The Rab family of small GTPases:

Structural determinants of lipid modification and function

José Bártholo Pereira Leal

Dissertation submitted to Instituto de Ciências
Biomédicas de Abel Salazar, Universidade do Porto

Thesis supervised by Prof. Miguel C. Seabra
Thesis sponsored by Prof. Maria João Saraiva

Laboratory of protein prenylation, pigment and ocular diseases
Imperial college School of Medicine – Division of Biomedical Sciences
London (2001)

To my father and my stepmother

To the memory of my mother

Abstract

The Rab/Ypt/Sec4 family forms the largest branch of the Ras superfamily of GTPases, acting as essential regulators of vesicular transport pathways. They are membrane associated molecular switches. However, they are intrinsically soluble proteins thus requiring a lipid modification for membrane association. This is accomplished by the enzyme Rab Geranylgeranyl Transferase (RGGT) which catalyses the transfer of two geranylgeranyl isoprenoids to the Rab C-terminus. RGGT absolutely requires an accessory factor termed Rab Escort Protein (REP) for activity, which binds non-prenylated Rabs and presents them to RGGT.

I used the large amount of information in the databases resulting from the different on-going genome projects to analyse the Rab family and identify the structural determinant for REP binding. I defined Rab-conserved sequences that we designate "Rab family" (RabF) motifs using the conserved GTP-binding motifs as "landmarks". The Rab-specific regions were used to identify new Rab proteins in the databases and suggest rules for nomenclature. I found that the RabF motifs are conserved across evolution and propose that positions in the RabF motifs mediate (conserved) interactions with general regulators as REP. Sequence and structure analysis of the REP family highlights the existence of a conserved surface that is likely involved in mediating Rab binding. I used the information gathered from analysis of the Rab and REP families to address the formation of the REP:Rab complex. A yeast two hybrid REP:Rab binding assay was designed and I used it to test a site directed mutagenesis strategy in Rab3 as a model. The result obtained suggest that positions in the RabF motifs mediate interactions with REP. Additionally, I designed methods to form, purify and analyse REP:Rab complexes *in vitro*, which can be used either on a analytical or preparative scale.

From the analysis of the Rab family, I found that RabF regions cluster in and around switch I and switch II regions, *i.e.* the regions that change conformation upon GDP or GTP binding. This finding suggest that specificity of Rab-effector interaction cannot be conferred solely through the switch regions as is usually inferred. Instead, I propose a model whereby an effector binds to RabF (switch) regions to discriminate between nucleotide-bound states and simultaneously to other regions that confer specificity to the interaction, possibly Rab subfamily (RabSF) specific regions that we also define here. These observation hold true for Rab proteins in all organisms studied, are supported by experimental evidence and form the basis of criteria for definition of putative orthologies and nomenclature. Furthermore, I observed that Rab proteins that exhibit similar functional properties (*eg* localisation to a given compartment), co-segregate in phylogenetic trees and display related gene structures, thus suggesting a shared ancestry. I introduce the concept of "Rab functional groups" to describe these groups of Rabs which have shared ancestry and similar functional properties. This concept will permit informative correlations between similar functional properties and sequence conservation, and shed light into the multiplication of Rab proteins that accompanied multicellularity.

Sumário

A família de proteínas Rab/Ypt/Sec4 é a mais numerosa das famílias que constituem a super-família de pequenas GTPases, e é constituida por proteínas que regulam vias de transporte vesicular. As Rab embora sejam proteinas solúveis, funcionam como "switch" molecular, associando com o folheto citoplasmático. Esta associação depende de uma modificação lipídica catalisada pelo enzima Rab Geranylgeranyl Transferase (RGGT). Este enzyme requer uma proteína acessória chamada "Rab Escort Protein"(REP), que se liga a Rabs não preniladas e as apresenta ao enzima.

Eu analisei a família Rab fazendo uso de métodos computacionais e da acessibilidade de genomas completamente sequenciados. Eu observei a existência de sequências apenas presentes nas proteínas Rab que designei motivos RabF. Estes motivos foram utilizados para pesquisar nas bases de dados e identificar novas proteínas desta família em vários organismos. Os motivos RabF são conservados em todas as proteínas encontradas. Eu proponho que posições conservadas nestes motivos medeiam interacções igualmente conservadas entre Rabs e REPs. Análise de sequências e estructuras de proteínas REP confirma a existência de uma superfície conservada que toda a evidência experimental sugere estar envolvida em ligação a Rabs. Na posse desta informação, um ensaio de ligação Rab:REP foi concebido, baseado no sistema "yeast two hybrid", e usado para testar mutações em uma Rab modelo, Rab3a. Os resultados obtidos indicam que a interacção entre as duas moléculas é de facto mediada por posições nos motivos RabF. Ainda relativamente à intreacção entre Rab e REP, eu concebi e implementei métodos que permitem, tanto em escala preparativa como analítica, preparar, purificar e analisar complexos entre estas duas proteínas *in vitro*.

Ao analisar a família Rab, eu observei que os motives RabF mapeiam às regiões da proteína que mudam de conformação de acordo com a ligação ao GTP ou GDP, as regiões "switch". Esta observação sugere que, ao contrário do que era aceite, a epecificidade das interacções entre Rabs e efectores não pode ser só determinada por estas regiões. Outras regiões são necessariamente importantes: eu identifiquei regiões que são especificamente conservadas em sub-famílias (*ie* interagem com os mesmos efectores) e que eu proponho serem responsáveis pela especificidade das interacções. Esta observação é válida para todas as proteínas Rab estudadas, e é incorporada nos critérios de numenclatura que eu proponho para esta família.

Eu observei que Rabs que co-segregam em árvores filogenéticas, tem estructuras genéticas semelhantes e, surpreendentemente, propriedades funcionais em comum. Eu proponho que estas Rabs formam "grupos funcionais", apresentam uma história evolutiva comum e são relacionadas em termos de função. Este novo coneito permite fazer correlações informativas entre função e padrões de conservação de sequência, e permitem compreender a multiplicação desta família ao longo da evolução.

Sommaire

La famille de protéines Rab/Ypt/Sec4 est la plus nombreuse parmi les familles de la super-famille des petites GTPases et est composée de protéines qui régulent les voies de transport vésiculaire. Les Rab sont des protéines solubles qui fonctionnent comme des "switch" moléculaires, en association avec les membranes cellulaires. Cette association est dependante de l'enzyme "Rab Geranylgeranyl Transferase". Cette enzyme nécéssite la présence d'une protéine auxiliaire appelé "Rab Escort Protein (REP)" qui s'attache aux Rabs non prénilées et les présente à l'enzyme.

J'ai analysé la famille des Rab avec des méthodes informatiques. J'ai observé l'existence de séquences seulement présentes sur les Rabs, que j'ai appelé motifs RabF. Ces motifs ont étés utilisés pour chercher dans des bases de données et identifier de nouvelles protéines de cette famille. Les motifs RabF sont conservées dans toutes les protéines trouvées. Je suggère que les positions conservées dans ces motives détermine les interactions entre REP et Rab. L'analyse des séquences et structures de protéines REP confirme l'existence d'une surface conservée qui l'évidence expérimentale suggére qu'elle soit impliquée dans l'attachment aux Rabs. Avec cette information un essai de attachment Rab :REP à été developpé basé sur le système "yeast two hybrid" et utilisé pour tester des mutations dans les Rab avec la modède la Rab3a. Les résultats indiquent que l'interaction entre deux molécules est en effet déterminé par des positions dans les motifs RabF. Concernant l'interaction entre Rab et REP, j'ai formulé et établi des méthodes qui permetent de préparer, à l'échelle préparative ou analytique, purifier et analyser les complexes Rab :REP *in vitro*.

J'ai observé que les motifs RabF sont localisés dans les régions "switch". Cette observation suggère que, contrairement avec ce qui était accepté, la spécificité des interactions entre Rabs et effecteurs ne peut pas être déterminée par ces régions. Autre régions sont également importantes : j'ai identifié des régions qui sont spécifiquement conservées dans les sous-familles et dont je suggère sont responsables par la spécificité de leurs interactions. Cette observation est valide pour toutes les protéines Rab étudiées.

J'ai observé que les Rabs qui co-ségrèguent en arbres philogénétiques présentent des structures génétiques similaires et, étonnament, des fonctions apparentées. Je suggère que ces Rabs forment des "groupes fonctionnels", présentnte une histoire évolutive communne et sont fonctionnellement apparentées. Ce nouveau concept permet faire des corrélations informatives entre fonctions et modèles de conservation de séquences et permettent de comprendre l'évolution des protéines de la famille Rab.

Declaração

De acordo com o nº 2 do artigo 8º do Decreto-Lei nº 388/70, utilizaram-se neste trabalho resultados já publicados, ou em vias de publicação, que a seguir se discriminam:

Pereira-Leal, JB and Seabra, MC (2000) The mammalian Rab family of small GTPases: Definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily *J. Mol. Biol.* **310**: 1077 – 1087

Pereira-Leal, JB; Gomes, AR, Seabra, MC (2001) Analysis and preparation of stable complexes between Rab GTPases, Rab Escort Protein and Rab Geranylgeranyl Transferase *Meth. Mol. Biol. In press*

Pereira-Leal, JB; Hume, AN and Seabra, MC (2001) Prenylation of RabGTPases: molecular mechanisms and involvement in genetic disease *FEBS lett* . **498**: 197-200

Pereira-Leal, JB and Seabra, MC (2001) Evolution of the Rab family of small GTP-binding proteins *submitted*

No cumprimento do Decreto-Lei mencionado supra, declaramos ser de nossa responsabilidade, excepto quando indicado, a execução das experiências que estiveram na base dos resultados aqui apresentados. São igualmente da nossa responsabilidade a interpretação dos resultados, sua discução e redacção.

Acknowledgements

I am indebt to many different people, who helped me both at the personal and scientific level during the period I spent preparing this thesis. To all of them I whish to thank. In particular, I would like to mention and thank....

***Miguel C. Seabra**, for his excellent supervision, for helping me to explore different ideas, for his friendship. A very special thanks for trusting and encouraging me when I followed a different approach, which involved sitting all day long at the computer .*

***Maria João Saraiva** who accepted to sponsor my thesis, for her support and help (and patience!) throughout these years.*

*All those who made possible the "**Programa Gulbenkian de Doutoramento em Biologia e Medicina**", which provided me a unique learning opportunity.*

***Fundação para a Ciência e Tecnologia** and **PRAXIS XXI**: which supported me financially for most of the duration of my thesis research, and the British **Medical Research Council** which provided financial support in the final months of my work.*

*All the present and past members of the **prenylation group** have provided for a stimulating working environment and helped me in my work in many occasions. A special mention to **Molly Strom** and **Alistair Hume**, for being always ready to discuss and actually do some crazy experiments. It has been most enjoyable!*

***Thomas Sippel-Dau**, from the Imperial College Center for Computing Services, without whose help I would have never survived the UNIX world (or recovered from the time I accidentally deleted the operating system from my workstation!).*

*Present and past members of the Sections of Molecular Genetics both in the **Imperial College School of Medicine** (UK) and in the **Southwestern Medical Center at Dallas** (USA) have in many occasions helped me in many different ways. **Axel** and **Herbert** in particular, with whom science was always fun.*

*All the technical and administrative staff that in many ways made my life as a PhD student possible, in particular **Joy Dexter** and **Carl Powel**, who helped me in countless occasions.*

*All the "**Portuguese crowd**" in London, that regularly on Friday evenings, in between beers, cigarettes and cheap curries, came to be a sort of "support group for depressed scientists".*

*To my wife **Monica**, who has walked the troubled waters of PhD studentship with me, for her support through the years.*

*Finally, a very special thank you to my father **Jacinto** and my stepmother **Lourdes**, for helping me pursue my dreams. For always reminding that this is just the beginning. For everything.*

Contents

Abstract iii
Sumário iv
Somaire v
Declaração vi
Acknowledgements vii
Contents viii
List of Figures xi
List of Tables xiii
List of Acronyms xiv
The progression of the research and the structure of this dissertation xv
Part I - Introduction I-1
I.1. Rab Small GTPases I-2
I.1.1. History and nomenclature I-2
I.1.2. The Small GTPase Molecular switch I-4
I.1.3. Switch ON: GEFs I-7
I.1.3.1. GEF catalysed exchange I-7
I.1.3.2. RabGEFs I-8
I.1.4. Switch OFF: GAPs I-10
I.1.4.1. Switching off I-10
I.1.4.2. RabGAPs I-12
I.1.5. Rab proteins in action I-13
I.1.5.1. What do they do? I-13
I.1.5.2. Is GTP hydrolysis really required? I-18
I.1.6. Rab:GDP binding proteins: General Regulators I-18
I.1.6.1. Rab Escort Proteins I-19
I.1.6.2. Rab GDP Dissociation Inhibitor I-19
I.1.6.3. MSS4, DSS4 and PRA1 ..... I-20
I.2. Lipid modification of Rab proteins I-21
I.2.1. Lipid modifications I-22
I.2.2. Protein Prenylation I-23
I.2.3. Prenylation of Rab proteins I-25
I.2.4. Post-prenylation processing I-26
I.2.4.1. Post-prenylation processing of CAAX proteins I-26
I.2.4.2. Post-prenylation processing of Rab proteins I-29
I.2.5. Deficient Rab prenylation and human disease I-30
I.3. The questions this work attempts to address I-33
Part II – Rab Proteins II-1

II.1. The Mammalian Rab Family of Small GTPases: **II-2**
Definition of Family and Subfamily Sequence Motifs Suggests a Mechanism for Functional Specificity in the Ras Superfamily II-2
II.1.1. Introduction II-2
II.1.2. Materials and Methods II-3
II.1.3. Results II-3
II.1.3.1. Rab-specific sequences (RabF1 to RabF5) II-3
II.1.2.2. New Rabs II-7
II.1.2.3. Rabs subfamilies and subfamily-specific sequences (RabSF) II-8
II.1.3. Discussion II-12
II.2. Evolution of the Rab family of small GTP binding proteins **II-15**
II.2.1. Introduction II-15
II.2.2. Methods II-16
II.2.3. Results and Discussion II-16
II.2.3.1. Identification and annotation of complete Rab families II-16
II.2.3.2. Rab familes across evolution II-22
II.2.3.3. Conserved interactions with general regulators and effectors II-24
II.2.3.4. Prenylation and targeting motifs II-26
II.2.3.5. Rab functional groups II-27
II.2.4. Conclusions II-30
II.2.5. Supplementary Material II-32
Part III – REP Proteins **III-1**
III. The Rab Escort Protein Family: **III-2**
Sequence and structural divergence in a conserved function **III-2**
III.1. Introduction III-2
III.2. Methods III-4
III.3. Results and Discussion III-5
III.3.1. The REP family III-5
III.3.2. Rab Binding: conserved property between REPs and RabGDIs III-8
III.3.2.1. Reassessment of REP/RabGDI SCRs III-8
III.3.2.2. Role of REP C-terminus in Rab binding III-11
III.3.3. Where do isoprenoids bind in REP and RabGDI? III-12
III.3.4. Prenylation of Rab proteins: unique REP property III-12
III.3.4.1. REP specific regions III-12
III.3.4.2. REP-specific insert region III-14
III.4. Conclusion III-17
Part IV – REP:Rab **IV-1**
IV.1. Analysis and preparation of stable complexes between Rab GTPases, Rab Escort Protein and Rab Geranylgeranyl Transferase **IV-2**
IV.1.1. Introduction IV-2

IV.1.2. Materials IV-3
IV.1.2.1. Geranylgeranyl Pyrophosphate IV-3
IV.1.2.2. Analytical scale preparation and purification of REP:Rab complexes IV-3
IV.1.2.3. Analytical scale preparation and purification of REP:Rab-GG complex IV-4
IV.1.2.4. Analytical scale preparation and purification of REP:RabGG:RGGT complex IV-4
IV.1.2.5. Analytical scale preparation and purification of REP:Rab[^{3}H]GG complex IV-4
IV.1.2.6. Large scale preparation and purification of REP:RabGG:RGGT complex IV-5
IV.1.3. Methods IV-5
IV.1.3.1. Expression and purification of recombinant proteins IV-5
IV.1.3.2. Analytical scale preparation and purification of REP:Rab complexes IV-5
IV.1.3.3. Analytical scale preparation and purification of REP:RabGG complex IV-6
IV.1.3.4. Analytical scale preparation and purification of REP:RabGG:RGGT complex IV-7
Iv.1.3.5. Analytical scale Preparation and Purification of REP:Rab[^{3}H]GG complexes IV-8
IV.1.3.6. Large scale preparation and purification of REP:RabGG:RGGT complexes IV-9
IV.1.4. Notes IV-10
IV.2 - Requirement for GTP hydrolysis prior to REP:Rab complex formation IV-11
IV.2.1 - Introduction IV-11
IV.2.2 - Materials and Methods IV-12
IV.2.3 - Results IV-13
IV.2.3.1 - Partial Purification of a RabGAP activity that acts on non-prenylated Rab1a IV-13
IV.2.3.2 - RabGAP insensitive Rab1 mutants are prenylated in vivo IV-20
IV.2.4 - Discussion IV-22
IV.3. RabF Motifs define the interaction surface in the RabREP complex IV-24
Introduction IV-24
Materials and Methods IV-26
Results and Discussion IV-27
V – Concluding remarks V-1
VI - References VI-1

List of Figures

Figure I.1- *Unrooted distance diagram of selected sequences from the mammalian Ras super-family.*
Figure I.2 - *The small GTPase molecular Switch*
Figure I.3 - *Topology diagram of the core structure of GTP binding proteins.*
Figure I.4 - *Alignment of model sequences representing selected families of small GTPases*
Figure I.5 - *Structure of p21 H-Ras:GTP and p21 H-Ras:GDP*
Figure I.6 - *Complementation of the active site of small GTPases by their GAPs: the "Arginine finger" model".*
Figure I.7 – *Simplified, schematic representation of the major steps that comprise a vesicular trafficking cycle.*
Figure I.8 - *Map of intracellular localization of Rab proteins.*
Figure I.9 – *(A) chemical structure of farnesyl pyrophosphate and genarylgeranyl pyrophosphate (B) Simplified scheme of the intermediates in the synthesis of cholesterol.*
Figure I.10 – *The Rab prenylation cycle*
Figure I.11 – *Model of Ras post-prenylation processing and trafficking.*
Figure I.12 - *Rab prenylation and disease of mice and man.*
Figure II.1.1 – *Frequency of the most common amino acid in the Rab family alignment.*
Figure II.1.2 – *Alignment of profile HMM model sequences*
Figure II.1.3 – *Alignment of RabF regions for novel and selected Rab proteins, and the corresponding regions in other representative small GTPases.*
Figure II.1.4 – *Phylogenetic tree of the 52 members of the mammalian Rab family.*
Figure II.1.5 – *Cartoon representing the Rab3a-GTP 3D structure.*
Figure II.2.1 – *Neighbour-Joining tree of the C. elegans and D. melanogaster Rab families*
Figure II.2.2 – *Neighbour-Joining tree of the H. sapiens Rab family*
FigureII.2. 3 – *Neighbour-Joining tree of the A. thaliana Rab family*
Figure II.2.4 – *Neighbour-Joining dendogram of the human, nematode, fly and fission yeast Rab families.*
Figure II.2.5 - *Partial alignment of model sequences for each family, calculated from pHMM describing the multiple sequence alignment of the complete Rab family in each organism.*
Figure II.2.6 – *Representation of the coding exons of available "functional group" members.*
Figure III.1 – *Neighbour-Joining dendogram of the GDI/REP superfamily.*
Figure III.2 – *Maximum parsimony tree of the REP family.*
Figure III.3 – *Domain organisation of the REP and GDI families*
Figure III.4 – *Molecular surface of RabGDI (1GND) and modelled human REP-1.*
Figure III.5- *Alignment of selected residues of some species of REPs and GDIs.*
Figure III.6 – *Alignment of model sequences emited from pHMM statistical models calculated for the REP and RabGDI family.*
Figure III.7 – *Alignment of the C-terminal portion of the REP family.*
Figure III.8 - *Modelled structures of REPs and αRGGT of the corresponding species.*
Figure IV.1.1 – *Separation of the REP:RabGG and REP:RabGG:RabGGTase complexes by ion exchange chromatography.*
Figure IV.2.1 – *Porcine Liver contains a RabGAP activity that acts on non prenylated Rab1a.*
Figure IV.2.2 – *Anionic exchange chromatography of porcine liver soluble fraction separates two distinct activity peaks.*
Figure IV.2.3 – *Activity profile of the eluate of a hydroxylapatite column chromatography of Q-sepharose activity peak QA.*
Figure IV.2.4 – *Activity profile of the eluate of a hydroxylapatite column chromatography of Q-sepharose activity peak QB.*

Figure IV.2.5 *– Partial purified RabGAP activities.*
Figure IV.2.6 *– Enzyme titration of QA activity peak, showing initial linear response to enzyme concentration and revealing a possible inhibition at higher concentrations of extract.*
Figure IV.2.7 *– Substrate saturation curve for QA activity peak*
Figure IV.2.8 *– Time curve of GTP hydrolysis in the presence and absence of RabGAP activity*
Figure IV.2.9 *– RabGAP-insensitive single point mutations mapped to canine Rab1a sequence.*
Figure IV.2.10 *– GFP-Rab1(D47N) and (I44M) do not accumulate unprenylated in the cytosol but associate with the golgi in a way that is not distinguishable form the wild type protein.*
Figure IV.3.1 *– Mapping of the mutated positions (in red) used in this study to a cartoon representing the molecular surface of Rab3a.*
Figure IV.3.2 *– Positions involved in REP binding (in blue), mapped to cartoon representing the molecular surface of Rab3a.*
Figure IV.3.3 *- Expression of REP-1 and Rab3 in the yeast two hybrid REP:Rab binding assay*
Figure IV.3.4 *– Alignment of select human proteins, indicating the REP-binding positions*

List of Tables

Table I.1. *RabGEPs*
Table I.2 - *RabGAPs*
Table I.1 - *Effectors of Rab proteins.*
Table II.1.1 - *Amino-acid identity within the different Rab Sub-Family Specific regions (RabSF).*
Table II.2.1 – *Rab families in six different organisms*
Table II.2.2 – *Structure of the Rab family in six organisms.*
Table S.1 – *S. pombe Rab family*
Table S.2 – *S.cerevisiae Rab family*
Table S.3 – *C. elegans Rab family*
Table S.4 – *D. Melanogaster Rab family*
Table S.5 – *A. thaliana Rab family*
Table S.6-*H. sapiens Rab family*
Table III.1 – *REP family members in different species*
Table IV.2.1 – *Purification table of the partial purification of the different Rab1GAP activities*
Table IV.2.1 – *Purification table of the partial purification of the different Rab1GAP activities*
Table IV.3.1 – *Yeast two hybrid Rab3a:REP binding assay results*

List of Acronyms

FPP	Farnesyl pirophosphate
FTase	Farnesyl transferase
GAP	GTPase Activating Protein
GDI	GDP dissociation Inhibitor
GDP	Guanosine diphosphate
GEF	Guanosyl nucleotide Exchange Factor
GGPP	geranylgeranyl pirophosphate
GGTaseI	Geranylgeranyl transferase
GPI	Glycosyl-phosphatidylinositol
GTP	Guanosine triphosphate
pHMM	profile Hidden Markov Models
PCR	Polymerase Chain Reaction
REP	Rab Escort Protein
RabGGTase	Rab Geranylgeranyl Transferase
RGGT	Rab Geranylgeranyl Transferase
XTP	Xantosine triphosphate
XDP	Xantosine diphosphate

The progression of the research and the structure of this dissertation

The present thesis represents part of the work performed in the past 4 years to complete my PhD dissertation. The first year of work was done at the Southwestern Medical Center at Dallas, USA, then the group moved to the UK and the second year of research was done at the Imperial College School of Medicine at St. Mary's Hospital. We moved again across Hide Park, within London, to a new purpose-built research building in South Kensington that now houses the division of Biomedical Sciences of the Imperial College School of Medicine.

This dissertation is organised in five major parts that I hope reflect a logical progression for the reader of this work, but that do not always reflect the chronological order in which the work was done. I started my experimental work using essentially lab bench-based techniques to address questions about the biochemistry of the lipid modification of Rab proteins, mainly concentrating on several aspects of the formation of the complex between Rabs and Rab Escort Proteins. As the work progressed, I realised that a substantial amount of the foundation work about the structure/function relationships of the proteins involved in this reaction was still to be done. I decided then to start exploring those structure/function issues, using computational based approaches. However, instead of obtaining simple and quick answers, I uncovered even more questions. As the time (and funding!) I had available was limited, I decided to concentrate my efforts in making a study of the primary structure of the proteins of interest to this thesis (Rab small GTPases and Rab Escort Proteins) and then trying to clarify some of the questions that these studies raised.

Part I is an introduction to the thesis, and consists of a review of the relevant literature followed by a statement of the aims of the research.

Part II of this thesis concerns the Rab family of small GTPases, and is divided in two chapters. The first chapter (chapter II.1) concerns what distinguishes Rab proteins from other small GTPase families, which parts of the protein could be interacting with specific effectors and general regulators, uncovering a mechanism of specificity of binding determination different form the previously accepted. I take this analysis a step further in the second chapter (chapter II.2), where I identify and annotate the complete Rab families in all organisms which had their genome

substantially sequenced and use this information to investigate if the models proposed for Rab interactions in the previous chapter are extensible to Rab families in other organisms. Also, I attempt to reconstruct the multiplication of Rab proteins that accompanies multicellularity.

Part III concerns Rab Escort Proteins, general regulators of the lipid modification and function of the Rab family. I investigate the primary structure determinants for the function of these proteins.

In Parts II and III predictions are made concerning how Rab proteins will interact with Rab Escort Proteins. Part IV of this thesis concerns the formation of the REP:Rab complex. A substantial amount of work was dedicated to creating tools for the study of how lipid and non-lipid modified small GTPases will interact with REPs and RabGGTase. These methods are presented as the first chapter of part IV (chapter IV.1). The next chapter (IV.2) is the testing of the previously proposed requirement for a step of GTP hydrolysis catalysed by a RabGAP prior to REP:Rab complex formation. Finally, in the last chapter of Part IV, a combination of *in silico* methods and experimental methods is used to determine the interface of the REP:Rab complex, thus testing the predictions resulting from the work described in parts II and III.

Part I - Introduction

I.1. Rab small GTPases

GTP binding proteins (G proteins) are involved in a variety of biological phenomena and diverge markedly in their primary structure. Well known examples of G proteins are members of the Ras-like small GTPases, of which the Rab family is part, heterotrimeric G-proteins involved in signal transduction from G-protein coupled receptors to second messenger generating effectors (see ref for review Mumby, 2000), and translation factors like EF-Tu and EF-G (reviewed in Abel and Jurnak, 1996).

The present work concerns the Rab family of small GTPases, which regulate vesicular trafficking pathways. This introduction will thus make a brief review on the history, structure, regulation and function of members of the Rab family. Mechanistic aspects of the function of small GTPases were first elucidated in members of the Ras family and these results will also be discussed when they are essential for the understanding of Rab function.

Rabs, like other small GTPases are subject to lipid modifications to their C-termini which are essential for membrane association and function. For clarity sake, this will be discussed independently later in this introduction.

The motivations and highlighting of the main questions which are address in this thesis are presented at the end of this introduction.

I.1.1. History and nomenclature

Small GTPases were identified from the Harvey and Kirsten sarcoma virus. These were the result of passage through rat cells of virus which lacked oncogenic potential, but through recombination events gained oncogenic sequences that were named H-Ras and K-Ras (Ellis et al., 1981; Ellis et al., 1980; Kirsten and Mayer, 1967; Harvey, 1964). Further characterisation of the v-H-ras gene revealed that it corresponded to a mutated, intronless form of a cellular protein (Chang et al., 1980), able to bind (Shih et al., 1981) and hydrolyse (Gibbs et al., 1984) guanine nucleotides. H-Ras was the first oncogene to be found in human tumours, indicating that proto-oncogenes can be activated by two different routes: transducing of proto-oncogenes by retroviruses or through somatic or replicative mutations (Der et al., 1982; Parada et al., 1982; Santos et al., 1982).

The first Rab was identified serendipitously as a gene with homology to Ras which lied between the actin and tubulin genes of *Sacharomyces cerevisae*. It was originally named YP2 (for Yeast Protein 2) and encoded a protein sharing about 30% identity with mammalian Ras protein (Gallwitz et al., 1983). Later the Rho genes were identified first in Aplysia and then in humans (Madaule and Axel, 1985). The realisation that Ras and Ras-like

proteins belonged to a possibly large and diverse family of proteins prompted researchers to employ a variety of approaches to identify new members of this family.

YP2 was later renamed to YPT1, (standing for Yeast Protein Transport) (Schmitt et al., 1986) and defined a new family of small GTPases. The involvement of members of this new family in vesicular transport however was only established with the identification of the SEC4, the gene mutated in the secretory mutant sec4. This mutant accumulates post-golgi vesicles and displays blocked transport to the plasma membrane (Goud et al., 1988; Salminen and Novick, 1987). Mammalian homologues of Sec4 and Ypt1 were named Rab proteins, describing the organ they were initially identified in (**RA**t **B**rain) (Touchot et al., 1987).

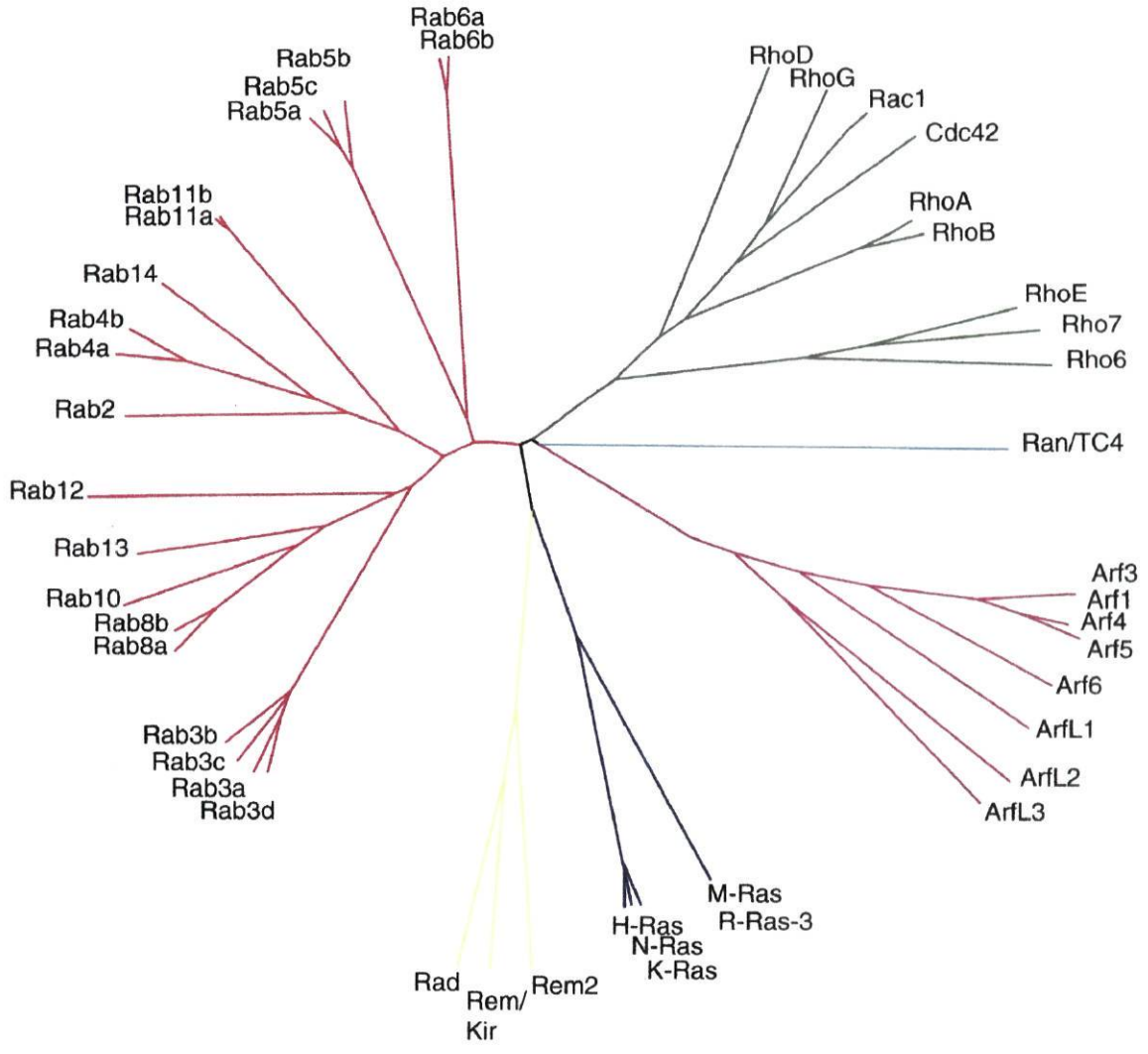


***Figure I.1**- Unrooted distance diagram of selected sequences from the mammalian Ras super-family. Distance matrix and tree calculated using the Neighbour-Joining method and based on an un-edited Clustal W 1.80 multiple sequence alignment of protein sequences.*

Today, the Ras superfamily of small GTPases comprises a large group of proteins that regulate important cellular processes such as growth, motility and protein trafficking. Members of this superfamily are also known as ras-like GTPases, small GTPases or small G proteins. A formal definition of the members of this super-family was accorded at the FASEB summer research Conference on "Low Molecular Weight GTP-Binding Proteins" (Kahn et al., 1992) and defines proteins which have molecular weights in the range of 20-29 KDa, present the consensus motifs for GTP-binding proteins (which will be discussed bellow), and have recognisable family specific motifs. The division in families is also based on purely structural grounds. Five families are recognised (see figure I.1), but recently a new family has been suggested, including the small GTPases Rem/Kir, Rad and Gem.

Different small GTPases are included into one of the existing families if they show above 30% identity with members of that family and if they display specific sequence motifs that characterise each family. For Rab proteins, the only family specific characteristic is the presence of two C-terminal cysteine residues, the Rab prenylation motif which will be discussed later in this introduction. Rabs are numbered according to their discovery, but by the time the eight mammalian Rab protein was identified (Rab8a), this criteria failed because this proteins displays only one C-terminal cysteine residue which resembles those found in the Rho and Ras families (the CAAX box, discussed in the context of Protein Prenylation). The identification of Rab-specific sequence motifs that permits the distinction between members of this family and any other small GTPase family is the subject of chapter II.1.

I.1.2. The Small GTPase Molecular switch

Small GTP binding proteins share the common characteristic of behaving as molecular switches by alternating between two different conformations. When bound to GTP the protein is said to be ON, or in the "Active" conformation. Hydrolysis of GTP to GDP brings the molecule to the "Inactive" or OFF conformation.

In the active conformation, small GTPases can bind effectors and transduce biological signals. By definition, effectors of small GTPases are proteins which bind only one specific GTPase, or a restricted set of GTPases, distinguish between active and inactive conformations and only bind to the active conformation (figure I.2). In the inactive conformation, small GTPases can only bind to general regulators, proteins that by definition bind all or the majority of the members of one family of small GTPases and recognise and bind to the inactive conformation (figure I.2). The transition between active and inactive conformations is tightly regulated by a class GTPase interacting proteins which, for clarity of discussion, we term here regulators of nucleotide status, and which will be discussed bellow (figure I.2).

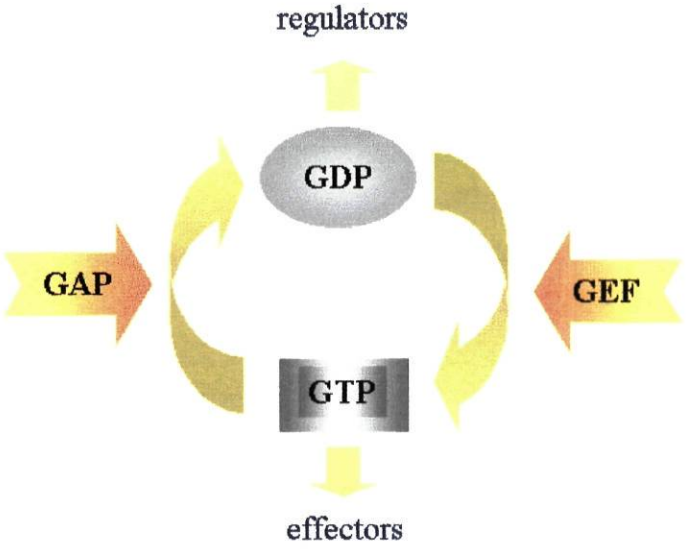


Figure I.2 *- The small GTPase molecular Switch*

Sequence analysis of small GTPases and the availability of crystal structures for some members of this family permitted the identification of consensus GTP binding motifs that are conserved throughout the super-family and a conserved fold that is characteristic GTP binding proteins. This conserved fold, represented in figure I.3, consists of a core structure of five α-helixes, 5 parallel β-sheets and one anti-parallel β-strand (Pai et al., 1989; for review see Kjeldgaard et al., 1996).

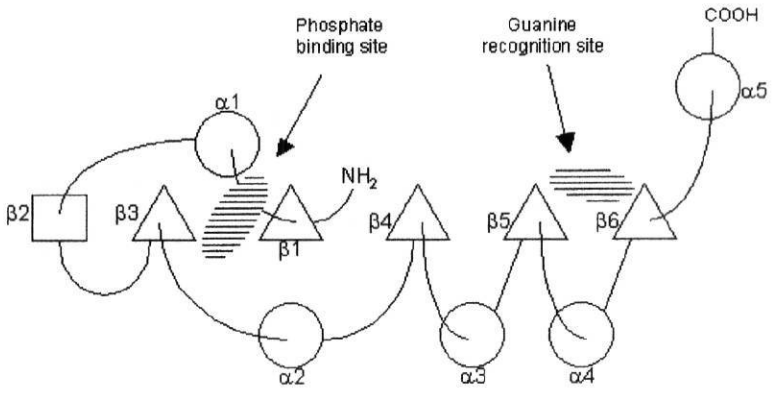


Figure I.3 *- Topology diagram of the core structure of GTP binding proteins. Adapted from (Kjeldgaard et al., 1996).*

Figure I.4 *- Alignment of model sequences representing selected families of small GTPases (Adapted from Pereira-Leal and Seabra, 2000). The PM and G motifs are highlighted in green (see text). PM1 has the consensus sequence GxxxxGK[S,T] where [S,T] means S or T can be found in that position, and localises in the loop 1, also known as the "P-loop". A single conserved T residue localised in loop 2, also known as "effector loop" defines PM2, and PM3 localises in β3/loop4 with the consensus sequence DTAGQ. The G1 motif is a single conserved F or Y residue in loop 2 (effector loop), G2 localises in β5/loop8 and has the consensus GNKxD , and G3 resides in β6/loop10 with the consensus sequence SAK.*

In small GTPases the conserved motifs, highlighted in figure I.4, are named PM1 to PM3 and G1 to G3 motifs (see figure I.4), involved in phosphate and Mg2+ binding and guanine binding, respectively (see (Valencia et al., 1991) for review). In Ras proteins, there are two regions that change conformation upon GTP binding and hydrolysis, highlighted in figure I.5: Switch I resides in loop 2 (effector loop) and switch II in loop4/α2/loop5. The first evidence that Rabs behave in the same way came from limited proteolysis studies in Rab5, in which nucleotide dependent conformational changes were detected in the putative Rab Switch II region (Nikolova et al., 1998).

The solution structure of murine Rab3a in the active conformation (Dumas et al., 1999), followed by the solution structure of Rab6 from *Plasmodium falciparum* bound to GDP (Chattopadhyay et al., 2000) provided evidence for Rabs displaying, like Ras proteins, two switch regions. The exact boundaries of these switch regions were recently determined with the solution structure of one Rab proteins (Sec4) both in the active and inactive conformation (Stroupe and Brunger, 2001). Later in this introduction we will return to the "molecular switch" in Rab proteins.

Figure I.5 *- Structure of p21 H-Ras:GTP (accession number 121P) and p21 H-Ras:GDP (accession number 4Q21), highlighting the Switch regions in red (Switch I) and green (switch II).*

I.1.3. Switch ON: GEFs

The reaction of dissociation of GTP and binding of GTP, termed "exchange", is usually very slow, and so is in general the hydrolysis of GTP to GDP . Exchange is catalysed by guanine-nucleotide exchange factors (GEFs). Exchange not assisted by these proteins is usually termed "intrinsic exchange".

I.1.3.1. GEF catalysed exchange

Exchange factors bind exclusively to the GDP bound small GTPases and are thought to destabilise the binding to GDP, thus promoting an increase in the off rate of GDP (Wittinghofer, 1998; Pan and Wessling-Resnick, 1998). Due to the excess of GTP over GDP in the cytosol (Bourne et al., 1991), the increase in the exchange rate has the consequence of promoting the binding to GTP. GTP binding results in conformational changes which cause the GEF to dissociate from the GTP:small GTPase complex (Wittinghofer, 1998).

The first insight into how this is accomplished came from the recent solution of the crystal structure of the binary complex of H-ras with its exchange factor Sos (Boriack-Sjodin et al., 1998). Through interactions with the Switch I, Sos opens the guanine nucleotide-binding pocket. Through interactions with the Switch II, Sos alters the chemical environment of the binding site for the phosphate groups of the nucleotide and the associated magnesium

ion, so that their binding is no longer favoured. As Sos does not affect the binding to the ribose and base of GTP or GDP, it favours nucleotide release and binding.

GEFs for different small GTPases are very divergent at the primary and tertiary structure level. Not all GEFs have the same structural motifs found in Sos, nor have they recognisable conserved sequence motifs. This suggests that different GEFs may promote exchange in different ways, and raises the possibility that GEFs for different small GTPases evolved independently (see (Cherfils and Chardin, 1999) for review). However, a conserved mode of interaction between GEFs and small GTPases has been suggested (Day et al., 1998), raising the possibility that although the different GEFs may be structural distinct, some still undetectable conserved sequence or structural elements may exist, underlying a conserved exchange mechanism.

I.1.3.2. RabGEFs

Rab activation is a membrane associated event that follows membrane association of the prenylated Rab protein and displacement of the general regulator RabGDI (Soldati et al., 1994; Ullrich et al., 1994). However, exchange factors that act on members of the Rab family (RabGEFs) have proved elusive to identify.

DSS4 and its mammalian counterpart MSS4 were the first molecules to be proposed as Rab exchange factors (Moya et al., 1993; Burton et al., 1993). However, there is strong evidence that supports a different role for these proteins as general regulators of Rab function. They will be discussed together with other general regulators in section I.1.6.

Table I.1 *- RabGEPs*

RabGEF	Rab specificity	Reference
p619*(human)*	Rab5	(Rosa et al., 1996)
Rab3-GEP*(rat)*	Rab3 Isoforms	(Wada et al., 1997)
Aex3*(nematode)*	Rab3	(Iwasaki et al., 1997)
Sec2(yeast)	Sec4	(Walch-Solimena et al., 1997)
Rabex-5(mouse)	Rab5a	(Horiuchi et al., 1995)
Vps9(yeast)	Ypt5	(Hama et al., 2001)
Ric1p:Rgp1p complex(yeast)	Ypt6	(Siniossoglou et al., 2000)
TRAPP complex(yeast)	Ypt1	(Wang et al., 2000)
Vps39(yeast)	Ypt7	(Wurmser et al., 2000)
SopE(salmonela)	Rab5	(Mukherjee et al., 2000)

The protein p619 is an unusually big protein which is able to catalyse nucleotide exchange in both Arf1 and Rab proteins (Rosa et al., 1996). It interacts with clathrin, thus suggesting a role in endocytosis (Rosa and Barbacid, 1997). The exact role of p619 is still unclear, particularly in the light of its sequence homology to RCC1, an exchange factor for the small GTPase Ran which is involved in nuclear-cytoplasmic transport.

Rab3-GEP is specific for prenylated members of the Rab3 sub-family. This requirement for lipid modification is consistent with the observation that exchange happens after membrane association of the Rab protein (Soldati et al., 1994; Ullrich et al., 1994). However, the same requirement in not observed for all other RabGEFs.

Rab3-GEP has high sequence homology to the gene product AEX-3, and the fact that mutations in the AEX-3 gene cause pre-synaptic defects, namely the accumulation of Rab-3 containing vesicles in the cell body, supports an essential, physiological role for the Rab3 exchange factor in the regulation of Rab3 activity (Iwasaki et al., 1997).

In yeast, the null Vps39 mutant exhibits the same phenotype as the ypt7 deletion (Nakamura et al., 1997; Wichmann et al., 1992), and the same is true for Vps9 which results in the same accumulation of 40-50 nm vesicles as observed for Ypt51 (Burd et al., 1996; Horazdovsky et al., 1994). A more dramatic effect is caused by the deletion of sec2 which is lethal (Nair et al., 1990).

RabGEFs for homologous Rab proteins in different organisms seem to be related to one another, as is exemplified by Rabex-5 and Vps9, exchange factors for the Rab5/Ypt5 GTPases, by Rab3-GEP and aex-3, exchange factors for Rab3 and by the fact that Ric1p and Vps39 have homolgies to proteins in other organisms. In contrast, RabGEFs for different Rab proteins show no common trait that could be used to identify novel RabGEFs or that could suggest a mechanism of Rab activation. Interestingly, SopE, which also acts as an exchange factor for Rab5, does not share any significant homologies with Vps9 or Rabex-5, suggesting different evolutionary origins.

SopE is a secreted salmonella protein which recruits non-prenylated Rab5 to the infected phagosome membrane, promoting its fusion with early endosomes, thus avoiding transport to lysosomes (Mukherjee et al., 2000). This observation, together with the observation that mutations in the exchange factors aex-3, Vps9 and Ric1p/Rgp1p all produce mislocalisation of its target Rab, supports the hypothesis that exchange factors may play a role in recruiting Rabs to their target membranes.

I.1.4. Switch OFF: GAPs

Switching off the signal being transduced by an active GTPase is acomplished through conformational changes resulting from GTP hydrolysis. Intrinsic hydrolysis in some GTPases is fast and the GTPase is thought to work as a "time switch" that will switch off after some time after activation. Small GTPases fall in the general category of "triggered switches" where the intrinsic hydrolysis is slow and GAPs (GTPase Activating Proteins), by accelerating the intrinsic hydrolysis rate, shut off the switch (Kjeldgaard et al., 1996).

Hydrolysis in the absence of GAP is, as mentioned for the exchange reaction, termed "intrinsinc hydrolysis". Intrinsinc hydrolysis rates in Rab proteins are in the range of (10^{-3} min^{-1} to 10^{-2} min^{-1}) (see for example (Zerial and Huber, 1995). GAP activation can increase these rates by a factor of three orders of magnitude (eg (Clabecq et al., 2000).

GAPs and GEFs provide the cell with a way of regulating when and where the molecular switch should be turned on or off.

I.1.4.1. Switching off

Several GAPs have been identified and crystallised for different families of small GTPases, but until recently no common feature was observed between them that suggested a common functional mechanism. However, the observation that oncogenic mutation in Ras that lock the GTPase in its active conformation are insensitive to GAP activation, but do not affect the formation of the GAP:GTPase complex, indicates that GAP action requires more than just binding to the GTPase.

Experiments using aluminium fluoride (AlF_4^-), a molecule which binds the small GTPase:GDP complex, mimiquing the transition state of the GTPase reaction, showed that although the GTPase:GDP complex cannot bind AlF_4^- on its own (Kahn, 1991), it does so in the presence of a GAP activity (Ahmadian et al., 1997; Mittal et al., 1996), thus suggesting that the GAP acts by stabilising the transition state of the GTPase reaction.

The realisation that GAPs exhibit conserved Arg residues (see for review Scheffzek et al., 1998) and the fact that a computational model of the interaction between p120GAP and Ras suggested that two invariant Arginine residues in the GAP protein were brought in close proximity of the nucleotide (Scheffzek et al., 1996) implied these Arg residues in the catalysis mechanism.

This was confirmed with the solution structure of the complex between the GAP domain of p120GAP and H-Ras, which revealed that an Arginine side chain (R789 – the "Arginine Finger") protrudes into the active site of Ras and neutralises the charges

developing in the transition state by specific interactions with the β-phosphate of the guanine nucleotide (Scheffzek et al., 1997) (see figure I.6). In addition, the main chain carbonyl oxygen of this Arg residue forms an hydrogen bond with a side chain of Gln61 in Ras, thus stabilising this residue which is important in catalysis. A positively charged residue in a "secondary" loop (as opposed to the Arginine finger which is considered the primary loop) in the GAP stabilises the primary loop by side chain – main chain interactions (see figure I.6). The same interactions are found in the complex between the catalytic domain of p50RhoGAP and RhoA (Rittinger et al., 1997). Mutational analysis in RabGAPs in yeast (Albert et al., 1999) suggests that these proteins will also obey the "arginine finger" model.

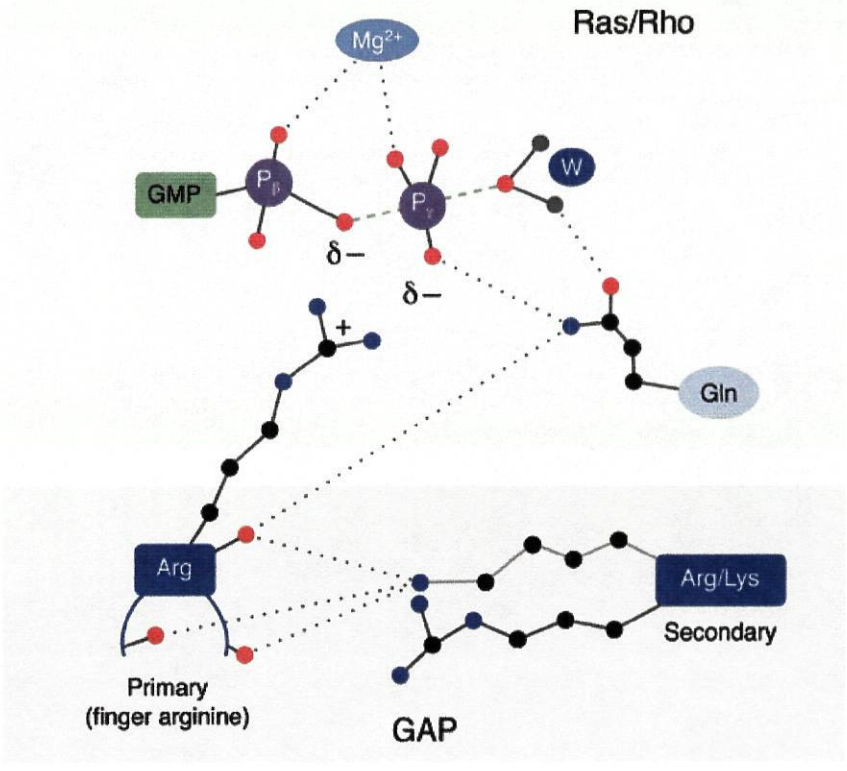


Figure I.6 - *Complementation of the active site of small GTPases by their GAPs: the "Arginine finger" mode". (reproduced from Scheffzek et al., 1998).*

Although GAPs for small GTPases are very divergent, a conserved core structure of seven α-helices was identified in RasGAP and RhoGAP (Bax, 1998; Rittinger et al., 1998). In both works the evolutionary implications of this finding is discussed, but for one it reflects convergent evolution of RasGAP and RhoGAP (Bax, 1998), while for the other it supports a shared ancestry between the two proteins (Rittinger et al., 1998). The recent solution structure of Gyp1, a yeast RabGAP reveals an protein with 16 α-helices, but there is no super-impositions of these and the α-helical core described above (Rak et al., 2000), thus limiting the scope of mentioned the evolutionary speculations to RasGAPs and RhoGAPs.

However, the Arginine finger model is not the only solution found in evolution for small GTPase activation. The solution structure of the complex between Arf1 and ArfGAP suggests that ArfGAP acts instead by inducing Switch II into a catalitically competent conformation (Goldberg, 1999).

I.1.4.2. RabGAPs

The complete yeast complement of RabGAPs has been identified (see table I.2), but only a few mammalian RabGAPs have been described so far.

Table I.2 - *RabGAPs*

RabGAP	Rab Specificity	Reference
Gyp6	Ypt6, Ypt7	(Strom et al., 1993)
Gyp7	Ypt7, Ypt31, Ypt32, Ypt1	(Vollmer et al., 1999)
Gyp1	Ypt1, Sec4	(Du et al., 1998)
Mdr1/Gyp2	Ypt6 and sec4	(Albert and Gallwitz, 1999)
Msb3/Gyp3	Sec4, Ypt6, Ypt51, Ypt31, Ypt32, Ypt32	(Albert and Gallwitz, 1999)
Msb4p/Gyp4	Sec4, Ypt6, and Ypt7	(Albert and Gallwitz, 2000)
Rab3-GAP	Rab3 isoforms	(Fukui et al., 1997)
GAPCenA	Rab6, Rab4, Rab2	(Cuif et al., 1999)
Tuberin	Rab5	(Xiao et al., 1997)
p120 RasGAP	Ras, Rab5	(Liu and Li, 1998)

All known RabGAPs are cytosolic proteins. The yeast proteins share sequence similarity (Neuwald, 1997), but with the exception of GAPCenA, this does not happen in mammalian RabGAPs (Cuif et al., 1999). This divergence is particularly illustrated by the observation that Rab3-GAP is an heterodimeric protein, composed of a catalytic and a regulatory subunit (Nagano et al., 1998; Fukui et al., 1997), unlike all other RabGAPs which are monomeric proteins.

The yeast RabGAPs are fully functional on non-prenylated Rabs (Albert and Gallwitz, 2000; Albert and Gallwitz, 1999; Vollmer et al., 1999; Du et al., 1998; Strom et al., 1993), and so are tuberin and p120RasGAP (Liu and Li, 1998; Xiao et al., 1997). However GAPCenA exhibits a strict requirement for lipid modification (Cuif et al., 1999). Rab3-GEP was described as a a GAP specific for prenylated substrates (Fukui et al., 1997) but recently this requirement was questioned on the grounds that no difference in Rab3-GAP preference and activity between prenylated and non-prenylated substrates was detectable (Clabecq et al., 2000).

RabGAPs are thought to activate GTP hydrolysis by the "Arginine finger" type mechanism described before. The active domains in Gyp1 and Gyp7 were identified by deletion analysis and by site directed mutagenesis one critical Arg residue in the active domain identified (Albert et al., 1999). Another supportive evidence for a conserved

mechanism of GAP action came from the solution structure of Gyp1 (Rak et al., 2000). Manually docking the crystal structures of Gyp1 and Ypt51 suggests that the best docking solution results in the alignment of the same arginine residue with Gln66 in Ypt51 (equivalent to Gln61 in p12 Ras) (Rak et al., 2000). Additional evidence for a conserved mechanism of GAP action came from the biochemical characterisation of the activation of GTP hydrolysis in Rab3 by Rab3-GAP. Mutation of conserved arginines in Rab3-GAP abolishes GAP activity, and the kinetics of interaction between Rab3-GAP and the different Rab3 mutants is consistent with a mechanism similar to that of RasGAP and RhoGAP (Clabecq et al., 2000).

The sequence conservation between yeast RabGAPs surprisingly is also shared by spindle assembly checkpoint proteins in yeast (Neuwald, 1997). The biological significance of this finding is still unknown but may indicate that RabGAPs provide a link between vesicular transport and other cellular processes. Evidence towards this comes from the only mammalian RabGAP which shares homology with yeast RabGAPs, GAPCenA. This protein is mainly cytosolic, but a minor fraction associates with the centrosome (Cuif et al., 1999). As GAPCenA can form complexes with γ-tubulin this may suggest that GAPCenA may provide a link between microtubule dynamics and vesicular transport (Cuif et al., 1999).

I.1.5. Rab proteins in action

I.1.5.1. What do they do?

Eukaryotic cells transport proteins between different compartments. This is accomplished by a complex series of events which is schematised in figure I.7. Vesicles, in which the correct contents are sorted and packaged, bud and detach from one compartment (donor membrane), then move to its target membrane where fuse and release their contents into the lumen of the organelle (for review see Zerial and McBride, 2001; Guo et al., 2000; Kamal and Goldstein, 2000; Hawes et al., 1999).

Rab proteins were first implicated in vesicular trafficking in the late 1980ies with the identification of SEC4 as the gene mutated in a yeast mutant which displays blocked transport to the plasma membrane (Goud et al., 1988; Salminen and Novick, 1987).

More than fifty Rab proteins have been identified to date in mammalian cells (Pereira-Leal and Seabra, 2000). Each Rab is believed to be localised to a specific subcellular compartment (see figure I.8) and some show tissue-specific variation in expression level, reflecting the complexity and variety of trafficking events found in mammalian cells (for review see Zerial and McBride, 2001; Guo et al., 2000; Chavrier and Goud, 1999; Olkkonen and Stenmark, 1997).

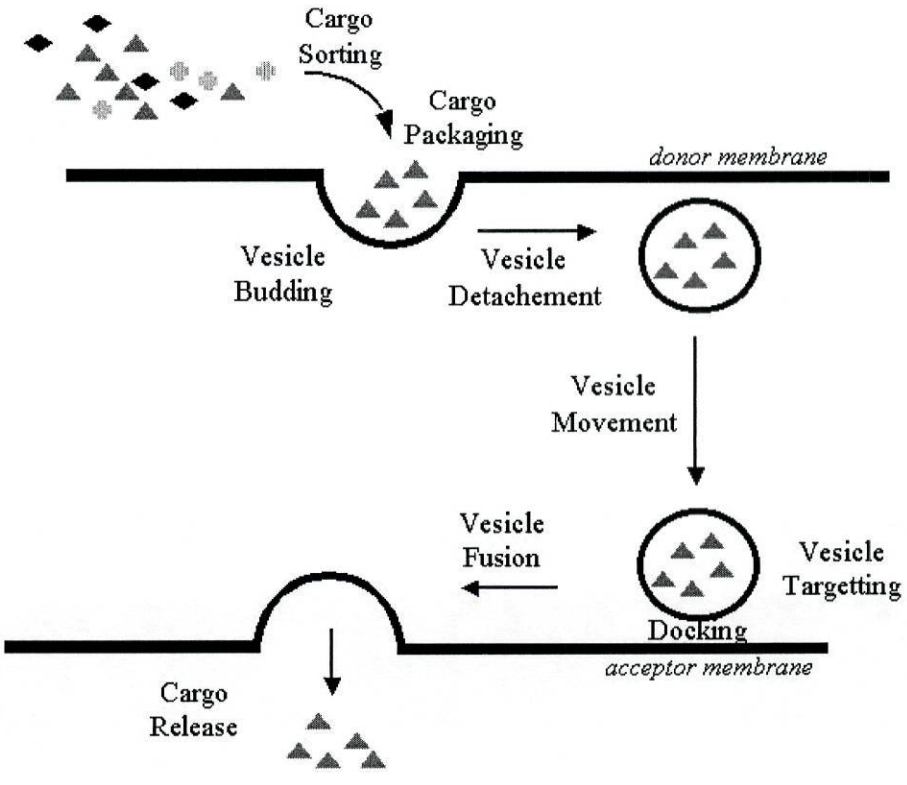


Figure I.7 *– Simplified, schematic representation of the major steps that comprise a vesicular trafficking cycle.*

The sec4 mutant exhibits accumulation of vesicles which lead to the proposition that Rab proteins work at the docking or fusion stage. Evidence which supports this view has been produced for a variety of Rab proteins (see for review Zerial and McBride, 2001; Olkkonen and Stenmark, 1997). For example antibodies agains Rab5 inhibit *in vitro* homotypic fusion of endosomes, in cultured cells, over-expression of dominant negative Rab5 mutations inhibits early endosome fusion, and finally over-expression of dominant activated Rab5 mutations stimulates early endosome fusion (Stenmark et al., 1994; Bucci et al., 1992; Gorvel et al., 1991). All this indicates a role for Rab5 in endosome fusion. Another example is Ypt1p. Antibodies against this protein inhibit ER to Golgi transport, resulting in the accumulation of vesicles, which indicates that vesicle formation is not affected (Baker et al., 1990). Furthermore, in recent work by Cao and Barlowe (Cao and Barlowe, 2000) it was observed that, in an *in vitro* system, vesicles prepared from yeast containing a mutant allele for Ypt1p which causes blocking of transport *in vivo*, are able to fuse with vesicle prepared from wild type acceptor membranes, but wild type vesicles are not able to fuse with membranes prepared from mutant yeasts. This strongly indicates that Ypt1p is required in the acceptor membrane and not in the donor membrane.

Additionally several effectors have been identified which support the widely accepted role of Rab proteins in the docking and fusion stage (see table I.1).

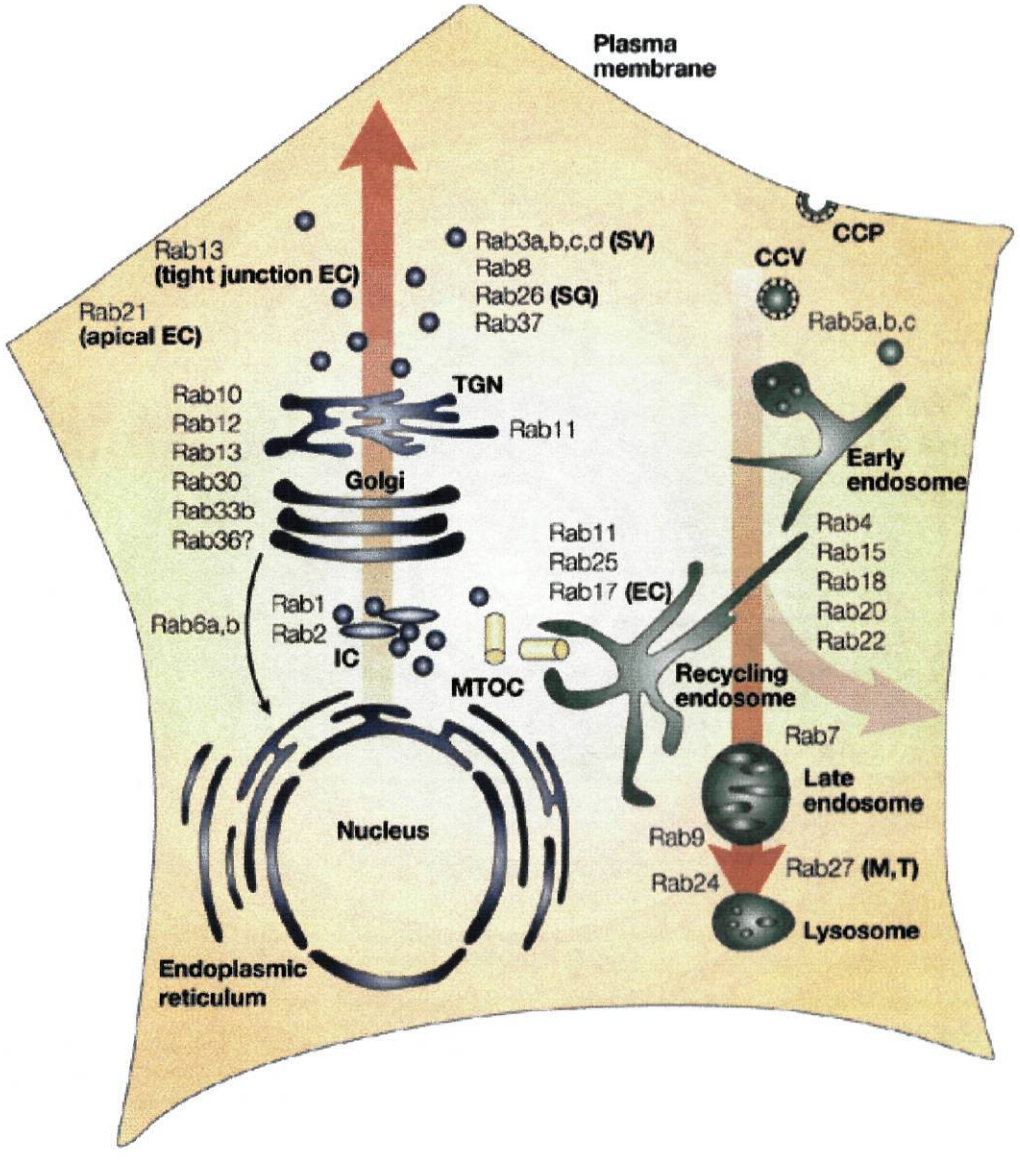


Figure I.8 *- Map of intracellular localization of Rab proteins, reproduced from (Zerial and McBride, 2001). (CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; EC, epithelial cells; IC, ER–Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granules; SV, synaptic vesicles; T, T-cell granules; TGN, trans-Golgi nextwork.).*

However, some evidence as been advanced which is suggestive of Rabs also playing a role in budding of vesicles from the donor compartment. Studies in *Sacharomyces cerevisiae* have shown that inactivation of Ypt31 and Ypt32 results in the enlargement of the Golgi complex, but not in the accumulation of vesicles (Jedd et al., 1997; Benli et al., 1996).

This suggests an inability to bud vesicles from the Golgi (Jedd et al., 1997; Benli et al., 1996). Also, clathrin coated pits can be formed in the absence of Rab5, but this protein is absolutely required for efficient cargo (transferin) sequestration (McLauchlan et al., 1998). This result came as a surprise at the light of the evidence described above which implicates Rab5 in endosome fusion. Rab5 thus seems to be a regulatory factor both for the early steps of vesicle formation and of vesicle fusion. Additionally, Nuoffer and co-workers (Nuoffer et al., 1994) observed that a dominant negative mutant Rab1a inhibits the formation of vesicles from the ER, suggesting a role in vesicle budding. However, no effector protein has been identified which supports a role for Rabs in membrane budding.

Another role for Rab proteins has been identified recently -mediating vesicle motility (see Deacon and Gelfand, 2001 and Kamal and Goldstein, 2000 for review). Echard and co-workers using the two-hybrid system, identified a kinesin-like molecule which they named Rab kinesin6, as a Rab6 interacting protein (Echard et al., 1998). This suggests a role for Rab6 in microtubule-dependent transport. More recently Rab5 was found to mediate attachment and mobility of endosomes along microtubules (Nielsen et al., 1999), and Rab27 was found to regulate the peripheral localisation of melanosomes via interactions with myosinVa (Hume et al., 2001; Bahadoran et al., 2001; Wilson et al., 2000). Furhtermore, a genetic interaction has been described between Sec4 and the myosin heavy chain Myo2 (Pruyne et al., 1998), thus establishing another link between Rab proteins and the actin cytoskeleton.

Rab proteins can also regulate more than one transport step. For example mutant Ypt1p in *Sacharomyces cerevisiae* results in blockage both in ER to cis-Golgi transport and cis-Golgi to medial-Golgi transport (Jedd et al., 1997).

In summary, different Rab proteins seem to act on different transport steps, regulating different events in vesicle budding, transport and fusion, via interactions with a diverse set of effector proteins. However some Rab proteins can regulate more than one transport step and/or regulate different events in vesicle budding, transport and fusion. To add even another layer of complexity to this problem, some evidence exists that questions if Rab proteins that are considered orthologs have indeed the same function in different organisms. For example, yeast Ypt1p seems to be required exclusively in the acceptor membrane, indicating a role solely in docking or fusion (Cao and Barlowe, 2000) but in mammals, the putative Ypt1p ortholog Rab1 seems to be required for vesicle budding (Allan et al., 2000; Nuoffer et al., 1994).

Table I.1 Effectors of Rab proteins (table expanded from the review (Zerial and McBride, 2001))

Effector	Rab	Proposed Effector function	References
p115	Rab1	Tethering Recruiting of SNAREs	(Moyer et al., 2001; Allan et al., 2000)
Rabphilin-3	Rab3	Potentiates fusion	(Burns et al., 1998)
RIM1	Rab3	Membrane fusion	(Wang et al., 2000)
RIM2	Rab3	Membrane fusion	(Wang et al., 2000)
Calmodulin	Rab3	Calcium sensibility	(Coppola et al., 1999; Park et al., 1997)
Noc2	Rab3	unclear	(Haynes et al., 2001)
Rabaptin-4	Rab4	Implicated in protein sorting and recycling	(Nagelkerken et al., 2000)
Rabip4	Rab4	Vesicle budding(?)	(Cormont et al., 2001)
Rabaptin-5	Rab4, Rab5	Activates Rab5	(Stenmark et al., 1995)
Rabaptin-5β		Stabilises Rabex-5 recruitment	
EEA1	Rab5	Tethering Core fusion component	(Christoforidis et al., 1999; McBride et al., 1999)
Rabenosyn-5	Rab5	Required for endosome fusion	(Nielsen et al., 2000)
p150	Rab5	Class III PI(3)K regulatory subunit	(Simonsen et al., 1998)
p110β	Rab5	Class I PI(3)K catalytic subunit	(Simonsen et al., 1998)
Rabkinesin-6	Rab6	Vesicle motility Cytokenesis	(Echard et al., 1998)
RILP	Rab7	Positioning of lysosomes (?)	(Cantalupo et al., 2001)
Rab8IP	Rab8	Stress activated protein kinase	(Ren et al., 1996)
p40	Rab9	Stimulates fusion	(Diaz et al., 1997)
Tip47	Rab9	Cargo selection	(Diaz and Pfeffer, 1998)
Rab11BP	Rab11	Vesicle recycling(?)	(Mammoto et al., 2000; Mammoto et al., 1999)
Rilp11	Rab11	Vesicle targeting(?)	
δ-PDE	Rab13	Extracts Rab13 from membranes	(Marzesco et al., 1998)
MyoV	Rab27	Melanosome motility	(Hume et al., 2001; Bahadoran et al., 2001; Wilson et al., 2000)
Rab33BP	Rab33	Probably regulates vesicle motility	Cited in (Zerial and McBride, 2001)
Uso1p	Ypt1	Tethering of vesicles	(Cao et al., 1998; Sapperstein et al., 1996)
Yp1p-Yif1p	Ypt1	Essential for docking and fusion	(Matern et al., 2000)
Sec15	Sec4	Tethering	(Guo et al., 1999; Bowser and Novick, 1991)
Vac1p	Ypt51p	TGN-Golgi transport	(Tall et al., 1999)
Vamp2p/Vamp6p complex	Ypt7p	Tethering and GEF Budding(?)	(Seals et al., 2000)

I.1.5.2. Is GTP hydrolysis really required?

Rab proteins are traditionally viewed as "triggered" molecular switches, regulated by GAPs and GEFs, as their intrinsic exchange and hydrolysis rates are in general very slow. A variety of RabGEFs and RabGAPs has been identified in recent years (discussed above), which lend support to this view. However, the realisation of the diversity in the rates of hydrolysis has led to the suggestion that not all Rabs have the same requirement for GTP hydrolysis.

Richardson and co-workers (Richardson et al., 1998) tested the importance of nucleotide hydrolysis in the function of one yeast Rab protein: Ypt1p. Using a dominant activated mutation (and partially purified RabGAP activity), they observed that the cells could grow normally at 37°C and were not defective in the secretory pathway, as tested by secretase and CPY secretion. Rybin and co-workers, also observed a similar lack of requirement for GTP hydrolysis for Rab5 function in regulating endocytic membrane fusion (Rybin et al., 1996). This lack of requirement for GTP hydrolysis however questions the role of RabGAPs, particularly for Rab1 and Rab5 GAPs. Rybin and co-workers propose that Rab5 acts as a timer for fusion. According to this model, Rab5 undergoes futile cycles of activation-deactivation, but when required, Rab5 recruits Rabaptin-5 to the membrane and the binding to this effector secures GAP insensitivity, i.e., that Rab5 is maintained in the active conformation for the time required for fusion to occur, time that would be defined by the intrinsinc hydrolysis rate of Rab5 (Rybin et al., 1996). As Rab5 has an unusually high intrinsic hydrolysis rate (0.05 min^{-1}) when compared to Ypt1 (0.006 min^{-1}), it is difficult to generalise this mechanism to other Rabs .

The role played by RabGAPs will need to be reassessed, particularly in the light of the finding that they are not essential genes in yeast (eg Strom et al., 1993).

I.1.6. Rab:GDP binding proteins: General Regulators

Two major types of proteins are defined as general regulators of Rab proteins: Rab Escort Proteins (REPs) and Rab GDP Dissociation Inhibitors (RabGDIs). These proteins are related at the primary sequence level and thought to have similar three dimensional fold (Schalk et al., 1996; Wu et al., 1996). A third unrelated protein, MSS4, has also been suggested to be a general regulator of Rab function.

I.1.6.1. Rab Escort Proteins

Two independent groups originally reported the partial purification of an enzyme activity, distinct from other protein prenyl trasferases that catalysed double geranylgeranylation (Moores et al., 1991; Horiuchi et al., 1991), but all attempts to purify this activity to homogeneity failed as the enzyme lost its activity after a few steps of purification. This was then interpreted as being caused by enzyme instability. However, biochemical complementation experiments revealed that two distinct components, termed component A and B, separable under conventional chromatography, compose this new enzyme, termed Rab Geranylgeranyl Transferase (RGGT or GGT-II) (Seabra et al., 1992). Component B is related to the other Protein Prenyl Transferases (Armstrong et al., 1993; Seabra et al., 1992), but component A coded for a novel protein with homology to RabGDI (Andres et al., 1993; Seabra et al., 1992), a regulator of Rab function. The discovery that component A was not only required for prenylation of Rab proteins but that it remains in complex with prenylated Rabs and delivers them to cellular membranes led to its renaming to Rab Escort Protein (REP) (Alexandrov et al., 1994; Andres et al., 1993)

Two REP proteins have been described in mammals, but only one is known in *D. melanogaster*, plants and unicellular eukaryotes. They function as essential activators of the prenylation reaction and differ only in the affinity for different Rab proteins (Anant et al., 1998; Desnoyers et al., 1996). The role of REP in the prenylation of Rab proteins is discussed in section I.2. More details will be given later on Part III of this thesis , which concerns the structural determinants of REP function.

I.1.6.2. Rab GDP Dissociation Inhibitor

RabGDI was originally identified by Takai and co-workers as a protein that interacts with the GDP-bound form of Rab3a, thereby inhibiting the dissociation of GDP from Rab3a, and thus it's binding to GTP (Sasaki et al., 1990). RabGDI was then observed to be active with other Rab proteins (Sasaki et al., 1990; Ueda et al., 1990). In addition to its properties as a negative regulator of the nucleotide cycling of Rabs, RabGDI was shown to inhibit association of GDP-bound Rabs with cellular membranes (Araki et al., 1990) and also to extract Rabs from membranes (Beranger et al., 1994; Garrett et al., 1993; Soldati et al., 1993; Araki et al., 1990). Only lipid modified Rabs can associate with RabGDI (Araki et al., 1991). In the light of these findings, RabGDI can be seen as a negative regulator of Rab function, both by inhibiting nucleotide cycling as well as Rab membrane association.

Mutations in the brain specific RabGDI-α isoform cause non-specific mental retardation in humans (D'Adamo et al., 1998), indicating an essential function, at least for

this isoform in this tissue, and the deletion of the GDI gene in yeast is lethal, again indicating that this protein performs an essential role (Garrett et al., 1994). However the exact biological role of RabGDI has been a subject of contention.

It has been proposed that RabGDI retrieves Rabs from acceptor membranes and recycles them back to donor membranes (Pfeffer et al., 1995). Supporting this model is the observation that a mutant Rab1b which cannot bind RabGDI, accumulates in the membranes instead of partitioning between membranes and cytosol (Wilson et al., 1996), and mutations in RabGDI domain II result in accumulation of Rabs in the membrane fraction (Luan et al., 2000). Additionaly, it was observed that RabGDI can mediate *in vitro* delivery of Rab5, Rab4, Rab9 and Rab11b into specific cellular membranes (Ayad et al., 1997; Soldati et al., 1994; Ullrich et al., 1994; Peter et al., 1994).

On the other hand, chimerical Sec4 and Ypt1, where the prenylation motif was substituted by a trans-membrane domain (thus rendering these proteins unavailable for RabGDI extraction), could rescue the loss of the corresponding wild-type protein (Ossig et al., 1995). This questions whether the dramatic effects that result from RabGDI deletion are indeed caused by lack of Rab recycling.

Interestingly, it is possible that RabGDIs only act on a subset of Rab proteins. Marzesco and co-workers identified the rod cGMP phosphodiesterase delta subunit (δ-PDE) as a Rab13 binding protein (Marzesco et al., 1998). Surprisingly δ-PDE displays the ability to specifically dissociate Rab13 from cellular membranes, which RabGI does not. If this is due to the atypical ras-like CAAX box displayed by Rab13, as opposed to the characteristic double cysteine prenylation motif remains to be addressed.

Another layer of complexity is added by the recent work of Erdman and Maltese (Erdman and Maltese, 2001), which observed that in human terato-carcinoma cells, different Rabs associate differentially with the two isoforms of RabGDI, independently of their relative abundance.

I.1.6.3. MSS4, DSS4 and PRA1

DSS4 and its mammalian counterpart MSS4 were originally described as exchange factors for members of the Rab family (Moya et al., 1993; Burton et al., 1993). However, several arguments can be raised to contest this classification.

Unlike other exchange factors, MSS4 has a broad range of Rab specificity, acting at least on Rab1, Rab3, Rab8, Rab10, sec4 and Ypt1 (Burton et al., 1994). MSS4 is a week GEF when compared with other RabGEFS (Moya et al., 1993; Burton et al., 1993), and finally, DSS4 is not an essential gene nor does its deletion cause any remarkable effect which can be associated with the non-activation of any particular Rab protein (Nuoffer et al.,

1997; Moya et al., 1993). This does not support a role as an exchange factor *in vivo.* Instead it was proposed that MSS4 and DSS4 represent a new class of general regulators of Rab proteins which bind and stabilise Rab proteins in the nucleotide free conformation, with a 1:1 stoichiometry (Nuoffer et al., 1997). The observation that the binding of these proteins to Rabs is not prenylation dependent suggests they might interact with nascent unprenylated Rab proteins (Nuoffer et al., 1997).

PRA1, standing for Prenylated Rab acceptor, was identified in a two hybrid screen as a Rab1 and Rab3 interacting protein, specific for prenylated Rab proteins and exhibiting two putative trans-membrane segments (Martincic et al., 1997). PRA1 can also bind RabGDI1, albeit weakly, and inhibits the extraction of membrane-bound Rab3 by RabGDI1 (Hutt et al., 2000). Recently another isoform of PRA1 was described and it was observed that the two isoforms display distinct sub-cellular localisations (one in the Golgi and the other in the ER) (Abdul-Ghani et al., 2001). The results above suggest that PRA1 may be a receptor for Rab proteins. However, in contradiction with the work of Martincic and co-workers (Martincic et al., 1997), PRA1 was recently shown to interact *in vitro* and *in vivo* also with prenylated members of the Ras and Rho families (Figueroa et al., 2001). In face of this, it is questionable if PRA1 can be indded a Rab receptor. Figueroa and co-workers suggest that PRA1 may act as an escort protein for small GTPases, by binding to the hydrophobic isoprenoid moieties of the small GTPases and facilitating their trafficking through the endomembrane system. An alternative possibility is that PRA1 is part of a protein complex that forms the Rab, Ras and Rho membrane receptors. PRA1 would provide for the prenyl protein specificity and the protein specificity would be decided by other components of this "receptor complex". In the Rab family, the soluble exchange factors could play this role, since, as discussed, they seem to be involved in membrane association of their target Rab protein (see I.1.3.2). In this case, PRA1 can be seen as a general regulator of membrane association, instead of a specific Rab effector.

I.2. Lipid modification of Rab proteins

Expanded and adapted from Pereira-Leal, Hume and Seabra (2001) FEBS lett. *In press*

Rab proteins, like other small GTPases, require association to cellular membranes for proper activity. This is accomplished by modification of the protein with lipid groups. Here a brief overview of the different types of lipid modifications is done, followed by a discussion on protein isoprenylation with particular emphasis on Rab isoprenylation.

I.2.1. Lipid modifications

Lipid modification of proteins is an expression used to describe the co- or post-translational covalent attachment of a lipid group to a protein, in general to permit the association of hydrophilic proteins to the hydrophobic lipid bilayers of biological membranes.

Four major types of lipids are known to be involved in lipid modification of proteins. These are fatty acids (myristic acid and palmitic acid), glycosyl-phosphatidylinositol, cholesterol and isoprenoids. Of particular relevance to this work is protein prenylation, which will be discussed in more detail. Here we will briefly mention the other major classes of lipid modification.

Glycosyl-phosphatidylinositol (GPI) is involved in mediating anchorage to the plasma membrane of secreted proteins as diverse as CD55, acetylcholinesterase, alkaline phosphatase, CD48, urokinase receptor among others, via "GPI anchors". Synthesis of GPI precursors and the addition of GPI to a GPI-signal sequence containing protein occurs in the endoplasmic reticulum. GPI anchor-containing proteins are then transported to the external leaflet of the plasma membrane via the secretory pathway, and cluster in sphingolipid-sterol micro-domains (for review see Muniz and Riezman, 2000; Udenfriend and Kodukula, 1995)). Deficiency in GPI synthesis results in a human disease, paroxysmal nocturnal hemoglobinuria (reviewed in Kinoshita et al., 1996).

Another type of lipid modification that seems to affect a family of secreted proteins is modification by cholesterol. It is a recent observation, and so far has only been observed in the fruit fly protein *sonic hedgehog* (Porter et al., 1996a; Porter et al., 1996b) and recently, in the human ortholog of this protein (Pepinsky et al., 1998). Interestingly, this modification is accomplished in the fly protein via an auto-catalytic process where after signal peptide cleavage, a precursor form of hedghog undergoes an autocatalytic cleavage that produces a lipid modified N-terminal fragment, the autocatalytic activity residing in the C-terminal portion of the precursor protein. The human form of this protein has also been reported to be acylated in the N-terminus by palmitate (Pepinsky et al., 1998).

Modification of proteins by fatty acids is termed acylation. N-Myristoylation refers to the attachment of the $C_{14:0}$ saturated fatty acid to an N-terminal glycine residue exposed after removal of the initiation methionine residue. It affects, among other proteins, members of the Arf family of small GTPases, proteins involved in the regulation of membrane transport (reviewed in Roth, 2000). Myristate is attached co-translationally to an N-terminal glycine residue, via an amide bond (Bhatnagar and Gordon, 1999) by one of at least two N-myristoyltransferases (Giang and Cravatt, 1998). Acylation of Arfs has been observed to be essential not only for membrane association but also for activation by exchange factors as

ARFs need to undergo a lipid mediated conformational switch before they can interact with GEFs (Donaldson and Jackson, 2000; Roth, 2000; Franco et al., 1996).

Palmitoylation, another type of acylation (thyoacylation) will be further discussed in the context of the processing of CAAX small GTPases.

I.2.2. Protein Prenylation

Protein prenylation refers to the stable covalent addition of 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenoids via thioether bonds to carboxyl terminal cysteine residues. This type of lipid modification was first reported in 1978 when peptide mating factors secreted by some fungi were observed to have a covalently attached farnesyl group (Kamiya et al., 1978). Protein prenylation was later found to also affect mammalian proteins. Mammalian epithelial cells incubated with radioactively labelled mevalonate, a precursor in the biosynthetic pathway of cholesterol (see figure I.9), were shown to accumulate proteins labelled with mevalonate derivatives (Schmidt et al., 1984).

The first prenylated mammalian protein identified was the nuclear envelope protein lamin B, which was shown to possess a farnesyl group covalently attached to a C-terminal cysteine via a thioether bond (Farnsworth et al., 1989; Wolda and Glomset, 1988). Since then, a wide range of proteins have been found to be prenylated, and prenylation has been recognised as a frequent post-translational modification in eukaryotic cells, estimated to affect about 0.5% of the cellular proteins (Epstein et al., 1991).

Isoprenoids are produced by the mevalonate pathway, a metabolic pathway that produces a wide variety of compounds, namely cholesterol, dolichol and ubiquinone (Grunler et al., 1994) (see figure I.9).

Protein prenylation is catalysed by a family of enzymes termed Protein Prenyl Transferases where all the enzymes are heterodimers consisting of an α and β-subunits (Casey and Seabra, 1996).

Protein Farnesyl Transferase (PFT) and Protein Geranylgeranyl Transferase I (PGGTI), which share a common α-subunit, define the functional class of CAAX prenyl transferases. They recognise a CAAX tetrapeptide prenylation motif at the C-terminus of their substrates, where C represents a cysteine residue, A an aliphatic residue and X any amino acid. The identity of the residue at position X is sufficient to determine which enzyme will modify which protein. When X is a methionine or serine residue, as in Ras proteins, then the protein is farnesylated by PFT. However, when X is a leucine residue, as in Rho proteins, then the protein is geranylgeranylated by PGGTI (Spence and Casey, 2001; Yokoyama and Gelb, 2001). CAAX prenylation motifs are also found in a variety of other

proteins such as γ-subunits of heterotrimeric G-proteins, nuclear lamins, G-protein coupled receptor kinases, fungal mating pheromones and retinal cyclic GMP phosphodiesterase (Spence and Casey, 2001; Yokoyama and Gelb, 2001).

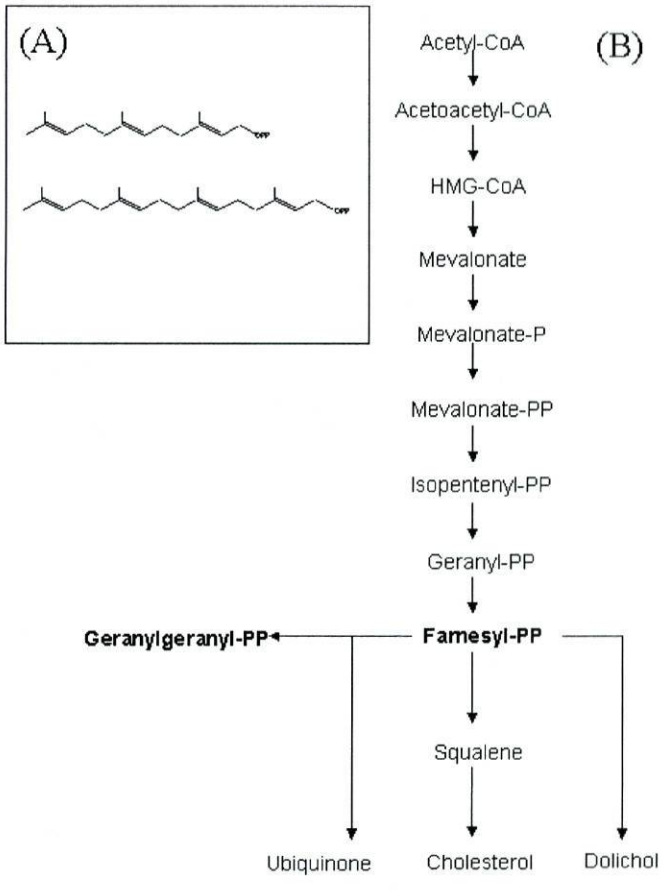


Figure I.9 *– (A) chemical structure of farnesyl pyrophosphate and genarylgeranyl pyrophosphate (B) Simplified scheme of the intermediates in the synthesis of cholesterol (adapted from (Goldstein and Brown, 1990) and (Grunler et al., 1994)).*

A second class of prenyl transferases is defined by RabGeranylgeranyl Transferase (RGGT or PGGTII), an enzyme that catalyses the addition of two geranlgeranylgroups to members of the Rab family. This enzyme will be discussed below.

Until recently only isoprenoids in C15 and in C20 were thought to be involved in protein prenylation, but Parmyd and Dallner (Parmyd and Dallner, 1999) reported they observed protein modified by penta- and hexaprenyl groups in rats injected with labelled mevalonate. The nature of the putative Protein penta and hexa prenyl Transferases, and their substrates, remains to be elucidated.

I.2.3. Prenylation of Rab proteins

Rab Geranylgeranyl Transferase (RGGT) defines a second class of prenyl transferase which differs in many ways from the CAAX Prenyl Transferases. RGGT was originally identified as the enzyme that modified Rab proteins, and so far no other substrates are known (Seabra, 2000; Seabra et al., 1992). The structure of RGGT has been solved recently (Zhang et al., 2000). The β-subunit consists of an α-α barrel and forms the lipid-binding site as shown for PFT (Seabra, 2000; Terry et al., 2001; Zhang et al., 2000). The α-subunit is larger than those found in the CAAX prenyltransferases, consisting of a helical domain similar to PFT plus two other domains of unknown function: a leucine-rich repeat and an immunoglobulin-like domain (Zhang et al., 2000).

Rab proteins, unlike other small GTPases, exhibit a variety of prenylation motifs at their C-termini, containing either one or, more frequently, two cysteine residues both of which are modified by geranylgeranyl groups (Farnsworth et al., 1994). Also, the prenylation motif in Rab proteins is insufficient by itself for substrate recognition by RGGT. Biochemical evidence indicate that unprenylated Rab must first bind to a 95kDa protein termed Rab Escort Protein (REP, originally termed component A of RGGT) in order to be recognised as a substrate for RGGT (Seabra, 2000; Anant et al., 1998). The role of REP in prenylation is supported by the finding that deficiency of REP results in hypoprenylation of Rab proteins in vivo (Bialek-Wyrzykowska et al., 2000; Seabra et al., 1995). Together these observations suggest that the REP:Rab complex is the true substrate of RGGT (Seabra, 2000; Anant et al., 1998).

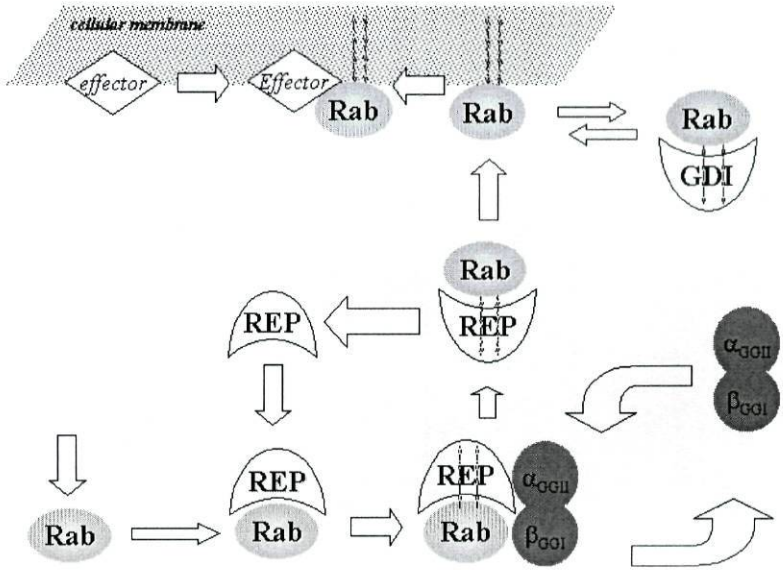


Figure I.10 – *The Rab prenylation cycle*

Two REP proteins are found in mammals (Desnoyers et al., 1996). Both REP1 and REP2 are ubiquitously expressed and are structurally related to RabGDI (Seabra, 2000; Wu et al., 1996). Recent results suggest that REPs and GDIs interact with Rab proteins in a conserved way, using amino acid residues in the conserved "Rab-binding platform" (Alory and Balch, 2000). Part III will deal with these issues.

Rab prenylation occurs in several steps (see figure I.10). Newly synthesised Rabs bind to one of the two REP proteins and this complex then associates with RGGT. This enzyme contains one GGPP binding site and recent studies indicate that GGPP binding increases the affinity of RGGT for the REP:Rab complex suggesting that the enzyme may already be bound to a single GGPP group at this stage (Thoma et al., 2000; Desnoyers and Seabra, 1998). Geranylgeranylation of the most N-terminal cysteine follows (Shen and Seabra, 1996). In the case of some Rabs e.g. Rab8 or Rab13, which contain a lone cysteine residue available for prenylation, dissociation of the enzyme is thought to occur at this point. However, a second cysteine residue is available in most cases and RGGT catalyses another round of lipid binding and transfer. In this case, it is unclear whether RGGT dissociates from the monoprenylated REP:Rab-GG complex, due to the reduction in the affinity of RGGT for the mono-prenylated complex in the absence of GGPP, or whether it can recruit a second geranylgeranyl group while bound to the REP:Rab-GG complex. Finally, after the second GG group is transferred, binding of an additional lipid group to the β-subunit destabilises the complex and the enzyme dissociates from the di-prenylated REP:Rab complex. (Thoma et al., 2000). REP may then deliver the geranylgeranylated-Rab to cellular membranes (Wilson et al., 1996; Alexandrov et al., 1994) where it can interact with specific effectors and perform its biological role.

I.2.4. Post-prenylation processing

I.2.4.1. Post-prenylation processing of CAAX proteins

CAAX proteins, after prenylation, see the proteolytical removal of the most C-terminal three amino-acids (see figure I.11). This reaction is catalysed by a isoprenylprotein endoprotease and is followed by carboxyl methylation of the prenylated cysteine, in a reaction catalysed by a prenylcysteine carboxyl methyltransferase. Some CAAX proteins, like N- or H-Ras can be also S-acylated in cysteine residues close to the prenylated cysteine residue.

Isoprenylprotein endoproteases were originaly cloned in *Saccharomyces cerevisiae*, in a genetic screen for sterile mutants that could not process the **a** mating factor (Boyartchuk et al., 1997). Two genes were identified, RCE1 and STE24, which were required for the processing of the **a**-mating factor and at least another protein, Ras2p. STE24, also known as AFC1 (**a**-factor convertase 1) is a zinc metallo-protease that participates in the endoproteolytical processing of **a**-factor, whereas RCE1, which stands for "Ras and **a**-factor converting enzyme" participates in the processing of both Ras and **a**-factor. The mammalian counterpart of RCE1 was cloned based on sequence similarity to the yeast protein (Otto et al., 1999). Further work by the same group revealed that inactivation of this protease by gene targeting results in complete loss of Ras proteolytical processing, suggesting that in mammals this protease is the sole protease involved in Ras post-prenylation removal of the C-terminal tripeptide (Kim et al., 1999). Interestingly, Ras proteins were found to be mainly cytosolic in RCE1 -/- cells, in contrast with the normal plasma membrane association found in RCE1 +/+ cells, suggesting an important role of post-prenylation processing in membrane association and/or targeting of Ras (Kim et al., 1999).

After endoproteolysis of the tripeptide AAX, the prenylated cysteine will then be methylated by a prenylcysteine Carboxyl Methyltransferase (pcCMT) (see figure I.11). The identification of the yeast pcCMT was made also from sterile mutants that could not process **a** mating factor. The mutant Ste14 was found to lack any pcCMT activity towards a Ras derived peptide (Hrycyna and Clarke, 1990) which suggested that the same pcCMT was responsible for processing of both **a**-factor and Ras proteins. Later, the same group cloned the STE14 gene and found that the Ste14 mutant completely lacked the ability to process Ras, thus showing that Ste14 is the pcCMT that methylates Ras proteins (Hrycyna et al., 1991). Lack of prenylcysteine carboxyl methylation, surprisingly, did not affect Ras function (Hrycyna et al., 1991). It did however have an effect in Ras levels and in its membrane association, resulting in reduction in total levels of Ras and reduction in the membrane fraction (Hrycyna et al., 1991).

The mammalian prenylcysteine Carboxyl Methyltransferase (pcCMT) was cloned recently by Dai and co-workers (Dai et al., 1998) which searched the EST databases for sequences homologous to Ste14. The pcCMT gene was able to complement an Ste14 mutant and is predicted to be a multiple membrane-spanning 33 KDa protein. This enzyme, like its yeast counterpart (Romano et al., 1998) was localised, by immuno-fluorescence microscopy to the ER (Dai et al., 1998). For a comprehensive review on proteolysis and carboxyl methylation of CAAX proteins see (Young et al., 2000).

All CAAX small GTPases are believed to undergo post-prenylation proteolysis and carboxyl methylation, but for some, like N- and H-Ras and RhoB and TC10, further processing is required (see figure I.11). These proteins display cysteine residues close to the

prenylated cysteine that can be modified by a saturated $C_{16:0}$ fatty acid via thioester bond. This type of lipid modification is called palmitoylation, or more correctly, protein thioacylation (see (Linder, 2001) for review)

Palmitoylated proteins include several families of small GTPases, heterotrimeric G proteins, rhodopsin, phospholipase D, the HIV-1 envelope glycoprotein, among others (Linder, 2001). Palmitoylation occurs frequently in conjunction with other lipid modifications, like prenylation in Ras, cholesterol modification in hedgehog and N-myristoylation of α-subunits of heterotrimeric G proteins. The most striking difference between thioacylation and other forms of lipid modification resides in its dynamic, reversible character. Several protein acyltransferases have been purified (for example (Das et al., 1997; Liu et al., 1996)), but it is still unclear if they perform this function *in vivo*, particularly in the light of the observed spontaneous and efficient transfer of fatty acid from acetyl-CoA to proteins (for example (Bizzozero et al., 1987)). Removal of acylgroups is better understood, with two enzymes responsible for this reaction already described and characterised (Duncan and Gilman, 1998; Camp et al., 1994). Mutations in one palmitoyl thioesterase (PPT1) cause infantile neuronal ceroid lipofuscinosis, a severe neurodegenerative disorder (see (Linder, 2001) for review).

Recent work by the groups of Mark Philips and John Hancock (Apolloni et al., 2000; Choy et al., 1999) revealed that the pos-prenylation modifications are part of a hierarchy of signals that determine the trafficking and targeting of Ras proteins to the plasma membrane. Prenyltransferases are believed to be solube enzymes (Casey and Seabra, 1996), but both the protease and the pcCMT localise to the ER membrane. Choy and co-workers (Choy et al., 1999) showed that prenylated Ras associates with the ER before reaching the plasma membrane, and that the targeting to the ER is determined solely by the CAAX motif. Trafficking from the ER to the plasma membrane for N- and H-Ras, happens through the exocytic pathway, but is dependent on a second signal, the cysteine residue which is modified by palmitoylation (Apolloni et al., 2000). In contrast, K-Ras, which does not posses a palmitoylatable cysteine residue, but instead has a polybasic stretch near the C-terminus, was found to undergo initial targeting to the ER, but traffics to the plasma membrane by an unknown route. Some evidence is presented which suggests that it might be a microtubule-dependent pathway (Apolloni et al., 2000). Together these two studies suggest that the CAAX motif targets the GTPase to the prenyltransferase, that the prenylated CAAX motif then targets the protein to the ER for proteolysis and carboxyl methylation, and that the presence of a palmitoylation motif directs trafficking of the protein, via the exocytic pathway to the plasma membrane, but that the absence of this second signal and/or the presence of a poly-basic signal directs trafficking to the plasma membrane via different route.

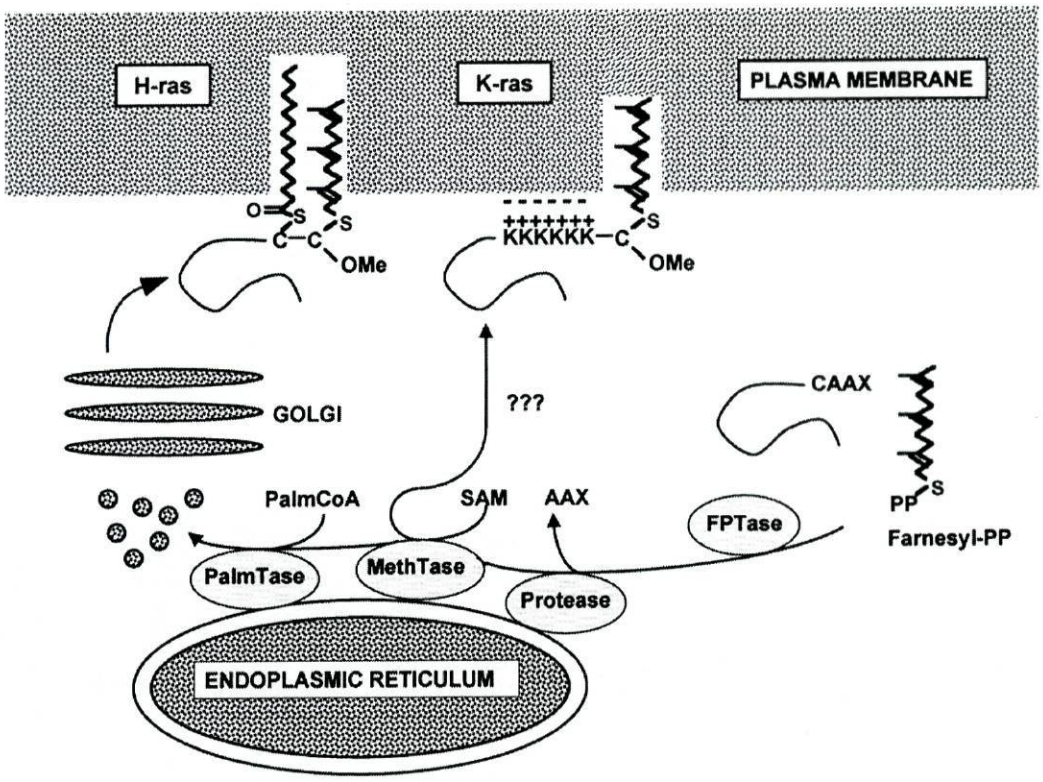


Figure I.11 *– Model of Ras post-prenylation processing and trafficking, reproduced from (Apolloni et al., 2000). After the common processing steps of farnesylation, AAX proteolysis, and methylation which are completed on the cytosolic surface of the ER, the trafficking routes of the different Ras proteins diverge. K-ras, by virtue of its C-terminal polybasic domain, is sorted out of the conventional exocytic pathway and takes an undefined pathway to the cell surface that bypasses the Golgi. H- and N-ras are palmitoylated by an ER-localized palmitoyltransferase and enter the exocytic pathway; they traffic to the cell surface via the Golgi. OMe, carboxymethyl; PalmCoA, palmitoyl coenzyme A; SAM, S-adenosyl methionine; PalmTase, palmitoyltransferase; MethTase, methyltransferase; FPTase, farnesyl protein transferase; Farnesyl-PP, farnesyl pyrophosphate.*

Recent work suggests that this simple model does not apply to members of the Rho family, where the existence of general regulators like RhoGDIs and possibly of other targeting signals play a role both in trafficking and targeting of members of this family (Michaelson et al., 2001).

I.2.4.2. Post-prenylation processing of Rab proteins

The only post-prenylation processing of Rab proteins that has been described so far is carboxyl methylation of the C-terminal prenylated cysteine residue in the contex of a CXC prenylation motif, but not in CC motifs (Smeland et al., 1994). The enzyme that catalyses this reaction is the same pcCMT that carboxyl methylates CAAX proteins (Bergo et al.,

2001), but unlike CAAX proteins, doubly-geranylgeranylated CXC proteins do not seem to require carboxyl methylation for efficient membrane association (Bergo et al., 2001; Smeland et al., 1994), nor does it seem to play a role in the trafficking or targeting (Bergo et al., 2001).

As mentioned before, some Rab proteins exhibit a single C-terminal cysteine residue, in the context of a CAAX or CAAX like prenylation motif. It has not been addressed, to our knowledge, if these Rab proteins will undergo the same type of post-prenylation processing that was described above. We have noticed though that some recent additions to the Rab family (see Part II of this thesis) display C-terminal sequences that resemble the palmitoylation signals in N- and H-Ras or the poly-basic signals in K-Ras. It is tempting to speculate that although members of the Rab family have evolved a different strategy for membrane association, which involves double geranylgeranylation by RGGT, some of the components of the Ras signals could have been re-utilized in this family.

I.2.5. Deficient Rab prenylation and human disease

Choroideremia (CHM) is an X-linked chorioretinal degeneration characterised by progressive night blindness and loss of peripheral vision starting in the second or third decade of life which invariably results in complete blindness within two or three decades of the initial manifestation of symptoms (van den Hurk et al., 1997; Seabra, 1996). The gene mutated in this disease was identified by Cremers and co-workers (Cremers et al., 1990) and named CHM. All known CHM mutations are predicted to result in loss of CHM protein function due to the production of truncated, non-functional or unstable polypeptide (van den Hurk et al., 1997).

A function of CHM became apparent when REP1 was purified and cloned, and observed to correspond to the CHM gene product (Seabra, 1996; Seabra et al., 1993). This suggested that CHM was caused by improper Rab prenylation, but it remained unclear how loss of a function essential for every cell type resulted in an eye-specific phenotype. The observation that lymphoblasts from CHM patients still contained RGGT activity led to the identification of a second REP protein, REP2 and to the hypothesis that REP2 incompletely compensates the loss of REP1 in CHM patients thus leaving a subset of Rab proteins unprenylated (Cremers et al., 1994). Consistent with this hypothesis, one Rab protein was observed to be selectively unprenylated in CHM lymphoblasts (Seabra et al., 1995). This Rab was subsequently identified as Rab27a and shown to be highly expressed in the same layers of the rat eye that are observed to degenerate in CHM patients, the retinal pigment epithelium (RPE) and the choroid (Seabra et al., 1995).

Rab27a, one of two isoforms of the Rab27 subfamily, was recently shown to localise to melanosomes, melanin containing granules present in melanocytes, and to be involved in the transport of these organelles to the periphery of melanocytes (Hume et al., 2001; Bahadoran et al., 2001; Wilson et al., 2000). Therefore, one possibility is that retinal degeneration in CHM patients might be triggered by defects in transport of melanosomes in RPE and choroidal melanocytes. However, recent data call into question the relationship between Rab27a hypoprenylation and chorioretinal cell death. First, Rab27a is highly expressed in a number of other tissues which are apparently unaffected in CHM patients (Hume et al., 2001; Ramalho et al., 2001; Chen et al., 1997). Second, loss-of-function mutations in the human and murine *RAB27A* genes result in Griscelli syndrome (GS) and the *ashen* (*ash*) mutant mouse, respectively. GS is characterised by partial cutaneous albinism and immunodeficiency, neither of which is reported as affecting CHM patients (Menasche et al., 2000; Wilson et al., 2000). In both GS patients and the *ash* mouse, the absence of Rab27a protein results in immunodeficiency due to failure of cytotoxic T lymphocytes to secrete the contents of their lytic granules (Stinchcombe et al., 2001; Haddad et al., 2001; Menasche et al., 2000), and albinism due to defects in melanosome transport (Hume et al., 2001; Bahadoran et al., 2001; Wilson et al., 2000).

In spite of these observation the involvement of Rab27a in the pathogenesis of CHM cannot be discounted at present as the *ash* mutant examined in the above studies is maintained in the inbred laboratory mouse strain (C3H) which is homozygous for the *rd* mutation (Pittler and Baehr, 1991). This mutation of the β-subunit of cyclic GMP phosphodiesterase causes complete degeneration of photoreceptors shortly after birth. The absence of observed retinal degeneration in GS is understandable as their mean life expectancy is 5 years in the absence of early bone marrow transplantation (Klein et al., 1994) and to our knowledge no reports of long term health of transplanted patients are published. In our view, it is still likely that dysfunction of Rab27a contributes to CHM pathogenesis perhaps in combination with other retinal Rabs similarly hypoprenylated and partially dysfunctional. Another possibility is that REP-1 may have other functions yet undiscovered required for survival of retinal cells.

Hermansky-Pudlak syndrome (HPS) is a group of recessively inherited disorders caused by mutation in multiple genes whose products are involved in the biogenesis and function of three related organelles; melanosomes in melanocytes, platelet dense granules and lysosomes. The disease manifests as hypopigmentation (partial albinism), prolonged bleeding and ceroid deposition in lysosomes (Huizing et al., 2000; Swank et al., 1998).

The *gunmetal* (*gm*) mutant is one of 14 murine models of HPS. In addition to the above defects, the *gm* mutant exhibits other abnormalities relative to wild-type animals; they have fewer, larger platelets, reduced platelet alpha granule content, and reduction in killing

capacity of cytotoxic T cells (Stinchcombe et al., 2001; Swank et al., 1993). Recently, the *gm* mutation was identified as a G to A substitution in a splice acceptor site in the gene encoding the murine RGGT α-subunit (Detter et al., 2000). As a consequence, the major RGGT transcript in the *gm* mutant lacks a start codon and is nonfunctional. However, the *gm* mutant contains a reduced level of RGGT activity due to the low frequency activation of a cryptic splice acceptor site within RGGT- α RNA which results in the production of a transcript containing a start codon.

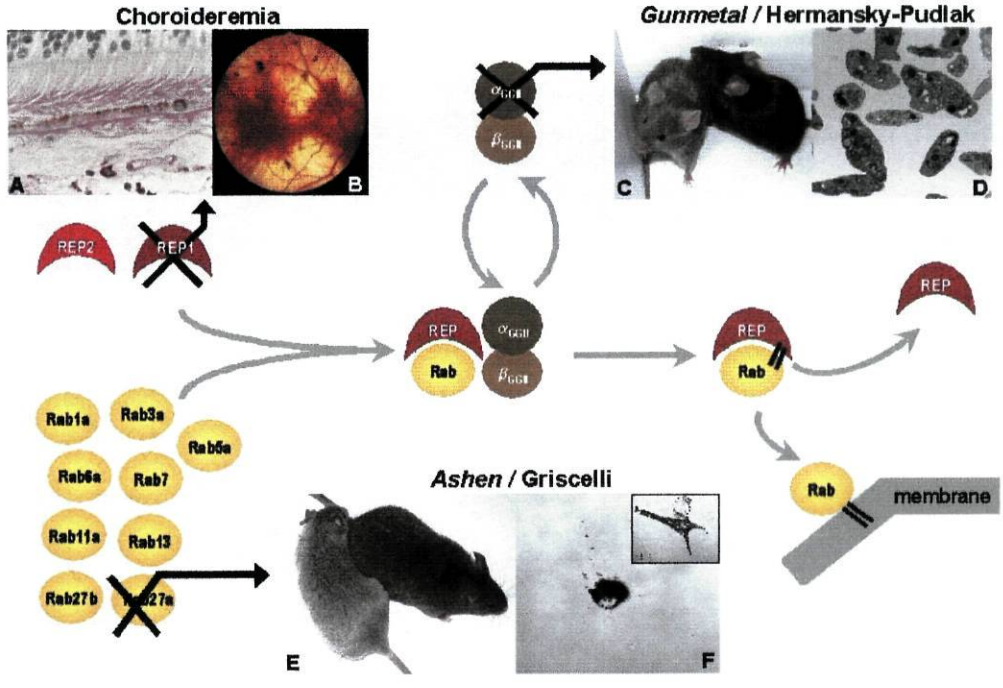


Figure I.12 *- Rab prenylation and disease of mice and man. Newly synthesised Rabs bind to one of two REP proteins. Mutations in REP1 cause Choroideremia, an X-linked retinal degeneration. Histological sections of retina reveal early pathological changes in a female carrier of the CHM mutation (A), and the fundus of the eye of an affected male show marked peripheral retinal degeneration (B). The REP:Rab complex is the substrate for the enzyme RGGT which catalyses geranylgeranylation of the Rab protein. Mutations in the α-subunit of RGGT result in hypopigmentation as observed in the gunmetal mouse model of Hermansky-Pudlak syndrome (C) and platelet dysfunction characterised by macrothrombocytopenia and reduced number of α – and dense-granules (D). Mutations in one Rab protein, Rab27a result in hypopigmentation as observed in the ashen mouse model of Griscelli syndrome (E), due to clustering of melanosomes in the perinuclear region of melanocytes (F). Inset in panel F shows normal distribution of melanosomes in wild-type melanocytes.*

This observation suggested that deficiency of Rab(s) prenylation results in the HPS phenotype, a hypothesis remarkably similar to the one proposed for CHM. Consistent with this possibility, a subset of Rabs accumulate unprenylated in the *gm* cytosol, including

Rab27a (Stinchcombe et al., 2001; Detter et al., 2000; Swank et al., 1993). Interestingly, Rab27 isoforms are highly expressed in the tissues affected in the *gm* mouse suggesting that the *gm* phenotype is at least in part a result of reduced Rab27 function. However, a number of observations indicate that hypoprenylation-induced defects in the function of other unknown Rabs contribute significantly towards more general disruption of membrane transport which triggers the pathological changes observed in this mutant. The most obvious of these is that although both the *gm* and *ash* mutants exhibit hypopigmentation and immunodeficiency, the fundamental cell biological defects underlying these phenotype differ. In *gm*, immunodeficiency appears to result from defects in T cell cytotoxic granule polarisation rather than in secretion of granule contents as observed in *ash* (Stinchcombe et al., 2001). Our preliminary observations of melanosome distribution in melanocytes derived from *gm* neonates indicate that hypopigmentation in this mutant does not result from failure to transport melanosomes to the peripheral cytoplasm (E Sviderskaya, AN Hume, DC Bennet and MC Seabra, personal communication) as is observed in *ash* (Wilson et al., 2000). Identification and characterisation of the other Rabs which accumulate unprenylated in the cytosol of gunmetal mutant should provide further insight into Rab prenylation and function, the pathogenesis of HPS and other human lysosomal storage disorders.

In conclusion, deficiencies in the Rab prenylation machinery can lead to different diseases (Figure I.12). Hypoprenylation of one or more Rab proteins seems to be the cause of the different diseases, although it is impossible to rule out at this point other molecular mechanisms. It is intriguing that while prenylation is required for the function of most Rab proteins, deficiencies in the prenylation machinery only affects certain tissues. A possible explanation is that these restricted pathologies result from members of the Rab family having a range of affinities for REP1, REP2 and/or RGGT, together with a differential tissue requirement.

Rab27 appears to be a common denominator in CHM and *gunmetal*, suggesting that it is not a good substrate for prenylation. As we cannot detect significant differences in binding of REP1 and REP2 to Rab27 in vitro (B Larijani and MC Seabra, personal communication), other yet undescribed factors may regulate Rab prenylation in vivo. If this is the case, elucidation the nature of these factors will bring further insight to the understanding of Rab prenylation and these pathologies.

I.3. The questions this work attempts to address

For almost a decade Miguel Seabra's lab has been interested in the lipid modification of small GTPases of the Rab family. A significant amount of work has been done on the

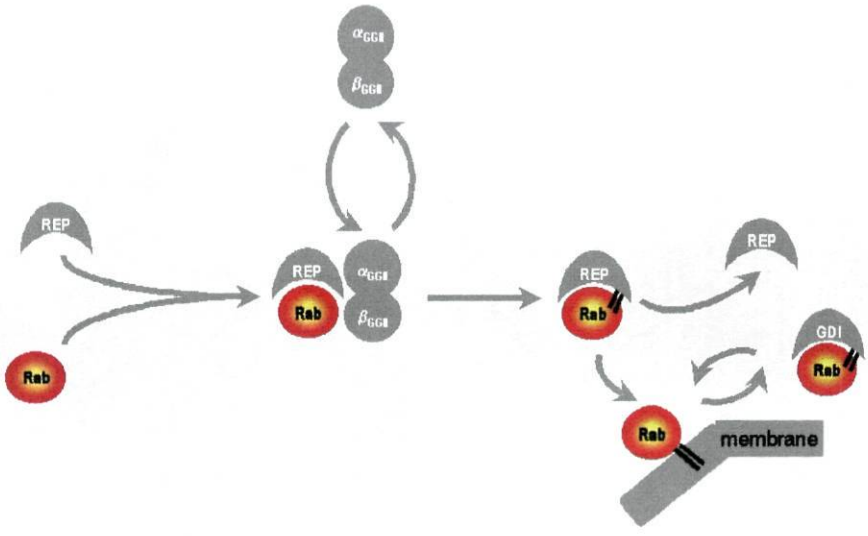
biochemistry of lipid modification of Rab proteins, but a number of questions dealing with the early steps of prenylation remain unanswered. The formation of the REP:Rab complex, the substrate of the enzyme RGGT, in particular hasn't been properly characterised. What are the structural determinants of REP:Rab binding?

This work tries to address this question, by dividing it into smaller questions:

- What defines a Rab protein?? What do they all have in common that could be recognised by 2 REPs
- What defines a REP protein? What do they have in common that different structures serve the same role in different organisms
- How do Reps and Rabs interact?
- Are there additional factors that regulate association of REP and Rab?

Several observations made in the course of this work prompted us to also pursue structural determinants of events after the prenylation reaction as natural consequences of our work, and to look into the proteins here studied from an evolutionary perspective in order to gain insights into the conserved and divergent features of these proteins.

Part II – Rab Proteins



ICBAS

II.1. The Mammalian Rab Family of Small GTPases:

Definition of Family and Subfamily Sequence Motifs Suggests a Mechanism for Functional Specificity in the Ras Superfamily

Reproduced from: Pereira-Leal and Seabra (2000) J.Mol.Biol. 301: 1077-1087

II.1.1. Introduction

The Ras superfamily of GTPases encompasses a large group of proteins that bind GTP and GDP and serve as molecular switches to regulate important cellular processes such as growth, motility and protein trafficking (Barbacid, 1987; Bourne et al., 1990). General guidelines for nomenclature on Ras-like GTPases were set in 1992, following a FASEB Summer Conference (Kahn et al., 1992). The proposal that there are five different families within the superfamily (Ras, Rho/Rac, Rab, Arf, Ran) is now widely accepted.

The largest branch of this superfamily is formed by the Rab/Ypt/Sec4 family, proteins that act as essential regulators of vesicular transport pathways (Lazar et al., 1997; Novick and Zerial, 1997; Olkkonen and Stenmark, 1997; Schimmoller et al., 1998; Chavrier and Goud, 1999; Brennwald, 2000; Rodman and Wandinger-Ness, 2000). Rabs have been traditionally numbered in order of discovery, Rab1, Rab2, Rab3, through to Rab37 at present. The number of Rab-like sequences has been growing steadily over the last decade and we noted that a significant number of Rabs have been deposited in the databases under different names. The problem is that there is no comprehensive definition of what distinguishes a Rab from other small GTPases. Simple criteria such as the presence of a double-cysteine prenylation motif at the C-terminus is insufficient as some *bona fide* Rabs such as Rab8 or Rab13 contain only a single cysteine residue (Casey and Seabra, 1996).

In this study, we attempt to identify mammalian Rab-specific regions that serve as diagnostic Rab sequences in order to define a Rab protein on the basis of its primary structure. We found five Rab-specific regions that we termed Rab family (RabF) motifs. Using the RabF motifs, we were able to identify new Rabs from the databases as well as find all the known Rabs, thus validating these motifs. We also analysed Rab subfamily sequence motifs (RabSF) and propose a new nomenclature for the family. These new findings suggest general mechanisms by which Rabs and possibly other families of Ras-like GTPases are able to bind such a myriad of regulators and effectors.

II.1.2. Materials and Methods

We selected a mammalian representative of each Rab sequence from the databases. Where more than one sequence was available, we chose the following arbitrary priority of species: human, rat, mouse, canine, bovine and rabbit. We performed alignments of the sequences retrieved from Genbank using the Clustal W 1.80 algorithm (Thompson et al., 1997) and the multiple sequence alignment with hierarchical clustering algorithm (Corpet, 1988). The complete sequence alignment data can be viewed at http://www.med.ic.ac.uk/db/dbbm/ rab_family.html. The software package HMMER 2.0 (available at http://hmmer.wustl.edu/) was used to calculate profile HMM and generate model sequences for each family. The alignment of model sequences was manually adjusted to accommodate structural considerations.

The search for new mammalian Rab sequences was performed by Ψ-BLAST (Altschul et al., 1997) of known Rab sequences, or by searching the databases with combinations of the Rab family specific motifs (RabF) defined in this work, using the PatternFind Server at http://www.isrec.isb-sib.ch/software/PATFND_form.html.

The final alignments were used to calculate phylogenetic trees using the Neighbour-Joining method excluding positions with gaps. Bootsraping (Felsenstein, 1985) involved 1000 trials. Trees were calculated using the program ClustalX 1.80 (Thompson et al., 1997) and plotted using the program Njplot (Perriere and Gouy, 1996). PDB files were retrieved from the Protein Data Bank and the mapping of the different regions identified in this work done using the Swiss-PDBviewer v3.51 program available on http://www.expasy.ch/spdbv/mainpage.htm (Guex and Peitsch, 1997).

II.1.3. Results

II.1.3.1. Rab-specific sequences (RabF1 to RabF5)

Many previous studies have highlighted conserved regions in all members of the Ras superfamily that are involved in guanine and phosphate/Mg^{2+} binding (Barbacid, 1987; Bourne et al., 1990). These have been referred to as G for guanine (G1-G3) and PM for phosphate/Mg^{2+} (PM1-PM3) (Valencia et al., 1991). We first analysed these sequences. As predicted, we found that these GTP-binding regions are not useful to distinguish Rabs because they are extremely conserved between all Ras-like proteins. Also, the variations are

not typical of one family. For example, the Rab9 PM1 motif GDGGVGKT is much closer to Rho protein PM1 sequences than Rab. This principle applies to the other PM/G motifs.

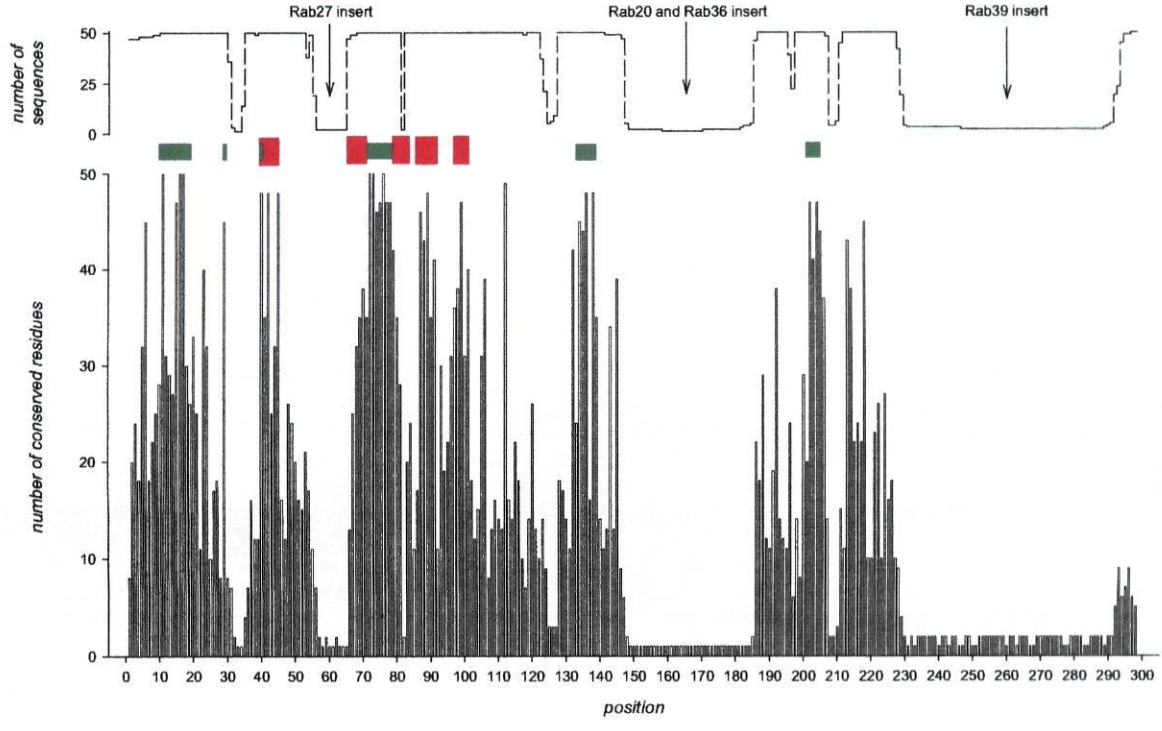


Figure II.1.1 *– (Panel A) Number of mammalian Rab sequences that align at a given position, highlighting the existence of some insert regions in specific Rabs. (Panel B) Frequency of the most common amino acid in the Rab family alignment. Green and red boxes indicate, respectively, the conserved GTP binding motifs and the RabF regions.*

Rabs have been shown to be substrates for prenylation by the enzyme Rab Geranylgeranyl Transferase (Seabra et al., 1992). They present prenylation motifs distinct from the motifs found in Ras and Rho, substrates for the *CAAX* prenyltransferases, Protein Farnesyl Transferase or Protein Geranylgeranyl Transferase-I. The Rab prenylation motifs consist of two C-terminal cysteines, found in one of the following combinations: *XXX*CC, *XX*CC*X*, *X*CC*XX*, CC*XXX* or *XX*C*X*C. The presence of the double-cysteine motif in the C-terminus is in general a very good diagnostic of a Rab protein. However, some Rabs present a C*XXX* box, where only one cysteine is available for prenylation. Therefore, the double-cysteine prenylation motif may confirm that a given small GTPase is a Rab but its absence should not be used to prove otherwise.

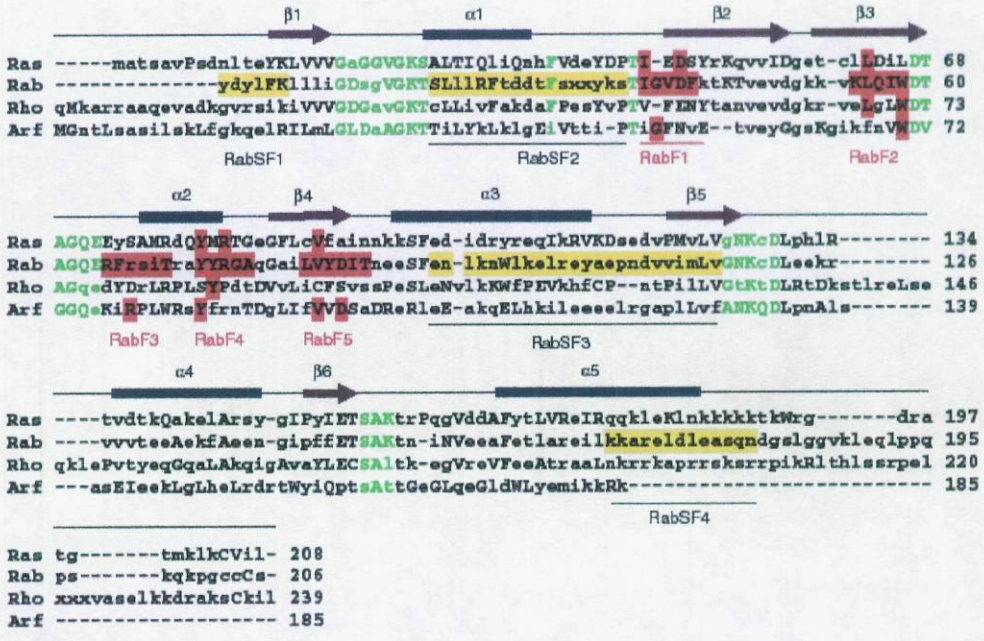


Figure II.1.2 *– Alignment of profile HMM model sequences (manually adjusted to accommodate structural considerations). The uppercase/lower case coding represents the results of the profile HMM method, in which uppercase characters were found at p>0.5. Residues found to be Rab specific are highlighted in red. When a position is conserved in other families, the corresponding position is also highlighted in red. Green characters denote the conserved nucleotide binding (PM/G) motifs.*

To address the existence of Rab-specific sequences, we first aligned all the known mammalian Rabs using the ClustalW 1.80 algorithm. The alignment of the complete mammalian Rab family (gap opening and extension penalties, respectively 10.00 and 0.20) can be found at http://www.med.ic.ac.uk/db/dbbm/rab_family.html.

Based on this alignment we followed two complementary approaches to describe a "model Rab" sequence. The first approach consisted in manually plotting the frequency of the most abundant amino acid for any given position in the alignment (Figure II.1.1). We observed the existence of regions of high amino acid identity, some corresponding to the conserved GTP-binding motifs, and some corresponding to other regions. In order to identify the Rab-specific regions, we defined variable length windows around the most promising areas, calculated the average amino acid identity for all the windows and compared it with the identity calculated, for the same window, for the entire Ras superfamily. We found five regions that appeared to be Rab specific (Figure II.1.1, red squares).

The second approach was based on profile Hidden Markov Models (HMM) (Eddy, 1996). A statistical model of each family consensus was calculated using the software package HMMER 2.0. The aligned model sequences revealed positions conserved throughout the superfamily as well as candidate family-specific regions. Figure II.1.2 summarises the results of the two strategies. The uppercase/lowercase code represents the

results of the profile HMM, in which uppercase amino acids were found at p>0.5. This analysis confirmed the existence of five conserved short stretches of residues that seem to be diagnostic for the Rab-family (RabF) that we numbered RabF1 to RabF5 (Figure II.1.2). The RabF motifs include mostly Rab-specific positions but in some cases residues that are also highly conserved in other small GTPase families. These residues are also helpful in that they help rule out specific families, for example the G residue in RabF1 helps rule out Ras and Rho.

RabF1 localises to the so-called effector domain (loop 2 – β2), in the putative switch I region. The prototypical sequence is IGVDF (Figure II.1.2). Olkkonen & Stenmark (Olkkonen and Stenmark, 1997) previously suggested that this region was Rab-specific, but by itself this sequence is not large or specific enough to serve as the sole criteria to identify a Rab protein. The G position is almost absolutely conserved in Rabs, Arfs and Ran, and represents an insertion relatively to Ras and Rho proteins. The solution structure of Rab3a-GTP revealed that this residue plays an essential role in forcing the main chain in the putative switch I region to bulge in the direction of the helix α2, promoting a closer interaction with the putative switch II region. This results in a more rigid conformation in the putative switch regions, apparently characteristic of the Rab family (Dumas et al., 1999). While we have been referring to "switch" regions in Rabs, it is important to note that there is only one known crystal structure form of Rab to date. In the absence of a Rab-GDP structure, we have no information on the conformational changes between the two nucleotide-bound configurations in Rabs. Whilst it is almost certain that Rab switch regions exist, their precise boundaries as compared to those well defined in Ras are difficult to predict at present (Pai et al., 1989; Tong et al., 1991), and hence it is more prudent to refer to "putative" switch regions.

All the other RabF regions cluster in and around the putative switch II region. The RabF2 prototypical sequence is KLQIW (β3) (Figure II.1.2). The W position is not Rab-specific as it is conserved in all small GTPases except Ras. The RabF3 prototypical sequence is RFrsiT (loop 4) (Figure II.1.2). In the Rab3a crystal structure, the hydrophobic residues in this motif and the adjacent hydrophobic T residue (corresponding to α2) reinforce the effect of the bulge in β2, contributing to the higher rigidity of the switch I/switch II interface (Dumas et al., 1999). The RabF4 prototypical sequence YYRGA (α2 - loop 5) is almost adjacent to RabF3 (Figure II.1.2) followed closely by the RabF5 motif, LVYDIT (β4 - loop 6). Therefore, RabF2 to RabF5 cluster between sheets β3 and β4 in a region that includes the putative switch II region.

II.1.2.2. New Rabs

Based on the criteria described, we found 52 sequences in Genbank that we consider Rabs. The submissions have been numbered in order of discovery, starting with Rab1a through Rab40. The gaps in the numbering are Rab16, which is Rab3d, and Rab31, which is Rab22b.

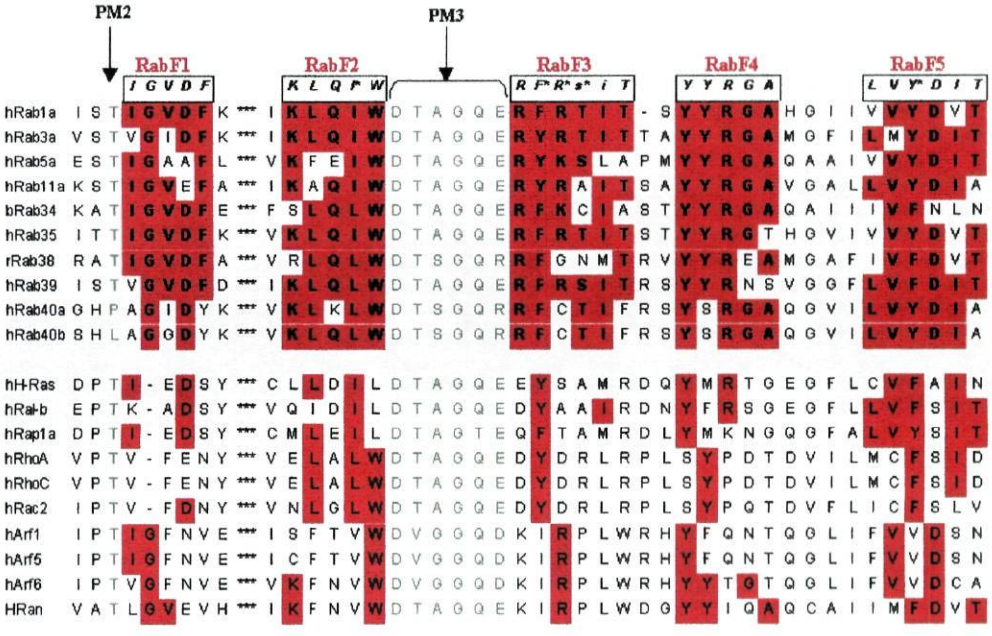


Figure II.1.3 *– Alignment of RabF regions for novel and selected Rab proteins, and the corresponding regions in other representative small GTPases. Conserved residues are highlighted in red. The prototypical sequence for each of the RabF motifs is indicated on the top of the alignment, in italics. An asterisk indicates that the position is frequently occupied by a conservative substitution and the second most common occurrence is also highlighted in the alignment.*

The newly identified Rabs are shown in Figure II.1.3. Two "old" entries, called Rah (accession AAC83182) and Ray (accession AAD25874) are clearly Rabs. Both possess all the RabF motifs, either with the prototypical sequence or in variations that are recognisable as Rab specific, possess the unique double cysteine prenylation motif and have overall average identities to other Rabs of respectively 23.9% and 36.3%. We propose that they should be renamed Rab34 (rah) and Rab35 (ray) and on our recommendation, the latter has already been renamed Rab35.

Our analysis suggests that a recent entry called Rab36 is indeed a Rab despite being peculiar. Rab36 presents a 125-residue long N-terminal extension (counted from the conserved K residue on position 13 in hRab1a) (Mori et al., 1999). Interestingly, one yeast protein known as Ypt11p or Ybj9 (Garcia-Ranea and Valencia, 1998; Lazar et al., 1997) also possesses a large N-terminal extension. It is not clear whether this protein is the Rab36

ortholog as both proteins share only 10% identity to each other. No functional information is available on either protein other than the deletion of the yeast gene produces no phenotype.

Two "Rab-related" sequences are found in the databases, with accession numbers AAA42000 and X99962 (Stankovic et al., 1997). We propose to rename Rab38 the first protein. The second protein we propose to be renamed Rab39. Its closest mammalian relative is hRab2 which is 41% identical..

Several proteins have been named "Rab-like". The human protein Rab7L1 (accession number NP_003920) (Shimizu *et al.*, 1997) is 94% identical to the protein Rab29 from rat, suggesting that Rab7L1 is the human ortholog of rat Rab29 and should be renamed accordingly. The proteins with accession numbers NP_009012 and NP_009013 (RabL2B and RabL2A) (Wong et al., 1999) do not present any prenylatable cysteine in the C-terminus, have low overall identity to the rest of the family (average identity respectively 21% and 23%) and have sequences that do not conform to the RabF motifs described above. They are clearly not Rab proteins but they are also not obvious members of another family. It is possible that RabL2 may represent a new family of small GTPases.

Using Ψ-BLAST of different Rabs, and searching the databases using combinations of the RabF motifs described above, we found two more mammalian sequences that we consider Rabs. The two related proteins CAB09136 and AAA17031 (originally called Rar) are more related to Rabs (27.5% average identity to the Rab family) than to any other small GTPase family. Both possess recognisable RabF motifs and none of the sequences presents structural motifs that suggest they belong to any of the other small GTPase families. We propose that they should be renamed Rab40a and Rab40b, due to their high identity to each other. Both protein present unusual substitutions at the conserved PM2 position. This residue, typically a threonine is replaced by proline or alanine. The role of the threonine residue has been extensively studied and shown to be involved in coordination of the Mg^{2+} ion and in stabilisation of the γ-phosphate (Valencia et al., 1991). Its absence in the Rab40 subfamily may imply that these proteins do not cycle between two conformations, but are permanently locked in an inactive state. A precedent for a Rab that appears not to cycle efficiently between two conformations was set by Rab24. Rab24 appears to be locked in the GTP bound conformation due to variations in two PM/G motifs (Erdman et al., 2000).

II.1.2.3. Rabs subfamilies and subfamily-specific sequences (RabSF)

Phylogenetic analysis of the complete mammalian Rab family using the Neighbour Joining algorithm (disregarding the gaps in the alignment caused by some Rabs having specific "insert regions"), revealed several clusters of "related" Rabs (Figure II.1.4). Within these clusters there are Rabs that show unusually high homology and are termed isoforms,

defining Rab subfamilies. The problem is where to draw the line between isoforms (named Rab1a and Rab1b, for example) and simply "related" Rabs. Since isoforms are believed to be functionally related and are thought to interact with the same type of effectors (Lazar et al., 1997; Novick and Zerial, 1997; Olkkonen and Stenmark, 1997; Schimmoller et al., 1998; Chavrier and Goud, 1999; Brennwald, 2000; Rodman and Wandinger-Ness, 2000), it is plausible that this functional conservation reflects conservation in specific regions, as opposed to complete sequence conservation. If such regions exist they are the best criteria to decide if two or more Rabs are isoforms.

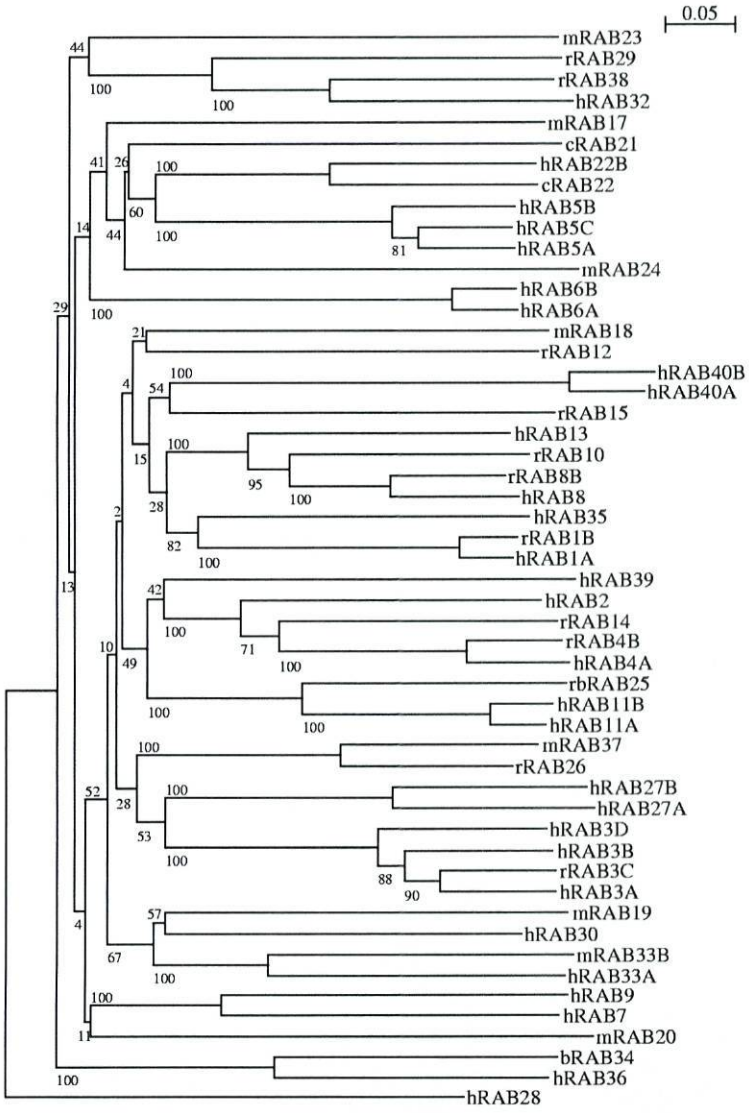


Figure II.1.4 – *Phylogenetic tree of the 52 members of the mammalian Rab family, calculated using the Neighbour-Joining method and excluding the gaps. Bootstraping involved 1000 trials and is represented as percentage in the figure.*

Table II.1.1 *- Amino-acid identity within the different Rab Sub-Family Specific regions (RabSF).*

	Complete sequence	*RabSF1*	*RabSF2*	*RabSF3*	*RabSF4*
Rab family Average identity	**32.7%**	**34.2%**	**28.6%**	**23%**	**14.4%**
	78.3%	**92.3%**	**90.1%**	**87.5%**	**58.4%**
Subfamilies					
1	91%	100%	100%	96%	84%
3	73%-82%	100%	83%-100%	88%-96%	46%-76%
4	82%	100%	88%	76%	84%
5	81%-86%	85-100%	94%-100%	88%-96%	69%-76%
6	90%	100%	100%	95%	53%
8	82%	100%	88%	88%	30%
11	90%	100%	100%	100%	84%
22	70%	85%	72%	76%	23%
27	71%	100%	72%	84%	76%
40	88%	100%	72%	86%	54%

Moore and co-workers (Moore et al., 1995) observed that there was high amino acid conservation within subfamilies in three regions designated here RabSF2, RabSF3 and RabSF4 and corresponding, respectively, to α1/loop2, α3/loop7 and α5 (Figure II.1.2). We verified the corresponding regions in all our mammalian Rab sequences and in general we confirmed that these regions show higher identity within the sub-families than the overall sequence (Table II.1.1). This is particularly remarkable with RabSF4, which is located in the so-called hypervariable domain, a region characterised by its sequence divergence: the average identity of this region among Rabs is 14.4% while subfamilies show an average of 58.4%.

Recently, the 3D structure of Rab3a in complex with its effector Rabphilin-3A revealed that three regions in Rab3a contribute to form a "pocket" that mediates binding to this and possibly other effectors (Ostermeier and Brunger, 1999). These three regions were named RabCDRs (Rab complementary-determining region) and we refer to them as RabCDRI to RabCDRIII, counting from the N-terminus of the protein. Interestingly, these regions correspond to subfamily specific sequences: RabCDRII and RabCDRIII correspond to RabSF3 and RabSF4. RabCDRI includes the N-terminal sequence upstream of PM1 (YXYLFK) previously proposed to be diagnostic of the Rab family (Sanford et al., 1995). However, the overall identity within the region is only 34% and one residue (K13 in hRab1a) is highly conserved throughout the superfamily. We propose instead that this sequence is a good indicator of Rab subfamily and termed it RabSF1 (Figure II.1.2).

We mapped both the RabF and RabSF regions into the crystal structure of Rab3a (Figure II.1.5). We observed that Rabs present two subfamily-specific surfaces: RabSF1, RabSF3 and RabSF4 form a surface that mediates specific interactions between Rab3a and

Rabphilin (Ostermeier and Brunger, 1999). Almost on the opposite side of this surface RabSF2 forms a second subfamily-specific surface near or within the switch I region that could mediate interaction with other effectors. The existence of these two distinct subfamily-specific surfaces suggests that different effectors will bind different RabSF regions. Taken together, this analysis is consistent with the hypothesis that isoforms will interact with the same type of effectors/auxiliary molecules via subfamily specific regions.

Criteria in addition to sequence homology at the RabSF regions need to be taken in consideration when defining a Rab subfamily. These include specific variants of PM, G or RabF motifs and small characteristic diversions from the consensus. For example, only Rab27 isoforms present the RabF4 variant FFRDA and have a 10-residue insertion in loop3, supporting the definition of the Rab27 subfamily. These new criteria allowed the definition of one more subfamily, Rab40a and Rab40b (Table II.1.1). The overall sequence identity is 88%. Both sequences present a high identity within all the RabSF regions as well as all the PM/G and RabF motifs, and both present a long C-terminal insertion.

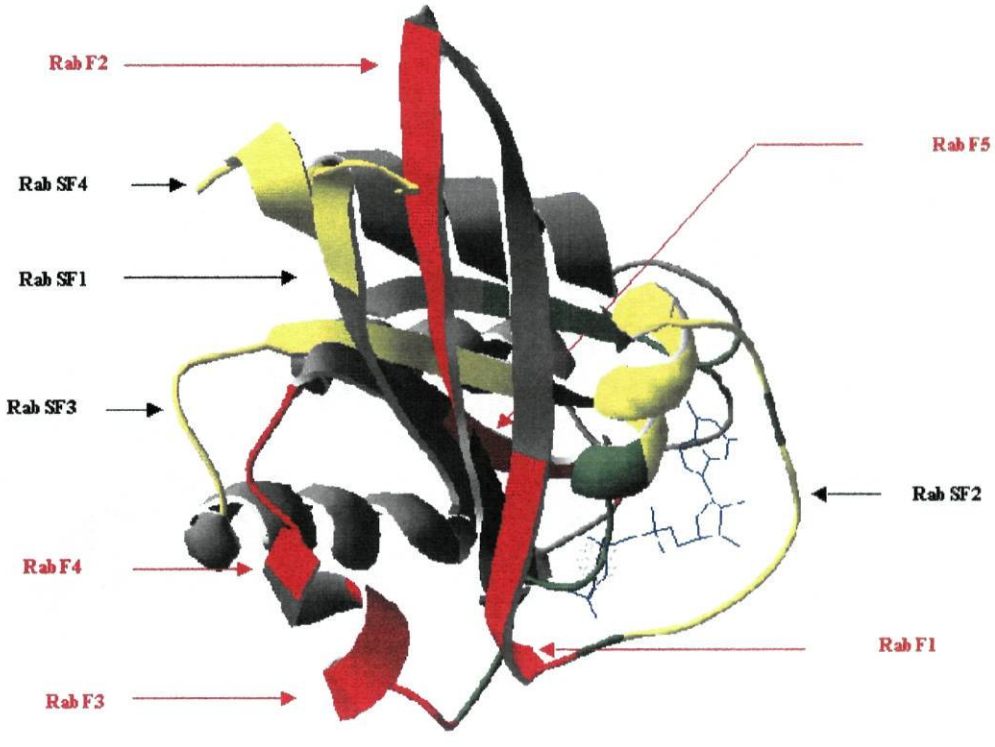


Figure II.1.5 *– Cartoon representing the Rab3a-GTP 3D structure (PDB code 3RABA). RabF regions are depicted in red, RabSF regions in yellow, and the conserved nucleotide binding (PM/G) motifs in green. The nucleotide and the Mg^{2+} atom are both represented in blue.*

A number of related Rabs present a problem of classification, in that they are clearly related but should they be considered isoforms? These related Rabs include Rab4a/b and

Rab14, Rab8a/b and Rab10, Rab11a/b and Rab25, Rab32 and Rab38, Rab33a and Rab33b (Figure II.1.4). Considering that in all cases the overall identity is lower than 70% and that there is no consistent conservation in more than two out of the four RabSF motifs, we suggest that they should not be considered isoforms (Table II.1.1).

II.1.3. Discussion

In this study, we identified five Rab specific motifs, named RabF1 to RabF5, that in conjunction with the conserved PM/G motifs and a double-cysteine C-terminal prenylation motif allow the definition of a Rab GTPase. As of June 2000, we found 52 sequences in the database that fit our criteria. Unfortunately, it is impossible to use the information in the EST database to predict whether this number is close to the real number of genes in a mammalian genome as only full length (or near full length) sequences are useful in this regard. Despite this problem, it is clear that the mammalian Rab family is much larger than the eleven-member orthologous gene family in *S. cerevisiae* (Garcia-Ranea and Valencia, 1998; Lazar et al., 1997), possibly reflecting the higher intracellular specialisation of mammalian cells. In order to classify a Rab as an isoform, we propose that the sequences must be at least 70% identical and this value should be supported by conservation at the RabSF and RabF motifs as well as unique characteristics. Currently, we recognise ten Rab subfamilies (Rab1, 3, 4, 5, 6, 8, 11, 22, 27, 40).

When we mapped these regions into the crystal structure of Rab3a, we noted surprisingly that all RabF motifs localise in and around the switch I and switch II regions. The switch regions change conformation significantly upon GTP binding and hydrolysis and therefore mediate interaction with effectors and regulators. If the switch regions are conserved among all Rabs, how are Rabs able to bind specific effectors and regulators, given that these proteins must discriminate between the two nucleotide-bound states of Rabs?

One possibility is that effectors and regulators will bind both RabF regions to discriminate between active/inactive conformations and RabSF regions for specificity. The existence of two distinct subfamily-specific surfaces further suggests that different effectors/regulators bind to different combinations of RabSF regions. Different lines of evidence support this model. First and foremost, the recent solution structure of the Rab3a-Rabphilin complex (Ostermeier & Brunger, 1999) revealed that the binding surface of this complex involves both switch regions and a Rab-subfamily specific surface composed by the regions we defined herein as RabSF1, RabSF3 and RabSF4. Second, both switch regions and the region corresponding to helix α3/loop 7 (RabSF3) have been shown by site directed

mutagenesis to be involved in interaction with regulators such as GEFs and GAPs (Becker et al., 1991; Burstein et al., 1992; Brondyk et al., 1993; Day et al., 1998; McKiernan et al., 1993). Third, helix α3/loop 7 (RabSF3), the effector domain (RabF1) and the hypervariable domain (including RabSF4) are key regions in determining both localisation and function of ypt1/sec4 chimeras (Brennwalt & Novick, 1993; Dunn *et al.,* 1993), Rab2/Rab5/Rab7 chimeras (Chavrier *et al.*, 1991) and Rab5/Rab6 chimeras (Stenmark et al., 1994). Fourth, the N-terminus (RabSF1) and helix α2/loop5 (RabF4) have also been shown to be essential in producing functional Rab5/Rab6 chimeras (Stenmark et al., 1994).

Another prediction of this model is that the binding of general regulators such as Rab Escort Protein (REP) and Rab GDP Dissociation Inhibitor (RabGDI) is nucleotide sensitive and occurs via the newly identified RabF regions. There is some evidence to support this hypothesis (Beranger et al., 1994; Burstein et al., 1992; Overmeyer et al., 1998; Wilson and Maltese, 1993; Wilson et al., 1996). It is also likely that binding of general regulators and specific regulators/effectors is mutually exclusive since both are able to sense the two alternative nucleotide-bound conformations and thus must interact with the switch regions.

We questioned if this could be a general model that applies to other Ras-like GTPases. The answer is generally yes but not in all cases. The most notorious exception is with Ras proteins. In Ras, the switch regions are also quite conserved (Figure II.1.2) but they appear to be the only contact point in some interactions such as with RalGDS (Vetter et al., 1999b) and the Ras binding domain of Raf1 (Nassar et al., 1995). The strategy to determine specificity seems to be that distinct Ras effectors bind different subsets of residues within the switch regions. This is particularly well characterised for the switch I region (also called effector loop) (White et al., 1995). In other cases, helix α3 (corresponding roughly to RabSF3) is also involved in defining the binding surface, as with the exchange factor SOS (Boriack-Sjodin et al., 1998) and p120GAP (Scheffzek et al., 1997). Similar conclusions may be drawn from the limited data available for the Arf family, where interaction with effectors and regulators appear in most cases to be restricted to the switch regions and helix α3 (Beraud-Dufour et al., 1998; Goldberg, 1998; Mossessova et al., 1998).

The picture is more complex in the Rho family probably because of the existence of a staggering number of Rho regulators/effectors. The switch regions are involved in binding regulators/effectors in every case we found, but other regions have been shown to be involved. RhoA and Cdc42 use switch I and/or switch II in combination with helix α5 (reminiscent of RabSF4) to bind the effector domain of PKN/PRK1 (Maesaki et al., 1999), Wiskott-Aldrich syndrome protein (WASP) (Abdul-Manan et al., 1999) and ACK (Mott et al., 1999). On the other hand, the binding of Cdc42 to Cdc42GAP appears to involve mainly residues in the switch regions (Nassar et al., 1998). As in Ras, different residues in the switch regions appear to confer specificity to the interaction in some cases. Examples for

this type of interaction are RhoA/Cdc42 to Lbc/Cdc24 (Li and Zheng, 1997) and RhoA/Cdc42 to PLD (Bae et al., 1998). In other cases, it seems that the interaction requires both the switch regions (possibly RhoF regions) and other regions (possibly RhoSF regions) such as helix α3 (Zhong et al., 1999), helix α5 (Abdul-Manan et al., 1999; Maesaki et al., 1999; Mott et al., 1999), and the specific Rho insert region (Freeman et al., 1996).

As for Ran, structural and biochemical data also implicate the switch regions and the helix α3 in binding effectors, such as binding to RCC1 and NTF2 (Azuma et al., 1999; Stewart et al., 1998) but other regions also contribute to binding, such as the α4/β6/α5 region with Importin β and the C-terminal helix α6 with RanBD1 (Vetter et al., 1999a; Vetter et al., 1999).

Overall, the available data suggest the interesting possibility that primitive interactions between Ras-like GTPases and effectors/regulators depended upon the switch regions. As the range and complexity of cellular mechanisms controlled by these GTPases grew, so did the interaction surfaces between them and the increasing number of specific effectors/regulators. The future analysis of the Ras, Rho and Arf families as presented here for the Rab family may help clarify this hypothesis.

II.2. Evolution of the Rab family of small GTP binding proteins

Reproduced from Pereira-Leal and Seabra (2001) *submitted.*

II.2.1. Introduction

The recent availability of substantially completed genome sequences for several eukaryotic organisms creates new opportunities for the study of protein evolution and function. At present, the nematode (*C. elegans*), fruit fly (*D. melanogaster*), the budding yeast (*S. cerevisiae*) and the fission yeast (*S. pombe*) have had their genome sequenced, and recently the first drafts of the complete genome of *H. sapiens* and *A. thaliana* were released. With six complete or near complete genomes of evolutionary distant organisms, it is now possible to start addressing the evolution of primary structure and function in the Rab protein family.

Rab proteins form the largest family of the Ras-like superfamily of small GTP-binding proteins and regulate intracellular trafficking pathways. More than fifty Rab proteins have been described in mammalian cells, each with a specific subcellular localisation and many with specific patterns of tissue distribution (Chavrier and Goud, 1999; Zerial and McBride, 2001; Stenmark and Olkkonen, 2001). Rabs behave as membrane-associated molecular switches to regulate budding, transport and fusion reactions in vesicular transport.

In a previous study, we analysed sequence conservation in the mammalian Rab family (Pereira-Leal and Seabra, 2000) and observed the existence of mammalian Rab-specific motifs (RabF motifs) that clustered in and around the switch regions. This allowed us to propose criteria for Rab family classification, and to identify novel Rab sequences from the databases. We also suggested that Rab proteins use the switch regions (Merithew et al., 2001; Dumas et al., 1999) in addition to other regions to determine specificity of binding to protein partners, unlike Ras proteins where specificity of binding is determined mainly by the switch regions (White et al., 1995; Vetter et al., 1999; Nassar et al., 1995). These specificity-determining regions were named RabSF regions (for Rab subfamily) (Pereira-Leal and Seabra, 2000; Moore et al., 1995; Ostermeier and Brunger, 1999).

In the present study, we identify and annotate the complete Rab family in *H. sapiens, D. melanogaster, C. elegans and A. thaliana*, and use this dataset, complemented with the complete Rab families in *S. pombe* and *S. cerevisiae* to study their evolution. We test the

hypothesis that there is a conserved mechanism of Rab interaction with regulators and effectors across evolution and we attempt to reconstruct the multiplication of Rab proteins. This analysis suggested the existence of a higher-order hierarchy in the Rab family with implications for the function and evolution of these proteins.

II.2.2. Methods

We retrieved protein sequences of known Rab families from GenBank. To identify the complete Rab families in *H. sapiens, D. melanogaster, C. elegans and A. thaliana*, we downloaded the latest releases of the calculated open reading frames of each organisms from the public databases. We then used a profile Hidden Markov Model (pHMM) calculated from the alignment of the mammalian Rab sequences presented previously (Pereira-Leal and Seabra, 2000) to query each database using the software HMMER 2.1.1 found at hmmer.wustl.edu (Eddy, 1998).

All the positive hits were then visually inspected, compared to pHMMs representing other small GTPase families and individually BLASTed against the non-redundant database in GenBank to assert if they were indeed Rab proteins, and to insure that no sequence was missed from our analysis due to a possible bias created by the query sequences.

Protein sequences were aligned using the Clustal W 1.80 (Thompson et al., 1994) multiple sequence alignment program with default parameters. Phylogenetic trees were reconstructed by the distance methods of Neighbour-Joining (Saitou and Nei, 1987) scoring for observed amino acid difference and were always bootstrapped with 1000 replicates (Felsenstein, 1985), using the software Phylo_Win (Galtier et al., 1996) . Genomic structures were obtained from GenBank, via the interface MapView.

II.2.3. Results and Discussion

II.2.3.1. Identification and annotation of complete Rab families

Previous studies have identified the Rab families in the budding and fission yeasts (Armstrong, 2000; Lazar et al., 1997; Armstrong, 2000) (shown in tables S1 and S2 of supplementary material). We first attempted to identify the complete Rab families in the human, nematode, fly, and Arabidopsis genomes. We searched the public databases with pHMM described in a previous study (Pereira-Leal and Seabra, 2000). This criteria considers conservation of GTP-binding motifs, presence of double-cysteine prenylation

motifs, and conservation of the RabF motifs. The fact that all the budding yeast Rab (Ypt/Sec4) proteins were correctly identified validated our method.

In the *C. elegans* genome, we identified 29 independent open reading frames which conform to our criteria (tables II.2.1, S3 and figure II.2.1) (Pereira-Leal and Seabra, 2000). Comparison of each sequence with pHMM describing other small GTPase families indicated that they were clearly not members of any other Ras-like small GTPase family. Using the same criteria, we identified 29 independent open reading frames in the *D. melanogaster* genome that we consider Rabs (tables II.2.1, S4 and figure II.2.1). Our strategy led to identification of more Rab sequences than two previous attempts (Bock et al., 2001; Armstrong, 2000), suggesting our analysis was more thorough and/or a recent improvement in the databases.

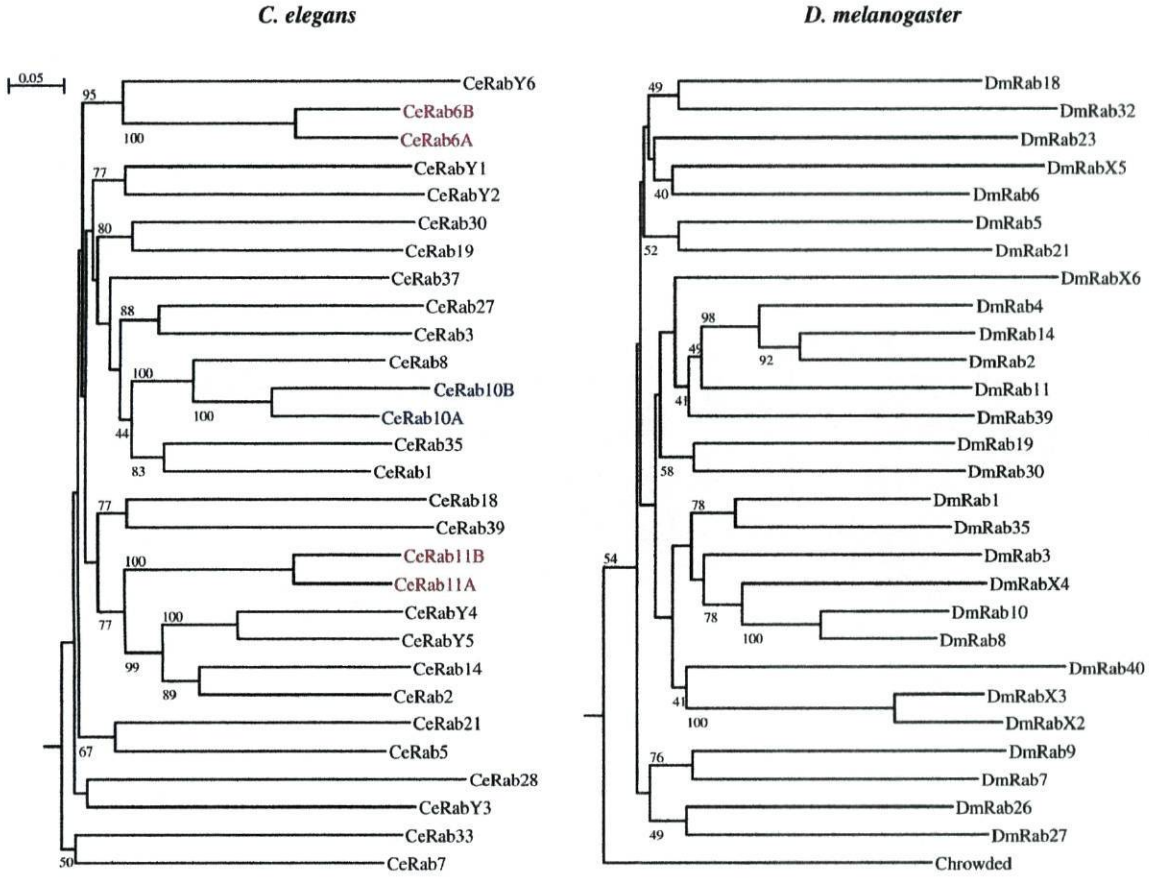


Figure II.2.1 *– Neighbour-Joining tree of the C. elegans and D. melanogaster Rab families, rooted with H-Ras (not shown), scoring for amino acid difference. The numbers on the branches represent the percentage of 1000 bootstrap pseudo-samples supporting that branch; only values ≥ 40% are shown. For clarity, subfamilies are represented in blue or red.*

In *A. thaliana*, we identified 56 proteins that we consider *bona fide* Rabs (tables II.2.1, S5 and figure II.2.2). One additional protein, named Ara6 (accession BAB32953), exhibits some peculiar features. It possesses putative N-terminal myristoylation and palmitoylation motifs, but not a C-terminal prenylation motif. Although this type of lipid

modification has not been described in the Rab family before, the presence of consensus sequences in the RabF motifs suggests that this is likely a Rab protein, albeit an unusual one.

Table II.2.1 *– Rab families in six different organisms, represented in relation to the human Rab family. Presence on the same row indicates that proteins are putative orthologues or are the closest homologs. Below the doted line are represented Rabs for which no clear homologues are found in the human Rab family, except for A. thaliana for which the family members are respesented according to their "homology group". Accession numbers for all these proteins can be found in the supplementary material*

H.sapiens	***D.melanogaster***	***C.elegans***	***S.cerevisiae***	***S.pombe***	***A.thaliana***
Rab1A,B	DmRab1	CeRab1	Ypt1	Ypt1	AtRabD1-D3b
Rab2A,B	DmRab2	CeRab2			AtRabB1a-B1c
Rab3A,B,C,D	DmRab3	CeRab3			
Rab4A,B	DmRab4				
Rab5A,B,C	DmRab5	CeRab5	Ypt5152,53	Ypt5	AtRabF1a-F2b
Rab6A,B,C	DmRab6	CeRab6A,B	Ypt6	Ryh1	AtRabH1a-H1e
Rab7	DmRab7	CeRab7	Ypt7	Ypt7	AtRabG1a-G3f
Rab8A,B	DmRab8	CeRab8		Ypt2	AtRabE1a-E1e
Rab9A,B	DmRab9				
Rab10	DmRab10	CeRab10A,B			
Rab11A,B	DmRab11	CeRab11A,B	Ypt31,32	Ypt3	AtRabA1a-A6b
Rab12					
Rab13					
Rab14	DmRab14	CeRab14			
Rab15					
Rab17					
Rab18	DmRab18	CeRab18			AtRabC1-C3
Rab19	DmRab19	CeRab19			
Rab20					
Rab21	DmRab21	CeRab21			
Rab22A,BC					
Rab23	DmRab23				
Rab24					
Rab25					
Rab26	DmRab26				
Rab27A, B	DmRab27	CeRab27			
Rab28		CeRab28			
Rab29					
Rab30	DmRab30	CeRab30			
Rab32	DmRab32				
Rab33A,B		CeRab33			
Rab34					
Rab35	DmRab35	CeRab35			
Rab36					
Rab37		CeRab37			
Rab38					
Rab39A,B	DmRab39	CeRab39			
Rab40A,B,C	DmRab40				
Rab41					
	Chrowded/ DmRabX1	CeRabY1	Sec4	Ypt4	
	DmRabX2	CeRabY2	Ypt10		
	DmRabX3	CeRabY3	Yp11		
	DmRabX4	CeRabY4			
	DmRabX5	CeRabY5			
	DmRabX6	CeRabY6			

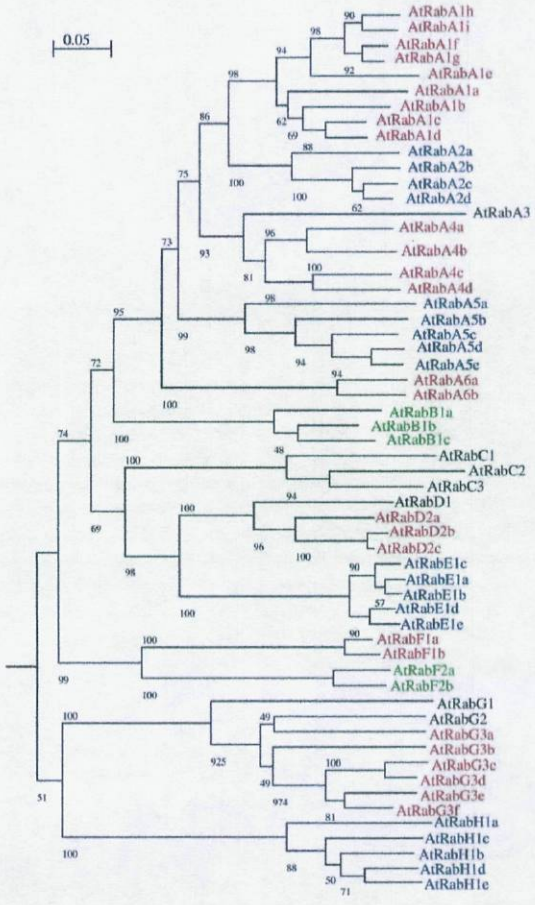


Figure II.2. 2 *– Neighbour-Joining tree of the A. thaliana Rab family, rooted with H-Ras (not shown), scoring for amino acid difference. The numbers on the branches represent the percentage of 1000 bootstrap pseudo-samples supporting that branch; only values ≥ 40% are shown. For clarity, putative subfamilies are represented in blue or red.*

Finally, we identified 60 independent open reading frames encoding Rab proteins in the *H. sapiens* genome (tables II.2.1, S6 and figure II.2.3). We caution that this analysis represents our best guess as of May 2001 and it is likely that the present list needs to be updated as the quality of the sequences in the databases improves. Surprisingly, our analysis differs considerably from a previous report published in early 2001 (Bock et al., 2001). First, we identify human proteins not previously reported, namely Rab24 and Rab33b. Secondly, we chose to discard sequences that differ from established Rab sequences only by the presence of insertions (for example, IGI_M1ctg4256_3 from Rab4a) or are virtually identical (for example, GI7705963 and Rab9), as these are suggestive of putative splice variants, misidentification of splice sites or pseudogenes. Thirdly, we renamed a few Rabs to fit our nomenclature criteria (Pereira-Leal and Seabra, 2000). A direct comparison between

the present study and the Bock *et al.*, study (Bock et al., 2001) is shown in table S6 of supplementary material.

We detect inconsistencies not just between these two studies on the human genome but also with annotation in Genbank. For example, the protein NP_003920 is annotated in GenBank as Rab7 like 1, renamed Rab42 by Bock *et al.*, (Bock et al., 2001) but we find it to be 93% identical to rat Rab29 (BAA65444), indicating that it represents the human homolog of Rab29. Another example is the protein AF322067, which is clearly the human Rab34 protein but is annotated in GenBank as Rab39 (direct submission). This is particularly problematic in the Rab family as the numbering of the different family members is taken frequently as indicative of function.

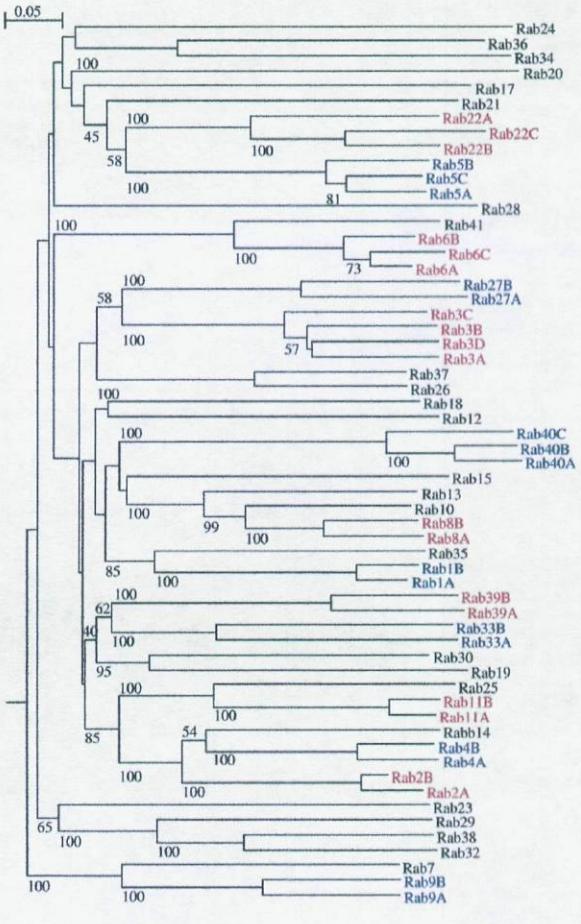


Figure II.2.3 *– Neighbour-Joining tree of the H. sapiens Rab family, rooted with H-Ras (not shown), scoring for amino acid difference. The numbers on the branches represent the percentage of 1000 bootstrap pseudo-samples supporting that branch; only values ≥ 40% are shown. For clarity, subfamilies are represented in blue or red.*

We observed that in phylogenetic trees of Rab proteins of different species, known orthologues always co-segregated, e.g., human Rab1 and yeast Ypt1p. This observation suggests that the strict phylogeny of function in the Ras superfamily previously observed by

Valencia and co-workers (Valencia et al., 1991) also applies within the Rab family (figure II.2.4). We thus propose that co-segregation in phylogenetic trees, together with specific conservation in the RabSF regions be used as the criteria to assign putative orthologues (Pereira-Leal and Seabra, 2000; Ostermeier and Brunger, 1999; Moore et al., 1995). The results of our analysis, including the proposed names for all members of the Rab families, are summarised in table II.2.1, and can be found cross-referenced with the respective accession numbers in tables S.3 to S.6 of supplementary material.

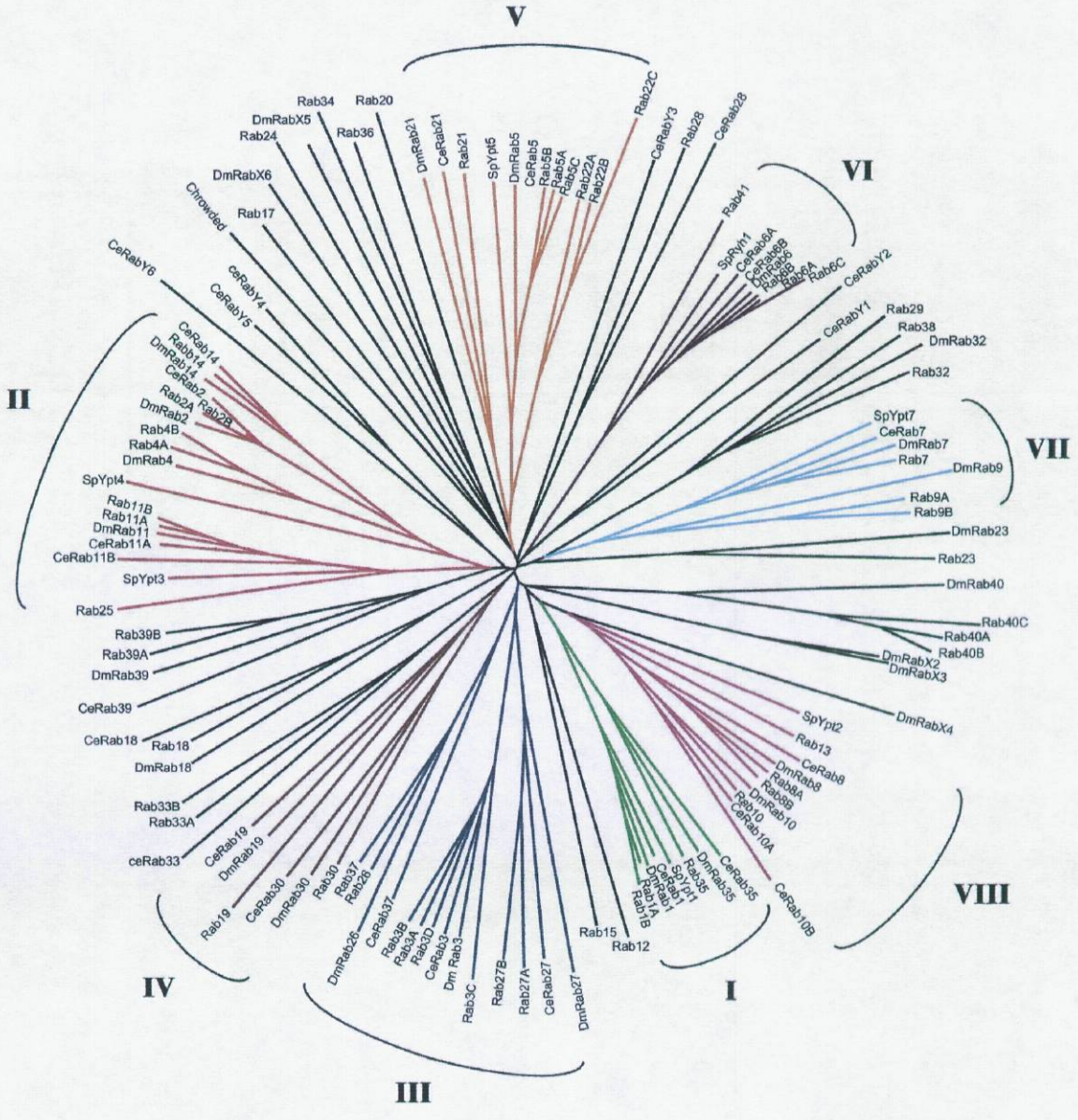


Figure II.2.4 *– Neighbour-Joining dendogram of the human, nematode, fly and fission yeast Rab families. For clarity, human Rabs are not preceded by the species initials. The romanic numerals (I - VIII) and the coloured branches represent Rab functional groups (see text for further details).*

Several proteins defied our annotation attempts. The nematode protein AAB04568, for example, has no clear homolog, presenting only vague homology to human Rab8. Protein AAB52431 is the clear ortholog of mammalian Rab2, however two other proteins (CAB07356 and CAB07357) are also most similar to mammalian Rab2, but all three are not similar enough between them (i.e., < 70% identity) to be considered isoforms. In these difficult cases, we decided to assign letters, rather than the usual numbering system to avoid confusions. For example, we propose AAB04568 to be named CeRabY1.

The Rab family of *A. thaliana* contains large putative subfamilies (up to 9 isoforms in each subfamily), and in several cases the similarities to proteins of known function are not sufficiently high to allow us to ascribe putative orthologies. Unfortunately, some of the proteins have already been named, based on weak similarities, order of discovery or other undefined criteria. We now propose different criteria for annotation of plant Rab families. Inspection of the tree in figure II.2.2 reveals that there are 8 major groups of Rab proteins in *A. thaliana* genome which can be broadly related to mammalian Rabs by homology (Ian Moore, personal communication). Each of these groups should be labelled with capital letters, from A to H. Within these groups, there are subfamilies which can be defined based on co-segregation and conservation in the RabSF regions. Subfamilies should be defined by a number after homology group letter, which in turn is followed by a letter when there is more than one isoform (figure II.2.2). As Rabs should be preceded by the species initials (e.g. AtRab), protein BAB09078 for example should be called AtRabA6a, which should be read as *Arabidopsis thaliana* Rab, group A, subtype 6, isoform a.

II.2.3.2. Rab familes across evolution

Of all available Rab families, the fission yeast (*S. pombe*) presents the smallest number of Rab proteins (table II.2.2). In this organism, all the Rabs produce a detectable phenotype when the corresponding gene is disrupted, ranging from lethality (Ypt1p, Ypt2p, Ypt3p), temperature sensitive growth (Rhy1p), fragmentation, size increase or reduction in number of vacuoles (Ypt7p andYpt4p, respectively) and vesicle accumulation (Ypt5p) (Lazar et al., 1997; Olkkonen and Stenmark, 1997). Furthermore, all *S. pombe* Rabs, with the exception of Ypt4p, are conserved in all other organisms.

The evolutionary divergent budding yeast (*S. cerevisiae*) on the other hand, displays an increased number of Rab genes, many of which produce no phenotype when they are disrupted (Ypt31, Ypt32, Ypt51, Ypt52, Ypt53, Ypt10, Ypt11). This increased number is in part due to the appearance of subfamilies, i.e., functionally redundant isoforms (Ypt51, Ypt52,Ypt53 and Ypt31, Ypt32), but also due to the appearance of Rab proteins without clear functional or sequence homology to known Rab proteins in other organisms (Ypt10,

Ypt11, and Sec4). The genome of the budding yeast contains several duplicated chromosomal regions that could underlie the appearance of subfamilies, which is consistent with the non-essentiality of the members of the different isoforms (Seoighe and Wolfe, 1998; Wolfe and Shields, 1997; Achaz et al., 2000).

Table II.2.2 *– Structure of the Rab family in six organisms. The listing of the family members and their accession numbers can be found as supplementary material.*

Species	Cell #	Rab #	Sub-families
S. pombe	1	7	0
S. cerevisiae	1	11	2
C. elegans	$\sim 1 \times 10^3$	29	3
D. melanogaster	$\sim 3 \times 10^9$	29	0
H. sapiens	$\sim 1 \times 10^{13}$	≥60	11
A. thaliana	-	≥57	11

The human Rab family is the largest of all Rab families studied here and reflects the increased family size that accompanies multicellularity. Many of the Rab proteins have close homologues and form subfamilies. If we consider, in a simplistic view, that each subfamily corresponds to one function, then 39 functions are required in mammals. A significant number of these functions may involve specialised, tissue-specific trafficking pathways, as many Rabs are not ubiquitously expressed.

The nematode and the fly Rab families contain an intermediate number of Rabs between the yeasts and the mammals. Interestingly, we observe the existence of subfamilies in the nematode, but not in the fly Rab family, consistent with the recent genomic duplications observed in the nematode (Friedman and Hughes, 2001). Analysis of these genomes suggests that there is not a linear increase in the number of Rab proteins with the number of cells as the nematode is made of less than a thousand cells and the fruit fly contains more than three billion, and both organism have a similar number of Rab proteins. This is true even if we discard "redundant" Rabs (i.e. Rabs forming subfamilies), assuming they represent a single function (26 Rab functions in *C. elegans* compared with 29 in *D. melanogaster*).

The Rab family of *A. thaliana* is quite different from the other Rab families considered here. It exhibits large subfamilies, which can be grouped in terms of homology to a small set of animal Rabs: Rab1, Rab2, Rab5, Rab6, Rab7, Rab8, Rab11 and Rab18. Surprisingly, the Rab11-like group in *A. thaliana* contains 26 proteins, but the significance of this fact is unclear. This Rab family organisation seems to be found also in other plant Rab families (Ian Moore, personal communication). It seems obvious to suggest that Rab proteins in plants followed a different evolutionary pathway than animals or fungi. The rationalisation of this

observations however will have to wait for more functional information on the different plant Rab proteins, as very little is known at present.

So what is the minimum number of Rab proteins required in a eukaryotic cell? One possibility is seven, as this is the number of Rabs found in the fission yeast (Lazar et al., 1997). Multicellularity and cellular specialisation may require more Rab proteins, possibly those corresponding to the conserved Rab proteins between the animal genomes considered here (table II.2.1). Such candidate Rabs include Rab3 and Rab27, two examples of tissue-restricted with specialised functions. The future availability of more sequenced genomes will allow a more accurate definition of the basic vesicular transport steps required in a multicellular organism.

II.2.3.3. Conserved interactions with general regulators and effectors

We recently proposed in mammalian Rabs that the RabF motifs, clustering in and around the putative switch regions, determine the interaction with Rab-specific general regulators such as Rab Escort Proteins (REP) and GDP Dissociation Inhibitors (RabGDI). We sought to determine whether this mode of interaction with general regulators is conserved across evolution. To do so, we asked if the same regions are conserved in the different Rab familes considered in this study. We calculated pHMMs for each organism Rab family and generated model sequences for each organism. Upon alignment we observed that the same regions are conserved in all organisms and that there is no organism-specific consensus (figure II.2.5). Thus, the RabF motifs seem to be a feature conserved in evolution and may indicate a conserved mode of interaction between Rabs and general regulators. Recent work showing that similar positions in yeast REP and RabGDI mediate interactions with Rabs further supports this possibility (Alory and Balch, 2000).

We worried that this observation could have been biased by the Rab identification strategy followed, as the presence of RabF motifs was one of the criteria used. However, this was by no means the only criteria. We BLASTed each individual "putative" Rab sequence against the non-redundant and organism-specific databases to confirm the similarity with other Rabs, and to look for further members of the Rab family. Also, we checked every sequence against pHMM of other small GTPase families (Ras, Rho, Arf, Ran, Gem).

We proposed previously the existence of Rab subfamily (RabSF) regions in mammalian Rabs, possibly involved in determining binding specificity to effectors. These RabSF regions are conserved across species(Moore et al., 1995). Consistently, Rabs from evolutionarily distant organisms exhibit functional complementation. For example, yeast Ypt1 deletions or temperature sensitive mutations can be complemented by small GTPases from

Volvox carteri, *Chlamidomonas reinhardtii* (Fabry et al., 1995), *Brassica napus* (Park et al., 1994) and *Mus musculus* (Haubruck et al., 1989), and ypt6 null mutants can be complemented by a small GTPase from *Arabidopsis thaliana* (Bednarek et al., 1994).

Figure II.2.5 *- Partial alignment of model sequences for each family, calculated from pHMM describing the multiple sequence alignment of the complete Rab family in each organism. Positions in uppercase occur at p>0.5. The PM/G motifs are highlighted in green, the RabF motifs in red boxes or red characters when the position is often occupied by a conservative substitution, and the RabSF regions in yellow boxes. Black bars above the alignment represent the switch regions*

Taken together, the absence of organism-specific consensus, the conservation of RabF regions, the cross-species functional complementation and the conservation of RabSF regions make a strong argument for a highly conserved mechanism of effector and general regulator recognition, likely to be present at the point of divergence from other small GTPases. It also suggests that this family originated by a single divergence event and that these interaction mechanisms represent a major constrain to the evolution of Rab proteins. Furthermore, the conservation of this effector recognition mechanism is indicative of effector conservation, an assumption that is supported by some recent evidence. While exchange factors for the Rab family form a very divergent class of proteins, there is a striking conservation of these proteins across evolution for known orthologues in the few known cases. For example, the mammalian and nematode exchange factors for Rab3 (Rab3GEP and Aex3) are highly conserved (Iwasaki et al., 1997; Wada et al., 1997), and so are the exchange factors for Ypt51 and Rab5 (Vps9 and Rabex-5) (Hama et al., 1999; Horiuchi et al., 1997).

II.2.3.4. Prenylation and targeting motifs

Rab proteins contain one or two C-terminal cysteine residues that undergo post-translational prenyl modification (Seabra, 2000). These cysteine residues are arranged in a variety of prenylation motifs. Some Rabs (HsRab8 and HsRab23), have a single cysteine residue, fourth from the C-terminus, sometimes within a CAAX box (C-cys, A-aliphatic, X-any), a motif commonly observed in the Ras and Rho families (Casey and Seabra, 1996). However, most Rabs have two cysteine residues arranged in different double cysteine prenylation motifs (e.g. XXCC, XCXC, CCXX, CCXXX, XCCX), both of which are modified by geranylgeranyl moieties. Unlike Ras and Rho proteins, the prenylation motif in Rabs does not determine which prenyl transferase (and consequently which type of prenyl moiety) will modify the C-terminal cysteine residues. All Rabs appear to be substrates for a unique enzyme, Rab Geranylgeranyl Transferase (RGGT), following the binding to REP, a general regulator of Rabs.

It is conceivable that the diversity of Rab prenylation motifs arises from lack of functional constrains other than those imposed by the geranylgeranylation reaction mechanism. When we compared the prenylation motifs in Rabs from all organisms, we observed that the number of cysteines is frequently conserved, and in many cases the topology of the prenylation motif is also retained (e.g., Ypt1(CC)→Rab1(CC), Ypt6(CXC)→Rab6(CXC)). Conservation of these topologies indicates constrains to evolution possibly due to the requirement for carboxyl-methylation. The functional significance of Rab carboxyl-methylation affecting Rabs ending in CXC but not in CC is not understood (Smeland et al., 1994; Bergo et al., 2001). Furthermore, we observed conservation of the number of cysteines available for prenylation. This suggests that the number of prenyl groups (one or two) may be functionally important, possibly revealing the existence of two distinct membrane-association mechanisms or perhaps different membrane targeting strategies.

We observed some unusual Rab proteins in this regard. For example protein BAB32953 in *A. thaliana* exhibits putative N-terminal myristoylation and palmitoylation motifs, but no C-terminal prenylation motifs, and these motifs are also found in similar plant proteins, indicating a conserved feature. This type of lipid modification is novel within the Rab family. Another peculiar Rab protein is Rab24, which is thought to be cytosolic (Erdman et al., 2000). These unusual cases may represent recent evolutions of the Rab family, where motifs not normally present in this family are recruited to provide for new functions.

We caution that sequencing errors or artefacts complicate annotation attempts. For example, several Rab proteins in *D. melanogaster*, *C. elegans* and *A. thaliana* do not exhibit C-terminal prenylation motifs. However, their putative orthologs in other organisms do exhibit

prenylation motifs, suggesting either bad quality sequencing or deficient gene identification algorithms.

II.2.3.5. Rab functional groups

Phylogenetic trees for all the sequences considered here, reconstructed using the Neighbour-Joining method, revealed a clear phylogeny of function, as opposed to a phylogeny of species (figure II.2.s 1- 4). In other words, Rab proteins of similar function in different organisms always co-segregate. As mentioned above, this represents an extension of the strict phylogeny of function previously observed in the yeast Ras superfamily (Valencia et al., 1991; Garcia-Ranea and Valencia, 1998). Within the clades representing each putative Rab "function", we observed a phylogeny of species, with proteins segregating according to organism provenience (figure II.2.4).

Figure II.2.s 1-3 show the trees calculated for each organism Rab family. Some proteins always co-segregate, even if they don't conform to the criteria defining isoforms (Pereira-Leal and Seabra, 2000). For example, members of the Rab1 sub-family always segregate with Rab35 . Based on the tree topology, we can identify 8 possible groups of co-segregating proteins (figure II.2.4). The proteins in each of these groups are more similar at the amino-acid level than any two random Rab proteins . This may represent a higher order organisation in the Rab family, above the subfamily level, and may represent either a shared ancestry between co-segregating proteins, functional relatedness or both.

To test the hypothesis that this co-segregation of "unrelated" proteins represents co-segregation of functional properties, we sought to identify patterns of function and/or cellular localisation in each group of figure II.2.4. In group V, which includes the sub-families 5 and 22, we noted a pattern of sub-cellular localisation and possibly of function. Rab5a has been extensively studied, it localises to early endosomes and clathrin coated vesicles and regulates endosome budding and fusion (Zerial and McBride, 2001; Bucci et al., 1992; Barbieri et al., 1998; Roberts et al., 1999; Rybin et al., 1996; Stenmark et al., 1994). Rab22a localises to endosomes and the plasma membrane. Over-expression of Rab22a results in the formation of abnormal endosomal structures, which is suggestive of a role in endocytosis (Olkkonen et al., 1993). Rab21 also segregates with the sub-families 5 and 22, albeit showing less sequence relatedness. Rab21 seems to be specific for polarised cells where it localises to apical vesicles and shows partial localisation to an endosomal compartment, suggesting it may also be functionally related to Rab5 (Opdam et al., 2000). Interestingly other Rab proteins which broadly segregate with Rab5 isoforms also display an endosomal localisation, namely Rab17 (Hunziker and Peters, 1998; Zacchi et al., 1998) and Rab20 (Lutcke et al., 1994), but not Rab24 which is reportedly cytosolic (Erdman et al., 2000).

In group III, we also observe a pattern of subcellular localisation to secretory granules. Rab37 has recently been identified and localised to secretory granules in mast cells (Masuda et al., 2000), and Rab26 has been localised to secretory granules in pancreatic acinar cells (Yoshie et al., 2000). Rab27a was the subject of recent work by several groups and found to localise to secretory granules (lytic granules) of cytotoxic T lymphocytes. Defects in the *RAB27A* gene in Griscelli Disease lead to haemophagocytic syndrome due to loss of CTL activity (Haddad et al., 2001; Stinchcombe et al., 2001). In melanocytes, Rab27 is present in melanosomes (lysosome-like pigment-containing organelles destined for secretion)(Hume et al., 2001; Wu et al., 2001; Bahadoran et al., 2001; Haddad et al., 2001). Rab27 appears to recruit MyosinVa to regulate the transport of melanosomes to the cell periphery prior to secretion (Hume et al., 2001; Wu et al., 2001; Haddad et al., 2001). Rab3 isoforms co-segregate with other members of this group in the human and fly Rab tree, but not in the nematode Rab family tree, which is suggestive of a more distant relationship. Members of the Rab3 subfamily have been implicated in regulated secretory events such as neurotransmitter release and insulin secretion, and associate with secretory granules such as synaptic vesicles, secretory granules in mast cells (Iezzi et al., 1999; Tuvim et al., 1999; Al-Matubsi et al., 1999; Raffaniello et al., 1999; Fischer et al., 1990; Darchen et al., 1990; Oishi et al., 1998), thus exhibiting a similar type of cellular localisation as the other members of this group.

Group VII includes Rab7 and Rab9 isoforms. Both proteins show overlapping localisation to late endosomes (Feng et al., 1995; Lombardi et al., 1993; Meresse et al., 1995).

In group II there is no pattern of similar subcellular localisation. For example, Rab2 localises to the ER and Golgi (Tisdale et al., 1992), and Rab4 to endosomes and plasma membrane (Sonnichsen et al., 2000). However, there may be a pattern of functional similarity in this group. Rab11 and 25 are proposed to be involved in recycling of proteins through the recycling endosome (Casanova et al., 1999; Ullrich et al., 1996) and Rab4 is also proposed to be involved in endocytic recycling (van der Sluijs et al., 1992). No functional data is available for Rab14, but Rab2 has recently been proposed to regulate a recycling step in the retrograde Golgi-ER transport (Tisdale, 1999).

In conclusion, we suggest that there is a recognisable pattern of subcellular localisation and possibly of function, which supports the hypothesis of a phylogeny of function applying between the subfamily and the family level.

We next questioned the origin of the sequence relatedness, and functional/ localisation similarity underlying the groups shown in figure II.2.4. The simplest explanation is that the phylogeny of function also represents the evolutionary history of the Rab family and that members of one given branch have a shared ancestry. For example, Rab5 and

Rab22 would share the same ancestor, and this ancestral protein would also be the ancestor of the budding yeast Ypt5.

This hypothesis is based on the phylogenetic reconstruction methods, however these methods can be biased to functional similarities in highly conserved protein families. Therefore, different lines of evidence need to be obtained to substantiate it. Supporting this hypothesis is the observation that there is differential conservation in discrete regions such as the PM/G and RabF motifs in some cases. For example, human group II proteins Rab25, Rab14, and the sub-families Rab11, Rab4 and Rab2, all share identical IGVEF sequence at the RabF1 motif, while human group III proteins Rab3 and Rab27 isoforms, Rab26 and Rab37 display the conserved sequence VGIDF.

In order to complement this analysis, we searched all available genomic structures of human Rab genes for an indication of shared ancestry. If proteins from one branch indeed arose from a common ancestor, we expected to find similarities in exon organisation. We retrieved available intron-exon boundaries in Rab genes from GenBank, using MapView. In many cases the sequencing data is still of low quality, resulting in incomplete or no useful information at all. Using a limited number of genes, we observed many common features in genomic structures within Rab functional groups. The intron-exon boundaries are either absolutely conserved, or are close within a maximum of 5 codons (Figure II.2.6). Some Rabs within each group share highly similar genomic structures. For example, Rab11 and Rab25 are almost identical, while Rab4 and Rab14 are distinguished by the appearance of an intron in Rab14 splitting in two the 5th exon in Rab4. Within subfamilies, genomic structures tend to be highly conserved (data not shown), a fact already noted by others (Adachi et al., 2000; Bhartur et al., 2000; Ramalho et al., 2001; Tolmachova et al., 1999).

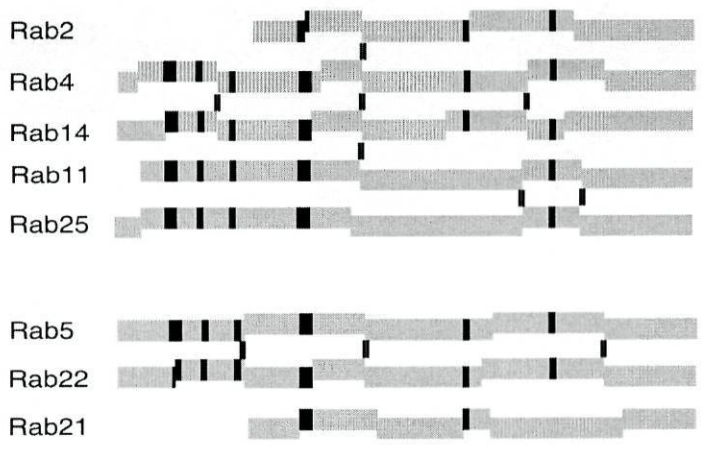


Figure II.2.6 *– Representation of the coding exons (grey boxes) of available "functional group members", mapped to a cartoon representing a generalised Rab protein sequence, aligned by the conserved PM and G motifs (black boxes).*

Based on these observations, we cannot discredit the hypothesis that co-segregating Rab proteins share a common ancestry. Consequently, the Rab family trees not only represent a separation according to function but may also reflect the evolutionary history of this family of proteins. A more comprehensive analysis of gene structures of Rab genes in different organisms is required to provide more clear evidence for shared ancestry.

II.2.4. Conclusions

We have identified and annotated complete Rab families in all eukaryotic organisms that had their genome substantially sequenced in May 2001. We propose here criteria for annotation of animal and plant Rab families. Our analysis suggests that interactions between Rab proteins and their general regulators and specific effectors is conserved across evolution, as the sequence determinants of this interactions (RabF motifs and RabSF regions) are conserved in all Rab families here studied.

We addressed the evolution of the Rab family and observed a higher-order organisation within the Rab family corresponding to Rab proteins which co-segregate in phylogenetic trees. Rabs within these groups exhibit similar function and/or cellular localisation and related genomic structures. It is tempting to speculate that early in eukaryotic evolution a minimum number of Rab proteins provided the "ancestral" Rab regulatory activities. Organism specialisation and multicellularity drove the multiplication of Rab family members from the initial set of "ancestral Rabs". These novel Rabs appear to have maintained one or more properties that defined their ancestry, allowing us to group Rab proteins according to their ancestry, i.e. according to their putative "ancestral Rab function". Thus, we propose that these related functions/cellular localisations form an intermediate level of classification between family and subfamily, better described as "Rab functional groups". One interesting and testable possibility is that this organisation level could have predictive value to suggest a function and/or localisation of a given Rab protein. Furthermore, we expect more functional groups to be defined as more functional information becomes available.

The minimal set of Rab proteins has been equated with the essential yeast Rab proteins (Lazar et al., 1997). These minimal Rab properties may represent localisation to a given cellular compartment, interaction with classes of related effectors/regulators, specific GTPase characteristics or a combination of these. A better understanding of this issue is essential to fully understand the nature of the "ancestral Rab functions", the way that they evolved to provide regulators for increasingly complex organisms, and to ascribe general functions to novel Rab sequences based solely on their segregation pattern in phylogenetic

trees. Furthermore, the understanding of the properties shared by groups of co-segregating Rabs identified here, will allow informative correlations between group-specific sequence conservation and localisation/function of Rab proteins.

II.2.5. Supplementary Material

Table S.1 – *S. pombe* Rab family

Name	Accession
Ypt1	S04590
Ypt2	S33900
Ypt3	S10026
Ypt4	CAA11239
Ypt5	S34729
Ryh1	S12789
Ypt7	O94655

Table S.2 – *S.cerevisiae* Rab family

Name	Accession
Ypt1	NP_011148
Sec4	NP_011185
Ypt31	NP_010948
Ypt32	NP_011305
Ypt51	S43399
Ypt52	NP_012939
Ypt53	NP_014306
Ypt6	NP_013363
Ypt7	NP_013713
Ypt10	NP_009823
Ypt11[¶]	NP_014095

[¶] Sequence exhibits large N-terminal extension.

Table S.3 – *C. elegans* Rab family

Accession	Proposed name
AAC69218	CeRab1
AAB52431	CeRab2
AAB16980	CeRab3
CAB04205	CeRab5
AAC69020	CeRab6A
CAA77590	CeRab6B
CAA91357	CeRab7
AAC78494[c]	CeRab8
AAC48200	CeRab10A
AAC48203[c]	CeRab10B
AAB54158	CeRab11A
CAB07678	CeRab11B
CAB01884	CeRab14
CAB60605	CeRab19
AAF60884[¶, c]	CeRab18
CAA91296	CeRab21
CAB54484	CeRab27
CAA21582[CAAX]	CeRab28
CAA21489	CeRab30
CAA84705[¶]	CeRab33
CAB57899	CeRab35
AAB52888	CeRab37
CAA87774[c]	CeRab39
AAB04568[CAAX]	CeRabY1
CAA20282[¶]	CeRabY2
AAC47067[c]	CeRabY3
CAB07357	CeRabY4
CAB07356	CeRabY5
AAA81090[¶,CAAX]	CeRabY6

[c] Sequence does not contain C-terminal cysteines
[CAAX] Sequence contains a single cysteine in the context of a CAAX box
[¶] Sequence exhibits large N- or C- terminal extension.

Table S.4 – *D. Melanogaster* Rab family

Accession	Proposed name
AAF55873	DmRab1
AAF57381	DmRab2
AAF58762	DmRab3
AAF57831	DmRab4
AAF51265	DmRab5
AAF53168	DmRab6
AAF56218	DmRab7
AAF49101[CAAX]	DmRab8
AAF53798	DmRab9
AAF50924	DmRab10
AAF55850	DmRab11
AAF53390	DmRab14
AAF46057	DmRab18
AAF50452[CAAX]	DmRab19
AAF45341	DmRab21
AAF51970[CAAX]	DmRab23
AAF51708[CAAX, ¶]	DmRab26
AAF45634	DmRab27
AAF52477[c]	DmRab30
AAF58970[c]	DmRab32
AAF45371	DmRab35
AAF46271	DmRab39
AAF48164[¶]	DmRab40
AAF47018	DmRabX1/chrowded
AAF46585[c]	DmRabX2
AAF47981[c]	DmRabX3
AAF56345[¶]	DmRabX4
AAF47546[c]	DmRabX5
AAF47406	DmRabX6

[c] Sequence does not contain C-terminal cysteines
[CAAX] Sequence contains a single cysteine in the context of a CAAX box
[¶] Sequence exhibits large N- or C- terminal extension

Table S.5 – *A. thaliana* Rab family

Accession number	Database name	Group	type	isoform	Proposed name
P28185	ARA2	A	1	a	AtRabA1a
Q39222	RAB11	A	1	b	AtRabA1b
BAB09217		A	1	c	AtRabA1c
T04872		A	1	d	AtRabA1d
T04539		A	1	e	AtRabA1e
BAB10106		A	1	f	AtRabA1f
BAA97069		A	1	g	AtRabA1g
F84750		A	1	h	AtRabA1h
AAF16749		A	1	i	AtRabA1i
O04486	RAB11C	A	2	a	AtRabA2a
AAF79570		A	2	b	AtRabA2b
Q96283	RAB11A	A	2	c	AtRabA2c
BAB09761	RAB11	A	2	d	AtRabA2d
AAF97325		A	3	-	AtRabA3
BAB11663		A	4	a	AtRabA4a
T06105	GB3	A	4	b	AtRabA4b
BAB09048		A	4	c	AtRabA4c
BAB01966		A	4	d	AtRabA4d
BAB09078		A	5	a	AtRabA5a
AAF02165		A	5	b	AtRabA5b
P28187	ARA4	A	5	c	AtRabA5c
G84723		A	5	d	AtRabA5d
P19892	ARA1	A	5	e	AtRabA5e
AAF24834		A	6	a	AtRabA6a
AAF97836[c, ¶]		A	6	b	AtRabA6b
D71440	RAB2A	B	1	a	AtRabB1a
H85191	RAB2 like	B	1	b	AtRabB1b
S71585	GB2	B	1	c	AtRabB1c
U75603	RAB18	C	1	-	AtRabC1
AAF23245		C	2	-	AtRabC2
T48379		C	3	c	AtRabC3
AAD00111	ATFp8	D	1	-	AtRabD1
P28188	ARA5	D	2	a	AtRabD2
BAA97153		D	2	b	AtRabD2b
CAB78756	RAB1C	D	2	c	AtRabD2c
T45901	RAB8	E	1	a	AtRabE1a
BAB08351	ARA3	E	1	b	AtRabE1b
P28186	ARA5 frag.	E	1	c	AtRabE1c
T48378		E	1	d	AtRabE1d
AAF23246		E	1	e	AtRabE1e
BAB32953[c]	ARA6	F	1	a	AtRabF1a
T06736[c]		F	1	b	AtRabF1b
P31582		F	2	a	AtRabF2a
T06157		F	2	b	AtRabF2b

Table S.5 – *A. thaliana* Rab family (continued)

Accession number	Database name	Group	type	isoform	Proposed name
BAB08894[c]	RAB7 Like	G	1		AtRabG1
AAD20423		G	2		AtRabG2
T04019	RAB7 Homolog	G	3	a	AtRabG3a
O04157	RAB7	G	3	b	AtRabG3b
BAB02676		G	3	c	AtRabG3c
AAG21568	RAB7D	G	3	d	AtRabG3d
AAD43167	RAB7	G	3	e	AtRabG3e
BAB01810		G	3	f	AtRabG1f
BAA97311		H	1	a	AtRabH1a
T01588		H	1	b	AtRabH1b
T06095		H	1	c	AtRabH1c
H84610		H	1	d	AtRabH1d
T50814		H	1	e	AtRabH1e

[c] Sequence does not contain C-terminal cysteines
[¶] Sequence exhibits large N- or C- terminal extension

Table S.6-*H. sapiens* Rab family

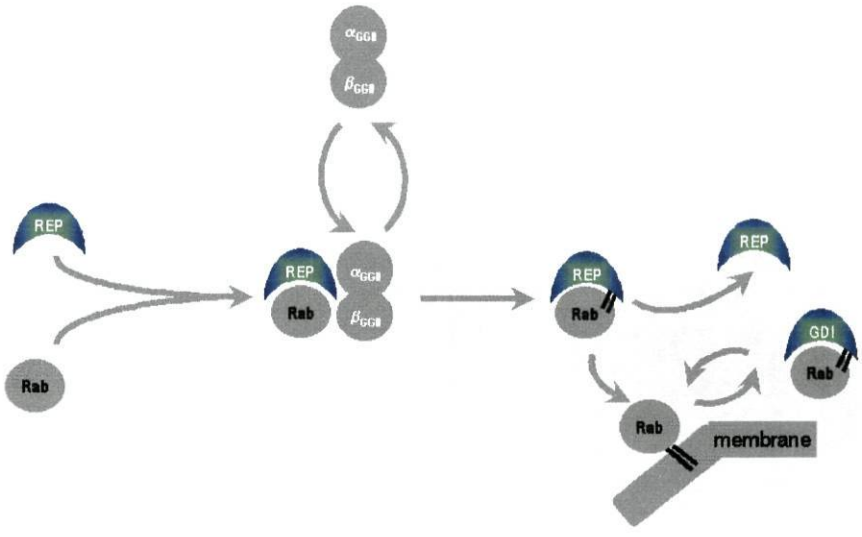
Accession	Proposed Name	Bock *et al.* Name	Bock *et al.* Accession
NP_004152	HsRab1A	1a	=
NP_112243	HsRab1B	1b	IGI_M1_CTG16159_31
NP_002856	HsRab2A	2a	=
IGI_M1_CTG53_358[b, c]	HsRab2B	2b	=
NP_002657	HsRab3A	3a	=
NP_002858	HsRab3B	3b	=
IGI_M1_CTG13513_30[b, c]	HsRab3C	3c	=
NP_004274	HsRab3D	3d	=
NP_004569	HsRab4A	4a	=
NP_057238	HsRab4B	4b	=
Splice variant of 4a(?)	-	4c	IGI_M1_CTG4256_3
NP_004153	HsRab5A	5a	=
NP_002859	HsRab5B	5b	=
NP_004574	HsRab5C	5c	=
NP_002860	HsRab6A	6a	=
NP_067661	HsRab6B	6b	=
CAB66661[c]	HsRab6C	6c	IGI_M1_CTG14521_66
NP_004628	HsRab7	7	=
NP_005361[CAAX]	HsRab8A	8a	=
NP_057614[CAAX]	HsRab8B	8b	=
NP_004242	HsRab9A	9a	=
NP_057454	HsRab9B	9b	IGI_M1_CTG16547_21
Same as 9a	-	9c	GI7705963
NP_057215	HsRab10	10	=
NP_004654	HsRab11A	11a	=
NP_004209	HsRab11B	11b	=
AC15676	HsRab12	12[i]	IGI_M1_CTG16793_17
NP_002861[CAAX]	HsRab13	13	=
NP_057406	HsRab14	14	=
AL139022	HsRab15	15	IGI_M1_CTG16579_4
BAB14121	HsRab17	17	IGI_M1_CTG16465_9
AAF61433[CAAX]	HsRab18	18	=
IGI_M1_CTG66_16[b, ¶]	HsRab19	41	=
NP_060287	HsRab20	20	=
NP_055814	HsRab21	21	=
AAF00047	HsRab22A	22a	=
NP_006859	HsRab22B	22c	GI583131
IGI_M1_CTG66_20[b]	HsRab22C	22b	=
NP_057631[c]	HsRab23	23	=
BAB13887[i, c]	HsRab24	-	-
NP_065120[c]	HsRab25	25	=
NP_055168	HsRab26	26	=
NP_004571	HsRab27A	27a	=
NP_004154	HsRab27B	27b	=
NP_004240[c]	HsRab28	28a	=

Table S.6-*H. sapiens* Rab family (continued)

Accession	Proposed Name	Bock *et al.* Name	Bock *et al.* Accession
Splice variant of 28(?)	-	28b	P51157
NP_003920	HsRab29	42	-
NP_055303	HsRab30	30	=
NP_006825	HsRab32	32a	=
NP_004785	HsRab33A	33	=
CAB66838	HsRab33B	-	-
AF322067*, ¶	HsRab34	34	IGI_M1_CTG15917_25
NP_006852	HsRab35	35	=
NP_004905¶	HsRab36	36	=
IGI_M1_CTG16279_23[b]	HsRab37	37	=
P57729[CAAX]	HsRab38	32b	IGI_M1_CTG15576_6
X99962	HsRab39A	39	Q14964
IGI_M1_CTG14748_14[b, c]	HsRab39B	38	=
CAB09136[CAAX, ¶]	HsRab40A	40a	O00407
NP_006813[CAAX, ¶]	HsRab40B	40b	=
O60795[b, i, CAAX, ¶]	HsRab40C	40c	=
IGI_M1_CTG19178_2	HsRab41	43	=

*Annotated in GenBank as Rab39(direct submission).
[b] Accession numbers given in Bock, J.*et al.*. (2001) Nature *409*, 839-841. which we could not find in any database. We used the sequences kindly provided by the authors. Note that our annotation differs from the one made in Bock, J.*et al.*. (2001) Nature *409*, 839-841
[i] Incomplete sequence
[c] Sequence does not contain C-terminal cysteines
[CAAX] Sequence contains a single cysteine in the context of a CAAX box
¶ Sequence exhibits large N- or C- terminal extension
= identical accession number

Part III – REP Proteins



ICBAS

III. The Rab Escort Protein Family:

Sequence and structural divergence in a conserved function

III.1. Introduction

Rab proteins form the largest family of small GTPases, with more than 50 members described in mammals to date (Pereira-Leal and Seabra, 2000). Rabs are regulators of intracellular trafficking pathways, many have a specific sub-cellular localisation and some show tissue specific variation in expression level, reflecting the complexity and variety of trafficking events found in mammalian cells (Novick and Zerial, 1997; Olkkonen and Stenmark, 1997; Schimmoller et al., 1998; Chavrier and Goud, 1999; Brennwald, 2000; Rodman and Wandinger-Ness, 2000). Several studies showed that Rabs are geranylgeranylated (Kinsella and Maltese, 1992; Khosravi-Far et al., 1991), but unlike other small GTPases they display two cysteine residues at or near the C-terminus, unlike the CAAX box that is usually found in other prenylated proteins. Both cysteine residues were shown to be geranylgeranylated (Farnsworth et al., 1991), and this type of lipid modification (di-gernaylgeranylation) was shown to be essential for Rab function (Walworth et al., 1989).

Geranylgeranylation of Rab proteins is catalysed by RabGeranylgeranyl Transferase (RGGT), a heterodimeric enzyme related to other Protein Prenyl Transferases (Seabra et al., 1992; Seabra et al., 1992) that is unique in its absolute requirement for an accessory protein termed Rab Escort Protein, a protein related to RabGDI (Andres et al., 1993; Seabra et al., 1992). Newly synthesised Rabs bind REP and the REP:Rab complex is the substrate of RGGT that catalyses the sequential addition of two geranylgeranyl groups to the C-terminal cysteine residues of the Rab protein (Shen and Seabra, 1996). After the transfer, the enzyme dissociates from the complex RabGG:REP and REP delivers the prenylated Rab to cellular membranes (Alexandrov et al., 1994; Wilson et al., 1996). Thus REP behaves as an essential activator of the Rab prenylation reaction (Anant et al., 1998).

Two REP proteins have been described in mammals, REP1 and REP2, sharing around 70% identity. They show similar tissue distribution patterns (Desnoyers et al., 1996) and seem to differ only in their affinity towards different Rab proteins (Anant et al., 1998; Seabra et al., 1995). REPs show a strict nucleotide requirement as they can only interact with Rabs in the inactive, GDP bound conformation (Seabra, 1996), thus falling into the category of general regulators of Rab function (for review on the biochemistry of Rab geranylgeranylation see (Seabra, 2000)).

REP1 is also known as Choroideremia protein, as its gene was found to be mutated in Choroideremia, an X-linked progressive retinal degeneration caused by complete absence of

REP1 (MacDonald et al., 1998; Seabra, 1996; Seabra et al., 1993; Seabra et al., 1995; Cremers et al., 1990; van den Hurk et al., 1997). Likewise, REP2 is also known as Choroideremia-like protein.

The gene MRS6 in *Saccharomyces cerevisiae*, identified as a suppressor of the respiratory deficient phenotype of the mrs-2 mutant (Ragnini et al., 1994) displayed homologies to REP-1/CHM and RabGDI (Waldherr et al., 1993), and was shown to be the functional homologue of REP-1 (Jiang and Ferro-Novick, 1994). MRS6 is an essential gene (Ragnini et al., 1994) and recent *in vivo* studies with a temperature-sensitive mutant of MRS6 reveals that reduction of MRS6 levels results in accumulation of unprenylated Rabs in the cytosol (Bialek-Wyrzykowska et al., 2000). This study, together with the observation that at least one Rab protein accumulates unprenylated in cytosol from patients suffering from CHM (Seabra et al., 1995), lends further support to the essential role of Rab Escort Proteins in the lipid modification of Rab proteins.

REPs are related to RabGDIs (Rab GDP Dissociation Inhibitors), proteins which form a highly conserved family that behave as negative regulators of Rab function, retrieving Rabs from cellular membranes into cytosolic complexes and maintaining them in an inactive conformation. RabGDIs also display the ability to re-insert Rab proteins into cellular membranes. This has led to the suggestion that RabGDI allows recycling of Rabs from an acceptor membrane to a donor membrane (Wu et al., 1998; Pfeffer et al., 1995). The solution structure of the alpha isoform of RabGDI showed a molecule composed of two distinct domains, termed domains I and II. The larger domain (domain I) consists of 20 β-sheets and 9 α-helixes. It shows structural similarity to FAD-dependent flavoproteins (Schalk et al., 1996) but the functional significance of this has yet to be elucidated. Domain II is smaller than domain I and is composed of five α-helixes.

REPs and RabGDIs sharing above 30% identity form a super-family of proteins believed to exhibit a conserved fold (Schalk et al., 1996; Wu et al., 1996). Sequence conservation between REPs and RabGDIs is restricted to regions termed SCRs (Sequence Conserved Regions) (Waldherr et al., 1993; Wu et al., 1996), in particular to regions SCR1A, SCR1B and SCR3B, which have been termed GDI-CHM consensus domain (GCD) (Schalk et al., 1996). Site directed mutageneses and structural studies have identified functionally important regions. One is domain II that is critical for Rab extraction from membranes, involving positions in both SCR2 and SCR3A (Luan et al., 2000; Gilbert and Burd, 2001), and the other is the region responsible for Rab binding, referred to as the "Rab Biding Platform", which involves positions in the SCR1A, SCR1B and SCR3 and localises in the apex of domain I (Alory and Balch, 2000; Luan et al., 2000; Schalk et al., 1996; Luan et al., 1999).

Although the biochemistry of Rab geranylgeranylation has been extensively characterised (Casey and Seabra, 1996; Seabra, 2000), comparatively little is known about the structural determinants of REP function. Bauer and co-workers (Bauer et al., 1996) performed the first mutational analysis of MRS6, showing that two mutations in the SCRs abolished Rab binding. Recent results suggest that MRS6 may also interact with Rab proteins through the conserved SCR1 and 3B, which form the "Rab binding platform" in RabGDI. Alory and Balch (Alory and Balch, 2000) observed that two mutations in SCR3B reduced the ability of MRS6 to bind Ypt1, as did the equivalent mutations in RabGDI. Since these regions are highly conserved between REPs and RabGDIs, and both groups of proteins are believed to have the same fold, it is possible that the apex of structural domain I represents a conserved Rab binding platform. Additionally, the same authors observed that two mutations in domain II affected prenylation of Ypt1 *in vitro*, although showing normal Rab binding properties. The authors interpret this as indicating that domain II may be involved in physical interactions with RGGT (Alory and Balch, 2000).

Here we analyse sequence conservation patterns in the REP family, attempting to relate and distinguishe members of the REP family and the RabGDI family, and discuss their functional significance.

III.2. Methods

Known REP sequences were retrieved from GenBank and used to identify novel elements of the super-family through Ψ-BLAST querying of GenBank (Altschul et al., 1997), pfam database (Sonnhammer et al., 1998; Sonnhammer et al., 1997), and of simple BLAST searches in GenBank using statistical models calculated from multiple sequence alignments. Sequences were aligned using the Clustal W 1.80 algorithm (thompson et al., 1994), and phylogenetic trees reconstructed with the methods of Maximum Parsimony and Neighbour-Joining (Saitou and Nei, 1987), with and without pairwise gap removal, scoring observed divergence, using program phylo_win (Galtier et al., 1996), which that can be found at pbil.univ-lyon1.fr/software/phylowin.html. Bootstrapping (Felsenstein, 1985) involved 1000 trials.

Identification of tree-determining residues was performed using Sequence Space analysis (Cassari et al., 1995), using the program SeqSpace, found at industry.ebi.ac.uk/SeqSpace/index.html, and multiple sequence alignment with hierarchical clustering (Livingston and Barton, 1993), using the AMAS program found at barton.ebi.ac.uk/new.software.html. The software package HMMER 2.0 (available at

hmmer.wustl.edu) was used to calculate profile Hidden Markov Models (pHMM) and generate model sequences.

Structures were retrieved from the PDB bank and manipulated using the program SwissPDBviewer (Guex and Peitsch, 1997) and modelling of protein structures used the Swiss-Model comparative modelling server (Guex and Peitsch, 1997; Peitsch, 1995; Peitsch, 1996), available at www.expasy.ch/swissmod/SWISS-MODEL.html.

III.3. Results and Discussion

III.3.1. The REP family

In order to identify all the available REP sequences, we searched the databases using a combination of methods based on profile Hidden Markov Models (Ψ-BLAST and the pfam database) using both known sequences and statistical models representing the multiple sequence alignments of the known sequences. Identification of novel sequences was followed by re-calculation of super-family multiple sequence alignment consensus. The Rab Escort Protein sequences found are listed in table III.1.

All the sequences were tested for high sequence identity/similarity to known members of the family, around the SCRs (sequence conserved regions), to confirm they were indeed members of the GDI-REP super-family.

In order to distinguish between REP and RabGDI sequences, we then compared each novel sequence to consensus calculated for both families (data not shown) and asked if proteins from this super-family would group according to similarity of function and/or in terms of similarity of species. We calculated distance diagrams based in the Clustal W 1.80 multiple sequence alignment of 11 candidate Rab Escort Protein sequences and 26 candidate RabGDI sequences from a variety of species. Figure III.1 shows the Neighbour-Joining distance diagram of the super-family clearly separating the two functional groups, a separation that was consistently observed when other algorithms are used to draw the distance diagrams (data not shown). Although there is little or no functional information for many of the molecules included in this analysis, the fact that there is consistent separation of two groups that include molecules of known distinct function, suggest that this separation reflects similarity of function as opposed to similarity of species. This further suggests that the positions that separate the two major groups are of functional and/or structural significance, and are conserved across evolution.

Table III.1 *– REP family members in different species.*

Species	Database name(s)	Accession number	Size (aa)
Schyzosaccharomyces pombe	Putative Rab geranylgeranyl-transferase escort protein (REP, GDP dissociation inhibitor)	CAA18894	459
Saccharomyces cerevisiae	MRS6	AAA34796	463
Candida albicans	REP	BAA36167	640
Arabidopsis thaliana	putative Rab Escort Protein	AAG51329	536
Oriza Sativa	ESTs(AU056916/5)	BAA89572	530
Drosophila Melanogaster	REP, REP homolog	AAD16891, AAF57544	511
Mus musculus	REP1/CHM	AF218084	661
Mus musculus	REP2/CHML	AF189156	621
Ratus Norvegicus	REP1/CHM/ component A of RGGT	AAA87626	650
Ratus Norvegicus	REP2	-	-
Homo sapiens	REP1/CHM/TCD protein/component A of RGGT	CAA55011	653
Homo sapiens	REP2/CHML/	CAA45979	656

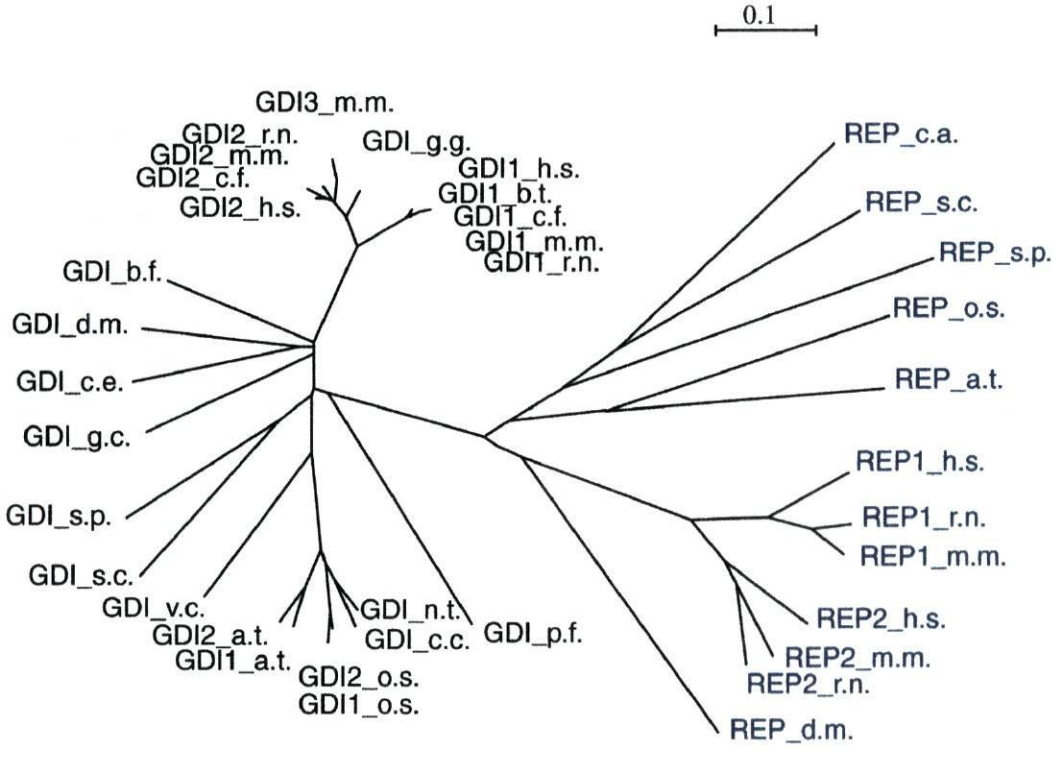


Figure III.1 *– Neighbour-Joining dendogram of the GDI/REP superfamily, highlighting the complete separation between different functions.*

Figure III.1 also highlights the high conservation we observe in the RabGDI family, with average identity/similarity between the family members of 62%/ 73%, and the lower conservation that the REP/MRS6 family presents, with average identity/similarity within the family of 31%/ 40%.

Within the Rab Escort Protein containing group we observe a clear hierarchy of species as can be seen in figure III.2. We recognise three major groups, corresponding to the three kingdoms fungi, plant and animals. Within the animal clade the mammalian isoforms form an independent branch of the tree that subdivides not according to species but according to isoform, in what seems to represent a phylogeny of function. This type of separation was also observed using distance based methods, as can be seen in figure III.1, and may represent subtle functional differences between the proteins in the different branches of the tree.

In order to distinguish between REP and RabGDI sequences, we then compared each novel sequence to consensus calculated for both families (data not shown) and asked if proteins from this super-family would group according to similarity of function and/or in terms of similarity of species. We calculated distance diagrams based in the Clustal W 1.80 multiple sequence alignment of 11 candidate Rab Escort Protein sequences and 26 candidate RabGDI sequences from a variety of species. Figure III.1 shows the Neighbour-Joining distance diagram of the super-family clearly separating the two functional groups, a separation that was consistently observed when other algorithms are used to draw the distance diagrams (data not shown). Although there is little or no functional information for many of the molecules included in this analysis, the fact that there is consistent separation of two groups that include molecules of known distinct function, suggest that this separation reflects similarity of function as opposed to similarity of species. This further suggests that the positions that separate the two major groups are of functional and/or structural significance, and are conserved across evolution.

Interestingly, we could not find any sequence in the latest release of *C. elegans* genome which could be considered a REP. One hypotheses to explain this is that RabGDI could perform both functions. However, the worm RabGDI sequence does not reveal any significant difference from other RabGDI sequences, which does not supports this hypotheses. More likely this is due to incomplete or bad quality sequencing which made it impossible to identify the REP gene.

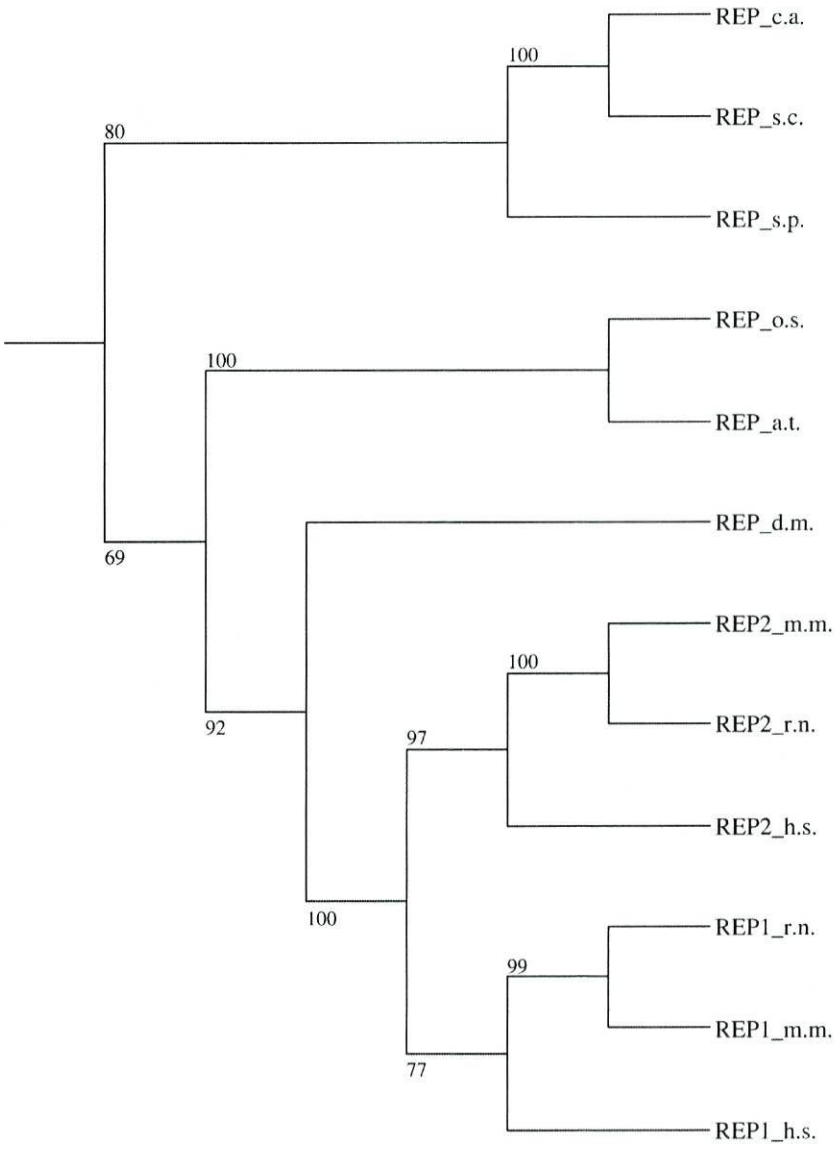


Figure III.2 *– Maximum parsimony tree of the REP family, rooted with yeast RabGDI (not shown). Bootstraping involved 100 trials*

III.3.2. Rab Binding: conserved property between REPs and RabGDIs

III.3.2.1. Reassessment of REP/RabGDI SCRs

REPs and RabGDIs share the ability to bind Rab proteins, and deliver them to cellular membranes (Soldati et al., 1993; Alexandrov et al., 1994; Wilson et al., 1996). REPs and RabGDIs display above 30% identity and are believed to have a conserved fold (Schalk et al., 1996; Wu et al., 1996). Sequence conservation was previously proposed to be restricted to regions termed SCRs (Sequence Conserved Regions) (Waldherr et al., 1993;

Wu et al., 1996), and in particular to the regions SCR1A, SCR1B and SCR3B, which have been termed GDI-CHM consensus domain (GCD) (Schalk et al., 1996) (figure III.3).

Figure III.3 – *Domain organisation of the REP and GDI families*

The solution structure of RabGDI revealed that GCD forms a conserved surface at the apex of domain I of RabGDI (see figure III.4) (Luan et al., 2000; Schalk et al., 1996). Restricted mutational analysis of RabGDI has identified residues important for Rab binding localising to this conserved surface (see figure III.5), which was termed "Rab binding platform" (Luan et al., 1999; Luan et al., 2000; Schalk et al., 1996).

As the definition of the SCRs was made using a limited set of sequences available at the time (Ragnini et al., 1994; Waldherr et al., 1993), and the increase in the number of available sequences has indicated that the REP family may be very divergent, we decided to asses the definition of the SCRs using the bigger data set presently available.

We tested the Clustal W 1.80 alignment of all the REP and RabGDI sequences represented in figure III.1 using hierarchical clustering analysis (Livingston and Barton, 1993), and compared statistical model sequences representing each of the families (Eddy, 1998) (figure III.6). We found that the majority of the conserved positions, concentrated in and around the previously identified SCRs that define the GCD. When we model the structures of REPs from each of the three major branches of the phylogenetic trees, and map these SCR regions to the structural models we observe a similar localisation (figure III.4), suggesting a conserved binding surface defining the interaction to Rab proteins, which would be defined by the exposed residues in the SCRs. This suggestion is supported by the fact that the MRS6 mutations E286S and R293A abolish binding to Rabs, the same effect caused by mutation of the the equivalent positions in RabGDIs (see figure III.5) (Alory and Balch, 2000). The suggestion that all Rabs will interact with general regulators in a conserved manner, through conserved positions clustering in and around the switch regions

(RabF motifs) (Pereira-Leal and Seabra, 2000) is also consistent with the existence of a conserved Rab binding platform in RabGDIs and REPS.

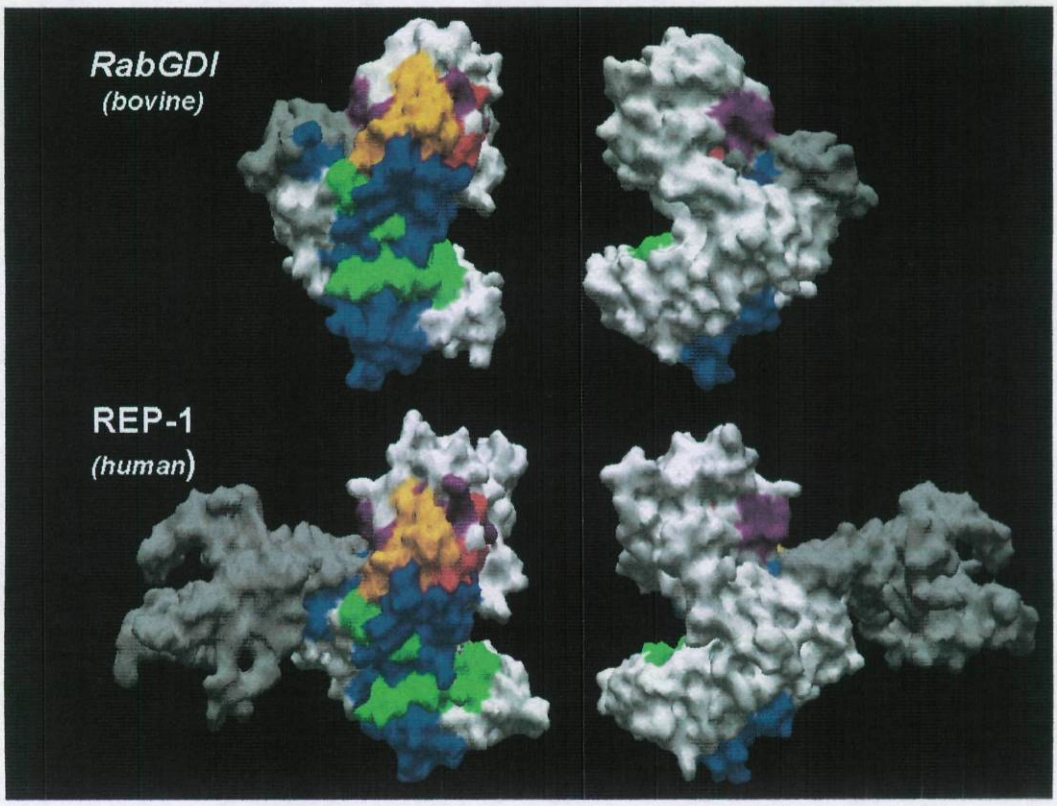


Figure III.4 *– Molecular surface of RabGDI (1GND) and modelled human REP-1. SCR regions coloured in red (SCR1A), purple (SCR1B), blue (SCR2), green (SCR3A) and orange (SCR3B). The residues corresponding to the sequence between SCR1B and SCR2 is coloured in dark grey. Images on the right of the image represent about 180° rotation of the figure on the left side of the image.*

The Rab binding platform in RabGDI was recently shown to display a high degree of flexibility around some positions in SCR3A (Luan et al., 2000). This is consistent with the hypothesis that common regions in Rabs are recognised by a structural theme present in RabGDIs and REPs, and that the flexibility of this regions allows RabGDIs and REPs to accommodate for the variability that is found in the Rab proteins.

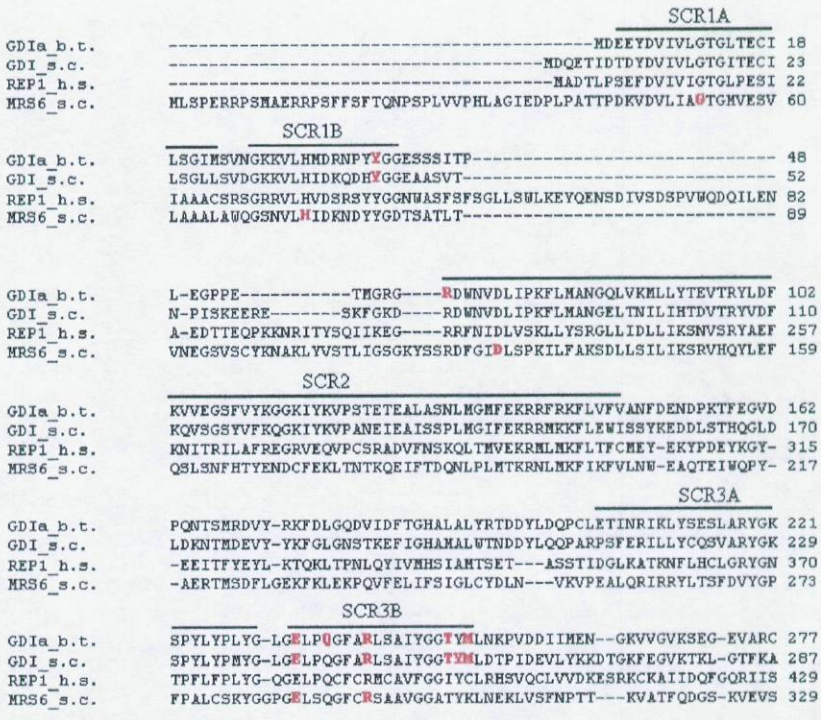


Figure III.5- *Alignment of selected residues of some species of REPs and GDIs. Sequence conserved regions (SCRs) are indicated above the alignment and positions that have been shown to be involved in REP binding are highlighted in red.*

III.3.2.2. Role of REP C-terminus in Rab binding

It has been argued that in REPs, the SCR regions are not sufficient for Rab binding, but REP-specific C-terminal sequences are also required (Bauer et al., 1996). This was based on the fact that deletion of C-terminal portions MRS6 failed to support growth of yeast, antibodies against MRS6 were not able to immunoprecipitate Ypt1 in strains expressing this deletion mutant and that by yeast two hybrid binding assays no binding was detected using just the first 322 a.a. of MRS6, but could be detected if the first 570 a.a. were used. However, inspection of the modelled structure of MRS6 indicates that it is unlikely that the mutant proteins would fold properly, as the two expected domains of MRS6 do not correlate in a linear from with the sequence. The authors did not address this issue. Although they show that in smaller C-terminal deletions the truncated protein is produced, the fact that point mutations in human REP-1, found in patients suffering from CHM result in complete absence of detectable protein in samples from patients (MacDonald et al., 1998), and that the deletion of the C-terminal portions of MRS6 places all the protein in the 100,000xg pellet (Miaczynska et al., 1997) suggests that the C-terminus plays an important role in the folding stability of REP proteins.

III.3.3. Where do isoprenoids bind in REP and RabGDI?

Binding of Rab proteins implies, both for REPs and RabGDIs, the ability to interact with the prenyl chains of the lipid modified Rab protein, and to shield them from the aqueous environment of the cytosol. Both molecules must contain a hydrophobic region that can accommodate two (or in some cases just one) 20 carbon geranylgeranyl isoprenoids. The nature of this hydrophobic regions has remained illusive.

Inspection of the structure of RabGDI and of structural models of different REP proteins reveals no evident hydrophobic patch. This suggests that binding of prenylated Rabs will involve conformational changes in REPs and RabGDIs. Supporting the ability of REP to undergo structural rearrangements are the different affinities of free REP and REP complexed with Rab to RGGT, which have been interpreted as resulting from different REP conformations (Anant et al., 1998). Different affinities for RGGT have also been reported for the prenylated and unprenylated REP:Rab complex, but it is not clear if this can also reflect changes in the REP:Rab complexes, or only in conformational changes in RGGT caused by binding to GGPP (Anant et al., 1998; Alexandrov et al., 1998; Thoma et al., 2000). Any structural rearrangement however will have to accommodate for a variety of C-terminus lengths, i.e., distance between the common Rab fold that binds the Rab binding platform, and the lipid modifiable/modified cysteines.

III.3.4. Prenylation of Rab proteins: unique REP property

Unlike RabGDI, REP may bind unprenylated Rab proteins and mediate their prenylation by RGGT. Sequence or structural motifs that are unique of REP proteins are then likely to determine any of these REP specific properties.

III.3.4.1. REP specific regions

To identify REP specific sequences we combined three different approaches: sequence space analysis, statistical models based on profile Hidden Markov Models and hierarchical conservation analysis. The results of this analysis are summarized in figure III.6, an alignment of single majority rule consensus calculated for each family, highlighting the positions that are specifically conserved in each family.

Bauer and collaborators (Bauer et al., 1996) observed three conserved regions between human REP1 and REP2 and yeast REP (MRS6). Our analysis that considers more REP sequences and different methods failed to identify such conservation in the REP family,

as can be see in figure III.7. Instead we were surprised to observe that very few REP specific positions could be detected. Most of these positions tended to cluster in the SCR regions, which was another surprise since we expected that unique REP functions would be determined by positions in regions that were not conserved in RabGDI.

In evolutionary terms this may reflect the existence of few "functional regions" in REPs and RabGDIs that the different families evolved and use to mediate interactions with different binding partners or to accommodate for family specific functions or functional variations.

One example of a family specific functional variation may be Rab binding. In both families binding to Rabs is mediated by the RabGDI/CHM conserved (GCD) domain, however In these regions there are several positions that are differentially conserved between the two functional families (see figure III.6). These differences could underlie the fact that although RabGDI can only bind prenylated Rabs, while REP also has to bind unprenylated Rabs, and it has to accommodate the addition of the two lipid groups during the prenylation reaction. This possibility could be easily tested *in vitro* by replacing the RabGDI GCD with that of REP and test the binding of non-prenylated Rabs to the mutant RabGDI. A non-exclusive alternative to explain the differential conservation in the GCD is the requirement for regulation of the prenylation machinery by other molecules that could interact with or even modify some of these consensus positions.

One example of a "functional domain" that has different functions in RabGDIs and REPs is Domain II. This domain has been proposed to be involved in RabGDI recycling of Rabs and in REP binding to the RGGT.

In RabGDI, mutations in this region result in increased levels of Rabs associated with membranes and reduced cytosolic pool, while another set of mutations results in the accumulation of a sub-set of Rab proteins in the cytosol, suggesting that Domain II mediates Rab membrane delivery and retrieval (Gilbert and Burd, 2001). However the precise nature of these mutations was not identified by the authors. In another study, combined mutations in SCR3A (also in domain II) and the Rab binding platform (R226A/R248A and Y227A/R248A) impair the ability of RabGDI to retrieve Rabs from membranes (Luan et al., 2000). Together, this results suggest that domain II plays a role in recycling Rabs from and to cellular membranes, possibly via interactions with the previously postulated Rab Recycling Factor (RRF) (Luan et al., 1999). SCR3A (represented in green in figure III.4) includes a flexible domain that according to Luan and co-workers interacts with the putative RRF (Luan et al., 2000).

SCR3A is one of the least conserved SCRs and contains at least 8 REP specific positions (see figure III.7). REPs have been shown to deliver and extract Rabs to and from cellular membranes after prenylation (Alexandrov et al., 1994; Wilson et al., 1996; Pfeffer et

al., 1995), and to associate with cellular membranes *in vitro* and *in vivo* (Miaczynska et al., 1997), Hume and Seabra, *personal communication*). Mutations in MRS6 putative domain II (R195, K199D, T185D, L226Y, D269A, V270R, Y271F) have no effect on yeast growth, except for R195A (Alory and Balch, 2000). However, the mutations R195A and L226Y severely affect *in vitro* prenylation of Rab proteins, without affecting Rab binding. This observation may suggest that positions in the putative domain II of REP mediate interactions with RGGT. It is interesting to note that none of these two positions correspond to REP specific positions: R195 is highly conserved in the super-family, and L226 is not a conserved position.

However parallels between yeast growth and Rab prenylation may not be appropriate. Although REP function is essential in yeast (Ragnini et al., 1994), different studies point to prenylation of Rab proteins being only transiently required to maintain cellular viability, and that only a small proportion of Rab protein needs to be, at any time, prenylated (Bialek-Wyrzykowska et al., 2000; Alory and Balch, 2000).

Even though the results of Allory and Balch (Alory and Balch, 2000) concerning the interaction of MRS6 with RGGT are inconclusive, it is still possible that domain II, where both R195 and L226 localise, may mediate interactions with RGGT, but further mutational analysis should be designed based on analysis of patterns of sequence conservation within each family.

III.3.4.2. REP-specific insert region

The most obvious difference between REPs and RabGDIs is the expansion of the intervening sequence between SCR1B and SCR2, (figure III.3). The length of this sequence varies between 10 and 140 a.a. Their sequences are not conserved and no obvious putative function can be assigned just based on homology as querying of databases reveals no meaningful homology (data not shown).

When we model the structures of different REP proteins based on the known structure of α-RabGDI (see panel A of figure III.8), the REP specific insertion is always placed between the equivalents of domains I and II of RabGDI, either as secondary structure elements or as what seems to be an independent folding unit in mammalian REPs. The fact that no significant homologies are detected between the mammalian REP insert and other sequences in the databases may mean that it represents a novel fold.

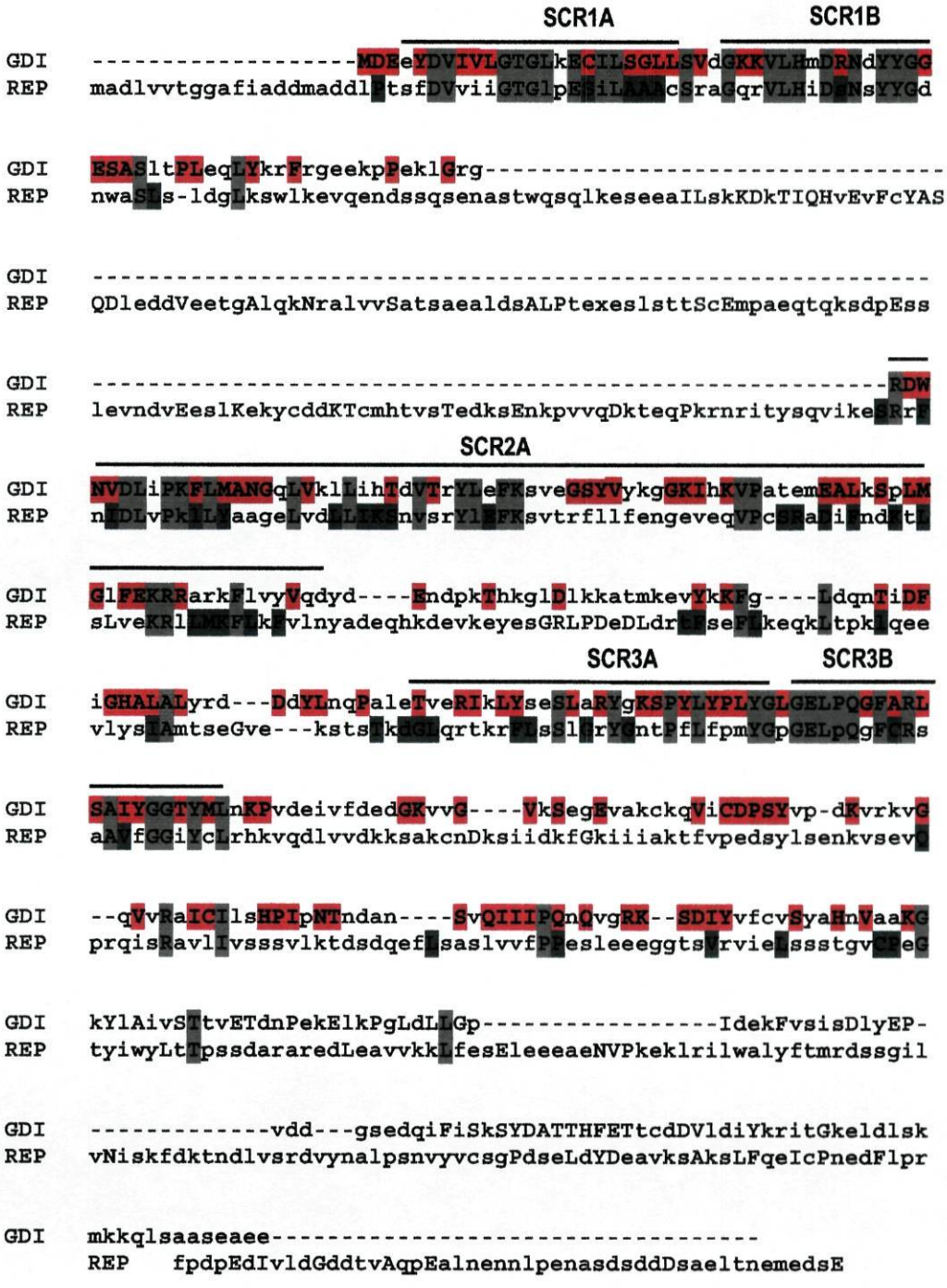


Figure III.6 *– Alignment of model sequences emited from pHMM statistical models calculated for the REP and RabGDI family. Positions that are common to both families are highlighted in grey. Positions that are family specific are highlighted in red (RabGDI family) and green (REP family). Uppercase positions are emitted at p>0.5.*

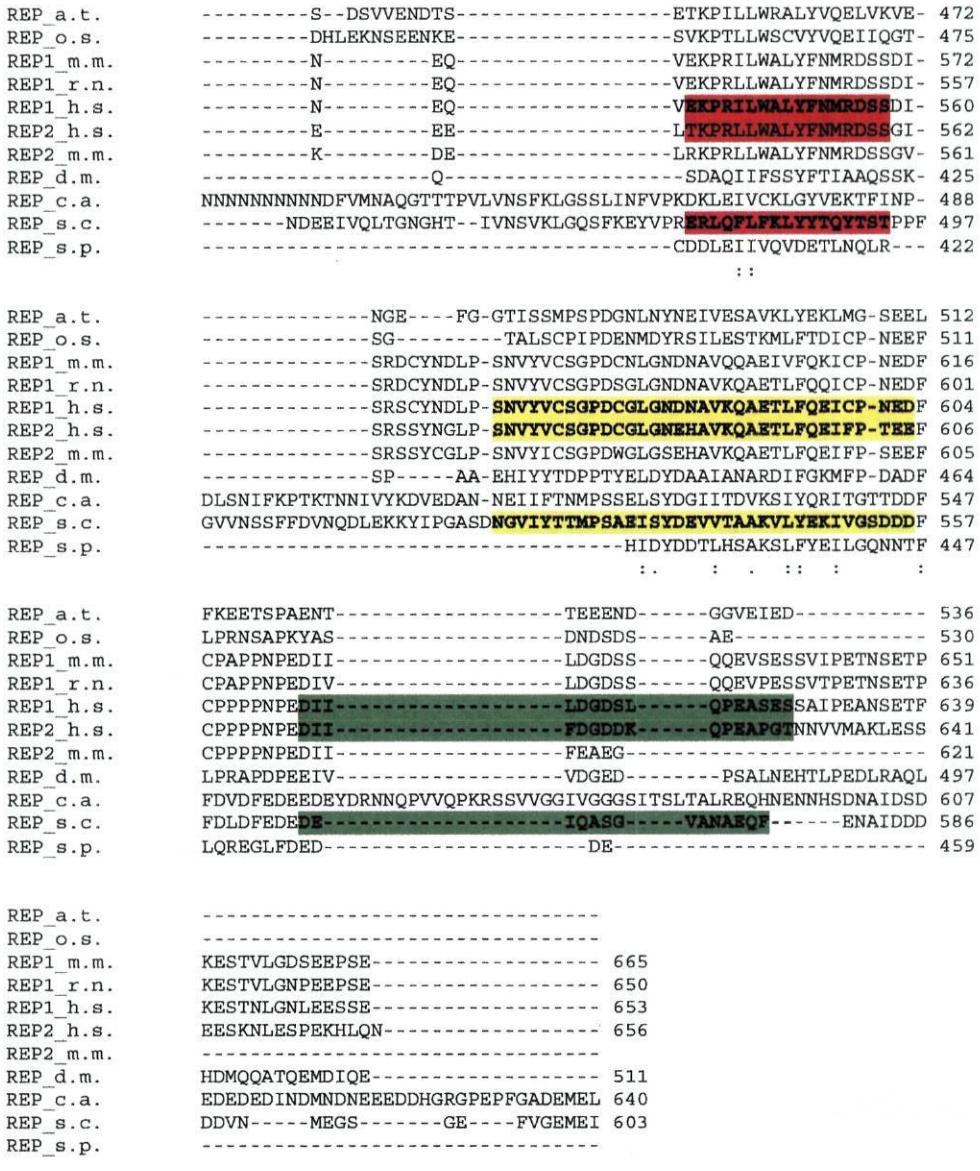


Figure III.7 *– Alignment of the C-terminal portion of the REP family. Proposed RCR are highlighted in colour, for the sequences they were defined for, in bold. With eleven sequences no significant conservation is detected in this regions*

The REP-specific insert region, representing a unique REP element, is a natural candidate for RGGT binding. We looked into the sequences of RGGTs in different organisms to identify any feature that could be correlated with the variation in size of the REP insert region.

We observed before that mammalian RabGGTase alpha sub-unit (αRGGT) exhibit two insertions relative to the yeast counterparts, corresponding to two structural domains: an IgG like domain, and a LRR domain (Zhang et al., 2000). As MRS6 shows the smaller REP insertion, we wondered if the size o the REP-specific insertion did correlate with the

appearance of the RGGT extra domains. Sequence comparison of RGGT α subunits revealed that the increase in size of the REP insertion observed in plants and mammals relatively to fungi REPs, parallels the appearance of insertions in alignment of the sequences of different RGGTα, which correspond to the structural motifs found in mammalian RGGTα (data not shown).

Modelling of the structures of RGGTα in selected species places these insertions as variations of the LRR and IgG-like domains (figure III.8, panels B). It is tempting to speculate either an interaction between the two insertion domains or a regulatory mechanism that requires the presence of the extra domains/ secondary structure motifs in both proteins. One way to test this hypothesis would be through cross-species complementation experiments with different REPs and RGGTα.

III.4. Conclusion

We analysed patterns of sequence conservation in the REP family of essential activators of the prenylation of Rab proteins, for a data set of eleven proteins all eukaryotic kingdoms: fungi, plants and animals. Previous analysis of this family of proteins had relied in a small data sets (n=3), comprising only human and *S. cerevisiae* proteins. The REP family is less conserved than the RabGDI family, and a separation into three sub-families, corresponding to the three major clades is suggested by our phenetic analysis, but no functional data is available that can support this claim. Structural diversity in the REP family is further illustrated by the variable size of the REP specific insertion between SCR1B and SCR2.

We observed what appears to be a correlation between the size increase of the REP-specific insert and the appearance of extra domains in RGGT, which we speculate may signify a physical interaction between insert domains of REP and RGGT. Interestingly, the size of this REP-specific insert does not correlate with the tree of life.

We found that even with an increased data set, the sequence conserved regions (SCRs) previously defined for the REP/RabGDI super-family still are the major conserved regions between the two proteins, particularly the regions SCR1A, SCR1B and SCR3B that define the RabGDI/Choroideremia consensus domain (GCD). These regions when mapped to the crystal structure of RabGDI or of modelled structures of REP-1 (see figure III.4) form a conserved surface on the apex of domain I. Mutational analysis both in RabGDIs and MRS6 indicates these regions may be involved in Rab binding. The fact that the GCD defines the

Rab binding surface is suggestive of a very conserved mechanism of Rab recognition between members of the two families, and across species

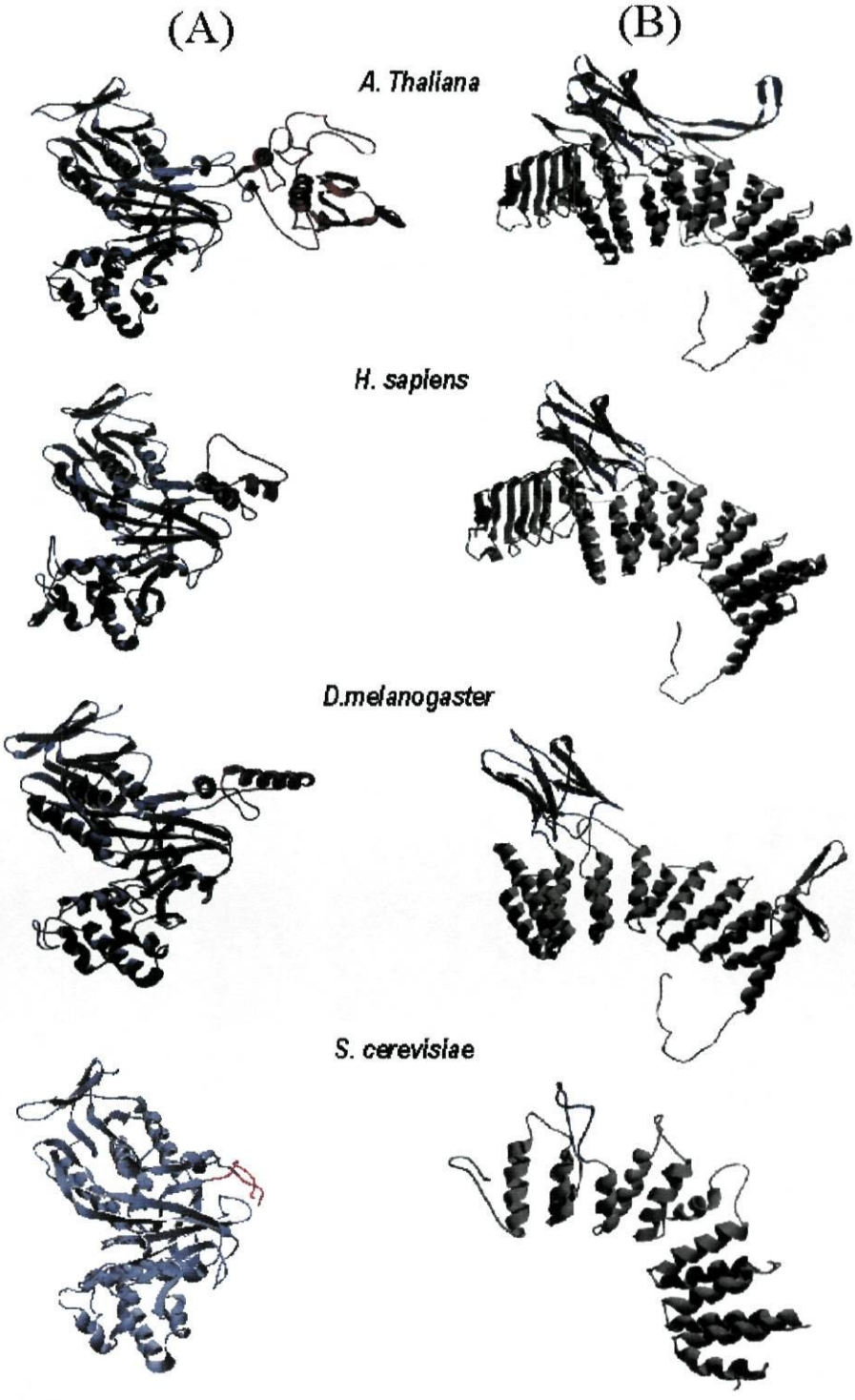
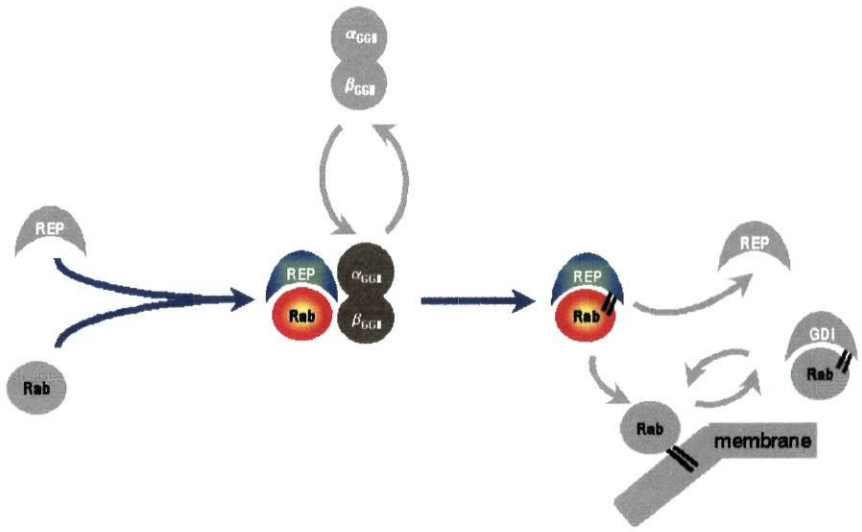


Figure III.8 *- (A) modelled structures of the modelled structures of REPs from A. thaliana, H. sapiens, D. melanogaster and S. cerevisiae. The insert domain in REP-1 is highlighted in red (B) modelled structures of αRGGT of the same species except for the human protein where it represent a crystal structure (accession number 1QBQ). The LRR and IgG-like domain are highlighted in green and blue respectively*

When we looked for specific structural determinants of REP function, by looking for REP specific sequences, we didn't observe any REP specific motifs, and actually refute the previous definition of their existence by others, but instead observe that specific REP positions cluster inside the SCRs, in between super-family conserved positions. In the absence of mutational analysis studies we can only speculate that these REP-specific positions underlie REP-specific functions, and that these functions may have arisen by divergence from a common ancestor that already had these defined functional domains.

Further structural and biochemical studies of REP proteins are still required to understand the structural basis of REP function.

Part IV – REP:Rab



ICBAS

IV.1. Analysis and preparation of stable complexes between Rab GTPases, Rab Escort Protein and Rab Geranylgeranyl Transferase

Reproduced from: José B. Pereira-Leal, Anita R. Gomes and Miguel C. Seabra (2001) *Methods in Molecular biology,* in press

IV.1.1. Introduction

Rab proteins are small Ras-like GTPases that regulate vesicular trafficking events in the cell. More than 50 Rabs have been described in mammalian cells (Pereira-Leal and Seabra, 2000) each with a specific sub-cellular localisation, reflecting the functional specificity of Rabs to specific trafficking steps (Lazar et al., 1997; Novick and Zerial, 1997; Schimmoller et al., 1998; Chavrier and Goud, 1999). Rabs contain two cysteine residues at or near the carboxyl terminus, arranged in a variety of motifs. Both cysteine residues are modified by the attachment of geranylgeranyl groups via thioether bonds, in a reaction catalysed by Rab Geranylgeranyl Transferase (also known as GGTase type II, RGGT) (Seabra, 2001). This enzyme is a tightly bound heterodimer, composed by 60 kDa α-subunit and a 38 kDa β-subunit, both related to the α- and β-subunits of the other known protein prenyltransferases, Farnesyl Transferase (FTase) and Caax Geranylgeranyl Transferase (also known as GGTase type I).

RGGT is unique among prenyl transferases. It cannot catalyse prenylation of Rabs on its own, but requires the presence of an additional component designated Rab Escort Protein (REP). REP binds unprenylated Rabs and the complex REP:Rab is the true substrate for the enzyme. Unlike what happens with other prenyl transferases, substrate recognition is not mediated by the prenylation motif of the substrate. RGGT binds the REP:Rab complex and then catalyses the covalent addition of two geranylgeranyl groups to the carboxyl terminal cysteines of the Rab, in a sequential process that is unique for RGGT. After prenylation diGG-Rab remains associated with REP (Andres et al., 1993), presumably because the addition of two GG groups renders the protein too hydrophobic to exist in aqueous solution. DiGG-Rab can then be directly delivered to their target membranes by REP (Alexandrov et

al., 1994). REP has the dual function of acting as an essential activator of the prenylation reaction and escorting newly prenylated Rabs to the membranes.

This chapter describes *in vitro* methods to form and purify prenylated and unprenylated complexes of Rab and REP, and to form and purify complexes of prenylated Rab, REP and RGGT. The methods presented in this chapter were optimised for REP1 and Rab1a, but they should be easily adaptable to other Rabs and REPs. These complexes are used in our lab to study the structural and biochemical factors that determine the assembly of both REP:Rab and the catalytic complexes, and to analyse membrane delivery of Rab proteins.

IV.1.2. Materials

IV.1.2.1. Geranylgeranyl Pyrophosphate

Tritium-labelled and unlabelled geranylgeranyl pyrophosphate are comercially available from several sources and are always stored at -20^{o}C. We typically use:

-unlabelled all-*trans*-GGPP (Sigma, G-6025).

-[1-^{3}H] all-*trans*-GGPP, 15-30 Ci/mmol (DuPont-New England Nuclear, NET-1052).

IV.1.2.2. Analytical scale preparation and purification of REP:Rab complexes

Stock solutions: 1 M sodium HEPES pH 7.2, 1 M $MgCl_2$, 1 M DTT, recombinant 6xHisREP1 and recombinant 6xHisRab1a.

SMART System Gel Filtration buffer (GF buffer): 50 mM sodium HEPES pH 7.2, 1 mM DTT.

SMART System chromatography system (Amersham Pharmacia) and gel filtration column Superdex 200 3.2/30 (Amersham Pharmacia).

IV.1.2.3. Analytical scale preparation and purification of REP:Rab-GG complex

Stock solutions: 1 M sodium HEPES pH 7.2, 1 M $MgCl_2$, 1 M DTT, 175 mM (10%) NP-40 protein grade (Calbiochem, 492017), 1 M TrisCl pH 8, 4 M NaCl, recombinant 6xHisREP1, recombinant 6xHisRab1a, recombinant RGGT.

SMART System monoQ buffer A: 50 mM TrisCl pH 8.0, 1 mM DTT.

SMART System monoQ buffer B: 50 mM TrisCl pH 8.0, 1 mM DTT, 1 M NaCl.

SMART System chromatographic system (Amersham Pharmacia) and ion exchange column monoQ PC 1.6/5 (Amersham Pharmacia).

IV.1.2.4. Analytical scale preparation and purification of REP:RabGG:RGGT complex

1. Stock solutions: 1 M sodium HEPES pH 7.2, 1 M $MgCl_2$, 1 M DTT, 175mM (10%) NP-40 protein grade (Calbiochem, 492017), 1 M TrisCl pH 8, 4 M NaCl, recombinant 6xHisREP1, recombinant 6xHisRab1aCS (mutant Rab1a with the C-terminal cysteine residue mutated to a serine), recombinant RGGT.
2. SMART System monoQ buffer A: 50 mM TrisCl pH 8.0, 1 mM DTT.
3. SMART System monoQ buffer B: 50 mM TrisCl pH 8.0, 1 mM DTT, 1 M NaCl.
4. SMART System chromatography system (Amersham Pharmacia) and ion exchange column monoQ PC 1.6/5 (Amersham Pharmacia)

IV.1.2.5. Analytical scale preparation and purification of REP:Rab[^{3}H]GG complex

1. Stock solutions: 1 M sodium HEPES pH 7.2, 1 M $MgCl_2$, 1 M DTT, 175 mM (10%) NP-40 protein grade (Calbiochem, 492017), recombinant 6xHisREP1, recombinant 6xhisRab1a, recombinant RGGT.
2. G75 Sephadex Gel Filtration buffer (G75): 50 mM sodium HEPES pH 7.2, 50 mM $MgCl_2$, 1 mM DTT, 0.05 mM NP40, 100 mM NaCl.
3. Disposable 2 ml Bio-Rad columns, 1 ml G75 Sephadex matrix (Amersham Pharmacia, 17-0051-01).
4. India His-Probe-HRP (Pierce, 15165ZZ).

5. 1.2 µm glass fibre filters (Whatman, 1822025).

IV.1.2.6. Large scale preparation and purification of REP:RabGG:RGGT complex

1. Stock solutions: 1 M sodium HEPES pH 7.2, 1 M $MgCl_2$, 1 M DTT, 175 mM (10%) NP-40 protein grade (Calbiochem, 492017), 1 M TrisCl pH 8, 4 M NaCl, recombinant 6xHisREP1, recombinant 6xHisRab1aCS, recombinant RGGT.
2. FPLC monoQ buffer A: 50 mM TrisCl pH 8.5, 1 mM DTT.
3. FPLC monoQ buffer B: 50 mM TrisCl pH 8.5, 1 mM DTT, 1 M NaCl.
4. FPLC chromatography system (Amersham Pharmacia) and ion exchange column monoQ HR 5/5 (Amersham Pharmacia)

IV.1.3. Methods

IV.1.3.1. Expression and purification of recombinant proteins

We have previously described in detail the protocols used in our lab to express and purify recombinant Rabs, REPs and RGGT (Armstrong et al., 1995; Seabra and James, 1998). Rab1a is expressed as an N-terminal six histidine-tagged fusion protein in *E. Coli* and purified by affinity chromatography using a Ni^{2+}-sepharose resin. RGGT is expressed in Sf9 insect cells after co-infection with baculovirus coding for both subunits, purified by cation exchange chromatography followed by gel filtration chromatography (*see* **Note 1**). REP is also expressed in Sf9 insect cells, produced as a C-terminal six histidine-tagged fusion protein and purified by Ni^{2+} affinity chromatography.

IV.1.3.2. Analytical scale preparation and purification of REP:Rab complexes

REP and Rab form stable complexes held together mainly by electrostactic interactions (Anant et al., 1998; Alexandrov et al., 1998). An important consideration is therefore the concentration of salt used in the preparation of the complex and the methodology to be used to purify the complex away from the free recombinant proteins. We developed a method based on gel filtration chromatography. REP migrates upon gel filtration with an apparent molecular weight of 150 kDa, both in the

free and complexed forms. As such, it is advisable to always use limiting REP concentrations to avoid contamination of the complex with free REP. The yield from this method is not great. This is a simple way to address the binding of the two molecules in a semi-quantitative way but it is unlikely to be useful for preparative purposes.

1. Mix, in a final volume of 50 µl, 50 mM sodium HEPES pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 4 µM Rab1a, 2 µM REP-1.
2. Incubate for 15 min at 37 ^{o}C.
3. Load the reaction mixture onto a Superdex 200 3.2/30 column (Amersham Pharmacia) pre-equilibrated with GF buffer and run at a flow rate of 50 µl/min. Fractions of 100 µl are collected between 0.75 and 1.95 ml. Elution of proteins from the column is monitored by absorbance at 280 nm. The complex elutes at around 1.6 ml corresponding to a predicted Mw of 150 Kda.
4. Run an aliquot of each fraction on SDS-gel electrophoresis and visualise proteins by silver (or Coomassie) staining. In the presence of REP1, Rab1a co-elutes with REP, indicating the formation of the complex.

IV.1.3.3. Analytical scale preparation and purification of REP:RabGG complex

After prenylation, the resulting REP:RabGG complex is more stable than the non-prenylated complex (Shen and Seabra, 1996; Anant et al., 1998). The REP:RabGG interaction involves both ionic as in the REP:Rab complex and hydrophobic interactions probably due to interactions between the prenyl groups and hydrophobic amino acids in REP.

The first step in the formation of the REP:RabGG complex is *in vitro* prenylation. Rab geranylgeranylation occurs upon incubation of REP and Rab with geranylgeranyl pyrophosphate (GGPP) and RGGT. Detailed protocols for *in vitro* geranylgeranylation of Rabs were published in a previous book in this series (Seabra and James, 1998) (see **Note 2**). After the reaction, the mixture is separated by ion exchange chromatography and Rab1a co-elutes with REP1 indicating the formation of the prenylated complex. This separation procedure has the advantage of not only separating the REP:RabGG complex from the uncomplexed proteins, but also to separate the complex from any non-prenylated REP:Rab complex as the presence of salt in the elution buffer promotes the dissociation of the latter complex. REP elution does not change significantly between free and complexed forms so the use of

limiting concentrations of REP in the reaction mixture as suggested above is recommended. Also, one should use optimal conditions for the *in vitro* prenylation reaction to maximise the amount of RabGG obtained. The majority of the reaction product is REP:RabGG complex but some ternary complex REP:RabGG:RGGT is produced. However, the ternary complex elutes differentially and is therefore easily separated away (Fig. 1).

1. Mix the following components on ice, in a final volume of 50 µl: 50 mM sodium HEPES pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 0.05 mM NP-40, 20 µM Rab1a, 5 µM REP1, 5 µM RGGT, 50 µM GGPP.
2. Incubate at 30^{o}C for 1 hour.
3. Load the reaction mixture on a 0.1 ml monoQ column pre-equilibrated in monoQ (SMART system) buffer A.
4. Wash the column with 0.3 ml of monoQ (SMART system) buffer A, follow with a 4 ml linear gradient from 0%-35% monoQ (SMART system) buffer B and then a 0.7 ml linear gradient from 35%-100%, all at a flow rate of 0.3 ml/min collecting 0.2 ml fractions.
5. Run an aliquot of each fraction on a 12% SDS-PAGE gel and visualise proteins by silver (or Coomassie) staining. Free Rab elutes in the flow through and a small fraction binds weakly to the column and is eluted at very low salt concentrations (fractions 7 and 8). The dimeric complex REP:RabGG elutes between fractions 17 and 20 with RGGT eluting in fractions 21 and 22 (Fig IV.1.1).

IV.1.3.4. Analytical scale preparation and purification of REP:RabGG:RGGT complex

As discussed above, the ternary REP:RabGG:RGGT complex is not stable and is therefore difficult to purify. We have shown previously that a mutant Rab1a, Rab1aCS (C-terminal cysteine residue mutated to a serine residue) that can only undergo single geranylgeranylation forms a much tighter complex with REP than wild-type doubly geranylgeranylated Rab1a, Rab1aCC (Shen & Seabra, 1996). By using this mutant as the substrate of the prenylation reaction, we favour the formation of the heterotrimeric complex. Apparently, RGGT has higher affinity for the REP:Rab-monoGG complex than to the REP:Rab non-modified complex and the equilibrium is shifted to the formation of ternary complex (unpublished observations). The procedure is identical to the one described above. When Rab1aCS is used, we

observed that REP and Rab co-elute with RGGT in fractions 21 and 22, with some dimeric complex eluting, as before, between fractions 17 and 20.

Iv.1.3.5. Analytical scale Preparation and Purification of REP:Rab[^{3}H]GG complexes

REP:Rab[^{3}H]GG complexes can be formed by modifying Rab *in vitro* in the presence of radiolabelled tritiated GGPP. Radiolabelling allows to monitor the formation of the reaction product and to follow the prenylated Rab in subsequent studies. The prenylated REP:Rab[^{3}H]GG is purified by manual gel filtration as it is undesirable to contaminate the chromatography systems with radioactivity.

In order to ensure that all prenylated Rabs are bound to REP, the same principles apply. We perform *in vitro* reactions by incubating excess Rab with RGGT and GGPP under limiting REP conditions. The free Rab that is not prenylated can then be easily separated from the REP:Rab[^{3}H]GG complex by gel filtration. It would also be possible to use limiting amounts of Rab and excess REP to insure that all Rabs would be prenylated and bound to REP. There are two main reasons why this is not a good idea. First, it has been shown that excess REP inhibits the geranylgeranylation reaction, probably as a result of the formation of non-productive REP:RGGT complexes (Anant et al., 1998). Secondly, the separation of free REP from the prenylated Rab:REP complex would be impossible by gel filtration as both free REP and REP:RabGG complex elute with the same apparent molecular weight on gel filtration chromatography.

1. Mix, on ice, in a final volume of 50 µl the following reagents: 50 mM sodium HEPES pH 7.2, 5 mM $MgCl_2$, 1mM DTT, 0.05 mM NP-40, 10 µM Rab, 2.5 µM REP1, 0.7 µM Rab GGTase, 20 µM GGPP (0.2 µM [^{3}H]GGPP mixed with 19.8 µM unlabelled GGPP).
2. Incubate at 37°C for 45 minutes.
3. At 4^{0}C, load the reaction mixture in a G75 Sephadex column (1ml) previously equilibrated in G75 buffer. Run the column by adding 20 x 50 µl of G75 buffer and collecting the respective 20 x 50 µl fractions (see **Note 3**).
4. Quantify each fraction as described previously (Seabra & James, 1998). Briefly, precipitate the proteins in a mixture of EtOH/HCl (10:1), filter the precipitable radioactivity on 1.2 µm glass fiber filters, add 5 ml of scintillation fluid and count in

the scintillation counter.

5. Run aliquots from each fraction on 12% SDS-gel electrophoresis. Visualise the proteins by silver staining on one gel. Use a replicate gel for western blotting with the India-His probe. This probe will allow visualisation of histidine-tagged Rab and REP proteins. If necessary, use a third gel for autoradiography to visualise Rab[^{3}H]GG (see **Note 4**).
6. REP:Rab[^{3}H]GG complex elutes in fractions 8-10 away from the free Rab that elutes in fractions 12-15, but co-eluting with RGGT. These fractions are pooled and used in subsequent *in vitro* studies (see **Notes 5** and **6**).

Figure IV.1.1 *– Separation of the REP:RabGG and REP:RabGG:RabGGTase complexes by ion exchange chromatography. Silver stained SDS-PAGE (4-15%)*

IV.1.3.6. Large scale preparation and purification of REP:RabGG:RGGT complexes

This method is essentially a scale-up of procedures 3.3 and 3.4. As discussed above, both ternary and dimeric complex are produced. The choice of wild-type Rab1a versus Rab1aCS will determine the enrichment in either the ternary or binary complex. If wild type Rab is used (RabCC), then the formation of the dimeric complex is favoured. If on the other hand, a mutant Rab1a with only one prenylatable cysteine (Rab1aCS) is used, then the heterotrimeric complex is favoured. We describe here the conditions to form the ternary complex REP:RabGG:RGGT.

1. Mix, on ice, in a final volume of 500 µl the following reagents: 50 mM sodium HEPES pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 0.05 mM NP-40, 20 µM Rab, 5 µM REP1, 5 µM RGGT, 50 µM GGPP.
2. Incubate at 30^{o}C for 1 hour
3. Load reaction mixture in a monoQ column previously equilibrated in monoQ (FPLC) buffer A

4. Wash the column with 3 ml of monoQ (FPLC) buffer A, then run a 20 ml linear gradient from 0%-30% of monoQ (FPLC) buffer B at a flow rate of 1 ml/min, collecting fractions of 1.5 ml until 30 ml.
5. Run an aliquot of each fraction on a 12% SDS-PAGE gel, and visualise by silver (or Coomassie) staining. Ternary complex typically elutes around 24 ml under these conditions and no further purification is usually needed (the complex is stable enough to allow a further purification/concentration step if required for specific applications) (see **Note 7**).

IV.1.4. Notes

1. Others have successfully expressed geranylgeranyl transferase in bacteria (K Alexandrov, personal communication). It is potentially a simpler way of expressing RGGT but a direct comparison of the properties of both recombinant enzymes is not available yet.
2. It is advisable, when working with Rab proteins, to use the utmost care in always maintaining the proteins on ice and avoiding freeze-thawing cycles. Rab proteins will undergo partial proteolysis in the carboxyl terminus and generate a truncated protein without the prenylatable cysteines. These truncated proteins will effectively act as a competitor to the binding of REP in the prenylation reaction.
3. The equilibration of the column and subsequent purification steps should be performed at 4^0C to avoid aggregation of the complex.
4. Blotting with India His-Probe allows a direct comparison between REP and Rab to determine whether fractions have equimolar amounts of both proteins.
5. The reaction can be scaled up to the amount of product desired, but the separation should always be performed as mentioned (50 µl of reaction mixture per 1 ml-G75 column).
6. The complexes should be stored at 4^0C with 1mg/ml of BSA as a carrier protein. They are stable for 5 days under these conditions.
7. The heterotrimeric complex can be kept froozen at –80 °C, although repeated freezing and thawing is not advisable.

IV.2 - Requirement for GTP hydrolysis prior to REP:Rab complex formation

IV.2.1 - Introduction

Rab proteins require lipid modification for membrane association and function (Seabra, 2000; Seabra, 1998). This lipid modification (prenylation) is accomplished by the enzyme Rab GeranylGeranyl Transferase (RGGT) which requires an accessory component for proper activity, Rab Escort Protein (REP) (Seabra et al., 1992; Armstrong et al., 1993; Andres et al., 1993) (see part I.1.5 and I.2).

REPs are essential activators of the prenylation reaction, binding non-prenylated Rab proteins and presenting them to RGGT for prenylation. After lipdi transfer, Rab is still in complex with REP which delivers it to cellular membranes (Alexandrov et al., 1994; Wilson et al., 1996).

The first step in Rab prenylation is the formation of the Rab:REP complex (Anant et al., 1998; Shen and Seabra, 1996). REPs are sensitive to the nucleotide status of the Rab proteins and can only bind in the GDP bound form, being unable to bind Rabs in the GTP bound form (Seabra, 1996). Since the GTP and GDP association and dissociation constants are in the same order of magnitude (Zerial and Huber, 1995), and it has been estimated that there is a 10 fold excess of free GTP over free GDP in the cytosol (Bourne et al., 1991), this would imply that Rab proteins bind GTP during or after synthesis, and fold into a conformation in which they cannot bind REP. With a hydrolysis rate in the order of 10^{-2} to 10^{-3} min^{-1}, it is expected that it would be possible to find at any given moment a pool of unprenylated Rab proteins in the cell, slowly hydrolysing GTP to GDP before binding REP and being prenylated. To our knowledge no such pool has been identified in the majority of cell types tested.

Seabra proposed the existence of a Rab GTPase Activating Protein (RabGAP) that would act on non-prenylated, newly synthesised Rab proteins and accelerate the transition between the non-prenylated, GTP bound form and the GDP bound form in complex with REP (Seabra, 1996). This RabGAP would necessarily be able to act on non-prenylated Rab proteins. At the time this work was started, the only mammalian RabGAP identified was specific for the lipid modified form of members of the Rab3 sub-family (Fukui et al., 1997). However, a precedent for

RabGAPs acting on non-prenylated Rab proteins had been established in yeast (Strom et al., 1993), and also in mammalian systems (Tan et al., 1991).

Here attempt to identify a RabGAP that acts on non-prenylated Rab proteins, as its existence would support its requirement for Rab prenylation.

IV.2.2 - Materials and Methods

To prepare complexes or Rab1a with labelled GTP, 60 μM of recombinant protein were incubated for 30 minutes at 37° C in Exchange Buffer, consisting of 25 mM Tris:Cl pH= 7.5, 50 mM NaCl, 10 mM EDTA, 250 mM $(NH_4)SO_4$ and 600 μM [^{32}P-γ-GTP] (sa=220 dpm/pmol). After this the reaction mixture was twice diluted in 2ml ice cold Stop Buffer and concentrated in a Centricon 10 to Rab1a:[^{32}P-γ-GTP]≈ 50 μM. The Stop Buffer consisted of 50 mM Tris:Cl pH= 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT.

To measure GTPase activity, the sample to be assayed was incubated Rab1a:[^{32}P-γ-GTP] in GAP buffer for 5 minutes at 37° C, the reaction was then stopped by addition of ice cold 5% activate charcoal in 80 mM "phosphoric acid", which serves the purpose of denaturing the proteins in the reaction mixture and precipitating guanine nucleotides, and agitated for 5 minutes at room temperature. The reaction was then centrifuged to pellet the charcoal and the amount of free labelled phosphate in the supernatant determined by scintillation counting. The composition of the GAP buffer was 20 mM HEPES pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA, 1 mM ATP, 20 μM Rab1a:[^{32}P-γ-GTP].

Frozen samples from porcine liver were thawed in ice cold lyses buffer and then homogenized, on ice, using a Polytron PT 1200 (Kinematica). The lysis buffer composition was 20 mM TrisCl pH 8.0, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20 mM NaCl, 1 mM PMSF, and 0.5 mg/ml of aprotinin, leupeptin and pepstatin. The homogenate was spun at 500xg and the supernatant (S0.5) then centrifuged at 100,000xg to prepare a soluble fraction (S100) that was used for subsequent purification steps. All chromatographic purifications were performed using a FPLC machine from Pharmacia. Ion Exchange chromatography used a 10 ml colum of cationic exchanger matrix Q Sepharose HP and a buffer system of 25 mM TrisCl pH 8.5, 1 mM DTT, in a gradient of 0 to 1 M NaCl. Hydroxylapatite Chromatography was performed at pH 7.0 with a gradient of 20 to 400 mM K_2H:KH_2 buffer.

Recombinant protein purification and *in vitro* prenylation assays were performed as described before (Seabra and James, 1998).

The cDNA coding for canine Rab1a was used as the template for site directed mutagenesis. Point mutations were generated by Polymerase Chain Reaction, and sub-cloned into the vector pEGFP-3C (CLONTECH Laboratories Inc). The resulting plasmids encode fusion proteins that contain each Rab mutant attached to the COOH terminus of enhanced green fluorescent protein (EGFP)

HeLa cells were grown in DME supplemented with 10% Fetal Calf serum at 37°C with 5% CO_2. Cells for transfection were grown on cover slips for 24h, transfection using the liposomal transfecting reagent Fugene 6 (Roche) and then fixed 48 hours later. For immunofluorescence, coverslip grown cells were washed in PBS and then fixed in 3% paraformaldehyde in PBS for 15 min. Excess fixative was removed by extensive washing in PBS and quenched by incubation with 50 mM NH_4Cl for 10 min. Fixed cells were then observed using a Leica DM-IRBE confocal microscope and the images processed using Leica TCS-NT software associated with the microscope. All images are single sections on the *z*-plane.

IV.2.3 - Results

IV.2.3.1 - Partial Purification of a RabGAP activity that acts on non-prenylated Rab1a

At the time this work was initiated, only one mammalian RabGAP had been cloned, Rab3-GAP. We chose Rab1a as the purification substrate as a RabGAP activity acting on non-prenylated Ypt1p (the Rab1a yeast ortholog) had been described before (Tan et al., 1991).

To prepare Rab1a:[^{32}P-γ-GTP] we had to optimise the conditions of complex formation since in our initial experiments we observed that only a small fraction of Rab1 was being complexed with [^{32}P-γ-GTP] and there was contamination of the complexes with protein-free GTP and [^{32}P-γ-GTP]. Although excess GTP (and GDP) appeared not to have any effect in our GAP assays (data not shown), protein-free [^{32}P-γ-GTP] created a high background in our experiments. We tested a variety of conditions and managed to obtain routinely an efficiency of loading of more than

70%, recovering about 80% of the initial protein, and reducing the contamination of protein-free [^{32}P-γ-GTP] and GTP to undetectable levels (data not shown).

We started our purification using porcine brain as the protein source, and detected a RabGAP activity that acted on non-prenylated Rab1a (data not shown). Due to supply problems we had to switch to another tissue, porcine liver, which was easier to obtain. A RabGAP activity was detected in porcine liver extracts that was partially precipitated by centrifugation at 100,000xg (figure IV.2.1).

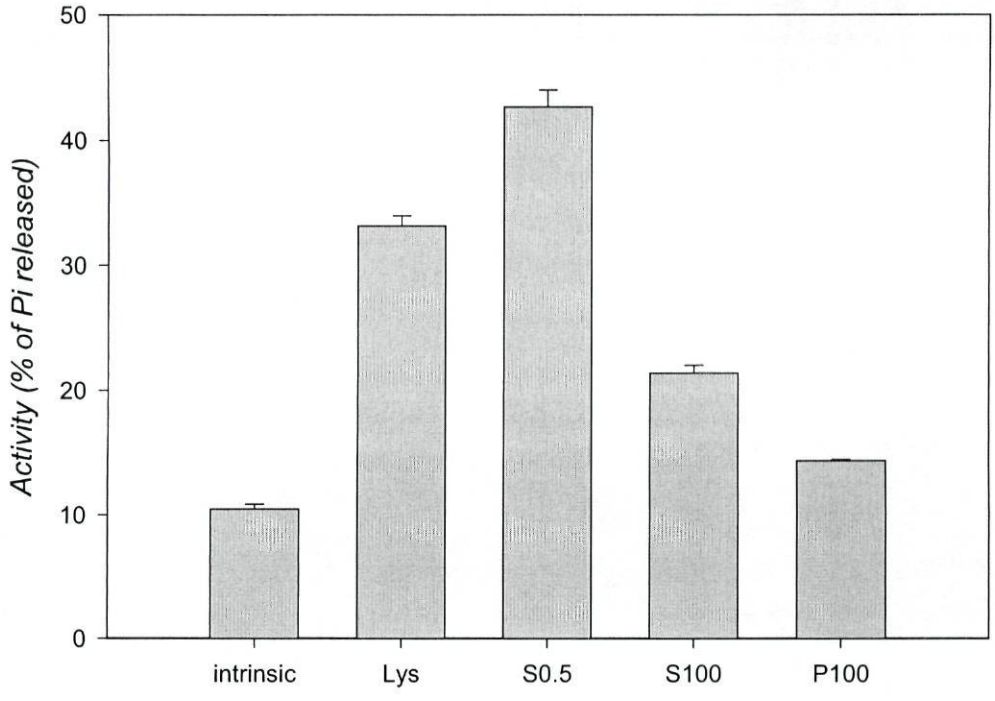


Figure IV.2.1 *– Porcine Liver contains a RabGAP activity that acts on non prenylated Rab1a. Intrinsic stands for hydrolysis in the absence of a RabGAP activity, Lys for lysate, S0.5 for supernatant of a 500xg centrifugation of Lys, S100 for supernatant of a 100,000xg centrifugation of S0.5 and P100 the resuspended pellet of the same centrifugation.*

We sought to solubilise the activity precipitated at 100,000xg using a variety of detergents in the resuspension buffer and different salt concentrations. Salt extraction was not successful at concentrations up to 1.25M NaCl. Of the detergents tested (Cholic acid,Tween20, NP40, CHAPS and Octyl-b-D-Glucopyranoside) all but NP-40 inhibited the GTPase activity assay (data not shown), and NP-40 was not effective in extracting the precipitated activity nor in decreasing the precipitation of this activity after centrifugation at 100,000xg (data not shown). Faced with this result we decided to concentrate solely on the soluble activity and test different chromatographic techniques in order further purify it.

Anionic exchange chromatography, using Q-sepharose resin, proved to be the most successful first column. Two peaks were purified from this column, peak 1 that did not bind the column and eluted in the first fractions, while peak 2 eluted at roughly 150 mM NaCl. We labelled the two peaks, respectively QA and QB. Both activity peaks represented a purification of 0.6 and 2.2 times respectively.

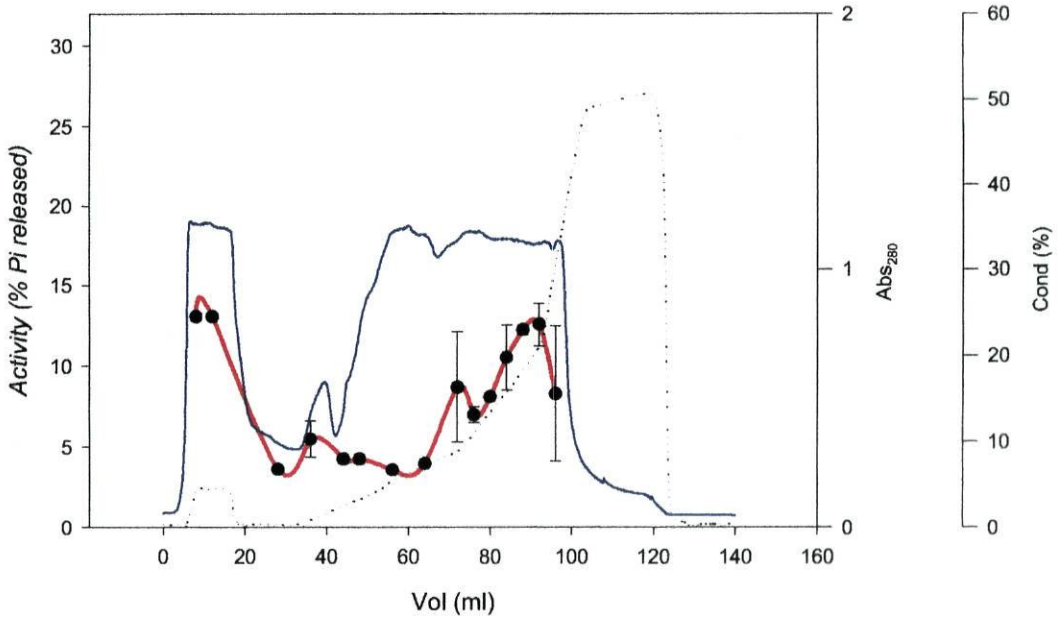


Figure IV.2.2 – *Anionic exchange chromatography of porcine liver soluble fraction separates two distinct activity peaks. Absorbance at 280 nm is represented as a blue line, conductivity as a doted black line and the activity as a red line.*

We next subjected both QA and QB activity peaks to hydroxylapatite column chromatography. QA fractionated in two further activity peaks, labelled H1 and H2 (figure IV.2.3 and table IV.2.1). Peak H2 corresponded to a ~34 fold purification of QB, but H1 was probably an artefact, as it contained little or no protein and was not reproducible. Peak QB also fractionated in two further peaks, labelled H3 and H4, corresponding to 10 and 3.4 fold purification, respectively (figure IV.2.4 and table IV.2.1).

It proved very difficult to further purify any of the hydroxylapatite column activity peaks, since they were extremely labile and would loose detectable activity after more than one round of freeze-thawing or a few hours at 4°C (data not shown). Additionally all the separation procedures tested resulted in either loss of activity (in a way that could not be reconstituted by mixing fractions) or just dilution of the starting activity rather than enrichment (data not shown). We tested several types of

purification procedures in the S100, both the QA and QB and in all the H peaks: ammonium sulphate fractionation, affinity chromatography with Rab1:GTPγS, cationic exchange chromatography, heparin, and hydrophobic interaction chromatography with no success, no matter the starting material or order of he purification procedures (data not shown).

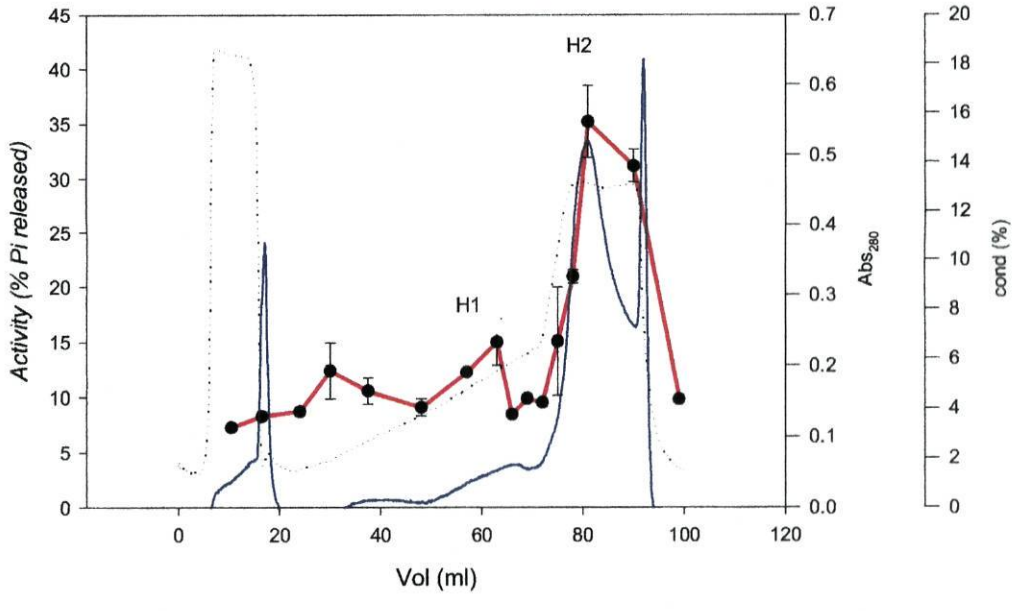


Figure IV.2.3 – *Activity profile of the eluate of a hydroxylapatite column chromatography of Q-sepharose activity peak QA. Activity is represented in red, protein concentration in blue and the conductivity is shown as a doted line.*

We sought to characterise the partial purified enzyme activities in order to determine if better assay conditions would allow us to proceed further with their purification. We first determined the best concentration of RabGAP activity to use in the GTPase activity assays by titrating the enzyme in the reaction mixture. We observe an initial linear increase of activity with increasing enzyme concentration in the assay (figure IV.2.6). At volumes bigger than 12 µl of partially purified enzyme we observe a slight decrease in activity. One explanation for this is the existence of some inhibitory reagent that, acting on the Rab protein, reaches a concentration that produces a detectable inhibition. The nature of this inhibitor is unclear to us.

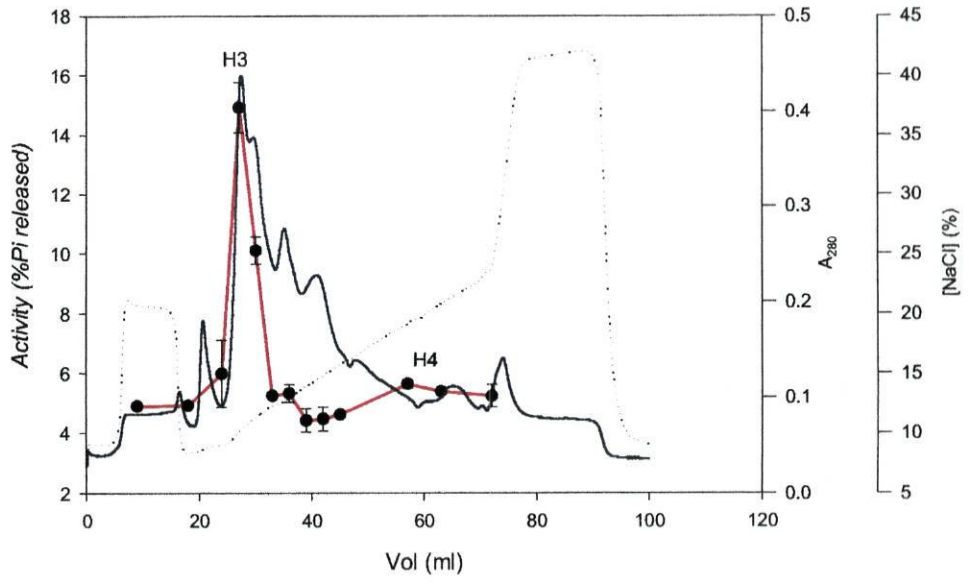


Figure IV.2.4 *– Activity profile of the eluate of a hydroxylapatite column chromatography of Q-sepharose activity peak QA. Activity is represented in red, protein concentration in blue and the conductivity is shown as a doted line.*

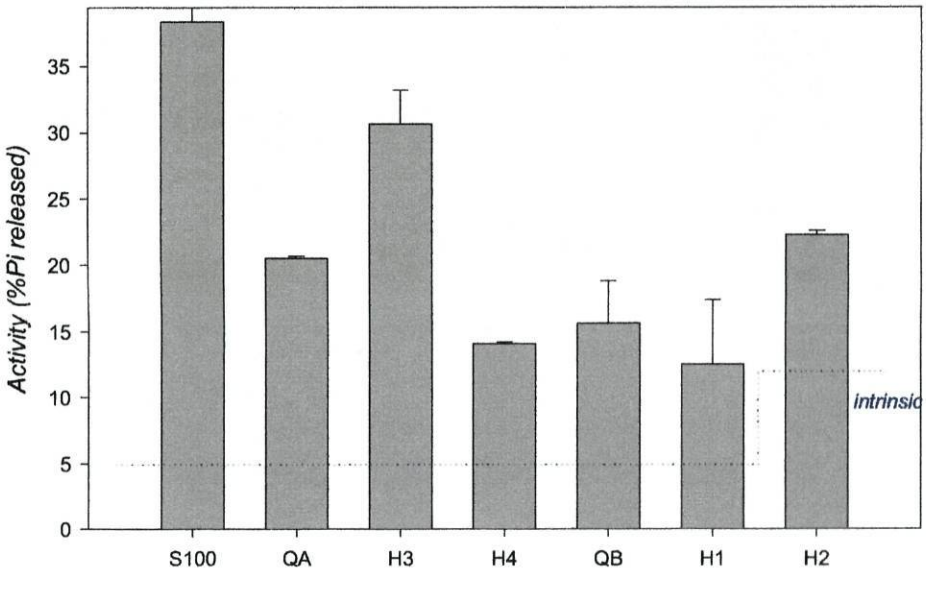


Figure IV.2.5 *– Partial purified RabGAP activities. The blue doted line represents intrinsinc hydrolysis (in the absence of added RabGAP activity).*

Table IV.2.1 *– Purification table of the partial purification of the different Rab1GAP activities*

	Total vol (ml)	Tot protein (mg)	Total activity	Specific Activity	Yield	Fold purification
S100	10	130.4	21777	167	1	1
QA	12	73.1	9503	105	0.43	0.6
H3	6	6.0	10026	1671	0.46	10
H4	6	5.8	3271	564	0.15	3.4
S100	10	130.4	21777	167	1	1
QB	9	21.4	7854	367	0.36	2.20
H2	9	2.6	5675	5675	0.67	33.9

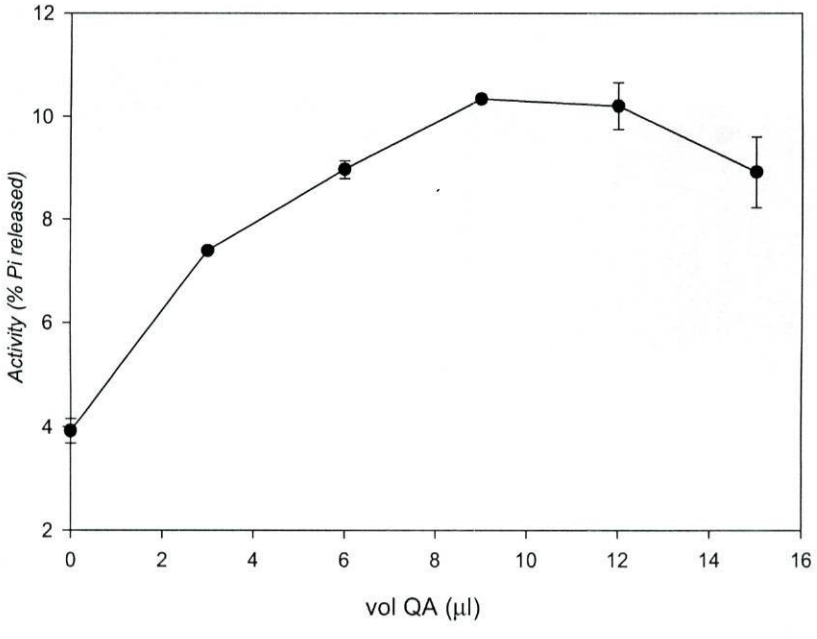


Figure IV.2.6 *– Enzyme titration of QA activity peak, showing initial linear response to enzyme concentration and revealing a possible inhibition at higher concentrations of extract.*

Having determined the best amount of partially purified RabGAP activity to use in the assays, we titrated the substrate so as to calculate the Henry-Michaelis-Menten constant, an indirect measure of the affinity of the RabGAP to Rab1a:GTP. This information is useful to determine minimum concentration of substrate required to be present in the assay. The results of this experiment are presented in figure IV.2.7. Finally, we determined the intrinsic hydrolysis rate of Rab1a in order to insure that our method produced constants in the same order of magnitude as published for Rab1a (figure IV.2.8).

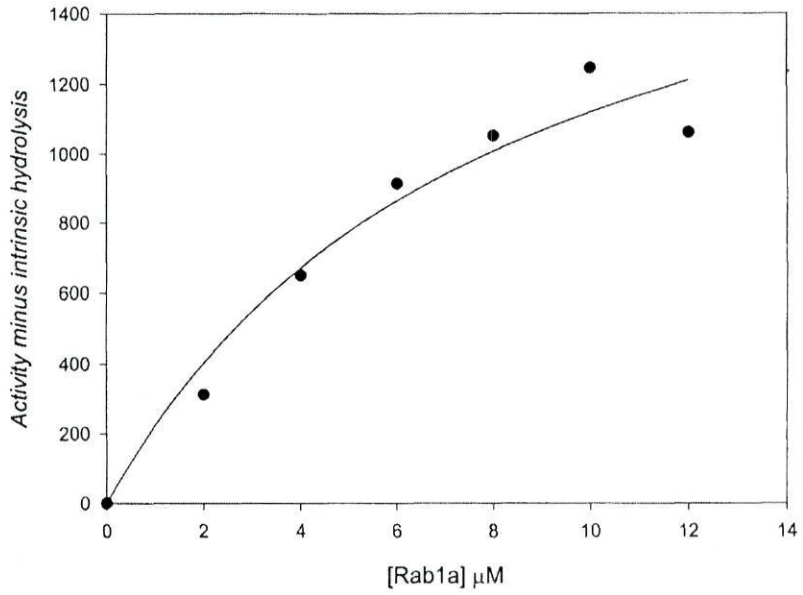


Figure IV.2.7 – *Substrate saturation curve for QA activity peak.* K_M= 8 µM +/- 3.4 at r^2=0.97.

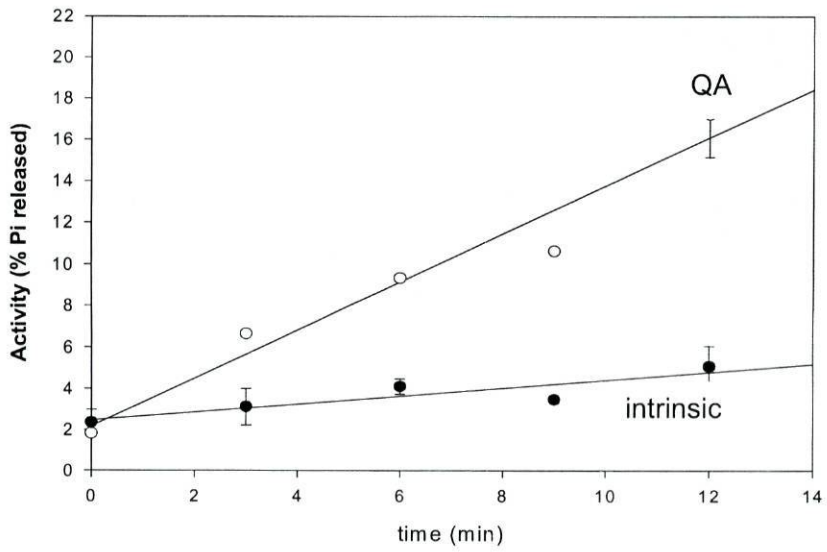


Figure IV.2.8 – *Time curve of GTP hydrolysis in the presence and absence of RabGAP activity QA. Intrinsinc:* $t^{1/2}$*=122.3 min, k=0.006 min*$^{-1}$*. QA activated:* $t^{1/2}$*=21.8 min, k=0.034 min*$^{-1}$*.*

Also we determined the RabGAP activated hydrolysis rate in order to compare it with those published for other mammalian and yeast RabGAPs (figure IV.2.8). These experiments indicate that the conditions we had selected for the GTPase activity assay are satisfactory (20 µM Rab1a:GTP and 10-15 µl of enzyme activity). Also, the intrinsic hydrolysis rate determined for Rab1a of 0.006 min^{-1} is

within the order of magnitude of the same constant determined by others (0.009 min^{-1}) (Touchot et al., 1989), which suggests our assay worked well.

Thus our inability to further purify this RabGAP activity cannot be attributed to assaying it far from ideal conditions. It is possible that the fact that we were using frozen as opposed to fresh tissues resulted in loss of enzyme activity. It was impossible for us to obtain fresh organs since the abattoirs that supplied us were too distant and sample collection implied a full day of travelling and sample washing and freezing. In fact in some cases we obtained tissues which completely lacked RabGAP activity. Although other possibilities could be discussed here, the fact that such activity was observed by others (Tan et al., 1991) suggests this was the major problem in this purification.

At this point we decided not to proceed with the purification attempts. Instead we concentrated on addressing the requirement for a RabGAP prior to prenylation in different way.

IV.2.3.2 - RabGAP insensitive Rab1 mutants are prenylated *in vivo*

Becker and colleagues (Becker et al., 1991) identified two positions in Ypt1p that when mutated render the protein either totally insensitive (I41M) or partially insensitive (D44N) to a partially purified Ypt1GAP, while retaining normal GTP binding properties (figure IV.2.9). Mutation of the corresponding positions in Rab3a (V55E and D58V) also reduced or eliminated sensitivity to a partially purified Rab3GAP (Burstein et al., 1993).

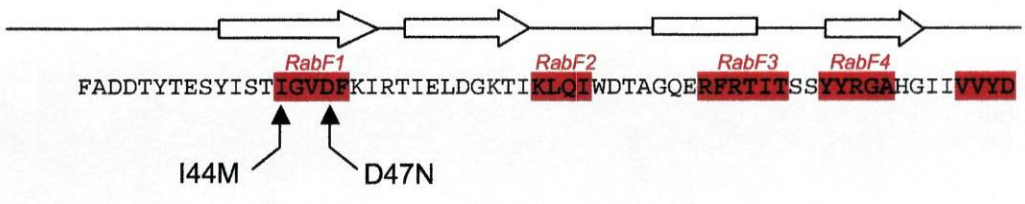


Figure IV.2.9 *– RabGAP-insensitive single point mutations mapped to canine Rab1a sequence, highlighting the sequence motifs (RabF) and structural motifs (extrapolated from the crystal structure of Rab3a, PDB entry 3RABA).*

These mutants are not prenylated *in vitro* (Wilson and Maltese, 1993) but D44N was shown to be prenylated *in vivo* (Wilson et al., 1996). If both GAP insensitive mutations were shown to be prenylated *in vivo*, then this would imply that

they could bind normally to REP even in the absence of RabGAP catalysed GTP hydrolysis.

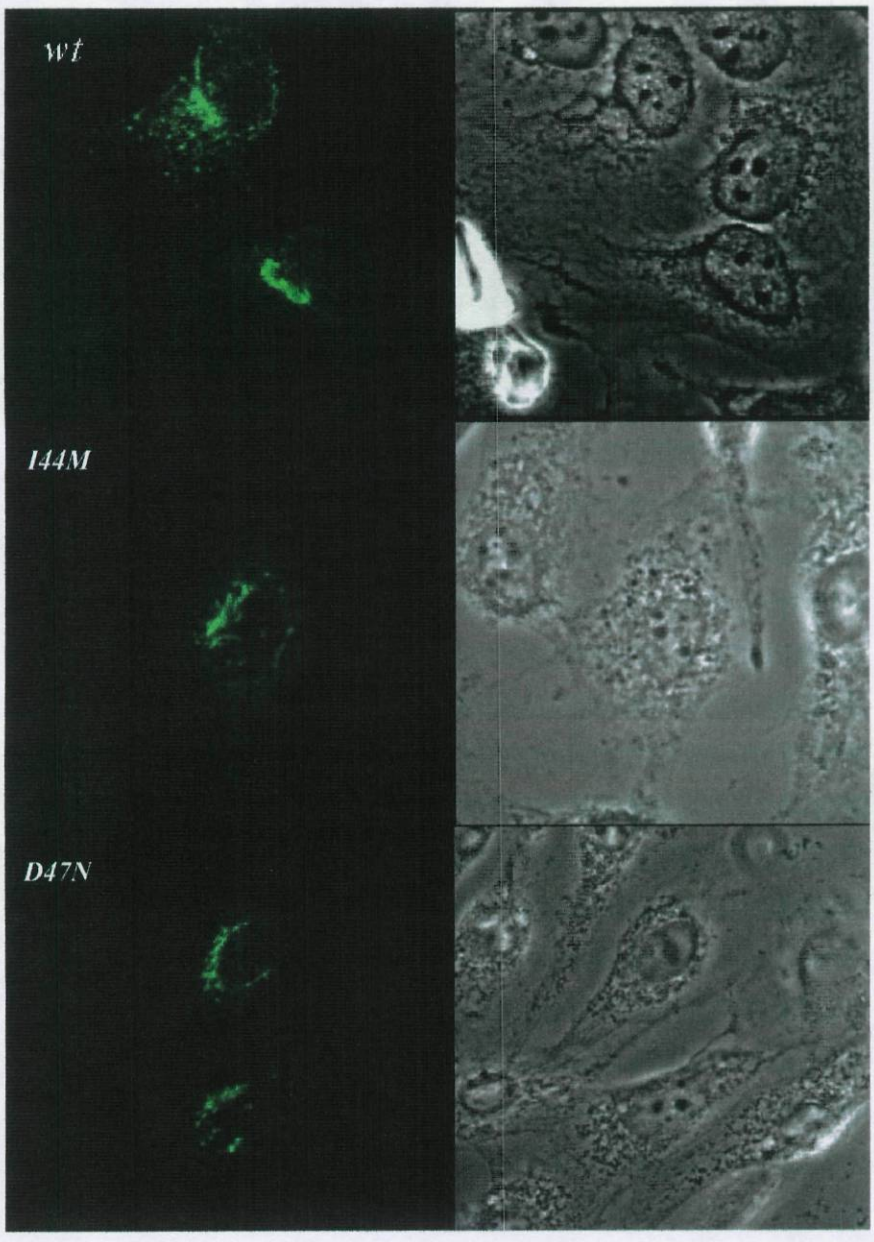


Figure IV.2.10 *– GFP-Rab1(D47N) and (I44M) do not accumulate unprenylated in the cytosol but associate with the Golgi in a way that is not distinguishable form the wild type protein. Left - Confocal section of HeLa cells transfected with pEGFP-Rab1a Right - phase-contrast .*

We chose to ask if the mutant proteins would associate with their target membranes, as this would indicate normal processing by RGGT. We made Enhanced Green Fluorescent Protein (EGFP) fusion constructs for both mutants (N-terminal fusions with GFP have are assumed undergo normal processing as they

associate with their target membranes, which wouldn't happen if they were not prenylated) and transfected them to NIH 3T3 (not shown) and HeLa cells (figure IV.2.10). The wild type protein and the mutant proteins were indistinguishable. In both cases we observed normal Golgi localisation, both in permeabilised and non-permeabilised cells, and failed to detect unusual levels of soluble protein in the transfected cells. This suggests that the mutants proteins are being prenylated with the same efficiency at steady state as the wild type protein.

The results of this experiment suggest that inability to bind RabGAPs does not impair membrane association and hence prenylation of Rab1a.

IV.2.4 - Discussion

To test if GTP hydrolysis catalysed by a RabGAP is required prior to REP binding and prenylation, we attempted to purify a RabGAP that acted on non-lipid modified Rab1a. This was based on the assumption that as Rab3GAP, all mammalian RabGAPs were prenylation-sensitive. We detected such activity but were unable to purify it to homogeneity. However, since this work was started, several mammalian RabGAPs were identified which are active on non-lipid modified Rab proteins (Xiao et al., 1997)(see I.1.4.2). Moreover, the prenylation requirement of Rab3GAP has been contested (Clabecq et al., 2000). Additionally, the complete RabGAP complement of the budding yeast has been identified, and none of its members display a requirement for lipid modification of its substrates (Strom et al., 1993). This leaves GAPCenA, a RabGAP for Rab6 isoforms, as the only mammalian RabGAP with clear preference for lipid modified Rab proteins (Cuif et al., 1999). Our initial assumption that mammalian RabGAPs were prenylation sensitive and that the identification of a RabGAP which acted on non-lipid modified Rab proteins supported its requirement prior to prenylation was thus proved wrong.

Next we introduced mutations in Rab1a that were known to reduce or abolish its sensitivity to RabGAP action (Becker et al., 1991; Burstein et al., 1993). We expressed these mutants as EGFP fusion proteins in mammalian cells and observed that they associate with the ER and Golgi compartments. Since mutations in the prenylation motif of Rab proteins renders them cytosolic, association with the proper cellular compartment of our mutant Rab proteins indicates that they are properly lipid modified. Thus a RabGAP does not seem to be required for the formation of the RabREP complex.

In conclusion it seems unlikely that GTP hydrolysis is required prior to the formation of the Rab:REP complex. Since concentration effects are expected to favour the formation of the Rab:GTP complex, then some mechanism must exist that prevents this. One possibility is that a chaperone binds the newly synthesised protein and stabilises it in the nucleotide free conformation and somehow also forces it to assume a GDP bound conformation. There is no evidence that suggest that REP can bind nucleotide free Rabs and that it can act as a chaperone for the newly synthesised polypeptide. On the other hand one molecule has been identified which has some properties similar to those described above: MSS4 (see part I.1.6.3) (Moya et al., 1993). MSS4 and its yeast ortholog DSS4 have broad substrate specificity, suggesting that their function is important for a large number or even all Rab proteins (Burton et al., 1994). They can bind and stabilise unprenylated Rab proteins in the nucleotide free conformation, with a 1:1 stoichiometry, which suggests that MSS4/DSS4 substrates are the nascent Rab proteins (Nuoffer et al., 1997). Furthermore Rab prenylation seems to be only transiently required to maintain cellular viability, and only a small proportion of Rab protein needs to be, at any time, prenylated (Bialek-Wyrzykowska et al., 2000; Alory and Balch, 2000) - accordingly, deletion of the DSS4 gene is not lethal (Moya et al., 1993). It remains to be determined if deletion of the DSS4 gene results in the accumulation of unprenylated Rab proteins in the cytosol.

IV.3. Structural determinants of Rab:REP binding:

Rab family conserved motifs define a conserved binding surface to REP-1

This work was done in collaboration with Dr. Molly Strom in the laboratory.

IV.3.1. Introduction

Rab proteins are Ras-like small GTP-binding proteins which regulate vesicular trafficking pathways. More than fifty Rab proteins have been identified in mammalian cells, forming the largest family of the Ras-like small GTPases (Pereira-Leal and Seabra, 2000). Rab proteins localise to specific sub-cellular membrane compartments and in some cases also show specific patterns of tissue expression, reflecting the variety of trafficking events found in mammalian cells (Zerial and McBride, 2001; Olkkonen and Stenmark, 1997; Guo et al., 2000).

Rabs are hydrophilic proteins, however their function depends on association with the cytoplasmic leaflet of cellular membranes . Membrane association is mediated by the covalent modification of C-terminal cysteine residues with geranylgeranyl isoprenoids (Seabra, 2000). This is accomplished by the enzyme Rab Geranylgeranyl Transferase (RGGT), a protein prenyl transferase which requires an accessory protein for proper function: Rab Escort Protein (REP) (Seabra, 2000). REP binds newly synthesised Rab proteins, and it is the Rab:REP complex that is the true substrate for RGGT (Anant et al., 1998). RGGT binds the REP:Rab complex and catalyses the transfer of one or two geranylgeranyl groups to one or two cysteines available in the prenylation motif, in what is believed to be a sequential reaction since there is only one lipid binding site in the enzyme (Anant et al., 1998; Desnoyers and Seabra, 1998). After lipid transfer, the enzyme dissociates from the REP:Rab complex and REP is believed to deliver the prenylated Rab to cellular membranes (Alexandrov et al., 1994).

REPs are related to Rab GDP Dissociation Inhibitors (RabGDIs), general regulators of Rab function which are though to recycle prenylated Rabs from and to cellular membranes (Pfeffer et al., 1995). Mammals exhibit two REP isoforms, REP-1 and REP-2. They are ~70% identical and display similar tissue expression patterns (Desnoyers et al., 1996; Seabra, 2001). In contrast, the budding yeast only possess one, MRS6. The essential role of REP for prenylation is illustrated by the accumulation of unprenylated Rab proteins in yeast where REP function is deficient, and the fact that its deletion is lethal (Bialek-Wyrzykowska et al., 2000). Also, mutations in the human REP-1 gene result in

Choroideremia, an X-linked progressive retinal degeneration (Cremers et al., 1990; Seabra, 1996). Improper compensation by REP-2 is a likely cause for the disease, possibly due the accumulation of a sub-set of unprenylated Rabs. Consistently, one Rab protein (Rab27) was found to be selectively unprenylated in lymphoblasts from Choroideremia patients (Seabra et al., 1995). Furthermore, Rab27 is expressed in the same layers of the rat eye which are observed to degenerate in Choroideremia patients (Seabra et al., 1995).

Comparison of the primary amino acid sequence of REP's and RabGDIs revealed three regions of conservation (SCRs – sequence conserved regions) (Ragnini et al., 1994; Waldherr et al., 1993). Furthermore, site directed mutagenesis has identified positions in the SCR1a, SCR1b and SCR3b of RabGDI as the main determinants for Rab binding (Schalk et al., 1996; Wu et al., 1998; Wu et al., 1996). The solution structure of the α isoform of RabGDI, which revealed a two domain organisation of the molecule, showed that SCR1a, SCR1b and SCR3b regions localize at the apex of domain 1, forming the "Rab binding platform" (Schalk et al., 1996; Wu et al., 1998; Wu et al., 1996). Finally, the same residues involved in RabGDI:Rab binding are involved in Mrs6p:Ypt1p binding, further suggesting a conserved Rab binding mechanism between REPs and RabGDIs (Alory and Balch, 2000; Bauer et al., 1996)

As to the critical elements in the Rab for binding to different general regulators, the picture is not so clear. Beranger and co-workers (Beranger et al., 1994) tested the role of different regions of Rab6 for interaction with RabGDI and REP by substituting predicted structural elements with the cognate regions from H-Ras. They observed that RabGDI binding is blocked by substitutions of the hypervariable domain, of the loop3/β3 region, or of the effector domain (which includes switch I residues), but that processing by RGGT (and hence REP binding) is unaffected by these substitutions. Rab6 prenylation was affected only by a combined hyper-variable domain and loop 3/β3 substitution, or by substituting the N-terminal sequence of Rab6 up to the beginning of the switch II region (which includes loop 3/β3), with the corresponding Ras residues. In contrast, Wilson and co-workers (Wilson et al., 1996) observed that the Switch I point mutation Rab1bD44N cannot bind αRabGDI *in vitro*, but is prenylated *in vitro* and *in vivo* at 40- 50% efficiency compared to the wt Rab1b. Recently, Overmeyer and co-workers (Overmeyer et al., 1998) showed that residues in the α2 helix, which localises in the switch II region, are essential both for Rab1b prenylation and for REP:Rab binding *in vitro*. Finally, Sanford and co-workers (Sanford et al., 1995) showed that the N-terminal amino acids in Rab5 are essential for efficient prenylation. Taken together, the observations made by these groups are in part contradictory and do not give a clear picture about how Rabs bind REPs or RabGDIs.

We have recently identified sequence motifs that distinguish the mammalian Rab proteins from other mammalian small GTPases, which we named RabF motifs (Pereira-Leal

and Seabra, 2000). This conserved protein sequences localise in and around the switch regions. As all Rab proteins bind REPs and RabGDIs in an nucleotide sensitive way, we proposed that conserved positions in the RabF motifs would define a general regulator binding surface in Rab proteins. This hypothesis is consistent with the data available for the Rab binding platform in RabGDI and MRS6 that suggest a conserved mechanism of Rab binding.

In collaboration with Dr. Molly Strom in the laboratory, here we test this model by site directed mutagenesis, using REP-1 and Rab3a, and identify Rab conserved positions which are critical for REP binding.

IV.3.2. Materials and Methods

Human Rab3a was cloned in frame to the LexA *E. Coli* DNA binding protein in pBTM116 (generous gift of S. Fields). Rat REP-1 was cloned into pGADGH so that REP-1 was fused in frame to the activation domain of GAL4. Mutations in Rab3a were created using Polymerase Chain Reaction (Pfu) mutagenesis, using pBTM116-Rab3a as template. Yeast strain L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was then transformed with combinations of pBTM116-Rab3a and pGADGH-REP1 constructs and colonies were assayed for their ability to grow 3-aminotriazole (SIGMA) and for β-galactosidase activity as described in (Durfee et al., 1993). Expression levels of the different constructs was assayed by western blotting.

Sequence alignments were performed using Clustal W 1.80 (Thompson et al., 1997) using default parameters. Modelling of the structure of the Rab3:GDP complex was performed using the SWISS-Model automated comparative modelling server (Guex and Peitsch, 1997; Peitsch, 1996; Peitsch, 1995), based on the structure of the Rab6:GDP complex (1D5C) (Chattopadhyay et al., 2000).

IV.3.3. Results and Discussion

In order to determine the positions in Rab3a which may be critical for REP binding, we decided to use a site directed mutagenesis approach, and test the resulting mutant Rab proteins in a Rab:REP binding assay. Based on our previous identification of Rab-specific sequence motifs (RabF motifs), we hypothesised that positions in these motifs would be critical for REP binding (Pereira-Leal and Seabra, 2000). Further, we eliminated positions that were more likely to be buried inside the protein and concentrated solely on potentially

exposed positions. To do so, and in the absence of crystal structures for our model Rab (Rab3a) in a REP-binding competent conformation, we modelled the structure of the Rab3a:GDP complex by homology modelling to identify potentially exposed residues within the RabF motifs, and positions which by their chemical nature and/or localisation could also be considered candidate REP-binding residues. The positions we selected are indicated in red in figure IV.3.1.

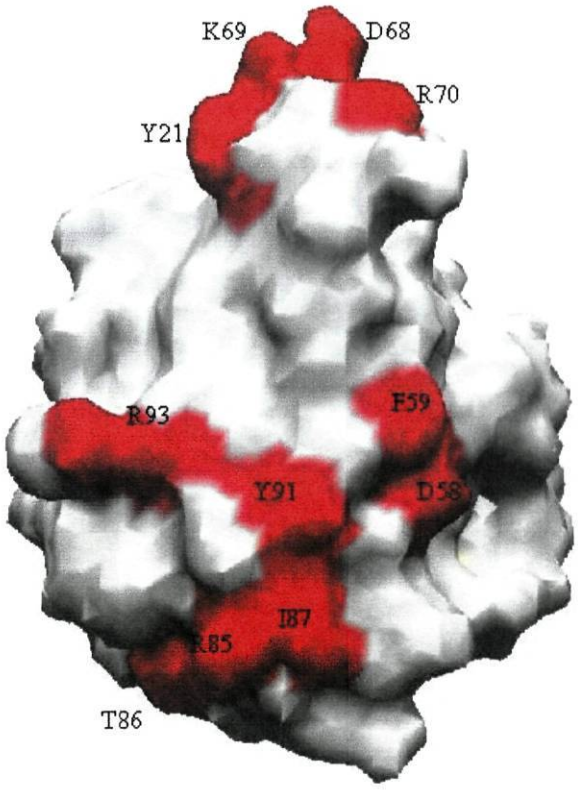


Figure IV.3.1 *– Mapping of the mutated positions (in red) used in this study to a cartoon representing the molecular surface of Rab3a.*

We then established a REP:Rab binding assay using on the yeast two hybrid system. This system is particularly suited for this type of study as it permits the screening of large number of mutations, it isolates REP:Rab binding from the end product of the prenylation reaction which is used in other studies as indicative of REP binding, and finally, it permits the testing of the binding of the two proteins *in vivo*.

. We tested the mutant Rab proteins in this assay and observed that of the chosen positions, only mutations in D58 and F59 (Switch I), and I87N/T86N and R93 (Switch II) resulted in the abolishing of REP:Rab binding in our assay (see table IV.3.1). When we map these mutations into the structure of Rab3a, we observe that all localise to a surface of the

molecule which includes the Switch I and II regions. This is consistent with the observation that REPs can only bind Rabs in a GDP bound conformation (Seabra, 1996).

Table IV.3.1 *– Yeast two hybrid Rab3a:REP binding assay results. For the β-galactosidase Induction:+ represents blue colour after 30 mins, ++ represents blue colour after 15 mins, 0 represents blue colour after 3 hours.*

Rab mutant	β-galactosidase induction	Growth on 3-aminotriazole
wt	+	≤50 mM
Y21A	+	≤50 mM
D58A	0	< 5 mM
F59A	0	< 5 mM
K69A	+	≤50 mM
R70A/D68L	+	≤50 mM
T86A	++	≥ 100 mM
I87A/T86N	0	< 5 mM
R85A	++	≥ 100 mM
Y91A	+	≤50 mM
R93A	0	< 5 mM
pBTM	0	< 5 mM

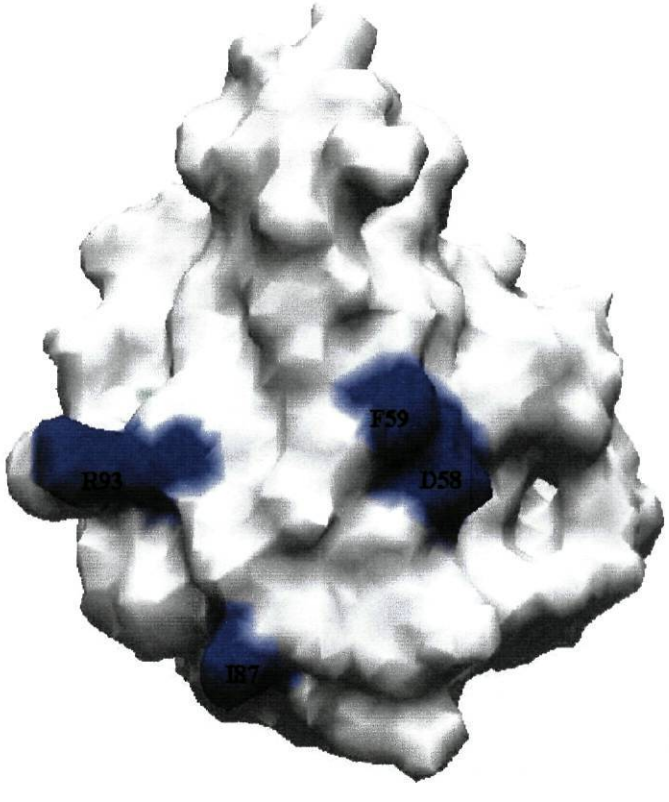


Figure IV.3.2 *– Positions involved in REP binding (in blue), mapped to cartoon representing the molecular surface of Rab3a.*

We observed that all Rab3 mutants were well expressed (figure IV.3.4), thus indicating that the protein is made and is stable. The negative signal we observed in some mutants then is not due to lack of protein expression, but is due to the inability of the mutant Rab protein to bind REP-1 in this assay.

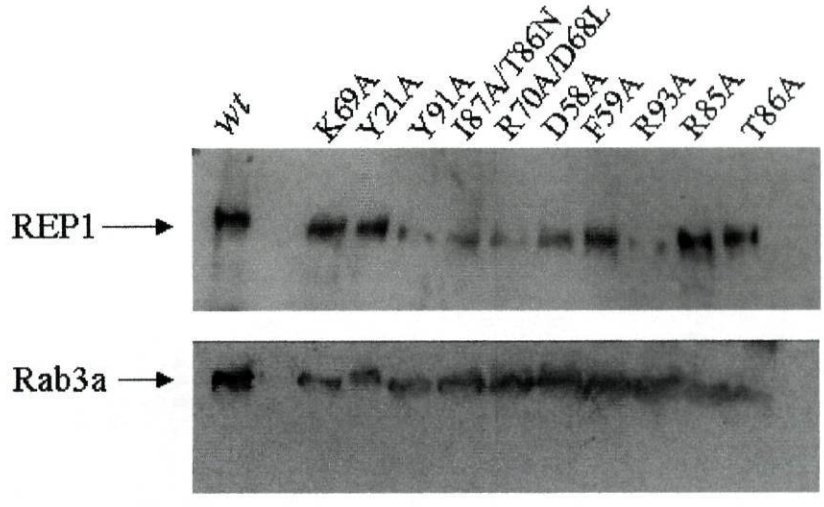


Figure IV.3.3 *- Expression of REP-1 and Rab3 in the yeast two hybrid REP:Rab binding assay*

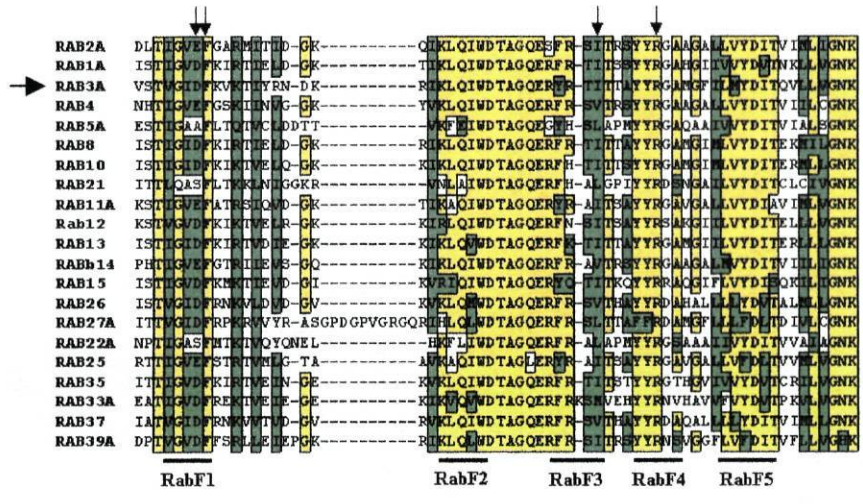


Figure IV.3.4 *– Alignment of select human proteins, indicating the REP-binding positions (arrows - top), the positions of the RabF motifs (black bars - bottom), and the identities (yellow) and similarities (green) in these regions*

.

We identified three novel positions involved in REP binding (D58, F59, R93), and confirmed the importance of the exposed residue I87, in accordance with the results of Overmeyer and co-workers (Overmeyer et al., 1998). Positions F79 and R93 are almost invariably conserved, and position I87, although not absolutely conserved, is frequently occupied by a conserved substitution. However, position D58 although in the majority of the

cases is occupied by either an aspartic acid residue or a glutamic acid residue (similar chemical nature), in a few cases can be occupied by a non-conservative substitution (fig IV.3.4). One of these cases is Rab5, which has an alanine residue in this positions. Interestingly, Rab5 was previously shown to be a poorer substrate for REP binding than Rab1a (Anant et al., 1998). Furthermore, in our assay we observed a weaker signal whenever we tested Rab5 (not shown). However, mutations in the positions in Rab5a corresponding to the Rab3a F59 and R93, abolish the binding to REP-1, like they do in Rab3a (not shown).

These results suggest that although there is a number of positions that are conserved and define the core "REP binding platform", it is possible that different Rabs may display different variations in this binding. It would be interesting to test the effect of naturally occurring variations to the RabF motifs that some Rabs display. For example, Rab5 has a D->A substitution in RabF1, while Rab21 and Rab22 have an S->A substitution in the corresponding position (figure IV.3.4). The differential binding of Rabs to REPs could for example account for the accumulation of unprenylated Rab27 in CHM patients that only possess functioning REP-2 (Seabra et al., 1995). Furthermore, it could also account for the differential binding *in vivo* of Rabs to different RabGDI isoforms (Erdman and Maltese, 2001). We tested the effect of the same Rab mutations in the binding of Rab3a to RabGDI (data not shown), and although the assay had a strong background signal, the results were similar to those obtained for Rab3a:REP binding, which is consistent with the proposed conserved mode of interaction between RabGDI and Rabs, and MRS6 and Rabs (Alory and Balch, 2000).

One interesting consequence from this work is the observation that both Switch I and Switch II will contribute to REP binding. This suggests that Rabs cannot simultaneously bind REPs and other proteins which require interactions with the switch regions, for example proteins which need to bind the switch regions to sense or alter the nucleotide status of the Rab. This is consistent with the observation that binding to RabGDI inhibits GDP dissociation from Rab proteins, a reaction catalysed by exchange factors which require interactions with the switch regions (Sasaki et al., 1991; Ullrich et al., 1993; Wittinghofer, 1998). Also, it consistent with the proposal that membrane association and RabGDI dissociation precedes GDP-GTP exchange (Ullrich et al., 1994).

When we compare the positions assayed here with those addressed in other studies, we observed that while we used modelling techniques to predict the structure of a Rab proteins in a REP-binding competent conformation and thus select only exposed residues, other studies have used mutations that were not exposed (for example Y89 in Rab1b used in (Overmeyer et al., 1998). This mutations are not likely to be involved in REP binding. However, they may play a role in the folding and stability of the Rab. In this case, the inability

to bind REP and/or be prenylated cannot be interpreted as proof of involvement in direct interactions with REP.

The method here described can be used to systematically analyse these issues, but it has one important limitations in which it is not quantitative: it can only be interpreted when yes or no answers are required. We are now following these results by studying the effect of the Rab mutations in the kinetics of *in vitro* Rab3a prenylation. Furthermore, the identification of the REP-binding positions will be now used as constrain for *in silico* docking of the structures of RabGDI and REP-1 with Rab3 by Richard Jackson at University College London, from which a prediction of the interaction surface between the two molecules can be inferred. In combination with patterns of sequence conservation these results can then be used to address systematically the differential binding of Rabs to REP and RabGDI isoforms, which may help us understand the Choroideremia, and the regulation of Rab membrane association by REPs and RabGDIs

V – Concluding remarks

ICBAS

The main objective of this thesis was to study the structural determinants of Rab prenylation and function.

Part II concentrates on the Rab family of small GTP-binding proteins. In chapter II.1 a study of primary structure conservation in the mammalian Rab family is presented and a sequence definition of a Rab protein is obtained. This is the first time that criteria for inclusion of novel proteins in the Rab family is presented, which will be useful for future assignments of GTPases. Also, a working criteria for the definition of sub-families is presented, which hopefully will help to clarify the ruling confusion in the nomenclature of the mammalian Rab family. We have been working with the HUGO nomenclature commission, using the criteria here defined, to help to rationalise the designations in the databases. Finally, the study of primary structure in Rab proteins led to the proposal of a novel mechanism for interaction with specific effectors and general regulators like REP. This proposal is supported by the available experimental evidence, and represents a departure from the accepted model that all small GTPases obey the rules determined for the Ras family. It is a Rab-specific mechanism that is testable, and provides a conceptual framework for further studies on the structural determinants of Rab function and lipid modification.

In chapter II.2 the analysis of sequence conservation patterns in the Rab family is taken a step further. Complete Rab families are identified and annotated in six different organisms which had their genomes sequenced in its entirety. For the first time a general criteria for the annotation of Rab proteins is proposed. Furthermore, in collaboration with Ian Moore at the Department of Plant Sciences of Oxford University, a classification system for plant Rabs is proposed. The evolutionary analysis of Rab proteins revealed that the interaction mechanisms proposed in chapter II.1 can be generalised to Rab proteins in other organisms. Existing experimental information suggests that this prediction is accurate. A new level of organisation in the Rab family is uncovered in the animal and fungi Rab families, which we termed "Rab functional groups". This new hierarchy reflects the evolutionary history of the Rab family in general and of a given Rab protein in particular, and reveals that Rabs arising from common protein ancestors maintain the basic property(ies) of the putative ancestral protein. This clustering of Rab proteins in groups paves the ground for analysis, both computational and experimental, of what are the functional properties shared by the different Rab proteins, what are the minimal Rab functional properties which are required in an eukaryotic cell, what are the structural determinants of the conserved functional properties and will in the future permit a better understanding of the evolution of trafficking pathways in the cell.

One unexpected observation made in chapter II.2 came from the analysis of the prenylation motif usage across evolution. Prenylation motifs for homologous Rabs in

different organisms are conserved in the number of prenylatable cysteine residues, and in many cases in the topology of the motif. This suggests that the prenylation motifs of Rab proteins may play a role in the function of these proteins, determining the way these proteins reach their target membranes and/or how they associate with the membranes. There are obvious experiments that can be done to test these predictions, including swapping prenylation motifs between Rab proteins and testing trafficking and targeting to the membranes, and also the effect of the type of prenylation motif in the efficiency of membrane association. Some of these experiments are now being done in the lab.

Part III concentrates on the REP/GDI superfamily of general regulators of Rab proteins in order to identify the primary and tertiary structure determinants of functional overlapping and difference between REPs and GDIs. Sequence and structure analysis, correlated with the restricted experimental information, supports that the proposed "Rab binding platform" of RabGDI is conserved in REP proteins. Furthermore, several REP specific positions were identified, providing a framework for future structure-function studies in members of this family.

Part IV concerns the formation of the REP:Rab complex. Chapter IV.1 describes methods designed and optimised for the formation and purification of such complexes *in vitro*. In this chapter we describe methods, which are simple, quick and allow different scale preparations. Moreover, it is the first time where methods to purify all the individual complexes corresponding to the different stages of the prenylation reaction is reported, providing tools for structural studies of the Rab prenylation reaction.

Chapter IV.2 addresses the requirement for GTP hydrolysis prior to the formation of the REP:Rab complex. I attempted to purify a RabGAP that acted on non-prenylated Rab proteins, as at the time it made sense to think that all RabGAPs that acted later on the Rab cycle would have a strict requirement for prenylation. While this project was being pursued, several reports appeared in the literature which questioned the RabGAP requirement for lipid modified Rabs. Furthermore, I was not able to purify the RabGAP activity for non-prenylated Rab1a, possibly due to problems with the starting material used. I have to conclude that this particular project was ill designed. A better way would have been to address the requirement for a RabGAP, and not try to find the RabGAP. Available evidence suggests an alternative mechanism that involves MSS4, a molecule that appears to have all the characteristics required to behave as a chaperone for unprenylated Rab proteins. Further experiments need to be done however to rule out the RabGAP requirement, test if MSS4 is a chaperone for newly synthesised Rab proteins, or if other, yet unidentified molecules, play a role in maintaining or bringing newly synthesised Rabs into a REP-binding competent conformation.

Finally, in chapter IV.3 I test the prediction made in chapter II.1, that the RabF motifs will determine the interface between REP and Rab when these two proteins are in complex.

In the absence of any known structure of a Rab protein in the GDP bound conformation and of REP proteins, I calculated homology structural models for this protein complex that were then docked *in silico* by Richard Jackson at University College London. The initial docking models required the creation of more constrains to the docking procedures, and based on both my primary structure analysis of Rab and REP proteins, and with Molly Strom in the lab we created mutants that were used in a yeast-two hybrid based binding assays and *in vitro* prenylation reactions. The results of these experiments suggest an interface between the two proteins that is consistent with the predictions made in parts II and III. This work provides the groundwork and a methodology to address now the differential binding of two REP proteins in mammals for different Rab proteins, an important issue to understand human disease that arises when REP1 is mutated.

Part VI – References

Abdul-Ghani, M., Gougeon, P.Y., Prosser, D.C., Da-Silva, L.F., and Ngsee, J.K. (2001). PRA isoforms are targeted to distinct membrane compartments. J Biol Chem *276*, 6225-6233.

Abdul-Manan, N., Aghazadeh, B., Liu, G.A., Majumder, A., Ouerfelli, O., Siminovitch, K.A., and Rosen, M.K. (1999). Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott-Aldrich syndrome' protein. Nature *399*, 379-383.

Abel, K. and Jurnak, F. (1996). A complex profile of protein elongation: translating chemical energy into molecular movement. Structure *4*, 229-238.

Achaz, G., Coissac, E., Viari, A., and Netter, P. (2000). analysis of intrachromosomal duplications in yeast *Saccharomyces cerevisiae*: a possible model for their origin. Mol Biol Evol *17*, 1268-1275.

Adachi, R., Nigam, R., Tuvim, M.J., DeMayo, F., and Dickey, B.F. (2000). Genomic organization, chromosomal localization, and expression of the murine RAB3D gene. Biochem Biophys Res Commun *273*, 877-883.

Ahmadian, M.R., Mittal, R., Hall, A., and Wittinghofer, A. (1997). Aluminum fluoride associates with the small guanine nucleotide binding proteins. FEBS Lett *408*, 315-318.

Al-Matubsi, H.Y., Breed, W., Jenkin, G., and Fairclough, R.J. (1999). Co-localization of Rab3B and oxytocin to electron dense granules of the sheep corpus luteum during the estrous cycle. Anat Rec *254*, 214-221.

Albert, S. and Gallwitz, D. (1999). Two new members of a family of Ypt/Rab GTPase Activating Proteins. J.Biol.Chem. *274*, 33186-33189.

Albert, S. and Gallwitz, D. (2000). Msb4p, a protein involved in Cdc42-dependent organization of the actin cytoskeleton, is a Ypt/Rab specific GAP. Biol.Chem. *381*, 453-456.

Albert, S., Will, E., and Gallwitz, D. (1999). Identification if the catalytical domains and their functionally critical arginine residues of two yeast GTPase-activating proteins specific for Ypt/Rab transport GTPases. EMBO J *18*, 5216-5225.

Alexandrov, K., Horiuchi, H., Steele-Mortimer, O., Seabra, M.C., and Zerial, M. (1994). Rab escort protein-1 is a multifunctional protein that accompanies newly prenylated rab proteins to their target membranes. EMBO J *13*, 5262-5273.

Alexandrov, K., Simon, I., Iakovenko, A., Holz, B., Goody, R.S., and Scheidig, A.J. (1998). Moderate discrimination of REP-1 between Rab7 x GDP and Rab7 x GTP arises from a difference of an order of magnitude in dissociation rates. FEBS Lett *425*, 460-464.

Allan, B.B., Moyer, B.D., and Balch, W.E. (2000). Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. Science *289*, 444-448.

Alory, C. and Balch, W.E. (2000). Molecular basis for Rab prenylation. J Cell Biol *150*, 89-103.

Altschul, S. F, Madden, T. L, Schaffer, A. A, Zhang, J, Zhang, Z, Miller, W, and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Anant, J.S., Desnoyers, L., Machius, M., Demeler, B., Hansen, J.C., Westover, K.D., Deisenhofer, J., and Seabra, M.C. (1998). Mechanism of Rab geranylgeranylation: formation of the catalytic ternary complex. Biochemistry *37*, 12559-12568.

Andres, D.A., Seabra, M.C., Brown, M.S., Armstrong, S.A., Smeland, T.E., Cremers, F.P., and Goldstein, J.L. (1993). cDNA cloning of component A of Rab geranylgeranyl transferase and demonstration of its role as a Rab escort protein. Cell *73*, 1091-1099.

Apolloni, A., Prior, I., Lindsay, M., Parton, R., and Hancock, J. (2000). H-ras bu not K-ras trafficks to the plasma membrane through the exocytic pathway. Mol Biol Cell *20*, 2475-2487.

Araki, S., Kaibuchi, K., Sasaki, T., Hata, Y., and Takai, Y. (1991). Role of the C-terminal region of smg p25A in its interaction with membranes and the GDP/GTP exchange protein. Mol Cell Biol *11*, 1438-1447.

Araki, S., Kikuchi, A., Hata, Y., Isomura, M., and Takai, Y. (1990). Regulation of reversible binding of smg p25A, a ras p21-like GTP-binding protein, to synaptic plasma membranes and vesicles by its specific regulatory protein, GDP dissociation inhibitor. J Biol Chem *265*, 13007-13015.

Armstrong, J. (2000). How do Rab proteins function in membrane traffic? Int J Biochem Cell Biol *32*, 303-307.

Armstrong, J. (2000). Membrane Traffic between genomes. Genome Biol *1*, 104.1-104.4

Armstrong, S.A., Brown, M.S., Goldstein, J.L., and Seabra, M.C. (1995). Preparation of recombinant Rab geranylgeranyltransferase and Rab escort proteins. Methods Enzymol *257*, 30-41.

Armstrong, S.A., Seabra, M.C., Sudhof, T.C., Goldstein, J.L., and Brown, M.S. (1993). cDNA cloning and expression of the alpha and beta subunits of rat Rab geranylgeranyl transferase. J Biol Chem *268*, 12221-12229.

Ayad, N., Hull, M., and Mellman, I. (1997). Mitotic phosphorylation of rab4 prevents binding to a specific receptor on endosome membranes. EMBO J *16*, 4497-4507.

Azuma, Y., Renault, L., Garcia-Ranea, J.A., Valencia, A., Nishimoto, T., and Wittinghofer, A. (1999). Model of the Ran-RCC1 interaction using biochemical and docking experiments. J.Mol.Biol. *289*, 1119-1130.

Bae, C.D., Min, D.S., Fleming, I.N., and Exton, J.H. (1998). Determination of interaction sites on the small G protein RhoA for phospholipase D. J.Biol.Chem. *273*, 11596-1604.

Bahadoran, P., Aberdam, R., Mantoux, F., Busca, R., Bille, K., Yalman, N., de Saint-Basile, G., Casaroli-Marano, R., Ortonne, J., and Ballotti, R. (2001). Rab27a. A key to melanosome transport in human melanocytes. J Cell Biol *152*, 843-850.

Baker, D., Wuestehube, L., Schekman, R., Botstein, D., and Segev, N. (1990). GTP-binding Ypt1 protein and Ca2+ function independently in a cell-free protein transport reaction. Proc Natl Acad Sci U S A *87*, 355-359.

Barbacid, M. (1987). Ras genes. Annu Rev Biochem. *56*, 779-827.

Barbieri, M.A., Hoffenberg, S., Roberts, R., Mukhopadhyay, A., Pomrehn, A., Dickey, B.F., and Stahl, P.D. (1998). Evidence for a symmetrical requirement for Rab5-GTP in in vitro endosome-endosome fusion. J Biol Chem *273*, 25850-25855.

Bauer, B.E., Lorenzetti, S., Miaczynska, M., Bui, D.M., Schweyen, R.J., and Ragnini, A. (1996). Amino- and carboxy-terminal domains of the yeast Rab escort protein are both required for binding of Ypt small G proteins. Mol Biol Cell *7*, 1521-1533.

Bax, B. (1998). Domains of rasGAP and rhoGAP are related. Nature *392*, 448

Becker, J., Tan, T.J., Trepte, H.H., and Gallwitz, D. (1991). Mutational analysis of the putative effector domain of the GTP-binding Ypt1 protein in yeast suggests specific regulation by a novel GAP activity. EMBO J *10*, 785-792.

Bednarek, S., Reynolds, T., Schroeder, M., Grabowski, R., Hengst, L., Gallwitz, D., and Raikhel, N. (1994). A small GTP-binding protein from Arabidopsis thaliana functionally complements the yeast ypt6 null mutant. Plant Physiol *104*, 591-596.

Benli, M., Doring, F., Robinson, D., Yang, X., and Gallwitz, D. (1996). Two GTPase isoforms, Ypt31 and Ypt32p, are essential for Golgi function in yeast. EMBO J *15*, 6460-6475.

Beranger, F., Cadwallader, K., Porfiri, E., Powers, S., Evans, T., de Gunzburg, J., and Hancock, J.F. (1994). Determination of structural requirements for the interaction of Rab6 with RabGDI and Rab geranylgeranyltransferase. J Biol Chem *269*, 13637-13643.

Beraud-Dufour, S., Robineau, S., Chardin, P., Paris, S., Chabre, M., Cherfils, J., and Antonny, B. (1998). A glutamic finger in the guanine nucleotide exchange factor ARNO displaces Mg^{2+} and the β-phosphate to destabilize GDP on ARF1. EMBO J. *17*, 3651-3659.

Bergo, M.O., Gordon, K., Ambroziak, P., Otto, J.C., Casey, P.J., Gomes, A.Q., Seabra, M.C., and Young, S.G. (2001). Isoprenylcysteine Carboxyl Methyltransferase deficiency in mice. J Biol Chem *276*, 5841-5845.

Bhartur, S.G., Calhoun, B.C., Woodrum, J., Kurkjian, J., Iyer, S., Lai, F., and Goldenring, J.R. (2000). Genomic structure of murine Rab11 family members. Biochem Biophys Res Commun *269*, 611-617.

Bhatnagar, R.S. and Gordon, J.I. (1999). Understanding covalent modifications of proteins by lipids: where cell biology and biophysics mingle. Trends in Cell Biology *7*, 14-20.

Bialek-Wyrzykowska, U., Bauer, B.E., Wagner, W., Kohlwein, S.D., Schweyen, R.J., and Ragnini, A. (2000). Low levels of Ypt protein prenylation cause vesicle polarization defects and thermosensitive growth that can be suppressed by genes involved in cell wall maintenance. Mol Microbiol *35*, 1295-1311.

Bizzozero, O.A., McGarry, J.F., and Lees, M.B. (1987). Autoacylation of myelin proteolipid protein with acyl coenzyme A. J.Biol.Chem. *262*, 13550-13557.

Bock, J., Matern, H., Peden, A., and Scheller, R.H. (2001). A genomic prespective on membrane compartment organization. Nature *409*, 839-841.

Boriack-Sjodin, P.A., Margarit, S.M., Bar-Sagi, D., and Kuriyan, J. (1998). The structural basis of the activation of Ras by Sos. Nature *394*, 373-343.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature *348*, 125-132.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. Nature *349*, 117-127.

Bowser, R. and Novick, P. (1991). Sec15 protein, an essential component of the exocytotic apparatus, is associated with the plasma membrane and with a soluble 19.5S particle. J Cell Biol *112*, 1117-1131.

Boyartchuk, V.L., Ashby, M.N., and Rine, J. (1997). Modulation of Ras and a-factor function by carboxyl-terminal proteolysis [see comments]. Science *275*, 1796-1800.

Brennwald, P. (2000). Reversal of fortune: do Rab GTPases act on the target membrane? J.Cell Biol. *149*, 1-3.

Brondyk, W.H., McKiernan, C.J., Burstein, E.S., and Macara, I.G. (1993). Mutants of Rab3A analogous to oncogenic Ras mutants. Sensitivity to Rab3A-GTPase activating protein and Rab3A-guanine nucleotide releasing factor. J Biol Chem *268*, 9410-9415.

Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell *70*, 715-728.

Burd, C.G., Mustol, P., Schu, P., and Emr, S.D. (1996). A yeast protein relatoed to mammalian Ras-binding protein, Vps9p, is required for localisation of vacuolar proteins. Mol Cell Biol *16*, 2369-2377.

Burns, M.E., Sasaki, T., Takai, Y., and Augustine, G.J. (1998). Rabphilin-3A: a multifunctional regulator of synaptic vesicle traffic. J Gen Physiol *111*, 243-255.

Burstein, E.S., Brondyk, W.H., and Macara, I.G. (1992). Amino acid residues in the Ras-like GTPase Rab3A that specify sensitivity to factors that regulate the GTP/GDP cycling of Rab3A. J Biol Chem *267*, 22715-22718.

Burstein, E.S., Brondyk, W.H., Macara, I.G., Kaibuchi, K., and Takai, Y. (1993). Regulation of the GTPase cycle of the neuronally expressed Ras-like GTP-binding protein Rab3A. J Biol Chem *268*, 22247-22250.

Burton, J., Roberts, D., Montaldi, M., Novick, P., and De Camilli, P. (1993). A mammalian guanine-nucleotide-releasing protein enhances function of yeast secretory protein Sec4. Nature *361*, 464-467.

Burton, J.L., Burns, M.E., Gatti, E., Augustine, G.J., and De Camilli, P. (1994). Specific interactions of Mss4 with members of the Rab GTPase subfamily. EMBO J *13*, 5547-5558.

Camp, L.A., Verkruyse, L.A., Afendis, S.J., Slaughter, C.A., and Hofmann, S.L. (1994). Molecular cloning and expression of palmitoyl-protein thioesterase. J.Biol.Chem. *269*, 23212-23219.

Cantalupo, G., Alifano, P., Roberti, V., Bruni, C., and Bucci, C. (2001). Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. EMBO J *20*, 653-693.

Cao, X. and Barlowe, C. (2000). Asymmetric requirement for a Rab GTPase and SNARE protein in fusion of COPII vesicles with acceptor membranes. J Cell Biol *149*, 55-65.

Cao, X., Ballew, N., and Barlowe, C. (1998). Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins. EMBO J *17*, 2156-2165.

Casanova, J.E., Wang, X., Kumar, R., Bhartur, S.G., Navarre, J., Woodrum, J.E., Altschuler, Y., Ray, G.S., and Goldenring, J.R. (1999). Association of Rab25 and Rab11a with the apical recycling system of polarized Madin-Darby canine kidney cells. Mol Biol Cell *10*, 47-61.

Casey, P.J. and Seabra, M.C. (1996). Protein prenyltransferases. J Biol Chem *271*, 5289-5292.

Cassari, G., Sander, C., and Valencia, A. (1995). A method to predict functional residues in proteins. Nature Struct.Biol. *2*, 171-178.

Chang, E., Maryak, J., Wei, C., Shih, T., Shober, R., Cheung, H., Ellis, R., Hager, G., Scolnick, E., and Lowy, D. (1980). Functional organization of the Harvey murine sarcoma virus genome. J.Virol. *35*, 76-92.

Chattopadhyay, D., Langsley, G., Carson, M., Recacha, R., DeLucas, L., and Smith, C. (2000). Structure of the nucleotide-binding domain of Plasmodium falciparum Rab6 in the GDP-bound form. Acta Crystallogr D Biol Crystallogr *56*, 937-944.

Chavrier, P. and Goud, B. (1999). The role of ARF and Rab GTPases in membrane transport. Curr Opin Cell Biol *11*, 466-475.

Chen, D., Guo, J., Miki, T., Tachibana, M., and Gahl, W.A. (1997). Molecular cloning and characterization of rab27a and rab27b, novel human rab proteins shared by melanocytes and platelets. Biochem Mol Med *60*, 27-37.

Cherfils, J. and Chardin, P. (1999). GEFs: structural basis for their activation of small GTP-binding proteins. Trends Biochem Sci *24*, 306-311.

Choy, E., Chiu, V., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I.E., and Philips, M.R. (1999). Endomembrane Trafficking of Ras: The CAAX Motif Targets Proteins to the ER and Golgi. Cell *98*, 69-80.

Christoforidis, S., McBride, H.M., Burgoyne, R.D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. Nature *397*, 621-625.

Clabecq, A., Henry, J.P., and Darchen, F. (2000). Biochemical characterization of Rab3-GTPase-activating protein reveals a mechanism similar to that of Ras-GAP. J.Biol.Chem. *275*, 31786-31791.

Coppola, T., Perret-Menoud, V., Luthi, S., Farnsworth, C.C., Glomset, J.A., and Regazzi, R. (1999). Disruption of Rab3-calmodulin interaction, but not other effector interactions, prevents Rab3 inhibition of exocytosis. EMBO J *18*, 5885-5891.

Cormont, M., Mari, M., Galmiche, A., Hofman, P., and Le Marchand-Brustel, Y. (2001). A FYVE-finger-containing protein, Rabip4, is a Rab4 effector involved in early endosomal traffick. Proc Natl Acad Sci U S A *98*, 1637-1642.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. *16*, 10881-10890.

Cremers, F.P., Armstrong, S.A., Seabra, M.C., Brown, M.S., and Goldstein, J.L. (1994). REP-2, a Rab escort protein encoded by the choroideremia-like gene. J Biol Chem *269*, 2111-2117.

Cremers, F.P., van de Pol, D.J., van Kerkhoff, L.P., Wieringa, B., and Ropers, H.H. (1990). Cloning of a gene that is rearranged in patients with choroideraemia [see comments]. Nature *347*, 674-677.

Cuif, M.H., Possmayer, F., Zander, H., Bordes, N., Jollivet, F., Couedel-Courteille, A., Janoueix-Lerosey, I., Langsley, G., Bornens, M., and Goud, B. (1999). Characterization of GAPCenA, a GTPase activating protein for Rab6, part of which associates with the centrosome. EMBO J *18*, 1772-1782.

D'Adamo, P., Menegon, A., Lo, N., Grasso, M., Gulisano, M., Tamanini, F., Bienvenu, T., Gedeon, A.K., Oostra, B., Wu, S.K., Tandon, A., Valtorta, F., Balch, W.E., Chelly, J., and Toniolo, D. (1998). Mutations in GDI1 are responsible for X-linked non-specific mental retardation [see comments] [published erratum appears in Nat Genet 1998 Jul;19(3):303]. Nat Genet *19*, 134-139.

Dai, Q., Choy, E., Chiu, V., Romano, J., Slivka, S., Steitz, S.A., Michaelis, S., and Philips, M.R. (1998). Mammalian Prenylcysteine Carboxyl Methyltransferase is in the Endoplasmic Reticulum. J.Biol.Chem. *273*, 15030-15034.

Darchen, F., Zahraoui, A., Hammel, F., Monteils, M.P., Tavitian, A., and Scherman, D. (1990). Association of the GTP-binding protein Rab3A with bovine adrenal chromaffin granules. Proc Natl Acad Sci U S A *87*, 5692-5696.

Das, A.K., Dasgupta, B., Bhattacharya, R., and Basu, J. (1997). Purification and biochemical characterization of a protein-palmitoyl acyltransferase from human erytrocytes. J.Biol.Chem. *272*, 11021-11025.

Day, G., Mosteller, R.D., and Broek, D. (1998). Distinct subclasses of small GTPases interact with guanine nucleotide exchange factors in a similar manner. Mol Cell Biol *18*, 7444-7454.

Day, G.J., Mosteller, R.D., and Broek, D. (1998). Distinct subclasses of small GTPases interact with guanine nucleotide exchange factors in a similar manner. Mol Cell Biol *18*, 7444-7454.

Deacon, S.W. and Gelfand, V.I. (2001). Of Yeast, Mice, and Men. Rab proteins and organelle transport. J Cell Biol *152*, F21-F24

Der, C.J., Krontiris, T., and Cooper, G. (1982). Transforming genes of human bladder and lung carcinomas are homologous to ras genes of Harvey and Kirsten sarcoma viruses. Proc Natl Acad Sci U S A *79*, 3637-3640.

Desnoyers, L., Anant, J.S., and Seabra, M.C. (1996). Geranylgeranylation of Rab proteins. Biochem Soc Trans *24*, 699-703.

Desnoyers, L. and Seabra, M.C. (1998). Single prenyl-binding site on protein prenyl transferases. Proc Natl Acad Sci U S A *95*, 12266-12270.

Detter, J.C., Zhang, Q., Mules, E.H., Novak, E.K., Mishra, V.S., Li, W., McMurtrie, E.B., Tchernev, V.T., Wallace, M.R., Seabra, M.C., Swank, R.T., and Kingsmore, S.F. (2000). Rab geranylgeranyl transferase alpha mutation in the gunmetal mouse reduces Rab prenylation and platelet synthesis. Proc Natl Acad Sci U S A *97*, 4144-4149.

Diaz, E. and Pfeffer, S.R. (1998). TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell *93*, 433-443.

Diaz, E., Schimmoller, F., and Pfeffer, S.R. (1997). A novel Rab9 effector required for endosome-to-TGN transport. J Cell Biol *138*, 283-290.

Donaldson, L.G. and Jackson, C.L. (2000). Regulators and effectors of the Arf GTPases. Curr Opin Cell Biol *12*, 475-482.

Du, L.L., Collins, R.N., and Novick, P.J. (1998). Identification of a Sec4p GTPase-activating protein (GAP) as a novel member of a Rab GAP family. J Biol Chem *273*, 3253-3256.

Dumas, J.J., Zhu, Z., Connolly, J.L., and Lambright, D.G. (1999). Structural basis of activation and GTP hydrolysis in Rab proteins. Structure *7*, 413-423.

Duncan, J.A. and Gilman, A.G. (1998). A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). J.Biol.Chem. *273*, 15830-15837.

Durfee, T., Becherer, K., Chen, P., Yeh, S., Yang, Y., Kilburn, A., Lee, W., and Elledge, S. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytical subunit. Genes Dev *7*, 555-569.

Echard, A., Jollivet, F., Martinez, O., Lacapere, J.J., Rousselet, A., Janoueix-Lerosey, I., and Goud, B. (1998). Interaction of a Golgi-associated kinesin-like protein with Rab6. Science *279*, 580-585.

Eddy, S.R. (1996). Hidden Markov models. Curr.Opin.Struct.Biol. *6*, 361-365.

Eddy, S.R. (1998). Profile hidden Markov models. Bioinformatics *14*, 755-763.

Ellis, R., DeFeo, D., Maryak, J., Shih, T., Gonda, M., Young, H., Tsuchida, N., Lowy, D., and Scolnick, E. (1981). The p21 scr genes to Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature *292*, 505-511.

Ellis, R., DeFeo, D., Maryak, J., Young, H., Shih, T., Chang, E., Lowy, D., and Scolnick, E. (1980). Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. J.Virol. *12*, 594-599.

Epstein, W.W., Lever, D., Leining, L.M., Bruenger, E., and Rilling, H.C. (1991). Quantitation of prenylcysteines by a selective cleavage reaction. Proc Natl Acad Sci U S A *88*, 9668-9670.

Erdman, R.A. and Maltese, W.A. (2001). Different Rab GTPases Associate Preferentially with α or β GDO-Dissociation inhibitors. Biochem Biophys Res Commun *282*, 4-9.

Erdman, R.A., Shellenberger, K.E., Overmeyer, J.H., and Maltese, W.A. (2000). Rab24 is an atypical member of the Rab GTPase family. Deficient GTPase activity, GDP dissociation inhibitor interaction, and prenylation of Rab24 expressed in cultured cells. J Biol Chem *275*, 3848-3856.

Fabry, S., Steigerwald, R., Berknlau, C., Dietmaier, W., and Schmidt, R.A. (1995). Structure-function analysis of small G proteins from volvox and Chlamidomonas by complementation of Saccharomyces cerevisiae YPT/SEC mutations. Mol Gen Genet *247*, 265-274.

Farnsworth, C.C., Kawata, M., Yoshida, Y., Takai, Y., Gelb, M.H., and Glomset, J.A. (1991). C terminus of the small GTP-binding protein smg p25A contains two geranylgeranylated cysteine residues and a methyl ester. Proc Natl Acad Sci U S A *88*, 6196-6200.

Farnsworth, C.C., Seabra, M.C., Ericsson, L.H., Gelb, M.H., and Glomset, J.A. (1994). Rab geranylgeranyl transferase catalyzes the geranylgeranylation of adjacent cysteines in the small GTPases Rab1A, Rab3A, and Rab5A. Proc Natl Acad Sci U S A *91*, 11963-11967.

Farnsworth, C.C., Wolda, S.L., Gelb, M.H., and Glomset, J.A. (1989). Human lamin B contains a farnesylated cysteine residue. J Biol Chem *264*, 20422-20429.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution *39*, 783-791.

Feng, Y., Press, B., and Wandinger-Ness, A. (1995). Rab7: an important regulator of late endocytic membrane traffic. J Cell Biol *131*, 1435-1452.

Figueroa, C., Taylor, J. and Vojtek, AB (2001) PRA1 is a receptor for prenylated small GTPases. J. Biol. Chem. *In press*

Fischer, v.M., Mignery, G.A., Baumert, M., Perin, M.S., Hanson, T.J., Burger, P.M., Jahn, R., and Sudhof, T.C. (1990). rab3 is a small GTP-binding protein exclusively localized to synaptic vesicles. Proc Natl Acad Sci U S A *87*, 1988-1992.

Franco, M., Chardin, P., Chabre, M., and Paris, S. (1996). Myristoylation-facilitated binding of the G Protein $Arf1_{GDP}$ to membrane phospholipids is required for its activation by a soluble nucleotide exchange factor. J.Biol.Chem. *271*, 1573-1578.

Freeman, J.L., Abo, A., and Lambeth, J.D. (1996). Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. J.Biol.Chem. *271*, 19794-19801.

Friedman, R. and Hughes, A. (2001). Gene duplication and the structure of the eukaryotic genomes. Genome Res *11*, 373-81.

Fukui, K., Sasaki, T., Imazumi, K., Matsuura, Y., Nakanishi, H., and Takai, Y. (1997). Isolation and characterization of a GTPase activating protein specific for the Rab3 subfamily of small G proteins. J Biol Chem *272*, 4655-4658.

Gallwitz, D., Donath, C., and Sander, C. (1983). A yeast gene encoding a protein homologous to the human c-has-bas proto-oncogene product. Nature *306*, 704-707.

Galtier, N., Gouy, M., and Gautier, C. (1996). SeaView and Phylo_win, two graphic tools for sequence alignment and molecular phylogeny. Comput.Applic.Biosci. *12*, 543-548.

Garcia-Ranea, J.A. and Valencia, A. (1998). Distribution and functional diversification of the ras superfamily in *Saccharomyces cerevisiae*. FEBS Lett. *434*, 219-225.

Garrett, M., Kabcenell, A.K., Kaibuchi, K., Sasaki, T., Takai, Y., Cheney, C.M., and Novick, P. (1993). Interaction of Sec4 with GDI proteins from bovine brain, Drosophila melanogaster and Saccharomyces cerevisiae. Conservation of GDI membrane dissociation activity. FEBS Lett *331*, 233-238.

Garrett, M., Zahner, J., Cheney, C.M., and Novick, P. (1994). GDI1 encodes a GDP dissociation inhibitor that plays an essential role in the yeast secretory pathway. EMBO J *13*, 1718-1728.

Giang, D.K. and Cravatt, B.F. (1998). A second mammalian N-Myristoyltransferase. J.Biol.Chem. *273*, 6595-6598.

Gibbs, J., Sigal, I., Poe, M., and Scolnick, E. (1984). Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. Proc Natl Acad Sci U S A *81*, 5704-5030.

Gilbert, P. and Burd, C.G. (2001). GDP Dissociation inhibitor domain II required for Rabd GTPase recycling. J Biol Chem *276*, 8014-8020.

Goldberg, J. (1998). Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. Cell *95*, 237-248.

Goldberg, J. (1999). Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatomer in GTP hydrolysis. Cell *96*, 893-902.

Goldstein, J.L. and Brown, M.S. (1990). Regulation of the mevalonate pathway. Nature *343*, 425-430.

Gorvel, J.P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991). rab5 controls early endosome fusion in vitro. Cell *64*, 915-925.

Goud, B., Salminen, A., Walworth, N., and Novick, P. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell *53*, 753-768.

Grunler, J., Ericson, J., and Dallner, G. (1994). Branch-point reactions in the biosynthesis of cholesterol, dolichol and, biquinone and prenylated proteins. Biochem.Biophys.Acta *1212*, 259-277.

Guex, N. and Peitsch, M.C. (1997). SWISS_MODEL and the Swiss-PdbViewer: an environment for comparative protein modelling. Electrophoresis *18*, 2714-2723.

Guo, W, Sacher, M, Barrowman, J, Ferro-Novick, S., and Novick, P. (2000) Protein complexes in transport vesicle targeting. Trends in Cell Biology 10(6), 251-255.

Guo, W., Roth, D., Walch-Solimena, C., and Novick, P. (1999). The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. EMBO J *18*, 1071-1080.

Haddad, E., Wu, X., Hammer, J.3., and Henkart, P. (2001). Defective granule exocytosis in Rab27a-deficient lymphocytes from ashen mice. J Cell Biol *152*, 835-842.

Hama, H., Tall, G., and Horazdovsky, F. (2001). Vps9p is a guanine nucleotide exchange factor involved in vesicle-mediated vacuolar transport. J.Biol.Chem. *274*, 15284-15291.

Hama, H., Tall, G.G., and Horazdovsky, B.F. (1999). Vps9p is a guanine nucleotide exchange factor involved in vesicle-mediated vacuolar protein transport. J Biol Chem *274*, 15284-15291.

Harvey, J. (1964). An unidentified virus which causes the rapid production of tumos in mice. Nature *204*, 1104-1105.

Haubruck, H., Prange, R., Vorgias, C., and Gallwitz, D. (1989). The ras-related mouse ypt1 protein can functionally replace the YPT1 gene product in yeast. EMBO J *8*, 1427-1432.

Hawes, C.R., Brandizzi, F., and Andreeva, A.V. (1999). Endomembranes and vesicle trafficking. Curr Opin Plant Biol *2*, 454-461.

Haynes, L.P., Evans, G.J., Morgan, A., and Burgoyne, R.D. (2001). A direct inhibitory role for the rab3-specific effector, noc2, in ca2+-regulated exocytosis in neuroendocrine cells. J Biol Chem *276*, 9726-9732.

Horazdovsky, B.F., Busch, G., and Emr, S.D. (1994). VPS21 encodes a Rab5-like GTP binding protein that is required for the sorting of yeast vacuolar proteins. EMBO J *13*, 1297-1309.

Horiuchi, H., Giner, A., Hoflack, B., and Zerial, M. (1995). A GDP/GTP exchange-stimulatory activity for the Rab5-RabGDI complex on clathrin-coated vesicles from bovine brain. J Biol Chem *270*, 11257-11262.

Horiuchi, H., Kawata, M., Katayama, M., Yoshida, Y., Musha, T., Ando, S., and Takai, Y. (1991). A novel prenyltransferase for a small GTP-binding protein having a C-terminal Cys-Ala-Cys structure. J Biol Chem *266*, 16981-16984.

Horiuchi, H., Lippe, R., McBride, H.M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997). A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell *90*, 1149-1159.

Hrycyna, C.A. and Clarke, S. (1990). Farnesyl cysteine C-terminal methyltransferase activity is dependent upon the STE14 gene product in Saccharomyces cerevisiae. Mol Cell Biol *10*, 5071-5076.

Hrycyna, C.A., Sapperstein, S.K., Clarke, S., and Michaelis, S. (1991). The Saccharomyces cerevisiae STE14 gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and RAS proteins. EMBO J *10*, 1699-1709.

Huizing, M., Anikster, Y., and Gahl, W. (2000). Hermansky-Pudlak syndrome and related disorders of organelle formation. Traffic *1*, 823-835.

Hume, A.N., Collinson, L.M., Rapak, A., Gomes, A.Q., Hopkins, C.R., and Seabra, M.C. (2001). Rab27a regulates the peripheral distribution of melanosomes in melanocytes. J Cell Biol *152*, 795-808.

Hunziker, W. and Peters, P.J. (1998). Rab17 localizes to recycling endosomes and regulates receptor-mediated transcytosis in epithelial cells. J Biol Chem *273*, 15734-15741.

Hutt, D.M., Da-Silva, L.F., Chang, L.H., Prosser, D.C., and Ngsee, J.K. (2000). PRA1 inhibits the extraction of membrane-bound rab GTPase by GDI1. J Biol Chem *275*, 18511-18519.

Iezzi, M., Escher, G., Meda, P., Charollais, A., Baldini, G., Darchen, F., Wollheim, C.B., and Regazzi, R. (1999). Subcellular distribution and function of Rab3A, B, C, and D isoforms in insulin-secreting cells. Mol Endocrinol *13*, 202-212.

Iwasaki, K., Staunton, J., Saifee, O., Nonet, M., and Thomas, J.H. (1997). aex-3 encodes a novel regulator of presynaptic activity in C.elegans. Neuron *18*, 613-622.

Jedd, G., Mulholland, J., and Segev, N. (1997). Two new YPT GTPases are required for exit from the yeast trans-Golgi compartment. J.Cell Biol. *137*, 563-580.

Jiang, Y. and Ferro-Novick, S. (1994). Identification of the yeast component A: Reconstitution of the geranylgeranyltransferase that modifies Ypt1p and Sec4p. Proc Natl Acad Sci U S A *91*, 4377-4381.

Kahn, A. (1991). Fluoride is not an actiator of the smaller (20-25 KDa) GTP-binding proteins. J.Biol.Chem. *266*, 15595-15597.

Kahn, R.A., Der, C.J., and Bokoch, G.M. (1992). The Ras superfamily of GTP-binding proteins: guidelines on nomenclature. FASEB J. *6*, 2512-2513.

Kamal, A. and Goldstein, L.S. (2000). Connecting vesicle transport to the cytoskeleton. Curr Opin Cell Biol *12*, 503-508.

Kamiya, Y., Sakurai, A., Tamura, S., and Takahashi, N. (1978). Structure of rhodotorucine A, a novel lipopeptide, inducing mating tube formation in Rhodosporidium toruloides. Biochem.Biophys.Res.commun. *83*, 1077-1083.

Khosravi-Far, R., Lutz, R.J., Cox, A.D., Conroy, L., Bourne, J.R., Sinensky, M., Balch, W.E., Buss, J.E., and Der, C.J. (1991). Isoprenoid modification of rab proteins terminating in CC or CXC motifs. Proc Natl Acad Sci U S A *88*, 6264-6268.

Kim, E., Ambroziak, P., Otto, J.C., Taylor, B., Ashby, M., Shannon, K., Casey, P.J., and Young, S.G. (1999). Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells. J Biol Chem *274*, 8383-8390.

Kinoshita, T., Inoue, N., and Takeda, J. (1996). Role of phosphatidylinositol-linked proteins in paroxysmal nocturnal hemoglobinuria pathogenesis. Annu.Rev.Med. *47*, 1-10.

Kinsella, B.T. and Maltese, W.A. (1992). rab GTP-binding proteins with three different carboxyl-terminal cysteine motifs are modified in vivo by 20-carbon isoprenoids. J Biol Chem *267*, 3940-3945.

Kirsten, W. and Mayer, L. (1967). Morphological responses to a murine erythroblastosis virus. J.Natl.Cancer Inst. *39*, 311-335.

Kjeldgaard, M., Nyborg, J., and Clark, B. (1996). The GTP binding motif: variations on a theme. FASEB J *10*, 1347-1368.

Klein, C., Philippe, N., Le Deist, F., Fraitag, S., Prost, C., Durandy, A., Fischer, A., and Griscelli, C. (1994). Partial albinism with immunodeficiency (Griscelli syndrome). J Pediatr *125*, 886-895.

Lazar, T., Gotte, M., and Gallwitz, D. (1997). Vesicular transport: how many Ypt/Rab-GTPases make a eukaryotic cell? Trends Biochem.Sci. *22*, 468-472.

Li, R. and Zheng, Y. (1997). Residues of the Rho family GTPases Rho and Cdc42 that specify sensitivity to Dbl-like guanine nucleotide exchange factors. J Biol Chem *272*, 4671-4679.

Linder, M.E. (2000). Reversible modification of proteins with thioester-linked fatty acids. In Protein Lipidation. F. Tamanoi and D.S. Sigman, eds. (San Diego: Academic Press), pp. 215-240.

Liu, K. and Li, G. (1998). Catalytic domain of the p120 Ras GAP binds to RAb5 and stimulates its GTPase activity. J Biol Chem *273*, 10087-10090.

Liu, L., Dudler, T., and Gelb, M. (1996). Purification of a protein palmitoyltransferase that acts on H-Ras protein and on a C-terminal N-Ras peptide. J.Biol.Chem. *271*, 23269-23276.

Livingston, C.D. and Barton, G.J. (1993). Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation. CABIOS *9*, 745-756.

Lombardi, D., Soldati, T., Riederer, M.A., Goda, Y., Zerial, M., and Pfeffer, S.R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. EMBO J *12*, 677-682.

Luan, P., Balch, W., Emr, S.D., and Burd, C.G. (1999). Molecular dissection of guanine nucleotide dissociation inhibitor function in vivo. Rab independent binding to membranes and role of Rab recycling factors. J.Biol.Chem. *274*, 14806-14817.

Luan, P., Balch, W.E., Emr, S.D., and Burd, C.G. (1999). Molecular dissection of guanine nucleotide dissociation inhibitor function in vivo. Rab-independent binding to membranes and role of Rab recycling factors. J Biol Chem *274*, 14806-14817.

Luan, P., Heine, A., Zeng, K., Moyer, B., Greasely, S., Kuhn, P., Balch, W., and Wilson, I.A. (2000). A new functional domain of guanine nucleotide dissociation inhibitor (a-GDI) involved in Rab recycling. Traffic *1* , 270-281.

Lutcke, A., Parton, R., Murphy, C., Olkkonen, V.M., Dupree, P., Valencia, A., Simons, K., and Zerial, M. (1994). Cloning and subcellular localization of novel rab proteins reveals polarized and cell type specific expression. J Cell Sci *107*, 3437-3438.

MacDonald, I.M., Mah, D.Y., Ho, Y.K., Lewis, R.A., and Seabra, M.C. (1998). A practical diagnostic test for choroideremia. Ophthalmology *105*, 1637-1640.

Madaule, P. and Axel, R. (1985). A novel ras-related gene family. Cell *41*, 31-40.

Maesaki, R., Ihara, K., Shimizu, T., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1999). The structural basis of Rho effector recognition revealed by the crystal structure of human RhoA complexed with the effector domain of PKN/PRK1. Mol.Cell *4*, 793-803.

Mammoto, A., Ohtsuka, T., Hotta, I., Sasaki, T., and Takai, Y. (1999). Rab11BP/Rabphilin-11, a downstream target of rab11 small G protein implicated in vesicle recycling. J Biol Chem *274*, 25517-25524.

Mammoto, A., Sasaki, T., Kim, Y., and Takai, Y. (2000). Physical and functional interaction of rabphilin-11 with mammalian Sec13 protein. Implication in vesicle trafficking. J Biol Chem *275*, 13167-13170.

Martincic, I., Peralta, M.E., and Ngsee, J.K. (1997). Isolation and characterization of a dual prenylated Rab and VAMP2 receptor. J Biol Chem *272*, 26991-26998.

Marzesco, A.M., Galli, T., Louvard, D., and Zahraoui, A. (1998). The rod cGMP phosphodiesterase delta subunit dissociates the small GTPase Rab13 from membranes. J Biol Chem *273*, 22340-22345.

Masuda, E.S., Luo, Y., Young, C., Shen, M., Rossi, A.B., Huang, B.C., Yu, S., Bennett, M.K., Payan, D.G., and Scheller, R.H. (2000). Rab37 is a novel mast cell specific GTPase localized to secretory granules. FEBS Lett *470*, 61-64.

Matern, H., Yang, X., Andrulis, E., Sternglanz, R., Trepte, H.H., and Gallwitz, D. (2000). A novel Golgi membrane protein is part of a GTPase-binding protein complex involved in vesicle targeting. EMBO J *19*, 4485-4492.

McBride, H.M., Rybin, V., Murphy, C., Giner, A., Teasdale, R., and Zerial, M. (1999). Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell *98*, 377-386.

McKiernan, C.J., Brondyk, W.H., and Macara, I.G. (1993). The Rab3A GTPase interacts with multiple factors through the same effector domain. Mutational analysis of cross-linking of Rab3A to a putative target protein. J Biol Chem *268*, 24449-24452.

McLauchlan, H., Newell, J., Morrice, N., Osborne, A., West, M., and Smythe, E. (1998). A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. Curr Biol *8*, 34-45.

Menasche, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulffraat, N., Bianchi, D., Fischer, A., Le Deist, F., and de Saint, B. (2000). Mutations in RAB27A cause Griscelli syndrome associated with haemophagocytic syndrome. Nat Genet *25*, 173-176.

Meresse, S., Gorvel, J.P., and Chavrier, P. (1995). The Rab7 GTPase resides on a vesicular compartment connected to lysosomes. J Cell Sci *108*, 3349-3358.

Merithew, E., Hatherly, S., Dumas, J.J., Lawe, D., Heller-Harrison, R., and Lambright, D. (2001). Structural plasticity of an invariant hydrophobic triad in the switch regions of Rab GTPases is a determinant of effector recognition. J Biol Chem *276*, 13982-13988.

Miaczynska, M., Lorenzetti, S., Bialek, U., Benito-Moreno, R.M., Schweyen, R.J., and Ragnini, A. (1997). The yeast Rab escort protein binds intracellular membranes in vivo and in vitro. J Biol Chem *272*, 16972-16977.

Michaelson, D., Silletti, J., Murphy, C., D'Eustachio, P., Rush, M.G., and Philips, M.R. (2001). Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. J Cell Biol *2001*, 1-111.

Mittal, R., Ahmadian, M.R., Goody, R., and Wittinghofer, A. (1996). Formation of the transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate, and GTPase-activating proteins. Science *273*, 115-117.

Moore, I., Schell, J., and Palme, K. (1995). *Subclass-specific sequence motifs identified in Rab GTPases.* Trends Biochem.Sci. *20*, 10-12.

Moores, S.L., Schaber, M.D., Mosser, S.D., Rands, E., O'Hara, M.B., Garsky, V.M., Marshall, M.S., Pompliano, D.L., and Gibbs, J.B. (1991). Sequence dependence of protein isoprenylation. J Biol Chem *266*, 14603-14610.

Mori, T., Fukuda, Y., Kuroda, H., Matsumura, T., Ota, S., Sugimoto, T., Nakamura, Y., and Inazawa, J. (1999). Cloning and characterization of a novel Rab-family gene, Rab36, within the region at 22q11.2 that is homozygously deleted in malignant rhabdoid tumors. Biochem.Biophys.Res.Commun *254*, 594-600.

Mossessova, E., Gulbis, J.M., and Goldberg, J. (1998). Structure of the guanine nucleotide exchange factor Sec7 domain of human Arno and analysis of the interaction with ARF GTPase. Cell *92*, 415-423.

Mott, H.R., Owen, D., Nietlispach, D., Lowe, P.N., Manser, E., Lim, L., and Laue, E.D. (1999). Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK. Nature *399*, 384-388.

Moya, M., Roberts, D., and Novick, P. (1993). DSS4-1 is a dominant suppressor of sec4-8 that encodes a nucleotide exchange protein that aids Sec4p function. Nature *361*, 460-463.

Moyer, B.D., Allan, B.B., and Balch, W.E. (2001). Rab1 Interaction with a GM130 Effector Complex Regulates COPII Vesicle cis-Golgi Tethering. Traffic *2*, 268-276.

Mukherjee, S., Gurevich, V.V., Jones, J.C., Casanova, J.E., Frank, S.R., Maizels, E.T., Bader, M.F., Kahn, R.A., Palczewski, K., Aktories, K., and Hunzicker-Dunn, M. (2000). The ADP ribosylation factor nucleotide exchange factor ARNO promotes beta-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor desensitization. Proc Natl Acad Sci U S A *97*, 5901-5906.

Mumby, S.M. (2000). G proteins I: Gs and Gi. In GTPases. A. Hall, ed. (Oxford: Oxford University Press), pp. 1-34.

Muniz, M. and Riezman, H. (2000). Intracellular transport of GPI-anchored proteins. EMBO J *19*, 10-15.

Nagano, F., Sasaki, T., Fukui, K., Asakura, T., Imazumi, K., and Takai, Y. (1998). Molecular cloning and characterization of the noncatalytic subunit of the Rab3 subfamily-specific GTPase-activating protein. J Biol Chem *273*, 24781-24785.

Nagelkerken, B., Van Anken, E., van Raak, M., Gerez, L., Mohrmann, K., Van Uden, N., Holthuizen, J., Pelkmans, L., and van der Sluijs, P. (2000). Rabaptin4, a novel effector of the small GTPase rab4a, is recruited to perinuclear recycling vesicles. Biochem J *346 Pt 3*, 593-601.

Nair, J., Muller, H., Peterson, M., and Novick, P. (1990). Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensable carboxy terminal domain. J Cell Biol *110*, 1897-1909.

Nakamura, N., Hirata, A., Ohsumi, Y., and Wada, Y. (1997). Vam2/vps41p and Vam6/Vps39p are components of the a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast Saccharomyces cerevisiae. J Biol Chem *272*, 11344-11349.

Nassar, N., Hoffman, G.R., Manor, D., Clardy, J.C., and Cerione, R.A. (1998). Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP. Nat Struct Biol *5*, 1047-1052.

Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995). The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. Nature *375*, 554-560.

Neuwald, A. (1997). A shared domain between a spindle assembly checkpoint protein and Ypt/Rab-specific GTPase activators. Trends Biochem Sci *22*, 243-244.

Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B., and Zerial, M. (2000). Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. J Cell Biol *151*, 601-612.

Nielsen, E., Severin, F., Backer, J.M., Hyman, A.A., and Zerial, M. (1999). Rab5 regulates motility of early endosomes on microtubules [see comments]. Nat Cell Biol *1*, 376-382.

Nikolova, L., Soman, K., Nichols, J.C., Daniel, D.S., Dickey, B.F., and Hoffenberg, S. (1998). Conformationally variable Rab protein surface regions mapped by limited proteolysis and homology modelling. Biochem J *336 (Pt 2)*, 461-469.

Novick, P. and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. Curr Opin Cell Biol *9*, 496-504.

Nuoffer, C., Davidson, H., Matteson, J., Meinkoth, J., and Balch, W. (1994). A GDP-bound Rab1 inhibits protein export from the endoplasmic reticulum and transport between golgi compartments. J Cell Biol *125*, 225-237.

Nuoffer, C., Wu, S.K., Dascher, C., and Balch, W.E. (1997). Mss4 does not function as an exchange factor for Rab in endoplasmic reticulum to Golgi transport. Mol Biol Cell *8*, 1305-1316.

Oishi, H., Sasaki, T., Nagano, F., Ikeda, W., Ohya, T., Wada, M., Ide, N., Nakanishi, H., and Takai, Y. (1998). Localization of the Rab3 small G protein regulators in nerve terminals and their involvement in Ca2+-dependent exocytosis. J Biol Chem *273*, 34580-34585.

Olkkonen, V.M., Dupree, P., Killisch, I., Lutcke, A., Zerial, M., and Simons, K. (1993). Molecular cloning and subcellular localization of three GTP-binding proteins of the rab subfamily. J Cell Sci *106 (Pt 4)*, 1249-1261.

Olkkonen, V.M. and Stenmark, H. (1997). Role of Rab GTPases in membrane traffic. Int Rev Cytol *176*, 1-85.

Opdam, F.J., Kamps, G., Croes, H.J., van Bokhoven, H., Ginsel, L.A., and Fransen, J.A. (2000). Expression of Rab small GTPases in epithelial Caco-2 cells: Rab21 is apically located GTP-binding protein in polarised intestinal epithelial cells. Eur J Cell Biol *79*, 308-318.

Ossig, R., Laufer, W., Schmitt, H., and Gallwitz, D. (1995). Functionality and specific membrane localization of transport GTPases carrying C-terminal membrane anchors of synaptobrevin-like proteins. EMBO J *14*, 3645-3653.

Ostermeier, C. and Brunger, A.T. (1999). Structural basis of Rab effector specificity: crystal structure of the small G protein Rab3A complexed with the effector domain of rabbphilin-3A. Cell *96*, 363-374.

Otto, J.C., Kim, E., Young, S.G., and Casey, P.J. (1999). Cloning and characterization of a mammalian prenyl protein-specific protease. J Biol Chem *274*, 8379-8382.

Overmeyer, J.H., Wilson, A.L., Erdman, R.A., and Maltese, W.A. (1998). The putative "switch 2" domain of the Ras-related GTPase, Rab1B, plays an essential role in the interaction with Rab escort protein. Mol.Biol.Cell *9*, 223-235.

Pai, E.F., Kabsch, W., Krengel, U., Holmes, K.C., John, J., and Wittinghofer, A. (1989). Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. Nature *341*, 209-214.

Pan, J. and Wessling-Resnick, M. (1998). GEF-mediated GDP/GTP exchange by monomeric GTPases:: a regulatory role for Mg^{2+}? Bioassays *20*, 516-521.

Parada, L., Tabin, C., Shih, C., and Weinberg, R.A. (1982). Human EJ bladder carcinoma oncogene is a homologue of harvey sarcoma virus ras gene. Nature *297*, 474-478.

Park, J.B., Farnsworth, C.C., and Glomset, J.A. (1997). Ca2+/calmodulin causes Rab3A to dissociate from synaptic membranes. J Biol Chem *272*, 20857-20865.

Park, Y., Song, O., Kwak, J., Hong, S., Lee, H., and Nam, H. (1994). Functional complementation of a yeast vesicular transport mutation ypt1-1 by a Brassica napus cDNA clone encoding a small GTP-binding protein. Plant Mol Biol *26*, 1725-1735.

Parmyd, I. and Dallner, G. (1999). In Vivo prenylation of rat protein: modificatio of proteins with penta- and hexaprenyl groups. Arch.Biochem.Biophys. *364*, 153-160.

Peitsch, M.C. (1995). Protein modelling by e-mail. BioTechnology *13*, 658-660.

Peitsch, M.C. (1996). ProdMod and Swiss-Model: internet-based tools for automated comparative protein modelling. Biochem Soc Trans *24*, 274-279.

Pepinsky, R.B., Zeng, C., Wen, D., Rayhorn, P., Baker, D.P., Williams, K.P., Bixler, S.A., Ambrose, C.M., Garber, E.A., Miatkowski, K., Taylor, F.R., Wang, E.A., and Galdes, A. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. J Biol Chem *273*, 14037-14045.

Pereira-Leal, J.B. and Seabra, M.C. (2000). The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. J Mol Biol *301*, 1077-1087.

Perriere, G. and Gouy, M. (1996). WWW-query: an on-line retrieval system for biological sequence banks. Biochimie *78*, 364-369.

Peter, F., Nuoffer, C., Pind, S., and Balch, W. (1994). Guanine nucleotide dissociation inhibitor is essential for Rab1 function in budding from the endoplasmic reticulum and transport through the golgi stack. J Cell Biol *126*, 1393-1406.

Pfeffer, S.R., Dirac-Svejstrup, B., and Soldati, T. (1995). Rab GDP dissociation inhibitor: putting Rab GTPases in the right place. J.Biol.Chem. *270*, 17057-17059.

Pittler, S.J. and Baehr, W. (1991). Identification of a nonsense mutation in the rod photoreceptor cGMP phosphodiesterase beta-sub-unit gene of the rd mouse. Proc Natl Acad Sci U S A *88*, 8322-8326.

Porter, J.A., Ekker, S.C., Park, W.J., von Kessler, D.P., Young, K.E., Chen, C.H., Ma, Y., Woods, A.S., Cotter, R.J., Koonin, E.V., and Beachy, P.A. (1996b). Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. Cell *86*, 21-34.

Porter, J.A., Young, K.E., and Beachy, P.A. (1996a). Cholesterol modification of hedgehog signaling proteins in animal development [see comments] [published erratum appears in Science 1996 Dec 6;274(5293):1597]. Science *274*, 255-259.

Pruyne, D., Schott, D., and Bretscher, A. (1998). Tropomyosin-containing actin cables direct the Myo2-dependent polarized delivery of secretory vesicles in budding yeast. J Cell Biol *143*, 1931-1945.

Raffaniello, R.D., Lin, J., Schwimmer, R., and Ojakian, G.K. (1999). Expression and localization of Rab3D in rat parotid gland. Biochim Biophys Acta *1450*, 352-363.

Ragnini, A., Teply, R., Waldherr, A., Voskova, A., and Schweyen, R.J. (1994). The yeast protein Mrs6p, a homolog of the rabGDI and human choroideremia proteins, affects cytoplasmic and mitochondrial functions. Curr.Genet. *26*, 308-314.

Rak, A., Fedorov, R.F., Alexandrov, K., Albert, S., Goody, R.S., Gallwitz, D., and Scheidig (2000). Crystal structure of the GAP domain of Gyp1p: first insights into interaction with Ypt/Rab proteins. EMBO J *19*, 5105-5113.

Ramalho, J.S., Tolmachova, T., Hume, A., McGuigan, A., Gregory-Evans, C., Huxley, C., and Seabra, M.C. (2001). Chromosomal mapping, gene structure and characterization of the human and murine RAB27B gene. BMC Genet. *2*, 2

Ren, M., Zeng, J., De Lemos-Chiarandini, C., Rosenfeld, M., Adesnik, M., and Sabatini, D.D. (1996). In its active form, the GTP-binding protein rab8 interacts with a stress-activated protein kinase. Proc Natl Acad Sci U S A *93*, 5151-5155.

Richardson, C.J., Jones, S., Litt, R.J., and Segev, N. (1998). GTP hydrolysis is not important for Ypt1 GTPase function in vesicular transport. Mol Cell Biol *18*, 827-838.

Rittinger, K., Taylor, W.R., Smerdon, S.J., and Gamblin, S.J. (1998). Support for shared ancestry of GAPs. Nature *392*, 449

Rittinger, K., Walker, P.A., Eccleston, J.F., Smerdon, S.J., and Gamblin, S.J. (1997). Structure at 1.65 A of RhoA and its GTPase-activating protein in complex with a transition-state analogue [see comments]. Nature *389*, 758-762.

Roberts, R.L., Barbieri, M.A., Pryse, K.M., Chua, M., Morisaki, J.H., and Stahl, P.D. (1999). Endosome fusion in living cells overexpressing GFP-rab5. J Cell Sci *112 (Pt 21)*, 3667-3675.

Rodman, J.S. and Wandinger-Ness, A. (2000). RabGTPases coordinate endocytosis. J.Cell Sci. *113*, 183-192.

Romano, J.D., Schmidt, W.K., and Michaelis, S. (1998). The Saccharomyces cerevisiae prenylcysteine carboxyl methyltransferase Ste14p is in the endoplasmic reticulum membrane. Mol Biol Cell *9*, 2231-2247.

Rosa, J.L. and Barbacid, M. (1997). A giant protein that stimulates guanine nucleotide exchange on ARF1 and Rab proteins forms a cytosolic ternary complex with chlatrin and Hsp70. Oncogene *15*, 1-6.

Rosa, J.L., Casaroli-Marano, R.P., Buckler, A.J., Vilaro, S., and Barbacid, M. (1996). p619, a giant protein related to the chromosome condensation regulator RCC1, stimulates guanine nucleotide exchange on ARF1 and Rab proeins. EMBO J *15*, 4262-4273.

Roth, M.G. (2000). Arf. In GTPases. A. Hall, ed. (Oxford: Oxford University Press), pp. 176-197.

Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M.C., Goody, R., and Zerial, M. (1996). GTPase activity of Rab5 acts as a timer for endocytic membrane fusion [see comments]. Nature *383*, 266-269.

Saitou, N. and Nei, M. (1987). The neighbour-joining method: a new method to reconstruct phylogenetic trees. Mol.Biol.Evol. *4*, 406-425.

Salminen, A. and Novick, P. (1987). A ras-like protein is required for a post-golgi event in yeast secretion. Cell *49*, 527-538.

Sanford, J.C., Pan, Y., and Wessling-Resnick, M. (1995). Properties of Rab5 N-terminal domain dictate prenylation of C-terminal cysteines. Mol.Biol.Cell *6*, 71-85.

Santos, E., Tronick, S., Aaronson, S., Pulciani, S., and Barbacid, M. (1982). T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. Nature *298*, 343-347.

Sapperstein, S.K., Lupashin, V.V., Schmitt, H.D., and Waters, M.G. (1996). Assembly of the ER to Golgi SNARE complex requires Uso1p. J Cell Biol *132*, 755-767.

Sasaki, T., Kaibuchi, K., Kabcenell, A.K., Novick, P., and Takai, Y. (1991). A mammalian inhibitory GDP/GTP exchange protein (GDP dissociatio inhibitor) for smg p25A is active on the yeast SEC4 protein. Mol Cell Biol *11*, 2909-2912.

Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isomura, M., Kuroda, S., and Takai, Y. (1990). Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein. J.Biol.Chem. *265*, 2333-2337.

Schalk, I., Zeng, K., Wu, S.K., Stura, E.A., Matteson, J., Huang, M., Tandon, A., Wilson, I.A., and Balch, W.E. (1996). Structure and mutational analysis of Rab GDP-dissociation inhibitor. Nature *381*, 42-48.

Scheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmuller, L., Lautwein, A., Scmitz, F., and Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science *277*, 333-338.

Scheffzek, K., Ahmadian, M.R., and Wittinghofer, A. (1998). GTPase-activating proteins: helping hands to complement an active site. Trends Biochem Sci *23*, 257-262.

Scheffzek, K., Lautwein, A., Kabsch, W., Ahmadian, M.R., and Wittinghofer, A. (1996). Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interactio with Ras. Nature *384*, 591-596.

Scheffzek, K., Reza, A., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic ras mutants. Science *277*, 333-338.

Schimmoller, F., Simon, I., and Pfeffer, S.R. (1998). Rab GTPases, directors of vesicle docking. J.Biol.Chem. *273*, 22161-22164.

Schmidt, R.A., Schneider, C.J., and Glomset, J.A. (1984). Evidence for post-translational incorporation of a product of mevalonic acid into Swiss 3T3 cell proteins. J.Biol.Chem. *259*, 10175-10180.

Schmitt, H., Wagner, P., Pfaff, E., and Gallwitz, D. (1986). The ras-related YPT1 gene product in yeast: a GTP-binding protein that might be involved in microtubule organization. Cell *47*, 401-412.

Seabra, M.C. (1996). New insights into the pathogenesis of choroideremia: a tale of two REPs [editorial; comment]. Ophthalmic Genet *17*, 43-46.

Seabra, M.C. (1996). Nucleotide dependence of Rab geranylgeranylation. Rab escort protein interacts preferentially with GDP-bound Rab. J Biol Chem *271*, 14398-14404.

Seabra, M.C. (1998). Membrane association and targeting of prenylated Ras-like GTPases. Cell Signal *10*, 167-172.

Seabra, M.C. (2000). Biochemistry of RabGeranylgeranyl Transferase. In Protein Lipidation. F. Tamanoi and D.S. Sigman, eds. (San Diego: Academic Press), pp. 131-154.

Seabra, M.C., Brown, M.S., and Goldstein, J.L. (1993). Retinal degeneration in choroideremia: deficiency of rab geranylgeranyl transferase. Science *259*, 377-381.

Seabra, M.C., Brown, M.S., Slaughter, C.A., Sudhof, T.C., and Goldstein, J.L. (1992). Purification of component A of Rab geranylgeranyl transferase: possible identity with the choroideremia gene product. Cell *70*, 1049-1057.

Seabra, M.C., Goldstein, J.L., Sudhof, T.C., and Brown, M.S. (1992). Rab geranylgeranyl transferase. A multisubunit enzyme that prenylates GTP-binding proteins terminating in Cys-X-Cys or Cys-Cys. J Biol Chem *267*, 14497-14503.

Seabra, M.C., Ho, Y.K., and Anant, J.S. (1995). Deficient geranylgeranylation of Ram/Rab27 in choroideremia. J Biol Chem *270*, 24420-24427.

Seabra, M.C. and James, G.L. (1998). Prenylation assays for small GTPases. Methods Mol Biol *84*, 251-260.

Seals, D.F., Eitzen, G., Margolis, N., Wickner, W.T., and Price, A. (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. Proc Natl Acad Sci U S A *97*, 9402-9407.

Seoighe, C. and Wolfe, K. (1998). Extent of genomic rearrangement after genome duplication in yeast. Proc Natl Acad Sci U S A *95*, 4447-4452.

Shen, F. and Seabra, M.C. (1996). Mechanism of digeranylgeranylation of Rab proteins. Formation of a complex between monogeranylgeranyl-Rab and Rab escort protein. J Biol Chem *271*, 3692-3698.

Shih, T., Papageorge, A., Stokes, P., Weeks, M., and Scolnick, E. (1981). Guanine nucleotide-binding and autophosphorylation ctivities associated with the p21 src protein of Harvey murine sarcoma virus. Nature *287*, 686-691.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion [see comments]. Nature *394*, 494-498.

Siniossoglou, S., Peak-Chew, S., and Pelham, R.B. (2000). Ric1p and Rgp1p form a complex that catalyses nucleotide exchange on Ypt6p. EMBO J *19*, 4885-4894.

Smeland, T.E., Seabra, M.C., Goldstein, J.L., and Brown, M.S. (1994). Geranylgeranylated Rab proteins terminating in Cys-Ala-Cys, but not Cys- Cys, are carboxyl-methylated by bovine brain membranes in vitro. Proc Natl Acad Sci U S A *91*, 10712-10716.

Soldati, T., Riederer, M.A., and Pfeffer, S.R. (1993). Rab GDI: a solubilizing and recycling factor for rab9 protein. Mol Biol Cell *4*, 425-434.

Soldati, T., Shapiro, A., Dirac-Svejstrup, B., and Pfeffer, S.R. (1994). Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. Nature *369*, 76-78.

Sonnhammer, E.E.L., Eddy, S.R., Birney, E., Bateman, A., and Durbin, R. (1998). Pfam:multiple sequence alignments and HMM-profiles of protein domains. Nucleic Acids Research *26*, 320-322.

Sonnhammer, E.E.L., Eddy, S.R., and Durbin, R. (1997). pfam:multiple sequence alignments and HMM-profiles of protein domains. Proteins *28*, 405-420.

Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol *149*, 901-914.

Spence, R. and Casey, P. (2001). Mechanism of catalysis by protein farnesyltransferase. In Protein Lipidation. F. Tamanoi and D.S. Sigman, eds. (San Diego: Academic Press), pp. 1-19.

Stankovic, T., Byrd, P.J., Cooper, P.R., McConville, C.M., Munroe, D.J., Riley, J.H., Watts, G.D., Ambrose, H., McGuire, G., Smith, A.D., Sutcliffe, A., Mills, T., and Taylor, A.M. (1997). Construction of a transcription map around the gene for ataxia telangiectasia: identification of at least four novel genes. Genomics *40*, 267-276.

Stenmark, H. and Olkkonen, V.M. (2001). The Rab GTPase family. Genome Biol *2*, REVIEWS3007

Stenmark, H., Parton, R., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994). Inhibition of Rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J *13*, 1287-1296.

Stenmark, H., Valencia, A., Martinez, O., Ullrich, O., Goud, B., and Zerial, M. (1994). Distinct structural elements of rab5 define its functional specificity. EMBO J *13*, 575-583.

Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M. (1995). Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion. Cell *83*, 423-432.

Stewart, M., Kent, H.M., and McCoy, A.J. (1998). Structural basis for molecular recognition between nuclear transport factor 2 (NTF2) and the GDP-bound form of the Ras-family GTPase Ran. J.Mol.Biol. *277*, 635-646.

Stinchcombe, J.C., Barral, D.C., Mules, E.H., Booth, S., Hume, A.N., Machesky, L.M., Seabra, M.C., and Griffiths, G.M. (2001). Rab27a is required for regulated secretion in cytotoxic t lymphocytes. J Cell Biol *152*, 825-834.

Strom, M., Vollmer, P., Tan, T.J., and Gallwitz, D. (1993). A yeast GTPase-activating protein that interacts specifically with a member of the Ypt/Rab family. Nature *361*, 736-739.

Stroupe, C. and Brunger, A.T. (2001). Crystal structures of a Rab protein in its inactive and active conformations. J Mol Biol *304*, 585-598.

Swank, R.T., Jiang, S., Reddington, M., Conway, J., Stephenson, D., McGarry, M., and Novak, E. (1993). Inherited abnormalities in platelet organelles and platelet formation and associated altered expression of low molecular weight guanosine triphosphate-binding proteins in the mouse pigment mutant gunmetal. Blood *81*, 2626-2635.

Swank, R., Novak, E., McGarry, M., Rusiniak, M., and Feng, L. (1998). Mouse models of Hermansky Pudlak syndrome: a review. Pigment Cell Res. *11*, 60-80.

Tall, G.G., Hama, H., DeWald, D.B., and Horazdovsky, B.F. (1999). The phosphatidylinositol 3-phosphate binding protein Vac1p interacts with a Rab GTPase and a Sec1p homologue to facilitate vesicle-mediated vacuolar protein sorting. Mol Biol Cell *10*, 1873-1889.

Tan, T.J., Vollmer, P., and Gallwitz, D. (1991). Identification and partial purification of GTPase-activating proteins from yeast and mammalian cells that preferentially act on Ypt1/Rab1 proteins. FEBS LETTERS *291*, 322-326.

Terry, K., Long, S., and Beese, L.S. (2001). Structure of Protein farnesyltransferase. In Protein Lipidation. F. Tamanoi and D.S. Sigman, eds. (San diego: Academic Press), pp. 20-47.

Thoma, N.H., Iakovenko, A., Kalinin, A., Waldmann, H., Goody, R.S., and Alexandrov, K. (2000). Allosteric regulation of substrate binding and product release in geranylgeranyl transferase type II. Biochemistry *40*, 268-274.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research *25*, 4876-4882.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673-4680.

Tisdale, E.J. (1999). A Rab2 mutant with impaired GTPase activity stimulates vesicle formation from pre-Golgi intermediates. Mol Biol Cell *10*, 1837-1849.

Tisdale, E.J., Bourne, J.R., Khosravi-Far, R., Der, C.J., and Balch, W.E. (1992). GTP-binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. J Cell Biol *119*, 749-761.

Tolmachova, T., Ramalho, J.S., Anant, J.S., Schultz, R.A., Huxley, C.M., and Seabra, M.C. (1999). Cloning, mapping and characterization of the human RAB27A gene. Gene *239*, 109-116.

Tong, L.A., de Vos, A.M., Milburn, M.V., and Kim, S.H. (1991). Crystal structures at 2.2 Å resolution of the catalytic domains of normal ras protein and an oncogenic mutant complexed with GDP. J.Mol.Biol. *217*, 503-516.

Touchot, N., Chardin, P., and Tavitian, A. (1987). Four additional members of he ras gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. Proc Natl Acad Sci U S A *84*, 8210-8214.

Touchot, N., Zahraoui, A., Vielh, E., and Tavitian, A. (1989). Biochemical properties of the YPT-related rab1B protein. Comparison with rab1A. FEBS Lett *256*, 79-84.

Tuvim, M.J., Adachi, R., Chocano, J.F., Moore, R.H., Lampert, R.M., Zera, E., Romero, E., Knoll, B.J., and Dickey, B.F. (1999). Rab3D, a small GTPase, is localized on mast cell secretory granules and translocates to the plasma membrane upon exocytosis. Am J Respir Cell Mol Biol *20*, 79-89.

Udenfriend, S. and Kodukula, K. (1995). How glycosylphosphatidylinositol-anchored membrane proteins are made. Annu Rev Biochem *64*, 563-591.

Ueda, T., Kikuchi, A., Ohga, N., Yamamoto, J., and Takai, Y. (1990). Purification and characterization from bovine brain cytosol of a novel regulatory protein inhibiting the dissociation of GDP from and the subsequent binding of GTP to rhoB p20, a ras p21-like GTP-binding protein. J Biol Chem *265*, 9373-9380.

Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994). Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange [see comments]. Nature *368*, 157-160.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R.G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol *135*, 913-924.

Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L.A., Kaibuchi, K., Sasaki, T., Takai, Y., and Zerial, M. (1993). Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins. J Biol Chem *268*, 18143-18150.

Valencia, A., Chardin, P., Wittinghofer, A., and Sander, C. (1991). The ras protein family: evolutionary tree and role of conserved amino acids. Biochemistry *30*, 4637-4648.

van den Hurk, J.A., Schwartz, M., van Bokhoven, H., van de Pol, T.J., Bogerd, L., Pinckers, A.J., Bleeker-Wagemakers, E.M., Pawlowitzki, I.H., Ruther, K., Ropers, H.H., and Cremers, F.P. (1997). Molecular basis of choroideremia (CHM): mutations involving the Rab escort protein-1 (REP-1) gene. Hum Mutat *9*, 110-117.

van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B., and Mellman, I. (1992). The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. Cell *70*, 729-740.

Vetter, I.R., Arndt, A., Kutay, U., Gorlich, D., and Wittinghofer, A. (1999a). Structural view of the Ran-Importin beta interaction at 2.3 A resolution. Cell *97*, 635-646.

Vetter, I.R., Linnemann, T., Wohlgemuth, S., Geyer, M., Kalbitzer, H.R., Herrmann, C., and Wittinghofer, A. (1999b). Structural and biochemical analysis of Ras-effector signaling via RalGDS. FEBS Lett. *451*, 175-180.

Vetter, I.R., Nowak, C., Nishimoto, T., Kuhlmann, J., and Wittinghofer, A. (1999). Structure of a Ran-binding domain complexed with Ran bound to a GTP analogue: implications for nuclear transport. Nature *398*, 39-46.

Vollmer, P., Will, E., Scheglmann, D., Strom, M., and Gallwitz, D. (1999). Primary structure and biochemical characterization of yeast GTPase-activating proteins with substrate preference for the transport GTPase Ypt7p. Eur J Biochem *260*, 284-290.

Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obaishi, H., Matsuura, Y., and Takai, Y. (1997). Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. J Biol Chem *272*, 3875-3878.

Walch-Solimena, C., Collins, R.N., and Novick, P. (1997). Sec2p mediates nucleotide exchange on sec4p and is involved in polarized delivery of post-Golgi vesicles. J.Cell Biol. *137*, 1495-1509.

Waldherr, A., Ragnini, A., Schweyen, R.J., and Boguski, M.S. (1993). MRS6-yeast homolog of the choroideremia gene. Nat Genet *3*, 193-194.

Walworth, N.C., Goud, B., Kabcenell, A.K., and Novick, P.J. (1989). Mutational analysis of SEC4 suggests a cyclical mechanism for the regulation of vesicular traffic. EMBO J *8*, 1685-1693.

Wang, W., Sacher, M., and Ferro-Novick, S. (2000). TRAPP stimulates guanine nucleotide exchange on Ypt1p. J Cell Biol *151*, 289-295.

Wang, Y., Sugita, S., and Sudhof, T.C. (2000). The RIM/NIM family of neuronal C2 domain proteins. Interactions with Rab3 and a new class of Src homology 3 domain proteins. J Biol Chem *275*, 20033-20044.

White, M.A., Nicolette, C., Minden, A., Polverino, A., Van Aeist, L., Marin, M., and Wigler, M.H. (1995). Multiple ras functions can contribute to mammalian cell transformation. Cell *80*, 533-541.

Wichmann, H., Hengst, L., and Gallwitz, D. (1992). Endocytosis in yeast: evidence for the involvement of a small GTP-binding protein (Ypt7p). Cell *71*, 1131-1142.

Wilson, A.L., Erdman, R.A., and Maltese, W.A. (1996). Association of Rab1B with GDP-dissociation inhibitor (GDI) is required for recycling but not initial membrane targeting of the Rab protein. J.Biol.Chem. *271*, 10932-10940.

Wilson, A.L., Erdman, R.A., and Maltese, W.A. (1996). Association of Rab1B with GDP-dissociation inhibitor (GDI) is required for recycling but not initial membrane targeting of the Rab protein. J Biol Chem *271*, 10932-10940.

Wilson, A.L. and Maltese, W.A. (1993). Isoprenylation of Rab1B is impaired by mutations in its effector domain. J Biol Chem *268*, 14561-14564.

Wilson, S.M., Yip, R., Swing, D.A., O'Sullivan, T.N., Zhang, Y., Novak, E.K., Swank, R.T., Russell, L.B., Copeland, N.G., and Jenkins, N.A. (2000). A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. Proc Natl Acad Sci U S A *97*, 7933-7938.

Wittinghofer, A. (1998). Caught in the act of the switch-on. Nature *394*, 317-320.

Wolda, S.L. and Glomset, J.A. (1988). Evidence for modification of lamin B by a product of mevalonic acid. J Biol Chem *263*, 5997-6000.

Wolfe, K. and Shields, D. (1997). Molecular evidence for an ancient duplication of the entire yeast genome. Nature *387*, 708-713.

Wong, A.C., Shkolny, D., Dorman, A., Willingham, D., Roe, B.A., and McDermid, H.E. (1999). Two novel human RAB genes with near identical sequence each map to a telomere-associated region: the subtelomeric region of 22q13.3 and the ancestral telomere band 2q13. Genomics *59*, 326-334.

Wu, S.K., Luan, P., Matteson, J., Zeng, K., Nishimura, N., and Balch, W.E. (1998). Molecular role for the Rab binding platform of guanine nucleotide dissociation inhibitor in endoplasmic reticulum to Golgi transport. J Biol Chem *273*, 26931-26938.

Wu, S.K., Zeng, K., Wilson, I.A., and Balch, W. (1996). Structural insights into the function of the Rab GDI superfamily. Trends Biochem Sci *21*, 472-476.

Wu, X., Rao, K., Bowers, M., Copeland, N., Jenkins, N., and Hammer, J. (2001). Rab27a enables myosinVa-dependent melanosomes capture. J Cell Sci *114*, 1091-1100.

Wurmser, A.E., Sato, T.K., and Emr, S.D. (2000). New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. J Cell Biol *151*, 551-562.

Xiao, G.H., Shoarinejad, F., Jin, F., Golemis, E.A., and Yeung, R.S. (1997). The tuberous sclerosis 2 gene product, tuberin, functions as a Rab5 GTPase activating protein (GAP) in modulating endocytosis. J Biol Chem *272*, 6097-6100.

Yokoyama, K. and Gelb, M. (2001). Protein Geranylgeranyltransferase Type 1. In Protein Lipidaion. F. Tamanoi and D.S. Sigman, eds. (San Diego: Academic Press), pp. 106-130.

Yoshie, S., Imai, A., Nashida, T., and Shimomura, H. (2000). Expression, characterization, and localization of Rab26, a low molecular weight GTP-binding protein, in the rat parotid gland. Histochem Cell Biol *113*, 259-263.

Young, S.G., Amboziak, P., Kim, E., and Clarke, S. (2000). Postisoprenylation protein processing: CXXX (CaaX) endoproteases and isoprenylcysteine caboxyl methyltransferase. In Protein Lipidation. F. Tamanoi and D.S. Sigman, eds. (San diego: Academic Press), pp. 155-213.

Zacchi, P., Stenmark, H., Parton, R.G., Orioli, D., Lim, F., Giner, A., Mellman, I., Zerial, M., and Murphy, C. (1998). Rab17 regulates membrane trafficking through apical recycling endosomes in polarized epithelial cells. J Cell Biol *140*, 1039-1053.

Zerial, M. and Huber, L.A. (1995). Guidebook to the Small GTPases (Oxford: Sambrook& Tooze publications at Oxford University Press).

Zerial, M. and McBride, H.M. (2001). Rab proteins as membrane organisers. Nature Rev- Mol.Cell Biol *2*, 107-119.

Zhang, H., Seabra, M.C., and Deisenhofer, J. (2000). Crystal structure of Rab geranylgeranyltransferase at 2.0 A resolution. Structure Fold Des *8*, 241-251.

Zhong, H., Raman, N., Mickleton-Young, L.A., Atkinson, S.J., and Quilliam, L.A. (1999). Loop 6 of RhoA confers specificity for effector binding, stress fiber formation, and cellular transformation. J.Biol.Chem. *274*, 4551-4560.

ofuka 5/11/01
4000500