Molecular Dissection of Guanine Nucleotide Dissociation Inhibitor Function in Vivo

Rab-INDEPENDENT BINDING TO MEMBRANES AND ROLE OF RAB RECYCLING FACTORS

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Peng Luan‡, William E. Balch‡§, Scott D. Emr¶, and Christopher G. Burd**

From the ‡Departments of Cell and Molecular Biology-IMM 11, The Scripps Research Institute, La Jolla, California 92037 and the §Division of Cellular and Molecular Medicine, The Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, California 92093-0668

Guanine nucleotide dissociation inhibitor (GDI) is an essential protein required for the recycling of Rab GTPases mediating the targeting and fusion of vesicles in the exocytic and endocytic pathways. Using site-directed mutagenesis of yeast GDI, we demonstrate that amino acid residues required for Rab recognition in vitro are critical for function in vivo in Saccharomyces cerevisiae. Analysis of the effects of Rab-binding mutants on function in vitro reveals that only a small pool of recycling Rab protein is essential for growth, and that the rates of recycling of distinct RabS are differentially sensitive to GDI. Furthermore, we find that membrane association of Gdi1p is Rab-independent. Mutant Gdi1 proteins unable to bind Rabs were able to associate with cellular membranes as efficiently as wild-type Gdi1p, yet caused a striking loss of the endogenous cytosolic Gdi1p-Rab pools leading to dominant inhibition of growth when expressed at levels of the normal, endogenous pool. These results demonstrate a potential role for a new recycling factor in the retrieval of Rab-GDP from membranes, and illustrate the importance of multiple effectors in regulating GDI function in Rab delivery and retrieval from membranes.

Rab proteins are membrane-associated small GTP-binding proteins which regulate the targeting and fusion of vesicle carriers transporting cargo between compartments of the exocytic and endocytic pathways of both yeast and mammalian cells (1). Following vesicle fusion, Rab proteins in the GDP-bound form are recycled by members of the guanine nucleotide dissociation inhibitor (GDI) gene family (reviewed in Ref. 2 and 3). The α isofrom of GDI, first discovered by Takai and colleagues (4) based on its ability to inhibit the intrinsic dissociation of GDP from Rab3A, is now recognized to be one of 2 major isofroms (α and β) found in mammalian cells that have nearly 85% identity and are highly related to yeast Gdi1p (>50% identity with α-GDI), the product of the single, essential GDI/SEC19 gene (5). Southern blot analyses of genomic DNA indicate that both mouse and rat contain at least five rab GDI genes (6). GDI family members are closely related to members of the CHM/REP gene family involved in Rab prenylation (reviewed in Ref. 7), thereby forming a GDI superfamily (reviewed in Ref. 3). While other Rab effector proteins, including guanine nucleotide exchange factors or GTPase activating proteins, exhibit specificity for individual Rab proteins, GDI family members recognize all Rab proteins examined to date (8, 9). This has led to the proposal that GDI/REP family members principally function in maintaining a cytosolic reservoir of Rab proteins for delivery to membranes.

We have solved the structure of bovine α-GDI at 1.8-Å resolution using x-ray crystallography (10). α-GDI is constructed of two main structural units, a large multisheet domain I and a smaller α-helical domain II. Domain I is largely composed of sequence conserved regions (SCRs) which are common to all members of the GDI superfamily. SCRs located in the NH₂-terminal and central portions of the molecule fold to form a compact structural unit at the apex of GDI. In particular, SCRs 1 and 3B contain tri- and tetrapeptide motifs that are invariant from yeast to man (11). The polar side chains of amino acids in these motifs are directed away from the α-carbon backbone, suggestive of a role in the recognition of other proteins. Selected residues in SCRs 1 and 3B have been implicated for the binding of Rab3A in vitro and for the ability of α-GDI to extract the GDP-bound forms of Rab3A from permeabilized rat brain synaptosomes (10). Recently, we have found that residues involved in Rab recognition have differential effects on Rab1 binding and ER to Golgi transport in vitro (12). These residues, in addition to other highly conserved residues distributed throughout the NH₂-terminal half and central half of GDI are grouped on only one face of the molecule, leading us to previously speculate that this face is involved in most, if not all, important features of GDI/CHM biological function (13).

The critical role of GDI family members in the regulation of membrane transport throughout the endocytic and exocytic pathways stresses the importance of understanding the contribution of individual amino acid residues in directing interaction of GDI not only with Rab, but importantly, upstream or downstream effectors involved in Rab recycling. The fact that the yeast S. cerevisiae contains only a single copy of GDI1 which is essential for cell growth (5) provides an excellent tool to extend our previous studies on GDI function in ER to Golgi transport in vitro using mammalian cells (12). To date, only one additional factor, a guanine nucleotide displacement factor has been proposed to function in the GDI-Rab cycle, in this case facilitating the delivery of Rab to membranes by interaction with the GDI-Rab cytosolic pool (14–16).

We now report evidence for a new factor involved in GDI...
function in vivo. We find that single point mutations in yeast GD11 codons encoding homologous residues of α-GDI involved in Rab3A binding and membrane extraction in vitro (10) exhibit reduced Rab binding in vivo yet retained partial Gdi1p function in support of growth and transport. These results establish for the first time the physiological importance of the putative Rab-binding platform found at the apex of GDI for function in vivo and that a functional excess of GD1-Rab complexes are present in living cells. Strikingly, selected residues involved in Rab binding in vitro, when combined in double mutants, had dominant effects on growth, were found to render GDI completely defective in Rab binding in vivo, failed to complement vesicular transport and growth defects of gdi1Δ and gdi1 temperature-sensitive mutations, and led to the accumulation of Rabs on membranes. Despite these changes, these double mutants bound membranes as efficiently as wild-type Gdi1p in vivo and in vitro. The implication of these results on the mechanism of GDI function in the delivery and retrieval of the Rab-GDP from membranes following vesicle fusion reveals a potential role for a rab recycling factor that mediates GDI-dependent retrieval of Rab-GDP.

MATERIALS AND METHODS

Strains, Media, and Microbiological Methods—Yeast strains were grown in standard yeast extract, peptone, dextrose (YPD) (17), yeast extract, peptone, dextrose (YPF), or synthetic media (SM) supplemented with 2% casamino acids and essential amino acid supplements (17) as required for maintenance of plasmids. Transformation of Saccharomyces cerevisiae strains was done by the lithium acetate method (18) with single-stranded DNA employed as carrier (19). Standard bacterial media was used for Escherichia coli cultures. E. coli transformations were done as described (20).

S. cerevisiae strains used for these studies are as follows. A gdi1Δ strain (CBY71) was constructed by transformation of a wild-type diploid strain (SEY 6210a/a) with a CEN URA3 plasmid containing GD11, and then transformation of this strain with the gdi1Δ::HIS3 deletion-disruption construct (described below). After confirming by polymerase chain reaction that this strain contained one intact and one disrupted copy of GD11, it was sporulated and colonies derived from Ura− His+ spores were identified. The sec19-1 strain, CBY47 (sec19-1 ura3-52 tcp1ΔΔS01 (eu3-3, 112 his3Δ200), was constructed by crossing NY420 (sec19-1 ura3-52) to a wild-type strain (SEY 6210), sporulating this strain and identifying colonies which did not grow at 37 °C.

DNA Methods—Standard DNA manipulations (21) were used with restriction endonucleases and modification enzymes from Roche Molecular Biochemicals, New England Biolabs, or U. S. Biochemical Corp. The gdi1Δ::HIS3 construct was generated by replacing the DNA fragment encoding amino acids 17 through 414 of Gdi1p with a DNA fragment containing the HIS3 gene. The gene SOEing technique (22) was used to make gdi1 point mutants with DNA oligonucleotides from Life Technologies, Inc. All mutants were sequenced to confirm that only the intended mutations were present.

Site-directed Mutagenesis of Yeast GD11—To map amino acid residues of yeast Gdi1p that are important for function, a plasmid shuffle-based complementation assay (17) was developed that allowed rapid screening of site-directed point mutants for the ability to complement a gdi1Δ null mutation. Plasmids (CEN LEU2) containing site-directed mutations were used to transform a gdi1Δ::HIS3 strain containing a CEN URA3 GD11 plasmid as the sole source of wild-type Gdi1p. Transformed strains were streaked to 5-fluoroorotic acid plates to select for loss of the URA3 GD11 plasmid leaving the mutant gdi1 gene as the only source of Gdi1p. Growth of the resultant strains was assayed after restreaking to rich medium. This method allowed us to assess if any gdi1 mutants constructed were able to supply the essential function of GD11. As a test to confirm the feasibility of this approach, two separate point mutations (I13R or T256P) were introduced into GD11. Based on the crystal structure (10), we predicted that each of these point mutations (Fig. 1) would affect the structural integrity of the Rab-binding region of Gdi1p and lead to a complete loss of function. As expected, each of these mutants failed to yield Leu+ Ura+ strains after streaking to 5-fluoroorotic acid plates, indicating non-complementation of the gdi1Δ null mutation and thus confirming the validity of this assay (Table I, single asterisk).

We have previously shown that mutation of some of the highly conserved residues in SCRs 1B and 3B found at the apex of GD1 affect the ability of the mutant GDIs to bind Rab3A in vitro (10) (Fig. 1). However, the physiological relevance of these observations remained to be determined. Two other SCRs (2 and 3A) contain conserved residues that are found on one face of GD1 located beneath the Rab-binding region (10) (Fig. 1). Using the crystallographic structure and the aligned sequences of all GDIs and REPs comprising the GDI superfamily as a
guide, point mutations changing conserved, surface-exposed amino acids of the various SCRs that were likely candidate residues for interactions with Rab or other molecules were constructed. Each was tested in the complementation assay. In addition, mutations were introduced to assess other structural features of Gdi1p. These included the GXG motif found at the base of the Rab-binding region that is potentially involved in the binding of a putative nucleotide cofactor. We also examined the importance of the carboxyl-terminal region containing helix N that supports the putative Rab-binding platform found at the apex of Gdi1p (10) (Fig. 1).

The results of our initial analysis is shown in Table I. Despite the extensive collection of point mutants tested, no single amino acid change was found which resulted in a complete loss of Gdi1p function other than point mutations predicted to disrupt structure (Table I, single asterisk). Although we did not observe complete loss of cell growth for the single point mutants, one mutation, R248A (Table I, double asterisks), clearly affected Gdi1p function as evident by the slower growth of this strain. This particular residue was previously implicated as one of the most important amino acids required for Rab binding in vitro, reducing binding approximately 60-fold (10). Because single point mutations of the homologous conserved residues in yeast Gdi1p did not result in lethal gdi1-1 phenotypes, it is apparent that even the reduced level of Rab3A binding measured in vitro for equivalent mammalian GDI mutants (10) is sufficient to maintain adequate levels of Rab recycling in vivo for cell survival. These results now establish a working relationship between in vitro binding assays (that provide relative measure of GDI-Rab interaction) and the ability of GDI to recognize Rab under physiological conditions in vivo. Given the observation that reduced binding is not lethal, we suggest that the recycling activity of wild-type GDI is likely to be in functional excess in living cells.

Because the R248A mutant resulted in a modest growth defect, this mutation was used as a starting point to construct a set of double mutants. Additional amino acid substitutions were made in either the same SCR containing R248 (SCR 3B) or in different SCRs, and each of these was tested in combination with R248A in the complementation assay (Table II). We also generated double mutants within and between SCRs that did not include the Arg248 residue to test whether multiple mutations in regions of Gdi1p flanking the putative Rab-binding platform would yield defective function. Three double mutant combinations did not complement the gdi1Δ mutation (Table II). One lethal construct was a triple mutant involving Arg248 and the residues GXG potentially involved in the binding of a cofactor to GDI (Table II, single asterisk) (10). Significantly, two of the lethal double mutant combinations (Y44V,R248A and R248A,E241S) (Table II, double asterisks) contained residues (Tyr44 and Glu241) which when mutated separately, have been previously implicated in Rab-binding in vitro (10). Thus, disruption of the physiological function of Gdi1p in vivo requires mutation of at least two surface residues in the putative Rab-binding region (10). All other double mutant combinations complemented the gdi1Δ mutation (Table II). Our results suggests that Rab interacts with GDI through multiple residues and demonstrates for the first time the physiological importance of the Rab-binding platform for the function of GDI in living cells.

The R248A,Y44V and R248A,E241S Double Mutants Cause a Growth Defect in sec19-1 Cells—To further define the function of the gdi1 mutant genes, we examined whether selected mutants could complement the growth defect of sec19-1 cells. The function of the protein encoded by sec19-1 is temperature-sensitive resulting from a stop codon near the carboxyl-terminal end of helix N, leading to the production of a truncated protein whose folding is thought to be unstable at slightly elevated (semi-permissive) temperatures (30 °C). Complete loss of function is observed at 37 °C (28). Consistent with this interpretation, further truncation resulting in the partial or complete removal of helix N leads to complete loss of function (data not shown).

### Table I

| Region   | Location | Yeast residue mutated | Growth at 30 °C* | Mammalian residue |
|----------|----------|-----------------------|------------------|------------------|
| Wild-type|          |                       | ±±               | 8                |
| SCR1A    | a1 strand| I13R                  |                  |                   |
| SCR1A    | a1 loop A| G16E                  | ++               | 11               |
| SCR1A    | a1 loop A| G18E                  | ++               | 13               |
| SCR1B    | a2 loop fl| Y44V                 | ++               | 39               |
| SCR2     | c2 strand| K37D                  | ++               | 55               |
| SCR2     | b2 strand| R76A                  | ++               | 70               |
| SCR2     | D loop E |                       | ±±               |                   |
| SCR3B    | 1 helix  | E241S                 | ++               | 233              |
| SCR3B**  | 1 helix  | R248A                 | +                | 240              |
| SCR3B    | a3 strand| T256V                 | ++               | 248              |
| SCR3B**  | a3 strand| T256P                 | −                | 248              |
| SCR3B**  | b4 strand| T322A                 | ++               | 311              |

** a, wild-type; +, sick; and −, dead.

### Table II

| Mutant     | Growth at 30 °C* |
|------------|------------------|
| Wild-type  | ++               |
| 44–16A     | ++               |
| 44–59      | ++               |
| 44–241     | +                |
| 44–241**   | −                |
| 16A–18A    | ++               |
| 16A–109    | ++               |
| 18A–109    | ++               |
| 126–137    | ++               |
| 241–256V   | ++               |

** a, wild-type; +, sick; and −, dead.
were transformed into the bly, when the R248A,Y44V and R248A,E241S double mutants the results of the gdi1 mutation at 37 °C, in complete accord with the sec19-1 strain, we observed significantly slower growth at 26 °C and a dramatic dominant growth defect at 30 °C (Fig. 2B), a semipermissive temperature for sec19-1. Interestingly, neither of the double mutants caused a growth defect when introduced into a wild-type strain, indicating that the double mutants exhibit an allele-specific genetic interaction with sec19-1. These results demonstrate that Gdi1p encoded by sec19-1 is likely to be weakly defective for function at both the permissive and semipermissive temperatures, and that this phenotype is clearly exacerbated by competition with a double mutant which fails to bind Rab (see below).

Mutants Are Deficient in Rab Binding—Mutant gdi1 alleles that failed to complement the gdi1Δ mutation or the sec19-1 ts phenotype could produce proteins that are defective for folding and therefore unstable and degraded. Alternatively, they could be deficient in any essential aspect of Gdi1p function, including Rab binding or interactions with membrane receptors possibly required for Rab delivery or extraction. To distinguish between these possibilities, a vector was constructed which allowed us to tag mutant and wild-type Gdi1p proteins with the influenza hemagglutinin (HA) epitope at the carboxyl terminus (HA-Gdi1p). This region in bovine GDI is disordered in the crystal structure (10) and the epitope tag would not be expected to interfere with Gdi1p function. Consistent with this conclusion, all HA-tagged constructs generated behaved in an identical fashion to their equivalent untagged versions.2

The double mutant genes and their corresponding single mutant genes cloned into the HA-vector were used to transform a sec19-1 strain. Immunoblotting of total cell lysates derived from these strains with an HA-specific antibody revealed that a single band of approximately 52 kDa was present in the R248A,Y44A and R248A,E241S double mutants (Fig. 3A). All other mutant proteins tested, with one exception (see below Fig. 4A), were stable when expressed in vivo (Fig. 3A and data not shown).

To determine if the stable double mutant Gdi1 proteins would bind Rabs, we used a native co-immunoprecipitation assay to examine their interaction with three different Rab GTPases that function at distinct steps of the secretory pathway: Ypt1p, required for ER/Golgi transport (29); Vps21p, required for Golgi/endosome transport (24, 30); and Ypt7, which functions in late endosome to vacuole trafficking and vacuole-vacuole fusion (31, 32). Extracts were prepared from strains grown at permissive temperature (26 °C), centrifuged at 100,000 × g for 1 h to yield P100 particulate fractions containing most intracellular membranes, and S100 cytosolic fractions. HA-Gdi1 proteins were immunoprecipitated from the S100 fractions under native conditions and the immunoprecipitates were probed with antibodies against Ypt1p, Ypt7p, and Vps21p.

While each of the Rabs was efficiently co-immunoprecipitated with wild-type HA-Gdi1p, none were co-immunoprecipitated with the double mutant HA-Gdi1 proteins (Fig. 3B). Notably, significant but reduced and variable amounts of each of these Rabs were co-precipitated by each of the corresponding single mutants (Fig. 3B), a result consistent with the observation each of these single mutants affect binding of Rab3A in vitro (10), yet still support cell growth. Significantly, the differential effect of various point mutants on the recovery of Rab raises the possibility that residues in GDI may play specific roles in the recognition of separate Rab species. Identical results were obtained when we inactivated the endogenous sec19-1 mutant Gdi1p by temperature shift to the restrictive temperature (37 °C) for 30 min before cell lysis, indicating that

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2 P. Loan, W. E. Balch, S. D. Emr, and C. G. Burd, unpublished observations.
the lack of Rab binding observed was not simply a consequence of competition by the endogenous temperature-conditional Gdi1 protein (data not shown).

A concern in the above experiments was that the immunoprecipitation conditions may not accurately reflect binding of Gdi1p to Rab in vivo due to dilution effects following cell homogenization and subsequent washing steps during immunoprecipitation. To address this concern, we first examined whether the membrane association of wild-type and double HA-tagged mutants was the same or different when membranes prepared these cell lines were washed in an identical fashion to that used for co-immunoprecipitation with Rabs. To determine if any precipitation of mutant Gdi1 proteins to membranes. S100 fractions of the corresponding proteins in the immunoprecipitates using immunoblotting, to show that identical amounts of HA-Gdi1p is present in each sample. In B, antibodies to Ypt1p, Ypt7p, and Vps21p were used to detect the corresponding proteins in the immunoprecipitates using immunoblotting. C, identical amounts of the pellet fraction of the 100,000 g centrifugation (P100) were either not washed (a-h) or resuspended and washed (a-d) in an identical way to that of the immunoprecipitations described for panel B. In a and e, the P100 was prepared from the Sec19-1 strain containing the vector only; in b and f, the P100 was prepared from cells expressing HA-wild-type Gdi1p; in c and g, the P100 was prepared from cells expressing HA-R248A,Y44A Gdi1p; in d and h, the P100 was prepared from cells expressing HA-R248A,E241S. HA-Gdi1p was detected using immunoblotting with an HA-specific antibody.

Molecular Dissection of GDI Function in Vivo

**FIG. 3.** Co-immunoprecipitation of Rabs with wild-type and mutant Gdi1 proteins. A and B, identical amounts of cell homogenates of sec19-1 cells expressing HA epitope-tagged wild-type (WT) or mutant Gdi1 proteins (indicated at the top of each lane) were cleared of membranes by centrifugation at 100,000 × g, and Gdi1p was immunoprecipitated from each supernatant fraction (S100) under native conditions. In A, HA-Gdi1p was detected using immunoblotting with a HA-specific antibody in cell homogenates (before immunoprecipitation), to show that identical amounts of HA-Gdi1p is present in each sample. In B, antibodies to Ypt1p, Ypt7p, and Vps21p were used to detect the corresponding proteins in the immunoprecipitates using immunoblotting. C, identical amounts of the pellet fraction of the 100,000 g centrifugation (P100) were either not washed (a-h) or resuspended and washed (a-d) in an identical way to that of the immunoprecipitations described for panel B. In a and e, the P100 was prepared from the Sec19-1 strain containing the vector only; in b and f, the P100 was prepared from cells expressing HA-wild-type Gdi1p; in c and g, the P100 was prepared from cells expressing HA-R248A,Y44A Gdi1p; in d and h, the P100 was prepared from cells expressing HA-R248A,E241S. HA-Gdi1p was detected using immunoblotting with an HA-specific antibody.

The experiments suggest that the failure to bind Rabs represents at least one critical defect responsible for the growth phenotype of single and double Gdi1p mutants.

**FIG. 4.** Distribution of wild-type and mutant Gdi1 proteins between cytosol and membranes. A, strains (sec19-1) expressing the indicated HA epitope-tagged Gdi1 proteins were lysed and subjected to centrifugation at 100,000 × g to generate a P100 membrane fraction and an S100 cytosol fraction. Gdi1p in each fraction was visualized after immunoblotting with the HA antibody. The vector only control is shown in lane g. B, in vitro binding of HA-tagged wild-type and R248A,E241S Gdi1p proteins to membranes. S100 fractions of the sec19-1 strain expressing vector only (control) (a and d), HA-wild-type (b and e), or R248A,E241S (c and f) were isolated and incubated at 30 °C for 30 min with cell homogenates (a-c) or P100 membrane fractions (d-f) prepared from sec19-1 transformed with vector only. Subsequently, a P100 fraction of each incubation condition was isolated, washed, and probed with a HA-specific antibody using immunoblotting. C, in vitro binding of HA-GDI to membranes is trypsin-sensitive. A P100 membrane fraction prepared from sec19-1 transformed with vector only was not treated (a and d), treated with trypsin premixed with a 2-fold molar excess of trypsin inhibitor (b and e) (1% weight/weight for 15 min on ice), or trypsin alone (c and f). In a and e, after trypsin digestion, a 2-fold molar excess amount of trypsin inhibitor was added. Subsequently, P100 samples were mixed with HA-Gdi1p (a-c) or the HA-tagged-R248A,E241S Gdi1p mutant as described in B and incubated at 30 °C for 30 min. A P100 fraction of each sample was isolated at the end of the incubation and probed with a HA-specific antibody using immunoblotting.
fractions from the experiment described above were probed with the anti-HA antibody using immunoblotting (Fig. 4A). Intriguingly, we found that while approximately 30% of wild-type Gdi1p was found associated with the P100 fraction, a nearly identical level was observed for the GDI mutants. A negligible signal was detected in the vector only control. Interestingly, the mutant containing substitutions at both Arg248 and the GXG motif (R248A,G16A,G18A) could not be detected. One possibility is that the expression of the protein from this vector is somehow inhibited, even though at least 15 plasmids that differed by only single point mutations expressed stable protein (Fig. 4A and data not shown). Alternatively, we suggest that the inability to detect protein suggests that the conserved GXG motif found at the base of the Rab-binding platform (10) is critical for the folding and/or stability of the protein.

To further address the ability of mutant GDI to recognize membranes in response to loss of Rab recognition in vivo, a cell homogenate was prepared from sec19-1 cells containing the vector only. Either the whole cell homogenate (Fig. 4B, a-c) or a P100 fraction containing membranes (Fig. 4B, e-f) were incubated for 30 min at 30 °C with cytosol prepared in an identical fashion from cells expressing HA-tagged wild-type GDI and HA-tagged 241/248 double mutant. Following incubation, P100 membrane fractions were isolated from each sample, washed, and the amount of HA-tag bound to membranes was determined by immunoblotting. As shown in Fig. 4B, the levels of binding of wild-type and the double mutant were identical even in the presence of cytosolic Sec19-1 Gdi1p (Fig. 4B, compare lane b and c (cytosolic Sec19-1 Gdi1p present) to e and f (cytosolic Sec19-1 Gdi1p absent)). Moreover, binding was dependent on a membrane-associated receptor. As shown in Fig. 4C, pretreatment of membranes with trypsin prevented binding of wild-type and mutant HA-Gdi1p. These in vitro results are consistent with the conclusion that mutant GDI that cannot recognize Rab in vivo, still retains the ability to bind efficiently to membranes. Moreover, binding is weakly dominant over wild-type and mutant HA-Gdi1p. These

Intracellular Membrane Trafficking in gdi1 Mutants—Gdi1p is required for numerous membrane trafficking steps that involve potentially 11 different Rab proteins in yeast (33). We therefore examined our collection of mutants for secretory transport function. Given the ability of most gdi1 single mutants to support growth, it was possible that there were selective defects in Rab function given the differential effects of various single mutants on Rab binding using the co-immunoprecipitation assay (Fig. 3B). We monitored Gdi1p function in the early secretory pathway (ER/Golgi) and the vacuolar protein sorting/endocytic (VPS) pathway by examining the processing of carboxypeptidase Y (CPY), and secretion by monitoring the release of Hsp150 into the medium. In pulse-chase immunoprecipitation assays, the conversion of the ER p1CPY precursor form (67 kDa) to the Golgi-modified p2CPY (69 kDa) precursor reflects transport through the ER and Golgi, and conversion of p2CPY to mature CPY (mCPY, 61 kDa) in the vacuole reflects transport through the vacuolar protein sorting pathway. CPY biosynthesis was assayed at 10- and 30-min chase points which allowed us to visualize each of these CPY biosynthetic transport steps.

For the viable Gdi1p mutants, pulse-chase assays were conducted with strains in which the mutant gdi1 alleles were the sole source of Gdi1p. Of those tested, the Y44V, E241S, and R248A single mutants exhibited defects in CPY biosynthesis which reflected partial kinetic delays in transport (34) (see below) consistent with reduced binding of Rab (Fig. 3B). However, in each case, nearly all of the CPY was properly processed by late time points for each mutant, a result consistent with the cell growth phenotypes.

To test the inviable gdi1 double mutants, plasmids encoding them and the corresponding single mutations were transformed into a sec19-1 strain and CPY biosynthesis was assayed. Cultures were incubated at permissive (26 °C) or restrictive temperature (37 °C) for 10 min prior to labeling. Cultures were labeled with [35S]methionine/cysteine for 10 min, then chased for 10 or 30 min. CPY was immunoprecipitated from extracts derived from each culture. The positions of p1CPY (ER and Golgi), p2CPY (Golgi and endosome), and mature CPY (vacuole) are indicated to the left. B, spheroplasts of the indicated strains were pulse-labeled for 5 min at 30 °C as in A, and then chased (30 °C) for 0 or 5 min. Cells were collected by centrifugation and Hsp150 was immunoprecipitated from the cell fraction (I, intracellular) and from the media fraction (E, extracellular).

Fig. 5. Analysis of vesicle-mediated trafficking in gdi1 mutants. The indicated strains were prepared for labeling at 26 °C, then the cultures were split and one-half was transferred to 37 °C 10 min prior to labeling. Cultures were labeled with [35S]methionine/cysteine for 10 min, then chased for 10 or 30 min. CPY was immunoprecipitated from the cell fraction (I, intracellular) and from the media fraction (E, extracellular).
significant kinetic defects in CPY biosynthesis, but only at the restrictive temperature, a result identical to those observed using the gdi1Δ strain mentioned above. The sec19-1 strains with each of the three single gdi1 mutant plasmids had more p2CPY present at the 10-min chase point compared with cells with wild-type GDI1. At the 30-min chase point in Y44V, E241S, and R248A containing cells, mCPY was the major form present. Notably, in R248A containing cells, a small amount of p1CPY persisted even at the 30-min chase point reflecting the reduced ability of this mutant to recognize Ypt1p (Fig. 3B). Overall, these results are fully consistent with the growth phenotypes associated with each of the single gdi1 mutants (as the only source of Gdi1p).

In contrast to the effects of single point mutations, CPY biosynthesis was severely affected at 37 °C in sec19-1 strains expressing each of the double gdi1 mutant proteins (R248A,Y44V and R248A,E241S). In the R248A,Y44V strain, p1CPY accumulated, indicating a potent ER to Golgi transport block with only a very small amount (<5%) of mCPY present at the 30-min chase point (Fig. 5A, bottom left panel). The transport block observed in the R248A,E241S strain was not as severe, as we observed partial complementation of the transport block imposed by the sec19-1 mutation. In both cases, no further processing of CPY was observed beyond the level seen after 30 min (Fig. 5A, data not shown). The fact these mutants fail to support growth (Table II) is consistent with the interpretation that they can only support an exceedingly limited level of recycling of a critical Rab(s).

Because the analysis of CPY biosynthesis in sec19-1 R248A,Y44V cells did not reveal any significant transport defects in the early secretory pathways at 26 °C despite the partial growth defects at the permissive temperature and complete block at the semipermissive condition (Fig. 2), we also tested for possible late secretory defects (Golgi to cell surface) by assaying secretion of Hsp150p from mutant cells at 30 °C (semipermissive condition). Hsp150p is a 341-amino acid O-glycosylated protein which is rapidly secreted into the media from wild-type cells (34). Mutant strains were pulse labeled, chased for various amounts of time, and Hsp150 was immunoprecipitated from extracts derived from cells or from the media fraction. If late transport is blocked by the R248A,Y44A mutant, we should expect to see a marked depression in secretion despite the fact that HSP150 is an extremely rapidly transported protein, largely appearing in the medium during the initial pulse period (Fig. 5B). As shown in Fig. 5B, we did not detect a decreased rate of secretion of HSP150 in response to the presence of the R248A,Y44V and R248A,E241S double mutants. All of the newly synthesized Hsp150p was rapidly secreted from cells by the end of the labeling period (Fig. 5B). Thus, there were no detectable differences in secretion from these mutant strains at 30 °C, implying an important function for GDI in an as yet untested Rab-dependent pathway other than the secretory pathway affecting cell growth.

**Endocytic Membrane Transport Is Impaired in gdi1 Mutant Cells**—To expand our understanding of the Rab-binding region to Rab-regulated events involved in internalization from the cell surface, we examined the effects of the Rab-binding deficient double mutants on the endocytic pathway by following the uptake and transport of the lipophilic dye FM4-64. When added to growing cells, FM4-64 intercalates into the plasma membrane and is transported to the vacuolar membrane in an energy-, time-, and temperature-dependent manner. Thus, it can be used to monitor endocytosis of bulk membrane (35). Wild-type and sec19-1 cells were incubated at either the permissive temperature or shifted to restrictive temperature (37 °C) for 10 min, then FM4-64 was added to the growth media, and the cultures were incubated for 10 min to allow labeling of the plasma membrane. Fresh, prewarmed media was then added to initiate a chase period during which aliquots of cells were removed at 10- and 45-min chase points and the FM4-64 dye was visualized by fluorescence microscopy (Fig. 6).

The uptake of FM4-64 to the vacuole of wild-type and sec19-1 cells was indistinguishable at the permissive temperature (data not shown). In sec19-1 cells carrying a complementing GDI1 plasmid, most of the FM4-64 had been delivered to the vacuolar membrane at the 10-min chase point at 37 °C, although a small amount still persisted in a few puncta distributed throughout the cytoplasm (Fig. 6, top panels). By 45 min of chase, nearly all of the FM4-64 was in the vacuolar membrane. In contrast, sec19-1 cells at the 10-min chase point contained numerous cytoplasmic puncta, with little FM4-64 being delivered to the vacuole. At the 45-min time point, the vacuolar membrane became labeled, although cytoplasmic puncta remained along with large vesicular structures clustered together near the vacuole (Fig. 6, top right panels). Thus, the sec19-1 mutation slows, but does not completely block the endocytic transport of FM4-64 to the vacuole.

When FM4-64 uptake was observed in sec19-1 cells expressing either of the double mutant proteins (R248A,Y44V or R248A,E241S), trafficking of FM4-64 to the vacuole was impaired much more severely than in sec19-1 cells alone (Fig. 6, bottom panels). At the 10-min chase point at 37 °C, the dye was distributed throughout the cytoplasm in a very diffuse pattern, indicating the accumulation of a multitude of small structures, in contrast to the relatively few cytoplasmic puncta present in sec19-1 cells. By the 45-min chase point, the dye was observed in vacuolar membranes, although numerous large non-vacuolar vesicles were also present, consistent with the phenotype of
Molecular Dissection of GDI Function in Vivo

In addition to the accumulation of large vesicles, the vacuoles of sec19-1 R248A,Y44V cells often appeared fragmented and irregularly shaped. In the most striking cross-sections, large electron-lucent bodies appeared to be clustered along the vacuolar membrane, giving the vacuolar membrane a punched in appearance (Fig. 7, panel C, arrow). The EM analyses are consistent with the numerous puncta observed during endocytosis of FM4-64. These results suggest that a severe defect in vesicular traffic leads to the accumulation of distinct transport intermediates as a consequence of the more dominant effect of the double mutants. This defect appears to be distinct from that of the sec19-1 temperature-sensitive defect.

**Rabs Are Loaded onto Membranes by Gdi1p at Different Rates**—In yeast, in vivo depletion of wild-type Gdi1p over a 15-h time period by shut-off of GDI1 expression under control of an inducible promoter, results in the loss of the cytosolic pool of Sec4p (a Rab present on post-Golgi secretory vesicles) and concomitant accumulation of Sec4p on membranes (5). While these results demonstrate the importance of recycling of Rab from membranes, they do not address whether Gdi1p is, in addition, required for membrane loading of Rab as shut-off would be expected to remove both the Rab-bound and free pools over the 15-h time period.

To address the above question, we used the sec19-1 strain to rapidly inactivate the endogenous Gdi1p by shift to the restrictive temperature and monitored the distribution of several Rabs by subcellular fractionation. In order to rigorously quantitate any change in distribution that might occur, rather than use immunoblotting, strains were labeled at the permissive temperature (26 °C) for 15 min, and then chased for 30 min to allow equilibration of the nascent radiolabeled Rab pools with the total intracellular Rab pools. The cultures were then split and one-half of each was transferred to the restrictive temperature (37 °C) while the other was maintained at the permissive temperature. After 30 min, cells were lysed, membrane (P100) and cytosol (S100) fractions were generated by centrifugation, and Ypt1p, Ypt7p, and Vps21p were immunoprecipitated from the membrane and cytosol fractions. During this short time period the endogenous sec19-1p pool is stable, but is completely inactive as is apparent by the block in transport of CPY (Fig. 5, panel A).

Inactivation of Sec19-1p by shift to the restrictive temperature and incubation for 30 min led to only a partial shift of the cytosolic pools of Ypt1p and Vps21p to the membrane (Fig. 8, top panel Ypt1p and bottom panel Vps21p, lanes a and b). In contrast, the entire cytosolic pool of Ypt7p was shifted to the membrane fraction within 30 min of transfer to 37 °C (Fig. 8, middle panel, lanes a and b). Note that the time of exposure of the gels for cytosolic Rabs were 3-fold longer than that of the membrane-bound pool, suggesting that the major pool of these Rab proteins is membrane-bound. These results are quantitated in Fig. 9. The strikingly distinct effects of rapid inactivation of Sec19-1 Gdi1p on the redistribution of individual Rab species may reflect differences in rates of retrieval of a Rab protein at a particular step of the secretory pathway (see “Discussion”). Moreover, the ability to detect a cytosolic Ypt1p and Vps21p after 30 min at 37 °C suggests that the primary defect in Sec19-1p is not due to a misfolding event occurring at the restrictive temperature following delivery of Rab to membranes. Rather, that the function of the cytosolic Sec19-1p-Rab complex is sensitive to the temperature-induced folding defect. Thus, GDI is essential for Rab loading of membranes.

**Double Mutants Reveal the Requirement for a Novel Factor in Rab Recycling**—A GDF has been proposed to be required for Rab9 delivery from the cytosolic pool complexed to GDI (14–16). The requirement for other factors, including a possible

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**Fig. 7. Electron microscopy of gdi1 mutant strains.** sec19-1 (A and C) and sec19-1 R248A,Y44V strains (B and D) were grown overnight at 26 °C, then each culture was divided and one-half was incubated at 37 °C for 1 h (C and D), and the other half was maintained at 26 °C (A and B). An enlargement of a sec19-1 R248A,Y44V at 37 °C field, highlighting the numerous large vesicles which accumulate in this mutant, is shown in E. For panels A-D, the scale bar represents 1 micron, and in E the scale bar represents 0.2 micron.

the sec19-1 cells at this time point. These results suggest that the prominent effects of sec19-1 and Gdi1p double mutants were on rapid kinetic events occurring during early steps in the transit of dye through endocytic compartments.

Electron microscopy was used in an effort to visualize membrane trafficking intermediates that might accumulate in gdi1 mutants. Strains were prepared for analysis by growing at the permissive temperature (26 °C), then each culture was divided and one-half was transferred to 37 °C and incubated for 1 h while the other half remained at 26 °C. Cells were then fixed and prepared for electron microscopy. Fig. 7 (panels A and B) shows that sec19-1 R248A,Y44V cells and sec19-1 cells grown at the permissive temperature were similar in appearance. This morphology is characteristic of wild-type cells (25).

In contrast to the above results, numerous aberrant but unidentified membrane structures were observed in the sec19-1 strain at the restrictive temperature. Surprisingly, we did not observe the striking accumulation of vesicles that were observed following slow depletion of Gdi1p (5). Moreover, at the restrictive temperature, there were prominent differences between the sec19-1 strain and sec19-1 R248A,Y44V strains (Fig. 7, panels C and D). Particularly evident in the latter was the appearance of numerous 200–250 nm diameter vesicular structures throughout the cytoplasm of double mutant cells (Fig. 7, panel E).
accessory recycling factor involved in extraction of Rab-GDP following vesicle fusion have not been implicated to date. To test for this possibility, we examined the affect of expressing \textit{gdi1} double mutants unable to bind Rabs on the membrane/cytosol distributions of Ypt1p, Ypt7p, and Vps21p using the protocol outlined above for the distribution of Rabs in the \textit{sec19-1} strain at the permissive and restrictive temperatures.

In the presence of either the R248A,Y44V (Fig. 8, lanes c and d) or R248A,E241S (Fig. 8, lanes e and f) double mutants, the soluble pool of Ypt7p (Fig. 8, center panel) completely shifted to the membrane-bound form at both the permissive temperature and at the restrictive temperatures (Fig. 2). A similar result was observed for Vps21 (Fig. 8, bottom panel, compare a and b to c and d). The distribution of Ypt1p (Fig. 8, top panel) was more modestly affected at the permissive temperature reflecting its apparent slower rate of recycling. However, shift to the membrane-bound form was enhanced by incubation at 37 °C reflecting inactivation of Sec19-1p (Fig. 8, top panel, compare b to f). These results are quantitated in Fig. 9.

In particular, it is clear that expression of the double mutants at levels similar to endogenous Gdi1p pool interferes with the recycling of all Rabs from the membrane to the cytosol. Thus, the double mutants, that are unable to bind Rab can trigger a shift in the steady-state distribution of Rab from the cytosol to the membrane even under permissive and semipermissive conditions where Sec19-1p is functional in growth. These results suggest that a Rab retrieval step is at least one target for the dominant effect of double mutants on growth. Moreover, given the fact that binding of Gdi1p to membranes is independent or Rab, but dependent on a trypsin-sensitive membrane-associated factor, this result provides evidence for a novel membrane-associated receptor in these events.

**DISCUSSION**

GDI is critical for vesicular membrane transport in both the endocytic and exocytic pathways. Our genetic analyses provide new mechanistic insight into the GDI-Rab cycle. Using site-directed mutagenesis to inactivate Gdi1p function we have now established the physiological importance of residues previously implicated in Rab3A binding \textit{in vitro}.

Previous biochemical studies based on the structure of bovine \textit{a}-GDI led us to suggest that conserved residues present in SCRs 1 and 3B fold to form a platform at the apex of GDI which participates in the binding of mammalian Rab3A \textit{in vitro}. Mutations in this region of bovine \textit{a}-GDI led to marked reduction in the recognition of Rab 3A, inactivated the ability of GDI to extract Rab proteins \textit{in vitro}.
and interfered with the ability of GDI to inhibit ER to Golgi in vitro (10, 12). Given the indirect nature of these previous in vitro studies, we have characterized for the first time the physiological importance of these and other residues in the function of yeast Gdi1p in vivo. Single point mutations in yeast GD1 resulted in mutant Gdi1p proteins that still complemented the gdiΔ mutation in vivo, although interactions with Rab were clearly affected (Fig. 3). The surprising ability of mutant D1 proteins with reduced affinity for Rab to support the essential function of GD1, lead us to suggest that in the yeast Gdi1p-Rab recycling pathway, the cytosolic Rab pools bound to Gdi1p are likely to be in functional excess. Consistent with this view, several Rab proteins were nearly exclusively membrane-associated in sec19-1 cells expressing Gdi1p double mutant at the permissive temperature (Fig. 8), yet growth and membrane trafficking of these strains were largely unimpaired. Thus, only a limited recycling cytosolic pool is essential for Gdi1p function in cell growth. These results suggest that in vitro biochemical assays of Rab binding by GDIs may not accurately reflect the ability of Gdi1p to function in vivo.

In contrast to the lack of striking effects of single gdi1 point mutations on Gdi1p function, combinations of mutations in regions of α-GDI previously implicated in Rab binding in vitro (10) rendered yeast Gdi1p completely inactive in vivo despite normal levels of expression and partitioning the protein between the cytosolic and membrane-associated pools. In particular, the identification of double mutants defective in Gdi1p function stressed the importance of Arg248 (Arg240 in mammalian α-GDI). The combination of the R248A mutation with mutations in other conserved residues of SCRs 1 and 3B, such as Y44V (Y39V in α-GDI) or E241S (E233S in α-GDI), previously implicated in Rabα3A binding in vitro (10) were unable to support growth of the gdiΔ strain.

Arg248 is found in the center of helix I which forms the front edge of the putative Rab-binding platform (10) (Fig. 1). Mutations of the equivalent residue in bovine α-GDI (Arg240) led to an ~60-fold reduction in the binding of Rabα3A to α-GDI in vitro and neutralized the ability of α-GDI to extract Rabα3A from synaptosome membranes in vitro. In the context of the structure of α-GDI (10), our results now provide strong physiological evidence that Arg248 plays a pivotal role along with other residues found at the apex of GDI to the formation of the functional Rab-binding “platform” (12).

**Distribution of Rabs Is Differentially Sensitive to Residues in the Rab-binding Platform**—Our studies have demonstrated that the binding and distribution of different Rab proteins between membrane and cytosolic fractions were differentially sensitive to mutations in GD1. One explanation for this result is that residues in the Rab-binding pocket of Gdi1p contribute in different ways to the strength of interaction between Gdi1p and distinct Rab species, a result consistent with in vitro binding studies involving α-GDI and mammalian Rab (12). A second possibility is that the differential effects of Gdi1p inactivation on Rab distribution could reflect, in addition to differences in Rab recognition, the overall kinetics of Gdi1p-dependent recycling. Recycling per se could be dependent on multiple factors including the amount of membrane being converted to vesicular carriers per unit time and the steady-state abundance of each Rab GTPase in the cell. It has been shown that a permanently membrane-associated mutant Ypt1 protein (with COOH-terminal isoprenylation sites substituted with a transmembrane domain) can fulfill the essential function of Ypt1 in ER to Golgi transport, albeit inefficiently (36). This led to the suggestion that a cytosol-dependent translocation step is not absolutely required for Ypt1 function. However, our results now demonstrate that only a very small pool of Rab is required for function. Therefore, even an ER-associated reservoir of newly synthesized Ypt1 may have contributed significantly to the success of this experimental approach (36). We suggest that GDI-dependent translocation through the cytosol is essential for Rab function.

**Morphological Effects of GDI Mutants**—After a brief incubation of sec19-1 at the restrictive temperature, we noted an accumulation of aberrant membranes, but few transport vesicles (Fig. 7). These results were surprising in light of the results of Garret et al. (5) who found that gradual depletion (over 15 h) of wild-type Gdi1p led to accumulation of ER, Golgi-like elements, and transport vesicles. The distinction between these experiments and ours (gradual depletion of functional Gdi1p versus rapid inactivation of Gdi1p) is important because it revealed a novel requirement for Gdi1p in the function of exocytic and endocytic compartments. One possibility is that Gdi1p is required for vesicle biogenesis. This conclusion is supported by recent experiments that have shown a requirement for GDI-Rab5 in clathrin-mediated endocytic vesicle formation (37). In addition, in vitro experiments following the requirement for Rab1 (38) and analysis of ypt31A ypt32 yeast mutants (39) indicates that Rab proteins are required for vesicle budding from the ER and Golgi, respectively. Alternatively, Rab-dependent homotypic fusion is now recognized to play a prominent role in the architecture of the ER, Golgi, and endosome. It is possible that the novel structures detected morphologically following rapid turn-off of Gdi1p function represent defects in these events.

**Rab Delivery to Membranes Requires GDI and Binding of GDI to Membranes Is Rab-independent**—While a variety of in vitro experiments have implicated an important role for the delivery of Rab to membranes through a cytosolic GDI-Rab complex (reviewed in Refs. 2 and 3), this point remains to be established physiologically. Our ability to detect a prominent cytosolic Sec19-1p-Rab pool under conditions in which Sec19-1p was rapidly rendered dysfunctional (Fig. 8) is consistent with this assumption. Importantly, we demonstrated that the association of Gdi1p with membranes was independent of its ability to bind Rab (Figs. 3 and 4). Thus, while targeting of the GDI-Rab to specific compartments is believed to involve the variable carboxyl terminus of different Rab species (40), we now suggest that stable membrane association of Gdi1p is possible under these conditions.

4 W. E. Balch, unpublished data.
Molecular Dissection of GDI Function in Vivo

Rab-independent. Consistent with this possibility, we have recently shown that mutation of Arg70 in bovine α-GDI resulted in loss of Rab1 binding, yet the α-GDI mutant bound membranes strongly and was a potent inhibitor of transport of protein between the ER and Golgi (12). One possible candidate receptor is “GDI displacement factor” or guanine nucleotide displacement factor, a protein suggested by Pfeffer and colleagues (14, 16) to promote release of endosomal Rab GTPases from the Rab-GDI complex during Rab delivery to membranes. Alternatively, receptors that function in other steps in the Rab GTPase cycle may be responsible for stable binding.

**Rab Recycling Factors Are Required for GDI Function in Vivo**—An important and unexplored aspect of Gdi1p function regards the molecular mechanism by which Gdi1p extracts Rabs from membranes. GDP-bound Rabs to be extracted from membranes could potentially serve as Gdi1p “receptors” and recruit free Gdi1p from the cytosol. Our in vivo and in vitro results, however, indicate that Rab binding is not exclusively responsible for the association of Gdi1p with membranes. Moreover, we noted that the sec19-1 strain containing the double mutant, when incubated at the permissive or restrictive temperatures, led to the enhanced redistribution of multiple Rabs from the cytosol to membranes despite the fact that the double mutant could not recognize Rabs. The residual cytosolic pools, in the case of Ypt1p, may reflect weak binding to either misfolded Sec19-1 Gdi1p or the double mutant that cannot be detected in vitro. The effect of the double mutant on steps involved in Rab recycling in vivo was corroborated with its observed ability to efficiently bind membranes in vitro, even in the presence of Sec19-1p.

One possibility to explain our results is that Gdi1p or Gdi1p-Rab complex functions as a multimer and the mutant Gdi1p interferes with this function. We feel that this explanation is unlikely given that all experimental evidence to date demonstrates that GDI is found in the cytosol as a monomer or as a heterodimer with Rab (3). An alternative explanation that we prefer is that our findings now imply the activity of a novel membrane-associated factor(s) that mediates the recruitment of Gdi1p for Rab-GDP recycling steps. This factor, referred to as Rab recycling factor or RRF (Fig. 10), we would propose directs the interaction of unbound Gdi1p with newly formed Rab-GDP species formed during or following membrane fusion. Consistent with this interpretation, we have observed a requirement for a membrane-associated factor to recruit mammalian α-GDI in ER to Golgi transport in vivo (12), although we were unable to assess whether it operated in Rab delivery or Rab retrieval. Our proposal for a requirement for RRF in the retrieval of Rab-GDP is supported by the observation that double mutant Gdi1 proteins interfered with recycling of Rabs by Sec19-1p at both the permissive and restrictive temperatures, directing a shift in the steady-state distribution from the cytosol to membranes (Fig. 8). Moreover, the double mutant Gdi1p also caused dominant growth defects in the sec19-1 background at the semipermissive temperature (Fig. 2), suggesting that a recycling factor(s) that is not a Rab becomes inaccessible to the partially functional sec19-1 Gdi1p pool, possibly through competition by the double mutant.

Of related interest to the above observations is that overexpression of wild-type Gdi1p in yeast or α-GDI in mammalian cells does not have the same potent inhibitory effect on ER to Golgi or intra-Golgi vesicle transport in vivo as is observed when permeabilized cells are preincubated with excess GDI in vitro (8, 41–43). However, other Rab proteins, such as Rab11 that mediates transport of vesicular stromatid virus glycoprotein from the trans Golgi network to the cell surface, is very sensitive to GDI overexpression (41). These results, combined with a potential requirement for RRF, raises the possibility that membrane-associated Rabs may be restricted to the GTP-bound form in vivo when bound to membranes and inaccessible to GDI removal except at a defined point in the Rab cycle when it encounters RRF during or following vesicle fusion. The relationship between these observations and the finding that Rab5 undergoes rapid GTP turnover in vitro could reflect reduced stability of Rab5 (GTP) under reconstitution conditions (44). The implications of our observations for the model that Rab functions as a molecular timer in vivo remains to be clarified (44). We are currently exploring the identity of residues in Gdip involved in recognition of RRF by extending our current mutagenesis to residues in other SCRs such as 2 and 3A that line the conserved face of GDI.

**Implications for Physiological Function of the GDI Family**—While a variety of studies have shown little difference in the ability of various GDI isoforms to distinguish between different Rabs (8, 9, 43, 45, 46), it is known that the expression level of a particular GDI isoform is highly variable between tissues. The α-isofomr of GDI is nearly exclusively found in brain tissue, whereas the β isofom is ubiquitously expressed (47, 48). The altered levels of tissue distribution of GDI isoforms combined with our observations that residues in the Rab-binding region may contribute to differential activity in Rab recognition (and recycling), leads us to suggest that GDI isoforms play specialized roles in the handling of subsets of tissue-specific Rabs. Such specialization may contribute to higher efficiency of specific types of endomembrane traffic in these tissues. Support for this proposal comes from our recent discovery in collaboration with Toniole and colleagues (49) that the α-GDI isoform of GDI is responsible for X-linked mental retardation. Patients who are null for α-GDI are phenotypically normal with the exception of reduced mental capacity, implying an important role for α-GDI in the recycling of Rab3 GTPases in the development of the synapse responsible for human intelligence.

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**REFERENCES**

1. Novick, P., and Zerial, M. (1997) Curr. Opin. Cell Biol. 9, 496–504
2. Pfeffer, S. R., Dirac-Sweirstrup, B., and Soldati, T. (1995) J. Biol. Chem. 270, 17057–17059
3. Wu, S.-K., Zeng, K., Wilson, I., and Balch, W. E. (1996) Trends Biochem. 21, 472–476
4. Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isomura, M., Kuroda, S., and Takai, Y. (1990) J. Biol. Chem. 265, 2333–2337
5. Garrett, M. D., Zahnner, J. E., Cheney, C. M., and Novick, P. J. (1994) EMBO J. 13, 1718–1728
6. Janonius-Le Ravey, J., Jolivet, F., Camonis, J., Marche, P. N., and Goud, B. (1995) J. Biol. Chem. 270, 14851–14858
7. Casey, P. J., and Seabra, M. C. (1996) J. Biol. Chem. 271, 5289–5292
8. Ullrich, O., Stenmark, H., Alexandrov, K., Hubert, L., Kaibuchi, K., Sasaki, T., Takai, Y., and Zerial, M. (1993) J. Biol. Chem. 268, 18143–18150
9. Yang, C., Stimpson, V. I., and Goud, B. (1994) J. Biol. Chem. 269, 31891–31899
10. Schalk, I., Zeng, K., Wu, S.-K., Stura, E. A., Matteson, J., Huang, M., Tandon, A., Wilson, I. A., and Balch, W. E. (1996) Nature 381, 42–48
11. Balch, W. E. (1998) J. Biol. Chem. 273, 26931–26938
12. Wu, S.-K., Luan, P., Matteson, J., Zeng, K., Nishimura, N., and Balch, W. E. (1994) J. Biol. Chem. 269, 17057–17059
13. Schalk, I., Stura, E. A., Matteson, J., Wilson, I. A., and Balch, W. E. (1998) J. Biol. Chem. 273, 26931–26938
14. Ullrich, O., Bucci, C., and Zerial, M. (1994) Nature 368, 157–160
15. Dirac-Sweirstrup, A. B., Sumizawa, T., and Pfeffer, S. R. (1997) EMBO J. 16, 465–472
16. Sherman, F., Fink, G. R., and Lawrence, L. W. (1979) Methods in Yeast Genetics: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

5 W. E. Balch, unpublished observations.
18. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. \textbf{153}, 163–168.
19. Schiestl, R. H., and Gietz, R. D. (1989) \textit{Curr. Genet.} \textbf{16}, 339–346.
20. Hanahan, D. (1983) J. Mol. Biol. \textbf{166}, 557–580.
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982).
22. Dascher, C., and Balch, W. E. (1994) J. Biol. Chem. \textbf{269}, 4953–4962.
23. Horazdovsky, B., Busch, G. R., and Emr, S. D. (1994) \textit{EMBO J.} \textbf{13}, 1297–1309.
24. Vida, T. A., and Emr, S. D. (1995) \textit{J. Cell Biol.} \textbf{128}, 779–792.
25. Klionsky, D. J., Banta, L. M., and Emr, S. D. (1988) Mol. Cell. Biol. \textbf{8}, 2105–2116.
26. Gaynor, E. C., and Emr, S. D. (1997) J. Cell Biol. \textbf{136}, 789–802.
27. Collins, R. N., Brennwald, P., Garrett, M., Lauring, A., and Novick, P. (1997) J. Biol. Chem. \textbf{272}, 18281–18289.
28. Segev, N., Mulholland, J., and Botstein, D. (1988) Cell \textbf{52}, 915–924.
29. Singer-Kruger, B., Stenmark, H., Dusterhoft, A., Philippen, P., Yoo, J. S., Gallwitz, D., and Zerial, M. (1994) J. Cell Biol. \textbf{125}, 283–298.
30. Haas, A., Scheffmann, D., Lazar, T., Gallwitz, D., and Wickner, W. (1995) \textit{EMBO J.} \textbf{14}, 5258–5270.
31. Wichmann, H., Hengst, L., and Gallwitz, D. (1992) Cell \textbf{71}, 1131–1142.
32. Gallwitz, D. (1997) Trends Biochem. Sci. \textbf{22}, 468–472.
33. Gaynor, E. C., and Emr, s. d. (1997) J. Cell Biol. \textbf{136}, 789–802.
34. Wendland, B., McCaffery, J. M., Xiao, Q., and Emr, S. D. (1996) J. Cell Biol. \textbf{135}, 1485–1500.
35. Ossig, R., Laufer, W., Schmitt, H. D., and Gallwitz, D. (1996) \textit{EMBO J.} \textbf{14}, 3645–3653.
36. McLauchlan, H., Newell, J., Merrice, N., Osborne, A., West, M., and Smythe, E. (1988) \textit{Curr. Biol.} \textbf{8}, 34–45.
37. Nuoffer, C. N., Davidson, H. W., Matteson, J., Meinkoth, J., and Balch, W. E. (1994) J. Cell Biol. \textbf{125}, 225–237.
38. Jedd, G., Mulholland, J., Segev, N. (1997) J. Cell Biol. \textbf{137}, 563–580.
39. Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K., and Zerial, M. (1990) Cell \textbf{62}, 317–329.
40. Chen, G., Peng, Y., Chen, D., and Wandinger-Ness, A. (1998) Mol. Biol. Cell \textbf{9}, 3241–3247.
41. Elazar, Z., Mayer, T., and Rothman, J. E. (1994) J. Biol. Chem. \textbf{269}, 794–797.
42. Peter, F., Nuoffer, C., Pind, S. N., and Balch, W. E. (1994) J. Cell Biol. \textbf{126}, 1395–1406.
43. Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M. C., Goody, R., and Zerial, M. (1996) Nature \textbf{383}, 266–269.
44. Soldati, T., Riederer, M. A., and Pfeffer, S. R. (1993) Mol. Biol. Cell \textbf{4}, 425–434.
45. Sasaki, T., Kailhoori, K., Kabsch, A., and Novick, P. J. (1991) Mol. Cell. Biol. \textbf{11}, 2909–2912.
46. Shisheva, A., Buxton, J., and Czech, M. P. (1994) J. Biol. Chem. \textbf{269}, 23865–23868.
47. Shisheva, A., Doxsey, S. J., Buxton, J. M., and Czech, M. P. (1995) Eur. J. Cell Biol. \textbf{68}, 143–158.
48. D’Adamo, P., Menegon, A., Le Negre, C., Grasso, M., Gulisano, M., Tamanini, F., Bienvenu, T., Gedeon, A. K., Oostra, B., Wu, S.-K., Tandon, A., Valtorta, F., Balch, W. E., Chelly, J., and Tenisio, D. (1998) Nature Genet. \textbf{19}, 134–139.