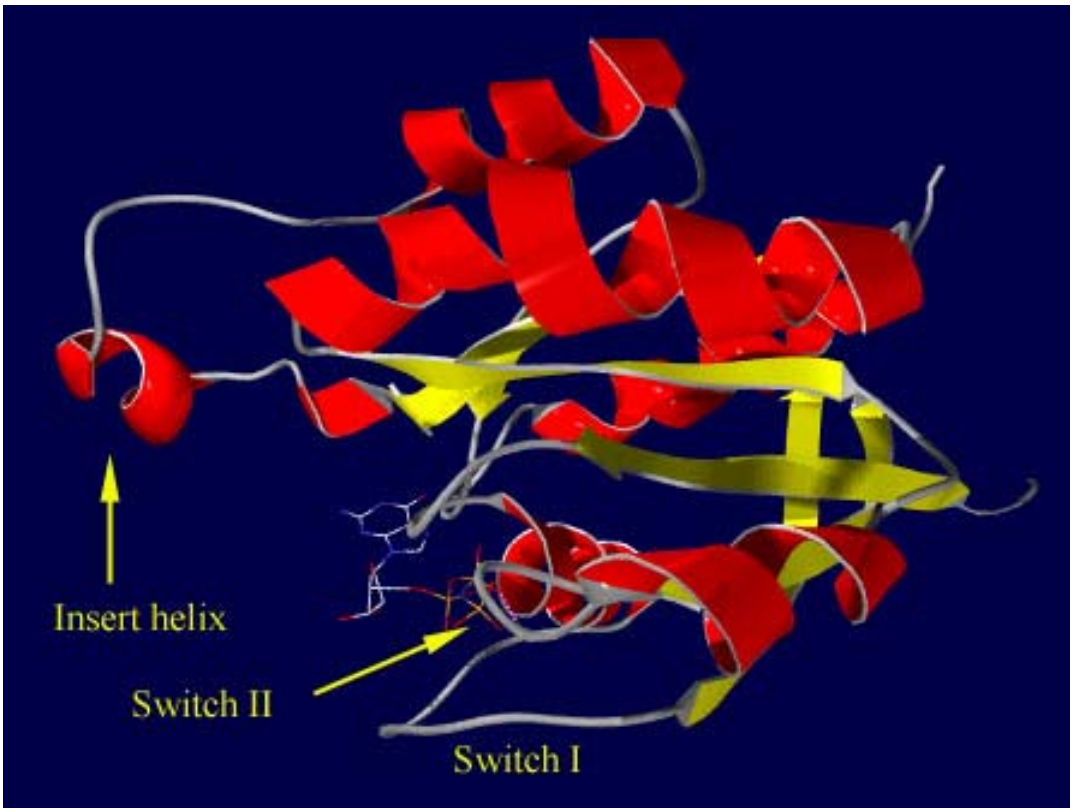

Figure 11. The three dimensional structure of a plant RAC GTPase. From this model the exposed nature of the insert helix is shown to the left. The figure was made with the GlaxoSmithKline Swiss-PdbViewer v3.7 (Guex, N. et al. 1997), and visualized with the raytracer program, Pow-Ray™ version 3.1g (http://www.povray.org/).

While the 3-D crystal structure of the Rho and Rac proteins has revealed a distinct α-helix in the insert region, NMR studies of human Cdc42 has suggested that the insert helix is both smaller and less stable than what was observed in the crystal structures. However, the protruding nature of the insert helix and its dynamic behavior suggests that it mediate interactions with other cellular proteins. The insert

region is found in most Rac/Rho proteins suggesting that it has distinct functions, but there are exceptions such as *Drosophila melanogaster* RhoL and *Dictyostelium discoideum* RacH, which have lost the region altogether.

The Rho insert region and NADPH oxidase complex.

The function of the insert region is still somewhat unclear and so far no proteins or protein motifs binding the insert region have been identified. Early studies of human Rac proteins suggested that this region interacted with components of the NADPH oxidase (Freeman, J. L. et al. 1996; Joneson, T. et al. 1998). In particular the human protein $p67^{phox}$, one of the components of the NADPH oxidase complex, was proposed to interact with the effector region while the insert region was suggested to interact with another component of the NADPH oxidase (Nisimoto, Y. et al. 1997). Later work revealed that the tetratricopeptide repeats (TPR) in $p67^{phox}$ was responsible for Rac binding and subsequent activation of the NADPH oxidase (Koga, H. et al. 1999). It was also shown that site-specific mutagenesis of an Arg residue in one of the TPR motifs disrupted the Rac-$p67^{phox}$ interaction and prevented the activation of the NADPH oxidase. Recently a co-crystal structure of human Rac1 in complex with the TPR domains from $p67^{phox}$ was published (Lapouge, K. et al. 2000).

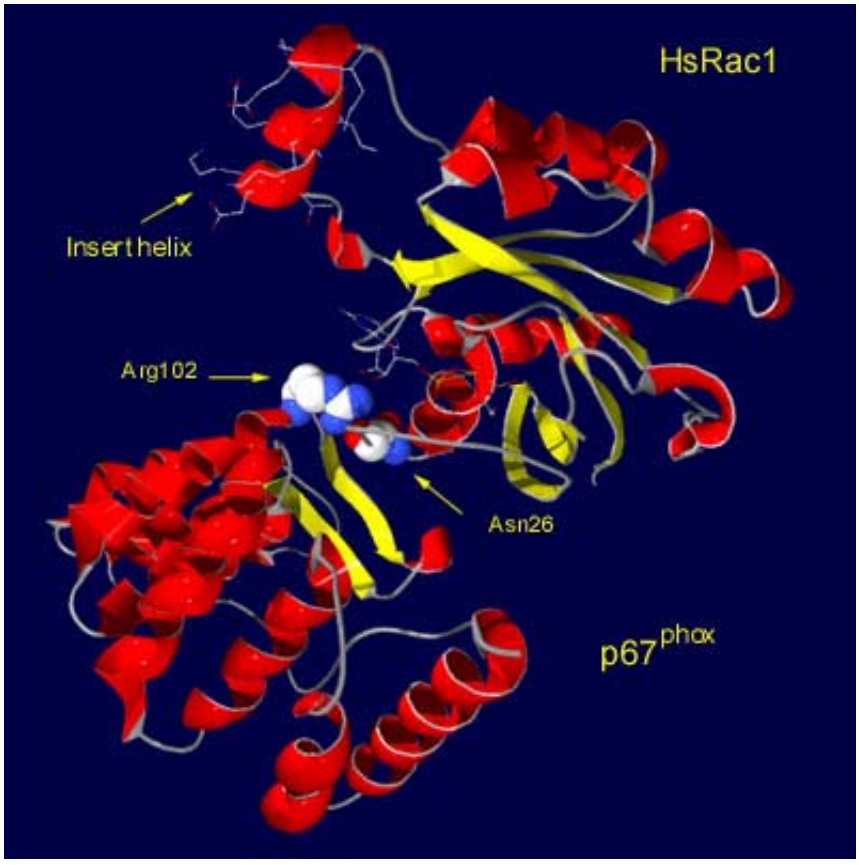

Figure 12. A 3D-structure of human Rac1 in complex with p67phox (pdb acc # 1E96). The model shows that the binding interface between Rac1 and p67phox does not include the insert region. The figure was made with the GlaxoSmithKline Swiss-PdbViewer v3.7 (Guex, N. et al. 1997), and visualized with the raytracer program, Pow-Ray™ version 3.1g (http://www.povray.org/).

From this model it was shown that the Arg residue in $p67^{phox}$ that abrogate Rac1 binding has an ionic interaction with the conserved Asn26 residue in Rac1, and it was also shown that there is no direct interaction between the insert region and the TPR domains of $p67^{phox}$, figure 12. The TPR motif is found in various proteins from prokaryots and eukaryots. More than 100 *Arabidopsis* proteins contain TPR repeats, one of them is the SPINDLY (SPY) protein, which may be a regulator of the gibberellin (GA) signal transduction pathway (Jacobsen, S. E. et al. 1998).

The importance of the insert region for activation of the NADPH oxidase is therefore questionable and mutational analysis of human Rac1 with deletion of the insert region has given conflicting results (Karnoub, A. E. et al. 2001). Studies of Rac chimeras, where domains in Rac has been replaced with corresponding domains from Ras, has suggested that other parts of the Rac protein than the insert and effector domain are responsible for the activation of the NADPH oxidase (Toporik, A. et al. 1998). In particular mutations of specific residues in α-helix 3 and 5 affect the activation of the NADPH oxidase. It is of particular interest that α-helix 3 is the one region apart from the switch I and II regions that is influenced by GDP/GTP switching. These results are also supported by "peptide walking" experiments, where synthetic peptides based on the Rac1 protein sequence have been used to assess their inhibitory effect on the NADPH oxidase (Joseph, G. et al. 1995). This has uncovered domains in the C-terminal part of Rac1 that are important for NADPH activation and one of inhibitory peptides maps to the polybasic region next to the CAAX motif.

Homologues of $p67^{phox}$ or $p47^{phox}$ have not been identified in plants even though experiments with various human antibodies have suggested their existence (Tenhaken, R. et al. 1995; Xing, T. et al. 1997). In fact the complete genomic sequences of *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Homo sapiens* have shown that $p47^{phox}$ and $p67^{phox}$ homologs are most likely restricted to vertebrates, and there is no indications similar proteins exist in invertebrates or yeast/fungi. However some the functional domains in $p47^{phox}$ and $p67^{phox}$ do exist in plant proteins. The N-terminal TPR repeats and the C-terminal SH3 domain in $p67^{phox}$, have corresponding motifs in several plant proteins. Similar, the TPR, SH3 and Phox homology (PX) domains in p47phox have homology to other plant proteins. Antibodies reacting to epitopes from these domains in $p47^{phox}$ and $p67^{phox}$ may recognize various plant proteins with cellular functions that differ from the animal Phox homologs (Tenhaken, R. et al. 1995; Xing, T. et al. 1997). At least 7

Arabidopsis proteins have PX domains, and it appears that this domain is distantly related to the NAM (No Apical Meristem) domain (personal observations). Recent studies have shown that the PX-domain specifically bind phosphatidylinositol phosphates and may regulate targeting of proteins to specific cellular compartments (Kanai, F. et al. 2001; Cheever, M. L. et al. 2001; Xu, J. et al. 2001). Interestingly, two phospholipase D homologues in *Arabidopsis* belonging to the PLDζ1 / PLD2 family, have a N-terminal PX domain and a pleckstrin homology (PH) domain (Qin, C. et al. 2002).

Another component of the NADPH oxidase that potentially could interact with the insert region is the α-subunit of cytochrome b558 ($p22^{phox}$), but no direct interactions have so far been reported (Heyworth, P. G. et al. 1994; Nisimoto, Y. et al. 1997). Interestingly, $p22^{phox}$ seems to have evolved only in Chordata and no homologues are found in invertebrates, yeast/fungi or plants. However*, Arabidopsis thaliana* has ten proteins that are related to animal $p91^{phox}$ proteins (Sagi, M. et al. 2001) plus a related group of proteins, the ferric-chelate reductases, which are involved in iron acquisition (Robinson, N. J. et al. 1999; Waters, B. M. et al. 2002). With so many components of the animal NADPH oxidase complex missing in plants, its regulation may entirely different.

Other functional roles of the insert region.

The insert region has also been implicated in the regulation of RhoGDIs (Wu, W. J. et al. 1997), but recent crystallographic data of human RhoA-RhoGDI and human Rac2-RhoGDI complexes show no direct interaction between the insert region and RhoGDI (Hoffman, G. R. et al. 2000b; Scheffzek, K. et al. 2000). A number of other proteins have been tested for interactions with the insert region. Site specific mutagenesis of the insert region of human Cdc42 proteins has shown that it does not interact with the Cdc42/Rac interactive binding motif (CRIB) from the protein kinases ACK and PAK or with the CRIB motif in RhoGAP and WASP (Owen, D. et al. 2000). However, from the crystal structure of the Cdc42 an Asn residue located at the end of the insert helix appear to interact with Cdc42GAP (Nassar, N. et al. 1998b), but the functional importance of this interaction is unknown.

Another enzyme probably depending on the insert region for its function is Phospholipase D (PLD), which catalyze the hydrolysis of phosphatidylcholine to choline and phosphatidic acid (PA) (Bae, C. D. et al. 1998; Yamazaki, M. et al. 1999; Walker, S. J. et al. 2000; Walker, S. J. et al. 2002). These reports suggest that there is a direct interaction between a human PLD, Cdc42 and RhoA, but that it does not

involve the insert region. However, deletions or mutations of the insert helix are affecting the PLD activity, indicating that other auxiliary factors may interact with the insert region. Deletion of the insert region apparently do not affect the GDP/GTP exchange of Rac, Rho and Cdc42 proteins, but nevertheless this modification affect cellular processes (Wu, W. J. et al. 1998; Karnoub, A. E. et al. 2001). So far there is little information of which part of the PLDs are binding Rho/Rac and Cdc42 proteins, but phage display studies indicate that the C-terminal part of PLD may be involved (Cai, S. et al. 2001). Another human protein depending on the insert region for its function is Rho kinase (Zong, H. et al. 2001). Results show that a deletion of the insert region does not affect Rho kinase binding, but similar to the PLDs, the deletion appear to influence its activity. Rho kinases do not have a Crib motif, instead they have another conserved Rho binding (RB) domain, also known as the HR1 domain (Flynn, P. et al. 1998; Maesaki, R. et al. 1999). No plant proteins with the HR1/RB domain are reported and it is probably restricted to animalia and fungi (personal observations). However, a distantly related motif may exist in plant kinesins (personal observations).

In another study, the deletion of the insert region in a human Rac protein, with an activating mutation (Q61L), has shown that it affect membrane ruffling in NIH 3T3 cells (Karnoub, A. E. et al. 2001). The Rac insert deletion did also potentiate the Rac induced production of ROS. This suggests that the insert region interact with unknown factors that are essential for regulating the actin cytoskeleton and/or production of superoxide.

The precise function of the insert region in Cdc42, Rac and Rho proteins is therefore still unclear. Compared to animal Rac and Rho proteins most plant RAC proteins have an insert region with an unusual two amino acid deletion. The AtRAC7 protein is an exception and has a four amino acid deletion (Winge, P. et al. 2000). In contrast to most Rac proteins the insert region in plant RAC proteins is more variable, indicating that it is under relaxed selection. However the region that corresponds to the insert helix is better conserved and suggest that plant RAC proteins have a similar, but shorter α-helix as animal Rac and Rho proteins. Even though no proteins are reported to interact with the insert region so far, the amino acid variations of this region may play an important role to differentiate between various RAC proteins and their interacting partners. Two candidate proteins that may interact with the insert region are ELMO and CDM-like proteins (after Ced-5, mammalian DOCK180 and Drosophila melanogaster myoblast city), see Rho guanine nucleotide exchange factors, RhoGEFs.

The C-terminal region of Rho-GTPases

The C-terminal part of Rho-GTPases is hyper-variable, except for the last four amino acids, CaaX (C=cysteine, a=aliphatic and X=any), which define the prenylation site. When X is methionine or serine a protein farnesyltransferase (FTase) transfers a farnesyl group, and when X is a leucine, isoleucine or phenylalanine a type I geranylgeranyl transferase (GTTase I) transfers a geranylgeranyl to the cysteine residue. Most Rho-GTPases have a C-terminal Caa(L/F) motif (L=Leu and F=Phe) and are modified with the prenyl group geranylgeranyl (Trainin, T. et al. 1996). After prenylation the C-terminal undergo two further modifications, which includes the removal of the last three amino acids, aaX, by a CAAX metallo protease and the methylation of the free carboxyl group by a isoprenylcysteine carboxyl methyltransferase (Bracha, K. et al. 2002; Crowell, D. N. et al. 2001).

Several RAC GTPases both in plants and in animals have a polybasic motif preceding the CaaL/F motif. Similar polybasic motifs are found in a number of GTPases, and are reported to be important for strengthening membrane associations through interaction with anionic lipids like phosphoinositides (Roy, M. O. et al. 2000). Thus, the polybasic residues and prenylation status (farnesylated or geranylgeranylated) gives the Rho-GTPases distinct affinities for lipid membranes (Silvius, J. R. et al. 1994; Ghomashchi, F. et al. 1995). An experiment where the polybasic residues in human Rac1 have been deleted, but leaving the C-terminal CAAX-box intact, has for instance shown that this affects its ability to activate the NADPH-oxidase (Di-Poi, N. et al. 2001). This may indicate that the targeting ability is compromised or that the prenylation of the GTPase is less efficient when the polybasic residues are missing. From the 3D-structure of the human FTase complexed with the C-terminal part of K-ras4B, the polybasic region is shown to interact with FTase and provide better affinity for the substrate (Long, S. B. et al. 2000). Whether the polybasic domain in Rho-GTPases has the same function in type I geranylgeranyl transferases are at present unknown (Liang, P. H. et al. 2002). Experiments where the CaaX motif in Rho-GTPases has been deleted have shown that it often render the protein non-functional and reduce the interaction with other proteins such as phospholipase D and RhoGDI (Chang, J. H. et al. 1998; Yamazaki, M. et al. 1999).

It has also been reported that the C-terminal part of Rho-GTPases has additional functions. In one report it is suggested that some Rho family proteins with an arginine residue at –2 from the C-terminal Cys residue may serve as a putative arginine finger with GTPase activating activity (Zhang, B. et al. 1999). However, the structure of Cdc42-GAP complexed to Cdc42 (with C-terminal intact) show that C-terminal part is very close to the active site of the GTPase and autoregulation by a C-terminal arginine can not be excluded. The same author has also suggested that the C-

terminal polybasic domain of human Rac1 mediate oligomerization and that this is the cause of the self-stimulatory GAP activity (Zhang, B. et al. 2001). However, neither oligomerization nor self-stimulatory GAP activity have independently been confirmed by other researchers. It is also conflicting evidence whether the polybasic domain of human Rac1 modulate its interaction with effector proteins such as PAKs (Kreck, M. L. et al. 1996; Knaus, U. G. et al. 1998).

Another group of proteins that form complexes with Rho-GTPases are the formins (Kohno, H. et al. 1996; Evangelista, M. et al. 1997). The formins do not have typical Rho-interacting motifs like CRIB-motif or DH-domain, but in some formins a Rho-binding domain have been identified (Yayoshi-Yamamoto, S. et al. 2000; Krebs, A. et al. 2001; Ishizaki, T. et al. 2001). How the Rho-GTPases interacts with formins are still not known in detail, but the polybasic domain in human Rac1 apparently interacts with the formin FHOS (Westendorf, J. J.2001). Interestingly, formins were recently shown to be important for regulating nucleation and polarization of unbranched filamentous actin structures and may be involved in Rac GTPase mediated regulation of the actin cytoskeleton (Pruyne, D. et al. 2002). The formins also have essential functions during cytokinesis in fission yeast, and probably regulate actin polymerization in concert with the Arp2/3 complex, profilin and WASP (Pelham, R. J. et al. 2002).

Predicted secondary structure of AtRAC proteins.

The N-terminal part of plant RAC proteins, including the P-loop and the two switch regions, are highly conserved and has best homology with yeast and animal Rac proteins. In addition to several unique amino acid substitutions that distinguish them from most Rho-GTPases, see table 4, the plant RAC proteins have a two amino acid deletion in the insert region that is unique, the exception being AtRAC7 which has a four amino acid deletion. Truncations of the insert region, although rare, are observed in other Rho-GTPases and suggest that its function to a certain extent is redundant. In our first paper we used the available structural data of the Ras protein to predict the secondary structure of the AtRAC proteins, figure 4 (Winge, P. et al. 1997) as the crystal structures of Rho-family GTPases were unknown at that time. In retrospect the structural prediction was pretty accurate and all helixes and β-sheets were detected, including the unique insert helix.

Amino acid	AtRAC/ ROP	RAC	CDC42	RHO	Amino acid	AtRAC/ ROP	RAC	CDC42	RHO
Thr11	X				Leu87	X	X		
Val17	X	X	X		Val/Ile88	X	X	X	
Ser25	X	X	X		Lys/Arg90	X			
Thr27	X	X	X		Tyr93	X	X	X	
Ser28	X				Lys98	X			
Asn29	X	X	X		Leu104	X			
Thr30	X			X	Ala108	X	X		
Thr33	X				Thr118	X	X	X	
Asp41	X	X	X		Glu145	X			
Phe43	X				Lys/Arg147	X			
Ser44	X	X			Ser160	X			
Asn46	X	X			Lys161	X			X
Thr53	X				Gln163	X	X	X	
Asn55	X	X			Val166	X			X
Asn68	X				Lys167	X	X	X	
Arg76	X				Ala168	X			
Gly77	X				Asp171	X	X	X	
Ala78	X				Ile174	X	X	X	
Phe81	X	X	X		Pro180	X	X	X	
Ala84	X								

Table 4. Analysis and comparison of conserved amino acids in plant RAC proteins that differ between AtRAC/ROP proteins and Rac, Cdc42 and Rho proteins from yeast and animals. Numbering reefers to type I plant RAC proteins. The close relationship between plant RAC/ROP proteins and the Rac/Cdc42 proteins is evident as just three amino acids are uniquely shared between RAC/ROP and Rho, while more than 12 are conserved between RAC/ROP and Rac/Cdc42, but not in Rho.

With the coordinates from several Rho-GTPases available it has been possible to refine and predict the structure of the plant RAC proteins through homology modeling using the GlaxoSmithKline Swiss-PdbViewer v3.7 (Guex, N. et al. 1997). The 3D-model is based on the GTP-bound form of Rac1 (PDB: 1MH1), Cdc42 (PDB: 1AJE) and RhoA (PDB: 1A2B). Modeling requests were submitted to a WWW-based server running the SWISS model program (http://www.expasy.ch/swissmod/cgi-bin/) (Rodriguez, R. et al. 1998) and the 3D-models were later manually refined and controlled with the WhatCheck program located at http://biotech.ebi.ac.uk:8400/ (Hooft, R. W. et al. 1996). The WhatCheck program performs several checks on Protein Data Bank Files (PDB files) that includes: verification of bond angles,

verification of bond lengths, bump check, His Gln Asn side chain conformation check, side chain planarity check and others. The quality check of the final 3D-models suggests that the model is valid with no packing problems, serious bond angles deviations or bond length problems. The 3D model was visualized using the raytracer program, Pow-Ray™ version 3.1g (http://www.povray.org/).

As expected the 3D-model of plant RAC proteins is highly similar to the published Rac1/Cdc42/RhoA models. In general, amino acids involved in nucleotide binding or which have essential roles during interaction with GAP, GDI or effector proteins are evolutionary conserved, while less conserved amino acids are often localized to solvent exposed loops and helixes, figure 13. Even though plant RAC proteins have a shorter insert region and do not have as distinct amphipathic insert helix as found in human Rac1, the models predict a similar protruding helix.

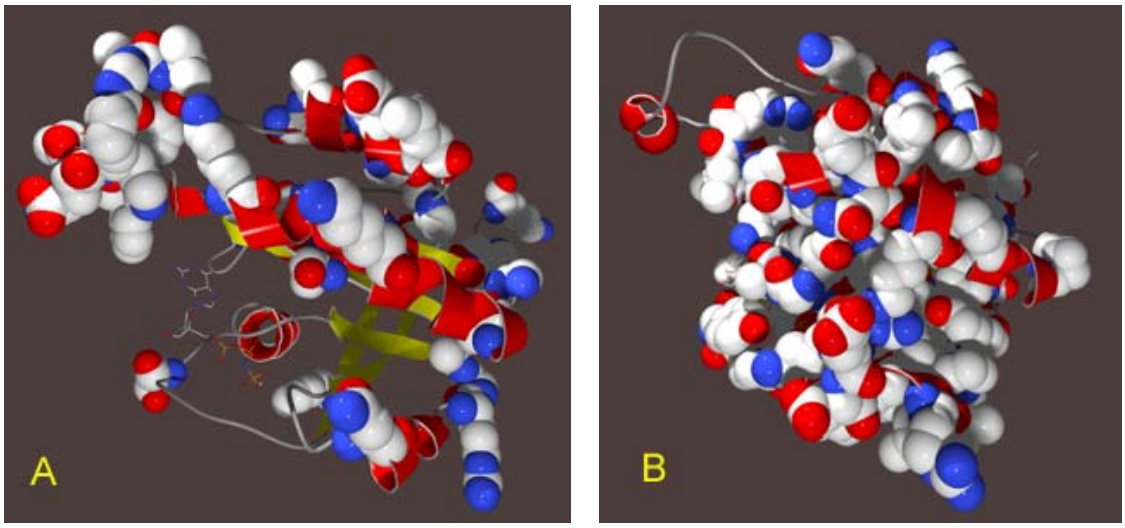

Figure 13. Structural similarities and differences in AtRAC proteins. Figure 13A shows amino acids that differ between animal and plant RAC proteins. Differences are indicated with space-filled residues. Figure 13B shows amino acids that are conserved between AtRAC and animal Rac proteins. Both figures are in same orientation. The figure show that most variations are found in surface exposed loops and helixes, while the nucleotide-binding region is highly conserved. The figures were made with the GlaxoSmithKline Swiss-PdbViewer v3.7 (Guex, N. et al. 1997), and visualized with the raytracer program, Pow-Ray™ version 3.1g (http://www.povray.org/).

Structural insights of Rho GTPase functions from domain and protein interactions

During the last few years crystal structures of the human RhoA protein in both GDP and GTP (GTPγS) bound form have been reported, which has allowed for detailed analysis of conformational changes that take place when the protein cycle between GDP/GTP bound form. A solution structure of the active and inactive conformation of

Cdc42Hs obtained through NMR studies is also available, although at a lower resolution (Feltham, J. L. et al. 1997). From these studies it is apparent that Rho-family GTPases, similar to the Ras proteins, display conformational changes in the switch I and II regions depending on their two nucleotide binding states. The conformational changes is however not as large as observed with the Ras proteins and it is mainly the switch I domain that changes conformation in Rho-family GTPases. Both switch regions are reported to interact with several effector proteins in addition to the common GAPs, GEFs and RhoGDIs (Li, R. et al. 1999).

RhoGAP proteins, structure and function.

The intrinsic GTPase activity of Rho family proteins is in general low, but can be accelerated up to 10^5 fold by their respective GAPs. In this system the active site is shared by two different proteins the GTPase and the GTP activating protein, and the function of the GAP is to stabilize the transition state of the reaction. The RhoGAP domain / Bcr-homology domain (BH-domain), which is responsible for the increased GTPase activity, is found in several types of proteins from protists, yeasts, animals and plants (Scheffzek, K. et al. 1998). Even though the RhoGAPs are highly variable, both in size and amino acid homology, they appear work through a common mechanism. Currently two crystal structures of p50rhoGAP / Cdc42GAP complexed with either RhoA or Cdc42 are known (Rittinger, K. et al. 1997b).(Rittinger, K. et al. 1997a; Nassar, N. et al. 1998b) In addition the crystal structure of the RhoGAP domains of the regulatory subunit of human phosphoinositide 3-kinase p85α and the GTPase regulator GrafGAP have been solved (Musacchio, A. et al. 1996; Longenecker, K. L. et al. 2000). These studies show that four highly conserved residues in the switch II region of the GTPase, Gln, Glu, Asp and Tyr, the QEDY motif, have specific interactions with the GAP, figure 14. In addition a conserved Tyr residue in the switch I region and two conserved residues, corresponding to Glu^{94} and Asn^{95} in AtRAC1, located in α-helix 3 interacts with the GAP. These residues are 100% conserved in the Rho-family, including the plant RAC proteins, and are also found in many other Ras-like proteins.

There appear to be a strict requirement of a catalytic arginine residue in all GTPase activating proteins. This arginine residue is placed directly into the active site of the GTPase and stabilize a conformation that mimic the transition state of the GTPase reaction (Scheffzek, K. et al. 1996). In addition, this arginine helps to coordinate a conserved Gln residue in the switch II domain that is involved in the GTP hydrolysis. Thus, the function of the GAP is probably to stabilize the switch I

and switch II domains of the GTPase and thereby induce a conformation that is suitable for catalysis (Nassar, N. et al. 1998b). Thus, the mechanism for binding and GTP hydrolysis is similar to the one proposed from the Ras-RasGAP structure.

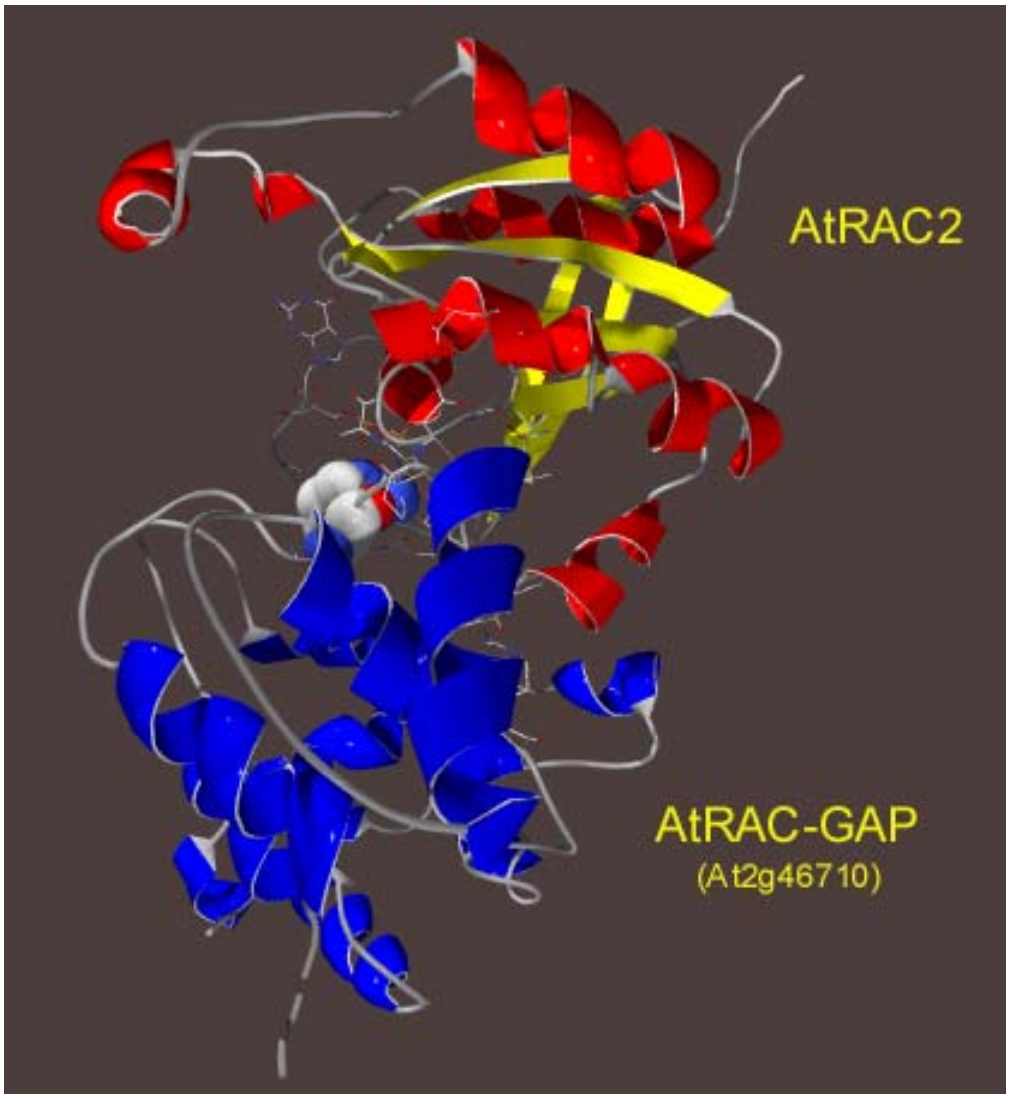

Figure 14. A 3D-homology model of AtRAC2 in complex with an *Arabidopsis* RAC-GAP protein (At2g46710), was made based on the 3D-structure of Cdc42 in complex with Cdc42GAP (pdb # 1GRN) (Nassar, N. et al. 1998a). The conserved Arg residue is shown as space filled. Several of the residues in RhoGAP / Cdc42GAP, which interact with the GTPase, are also conserved in the plant RacGAP/RopGAP proteins suggesting both structural and functional similarities.

Analysis of the *Arabidopsis thaliana* genome has shown that it encodes ten proteins with RhoGAP, which can be sub-divided into three distinct groups (personal observations). The largest group, consisting of 6 members, has a unique structure and contain a N-terminal CRIB motif (Borg, S. et al. 1999; Wu, G. et al. 2000), a motif which is mainly found in STE20/PAK like kinase and in WASP proteins in other

eukaryots. Thus, some plant GAP proteins have two RAC interacting motifs. Because CRIB and GAP binding do not overlap it is possible both interact with a single RAC protein simultaneously. Another distinct group of plant RhoGAPs genes, encode proteins with a N-terminal pleckstrin homology (PH) domain, a structure that is similar to the human GrafGAP, except that GrafGAP has a C-terminal SH3-domain (Longenecker, K. L. et al. 2000). PH domains are known to bind phosphoinositides and is found in various proteins, among them Rac/Rho exchange factors.

Instead of the CRIB domain, some animal RacGAP/Cdc42GAP proteins, and interestingly some RasGAP proteins, have a N-terminal BNIP-2 and Cdc42GAP homology (BCH) domain. This SEC14 related domain was first identified in a human protein, BNIP-2, which binds the adenovirus E1B 19-kDa protein and Bcl-2, and it was proposed that the BCH domain mediate protein-protein interactions (Boyd, J. M. et al. 1994). Interestingly, it has now been shown that BNIP-2 binds Cdc42GAP/p50rhoGAP and is able to stimulate intrinsic GTPase activity of Cdc42 through direct interactions (Low, B. C. et al. 1999). Apparently the BCH domain of BNIP-2 has GAP activity even though it lacks a proper arginine finger motif, possibly due to an arginine residue in the BCH domain (Low, B. C. et al. 2000a; Low, B. C. et al. 2000b). This suggests that GTPase activity can be stimulated in Cdc42/Rac proteins by other proteins than the regular GAP proteins. As the BCH domain is found in GAP proteins that regulate both Rho and Ras proteins, it may represent a new protein module that can regulate Rac-and Ras-like GTPases. The BCH domain is evolutionary conserved and can be found in most eukaryots, including three proteins from *Arabidopsis thaliana*, (At4g35750, At3g10210 and At1g69340).

Unique proteins with Rho-GTPase activating properties have also been detected in pathogenic bacteria. These proteins are essential for bacterial invasion and immobilization of the host cells by interfering with Rac/Rho/Cdc42 pathways. Even though these bacterial RhoGAPs have little or no homology to the RhoGAP domain they operate by a similar mechanism (Wurtele, M. et al. 2001; Evdokimov, A. G. et al. 2002). However, the argenine finger found in bacterial RhoGAPs is part of a helix, while in eukaryotic RhoGAPs it is part of a loop. Experiments with the *Yersinia pestis* RhoGAP YopE have also shown that it looses GTPase activating activity when this arginine residue is mutated (Von Pawel-Rammingen, U. et al. 2000).

RhoGDI, structure and function.

The Rho family specific guanine dissociating inhibitors, RhoGDIs, are important regulators of Rho, Rac and Cdc42 proteins (Ohga, N. et al. 1989; Abo, A. et al. 1991;

Hart, M. J. et al. 1992). Not only do they decrease the nucleotide dissociation from the GTPase they also are essential in retrieving them from membranes and keep them soluble in the cytosol (Nomanbhoy, T. K. et al. 1996). The RhoGDIs has therefore a shuttle function where they cycle the various Rho-family proteins between cytosol and cell membranes in response to external and internal stimuli. RhoGDIs binds with almost equal affinity to Rho proteins in GDP and GTP binding form and have therefore the capacity to also affect the interactions of various effector proteins that just binds the GTP bound form. The complex with the RhoGDI keeps the GTPase in an inactive form suggesting that the dissociation of the GTPase from the RhoGDI is an important regulatory step. In animals it has been suggested that this is partly controlled by the membrane bound ezrin/radixin/moesin (ERM) proteins (Hirao, M. et al. 1996; Takahashi, K. et al. 1997). ERM proteins do not exist in plants, but their N-terminal FERM domain, which is responsible for the dissociation activity, can be found in some plant kinesin-like proteins (Reddy, A. S. et al. 1996; Hamada, K. et al. 2001; Girault, J. A. et al. 1998). As mentioned previously, the *Arabidopsis* trichome mutant ZWICHEL has a T-DNA insertion in a gene encoding such a protein (Oppenheimer, D. G. et al. 1997).

Recently the 3D-structures of RhoGDI-RhoA, LyGDI-Rac2, RhoGDI-Rac1 and RhoGDI-CDC42 were solved (Grizot, S. et al. 2001; Scheffzek, K. et al. 2000; Lian, L. Y. et al. 2000; Hoffman, G. R. et al. 2000b). The 3D-structures of RhoGDIs show little resemblance to RabGDI that selectively binds prenylated GTP-bound Rab proteins that is involved in vesicle transport (Schalk, I. et al. 1996). Comparison of the RhoGDIs show that they are similar in structure and composed of two N-terminal α-helices followed by ten or more β-sheets which are folded into a immunoglobulin-like core. The interactions with the GTPase are mediated mainly through residues in two N-terminal α-helices. Most of the residues in the GTPase and the GDI that contributes to the binding interface are highly conserved. Figure 15 shows residues in Rho and Rac proteins that interact with the RhoGDI and RacGAP. This figure shows that the residues in Rac that interacts with the GDI are 100 % conserved in plant RAC proteins. The most extensive interactions with the GDI are found in the switch I and switch II regions, but also α-helix 3 contribute to the binding surface. The crystal structures of human Rho, Rac and Cdc42 proteins have shown that the switch II region consists of two 3_{10} helices. While the RhoGAP proteins mainly interact with first 3_{10} helix the RhoGDIs interacts with both. In addition, different residues in α-helix 3 interact with Cdc42/GAP and RhoGDI.

Another requirement for Rho-RhoGDI interaction is the isoprenylation of a C-terminal Cys residue of the GTPase. It had previously been suggested that a hydrophobic pocket in the core domain of the RhoGDI serves as the isoprenyl binding

region, (Gosser, Y. Q. et al. 1997), but neither of the co-crystals of Rac2-LyGDI or the RhoA-RhoGDI could confirm this. The structures of Cdc42-RhoGDI and Rac1-RhoGDI did however show that the geranylgeranyl moiety of Cdc42 and Rac1 is inserted into a hydrophobic pocket within the immunoglobulin-like domain of the GDI molecule. This was also confirmed by the fact that non-isoprenylated Rac1 binds more weakly to RhoGDI than the prenylated Rac1 or Cdc42.

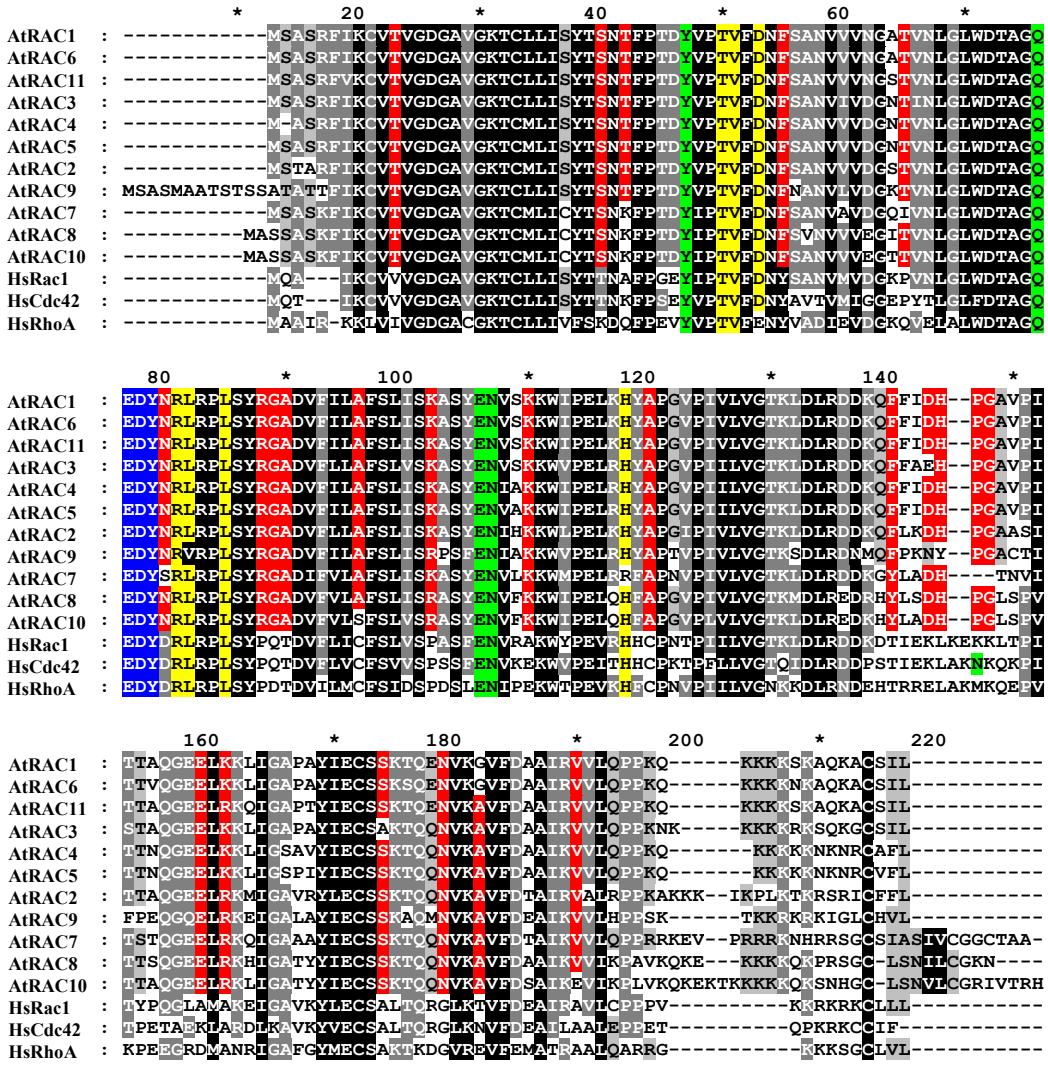

Figure 15. An alignment of AtRAC/AtROP proteins with human Rac1, Cdc42 and RhoA was made with the GeneDoc program. Residues highlighted in yellow interact with RhoGDI, green residues interact with RacGAP and blue residues interact with both RhoGDI and RacGAP. Amino acids that are "unique" for plant RACs/ROPs are marked in red. Residues interacting with RhoGDI and RacGAP is highly conserved in all Rac/Rho proteins. This figure also shows that RhoGAPs and RhoGDIs have a partly overlapping binding interface with the GTPase.

In the Cdc42/RhoGDI complex it was observed that the flexible C-terminal part of the Cdc42 protein, which starts after a series of prolines, forms a distinct

structure which extends away from the GTP binding domain and interacts with the RhoGDI. Two basic residues in the polybasic region of Cdc42 forms hydrogen bonds with some acidic residues in the RhoGDI and the geranylgeranyl moiety is buried within the hydrophobic pocket of RhoGDI, figure 16.

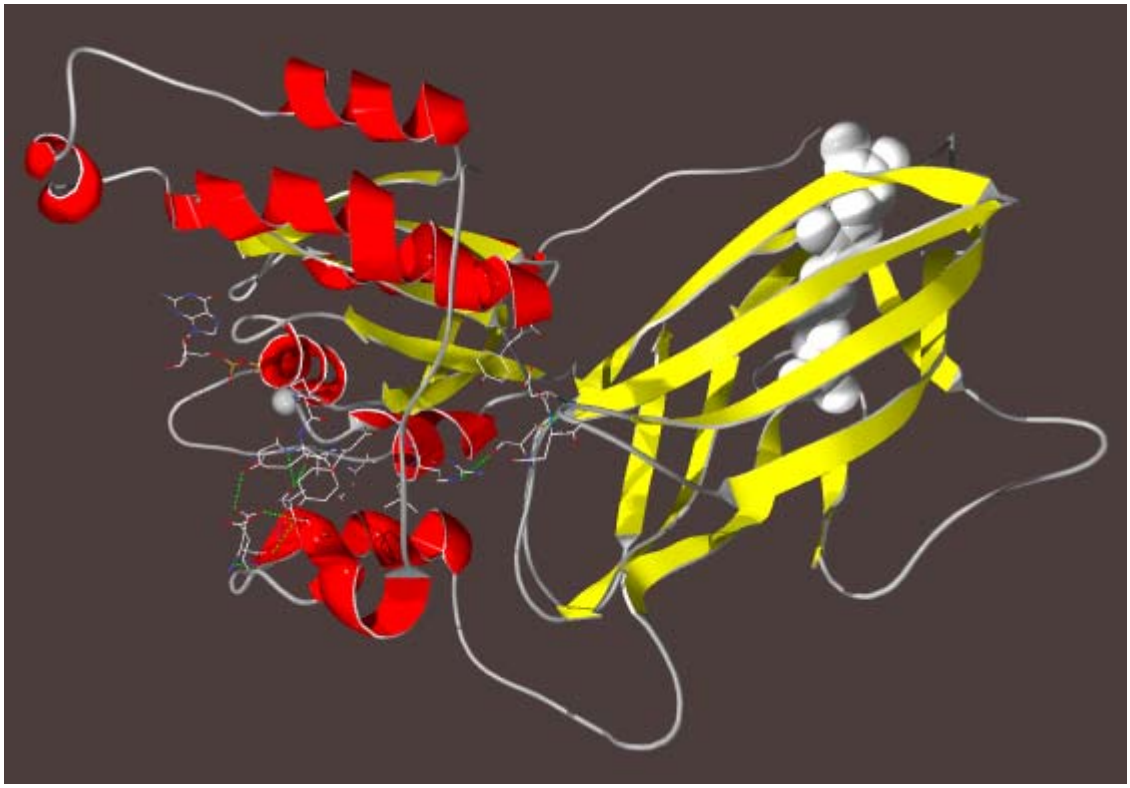

Figure 16. A 3D homology model of a complex between AtRAC2 and an *Arabidopsis* RACGDI (At1g62450) was constructed based on the known structures of RhoGDI-RhoA (pdb acc. # 1CC0) (Keep, N. H. et al. 1997), RhoGDI-Rac1 (pdb acc. # 1HH4) (Grizot, S. et al. 2001) and RhoGDI-Cdc42 (pdb acc. # 1DOA) (Hoffman, G. R. et al. 2000b). AtRAC2 is shown to the left, while the RACGDI protein with its immunoglobulin-like core is shown to the right. The geranylgeranyl moiety attached to the C-terminal of AtRAC is shown in space-fill mode and inserted into the hydrophobic cavity of the RACGDI.

Based on the 3D-structures of RhoGDIs bound to their respective GTPases a model have been suggested for GDI function. In this model the RhoGDI apparently block the switch I and II regions such that the GTPase is inaccessible for the activity of GEF and GAP proteins. In addition it appears as if the RhoGDI inhibits GDP dissociation by stabilizing a conformation of switch I that strengthen GDP binding. Competition for the conserved Thr residue in switch I, (corresponding to Thr38 in AtRAC1), which binds a Asp residue in RhoGDI, may be one of the factors that prevents nucleotide exchange (Hoffman, G. R. et al. 2000b). The two conserved Leu

residues in the switch II region, corresponding to Leu70 and Leu73 in AtRAC1, are also crucial for binding RhoGDI and other effector proteins.

RhoGDIs probably exist in all eukaryots, and they remain relatively conserved in all major kingdoms: protist, plant, yeast/fungi and animal. In contrast to *RAC/RHO* genes which has expanded to large gene families in many organisms the RhoGDIs are either single copy genes or exist as small gene families. For instance, just three RhoGDI genes are found in the *Arabidopsis* genome and this expansion has happened recently. Another distinct characteristic of RhoGDIs is that they are single domain proteins. This is in contrast with the RacGAP and RacGEF domains, which are often found in multi-domain proteins. Comparison of plant and animal RhoGDIs show that most of the residues that interacts with the Rho/Rac GTPases are conserved/identical, which indicate that they bind plant RAC proteins in a similar way. In a recent study one of the *Arabidopsis* RhoGDIs was shown to bind the AtRAC5/AtRop4 protein, and the interaction appeared to be independent of the nucleotide binding state of the GTPase (Bischoff, F. et al. 2000). RhoGDI has also been identified in other plants such as tobacco where it was found in a yeast two-hybrid screen using a RAC protein as a bait (Kieffer, F. et al. 2001).

Rho guanine nucleotide exchange factors, RhoGEFs

Spontaneous GDP release from GTPases is in general slow and guanine-nucleotide-exchange factors (GEFs) are required to promote fast activation of small G proteins through replacement of GDP with GTP in vivo. The relatively high concentration of GTP *in vivo* (normally ~20 times higher than GDP) favors binding of GTP. All known guanine nucleotide exchange factors for the Rho-family of GTPases have a Dbl homology (DH) domain of about 180 amino acids, that contain the catalytically active region of the protein, and many have a C-terminal pleckstrin homology (PH) domain (Stam, J. C. et al. 1999). Even though proteins with DH domains are found in animals, yeast and slime molds, this domain has never been reported in plant proteins and an extensive analysis of the *Arabidopsis* genome has so far not been fruitful (Valster, A. H. et al. 2000) and personal observations. However, it can not be excluded that the DH domains of plant GEFs are so divergent that they can not be found with standard bioinformatic tools.

While the DH domain can not be found in plants and probably no other bikonts, several plant proteins contain PH domains. Interestingly, many of these proteins are

good candidates as upstream or downstream effectors of plant RAC proteins. Among the PH containing plant proteins are: homologues of mammalian 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Deak, M. et al. 1999) dynamin-like proteins (Mikami, K. et al. 2000), phospholipase Dζ1 (Qin, C. et al. 2002), and Arf GAP and Rho GAP like proteins (personal observations). In general the PH domains function as membrane tethering devices with high affinity for inositol phosphates (Harlan, J. E. et al. 1994) and one of the functions of the PH domain in proteins proteins could therefore be to target the protein to lipid membranes which have "correct" phosphoinositide composition. Thus, several proteins involved in signal transducion and vesicle transport contain PH domains. While relatively few PH-domain proteins exist in plants, this domain is greatly expanded in yeast and animals, and humans, for instance, have more than 300 proteins with PH-domains. The PH domains have also been reported to increase the nucleotide exchange activity on Rac1 (Liu, X. et al. 1998; Russo, C. et al. 2001) while other report that exchange activity on nonprenylated Rac1 and Cdc42 is not affected when phosphoinositides binds the PH-domain (Snyder, J. T. et al. 2001).

Before 3D-structure of the Rho-GEFs were known mutation analysis of Rho and Cdc42 proteins had shown that the side chain of the conserved threonine residue, corresponding to amino acid 38 in AtRAC1, is essential for the nucleotide exchange reaction catalyzed by GEFs (Li, R. et al. 1997). This Thr residue is known to be involved in the coordination of the Mg^{2+} ion and is conserved in almost all members of the Ras-superfamily. Analysis of mutants of the human Rac2 protein has shown that in addition to Thr^{38} the residues Val^{39}, Asp^{41} and Gln^{64} affects GEF induced nucleotide exchange (Xu, X. et al. 1997). The 3D-structure of several proteins with GDP exchange activity towards Rho-family proteins are known (Soisson, S. M. et al. 1998; Liu, X. et al. 1998).(Aghazadeh, B. et al. 1998) Recently 3D-structures of the Dbl domain from TIAM1 complexed to human Rac1, (Worthylake, D. K. et al. 2000), the Dbl domain from intersectin complexed to Cdc42 and the Dbl domain from Dbs (Dbl's big sister) complexed with Cdc42 (Rossman, K. L. et al. 2002; Snyder, J. T. et al. 2002) have been solved, providing a unique opportunity to study their functions. Similar to the RasGEFs the DH domain in RhoGEFs is composed of ten or more α-helices connected by loops. The interface between the Rac/Cdc42/Rho proteins and the DH-domain is extensive and the two switch regions are the main interaction

domains. More than 20 amino acids in Rac are involved in complex binding and 17 of them are conserved between human Rac and *Arabidopsis* RAC/ROP proteins. The Rac1/TIAM1 complex shows that Ala^{59}, (Ala^{62} in AtRAC1), is affected by interaction with the DH-domain such that it displaces the Mg^{2+} ion in the Rac nucleotide-binding pocket and thereby prevent nucleotide binding. The 3D-stucture of Rac1 and TIAM1 also shows that the C-terminal part of the DH domain interact with both Rac1 and the PH domain, suggesting that the PH domain may stabilize the interaction. However, the precise role of the PH-domain in Rho-GEFs is still unclear.

Even though the close relationship and conserved structure of the small GTPases, the GEF proteins share little sequence homology and seems to belong to unrelated families. However, a comparison of the 3D-structure of Ras complexed to the exchange factor Sos (Boriack-Sjodin, P. A. et al. 1998), the Rac1/TIAM1 complex and Cdc42 complexed to the human exchange factor and oncoprotein Dbs (Rossman, K. L. et al. 2002) reveal several structural similarities. For instance, residues 57-63 in Rac1 have a conformation that is similar to the equivalent region in Ras when complexed to the exchange factor Son of sevenless (Sos) (Worthylake, D. K. et al. 2000; Cherfils, J. et al. 1998). Comparison of Ras, Rho and Arf GEFs suggest that the mechanism for nucleotide exchange is common for all GEFs and that their active sites have structural and functional similarities (Sprang, S. R. et al. 1998). The nucleotide exchange activity depends on GEF induced changes in switch I and II regions of the GTPase, which disrupt the coordination of the Mg^{2+} ion resulting in nucleotide release. Many Rho-GEFs have a complex structure with several distinct domains and some may be considered as scaffold proteins providing interaction with many proteins/ligands. For instance, in *Saccharomyces cerevisiae* Cdc24p the nucleotide-exchange factor for Cdc42p has a N-terminal F-actin binding Calponin homology domain and a C-terminal Phox and Bem1 (BP1) domain, but unlike many metazoan Rho-GEFs it lack a PH-domain.

GDP exchange activity may also exist in proteins lacking the Dbl-PH domains. Arfaptin2/Por1 was first identified as a protein that interacted with GTP bound Rac1 (Van Aelst, L. et al. 1996). Later studies revealed that Arfaptin2/Por1 also interacted with ADP ribosylation factor (ARF) family GTPases, in particular Arf1 and Arf6 (D'Souza-Schorey, C. et al. 1997). Arfaptins is therefore good candidates for mediating cross-talk between Rac and Arf signaling pathways. In the 3D-structure of

Arfaptin2 in complex with human Rac1, Arfaptin2 forms a dimer with three-helix coiled-coils, which interact with both switch regions of Rac1 (Tarricone, C. et al. 2001). Interestingly, TIAM1 and Arfaptin appear to have a common Rac-interacting domain, suggesting that Arfaptin has GDP exchange activity or may modulate interactions with GEFs, GAPs and GDIs (Cherfils, J.2001). Arfaptin homologues are restricted to metazooa but Arfaptin contain a motif found in plant proteins (personal observations). Many coiled-coil proteins are involved in maintaining cell shape, movement and organizing the cytoskeleton and it is not unlikely that some of them may be Rac effectors/GEFs. One possible link may be the actin binding myosins, which are two-headed dimers held together by a coiled-coil. In fact, an unconventional myosin, MyoM, from *Dictyostelium discoideum* has a C-terminal DH and PH-domain, suggesting that myosins may regulate Rac/Rho activity in other organisms as well (Geissler, H. et al. 2000).

Recently Rac exchange activity was reported in a protein complex composed of Dock180 (180-kDa protein downstream of CRK) and ELMO (Brugnera, E. et al. 2002). Dock180 belong to the CDM protein family named after, *Caenorhabditis elegans* CED-5, human Dock180 and *Drosophila melanogaster* Myoblast City (MBC), and it was first identified as a protein that affected cell morphology when it was translocated to the plasma membrane (Hasegawa, H. et al. 1996). The CED-12/ELMO gene was first identified in *C. elegans* as a protein required for engulfment of dying cells and for cell migrations, and is part of the Rac signaling pathway mediated by CED-2/Crkll, CED-5/DOCK180, and CED-10/Rac (Wu, Y. C. et al. 2001; Gumienny, T. L. et al. 2001). In addition to Dock180 another related CDM protein, Zizimin1, has nucleotide exchange activity against Cdc42 and Rac GTPases (Meller, N. et al. 2002). Interestingly the protein encoded by *Arabidopsis* SPIKE1, a protein involved in cytoskeletal reorganization during trichome development (Qiu, J. L. et al. 2002) is a close homologue to Zizimin1. Thus, SPIKE1 is probably one of the elusive plant Rac-GEFs. While the CDM proteins exist in most eukaryots, Ced-12/ELMO homologues are probably restricted to metazooa, however they contain a conserved motif, (apart from the PH-domain) which is found in some plant proteins.

Unconventional RacGEFs have also been found in bacteria. The bacterial pathogen *Salmonella typhimurium* modulates host cell physiology by injecting specific toxins into the cytoplasm of host cells that induce cytoskeletal reorganization

and apoptotic cell death. One of these cytotoxic proteins, SopE, has Rac/Cdc42 exchange activity despite the fact that it lacks a DH-domain (Hardt, W. D. et al. 1998; Buchwald, G. et al. 2002). The 3D-structure of the catalytic part of SopE complexed with Cdc42 shows that SopE interact with different residues in Cdc42 compared to TIAM1 and have an architecture that differ from the typical DH-domain. SopE is composed of six α-helices arranged in two three-helix bundles, a structural feature found in several proteins. Based on these latest findings and the fact that the *SPIKE1* mutant is viable, (*SPIKE1* is a single copy gene in *Arabidopsis*), it is likely that more activators of Rac and Rho GTPases will be found in both plants and other eukaryots.

The CRIB / RBD motif:

In addition to the common GAP, GEF and GDI proteins several proteins are known to bind Rac and Cdc42 in a GTP dependent fashion. Many of these proteins have a distinct motif, the Cdc42/Rac interactive binding (CRIB) motif or alternatively the Rac/Cdc42-binding domain (RBD), which is responsible for the protein interaction (Burbelo, P. D. et al. 1995). The CRIB motif, which first was detected as a motif in a p21 activated kinase $p65^{PAK}$, is approximately 16 amino acids in length and is found in various proteins in all eukaryotic kingdoms. Careful analysis of the Rac/Cdc42 interacting motif in $p65^{PAK}$ and related kinases has shown that the binding domain also extend outside this 16 amino acid CRIB motif (Thompson, G. et al. 1998).

Complexes of human Cdc42 and the RBD of the Wiskott-Aldrich syndrome protein WASP (Abdul-Manan, N. et al. 1999), p21 activated kinase (PAK) (Morreale, A. et al. 2000) and the tyrosine kinase ACK (Mott, H. R. et al. 1999) have shown that residues of the RBD interact with key amino acids within switch I and II. In particular switch I has extensive interaction with the CRIB-domain (7 amino acids), and Asp^{38} (Asp^{41} in AtRAC1) form hydrogen bonds with one of the conserved Histidines in the CRIB-domain figure 17. In addition, amino acids in the N-terminal part of the CRIB-motif interact with the last α-helix in Rac/Cdc42. CRIB-domain proteins in plants are expected interact with plant RAC proteins in a similar way as observed in the Cdc42:CRIB interactions. However, one difference may be the conserved Arg^{76} after the switch II domain, which is not found in animal and yeast Rac/Rho/Cdc42 proteins. This exposed Arg residue is positioned such that it may interact with residues in the

C-terminal “CRIB-helix” and thereby interact with or stabilize the RAC:CRIB complex, figure 17.

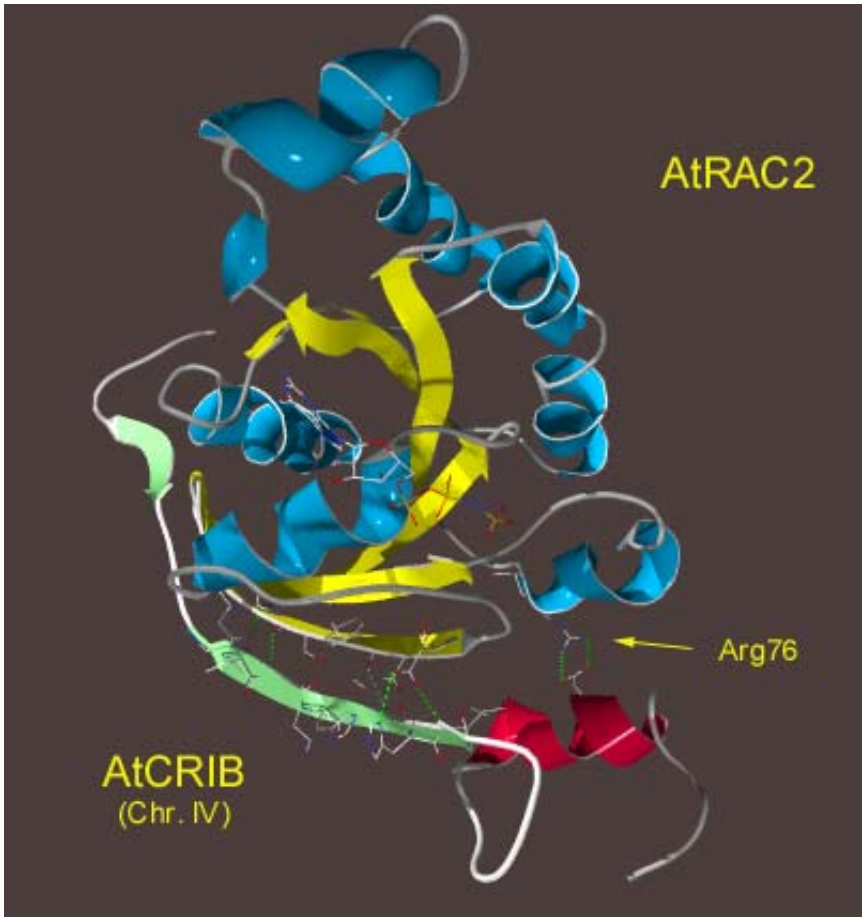

Figure 17. A 3D-homology model of a complex between AtRAC2 and an *Arabidopsis* CRIB motif protein on chromosome IV was made with Swiss-PdbViewer and visualized with the Pov-Ray program. The model is based upon the structure of Cdc42 in complex with WASP (pdb acc. # 1CEE) (Abdul-Manan, N. et al. 1999). The putative interaction between AtRAC2 Arg76 and an Asp residue in the “CRIB-helix” is shown in the figure.

CRIB-motif proteins.

CRIB-motifs are found in a variety of proteins that regulate cytoskeletal polarization and cell signaling. Examples are: myosin I heavy chain kinase from *Dictyostelium discoideum* / p21 activated kinases (PAKs), tyrosine kinases (ACK-like), *C. elegans* Par6, Genghis Khan *Drosophila melanogaster*, plexin-B1, WASP and WASP-like proteins / small binding proteins for Cdc42 (SPECs) and yeast GTPase interactive components (GICs) (Pirone, D. M. et al. 2001). In plants the CRIB-motif is found in RAC GTPase activating proteins and in ROP-interactive CRIB motif-containing proteins (RICs) (Wu, G. et al. 2001). In general CRIB-motif proteins are being

regulated by conversion between autoinhibited and uninhibited forms, so called intrasteric regulation (Hoffman, G. R. et al. 2000a). When an activated Rac/Cdc42 protein binds the CRIB-motif it induces a conformation change that exposes catalytic active sites or domains which recruit / activate other proteins (Lamson, R. E. et al. 2002).

One of the best studied CRIB-motif proteins is WASP. Human WASP encodes a proline-rich multi-domain protein, which was first identified as a mutated gene in persons suffering from the immunodeficiency disorder Wiskott-Aldrich syndrome (Derry, J. M. et al. 1994). Later WASP was identified in a yeast two hybrid screen as a protein that interacted with Cdc42 and was involved in regulating actin organization (Aspenstrom, P. et al. 1996). The WASP proteins is now known to transduce signals to the cytoskeleton through the Arp2/3 complex, an actin nucleating component that regulates the structure and dynamics of actin filament networks. Apart form the CRIB-domain the WASP protein has an N-terminal Arp2/3 binding WH1-domain (WASP Homology domain 1), and a C-terminal verprolin homology motif VHR/WH2 that binds actin. Binding of Cdc42 to WASP induces large conformational changes in WASP, which exposes the C-terminal part and enable interaction with actin (Kim, A. S. et al. 2000). Real homologues of WASP proteins do not exist in plants or yeasts, but yeast Gic2p has been shown to interact with several components that are known to regulate cell polarization and growth (Jaquenoud, M. et al. 2000). It is suggested that GIC proteins operate as adaptors to recruit proteins such as formins, spindle pole antigen Spa2p or Bud6/Aip1 to specific cellular locations. In plants the RIC proteins may have related functions and like the yeast GIC proteins they lack the distinct WH1 and WH2 motifs found in WASP proteins (Wu, G. et al. 2001). The RIC proteins however, have a conserved N-terminal motif of unknown function and some may also have a WH2-like motif (personal observations). Typical WH2 domain proteins are reportedly not found in plants (Paunola, E. et al. 2002).

Concluding remarks.

The RAC proteins are likely to regulate several important cellular processes in plants. Their expansion and diversification in embryophyta suggest that they have acquired novel functions when plants evolved vascular tissues, roots and flowers. As *Arabidopsis thaliana* have 11 RAC-like proteins, many of which have partial overlapping functions, it will be a challenge to uncover the processes they regulate. Gene inactivation experiments may answer some of the question and selective crossing of AtRAC/AtROP mutants into mutant lines with known cellular defects may give additional information. One of their prime functions for AtRAC/AtROP will probably be as regulators of vesicle transport and the actin cytoskeleton. Whether plant RAC proteins regulate the production of superoxide as seen in animals, or if they regulate signaltransduction pathways is an open question.

Comparison of the cellular processes regulated by Ras-family members such as Ras, Ral, Rac, Cdc42 and Rho will undoubtedly reveal many similarities, but also many differences as plants and animals evolved into multi-cellular organisms independent of each other. The expansion of these gene families have largely taken place in multi-cellular organisms, with a few exceptions, and they may reflect the different strategies employed by the organisms to achieve essential processes such as polarized cell growth/division and the development of cellular tissues. A process that probably is of ancient origin, namely polarized exocytosis is probably regulated by RAC proteins and could be conserved on the protein level. Similarities between plants and animals/yeast are also likely to be found when comparing the general regulation of actin cytoskeleton. Thus, the studies of these proteins will keep researchers occupied for many years to come and will probably reveal a deeper understanding how organisms operate at the cellular level.

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