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Chemical biology of protein lipidation: semi-synthesis and structure elucidation of prenylated RabGTPases

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Rab/Ypt guanosine triphosphatases (GTPases) represent a family of key membrane traffic regulators in eukaryotic cells. For their function Rab/Ypt proteins require double modification with two covalently bound geranylgeranyl lipid moieties at the C-terminus. Generally, prenylated proteins are very difficult to obtain by recombinant or enzymatic methods. We generated prenylated RabGTPases using a combination of chemical synthesis and protein engineering. This semi-synthesis depends largely on the availability of functionalized prenylated peptides corresponding to the proteins' native structure or modifications. We developed solution phase and solid phase strategies for the generation of peptides corresponding to the prenylated C-terminus of Rab7 GTPase in preparative amounts enabling us to crystallize the mono-prenylated Ypt1:RabGDI complex. The structure of the complex provides a structural basis for the ability of RabGDI to inhibit the release of nucleotide by Rab proteins and a molecular basis for understanding a RabGDI mutant that causes mental retardation in humans.

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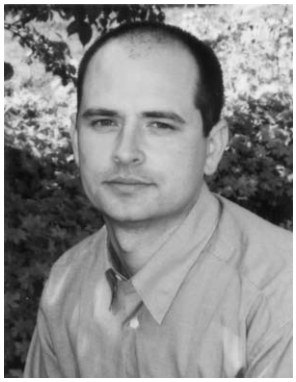
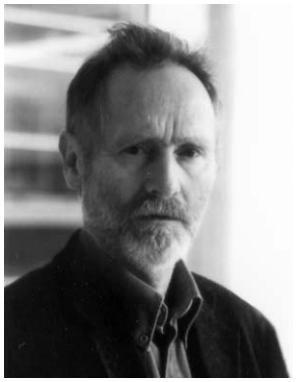


Alexey Rak



Roger S. Goody

Herbert Waldmann

Rab proteins

Rab (Ras gene from rat brain) proteins have emerged in the last decade as key regulators of intracellular vesicular transport in both the exocytic and endocytic pathways in eukaryotic cells. They belong to the Ras superfamily of guanosine triphosphate phosphatases (GTPases), forming the largest branch with more than 60 members in humans.[1] In yeast, the protein family analogous to the Rabs is called Ypt (yeast protein transport). While most Rab proteins are ubiquitous, some are cell type- or tissue-specific. Five Rab-specific sequences (RabF motifs) and four Rab subfamily-specific regions (RabSF) have been defined and used to identify other members of the family and Rab subfamily, respectively. Many proteins of the family have been localized to various compartements of the secretory and endocytic pathways, and it appears that they are involved in multiple stages of the transport processes, with their most prominent and well-characterized role in targeting, docking and fusion of transport vesicles with their acceptor membranes. As examples, the identified roles of 10 Rab proteins are summarized in Table 1.

In vivo and *in vitro* studies have led to the recognition that Rab proteins cycle between a GTP-bound active and a GDP-bound inactive form resulting in dramatic changes in their three-dimensional structure, in common with the properties of other members of the Ras superfamily.[16,17] In addition to this GTPase cycle, Rab GTPases cycle between membrane and cytosolic localizations (Fig. 1). Membrane association function, which is crucial for Rab, is achieved *via* two geranylgeranyl groups covalently attached to two cysteines at the C-terminus. The post-translational attachment of the geranylgeranyl lipid moieties requires the interplay of the GTPase with Rab geranylgeranyl-transferase (RabGGTase) and its accessory protein REP (Rab escort protein).[18] Thereby Rab/Ypt and RabGGTase (the latter is a heterodimer consisting of an α- and an β-subunit) and REP form a ternary complex. Transfer of geranylgeranyl groups from geranylgeranyl pyrophosphate occurs in this complex,

Table 1 Roles of some Rab GTPases in the exocytic or endocytic pathway in mammalian cells

Rab GTPases	Transport step[a]	Ref.
Rab1 and Rab2	ER to Golgi	2
Rab1b	*Cis* to medial Golgi	3
Rab3	Secretion of hormones and neurotransmitters	4
Rab6	Intra-Golgi; Golgi to ER	5; 6
Rab5A/B/C	Assembly of clathrin-coated vesicles (CCVs); homotypic fusion between endosomes	7; 8; 9
Rab7	EE to LE; EE to Lyosome	10
Rab9	LE to TGN	11
Rab4	Recycling of receptors from endosomes to PM	12
Rab11	TGN to PM; recyling to the Golgi; recycling from endosomes to the PM	13; 14; 15

[a] Exocytic pathway compartements: ER, endoplasmatic reticulum; Golgi; TGN, *trans*-Golgi network; secretory vesicles; PM, plasma membrane. Endocytic pathway compartements: EE, early endosome; LE, late endosome.

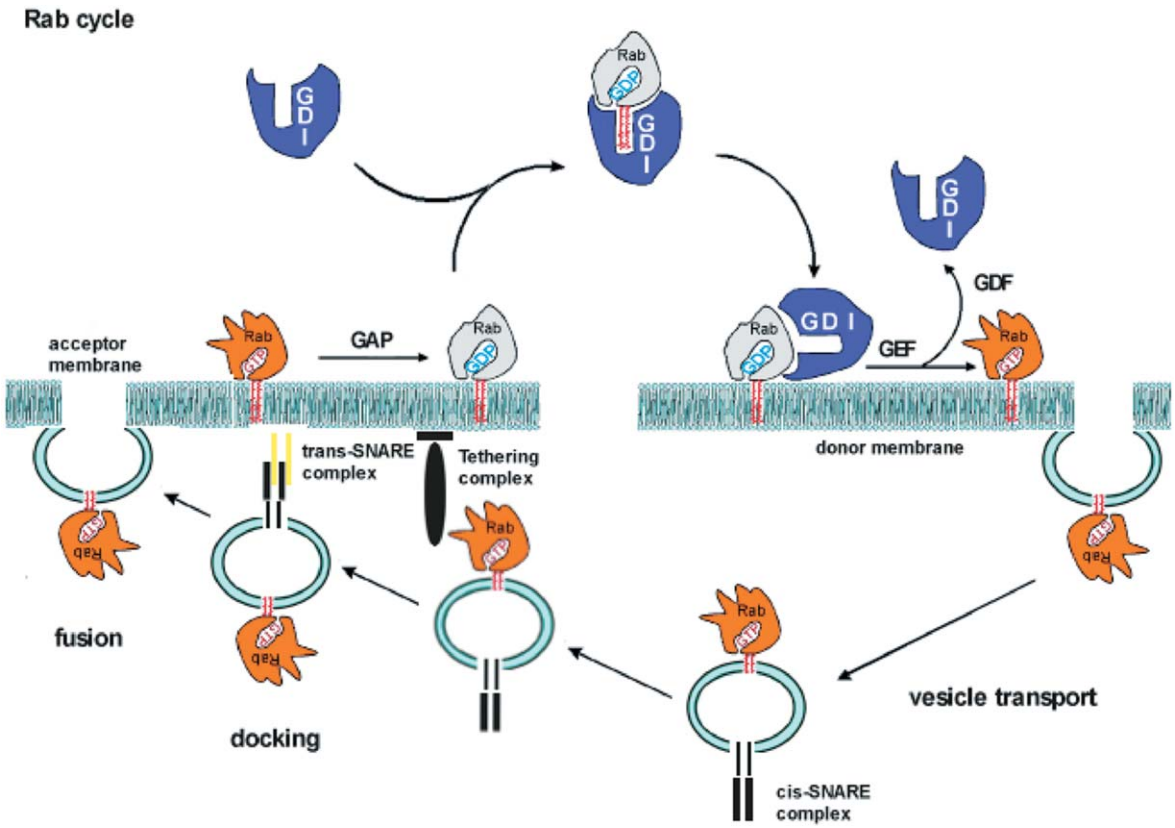


Fig. 1 Rab cycle and vesicular transport between the donor and acceptor membrane: Rab alternates between an inactive GDP-bound form (grey) and an active GTP-bound form (orange); GDI extracts Rab from the membrane and maintains it in the cytosol; the upstream regulators GAP and GEF regulate nucleotide exchange; SNAREs play an essential role in vesicle docking and fusion events.

yielding the modified Rab/Ypt proteins in a complex with the REP. Subsequently, REP escorts the Rab proteins to their donor membranes. For the reversible attachment of Rab proteins to membranes in subsequent cycles of activity, the well-characterized GDP dissociation inhibitor (GDI) and the less well-characterized GDI dissociation factor (GDF) are thought to be important. Prenylated Rab proteins are transported between the intracellular membranes and the cytosol by GDI, which interacts preferentially with the GDP-bound form of the proteins. GDI solubilizes the otherwise insoluble modified proteins and also inhibits GDP dissociation. At present it is not clear whether all known GDI isoforms have the same functions in mammalian cells.[19] In view of the central role of the RabGTPases and their interaction partners including REP and GDI proteins in cellular biogenesis, it is not surprising that loss or impairment of their activity is involved in a number of human diseases.[20] Deficiency in REP, for example, leads to choroideremia, a genetic disease leading to retinal degeneration and blindness.[21] According to a presently accepted model, GDF displaces GDI from the Rab-GDI complex, recruits the Rab protein to the donor membrane and localizes Rab to its correct cellular compartement. For the endosomal Rabs, Rab5, Rab7 and Rab9 such a GDI displacement factor has been identified.[22] However, details of the mechanism of specific localization of Rabs to specific membranes are unclear, although it is commonly accepted that the hypervariable C-termini of the Rab proteins are crucially involved.

In addition to these regulators of Rab activity, guanine nucleotide exchange factors (GEFs), important for the nucleotide exchange, and GTPase activating proteins (GAPs), crucial for GTP hydrolysis, are known. Analysis of Ypt/Rab GEFs reveals that they appear to be components of large protein assemblies, making their role in the regulation of Rab nucleotide cycling complex. In some cases, GEFs exist in a complex with an effector. Currently different models for the role of GEF/effector complexes exist with regard to their function of controlling the conversion of Rab to the active GTP-bound state.[23]

There is little definitive evidence in support of a role of Rab proteins in vesicle formation, but their role in subsequent transport and, in particular, targeting is well established. Thus, a number of studies imply that Rab proteins are responsible for vesicle tethering and docking to the correct target membrane (see Fig. 1). The final step of vesicular transport, vesicle fusion, is assigned to a family of membrane proteins conserved throughout evolution termed SNAREs, defined as SNAP (soluble NSF attachment protein) receptors, with NSF meaning *N*-ethylmaleimide-sensitive factor. For the fusion process, recognition of vesicular v-SNAREs (SNAREs that reside on vesicles) and target t-SNAREs (SNAREs that reside on target membranes) is essential. In the initial stage, the vesicular *cis*-SNARE complex is activated, leading to the formation of *trans*-SNARE complexes. Before the docking and the final fusion step take place, a relatively weak association between the vesicle and a large tethering complex has to occur, this being mediated by the Rab proteins.

The number of Rab proteins and the number of Rab interacting proteins reflect the complexity of the Rab-controlled network. There is a large number of open questions regarding Rab itself and its various effectors and regulators, specifically with regard to their mechanism of action, localization and specificity.

Rab structures

To shed more light on the mechanism of Ypt/Rab proteins and for the understanding of vesicular transport processes in cells at the molecular level, knowledge of the structures of Rabs/Ypts and their interacting proteins is a basic and essential requirement. The structures of Rab3A, Rab 6,[24] Ypt51, Sec4, Ypt7[25] and Rab7[26] have been determined. The overall structures of these three GTP-bound proteins are similar to each other and are also similar to that of Ras. The structures of Sec4, Ypt7 and Rab7 have been determined in the GTP and in the GDP-bound form, revealing that the conformational changes between the two forms are restricted mainly to the two switch regions common

to all proteins of the Ras superfamily (Switch I and Switch II).[27] The elucidation of the structure of Rab3A and its effector Rabphilin in a complex reveals the basis for the effector binding specificity of different Rabs.[28] Activated Rab3A, which plays an important role in the regulation of neurotransmitter release, reversibly recruits Rabphilin-3A to synaptic vesicles. The crystal structure of Rab3A in the active state bound to the effector domain of Rabphilin-3A reveals that Rabphilin-3A makes contact to Rab3A in two distinct areas: firstly to the conserved Switch I and II regions already mentioned, and secondly to a distinct pocket proposed to be the Rab complementary-determining region (RabCDR) mediating specific interactions with downstream effectors. The sequences of the RabCDR of other Rab proteins are different, suggesting this to be the origin of high specificity for Rab/effector interactions. The Rab3A–Rabphilin-3A complex is the only known Rab:effector structure. In view of the numerous interactions in which Rab proteins participate, the lack of structural information is striking.

The structures of the identified Ypt/Rab interacting proteins, GDI,[29] Rab geranylgeranyltransferase (RabGGTase)[30] and a Ypt GAP, the catalytic domain of Gyp1p,[31] have been solved. Concerning the binding of interacting proteins, the five conserved RabF motifs are proposed to be mainly recognized by general Rab regulators such as GDI and REP, whereas specific interactions with a certain subset of Rabs additionally require RabSF regions. Crystallization and X-ray diffraction analysis of the complex between RabGGTase and its accessory protein REP-1 [32] and of the ternary complex of mono-prenylated Rab7:REP-1:RabGGTase[33] provide access to a better understanding of the interaction of RabGGTase with its protein and lipid substrates.[34] In addition, the structure of mono-prenylated Rab in complex with REP-1 has been solved, extending the understanding of the Rab prenylation machinery and possibly providing new directions for the developement of therapies for treatment of choroideremia, a hereditary degenerative disease of the retina leading to blindness.[35] The structure reveals important information on the manner in which prenylated Rabs interact with REP. Rab7 interacts with the Rab binding platform of REP-1 *via* an extended interface involving the Switch I and II regions, which become ordered in the complex, in contrast to their disordered state when GDP is bound to Rab7 in the absence of other protein binding partners. The C-terminus of the REP-1 molecule functions as a mobile lid covering a conserved hydrophobic patch on the surface of REP-1 that coordinates the C-terminus of the Rab protein.

Although all members of the RabGTPase protein family must be geranylgeranylated to fulfil their biological role, structures of prenylated Rab proteins were not available until very recently.[33–35] This reflects the complexity of the Rab prenylation pathway and technical problems associated with the generation of prenylated Rab proteins. In addition, most methods for the engineering of post-translationally lipidated proteins suffer from low yields and inflexibility with respect to the lipid modification. Studies of Rab protein function require efficient methods to provide preparative amounts of prenylated Rab proteins.

Semi-synthesis of prenylated Rab proteins

Progress in protein ligation methods has widened the field of semi-synthetic protein synthesis to otherwise inaccessible post-translationally-modified proteins.[36–40] The combination of organic synthesis and intein-mediated *in vitro* protein ligation gives access to modified proteins which are generally not easily accessible *via* other, for example, enzymatic, processes.[20,41–45] The intein-mediated *in vitro* protein ligation methods allow the C-terminal attachment of large recombinant proteins to peptides generated by organic synthesis. This semi-synthetic approach provides the necessary platform to generate precisely modified proteins in substantial amounts in order to facilitate their application in protein-consuming methods such as protein crystallography or NMR spectroscopy. In order to study Rab functions in intracellular vesicular transport processes, the post-translationally-modified C-terminus of Rab has to be available. For the semi-synthesis of prenylated proteins, the development of an efficient synthetic method for hexapeptides **1** and **2** (Fig. 2) corresponding to the C-terminal amino acid sequence of the native Rab7 (A^{203}ESCSC) protein, with the exception of replacement of A^{203} by cysteine, is crucial.

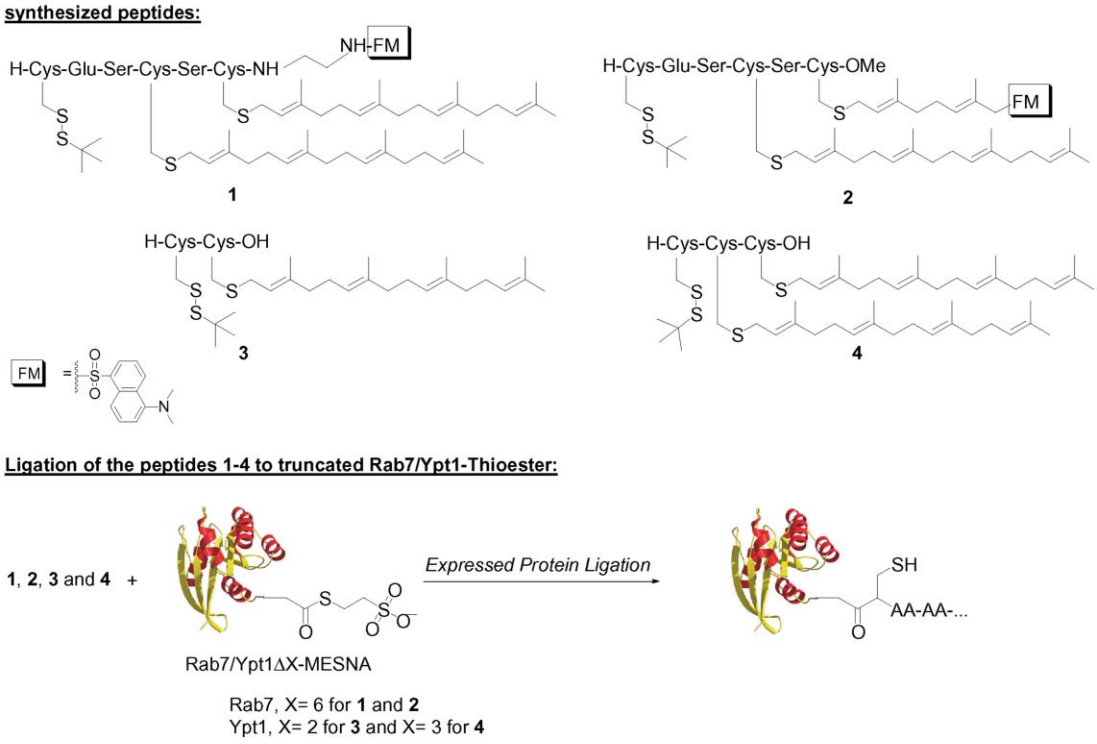


Fig. 2 Ligation of the fluorescently labeled and geranylgeranylated hexapeptides **1**, **2**, mono- and doubly-prenylated peptide **3** and **4** to Rab7 or Ypt1, that are truncated by the number of amino acids corresponding to the amino acid sequence of the peptides, by *Expressed Protein Ligation*.

In order to allow tracking of ligated protein during protein purification, a fluorescence marker [FM; here, the dansyl (5-(dimethylamino)-1-naphthalene sulfonyl) moiety] is required that can be connected to the peptides directly at the C-terminus (hexapeptide **1**) or at the prenyl side chain (hexapeptide **2**), since the native, post-translationally introduced methyl ester might influence the interaction of Rab proteins with other proteins. The semi-synthesis of the geranylgeranylated Rab7 protein *in vitro* bearing the fluorescence marker at the side chain of an amino acid of the C-terminus has also been realized.[46,47] Thereby the fluorescent mono- and doubly-prenylated Rab7 proteins display near-native properties providing a unique tool for studying the Rab prenylation mechanism.[47]

The functionalized Rab7 C-terminal peptides **1** and **2** can be successfully coupled to the thioester tagged Rab7/Ypt1 protein by *in vitro* protein ligation.[48] For the ligation, the technique of *Expressed Protein Ligation* (EPL), an extension of Kent and Dawson's native chemical ligation,[49] was applied. This technique requires a cysteine at the N-terminus of the peptide, which later attacks the C-terminal thioester of the protein to afford, after S–N acyl transfer, a protein with a native peptide link at the site of ligation.[50,51] The semi-synthesis of prenylated Rab proteins by means of EPL is generally applicable to different Rabs and different peptides and is capable of producing relatively large amounts of pure and homogeneous protein material. In order to simplify the preparation of suitable amounts of prenylated peptides, readily accessible mono-prenylated dipeptide **3** and doubly-prenylated tripeptide **4** were made available, providing ready access to preparative amounts of prenylated protein after their ligation to Ypt1Δ2 and Ypt1Δ3, respectively. These Rab proteins carrying a single prenyl group as well as the doubly-modified species are expected to be useful for a number of studies on Rab functions at the molecular level.

Synthesis of the prenylated Rab C-terminus

A synthesis of the functionalized peptides mimicking the Rab7 C-terminus needs to address the selective incorporation of the acid labile geranylgeranyl moiety to biologically relevant cysteine residues and the formation of the C-terminal methyl ester as transesterification and diketopiperazine formation have to be avoided. Considering these limitations the desired functionalized peptides were made available *via* a block coupling method (Fig. 3) with dipeptide **6** serving as the building block for all designed peptides. For the synthesis of the desired hexapeptide **1**, dipeptide **5** was reacted with dansyl-ethylenediamine yielding dipeptide **7**, which after deprotection subsequently was elongated with dipeptide **6** to give the corresponding tetrapeptide **8**.

The tetrapeptide was then elongated with the corresponding N-terminal dipeptide to yield the protected peptide **9**. After deprotection of the orthogonal protection groups, hexapeptide **1** was obtained. Generally, the solution phase peptide synthesis is suitable for the synthesis of larger amounts of prenylated peptides that are required for a variety of biological applications. In particular, the synthesis of mono-prenylated dipeptide **3** can easily be realized *via* a peptide coupling in solution. For the synthesis of the double-prenylated tripeptide **4** a solid phase peptide synthesis proved to be the better synthesis strategy. In general, solid phase peptide synthesis provides a faster and more flexible access to modified Rab-peptides.

In order to synthesize the prenylated peptides on the solid phase, the selective incorporation of the prenyl group has to be ensured. The use of prenylated cysteine derivatives as building blocks for the solid phase peptide synthesis provides an elegant synthesis strategy. The use of pre-synthezised prenylated cysteines avoids on-resin prenylation, which requires a large excess of prenyl halide and an extensive protecting group strategy as was performed previously for farnesylated peptides.[52,53] Fig. 4 shows the synthesis of the highly functionalized double-prenylated hexapeptides **1** and **2**. As a linker for connecting the peptides to the polymeric support, the oxidation-sensitive hydrazide linker **10** is employed allowing for cleavage of the peptide from the resin as a methyl ester for hexapeptide **2** and as an amide for hexapeptide **1**. The synthesis of the prenylated cysteines is based on the *S*-alkylation of cysteine with the appropriate prenyl chloride[54] followed by Fmoc protection of the amine function. The solid phase synthesis of the resin-bound tetrapeptide **12** can be performed by attaching the corresponding amino acid derivatives to the resin under standard solid phase peptide synthesis conditions. The tetrapeptide **12** is then elongated to the resin-bound hexapeptide **13** by coupling with the corresponding N-terminal dipeptide. The selected protecting groups for the side chains and the amino function have to be removed under relatively mild conditions that will leave the geranylgeranyl groups intact. The final cleavage from the resin and deprotection of the N-terminal amino function yielded hexapeptide **2** featuring the native methyl ester at its terminus. Hexapeptide **1** was obtained using dansylated ethylenediamine as the nucleophile for the cleavage reaction.

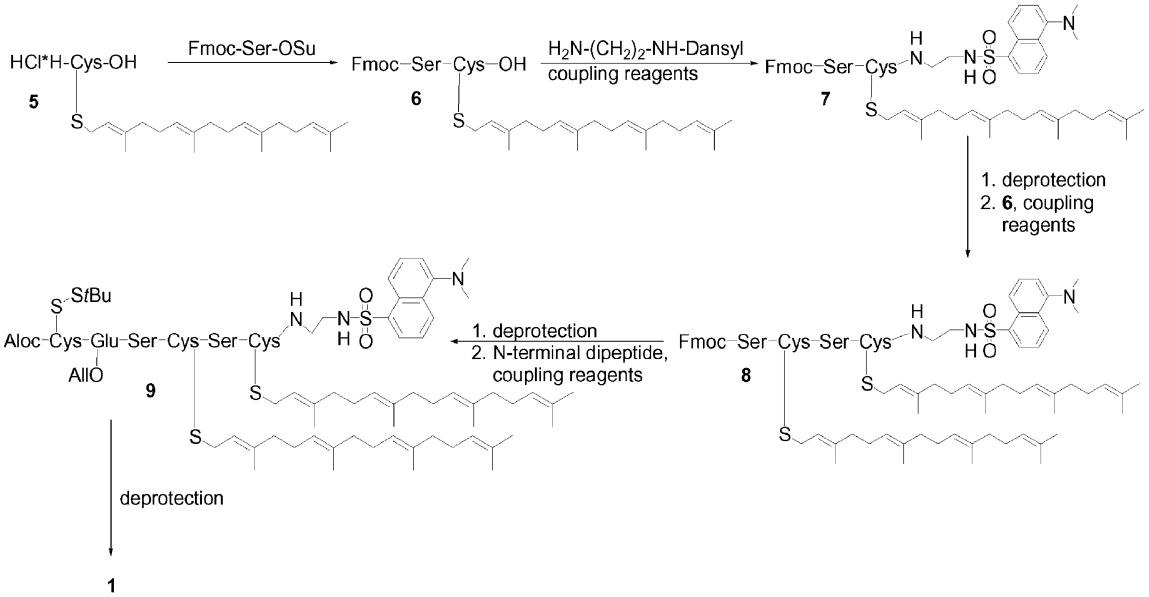


Fig. 3 Solution phase synthesis of the prenylated hexapeptide **1** corresponding to the C-terminus of human Rab7.

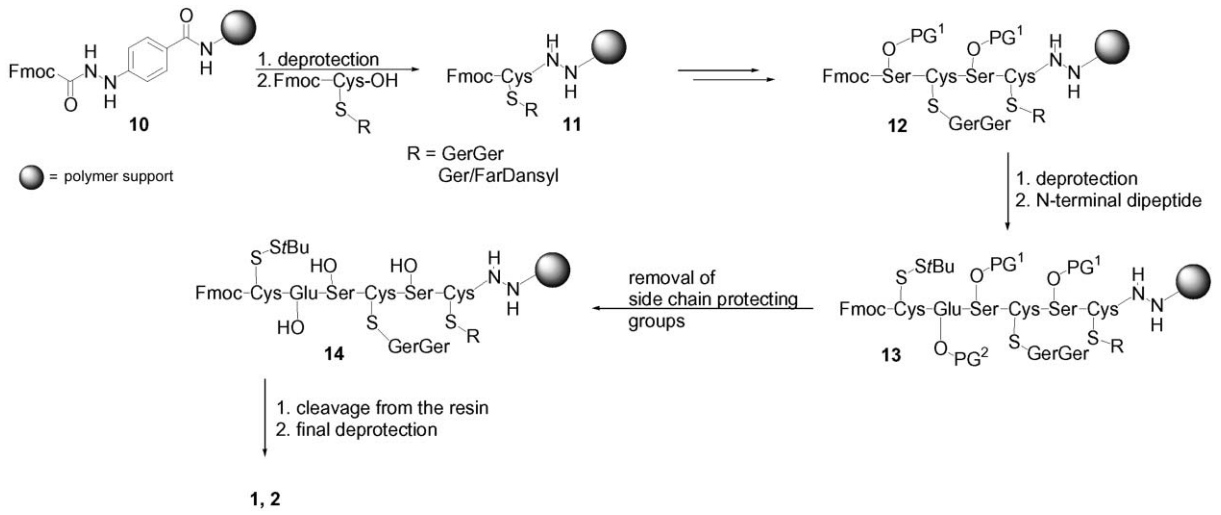


Fig. 4 Solid phase peptide synthesis of Rab7 C-terminal peptides with an additional fluorescent marker.

Peptides prepared by the methods described have been used for the synthesis of prenylated Rab proteins by the EPL strategy (see above). One of the most important applications of these proteins has been in structural studies of complexes between the lipidated proteins and partner molecules, an example of which is described briefly below. An extensive description can be found in ref. 55.

Crystallization of mono- and doubly-geranylgeranylated Ypt1:GDI protein complexes

For structural studies on prenylated Rab proteins, several different members of the Rab family from yeast and from mammalian sources were examined before finally concentrating on the yeast Rab protein Ypt1.[55] For the preparation of the mono- and di-prenylated proteins, it was truncated by two or three residues, respectively, and genetically fused to an intein domain and a chitin binding domain. After expression in *E. coli*, the protein was immobilized on a chitin-agarose column, which was eluted with a high concentration of a thiol reagent to cleave the fusion protein at the end of the truncated Rab protein, producing it with a C-terminal thioester. This was then ligated to the cysteine-containing dipeptide **3** or tripeptide **4** with geranylgeranyl groups on the C-terminal cysteine or (for the tripeptide) two cysteine residues. This led to native mono-prenylated Ypt1 or to di-prenylated Ypt1 with a cysteine instead of an asparagine at position 201 (*i.e.* three resdiues from the C-terminus). The ligation reaction was successful when carried out in one of three different detergents out of a total of *ca.* 80 tested (generally cetyltrimethylammonium bromide was used). After the addition of GDI and removal of detergent, a 1 : 1 complex could be isolated by gel filtration and crystallized to give crystals which diffracted X-rays in both cases to high resolution (1.5 and 1.4 Å, respectively). The structure of the complex of mono-prenylated Ypt1 and GDI is shown in Fig. 5.

The single prenyl group is sufficient to confer binding of Ypt1 to GDI and the obtained complex is homogeneously prenylated. Ypt1 contacts primarily the highly conserved Rab binding platform of GDI comprising the Switch I and II regions which are sensitive towards the nucleotide-bound state of GTPases. This explains the observed preference of GDI for the GDP-bound conformation of Rab proteins (Fig. 5).[55] Interactions of Ypt1 and GDI mainly rely on the contact of the last C-terminal 20 amino acid residues of Ypt1 including the hydrophobic isoprenoid with the apex of GDI domain I down to domain II which terminates in a hydrophobic pocket harbouring the geranylgeranyl moiety. The C-terminus binding

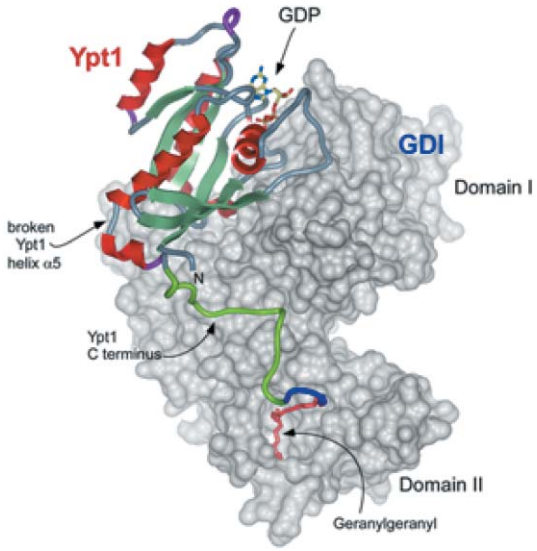


Fig. 5 Structure of the complex of mono-prenylated Ypt1 and GDI: ribbon representation of Ypt1 (α-helices in red and β-sheets in blue) bound to RabGDI, consisting of domain I and domain II, is displayed as a grey molecular surface. The C-terminus is displayed as a green worm. The C-terminal residues 199–205 are disordered and were modelled and displayed as a blue worm. The isoprenoid moiety (red) is displayed in a ball-and-stick representation. The GDP is shown in the nucleotide binding pocket in a ball-and-stick and space-filling representation, respectively.

region (CBR) induces a 90° turn in the C-terminus of Ypt1 enabling the geranylgeranylated C-terminus to coordinate to GDI *via* a hydrophobic patch (coloured in yellow in Fig. 6). Various mutation studies reveal that this patch is crucial for an efficient coordination.[56,57] Mutations in this area lead to erroneous Rab membrane extraction and delivery supporting the functional importance of the lipid binding sites identified in the Ypt1:GDI complex structure. The main role of the CBR is to direct the Ypt1 C-terminus containing the lipid moiety to the isoprenoid binding site on domain II.

The C-terminus of the Rab proteins determines their targeting to distinct subcellular compartments and their recognition by membrane-bound receptors which are responsible for the extraction and removal of Rab proteins.[58,59] The C-terminus of Ypt1 in the complex with GDI is coordinated in a deep cavity formed by hydrophobic residues of GDI domain II (Fig. 6). The formation of this hydrophobic pocket is the result of a conformational change upon complex formation. Owing to the fact that the hydrophobic cavity is limited in space, the question

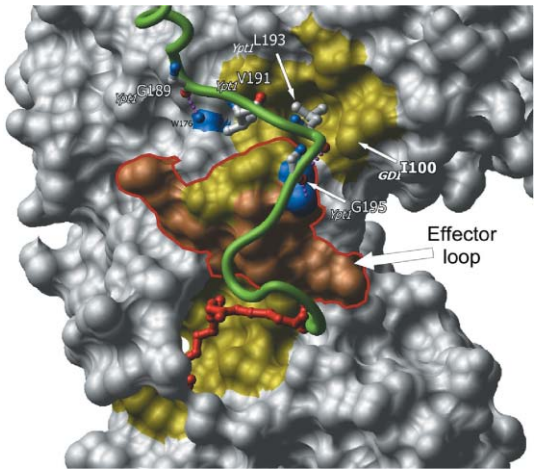


Fig. 6 Localization of the mono-prenylated C-terminus of Ypt1 in complex with GDI with the CBR, the effector loop and the lipid binding site of RabGDI. The RabGDI molecule is displayed as a grey molecular surface and the hydrophobic residues involved in binding of the C-terminus are coloured in yellow. The C-terminus of the Ypt1 molecule is displayed as a green worm and the residues and geranylgeranyl group (red) involved in interaction with RabGDI are displayed in ball-and-stick representation. The I100 that is mutated to proline (on GDI) in non-syndromic mental retardation is marked with a white arrow.

as to where the second isoprenoid is buried arises. A hydrophobic surface groove in close vicinity to the identified cavity on domain II seems to be the most likely alternative of accommodating the second geranylgeranyl group.

Structure elucidation of the complex of mono-prenylated Ypt1 and GDI revealed that non-polar residue $_{GDI}$I100 is located in the C-terminus coordinating region forming a hydrophobic patch. Mutations of $_{GDI}$I100 results in reduced Rab extraction from the membranes which finally leads to mental retardation in humans.[57] In general, mutations in members of the RabGDI/REP family serving as multifunctional regulators of the Rab family of GTP binding proteins lead to abnormalities including progressive retinal degradation (choroideremia) in humans by mutations in REP-1.[26]

Considering this C-terminal coordination, which is conserved between members of the Ypt/Rab and RabGDI/REP families, a similar arrangement is present in the Rab7:REP-1 complex.[26] In summary, the RabGDI/REP family is defined by conserved structural elements involved in complexation of prenylated RabGTPases that have been conserved throughout the evolution of eukaryotic organisms.

Conclusions

The strategy for the preparation of semi-synthetic proteins *via Expressed Protein Ligation* provides a powerful option to obtain access to post-translationally lipid-modified proteins in large quantities. For this semi-synthesis the availability of prenylated peptides is crucial. Therefore the development of a suitable synthesis strategy for these peptides has been realized. In this way the modified Rab proteins were made available in amounts sufficient for structure elucidation, which is crucial for the development of a better understanding of Rab function on the molecular level.

Abbreviations

GAP, GTPase activating protein; GDF, GDI displacement factor; GDI, GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; GTPase, guaninetriphosphate phosphatase; NSF, *N*-ethyl-maleimide-sensitive fusion protein; Rab, Ras-like (protein) from rat brain; RabCDR, Rab complementary-determining region; RabGGTase, Rab geranylgeranyltransferase; RabF, Rab family; RabSF, Rab subfamily; Ras, rat adeno sarcoma; REP, Rab escort protein; Sec, secretory protein; SNAP, soluble NSF attachment protein; SNARE, soluble NSF attachment protein receptor; Ypt, yeast protein transport.

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