Rab GTPases are localized to distinct subsets of organelles within the cell, where they regulate SNARE-mediated membrane trafficking between organelles. One factor required for Rab localization and function is Rab GDP dissociation inhibitor (GDI), which is proposed to recycle Rab after vesicle fusion by extracting Rab from the membrane and loading Rab onto newly formed transport intermediates. GDI is composed of two domains; Rab binding is mediated by Domain I, and the function of Domain II is not known. In this study, Domain II of yeast GDI, encoded by the essential GDII/SEC19 gene, was targeted in a genetic screen to obtain mutants that might lend insight into the function of this domain. In one gdi1 mutant, the cytosolic pools of all Rab tested were depleted, and Rab accumulated on membranes, suggesting that this mutant Gdi1 protein has a general defect in extraction of Rab from membranes. In a second gdi1 mutant, the endosomal/vacuolar Rab Vps21p/Ypt51p and Ypt7p accumulated in the cytosol bound to Gdi1p, but localization of Ypt1p and Sec4p were not significantly affected. Using an in vitro assay which reconstitutes Gdi1p-mediated membrane loading of Rab, this mutant Gdi1p was found to be defective in loading of Vps21p but not Ypt1p. Loading of Vps21p by loading-defective Gdi1p was restored when acceptor membranes prepared from a deletion strain lacking Vps21p were used. These results suggest that membrane-associated Rab may regulate recruitment of GDI-Rab complexes to the cytosol, possibly by regulating a GDI-Rab receptor. We conclude that Domain II of Gdi1p is essential for Rab loading and Rab extraction, and confirm that each of these activities is required for Gdi1p function in vivo.

Monomeric GTPases of the Ras superfamily regulate myriad cellular pathways, and the study of the mechanisms by which they confer regulation, and the mechanisms by which they are themselves regulated, are central problems in cell biology. Members of the Rab subfamily regulate trafficking of macromolecules between organelles by directly regulating the machinery responsible for vesicle-mediated trafficking. Each unique inter-organelle transport pathway within the cell is regulated by a distinct Rab GTPase, and this is reflected in the localization of each Rab to distinct organelles (1, 2). The enzymatic cycle of several Rabs is well characterized, and they work in much the same way as Ras. Guanine nucleotide exchange factors activate Rab by facilitating exchange of GDP for GTP, activating downstream signaling by specific recruitment of effectors proteins which bind activated Rab. GTPase activating proteins terminate Rab signaling by accelerating hydrolysis of GTP (1). Of the Rab guanine nucleotide exchange factors characterized to date, each is specific for a single Rab, but the characterized Rab GTPase activating proteins exhibit overlapping specificities in vitro (3–7).

Membrane association of Rab is mediated primarily by geranylgeranyl moieties covalently attached to C-terminal cysteine residues (8). In addition, however, a soluble pool of Rab also exists, associated with Rab GDP dissociation inhibitor (GDI), a protein first identified by its ability to inhibit activation of Rab (9). The importance of GDI function is reflected in the finding that mutations in the gene encoding the human GDI result in mental retardation (10). Rab GDI extracts inactive (i.e. GDP-bound conformation) Rab from membranes in what is thought to constitute the initial step of Rab recycling. In the second step of Rab recycling, Rab is loaded onto membranes. Little is known about the mechanism by which GDI-Rab complexes are targeted to membranes. Presumably, only a subset of intracellular membranes can serve as the target for GDI-mediated Rab loading, although this idea has not been rigorously tested. Accessory factors that regulate GDI function have been postulated to exist, although none have been identified. An activity that stimulates dissociation of Rab9 from GDI, but does not catalyze nucleotide exchange, has been termed GDI displacement factor and suggested to facilitate loading of Rab9 onto endosomal membranes (11). Hypothetical factors that facilitate Rab membrane extraction by GDI have been termed Rab recycling factors (12).

The crystal structure of the α isoform of bovine GDI has been solved to high resolution (13, 14). The molecule consists of two structural domains: Domain I contains all of the β strands present in the molecule, and a smaller domain, Domain II, is composed primarily of α-helix (13). Previous mutagenesis studies have clearly shown that Rab binds Domain I in a region termed the Rab-binding platform (12, 13, 15). The function of helical Domain II is not known, although it has been postulated to interact with protein receptors important for membrane targeting of GDI-Rab complexes (12–15). Domain II of the highly related Mrs6 protein, a component of the Rab prenylation machinery, has been implicated in binding enzymatic subunits of the prenylation machinery, consistent with the role of Domain II in proteins of the CHM/GDI family in recognition of other factors required for Rab function. Recently, a highly mobile loop in GDI, termed the “GDI effector loop,” was iden-
tifled in a region linking Domain I and Domain II, and this loop was also suggested to constitute an important element of the membrane targeting region of GDI (14).

In the study presented here, we have addressed several key issues regarding the mechanism by which GDI-Rab complexes are targeted to membranes using genetic and biochemical approaches with the budding yeast, Saccharomyces cerevisiae. Yeast contains a single, essential gene encoding Rab GDI (GDI1/SEC19) which has been shown to interact with multiple Rab5s by a variety of assays (16). We have focused our studies on two Rab5s that function in the vacuolar protein sorting pathways, Vps21p, required for biosynthetic transport between the Golgi and the late endosome (17, 18), and for early endosome-to-late endosome transport (19), and Ypt7p, a Rab required for Golgi and early endosome (17, 18), and for early endosome-to-late endosome transport (19), and Ypt7p, a Rab required for late endosome-to-vacuole transport (20, 21) and for homotypic fusion of vacuoles (22). Regarding Gdi1p, we have focused our efforts on investigating the function of the helical domain, as this domain has been implicated in membrane targeting.

MATERIALS AND METHODS

Strains and Media—S. cerevisiae strains used for these studies are listed in Table I. Yeast strains were grown in standard yeast extract, peptone, dextrose (YPD) (22), yeast extract, peptone, fructose (YPF), or synthetic media (SM) with essential amino acid supplements (23) as required for maintenance of plasmids. Standard bacterial media (24) was used for Escherichia coli cultures. Transformation of S. cerevisiae strains was done by the lithium acetate method of Ito et al. (25) with single-stranded DNA employed as carrier (28). E. coli transformations were done according to the method of Hanahan (27).

DNA Manipulations—Standard DNA manipulations (28) were carried out with restriction endonucleases, DNA modification enzymes, and Taq polymerase from Promega, Roche Biochemicals, or New England BioLabs. DNA was prepared using Qiagen prep column and DNA fragments were gel-purified using a Qiagen gel extraction kit.

The plasmid vector used for random mutagenesis of GDI1, pPG3, was constructed by digesting pRS414 with ScaI, then trimming the ends with T4 DNA polymerase, and reclosing the plasmid. Next, the wild-type GDI gene (12) was cloned into the SpeI and CioI sites. Versions of mutant Gdi1p tagged with the hemagglutinin (HA) epitope were generated by replacing the NcoI to NotI fragment of pPGdi11 or pPGdi29 with the NcoI to NotI fragment from wild-type, HA-tagged GDI1 (12). The membrane trafficking and Rab fractionation phenotypes of HA-tagged gdi1 mutant cells was indistinguishable from the phenotypes of native gdi1 mutants (data not shown).

Mutagenesis of GDI1—Gapped plasmid repair mutagenesis was used to restrict mutagenesis to codons encoding amino acids 81 to 175 of GDI1 (29). To prepare the vector for transformation, 50 μg of pPG3 was digested with NcoI and ScaI, and the gapped vector was gel-purified. For polymerase chain reaction amplification of GDI1, four separate PCR reactions were performed. The standard concentrations for each nucleotide in the mutagenic PCRs was 200 μM. Four PCRs were done, and in each PCR the concentration of one of the nucleotides was dropped as follows: dATP, 0.2 μM; dGTP, 0.13 μM; dCTP, 0.13 μM; dTTP, 0.2 μM. These concentrations were determined empirically to produce approximately equal yields of amplified product, and product from all four PCRs was pooled. For transformation, gapped plasmid and PCR-mutagenized DNA were transformed into CBY71. We used plasmid shuffling on 5-fluoroorotic acid plates to exchange the wild-type GDI1 allele on a URA3 vector for the gap-repaired allele (30). Two screens were done to identify mutants affected in GDI1 function. To screen for temperature-cometional growth, transformation plates were replicated to two YPD plates and one set of plates was maintained at room temperature and the other set was incubated at 37 °C for 3 days. Colonies were identified which grew well at room temperature, but not at 37 °C. To screen for gdi1 mutants defective in vacuolar protein sorting, transformation plates were replica-plated to two YP fructose plates and allowed to grow at room temperature overnight. The next morning, one set of plates was transferred to 37 °C for 3 h, and then both plates were screened for secretion of CPY-Invertase using a previously described colorimetric plate assay (31). Colonies were identified that secreted the CPY-Invertase fusion protein at both temperatures, or predominantly at 37 °C.

Subcellular Fractionation—Subcellular fractionation experiments were carried out as described (34). For experiments requiring temperature shift, cells were converted to spheroplasts, and then resuspended (5 A_{600} /ml) in SM media containing 1% sorbitol. Each culture was split in half, and one-half was incubated with shaking at 26 °C while the other was placed at 37 °C as indicated for 30 min before harvesting.

GDI Functional Assays—Coimmunoprecipitations of Rabs by HA-tagged Gdi1p were done as described in Luan et al. (12). Antisera used for immunoblotting various Rab proteins have been described (12, 17).

For in vitro GDI assays, acceptor membranes were prepared as follows. Cells relevant genotype indicated in the legends to the figures were converted to spheroplasts, and lysed on ice in reaction buffer (RB) (20 mM HEPES, pH 7.5, 200 mM sorbitol, 50 mM potassium acetate, 1 mM dithiothreitol, 1 mM EDTA, 5 mM magnesium chloride, and protease inhibitors (mini-complete, Roche Molecular Biochemicals)) at a concentration of 20 A_{600} /ml with 20 strokes of a glass tissue homogenizer. Cell extracts were cleared by centrifugation at 300 x g for 5 min. Then the extract was then loaded onto a 1-mJ cushion of sucrose (60% in RB), and centrifuged at 100,000 x g for 1 h in a Sorvall SS-34 rotor. The supernatant fraction was collected and the protein concentration determined.

Standard assays contained 80 μg of membrane protein in the acceptor fraction, 100 μg of S100 protein in the donor fraction, and GDP or GTPγS as indicated. The final assay volume was 300 μl, and standard assay conditions were 30 °C for 30 min. After the assay, 1 ml of RB was added to each reaction and mixed. Diluted assay mixtures were centrifuged for 1 h at 100,000 x g in a Sorvall SS-34A rotor. Supernatant fractions were collected, precipitated with trichloroacetic acid, washed with cold acetone twice, and then dried. Sample buffer (2.5 mM Tris, pH 6.8, 12% SDS, 6 M urea, 5% β-mercaptoethanol, 10% glycerol) was then added to each pellet. An equivalent amount of sample buffer was added to each pellet fraction. Rabs were visualized by immunoblotting and quantitated by densitometry using NIH Image software (developed at the U.S. National Institutes of Health and available on the Internet).

To test the effect of trypsin digestion on acceptor membranes, 200 μg of membranes (protein concentration), harvested using the standard protocol, were incubated with 90 μg of trypsin (Life Technologies) at 37 °C for 30 min. Protease inhibitor (AEBSF) (Calbiochem) was then added to 10 mM, and the membranes were harvested by centrifugation at 100,000 x g for 1 h. Mock-treated membranes were prepared exactly as above, except trypsin was omitted. The membrane pellets were resuspended in RB containing 1 mM AEBSF and protein concentrations were determined. Standard loading assays were set up using equivalent volumes of mock treated and trypsin-treated membranes.

For Rab loading saturation experiments (Fig. 6B), standard GDI assays were set up using only 20 μg of membrane protein, and different

| Table I |
| --- |
| S. cerevisiae strains used in this study |
| **Strain** | **Genotype** | **Source** |
| CBY71 | MAT a, gdi1Δ::HIS3 pRS416-GDI1, ura3–52, his3–Δ200, trpl-Δ901, lys2–801, suc2–Δ9, leu2–Δ3, 112; pPHY11(CPY-Invertase) | This study |
| CBY74 | MAT a, gdi1Δ::HIS3 pRS416-GDI1, ura3–52, his3–Δ200, trpl-Δ901, lys2–801, suc2–Δ9, leu2–Δ3, 112 | Luan et al. (12) |
| GBT10 | MAT a, vps2Δ1::HIS3, his3–Δ200, ura3–52, trpl-901, lys2–801, suc2–Δ9, leu2–Δ3, 112 | Horazdovsky et al. (17) |
Rab GTPase activating protein. B, a ribbon diagram of bovine GDI-a (13) is shown with several features highlighted. The region of yeast GDI1 mutagenized in this study is highlighted in dark gray, and the previously defined Rab-binding platform is contained in the dashed box. Both the Rab loading-defective (gdi1-29) and the Rab extraction-defective (gdi1-11) mutant Gdi1 proteins contained two mutations in common, M140I and G141S, contained in the rectangle. The Rab loading-defective mutant contained two additional mutations, Q55R and T105A, which are boxed. The Rab extraction-defective mutant contained four additional mutations, S116P, I155S, V179A, and R211G, which are circled.

amounts of cytosol prepared from wild-type cells. Each reaction contained 100 μM GTP-γ-S, Vps21p was visualized using an enhanced chemifluorescence kit (Pierce) and the signals were quantitated on a STORM PhosphorImaging system (Molecular Dynamics), or by densitometry of ECL films.

RESULTS

Mutagenesis of Gdi1p Helical Domain—Previous structure/function studies of mammalian and yeast GDI have identified the apex of Domain I as the region which binds Rab, and this region has been termed the Rab-binding platform (Fig. 1) (12-15). GDI also contains a highly conserved Domain II, composed primarily of α-helix, and the function of this domain is not known. Using a PC-based random mutagenesis procedure, we targeted amino acids 54–225 of yeast Gdi1p, which includes nearly all of Domain II (amino acids 128–228), to obtain new mutants that might provide insight into the function of this conserved domain. A small portion of Domain I was also targeted due to restraints imposed by the location of restriction enzyme sites. After a plasmid shuffle scheme to swap wild-type and mutagenized GDI1 genes, we screened for temperatureconditional loss-of-function mutants by identifying colonies which grew at 23 °C, but grew poorly or not at all at 37 °C, and in a second screen of the same transformants, we identified mutants which missorted vacuolar proteins at 23 °C and/or at

37 °C using a colorimetric-based plate assay (31). Two mutants were obtained which grew well at 23 °C, but extremely slowly at 37 °C. Twenty-eight mutants were obtained which missorted vacuolar proteins at 23, 37 °C, or at both temperatures. Each of the mutants obtained was characterized by pulse-chase analysis of newly synthesized carboxypeptidase Y (CPY). Biogenesis of CPY can be informative for identifying trafficking defects in multiple segments of the secretory and vacuolar protein sorting pathways due to compartment-specific modifications (addition of N-linked carbohydrate and proteolytic processing) which can be easily monitored by SDS-polyacrylamide gel electrophoresis. Based on this analysis, the collection of mutants could be categorized into two classes. Class I mutants (2 isolates) exhibited a rapid, temperature-conditional block in endoplasmic reticulum to Golgi transport similar to the original gdi1/sec19-1 mutant (Fig. 2), and did not secrete any CPY from the cell at restrictive temperature (data not shown). Class II mutants (28 isolates) accumulated Golgi-modified p2CPY, especially when assayed at 26 °C (Fig. 2), and a small amount of p2CPY (~5%) was secreted into the media (data not shown). Analysis of protein secretion in representative mutants from each class revealed a strong block in secretion from the Class I mutant (gdi1-11) cells, while secretion from the Class II mutant (gdi1-29) was similar to wild-type cells (Fig. 2). DNA sequencing of three mutants (2 Class I mutants and 1 Class II mutant) revealed that each had multiple mutations, but interestingly, all three mutants had changes in the same two codons encoding Met140 and Gly141 (Fig. 1).

Gdi1p-mediated Rab Recycling Is Affected in gdi1 Mutants—It is thought that the primary function of Rab GDI is to recycle inactive Rab GTTPases after vesicle fusion back to an appropriate donor membrane (e.g., a transport vesicle). Two biochemical activities of GDI contribute to this function, extraction of inactive Rab-GDP from the membrane and loading of Rab-GDP onto membrane (Fig. 1). To determine which activities of Rab GDI are affected in the mutants, we examined the distribution of multiple Rabs in wild-type cells and in the gdi1-11 and gdi1-29 mutants at permissive (26 °C) and restrictive temperatures (37 °C) (Fig. 3). Mutant cells were incubated at 26 or 37 °C for 30 min prior to cell fractionation, and the relative amounts of Rab in pellet fractions containing membranes and soluble cytosolic fractions were determined by immunoblotting with antibodies to Ypt1p (endoplasmic reticulum to Golgi transport), Vps21p (Golgi to endosome, and early endosome to late endosome transport), Ypt7p (late endosome to vacuole transport and vacuole-vacuole fusion), and Sec4p (Golgi to plasma membrane transport). In wild-type cells, the majority of each Rab fractionated with membranes in the 100,000 x g pellet, in agreement with previously published studies. In the Class I gdi1-11 mutant, the distribution of all four Rabs was affected compared with wild-type cells. When cells were preincubated at restrictive temperature (37 °C), the cytosolic fractions of each Rab were nearly completely depleted, with a concomitant increase in membrane-associated pools. Compared with wild-type cells, little change in Rab fractionation was observed when gdi1-11 cells were preincubated at permissive temperature (26 °C), suggesting that this Gdi1p mutant protein has a general, temperature conditional defect in membrane extraction of Rab.

In the gdi1-29 mutant, Vps21p and Ypt7p accumulated in the cytosol when cells were preincubated at 26 or 37 °C (Fig. 3A). Localization of Ypt1p (Fig. 3B) or Sec4p (data not shown) were not significantly affected. In three independent experiments, an average of ~60% of Vps21p, and ~90% of Ypt7p were detected in the cytosolic fractions of mutant cells compared with ~20 and 10%, respectively, in wild-type cells. Thus, in the
FIG. 2. Protein transport in gdi1 mutants. A, CPY sorting assays. The indicated cultures were incubated at 26 or 37 °C for 15 min prior to labeling for 10 min with Easy-Tag. Chase solution was then added and the cells were incubated for 30 min. Cultures (media and cells) were then harvested by trichloroacetic acid precipitation and processed for immunoprecipitation of CPY. B, secretion assay. The indicated cultures were labeled and chased as described above. After the chase period, cells were removed from the culture by centrifugation, and proteins in the media fraction were collected by trichloroacetic acid precipitation and visualized by autoradiography.

FIG. 3. Distribution of Rab GTPases in gdi1 mutants. A, Vps21 and Ypt7p. Spheroplasts of the indicated cultures were preincubated at 37 °C for 30 min prior to cell lysis. After lysis, cell extracts were centrifuged (100,000 g) for 1 h to separate membranes from soluble components, and equivalent amounts of pellet fractions containing cell membranes, and supernatant fractions containing soluble components, were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with antibodies to the indicated Rab. Membrane fractions were then recovered by centrifugation and the amounts of this Rab in the membrane fraction, with a concomitant increase in the cytosolic pool, indicated that Vps21p had been loaded onto membranes in the gdi1-29 mutant. The membrane-associated pools of the endosomal/vacuolar Rabs, Vps21p and Ypt7p, were depleted, with a concomitant increase in the cytosolic pools of these Rabs. These results suggest that the gdi1-29 mutant Gdi1 protein is defective preferentially in membrane loading of Vps21p and Ypt7p.

Mutant Gdi1 proteins unable to bind Rab due to mutations in the Rab-binding platform are not functional and do not support the essential function of GDI1 in vivo (12). Thus, it was important in the case of the gdi1-29 mutant to determine whether Rab accumulated in the cytosol bound to Gdi1p. To test this, we introduced HA epitope-tagged versions of wild-type and mutant Gdi1p that allowed us to immunoprecipitate Gdi1p under native conditions (12). Spheroplasts were incubated at 26 or 37 °C for 30 min, then lysed and membranes were removed by centrifugation. Gdi1p was immunoprecipitated from the soluble fraction under native conditions and the immunoprecipitated material was then probed with antibodies to Vps21p, Ypt7p (Fig. 3), Ypt1p, and Sec4p (data not shown). All four Rab proteins coimmunoprecipitated with wild-type and gdi1-29 mutant Gdi1 proteins, indicating that the gdi1-29 mutant Gdi1 protein is not defective in binding Vps21p or Ypt7p.

Mutant Gdi1 Proteins Are Defective in Rab Extraction and Loading—Accumulation of Rab in the membrane fraction, with a concomitant loss in the cytosol fraction, suggests that the gdi1-11 mutant protein is defective in membrane extraction of Rab, whereas accumulation of Rab in the cytosol bound to Gdi1p suggests that the gdi1-29 mutant protein is defective in Rab loading. To test these hypotheses, an in vitro assay was developed that monitored Rab loading and extraction. For these experiments, we focused on Vps21p because localization of this Rab in the gdi1 mutants was representative of the other three Rabs assayed in the experiments described above, and because several factors which regulate, or are regulated by, Vps21p are known. In the first experiment, membranes were collected from wild-type cells and served as “acceptor” membranes. Cytosol from wild-type cells, cleared of membranes by centrifugation, was used as a source of Gdi1p-Vps21p complexes (the “donor” fraction). The two fractions were mixed, incubated at 30 °C for 30 min with or without added GDP or GTPγS, then diluted with ice-cold reaction buffer. Membranes were then recovered by centrifugation and the amounts of Vps21p in the pellet and supernatant fractions, containing membranes and cytosol, respectively, were determined by immunoblotting (Fig. 4). Comparison of the amounts of Vps21p in the starting donor and acceptor fractions, versus the amounts in the membrane and cytosol fractions after incubation together revealed whether net loading or extraction had occurred during the incubation. When cytosol from wild-type cells was used, there was little change in the distribution of Vps21p between membranes and cytosol in the absence of added nucleotide, or in the presence of GDP, indicating that little loading or extraction of Rab occurred under these conditions. An alternative interpretation of this result is that Rab loading and extraction are balanced. When GTPγS was included in the reaction, Vps21p was recovered predominantly in the membrane fraction, indicating that Vps21p had been loaded onto membranes under these conditions. Rab loading required physiological temperature, as little Vps21p net loading was observed when the reaction was incubated on ice (Fig. 5). Immunoblotting of membrane fractions with antibodies to Gdi1p revealed that Gdi1p was not detected in the membrane frac-
A putative GDI receptor is expected to bind Gdi1p, Rab, or both. We adapted the in vitro GDI assay to test for a possible role of membrane-associated Rab on GDI-mediated Rab loading. To do so, we took advantage of the fact that VPS21 is not an essential gene, so we were able to prepare membranes from a vps21Δ null strain, and test them for acceptor activity, using cytosol prepared from wild-type cells. Vps21p loading required substantially more GTPγS compared with standard assays using membranes from wild-type cells which contain Vps21p (Fig. 7). Over three independent experiments, ~60% of Vps21p was loaded in the presence of 150 μM GTPγS, while greater than 90% of Vps21p was loaded under standard assay conditions (100 μM GTPγS) using membranes prepared from wild-type cells containing Vps21p. These results suggest that membrane-associated Vps21p strongly influences Gdi1p-mediated loading of cytosolic Vps21p.

We next examined loading by the gdi1-29 Gdi1p mutant, using vps21Δ membranes and cytosol prepared from gdi1-29 cells (Fig. 7). Surprisingly, when cytosol prepared from gdi1-29 cells was tested, no significant difference between wild-type and gdi1-29 loading of Vps21p was observed. These results indicate that membrane-associated Vps21p is required to recapitulate in vitro the Rab loading defect of the gdi1-29 mutant protein. Because loading of cytosolic, Gdi1p-associated Vps21p clearly does not require membrane-associated Vps21p, these
Gdi1p-mediated Rab Loading—Membrane-associated receptors which recruit Gdi1p-Rab complexes from the cytosol have been postulated to exist, however, no functional receptor for GDI-mediated loading of any Rab has been identified (35). Evidence supporting this hypothesis includes saturation loading of Rab (Fig. 6), and that protease treatment of membranes inhibits Rab loading activity (Fig. 6) (36–39). A candidate receptor for GDI-mediated Rab loading in mammalian cells is prenylated Rab acceptor protein, Pra1, a predicted membrane protein which was first identified in a two-hybrid screen with Rab3, and subsequently demonstrated to bind many other (but not all) Rabs (40, 41). Pra1 also has been shown to bind weakly to GDI and inhibit in vitro extraction of Rab3a by recombinant GDI (42). The yeast protein Yip3p is homologous to Pra1, and was identified in a two-hybrid screen using the yeast Rab Ypt31p, indicating that it can bind Rab, but little else is known about Yip3p function.2 Another Rab-binding membrane protein identified in the same screen was Yip1p, which is essential for secretion (43). We have found that overexpression of YIP1 or YIP3 does not suppress Rab mislocalization in the gdi1-11 loading-defective mutant (data not shown). Nonetheless, the sequence and functional similarities between human Pra1 and yeast Yip1p and Yip3p suggest that Yip1p and Yip3p are likely candidates for regulating Rab and possibly Gdi1p functions in yeast.

An important implication of the analysis of the gdi1-29 loading-defective mutant is that Gdi1p-dependent localization of Vps21p and Ypt7p appear to be coregulated. This conclusion is based on the observations that localization of Vps21p and Ypt7p (but not Ypt1p or Sec4p) were affected in the gdi1-29 mutant. One possible explanation for these results is that the rates at which different Rabs are recycled by GDI are not equivalent, so that in gdi1 mutants Rab localization is perturbed in nonequivalent ways. If, for example, Vps21p and Ypt7p are rapidly recycled compared with Ypt1p and Sec4p, then loss of Gdi1p-mediated loading would affect localization of Vps21p and Ypt7p more significantly than Ypt1p or Sec4p. An alternative explanation is that loading-defective Gdi1p fails to interact sufficiently with a membrane receptor that functions for only a subset of Rabs including Vps21p and Ypt7p. The target organelles for Vps21p and Ypt7p loading have not been identified, and this will be crucial information for distinguishing these possibilities.

Membrane-associated Vps21p had a dramatic effect on in vitro loading of Vps21p; in vitro membrane loading of Vps21p by the gdi1-29 mutant Gdi1p mutant was defective, but only when membranes containing Vps21p were used in the assays. Thus, the presence of Vps21p on membranes more faithfully reconstitutes the Gdi1p-Rab loading reaction. Because membranes lacking Vps21p were still active for Vps21p loading, we speculate that the activity of an as yet unidentified Gdi1p-Vps21p receptor may be regulated by direct association of Vps21p with the putative receptor. Under conditions where receptor is not bound to Rab (i.e. vps21Δ membranes), Gdi1p-mediated loading is facilitated, however, when receptor is limiting, due to association with Rab, differences in the abilities of wild-type and mutant Gdi1p to load Rab manifest. This mechanism could provide a means to regulate Rab loading according to the availability of receptor.

2 D. Gallwitz, personal communication.
to the amount of Rab engaged with membrane-associated proteins such as downstream effectors, which would in turn affect the amount of Rab available to bind receptor. An alternative possibility is that gross changes in the membrane composition of acceptor membranes prepared from vps21Δ cells facilitated loading by the gdi1-29 loading-defective Gdi1 protein. This possibility is unlikely because membranes prepared from other deletion strains which lack endosomal components that interact with Vps21p (Pep12p t-SNARE and Vac1p effector) behave like wild-type membranes in in vitro loading assays. Membranes from these cells are expected to have gross changes in composition similar to vps21Δ cells.

Membrane Extraction of Rab by GDI—Little is known about regulation of GDI-mediated extraction of Rab. Only Rab in the inactive, GDP-bound state is a ligand for GDI (44), so activation of Rab by GDP/GTP exchange, and hydrolysis of GTP, are presumed to be key factors regulating the size of cytosolic Rab pools. In this context, it is surprising that the amount of Vps21p in the cytosol of wild-type and vps2Δ cells (encoding a guanine nucleotide exchange factors for Vps21p (5)) is not significantly different compared with wild-type cells. Other factors must regulate extraction of Rab by Gdi1p, and these putative factors have been termed Rab recycling factors (12). Observations supporting this idea include the finding that expression of Gdi1p mutant proteins unable to bind Rab still associate with membranes in a protease-sensitive manner (12), and that expression of high levels of Rab binding-defective Gdi1p results in depletion of cytosolic Rab pools. The collection of new gdi1 mutants described here should be useful for identifying these and other factors that regulate Gdi1p function.

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