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Structural determinants of Rab and Rab Escort Protein interaction: Rab family motifs define a conserved binding surface

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Abstract

Rab proteins are a large family of monomeric GTPases with 60 members identified in the human genome. Rab GTPases require an isoprenyl modification to their C-terminus for membrane association and function in the regulation of vesicular trafficking pathways. This reaction is catalysed by Rab geranylgeranyl transferase, which recognises as protein substrate any given Rab in a 1:1 complex with Rab Escort Protein (REP). REP is therefore able to bind many distinct Rab proteins but the molecular basis for this activity is still unclear. We recently identified conserved motifs in Rabs termed RabF motifs, which we proposed to mediate a conserved mode of interaction between Rabs and REPs. Here, we tested this hypothesis. We first used REP1 as a bait in the yeast two-hybrid system and isolated strictly full-length Rabs, suggesting that REP recognises multiple regions within and properly folded Rabs. We introduced point mutations in Rab3a as a model Rab and assessed the ability of the mutants to interact with REP using the yeast two-hybrid system and an in vitro prenylation assay. We identified several residues that affect REP:Rab binding in the RabF1, RabF3, and RabF4 regions (which include parts of the switch I and II regions), but not other RabF regions. These results support the hypothesis that Rabs bind REP via conserved RabF motifs and provide a molecular explanation for the preferential recognition of the GDP-bound conformation of Rab by REP.

Keywords: GTP-binding proteins; Vesicular transport; Rab; Rab Escort Protein; Geranylgeranyl transferase; GDP dissociation inhibitor; Protein–protein interactions; Molecular modelling; Yeast two-hybrid system

Rab proteins are Ras-like small GTP-binding proteins that regulate vesicular trafficking pathways [1–3]. Sixty Rab proteins have been identified in the human genome, forming the largest family within the Ras superfamily of monomeric GTPases [4–6]. 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.

Rabs are hydrophilic proteins, however, their function depends on association with the cytoplasmic leaflet of cellular membranes [7]. Membrane association is mediated by the covalent modification of C-terminal cysteine residues with geranylgeranyl isoprenoids [8]. This is accomplished by the enzyme Rab geranylgeranyl transferase (RGGT), a protein prenyl transferase which, unlike other prenyl transferases, does not recognise the Rab substrates directly. Instead, the protein substrate for RGGT is a 1:1 complex between any given newly synthesised Rab protein and Rab Escort Protein (REP) [9,10]. RGGT binds the REP:Rab complex or alternatively RGGT binds REP first and then Rab binds the REP:RGGT complex [10,11]. RGGT then catalyses the transfer of geranylgeranyl groups to one or two cysteines present in the carboxyl-termini of Rab proteins [12]. The reaction is sequential reaction since there is only one lipid binding site in the enzyme [10,13–16]. After lipid

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transfer, the enzyme dissociates from the REP:Rab complex and REP is believed to deliver the prenylated Rab to cellular membranes [17,18].

The two known mammalian REP proteins are related to Rab GDP Dissociation Inhibitors (RabGDIs), which are though to recycle prenylated Rabs from and to cellular membranes [19]. REP1 and REP2 are ~70% identical and display similar tissue expression patterns. In contrast, the budding yeast possesses only one REP called Mrs6p [20]. 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 [21]. Also, mutations in the human REP1 gene result in Choroideremia, an X-linked progressive retinal degeneration [22,23]. Improper compensation by REP-2 is a probable cause for the disease, possibly due the accumulation of a subset of unprenylated Rabs [24].

Comparison of the primary amino acid sequence of REPs and RabGDIs revealed three regions of conservation (SCRs—sequence conserved regions) [20,25]. Furthermore, site directed mutagenesis has identified positions in the SCR1a, SCR1b, and SCR3b of RabGDI as the main determinants for Rab binding [26–28]. The solution structure of the α isoform of RabGDI revealed a two domain organisation of the molecule and showed that SCR1a, SCR1b, and SCR3b regions localise at the apex of domain 1, forming a "Rab binding platform" [27]. 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 [25,29].

Conversely, the critical elements in Rab proteins responsible for binding to REP remain ill defined. We have previously shown that REP interacts preferentially with the GDP-bound form of Rabs [30]. Beranger and co-workers 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 and observed that RabGDI binding was blocked by substitutions of either the hypervariable domain, the loop3/β3 region, or the effector domain, which includes switch I residues [31]. The kinetics of Rab6 geranylgeranylation was affected by hypervariable domain or loop3/β3 region substitutions, but not by effector domain substitutions. In contrast, Wilson and co-workers observed that the switch I point mutation Rab1bD44N cannot bind RabGDIα in vitro and is prenylated only in vitro and in vivo at 40–50% efficiency compared to the wild type protein [17]. More recently, Overmeyer and co-workers 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 [32].

We have recently identified sequence motifs that distinguish the mammalian Rab proteins from other mammalian small GTPases, which we named RabF motifs [5]. These motifs localise in and around the switch regions and are conserved across evolution [6]. As all Rab proteins bind REPs and RabGDIs in a nucleotide sensitive way, we proposed that conserved positions in the RabF motifs define a general binding surface in Rab proteins to general regulators. Here we demonstrate that conserved positions in the RabF motifs are critical for Rab binding to REP1, further supporting the hypothesis that the RabF motifs form a conserved binding platform to general regulators such as REPs and RabGDIs.

Materials and methods

Strains and media. The *Saccharomyces cerevisiae* reporter strain L40 (*MAT trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was described previously [33]. Yeast strains were grown at 30 °C in rich medium (1% yeast extract, 2% bacto-peptone, and 2% glucose) or in synthetic minimal medium with appropriate supplements.

Plasmids. The "bait" plasmid was constructed in pBTM116 (gift of S. Fields, University of Washington) such that the protein of interest was expressed as a C-terminal fusion with the DNA binding domain of LexA. pLexA-REP1 was constructed by inserting an *Eco*RI/*Xba*I fragment containing rat REP1 from pBS-REP1 [9], pLexA-Rab3a was constructed by inserting an *Eco*RI/*Xba*I fragment containing human Rab3a from pET14b-Rab3a [34] into pBTM116. Mutations in Rab3a were created by high-fidelity PCR with *Pfu* polymerase (New England Biolabs) mutagenesis, using pBTM116-Rab3a as template. The "prey" plasmids were as follows. cDNA libraries were expressing fusion proteins with the activation domain of either GAL4 (Human HeLa matchmaker library, Clontech) or VP16 (Rat Brain library, a gift from Thomas Südhof, University of Texas, Southwestern Med. Ct.). pGADGH-REP1 (using the GAL4 activation domain) was constructed by inserting an *Bam*HI/*Xho*I fragment containing rat REP1 from pBS-REP1 into pGADGH (Clontech). Human Rab3a wild type and mutants were cloned in frame into pGEX-2T (Amersham Pharmacia Biotech). All constructs were verified by sequencing.

Two-hybrid assays. The yeast reporter strain L40 was sequentially transformed with pLex4-REP1 and one of the two cDNA libraries is described above. Double transformants were plated in synthetic medium lacking histidine, leucine, tryptophan, uracil, and lysine in the presence of 3-aminotriazole (Sigma), where indicated. The plates were incubated at 30 °C for three days, surviving colonies were patched onto selective plates and assayed for β-galactosidase activity by a filter assay [35]. A positive assay was scored when a colony turned blue in less than 6 h. Plasmid DNA was prepared from the His+/LacZ+ colonies and used to retransform the L40 strains with pLexA-REP1 and pLexA-SRBP (a gift from Luca Barella, University of Texas Southwestern Med. Ct.) as a negative control. The cDNA inserts were sequenced with an automated Applied Biosystems Model 373A sequencer. Expression levels of the different constructs were assayed by immunoblotting [33].

Structural modelling. Modelling of the structure of the Rab3a:GDP complex was performed using the SWISS-Model automated comparative modelling server [36,37].

Production of recombinant proteins. BL21 *E. coli* (Stratagene) were transformed with pGEX-2T-Rab3a, grown at 37 °C until optical density reached 0.6, and then induced with IPTG (Sigma) for 3 h. The bacteria were then harvested, washed in ice-cold PBS, and lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1× protease inhibitor cocktail. The lysate was then centrifuged at 100,000*g* at 4 °C and the soluble fraction was subjected to chromatography on GSH–agarose (Sigma). The column was

equilibrated in running buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 1 mM DTT), the soluble fraction was loaded, the column was washed with 10 column volumes of running buffer, and the GST-Rab3A fusion protein was then eluted with a step gradient of free GSH. The eluate was dialysed in buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 1 mM GDP, concentrated using Centricon 10 (Amicon), and stored at −80 °C. Rat REP1 and human RGGT were prepared as described previously [38].

In vitro prenylation assays. In vitro prenylation of GST-Rab proteins in 25 μl reaction volumes was performed as described previously [39]. Briefly, 10 μM of GST-Rab3a was incubated with 25 nM RGGT and 5 μM [^{3}H]geranylgeranyl pyrophosphate (GGPP) in the presence of various concentrations of REP (0–6 μM) at 37 °C for 10 min. The [^{3}H]geranylgeranyl transferred to Rabs was measured by scintillation counting as the precipitable radioactivity following filtration of the reaction mixtures onto 1.2 μm glass fibre filters.

Table 1
Results of yeast two-hybrid screen using cDNA libraries

Clone name	cDNA Library	Independent clones	Linker size (codons)
Rab3a	Brain	2	46
			50
Rab3b	Brain	1	20
Rab4	Brain	1	44
Rab5b	Brain	1	16
Rab11b	Brain	1	20
Rab14	HeLa	1	85
Rab18	HeLa	2	18
			16
Rab32	HeLa	5	5
			6
			8
			9
			26

Results

We aimed at identifying regions within Rabs responsible for the interaction with REP. We started by using the two-hybrid system in the yeast *S. cerevisiae*. This system is particularly suited for this type of study as it permits the unbiased screening of regions involved in binding, allows screening of a large number of deliberate mutations, isolates REP:Rab binding from the prenylation reaction, and finally permits the testing of the binding of the two proteins in vivo.

The reporter strain L40 containing REP1 fused to the DNA-binding domain of the yeast transcription factor LexA was transformed with a library of human HeLa cell cDNA fragments expressed as fusion with the activation domain of the yeast transcription factor GAL4 (GAL4AD). The formation of a complex between REP1 and a polypeptide translated from the library of cDNAs confers histidine auxotrophy and β-galactosidase activity due to activation of LexA-dependent genes. Approximately 6×10^6 transformants were screened, of which 110 were selected on His-plates. Of those, 33 were also positive for β-galactosidase activity. Plasmid DNA was prepared from all 33 double positive colonies but only 16 showed strong β-galactosidase activity (<6 h) that was specific for REP1. The cDNA inserts were analysed by restriction digestion and sequencing. The screen produced four different proteins, three of which were full length Rab proteins (Table 1) and one cyclophilin-like fragment (data not shown). One of these sequences was novel and was named Rab32. The cDNA sequence for Rab32 was deposited in Genbank under Accession No. U71127.

We then repeated this approach using a different cDNA library constructed from rat brain. Approximately 8×10^6 transformants were screened, of which 90 were selected on His-plates. Of those, 23 were also positive for β-galactosidase activity. Plasmid DNA was prepared from all 23 double positive colonies but only 11 showed strong β-galactosidase activity (<4 h) that was specific for REP1. The cDNA inserts were analysed by restriction digestion and sequencing. The screen produced five different proteins, all of them Rab proteins (Table 1). Interestingly, all the Rab insert cDNAs obtained in both screens contained the entire coding region. Since the clones are carboxyl-terminal fusions with GAL4AD, they all contained a variable portion of the 5′ untranslated region of the mRNA between the GAL4AD and the full-length Rab sequence. This variable sequence may have served as a linker between the two domains of the fusion protein. In one extreme case the linker coded for 85 amino acids but the length was more typically between 5 and 26 amino acids.

The fact that only full length Rab sequences were identified in both screens strongly suggests that REP recognises multiple regions of Rabs, probably reflecting the requirement for properly folded, GDP-bound proteins [30]. We therefore decided to pursue a site-directed mutagenesis approach to identify structural motifs and specific residues in Rabs involved in the interaction with REP, using Rab3a as a model. We focused on the RabF motif regions and based our choice of positions to mutate using two criteria: the conservation pattern within the RabF motifs and determination of the likelihood that these residues were solvent-exposed. In the absence of structural information for the GDP-bound conformation of Rab3a, we modelled the structure of Rab3a:GDP on the structure of Rab6:GDP [40] by homology modelling.

Based on these criteria we chose to introduce the following point mutations (Fig. 1): Y21A (corresponding to RabSF1), D58A, F59A (corresponding to RabF1), D68L, K69A, and R70A (adjacent to RabF2), R85A, T86A, and T86N/I87A (corresponding to RabF3), Y91A, R93A (corresponding to RabF4), and then tested the mutant Rab3a proteins in the yeast two-hybrid assay for interaction with REP. We first verified that all Rab3a mutants were expressed at equivalent

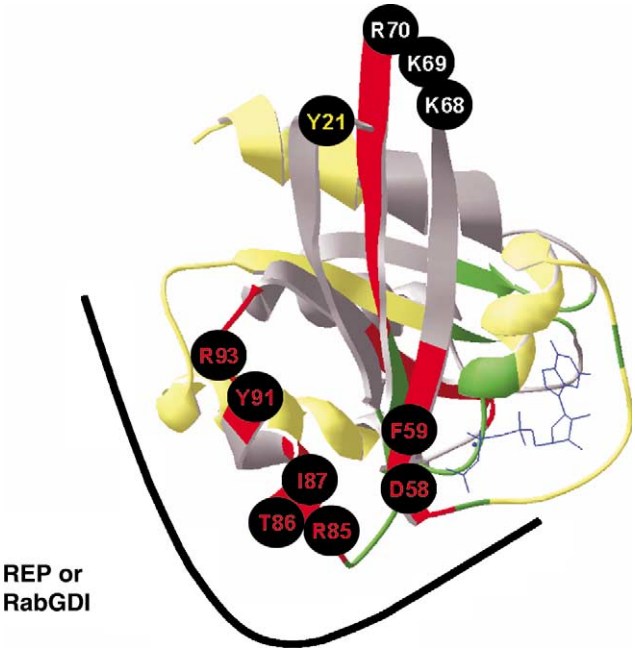

Fig. 1. Cartoon representing the Rab3a-GTP 3D structure (PDB code 3RABA). RabF regions are depicted in red, RabSF regions in yellow, and the conserved nucleotide binding (PM/G) motifs in green. The nucleotide and the Mg^{2+} atom are both represented in blue. The residues mutated in this work are indicated.

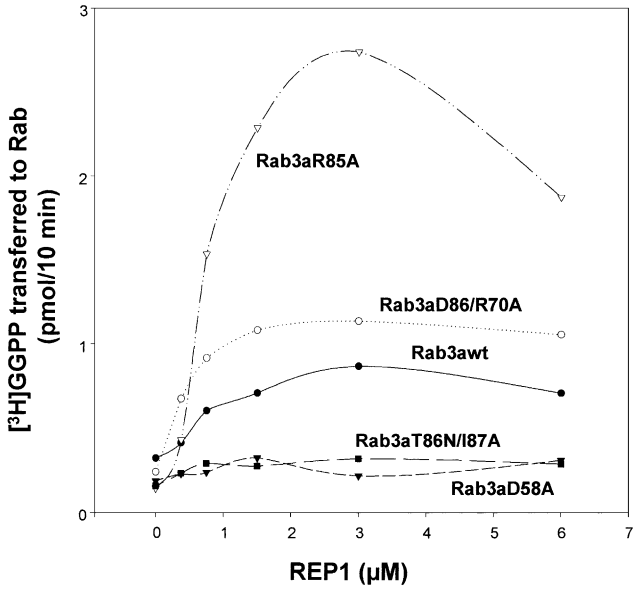

Fig. 2. In vitro prenylation kinetics of recombinant GST-Rab3a mutants. In vitro prenylation reactions containing 10 μM of the indicated GST-Rab3a proteins were incubated with increasing concentrations of REP1, 5μM [^{3}H]GGPP (3000 dpm/pmol), and 25 nM RGGT. After 37 °C incubation for 10 min, the amount of [^{3}H]GGPP transferred to each protein was determined as described under "Materials and methods." Each value is the average of duplicate determinations.

levels by immunoblotting of yeast extracts, thus indicating that the mutant proteins were made and were stable (data not shown). We observed that the D58A and F59A (RabF1), I87N/T86A (RabF3), and R93A (RabF4) mutations abolished REP:Rab binding as determined by this assay (Table 2). We thus identified three novel positions whose mutation abolishes REP binding (D58, F59, and R93), and confirmed the importance of the exposed residue I87, in accordance with the results of Overmeyer and co-workers [32]. Surprisingly, the single RabF3 mutations (R85A and T86A) resulted in increased binding to REP, as revealed by growth under high concentrations of 3-aminotriazole (Table 2). Conversely, Y21A, D68L/R70A, K69A, or Y91A did not affect significantly the interaction as measured by this assay. We further tested the effect of the same mutations in the binding of Rab3a to RabGDIα. Despite some background problems, we obtained similar results to those obtained for REP:Rab3a binding (data not shown), which is consistent with the proposed conserved mode of interaction between RabGDI, Mrs6, and REP with Rabs [26].

Table 2
Yeast two hybrid analysis of Rab3a:REP binding

Rab mutant	β-galactosidase induction	Growth on 3-aminotriazole (mM)
Wild type	+	⩽50
Y21A	+	⩽50
D58A	0	<5
F59A	0	<5
K69A	+	⩽50
D68L/R70A	+	⩽50
R85A	++	⩾100
T86A	++	⩾100
T86N/I87A	0	<5
Y91A	+	⩽50
R93A	0	<5
pBTM	0	<5

For the β-galactosidase induction: + represents blue colour after 30 min, ++ represents blue colour after 15 min, and 0 represents no blue colour after 3 h.

We then subjected the same mutant Rab3a proteins to in vitro prenylation. Neither Rab3aD58A nor Rab3aI87N/T86A mutants underwent significant geranylgeranylation while the Rab3aD68L/R70A mutant behaved essentially like the Rab3a wild type protein (Fig. 2). Conversely, the Rab3aR85A mutant exhibited unique kinetics, including a 3-fold increase over wild type in V_{max} and evidence of product inhibition at high concentrations of substrate. These in vitro prenylation assay results are consistent with those obtained with the REP:Rab binding assay.

Discussion

The present study provides a model to explain the molecular mechanism by which REP (and RabGDI) interact with 60 distinct Rab proteins. Our results sug-

gest that the interaction of REP (and RabGDI) with Rab GTPases involves the recognition of the conserved RabF motifs, namely RabF1, RabF3, and RabF4. These RabF motifs include parts of the switch I and switch II regions, which rearrange significantly upon GTP versus GDP-binding. Thus, REP binding to RabF sequences also provides a mechanism for preferential recognition of the inactive GDP-bound conformation [30]. Finally, our results further support the notion that REP requires the properly folded GDP-bound conformation of Rab proteins and the interaction involves multiple non-consecutive regions.

Our previous analysis of the Rab family resulted in the identification of conserved regions within the Rab family (but not in other families of the Ras superfamily) termed RabF, and regions conserved only among Rab isoforms that we termed RabSF regions [5]. We also predicted that RabF motifs would be recognised by general regulators of Rab proteins such as REP and RabGDI, and the present study confirms that prediction.

Maltese and co-workers [32] showed that mutation of the RabF3 and RabF4 residues in Rab1b, I73 (equivalent to Rab3aI87), Y78 (equivalent to Rab3aY92), and A81D (equivalent to Rab3aA95) prevented prenylation, suggesting reduced or abolished Rab:REP binding. Our results are consistent with these results as we show that in Rab3a the double mutant I87N/T86A and the neighbouring residue Y91 abolish REP binding. Interestingly, we found that the RabF3 mutations R85 and T86 in Rab3a result in a stronger signal in the binding assay, and increased Vmax in the prenylation reaction, suggestive of a stronger binding between Rab3a and REP-1.

Our results also implicate the RabF1 residues D58 and F59. In agreement with our data, mutation in the equivalent Rab1b residue D44 (and I41) resulted in partial prenylation defects, and the less severe effect could have been due to the nature of the mutation (D–N in Overmeyer et al., versus D–A in this study) [32]. RabF1 forms part of switch I and is not contiguous with RabF3 and RabF4 in the primary sequence. However, these RabF regions are adjacent in the native three-dimensional structure of Rabs (Fig. 1). Therefore, these results implicate this face of the Rab protein as the main binding surface towards REP and RabGDI.

The critical F59 and R93 positions are almost invariably conserved in the Rab family, and position I87, although not absolutely conserved, is frequently occupied by a conserved substitution. This is suggestive of a highly conserved and critical role for these residues in determining the interface of the Rab:REP complex. However, position 58, which is frequently an aspartic acid or a glutamic acid residue, can be also be occupied by a non-conservative substitution such as in Rab5, Rab17, Rab20, and Rab24, which possess an alanine residue at this position. Interestingly, Rab5 was previously shown to be a poorer substrate for REP binding than Rab1a [10]. These naturally occurring variations to the RabF motifs displayed by some Rabs may indicate differential binding to REPs and RabGDIs. This could account for example for the accumulation of unprenylated Rab27 in CHM patients that possess only functioning REP-2 [24]. Furthermore, it could also account for the differential binding in vivo of Rabs to different RabGDI isoforms [41]. We suggest that subtle variations in the RabF1, F3, and F4 of different Rabs will determine different binding affinities towards REP and RabGDI, which may be of functional significance.

In conclusion, our sequence conservation directed approach, complemented with structural considerations, led to the mapping of conserved Rab determinants of REP binding to RabF motifs, lending support to the idea that Rabs interact with general regulators in a conserved manner. Crystallographic studies of REP:Rab complexes will be necessary to confirm the present findings and to provide further insights into the mechanisms of Rab geranylgeranylation.

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