The Yeast Rab Escort Protein Binds Intracellular Membranes in Vivo and in Vitro*

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In both mammals and yeast, intracellular vesicular transport depends on the correct shuttling between membrane and cytosol of the Rab/Ypt small G proteins. Membrane association of these proteins requires prenylation by the Rab geranylgeranyl transferase that recognizes a complex formed by the Rab/Ypt protein and the Rab escort protein (REP). After prenylation the Rab/Ypt protein is delivered to the target membranes by REP. Little is known about the early steps of the Rab/REP complex formation and where this association occurs in the cell. Although prenylation is believed to take place in the cytosol, we show that the yeast Rab escort protein Mrs6 is present in both soluble and particulate fractions of cell extracts. Mrs6p is associated with the heavy microsomal fraction that contains endoplasmic reticulum-Golgi membranes but is absent in the plasma membrane, vacuoles, mitochondria, and microsomal subfraction associated with mitochondria. The solubilization pattern of the particulate pool of Mrs6p implies that this protein is peripherally but tightly associated with membranes via hydrophobic interactions and metal ions. We also report that the C terminus of Mrs6p is important for maintaining the solubility of the protein because its deletion or replacement with the C terminus of RabGDI results in a protein that localizes only to membranes.

Rab escort proteins (REP)† assist in the geranylgeranylation of the Rab/Ypt type of small G proteins, which is catalyzed by Rab geranylgeranyl transferase (GGTase; reviewed in Ref. 1). Prenylation of Rab/Ypt proteins is necessary for their function in the regulation of vesicular transport because it enables them to associate with intracellular membranes (2).

Rab GGTase is a heterodimeric enzyme consisting of two catalytic subunits, α and β, which adds a geranylgeranyl moiety to each of the two C-terminal cysteine residues of Rab/Ypt proteins. However, Rab GGTase on its own lacks the ability to stably bind Rab/Ypt substrates and is therefore inactive in the absence of the Rab escort protein (formerly called component A of Rab GGTase) (3, 4).

Two related mammalian REP proteins (REP-1 and REP-2) and one Saccharomyces cerevisiae homologue have been identified to date. REP-1 in humans is encoded by the choirderemia gene, and deletions in this gene cause a progressive retinal dystrophy leading to blindness (5, 6). The yeast REP is encoded by the MRS6/MSI4 gene, which is essential for viability and Rab GGTase activity in yeast (7–10). Both human and yeast REPs share structurally conserved regions with RabGDI, a protein that recognizes prenylated GDP-bound Rab and takes part in their membrane-cytosol recycling (11–13).

Based on studies of the REP-1 protein, a model for the function of Rab escort proteins in Rab membrane-cytosol cycling has been established (4, 14–16). According to this model, a newly synthesized Rab protein is bound by REP, and the complex is recognized by Rab GGTase, which allows a subsequent dimergeranylgeranylation of a Rab protein to take place. After prenylation, REP stays associated with the modified Rab and delivers it to its target membrane. Upon membrane association of Rab, REP is released into the cytosol for another round of prenylation and Rab undergoes activation due to GDP/GTP exchange. After GTP hydrolysis, prenylated GDP-bound Rab is subsequently extracted from the membranes by RabGDI (12). RabGDI can again deliver Rab to a donor membrane, thus restarting the cycle. However, the yeast REP cannot suppress the lethality due to the disruption of the yeast GDI1 gene (13, 17) implying that despite the structural and functional similarities, REP and RabGDI have unique and distinct functions in vivo.

There have been no systematic studies on the intracellular localization of the components of the Rab geranylgeranylation machinery. The catalytic subunits of Rab GGTase are believed to be soluble proteins, like the other prenyl transferases, suggesting that the reaction takes place in the cytoplasm (18, 19). Here we report that the yeast Rab escort protein Mrs6 is present not only in the soluble but also in the particulate fractions of cell extracts. The biochemical characteristics of the membrane-bound pool of Mrs6p imply that this peripheral association is mediated through hydrophobic interactions and possibly regulated by the availability of divalent cations. Only the membrane-associated form of Mrs6p can be detected in vivo upon deletion of the C terminus of the protein, suggesting that this part of the Mrs6p is essential for maintaining its solubility.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions—The following strains were used in this study: AR4-6A (MATa, ura3, leu2, his3, trpl, ade8, rho-), AR4-6B (MATa, ura3::Gal1-MRS6-URA3, mrs6::HIS3, leu2, his3, trpl, ade8, rho-) (8), and X2180–1A (MATa, SUC2, mal, gal2, CUP1). The media YPD, YPGal, SD, and SGal (supplemented with amino acids and bases as required) were previously described (20). Yeast transformations were performed according to Ref 21.

The Mrs6-GDI chimera was constructed as follows: the C-terminal fragment (amino acids 276–442) from the bovine RabGDI was amplified by polymerase chain reaction from the plasmid pGEX2TH-RabGDI (kindly provided by Dr. Y. Takeda) using the oligonucleotides: GDI-
Membrane Binding of the Yeast REP

BamHI (5′-AGGTGCGGATCCAGCAAGCTGACTGCTTGACC-3′) and GDIxhoI (5′-CCATCAGGTGCGCAGAGAATGAC-3′) and GDIxhoI (5′-CCATCAGGTGCGCAGAGAATGAC-3′) and digested with BamHI and HindIII (32) and cloned into the BamHI site at amino acid 322 of the MRs6 gene. The resulting construct, named M6GD1, was recloned into the EcoRI site of the YEpM6 plasmid and propagated in the yeast strain containing the YEpM6 plasmid (YEpM6GD1). The plasmids YEpM6PN, YEp351, YEpM63Nhe, and YEpM63Xba were previously described (7, 20). Plasmid amplification and propagation were performed as described previously (20).

For Mrs6p depletion, the strain AR4-6B was precultured in YPgal or, when carrying plasmids, in appropriate selective SGal medium for 48 h. Cells were then washed, diluted to an A600 = 0.0 in YPDO and incubated under the strain described above for 14 h before harvesting. Absence of the endogenous Mrs6p was determined by immunoblotting analyses as described previously (20). For Mrs6p overexpression, the strain AR4-6A was transformed with YEpM6PN (7), precultured in ~Ura SD medium for 48 h, diluted to A600 = 0.2 in YPD, and grown at 30 °C for 8 h.

Subcellular Fractionation—Protein extracts were prepared from the wild type strain AR4-6A by glass bead lysis as described (20) and subsequently centrifuged at 100,000 × g for 1 h to obtain the total membrane fraction (P100) and cytosol (S100). The membrane fraction was washed once with lysis buffer and resuspended in 10 mM Tris-Cl, pH 7.4. Plasma membrane, vacuoles, microsomes (30,000 × g, 40,000 × g, and 100,000 × g subfractions), mitochondria, and mitochondria-associted microsomal fraction (MAM: particulate fraction) from the strain X-2180-1A as described (22–24), were kindly provided by Dr. G. Daum. Briefly, plasma membrane was obtained by centrifugation of homogenate at 20,000 × g followed by sucrose gradient purification, and vacuoles were isolated by floating in Ficoll gradients (22). Mitochondria and MAM fraction were prepared by centrifuging homogenate at 9,000 × g followed by sucrose gradient purification (23, 24). Microsomes were isolated by differential centrifugation of 20,000 × g post-mitochondrial supernatant at 30,000 × g, 40,000 × g, and 100,000 × g (24) and were essentially devoid of mitochondria and plasma membrane as determined by immunoblot with porin and Pdr5 antibodies. Protein concentration was determined by the Bradford method (25).

Mrs6 and Ypt1 Protein Expression, Antibodies, Immunodetection, and Geranylgeranylation Assay—Mrs6p and Ypt1p were expressed as His-tagged proteins in Escherichia coli as described previously (20). The anti-Mrs6p polyclonal antibody SLM6-1 (20) was used in this study. The anti-Ypt1 polyclonal antibody was raised in rabbits against the His6-tagged Ypt1p, and the serum was purified on protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) according to the manufacturer’s instructions. The following polyclonal antibodies were gifts: the anti-glycerol-dehydrogenase-dehydrogenase and anti-porin antibodies from Dr. G. Daum; the anti-Ypt7 antibody from Dr. D. Gallwitz; the anti-Pdr6 antibody from Dr. K. Kuchler; the anti-hexokinase antibody from Dr. K. Kuchler; and the anti-Sec61 antibody from Dr. R. Schekman. Western blots and immunodetection were performed as described previously (20) by the ECL method (Amersham Corp.). Geranylgeranylation assay was performed as described (26).

Triton X-114 Partitioning—200 μl of total protein extract from the strain AR4-6A overexpressing Mrs6p were resuspended in 200 μl of TX buffer (1% Triton X-114, 1 mM dithiothreitol, 2 mM EDTA, 50 mM Tris-Cl, pH 7.4) incubated on ice for 30 min and subsequently at 37 °C for 5 min. Samples were spun at 16,000 rpm for 2 min at room temperature, and aqueous and detergent phases were separated. 200 μl of TX buffer was added to the aqueous phase, 200 μl of 50 mM Tris-Cl, pH 7.4, was added to the detergent phase, and the phase separation was repeated. Aqueous and detergent phases from both separations were pooled together and precipitated with trichloroacetic acid, and aliquots were analyzed by immunoblot.

In Vivo Membrane Binding of Mrs6p—For the binding assays, total microsomal plasma membranes were obtained by 100,000 × g centrifugation of the postmitochondrial supernatants as described (22), and mitochondria were isolated according to Ref. 23. Aliquots (500 μg of protein) of these preparations from the strain AR4-6B depleted of Mrs6p or from the wild type strain AR4-6A were resuspended in buffer A, and the indicated amount of the recombinant Mrs6p was added to a final volume of 100 μl. Samples were incubated for 30 min at 30 °C and centrifuged at 100,000 × g for 1 h at 4 °C. Pellets were washed with buffer A, recentrifuged at the same speed, dissolved in 2 × SDS sample loading buffer, and analyzed by immunoblot. The amounts of bound Mrs6p were determined by quantitative immunoblot using purified Mrs6p as a standard followed by densitometric scanning of the films with the BIO-PROFIL package BIO-1D software version 6.02e (Biotechnology Division, Vilber Lourmat, Marne-la-Vallée, France). X values were calculated based on the binding saturation curves using the program GraphPad Prism version 2.0 (GraphPad Software Inc., San Diego, CA).

EDTA pretreatment was performed as follows: total microsomal membranes from the strain AR4-6B depleted of Mrs6p were incubated with 25 mM EDTA on ice for 30 min and centrifuged at 100,000 × g, and the pellets were resuspended in 100 μl of reaction buffer (50 mM Tris-Cl, pH 7.5, 5 mM dithiothreitol, 1% PIM) to which 0.6 μM recombinant Mrs6p and 150 mM MgCl2, CaCl2, ZnCl2, CuCl2, or MnCl2 was added. Samples were incubated at 30 °C for 30 min and centrifuged, and pellets were washed in reaction buffer and processed as described above.

For the proteinase pretreatment, total microsomal membranes from the strain AR4-6B depleted of Mrs6p were incubated with the indicated increasing concentrations of trypsin (Boehringer Mannheim) in a final volume of 100 μl of buffer A for 30 min on ice, followed by the addition of PMSF to 5 mM and incubation for a further 30 min on ice. As a control, trypsin and PMSF were added simultaneously, and samples were incubated for 60 min on ice. After 100,000 × g centrifugation, pellets were resuspended in 50 μl of buffer A with 0.1 μM recombinant Mrs6p, incubated at 30 °C for 30 min, and centrifuged, and the pellets were washed in buffer A and processed as above.

For binding of the cytosolic Mrs6p to microsomal and mitochondrial membranes, aliquots of purified fractions (50 μg of protein) from the strain AR4-6B depleted of Mrs6p were incubated with increasing amounts of cytosol from the strain AR4-6A overexpressing Mrs6p in a final volume of 50 μl of buffer A for 30 min at 30 °C. The samples were centrifuged at 100,000 × g, and the pellets were washed and analyzed by immunoblot.

RESULTS AND DISCUSSION

The particulate or cytosolic distribution of the Mrs6 protein in the wild type yeast cells was determined by subcellular fractionation of yeast extracts and immunoblot analyses. Total protein extracts were centrifuged at 100,000 × g to separate the soluble (S100) and particulate (P100) material. As shown in Fig. 1A, Mrs6p is found predominantly in the cytosolic fraction (lane S), but a significant portion co-sediments with the particulate fraction (lane P), suggesting that some Mrs6p is membrane-associated.

Further analysis showed that the membrane-bound Mrs6p does not co-fractionate with mitochondria, plasma membranes, or vacuoles (Fig. 1A, lanes M, FM, and V, respectively). Because plasma membranes and vacuoles are the targets for Sec4 and Ypt7 proteins, respectively (26, 27), the presence of Mrs6p in the particulate fraction cannot be attributed to its Ypt membrane-delivering activity. This observation is in agreement with in vitro studies in which a purified mammalian REP-1-Rab5 complex was used to study the sorting activity of REP-1 (14). It was estimated that after Rab purification, a membrane fraction (less than 1%) of the REP-1 protein is associated with the membranes of either permeabilized or unpermeabilized MDCK cells. The membrane association of Mrs6p could therefore be an early event in the cycle involving the formation of the Mrs6-Ypt complex. It has been suggested that the mammalian REP, due to its preference for the GDP-bound form of Rab, was added to the detergent phase, and the phase separation was repeated.
could act as a true chaperone, binding the Rab protein as it is released from the translational machinery and possibly forcing the nascent Rab into the GDP-bound conformation (28). Our results are consistent with this hypothesis, because, as shown in Fig. 1B, we observed that Mrs6p co-fractionates with the microsomal membranes. In particular, Mrs6p is associated with the heavy microsomal fraction (30,000 × g and 40,000 × g fractions, lanes 30 and 40, respectively), which is enriched in endoplasmic reticulum and Golgi membranes (24, 29), as indicated by Ypt1p enrichment observed in our experiments. Interestingly, Mrs6p was not detected in the light microsomal fraction containing secretory vesicles (100,000 × g microsomes, lane 100) or in the MAM fraction (lane MAM). The MAM fraction was described previously as a specialized part of endoplasmic reticulum surrounding mitochondria that co-purifies with them and was shown to be enriched in a subset of enzymes involved in phospholipid biosynthesis (24). In fact our mitochondrial preparation was contaminated with endoplasmic reticulum membranes because Sec61 protein was detectable in mitochondria (Fig. 1A) and porin was detectable in the MAM fraction (Fig. 1B). In contrast, Mrs6p appears to be absent in both these fractions indicating that this protein, although it associates with microsomes, is not evenly distributed through these membranes like Sec61p is. Taken together, our localization data strongly suggest that the membrane-bound form of Mrs6p is present in a specific subcompartment of microsomes. Moreover, because no Mrs6 protein was found in the light microsomal fraction, it is likely that Mrs6p association with membranes is an early event in the Mrs6p cycle and not a consequence of its Rab delivery activity.

To further characterize the nature of the Mrs6p membrane association, the P100 membrane fraction was treated with reagents known to affect different types of protein-membrane interactions, such as electrostatic (treatment with high salt), hydrophobic (treatment with nonionic detergents), metal ion-mediated associations (treatment with chelating agents), or via a post-translational lipid modification such as palmitoylation (treatment with NH₂OH). As shown in Fig. 3A, the peripheral association of Mrs6p with membranes was confirmed by sodium carbonate treatment, which resulted in partial extraction of Mrs6p. Carbonate treatment is known to remove ribosomes from rough microsomes, to strip peripheral membrane-bound proteins, to convert sealed vesicles into open sheets, and to effectively release the contents of cisternae (30). Among the different reagents used to extract Mrs6p, the combined treatment with urea and Triton X-100 was the most effective (Fig. 3A). Each of these two reagents alone, as well as treatment of the membranes with 25 mM EDTA, was less efficient but still significant. No extraction was observed upon incubation with high salt.

The fact that Triton X-100 and/or urea caused solubilization of Mrs6p suggested that membrane binding is mediated by hydrophobic forces (31), either directly with lipids or through interactions with integral membrane proteins. Because 2.5 M urea causes partial protein denaturation, it is likely that the proper folding of Mrs6p is required for its binding to the membranes. The possibility that post-translational lipid modifications could account for the hydrophobic behavior of Mrs6p was
investigated by hydroxylamine treatment and by phase partitioning in Triton X-114. Hydroxylamine treatment at neutral pH is known to solubilize palmitoylated proteins by hydrolyzing the thioester bonds between a palmitate and a cysteine residue of a modified protein (32, 33). Such treatment, however, did not show any effect on the membrane association of Mrs6p (Fig. 3A). The possibility that another lipid modification or the overall hydrophobicity of Mrs6p could be responsible for its association with intracellular membranes was further excluded by phase partitioning in Triton X-114 solution of the total protein extracts containing Mrs6p (Fig. 3B). Mrs6p was found mainly in the aqueous phase, pointing to an overall hydrophilic character of the protein, in contrast to the lipid modified Ypt1p, which partitions in the detergent fraction.

Similar characteristics of membrane binding mediated by hydrophobic forces, despite the overall hydrophilic character of a protein, have been reported for a yeast protein, Vps17, in vivo and in vitro (34). By contrast, the hydrophobic character of the protein, in contrast to the lipid modified Ypt1p, have been reported for a yeast protein, Vps17, in vivo and in vitro (34). Similar characteristics of membrane binding mediated by hydrophobic forces, despite the overall hydrophilic character of a protein, have been reported for a yeast protein, Vps17, in vivo and in vitro (34).

To check if we are able to reproduce the binding of Mrs6p to membranes in vitro, we set up an assay using recombinant E. coli-expressed Mrs6p and membrane fractions from the strain AR4-6B depleted of Mrs6p (see “Experimental Procedures”) to avoid a background of the endogenous protein. Our in vitro binding studies show that the E. coli-expressed Mrs6p is able to associate with intracellular membranes in a concentration-dependent manner, reaching saturation at about 2 μM added Mrs6p (Fig. 4A). Membrane-bound recombinant Mrs6p could be solubilized by the same factors as demonstrated for the endogenous protein, pointing to similar biochemical characteristics of in vivo and in vitro association. However, the recombinant Mrs6p was found to be able to bind purified plasma membranes as well as mitochondria from the strain AR4-6B. Nonspecific binding was also observed when the cytosol from cells overexpressing Mrs6p was used as a source of the protein in a similar assay (data not shown). Thus, despite the same
behavior of recombinant and endogenous Mrs6p in the in vitro membrane binding assay, the membrane specificity observed in vivo was lost. A similar effect has been reported for the Rab3A protein, which localizes to synaptic plasma membranes and vesicles in rat brain synapses, but the protein purified from bovine brain membranes binds in vitro also to mitochondrial membranes and erythrocyte ghosts (35). Because it is possible that membranes prepared from the Mrs6p depleted strain AR4-6B could lack some factors or components required for the specificity of Mrs6p-membrane association, we repeated the binding assay with mitochondrial and microsomal membranes prepared from the wild type strain (Fig. 4B). Although Mrs6p was found to bind both types of membranes, the affinity of Mrs6p for the microsomes was higher ($K_d = 0.22 \pm 0.05 \mu M$) than for mitochondrial membranes ($K_d = 0.53 \pm 0.15 \mu M$). The existence of a saturation point in the Mrs6p membrane binding assay suggested a protein-mediated interaction; however, proteinase pretreatment of membranes did not abolish the Mrs6p association in vitro (Fig. 4C). The co-existence of two membrane-bound forms, a tightly bound and saturable form (protein-mediated binding), as well as a loosely bound and nonsaturable one (binding to lipid bilayers), has been reported for the ADP-riboseylation factor small G protein (36). Our results imply that the nature of the Mrs6p allows for hydrophobic interactions with lipid bilayers, whereas other factors are responsible for the specificity observed in vivo. Indeed, REP-1 can be found by gel filtration chromatography to be partly associated with phosphatidylcholine vesicles when the prenylation reaction is carried out in vitro in the presence of phospholipids (15). Therefore, we may speculate that a specific transport step and/or a compartmentalization system could be required to maintain the REP in its site of function and to avoid nonspecific membrane binding in vivo.

The fact that treatment of membranes containing endogenous Mrs6p with a chelating agent such as EDTA (Fig. 3A) results in partial extraction of Mrs6p suggests that its membrane binding in vivo depends to some extent on the presence of divalent cations. To better investigate the participation of divalent cations in the binding of Mrs6p to membranes, we used our in vitro membrane binding assay and observed that pretreatment of the membranes with 25 mM EDTA completely abolishes Mrs6p binding in vitro. Association was restored upon the addition of divalent cations to the assay (Fig. 5). We conclude that both in vivo and in vitro divalent cations participate in the Mrs6p membrane association. Changes in the concentration of intracellular calcium have been extensively reported to regulate secretion in both mammalian and yeast systems (37, 38). For example, a temperature-sensitive growth defect of the ypt1N121I/A161V mutant can be suppressed by high extracellular concentrations of Ca$^{2+}$ (39) as well as by overexpression of the MRS6 gene (7).

We addressed the question of whether the membrane and cytosolic Mrs6p pools could be altered if the activity or the integrity of the protein was affected. We observed that the balance between the membrane-bound and cytosolic Mrs6p is not significantly changed when the wild type Mrs6p is overexpressed (Fig. 6B). Subsequently, we tested the intracellular distribution of two C-terminal truncated forms of Mrs6p: deletion of 33 amino acids (ΔNhe construct) (20) and of 174 amino acids (ΔXba construct) (20) upon overexpression in yeast cells (Fig. 6A). The Mrs6pΔNhe protein, which is able to complement Mrs6p depletion and causes only a slight reduction in the Rab...
GGTase activity (20), was found mainly in the membrane pellet, but still a significant fraction was present in the cytosolic pool (Fig. 6B). The inactive Mrs6(3Xha) protein was instead detected exclusively in the 100,000 × g pellet (Fig. 6B). Previous studies have shown that this truncated protein is unable to interact with the Ypt1 protein (20). The double effect of the C-terminal deletion on Ypt binding and on Mrs6p partitioning suggests that the soluble Mrs6p pool is dependent on a properly conferred to the protein by the presence of the C terminus. The importance of the intact C terminus for proper intracellular distribution of Mrs6p was further supported by the observation that the replacement of the Mrs6p C terminus with the C-terminal part of the bovine RabGDI (Fig. 6A) does not restore the wild type distribution of the Mrs6p. Such chimeric protein is located in the membrane fraction, similarly to the Mrs6(3Xha) protein (Fig. 6B), whereas RabGDI itself is prevalently cytosolic (12). The C terminus of the REP/GDI protein family represents the most variable region among the family members, and crystallographic studies of the bovine RabGDI structure have shown that the RabGDI C terminus together with the structurally conserved region 1 and structurally conserved region 3B constitutes the Rab-binding site (40). Our results show that the C terminus of the Mrs6p cannot be replaced by the C terminus of bovine RabGDI. As summarized in Fig. 6A, the Mrs6-GDI protein was not able to substitute for the wild type Mrs6p in an in vitro geranylgeranylation assay. Because swapping of the Mrs6 C terminus with the RabGDI C terminus may have altered the Rab/Ypt binding site, it is possible that Ypt binding to Mrs6p is required for maintaining the Mrs6 protein in a soluble conformation.

Taken together, our data give new insights into the possible cycle of the REP-Rab/Ypt complex and are consistent with the idea that in the absence of intracellular compartmentalization or of regulated conformational switching, Mrs6p has a tendency to associate with all intracellular membranes. Conformational changes due to the formation of a functional heterodimer between Ypt and REP proteins could mediate the membrane association/dissociation properties of Mrs6p by forcing it into a more hydrophilic conformation. Changes in intracellular ion concentrations could also be a part of the mechanism by which Mrs6p binding to membranes in vivo is regulated.

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