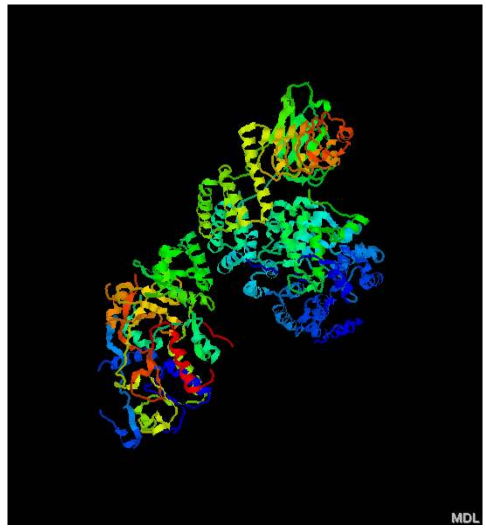
Please note these web pages are part of an assignment for a graduate course in Advanced Biochemistry and Molecular Biology BCMB8010 at the University of Georgia. Questions should be directed to K. Smith at kns@uga.edu.

Rab Geranylgeranyltransferase

Introduction (Abstract)



Prenylation of proteins is a prevalent post-translational modification in eukaryotic organisms. It confers a lipophilic nature to the modified protein via a covalent attachment of a polyisoprenoid group, typically a farnesyl (C15) or a geranylgeranyl (C20), to the sulfhydryl group of a cysteine residue near the carboxyterminus (1,2). In some proteins, modifications such as these are essential for accurate protein interactions as well as the correct membrane localization. Key signaling proteins such as $p21^{ras}$, of which the oncogenic forms are involved in 20-30% of all human cancers as well as various G-proteins require prenylation for function (5,8). G-proteins of the Rab family are of extreme importance in intracellular trafficking, and double geranylgeranylation of these proteins is essential for biological activity. Isoprenoid groups are transferred to cellular proteins by the small family of protein prenyltransferases. Farnesyltransferase (FTase) and geranylgeranytransferase I (GGTase I) are members of this group that recognize the CAAX prenylation signal. A third member, geranylgeranyltransferase II (GGTase II) or Rab geranylgeranyltransferase (RabGGTase) recognizes two C-terminal cysteines instead of the CAAX box, and requires the chaperon, Rab escort protein (REP-1) for recognition of substrate proteins and delivery of newly prenylated proteins to the target membrane after release from RabGGTase (5).
Focus on Rab prenylation increased with the observation that Hermansky-Pudlak syndrome (HPS) and the X-linked choroideremia (CHM) disease are a result of deficiences in Rab prenylation caused by various mutations in the genes encoding the enzyme complex (5).
The protein crystal structure of an isoprenoid bound RabGGTase-REP-1 complex at 2.7 Å indicated that the catalytic RabGGTase exists as a tightly associated α/β heterodimer of 68 kDa and 48 kDa respectively, and the active site, located on the β subunit has a zinc ion, and becomes occupied by

the isoprenoid moiety. The Rab binding platform is located on the 75 kDa REP-1 protein which exhibits protein-protein interaction with the α helix over an unusually small contact area. This is observed in very few proteins and is remarkably similar to that observed with the HIV gp120 envelope interaction with the CD4 receptor (33).

Background

Prenylation is a widespread post-translational modification that confers a lipophilic nature to proteins by the covalent addition of a 15-carbon farnesyl ($C_{15}H_{25}$) or more commonly, a 20-carbon geranylgeranyl ($C_{20}H_{33}$) isoprenoid unit via a thioether linkage (1, 2, 3). As many as 2% of cellular proteins may be modified with an isoprenoid unit and the attached lipid is essential for biological activity of the protein (4).

Protein prenylation was first observed in fungi in the late 1970s and early 1980s, and the first evidence that several proteins in animal cells were modified by isoprenylation was provided by Schmidt and others using Swiss 3T3 cells in 1984. Isoprenoid modified proteins were detected in several other cell types, and it was then shown that different proteins were either modified by a farnesyl group or they were geranylgeranylated (4, 5, 6, 7).

Protein prenyltransferases

Prenylation is carried out by the small family of protein prenyltransferases which all modify cysteine residues at or near the C-terminus of intracellular proteins, and three distinct enzymes can be differentiated based on the isoprenoid substrate and the protein sequence that is recognized (4, 8). The first two enzymes to be identified were protein farnesyltransferase (FTase[1]) and protein geranylgeranyltranserase I (GGTase-I). FTase attaches a farnesyl moeity to proteins such as Ras GTPases, nuclear lamins and fungal mating factors, while a geranylgeranyl group is attached to such protein substrates as heterotrimeric G-proteins by GGTase-I.

Both transferases recognize the CAAX box motif, where A refers to an aliphatic amino acid and X may be any amino acid, but is typically alanine, glutamine, methionine or serine for FTase and leucine or phenylalaninefor GGTase-I (4,9). Isoprenylation is usually the first in a series of post-

[1]Abbreviations: FTase - farnesyltransferase; GGTase - geranylgeranyltransferase; RabGGTase - Rab geranylgeranyltransferase; REP - Rab Escort protein; G-protein / GTPase - guanine nucleotide binding protein; Rabs-Rab proteins, GGpp-geranylgeranylpyrophospate.

translational modifications, and most prenylated proteins undergo further modifications. In general, after prenylation, the terminal tri-peptide is cleaved by the proteolytic action of an endonuclease, and the prenyl cysteine residue, now at the carboxy terminus has its free carboxyl group methyl esterified by a methyltransferase (4, 8).

Rab Geranylgeranyltransferase (EC.2.5.1.60)

The third prenyltransferase was first purified in 1991 from bovine brain cytosol and established as a novel prenyltranferase after differentiation from the other two previously described enzymes FTase and GGTase-I using chromatographic techniques and kinetic analyses (10,11). Two chromatographically separable components initially designated A and B were distinguished.

Structural and functional data led to the assignment of the B component, now referred to as Rab Geranylgeranyltransferase (RabGGTase) or protein geranylgeranyltranferase II (GGTase-II) as the catalytic unit, and the A component as the Rab-Escort protein (REP) (12, 13).

RabGGTase shares many characteristics with FTase and GGTase-I. It is a heterodimeric enzyme with tightly associated alpha and beta subunits analogous to the corresponding subunits of the other prenyltransferases, and association of both subunits of the dimer is required for isoprenoid transfer. Unlike the other enzymes however, this enzyme is inactive on its own and requires the additional chaperone-like component, REP for function (14, 15).

Another dissimilarity is evident in the protein substrates of the enzymes. Proteins that will be modified by FTase or GGTase-I terminate in the carboxy-terminal CAAX box motif, but most substrates of RabGGTase contain a double cysteine motif instead, usually in one of the forms - XXCC, XCXC or CCXX or (XCCX in plants) (11, 16, 35). In general, those that terminate in CXC are methylated while those ending with CC are not. (15).

Another contrast to the CAAX protein prenyltansferases which can prenylate peptides, if the CAAX box is present, is the inability of RabGGTase to do so. The actual substrate for the enzyme is a protein involved in a complex with REP (4 , 15). RabGGTase is also unique in that it carries out double prenylation, that is, modification at both cysteine residues, and each isoprenoid transfer is independently carried out. Additionally, although the order of the addition of the lipid moeity to acceptor residues is not fixed, the N-terminal cysteine is normally preferred (17).

Table 1: Properties of Protein prenyltransferases (Adapted from Casey and Seabra, 1996)

Property	**CAAX prenyltransferases**		**RabGGTase (GGTase-II)**
	FTase	**GGTase-I**	
Subunit composition	48 kDa (α)	48 kDa (α)	60kDa (α)
	46 kDA (β)	43 kDA (β)	38 kDA (β)
S.cerevisiae genes	*RAM*2 (α)	*RAM*2 (α)	*BET*4 (α)
	*RAM*1 (β)	*CDC43* (β)	*BETI2* (β)
Metal requirements	Zn^{2+},Mg^{2+}	Zn^{2+}	Zn^{2+}?,Mg^{2+}
Isoprenoid substrate	FPP	GGPP	GGPP
Protein substrates	Ras, nuclear lamins, transducin γ subunit,fungal mating factors	Rho, Rac, most trimeric G-protein γ subunit	Rep-Rab complex
Protein substrate motif	-CAAX X=M,S,Q,A,F	-CAAX X=L,F	Double Cys motifs (-CC,-CXC, CCXX, others)

Substrates : Rab Proteins

Many extracellular signals are transmitted across the cell membrane to changes in the levels of intracellular second messengers by means of GTP-binding proteins (G-proteins) interaction. The Ras superfamily of proteins is a large group of cytoplasmic G-proteins that are involved in intracellular signaling. Five large families - Ras, Rab, Rho/Rac, Arf and Ran are classified on the basis of structural and functional homologies (18, 19, 20). These proteins are characterized by their

GDP and GTP binding and are referred to as small GTPases due to their GTP- hydrolyzing activity, monomeric structure and relatively low molecular weight (19).

RabGGTase prenylates the Rab family (Ypt/Sec4 proteins in lower eukaryotes and plants) of small (21-25 kDa) proteins, which has at least 60 members. Rabs are the only known substrates for RabGGTase, and they are present in all eukaryotes, where they are essential for cell function (16, 35).

The evolutionarily conserved Rab proteins are usually ubiquitously expressed and regulate key transport pathways, while the less conserved Rabs tend to be found operating in specialized pathways in selected cell types (12).

Rab proteins are intrinsically hydrophilic and the post-translational lipidation is the means by which they are able to reversibly associate to the cytoplasmic leaflet of organelle membranes and vesicles. This membrane targeting is essential for Rab function and each Rab protein is specifically targeted to a specific subset of intracellular membranes. The precise localization of Rab proteins is required as they act as molecular on-off switches, cycling between inactive GDP-bound and active GTP-bound states inducing a conformational change in the GTPase which leads to the mediation of vesicular transport in endocytic and secretory pathways (20, 21, 22). Consistent with this is the localization of Rab proteins in membranes of the Golgi complex, endosomes and synaptic vesicles. Due to this role in proper membrane localization as well as its function in mediating specific protein-protein interactions, prenylation is crucial in vital cellular processes such as signal transduction and intracellular trafficking pathways. Protein prenylation has other noteworthy functions including cytoskeletal organization and cell cycle control. It has also been implicated in protein-protein interactions and an example is given by the association of RAM-GDP dissociation inhibitor (RabGDI) protein with specific Rab proteins for example. It has even been suggested that prenylation plays a role in the plant response systems such as those activated by pathogen attack and heat stresses (4, 16).

Mechanism

All known Rabs that have been tested are substrates of RabGGTase and prenylation occurs in several steps. After a soluble Rab protein is synthesized, it is bound by the REP preferentially in the GDP-bound form, presented to the RabGGTase and a tight ternary complex is formed. It is then that geranylgeranyl transfer to the Rab proteins occurs (14, 23, 24). After both geranylgeranyl (GG) groups are transferred, the binding of an additional geranylgeranyl moiety to the β subunit destabilizes the complex by weakening the binding of the product by approximately 10-fold, and RabGGTase dissociates from the di-prenylated REP-Rab complex. The Rab-GG is now hydrophobic and remains complexed to the REP for transport to the target. Free REP is then released for another round of geranylation. REP is an absolute requirement for even one round of catalysis as RabGGTase itself does not bind Rab proteins (14 23, 24).

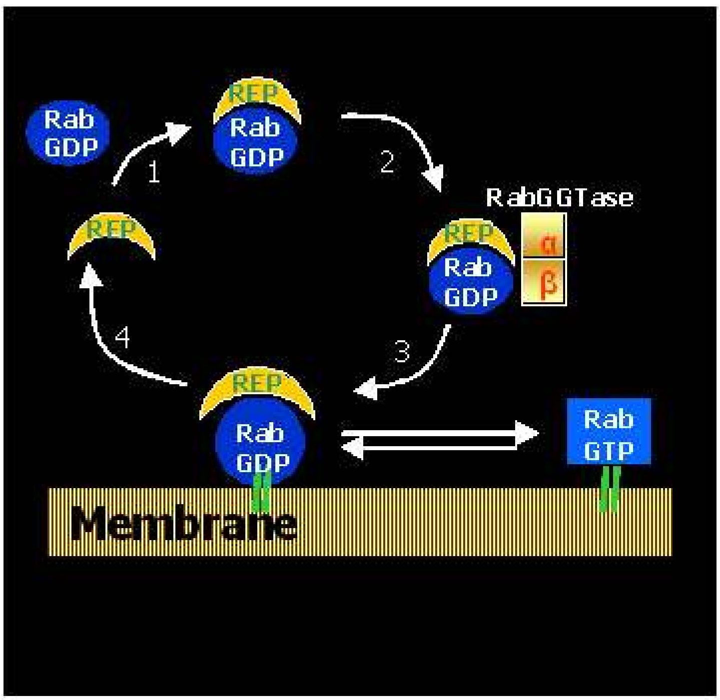


Fig. 1 Intrinsically soluble Rab proteins require prenylation for attachment to target membranes.(1) A complex forms between REP and newly synthesized Rabs.(2) The complex is the substrate for RabGGTase.(3) REP delivers the newly prenylated Rab to the membrane and (4) is then recycled to bind other Rabs.
Adapted from Seabra *et al.*,2002.

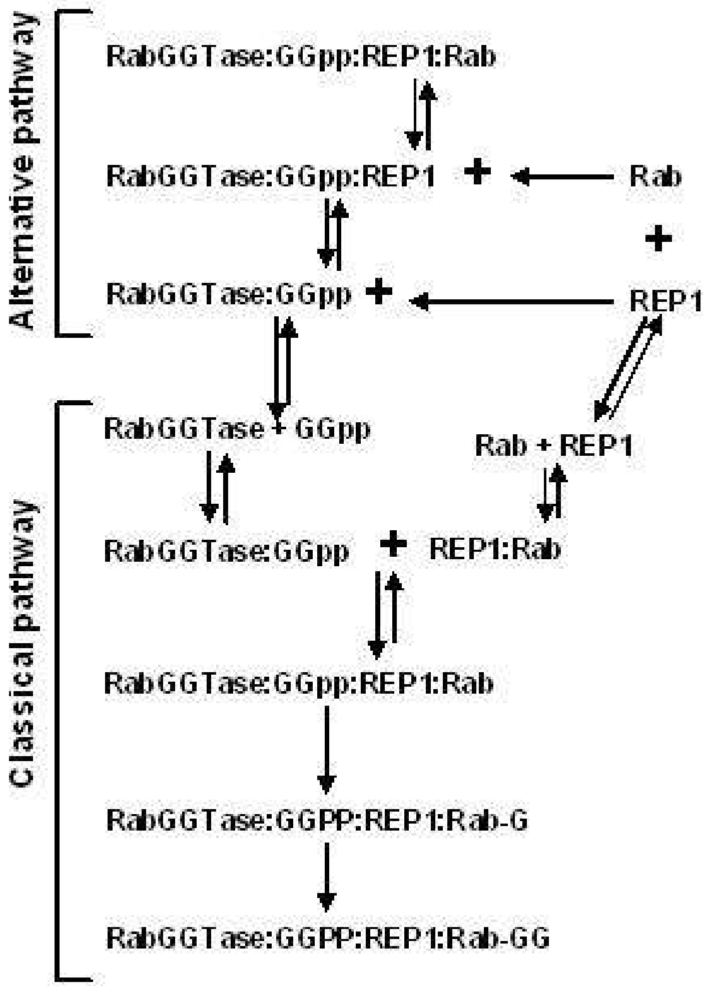
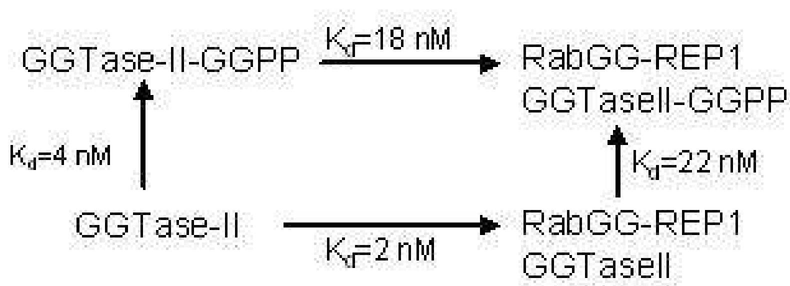


Fig. 2. Diagrammatic representations of possible pathways in binding of Rabs by the REP chaperone. In the classical pathway, the Rab:REP complex binds to RabGGTase that is already bound to the isoprenoid substrate. Alternatively, the REP may complex with RabGGTase, and then facilitate the binding of the Rab protein.
Adapted from Thoma *et al.*, 2001.

Three roles exist for the phosphoisoprenoid moiety in the catalytic cycle of RabGGTase. Firstly, it is the isoprenoid donor in the prenylation reaction, and second it is an allosteric activator, facilitating tighter substrate binding. In the presence of the geranylgeranyl unit, which is already bound to the enzyme, the affinity of RabGGTase for the substrate increases, as is illustrated by the dissociation constant (K_d=2nM) in Scheme 1. Finally, it enables substrate release by increasing the dissociation rate of the product (14, 23, 24)



Scheme 1. Mechanistic scheme fillustrating the effects of isoprenoid binding on the affinity of RabGGTase for its substrate.
Adapted from Thoma *et al.,2001b*.

The protein and lipid substrate specificity of RabGGTase II is strict. Only Rab proteins are prenylated, and the isoprenoid group is obtained only from geranygeranylpyrophosphate and no stable complex is formed with farnesylpyrophosphate nor geranyldiphosphate (23, 24).

Purification and expression of RabGGTase

RabGGTase is a soluble protein that has been characterized at the molecular level in lower eukaryotic and mammalian systems and various methods have been used in its expression and purification.

Van Bokhoven and others isolated and sequenced the coding sequences for the α and β subunits of the human enzyme (genes: α - pVL-RabGGTα and β subunit - pVL-RabGGTβ, American Type

Culture Collection Nos. 87154 and 87155 respectively) , and fluorescence *in situ* hybridization was used to map the α subunit of to human chromosome 14q11.2, and the β subunit to chromosome 1p31(25, 26).

In yeast, MAD2 is the counterpart of the α subunit, and the counterpart for the β subunit is BET2. Purification and characterization of the mammalian protein has been carried out by various groups using electrophoresis and chromatography. Recombinant bovine Rab3A and canine Rab1A were expressed in *Escherichia coli (E. coli)* and purified for use as acceptors of geranylgeranyl groups. Biological activity was determined by measuring the transfer of the geranylgeranyl unit from GGPP to Rab3A using radiolabeled isotopes. The B component was purified using ammonium sulphate precipitation, ion exchange chromatography and two gel filtration steps separated by a hydrophobic interaction chromatography step. The A component was partially purified using similar techniques combined with dialysis (12, 27, 28, 29).

Additionally, each subunit of the mammalian RabGGTase has been expressed separately using baculoviral systems. Limitations in this method were evident however, as each subunit could only be expressed individually, and the protein expression was dependent on viral efficiency and the practical limitations of the expression system in general. Genes used as templates in PCR amplification cloned have also been overexpressed in *E. coli* and after purification using a glutathione S-transferase tag, the specific activity and affinity of the expressed RabGGTase proteins were demonstrated in prenylation assays in which the transfer of ^{3}H-labeled prenyl groups from labeled geranylgeranylpyrophosphate to Rabs expressed was measured in *E. coli* . Additionally, in-vitro prenylation experiments which produced pure prenylated Rab7:REP-1 complex also illustrated that the expressed RabGGTase was biologically active (12, 27, 28, 29).

The α subunit gene coded for a 567 amino acid protein and the β subunit protein was 331 amino acids. The mammalian proteins are highly conserved, and 91 and 95% sequence identity were observed between the corresponding α and β subunits of rat Rab GGTase which was cloned previously. (25, 30). The corresponding protein for the α subunit (BET4) from *Saccharomyces cerevisiae* shows a lower sequence identity (50%) however, and consisted of only 290 amino acids (25).

There are two ubiquitously expressed REPs in mammalian cells showing approximately 75% sequence identity. REP-1 is the product of the choroideremia gene on the X chromosome, and the

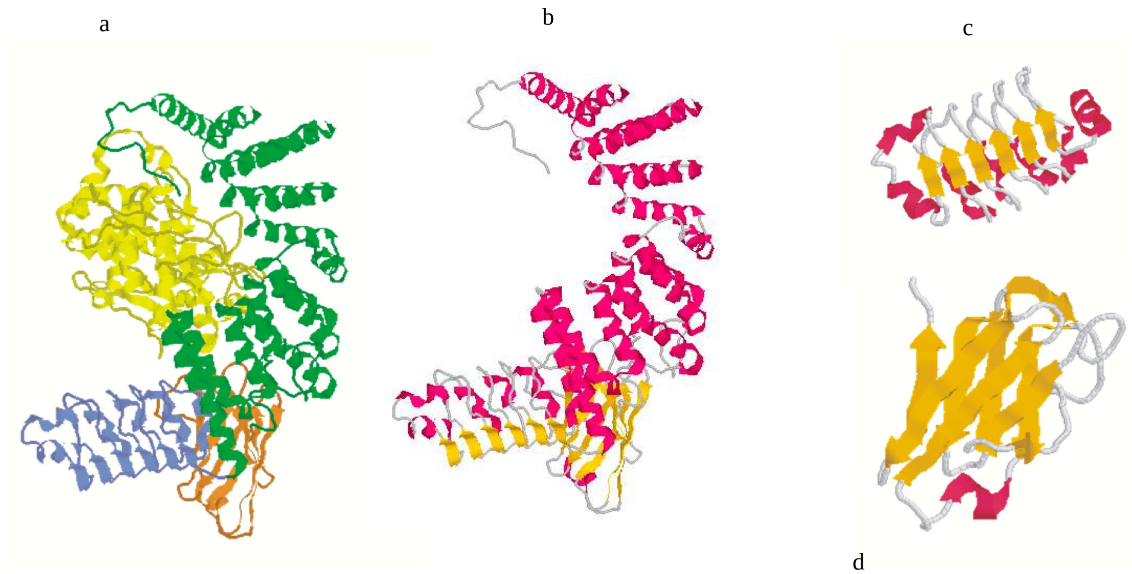
CHM-like REP-2 isoform is the product of an intronless gene on chromosome 1 (22).

The gene that codes for the yeast REP homologue is MS14/MRS6, and this protein is essential for the double geranylation of yeast Rab proteins as it is the only REP in yeast (27, 28 31).

Structural Biology and Complex formation

GGTase is a multicomponent enzyme composed of tightly associated α (68kDa) and β (45kDa) subunits (12).

The α subunit is larger than the corresponding unit of FTase and GGTase-I, and consists of a helical domain, which is similar to the other two prenyltransferases in some aspects, but also has two other domains, a leucine rich repeat (LRR) and an immunoglobulin-like (Ig-like) domain which [2]distinguishes the α subunit from that of the other two enzymes (32, 33).



[2]Fig 3. Ribbon representation of RabGGTase. (a) Complete structure of RabGGTase: green - α helical domain of the α subunit, orange - Ig-like domain of the α subunit, light blue - LRR domain of the α subunit, yellow - β unit embraced by the crescent α subunit. (b) α helical domain of the α subunit, with 15 helices. c) The LRR domain, which consists of five leucine rich repeats. (d) The Ig-like domain, which is predominantly comprised of β sheets.

[2] All molecular representations were created using Protein Explorer 2 Beta

A crescent-shaped right-handed superhelix is formed from 15 helices in the helical domain of the α helix. The Ig-like domain is folded into an eight stranded β sandwich and is formed from residues 244α to 345α, and this domain, without the eighth β strand would belong to the *h*-type immunoglobulin fold. This Ig-like domain is connected to the helical domain via two loops and is inserted between helices α11 and α12. One face of the Ig-like domain is packed against the LRR domain. The LRR domain, is comprised of residues 443α to 567α, and there are five LRRs in this domain each containing between 22-27 residues. The C-terminus of the α subunit is included in this domain which folds into a right-handed superhelix, with alternating β strands and 3_{10} helices, except in the last repeat which has an α helix instead (32).

The catalytic β subunit consists of an α-α barrel made up of 12α helices which forms the lipid binding site, a property analogous to FTase. The center of the α-α barrel forms a tunnel shaped cavity that is comprised mostly of aromatic residues including Trp52β, Phe147β, Tyr195β, Trp243β, Trp244β, Phe289β and Phe293β. Several positively charged residues form a cluster near the opening of the tunnel near the active site zinc ion which is required for catalytic activity and the interface with the α subunit. The top of the barrel is open, while the bottom of the barrel is blocked by a turn. The β subunit is enclosed by the crescent shaped helical domain of the α subunit, and although the LRR and Ig-like domains make no contact with the β subunit, a pronounced groove is formed between the 3_{10} helix side of the LRR domain and the bottom of the α-α barrel of the β subunit (32).

The zinc ion in the β subunit is coordinated by His2α, Asp238β, Cys240β and His290β. An extended conformation is formed between the N-terminal region of the α and β subunits with the His2α coordinating the zinc ion and Lys6α in an ionic interaction with Asp272β (32).

a

b

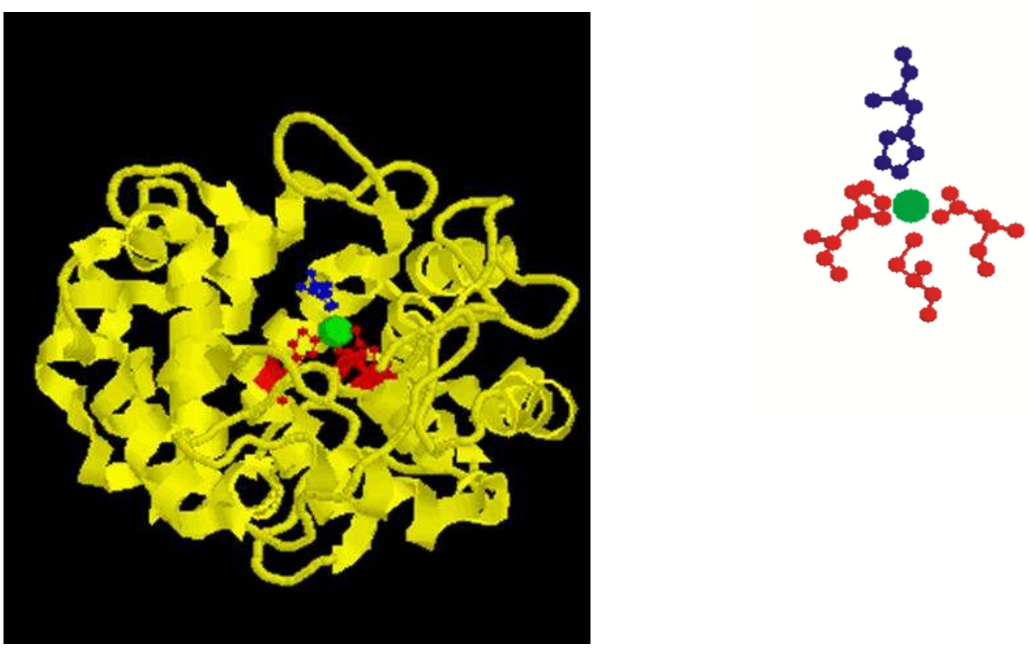


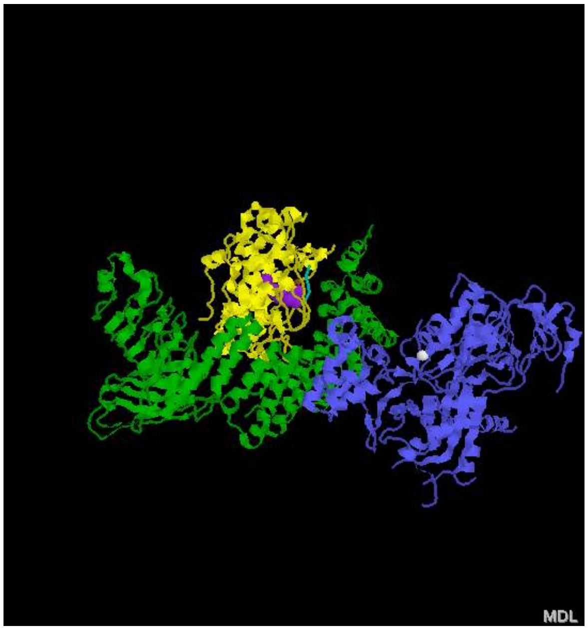
Fig.4. The catalytic β **subunit**, comprised of an alpha barrel. a) The alpha barrel of the β subunit showing key residues coordinating the zinc ion.b) Restricted view of only the amino acids coordinating the zinc ion .
Adapted from Zhang *et al*., 2000.

RabGGTase:REP-1 complex

The heterodimer is unusual in that the size and structure of the single binding interface is extremely small, with a surface area of approximately 680 $Å^2$, a property only previously observed with two other biological heterodimers - HIV gp120 envelope glycoprotein complexed with the CD4 receptor (742 $Å^2$) and the fibrinopeptide A complexed with thrombin (312 $Å^2$) (33).A binding interface, the single contact area in the RabGGTase:REP-1 complex, is found between helices 8, 10 and 12 of the α subunit of GGTase and helices D and E of domain II of REP-1. Hydrogen bonds and hydrophobic interactions contribute to the stability of this binding interface.

A shelf-like opening, presumably for Rab docking on one side of the complex is formed by domains I and II of REP-1 and both subunits of RabGGTase (33).

a



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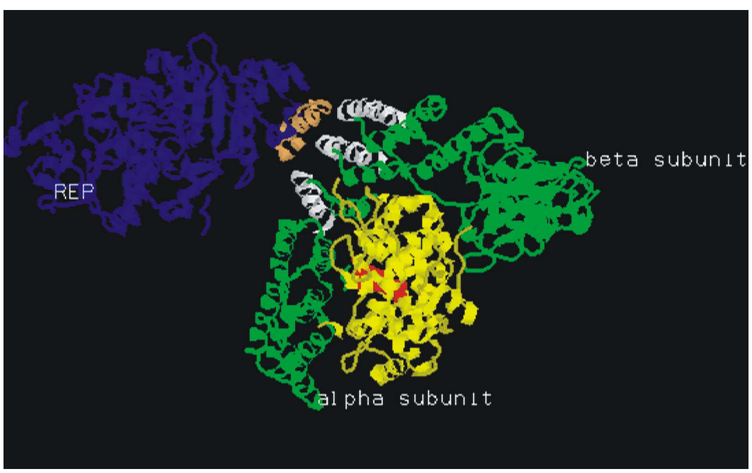


Fig 5. RabGGTase complexed with REP. a) The α and β subunits are shown in green and yellow respectively, and REP is colored blue. The purple spacefilled molecule is the isoprenoid unit. b) A representation of the unusually small binding interface between RabGGTase and REP. The α and β subunits are colored as before, and the isoprenoid is colored red. The interface is comprised of helices 8, 10 and 12 of the α subunit, colored white, and helices D and E of domain II of REP, colored light orange.
Adapted from Pylypenko *et al., 2003*.

The active site located on the β subunit is occupied by an isoprenoid unit which is positioned in an aromatic pocket in the α-α barrel of the β subunit, and a zinc ion. The aromatic patch borders on cavities that could accommodate the second isoprenoid moeity (33).

a b

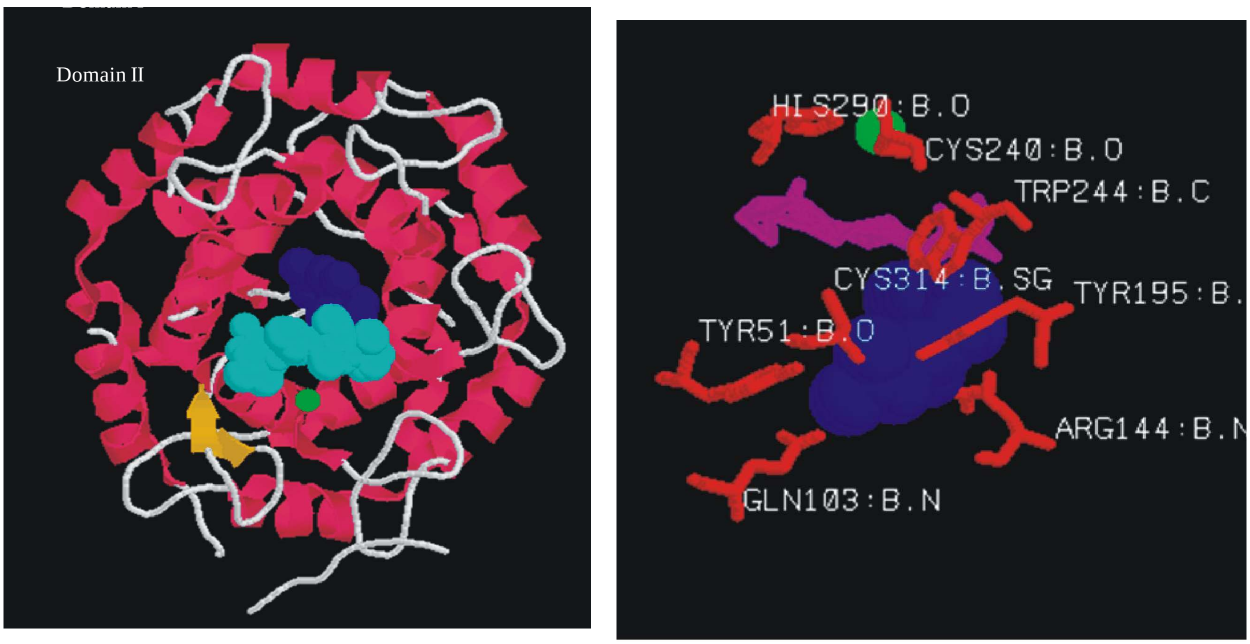


Fig. 6a. Illustration of the isoprenoid moeity in spacefill, and the zinc ion, also in spacefill colored green. Both are located in the active site. b) Important residues in the complex, coordinating the zinc ion and interacting via hydrogen bonds and

The REP-1 protein consists of two domains. Domain I consists of four β sheets and six α helices comprising a large cylindrical region. This domain is tilted towards domain II, which is composed of five α helices. The binding platform for the Rab protein as well as an effector loop are important structural regions on the escort protein (33).

Fig. 7. Illustration of the REP, showing two domains which consist primarily of α helices.

The largest conformational changes due to complex formation occurs with residues forming helix 8 and the link with helix 9 which is related to the intrinsic flexibility of α helices 8 and 10 of the α subunit. This functions to provide access for Phe279, a key residue in complex formation to the deep hydrophobic binding site, and this is illustrated by the failure of a mutant of REP-1 Phe279 to interact with RabGGTase.

By comparing the molecular surface of complexed versus unliganded transferase, it can be illustrated that the REP-1 binding site on the surface of RabGGTase can be divided into two parts (33).

Diseases associated with protein prenylation

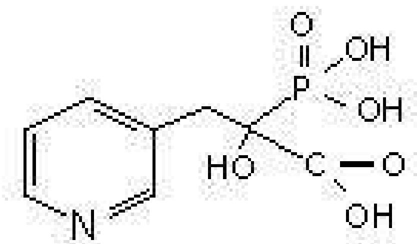
Defects in prenylation lead to flaws in the protein machinery involved in intracellular trafficking. Such malfunctions form the basis of several human diseases. For instance, a loss of function mutation in the REP-1 gene, an essential component in prenylation reactions, is the basis of the human hereditary disease choroideremia (CHM). CHM is an X-linked disorder caused by chorioretinal degeneration which is manifested by loss of peripheral vision and progressive night blindness leading to eventual blindness at quite an early age in afflicted males (20, 22,34). REP-1 is one of the two isoforms - REP-1 and REP-2 present in mammalian cells. Both have similar activities in the geranylgeranylation of many Rab proteins. Rabs can still be processed in most tissues by REP-2. This compensation for REP-1 deficiency is not absolute however in the case of Rab27a, as it remains unprenylated in the retinal pigment epithelium and choroid of CHM patients. This indicates that loss of Rab27a function alone, or in combination with some other general effect of REP-1 deficiency may be the causative factor in retinal deterioration in CHM (20).

In the Hermansky-Pudlak syndrome (HPS), the RabGGTase activity is reduced to 20% due to a splice site mutation in the gene that encodes the α subunit of the prenyltransferase. HPS is a rare genetically heterogenous disease that is recessively inherited, and the phenotype reflects defects in platelet-dense granules, lysosomes and melanosomes. Symptomatology includes lysosomal accumulation of ceroid, hypopigmentation and a tendency for bleeding (20, 22, 36).

Inhibitors of RabGGTase

Mutated forms of Ras are found in may human cancers - approximately 30% of all human cancers; 30-50% of colon cancers and 90% of pancreatic carcinomas. Due to the role of prenylated proteins such as Ras in neoplastic processes, prenyl modification as gained significant research interest (39). Today, the most promising drug candidates for anticancer as well as antikinetoplastid therapy include inhibitors of protein prenyltransferases, particularly, FTase and GGTase-I (37, 27). Due to the role of Ras in cancers, and evidence that GGTase-I prenylates N-Ras and K-Ras upon inhibition of FTase, resulting in fuctionally equivalent geranylgeranylated Ras proteins, several effective and specific inhibitors of both FTase and GGTase-I have been developed (38, 37, 39, 40).

Non-specific inhibitors of prenylation such as the metabolites of limonene, a monoterpene, generally inhibit one or more prenyltransferases. For instance, perillyl alcohol, inhibits both GGTase-I and RabGGTase in intact cells such as 3T3 fibroblasts and cell-free lysates. Additionally, the depletion of FPP and GGPP due to the inhibition of FPP synthase by some synthetic bisphosphonate drugs indirectly results in the prevention of protein prenylation by preventing the formation of FPP or GGPP which are required for protein prenylation. None of the abovementioned inhibitors specifically inhibit RabGGTase however. Recent work by Coxon and others, identified a phosphonocarboxylate inhibitor of RabGGTase - NE10790.



NE10790

Fig. 8. Structure of NE10790
Adapted from Coxon *et al.*, 2001

This compound is an analogue of bisphosphonate (2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonate)- BP RIS in which a phosphonate group is replaced by a carboxylate group. This compound selectively inhibits RabGGTase in several cell types including osteoclasts in vitro and in intact cells and may aid in the future design of potent therapeutic inhibitors as well as assist in the ongoing investigations of Rab protein function (41).

Conclusion

The Rab family of proteins is an important one, playing roles in endocytic and biosynthetic pathways involving memebrane vesicle docking and fusion. Prenylation is a key factor in these processes, as Rabs are stably isoprenylated by RabGGTase shortly after synthesis. Other processes effected by prenylation of Rabs, all of which are prenylated by RabGGTase include protein-protein interactions, and even plant defense mechanisms. This is indicative of the importance of RabGGTase in cellular life.

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