Molecular Role for the Rab Binding Platform of Guanine Nucleotide Dissociation Inhibitor in Endoplasmic Reticulum to Golgi Transport

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Guanine nucleotide dissociation inhibitor (GDI) regulates the recycling of Rab GTPases involved in vesicle targeting and fusion. We have analyzed the requirement for conserved amino acid residues in the binding of Rab1A and the function of GDI in transport of cargo between the endoplasmic reticulum (ER) and the Golgi apparatus. Using a new approach to monitor GDI-Rab interactions based on the change in fluorescence associated with the release of methylanthraniloyl guanosine di(tri)phosphate-GDP (mGDP) from Rab, we show that residues previously implicated in the binding of the synapse-specific Rab3A, including Gln-236, Arg-240, and Thr-248, are essential for the binding of Rab1A. Mutation of each of these residues has potent effects on the ability of GDI to remove Rab1A from membranes and inhibit ER to Golgi transport in vitro. Given the sequence divergence between Rab1A and 3A (35% identity), these residues are proposed to play a general role in GDI function in the cell. In contrast, several other residues found within or flanking the Rab-binding region were found to have differential effects in the recognition and recycling of Rab1A and 3A, and therefore direct selective interaction of GDI with individual Rab proteins. Intriguingly, mutation of one residue, Arg-70, led to a reduction of Rab1A binding, failed to extract Rab1A from membranes in vitro, yet bound membranes tightly and potently inhibited ER to Golgi transport. These results provide evidence that novel membrane-associated factor(s) mediate Rab-independent GDI interaction with membranes.

Rab GTPases regulate vesicle transport through both the exocytic and endocytic pathways by controlling the assembly of protein complexes involved in vesicle targeting and fusion (1). Rab proteins cycle between two pools: an “activated” (GTP) membrane-bound pool and a cytosolic, “inactive” GDP-bound pool complexed to members of the guanine nucleotide dissociation inhibitor (GDI)† gene family (reviewed in Refs. 2 3). The α isoform of GDI was first discovered by Takai and colleagues (4) based on its ability to inhibit the intrinsic dissociation of GDP from Rab3A. To date, 3 isoforms (α, β, γ; 85% identity) of GDI have been characterized biochemically with a potential fourth isoform detected on Northern blots. While the β isoform is ubiquitously expressed, the α isoform is largely limited in distribution to brain tissue (5). The γ isoform has been reported to have a unique membrane localization in adipocytes compared with the cytosolic distribution of β-GDI (6–8). The yeast Saccharomyces cerevisiae has only a single GDI protein (50% identity) which is essential for growth (9) demonstrating the conserved and essential role of GDI-Rab interactions during evolution of membrane trafficking pathways.

GDI family members appear to bind all Rab proteins examined to date (10, 11), leading to the suggestion that the various GDI species can have overlapping functions to form heterodimeric complexes with GDP-bound forms of Rab. However, the residues responsible for interaction with divergent Rabs remain to be determined. Moreover, it remains possible that tissue-specific isoforms may be specialized for subclasses of Rab proteins found in developmentally divergent cell lineages. In support of this possibility, we have recently shown in collaboration with Toniola and colleagues that the brain-specific α isoform of GDI is responsible for X-linked mental retardation and therefore important for the normal neural function in the brain (12).

The Rab GTPase cycle is intimately linked to GDI function. While the events that direct the recruitment of the cytosolic GDI-Rab(GDP) complex to the membrane are presently unknown, delivery to a specific subcellular compartment requires residues present in the hypervariable carboxyl terminus of each Rab protein (13). Membrane localization of Rab has been proposed to involve at least two accessory factors, a GDI displacement factor to dissociate GDI from Rab and a guanine nucleotide exchange factor to promote stable binding of the activated (GTP-bound) form of Rab to the membrane (14–16). Following GTP hydrolysis, presumably promoted by Rab-specific GTPase activating proteins, the membrane-bound form of Rab(GDP) is returned to the cytosolic reservoir through extraction by GDI. The role of GDI in the recycling of GDP-bound forms of Rab is supported by numerous in vitro studies in which GDI has been shown to extract different Rab proteins from a variety of membranes (10, 17–20).

GDI and a closely related family of proteins involved in Rab prenylation (referred to as Rab escort proteins or REP) together form a GDI superfamily (reviewed in Refs. 2 and 3). Two isoforms, REP1 and REP2, which have nearly 90% identity, have been identified (21–25). REP delivers newly synthesized Rab to the catalytic subunits of the geranylgeranyl transferase II (GGTase II) complex for addition of two geranylgeranyl groups to cysteine residues found at the carboxyl terminus. These are essential for Rab function. Subsequently, REP deliv-
ers prenylated Rab to membranes in a manner indistinguishable from GDI (22, 26). REP1 is identical to the choroideremia gene product involved in late onset X-linked retinal degeneration. Consistent with the proposal that different isoforms in the GDI family have unique function, loss of REP1/choroideremia1 results in the defective prenylation of one Rab protein, Rab27. As a consequence of loss of Rab27 function, the choroidal epithelial layer in the eye undergoes degeneration leading to loss of vision (24, 27).

The essential and ubiquitous roles of members of the GDI superfamily in regulation of membrane transport emphasizes the need for identifying and characterizing the role of specific residues that interact with Rab and those that function to deliver and/or extract Rab from membranes. To address this question, we have solved the structure of α-GDI at 1.81-Å resolution using x-ray crystallography (28). These studies revealed that α-GDI is constructed of two main structural units, a large complex multisheet domain I and a smaller α-helical domain II. Domain I includes sequence-conserved regions (SCRs) located in the NH2-terminal and central portions of the molecule common to members of both the GDI/REP families. These contain tri- and tetrapeptide motifs which are invariant from yeast to man (29). In addition, highly conserved residues are clustered on only one face of the molecule suggesting that this face is involved in most, if not all, important features of GDI/choroideremia function (28). The two most conserved SCRs, 1 and 3B, form a compact structure at the apex of GDI. Using site-directed mutagenesis, we have previously shown that surface residues with their polar side chains directed away from the α-carbon background in SCRs 1B and 3B are important for the binding of Rab3A and for the ability of α-GDI to extract the GDP-bound form of Rab3A from rat brain synaptosomes (28). Based on these studies we have speculated that this region recognizes Rab3A in vivo and is critical for function.

To date there has been no correlation between the role of residues involved in binding of Rab in vitro (28) and the activity of GDI in vesicular transport. We now provide functional evidence that select conserved residues required for the binding of Rab3A in the synapse also regulate the activity of the Rab1A GTPase involved in endoplasmic reticulum (ER) to Golgi transport. Like other Rab proteins, Rab1A forms a cytosolic complex with GDI and this pool is essential for transport in vivo (30 and in vitro (20). Given the evolutionary divergence between these two Rab species (35% identity), we propose that residues which mediate the binding of both Rab1A and Rab3A participate in general Rab function in vivo. Intriguingly, we have also identified residues that appear to discriminate not only between the role of GDI in the recognition of different Rabts, but in interacting with membranes, providing evidence for specialized receptor(s) directing Rab recycling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trans[35S]label (> 1,000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Rat liver cytosol used in transport assays was prepared as described previously (31). Endoglycosidase H (endo H) was obtained from Boehringer Mannheim. Antibody specific for Rab1 (p68) was prepared as described (32). His6-tagged Rab1 and Rab3A expressed from Escherichia coli were prepared as described (33). Prenylated His6-tagged Rab3A was purified as described (28). Site-directed mutagenesis and purification of E. coli expressed mutants and wild type GDI were performed as described (28). Methylanthraniloyl guanosine ditriphosphate-GDP (mGDP) was prepared as described (34).

**Fluorescence Assay to Measure GDI-Rab Interaction**—Rab-mGDP complexes were prepared by incubating mGDP and Rab protein at a 100 to 1 molar ratio in 50 mM Tris-HCl (pH 7.2), 10 mM EDTA, 1 mM dithiothreitol, 100 μM mGDP at 32 °C for 45 min. At the end of the incubation, the mixture was adjusted to 20 mM MgCl2 (to trap mGDP in Rab) and kept on ice for 1 h before use. The measurement of GDI-Rab interactions were performed in buffer A composed of 25 mM Tris-HCl (pH 7.2), 0.3 mM GDP, 0.5 mM MgCl2, 0.6 mM EDTA. Typically, 100 nM Rab mGDP was incubated with increasing concentrations of GDI in 300 μl of buffer A. The dissociation of mGDP from Rab was measured as a decrease in fluorescence using a Perkin-Elmer LS50B Fluorescence Spectrometer set at excitation and emission wavelengths of 360 and 440 nm, respectively.

Dissociation constants (Kd) for Rab binding to GDI wild-type and mutants were determined by the inhibition of mGDP dissociation from Rab protein in the presence of increasing concentrations of GDI. The calculation of dissociation constant (Kd) was based on the reaction (Scheme 1) where kobs is negligible.

![Scheme 1](https://example.com/scheme.png)

The observed dissociation rate constants (kobs) were obtained by fitting the data to single exponentials. The Kc can be obtained by fitting kobs and the GDI concentration to Equation 1.

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\text{Rab} \cdot \text{GDP} \xrightarrow{k_{-1}} \text{Rab} + \text{GDP} + \text{GDI} \quad (\text{scheme 1})
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**Semi-intact Cell Transport Assay**—Semi-intact cells used for the analysis of ER to Golgi transport were prepared from NRK cells infected with the tsO45 strain of vesicular stomatitis virus (VSV) using the swelling method and transport from the ER to Golgi measured as described (31, 35). Briefly, NRK cells were infected with the tsO45 strain of VSV at the restrictive temperature (39.5 °C) for 4 h to accumulate the VSV-G in the ER. The cells were then pulse-labeled with 100 μCi of Trans[35S]S-label and perfused by swelling and scraping. The assays were performed in a final volume of 40 μl in a buffer containing 25 mM Hepes-KOH (pH 7.2), 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl2, 1 mM N-acetylglucosamine, an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU of rabbit muscle creatine phosphokinase, final concentration), 2–6 μl of rat liver cytosol (15–30 mg/ml protein in 25 mM Hepes-KOH (pH 7.2), 125 mM KOAc), and 5 μl of semi-intact cells (25–50 mg of protein in 50 mM Hepes-KOH (pH 7.2), 90 mM KOAc). Recombinant wild-type and GDI were added to the mixture as indicated under “Results” and incubated for 32 °C for 1 h. After termination of the reaction by transfer to ice, membranes were collected by centrifugation, solubilized, digested with endo H, and processed for SDS-polyacrylamide gel electrophoresis and fluorography as described (31). Autoradiographs were quantitated using a Molecular Dynamics Densitometer (Molecular Dynamics, Sunnyvale, CA).

**Extraction of Rab Proteins from Membranes by GDI**—Extraction of Rab proteins from membranes by purified recombinant GDI was performed as described (20). Briefly, 300 μg of perforated cell membranes were incubated in a buffer containing 25 mM Hepes-KOH (pH 7.2), 75 mM KOAc, 1.8 mM CaCl2, 5 mM EGTA, 5 mM MgOAc, and a protease inhibitor mixture. Subsequently, 5 μg of His6-GDI were added and the incubation was continued for 30 min at 37 °C. At the end of the incubation, the samples were transferred to ice and centrifuged for 5 min at 16,000 × g to pellet membranes. The supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using GDI-specific and Rab1A-specific antibodies (20, 32). The plots were developed using an ECL™ kit and quantitated using a Molecular Dynamics densitometer calibrated using purified proteins.

**Binding of Wild-type and R70E Mutant to Membranes**—Measurement of the binding of mutant R70E and wild-type recombinant GDI to semi-intact cells was carried out as described above using unlabeled NRK cells. Briefly, purified mutant and wild-type GDI were added at the indicated concentration (see “Results”) to a standard 40 μl in vitro transport mixture and incubated for 30 min at 32 °C. Samples were transferred to ice to terminate the reaction. Membranes were collected by centrifugation and washed twice with 1 ml of phosphate-buffered saline to assure the complete removal of unbound GDI. The amount of membrane-bound GDI recovered in the pellet was detected by immunoblotting using GDI-specific antibody. The blots were developed using...
an ECL™ kit and quantitated using a Molecular Dynamics densitometer calibrated using purified proteins.

RESULTS

A New Fluorescence Assay to Measure α-GDI-Rab Interaction: Analysis of Binding to Rab3A—The dissociation constants ($K_d$) for the interaction of wild-type and mutant α-GDIs with Rab3A, a GTPase required for vesicle fusion in the synapse, were previously determined by measuring the differential elution of free Rab3A or the GDI-Rab3A complex from Q-Sepharose in response to increasing salt (28). This approach was not possible with Rab1A (~35% identity to Rab3A) involved in ER to Golgi transport given that both the free and GDI complexed form of Rab1A elute from Q-Sepharose at similar concentrations of salt. Therefore, we developed an indirect measure for binding. We used the well established property of GDI to prevent the intrinsic dissociation of GDP from Rab (4, 10) in combination with methylanthraniloyl guanosine di(tri)phosphate-GDP (mGDP) to monitor binding of guanine nucleotides to GTPases using fluorescence spectroscopy (36, 37). The dissociation of mGDP was determined by measuring the decrease in relative fluorescence ($\lambda_{excitation}$, 360 nm; $\lambda_{emission}$, 440 nm) that accompanies the release of mGDP from Rab, providing a measure of the strength of Rab and GDI interactions (see “Experimental Procedures”). As shown in Fig. 1, the addition of increasing concentrations of α-GDI prepared from Spodoptera frugiperda (Sf9) cells to Rab3A-mGDP led to a decrease in the loss of intrinsic fluorescence through formation of a GDI-Rab-mGDP ternary complex, demonstrating that α-GDI prevents GDP dissociation from Rab3A in this assay.

To relate the new approach to the Q-Sepharose assay (28, 38), we examined the ability of mutant GDI to bind Rab3A. Because all mutant GDIs are produced as recombinant protein in E. coli, we first compared the ability of wild-type GDI produced in insect cells to that produced in bacteria. Both significantly reduced the intrinsic dissociation rate of mGDP pre-bound to prenylated Rab3A (Fig. 2A). The stronger interaction of Sf9 GDI is consistent with its higher specific biological activity in vitro, possibly reflecting post-translational modifications not carried out by E. coli (39). Next, we examined the binding of the R240A mutant to Rab3A. Residue Arg-240, found in helix I on the front edge of the Rab-binding platform (Fig. 3), is essential for recognition of Rab3A by GDI. When mutated (R240A), the binding of GDI to Rab3A based on the Q-Sepharose assay is lost. This decrease in binding corresponds quantitatively to an ~60-fold reduction in $K_d$ (28). Correspondingly, the R240A GDI mutant in the fluorescence assay completely lost its ability to inhibit the intrinsic rate of dissociation of mGDP from Rab3A (Fig. 2A). This loss in binding to Rab3A corresponds quantitatively to an ~6-fold decrease based on kinetic analysis of the fluorescence data (see “Experimental Procedures”).

To determine whether Rab prenylation was necessary for Rab recognition, the binding of wild-type GDI and the R240A mutant to Rab3A generated in bacteria (and therefore lacking prenyl groups) was examined. While binding of wild-type GDI to unprenylated Rab3A was weaker than that observed for the prenylated form, significant interaction was detected judging from the decrease in the intrinsic dissociation of mGDP from Rab3A (Fig. 2B). As expected, the R240A mutant reduced binding to unprenylated Rab3A to background levels (Fig. 2B). These results suggest that while the prenyl group facilitates the interaction between GDI and Rab, other residues in Rab are required to stabilize formation of the ternary complex.

In general, the change in binding of wild-type and various mutant GDIs to Rab3A obtained using the fluorescence assay directly mirrored the change in $K_d$ as determined by the Q-Sepharose assay (28). Although the fluorescence assay has a more limited dynamic range (~6-fold compared with the ~60-fold dynamic range observed for the Q-Sepharose assay (28)), it is applicable to the entire spectrum of Rab GTPases. It, therefore, provides a more useful approach to assess the relative ability of GDI to recognize different Rab proteins. Given the ease of producing protein using bacteria, all further studies

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*S. K. Wu and W. E. Balch, unpublished observations.*

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Fig. 1. Effect of α-GDI on the intrinsic dissociation of mGDP from Rab3A. GDI was added at the indicated final concentrations (μM) and the change in GDP dissociation determined by measuring the decrease in relative fluorescence ($\lambda_{excitation}$, 360 nm; $\lambda_{emission}$, 440 nm) that accompanies the release of mGDP from Rab3A as described under “Experimental Procedures.”

Fig. 2. Comparison of the effects of the R240A GDI mutant on mGDP dissociation from prenylated and unprenylated forms of Rab3A. mGDP dissociation was determined as described under “Experimental Procedures.”
utilized recombinant GDI and unprenylated Rab purified to homogeneity following overexpression in E. coli (see “Experimental Procedures”).

**Binding of Rab1A to Wild-type and Mutant α-GDI**—To define the role of conserved residues in α-GDI in the regulation of Rab1A function in ER to Golgi transport, we first examined the ability of a range of GDI mutants to bind to Rab1A in vitro using the mGDP dissociation assay.

Residues Gln-236 and Arg-240 in SCR 3B are found on adjacent turns of helix I defining the front edge of the Rab binding platform (Fig. 3). Mutation of both of these residues eliminated the ability of GDI to inhibit the intrinsic dissociation rate of mGDP from Rab1A (Fig. 4, lanes m and n). Mutation of T248 (SCR3B), a residue found in β-strand a3 of sheet A which defines the upper edge of the Rab binding platform (Fig. 3) (28), resulted in a similar loss of GDI binding to Rab1A (Fig. 4, lane o). Because these results are identical to the complete loss of binding of GDI to Rab3A observed previously (28), we suggest that the Gln-236, Arg-240, and Thr-248 residues play critical roles in the general function of the Rab binding platform. They may potentially contribute polar side chains which recognize conserved residues in divergent Rab species. Interestingly, Tyr-39, a conserved residue located in SCR1A found in the center of the Rab binding platform (Fig. 3), and Glu-233 (a residue that is proximal to Arg-240 in helix I) (Fig. 3) are absolutely critical for recognition of Rab3A by GDI (28). Although important for Rab1A recognition (Fig. 4, lane i and l, respectively), they were clearly less essential than either Arg-240 or Thr-248 as significant levels of binding could be detected. Thus, Tyr-39 and Glu-233 and possibly other more variable residues found in the Rab binding cleft may have more specific roles in defining the strength of the interaction between α-GDI or other GDI isoforms with distinct Rab species.

Residues found more distant from Arg-240 in helix I, but also having polar side chains directed into the Rab binding cleft include Arg-70 found in the “insert” region (a more hypervari-

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**FIG. 3. Location of mutated residues in α-GDI.** The structure of α-GDI illustrating the location of selected residues which were mutated as described under “Results.” The Rab binding platform containing SCRs 1 and 3B is highlighted in dark gray; the insert region is highlighted in light gray.

**FIG. 4. Relative binding affinity of GDI mutants to Rab1A.** mGDP dissociation and binding was determined as described under “Experimental Procedures.” The effects of mutation of each of the indicated residues fall into 3 arbitrary categories. Mutants in category I lead to a fractional decrease in binding of less than 0.3 when compared with wild-type levels (set at a value of 1). Category II includes mutants with an intermediate level of binding (0.3 to 0.7 of wild-type). Category III includes those with a more complete loss of binding. The standard error of the mean for duplicate determinations was <10% of the reported value.
GDI from semi-intact cell membranes. Semi-intact NRK cells were incubated with the indicated wild-type or mutant GDI (1 μg) and the amount of Rab1A extracted into the supernatant determined as described under “Experimental Procedures.” Values are reported as % of amount of Rab1A extracted into the supernatant determined as described (28). Values are reported as % of the wild-type value. The standard error of the mean for duplicate determinations was 70% of total Rab1 on membranes. The standard error of the mean for duplicate determinations was <15% of the reported value.

![Diagram](image)

**Fig. 5.** Fraction of Rab1A extracted by wild-type and mutant GDI from semi-intact cell membranes. Semi-intact NRK cells were incubated with the indicated wild-type or mutant GDI (1 μg) and the amount of Rab1A extracted into the supernatant determined as described under “Experimental Procedures.” Values are reported as % of amount of Rab1A extracted into the supernatant determined as described (28). These residues may therefore define the outer boundaries of the Rab-binding region which contribute either direct or indirect (structural) features to general recognition of Rab. Other highly conserved residues flanking the Rab-binding region, notably those found within the insert region (Arg-55, Arg-68, and Lys-79) or SCRs 2 and 3 (Glu-136, Lys-142, and Asn-252) as well as K278S outside the conserved regions (Fig. 3) were also mutated. In general, they had no effect or only marginal effects on GDI binding to either Rab1A or Rab3A (28). These residues may therefore define the outer boundaries of the Rab-binding region which contribute either direct or indirect (structural) features to general recognition of Rab. Other highly conserved residues flanking the Rab-binding region, notably those found within the insert region (Arg-55, Arg-68, and Lys-79) or SCRs 2 and 3 (Glu-136, Lys-142, and Asn-252) as well as K278S outside the conserved regions (Fig. 3) were also mutated. In general, they had no effect or only marginal effects on GDI binding to either Rab1A or Rab3A (28).

To simplify classification and characterization of mutants, we have generated 3 arbitrary categories based on their ability to bind Rab1A in vitro (Fig. 4, dotted lines). The GDI mutants in the first category (I) have only a small change in binding. Mutants in this category lead to a fractional decrease in binding of less than 0.3 when compared with wild-type levels (set at a value of 1) (Fig. 4). Category II includes mutants with an intermediate level of binding (0.3 to 0.7 of the wild-type value). Category III includes those with a more complete loss of binding. Two of the residues in the latter category (III) (Arg-240 and Thr-248) are completely defective in recognition of Rab in vitro based on their inability to inhibit the intrinsic rate of dissociation mGDP from Rab (Fig. 4). The three classes provide a useful standard for comparing the effects of the various GDI substitutions on Rab1A binding to their role in modulating Rab1A recycling and function in vesicular transport.

**Effect of GDI Mutants on the Extraction of Rab1A from Membranes**—We previously reported that recombinant His6-tagged α-GDI or α-GDI purified from bovine brain were able to efficiently extract Rab1A from highly enriched Golgi membranes (20). To correlate the effects of mutations in GDI on Rab1A binding (Fig. 4) to GDI function in recognition of membrane bound Rab1A, we tested each mutant for its capacity to extract Rab1 from normal rat kidney (NRK) semi-intact cells. Semi-intact cells are a population of cells whose plasma membrane has been selectively disrupted to expose intact functional ER and Golgi compartments (31, 35, 40). Wild-type GDI extracted ~70% of the Rab1 from semi-intact cells. Setting this value to 100% (Fig. 5), we found that most type II and type III mutants (which showed either a partial or complete reduction in Rab1A binding (Fig. 4)) were either partly defective (30–70% of the wild-type value) or largely defective (less than 30% of the wild-type value), respectively, in extraction of Rab1A from membranes (Fig. 5). These results reinforce the general importance of residues involved in Rab binding in vitro in promoting the interaction of GDI with Rab bound to membranes.

Interestingly, the R55D, R66E, and R70E substitutions present in the insert region (Fig. 3) that showed either no or intermediate levels of binding to Rab1A (category I and II mutants) (Fig. 4, lanes b, c, and j), were largely inactive in Rab1 extraction from membranes (Fig. 5, lanes h-j). In contrast, the Y39L mutant located in the middle of the Rab binding platform, which has an identical level of binding to soluble Rab1A as the R70E substitution (Fig. 4, compare lanes i and j), was able to extract Rab1 as efficiently as wild-type GDI (Fig. 5, lane b). These results raise the possibility that conserved residues in the insert region play an important role in recognizing membrane-bound Rab1. Moreover, and in contrast to the results with Rab1A, the Y39L mutant is unable to either bind or extract Rab3A from synaptosomal membranes (28). This difference supports our interpretation that Tyr-39 and potentially other residues may play distinct roles in the recognition of different Rab species. Thus, while a number of the mutants showed an extraction phenotype consistent with their ability to bind Rab1A, others showed inconsistencies, highlighting a more specific role in either Rab binding or interaction with membrane-bound receptors potentially involved in Rab recycling.

**Effect of GDI Mutants on the Transport of Cargo between the ER and the Golgi Complex in Vitro**—To address the importance of the above results on GDI function in vesicular traffic, we examined the effect of each of the GDI mutants on the transport of the transmembrane protein VSV-glycoprotein from the ER to the Golgi in semi-intact cells. In this assay, transport is measured by processing of the N-linked oligosaccharides found on VSV-G from endoglycosidase H-sensitive ER forms, to endoglycosidase H-resistant Golgi forms following incubation in vitro in the presence of cytosol and ATP (31). Using this assay, we have previously demonstrated that transport requires recruitment of Rab1 from the GDP-Rab1 complex found in the cytosol (20). Moreover, incubation of semi-intact cells with excess wild-type GDI, which can extract the functional pool of Rab1 from membranes (Fig. 5, lane a), leads to nearly complete inhibition of transport (20).

In the absence of GDI, VSV-G was transported between the ER and Golgi compartments with nearly 80% efficiency. Setting this value to 100% (Fig. 6), we found that inhibition of transport by wild-type GDI was saturable with maximal inhibition in the presence of 1 μg (0.25 μM) of wild-type GDI (Fig. 6, open squares). In contrast, addition of the R240A mutant at similar or higher levels had no effect on transport (Fig. 6, closed squares), consistent with its inability to bind (Fig. 4, lane n) or extract (Fig. 5, lane l) from semi-intact cell membranes.

The effects of all mutants on VSV-G transport are summarized in Fig. 7. Mutants which largely lacked the ability to extract Rab1A from membranes, including Q236E, T248P, and M250Y (Fig. 5, lanes k, m, and n, respectively) had little effect on transport when added at a final concentration of 0.25 μM (Fig. 7, lanes j, l, n, and o, respectively). In contrast, mutants which partially (K79S, K142S, and E233S) (Fig. 5, c-g, i, respectively) or completely (Y39L, E136Q, and K278S) (Fig. 5, lanes b-d, respectively) extracted Rab1A from membranes were able to inhibit transport in a roughly correlative fashion (Fig.
FIG. 6. Effect of increasing amount of wild-type or R240A GDI mutant on ER to Golgi transport of VSV-G. VSV-infected semi-intact cells were incubated in transport mixture at 32 °C for 60 min in the presence of the indicated amount the wild-type or R240A GDI mutant, and the amount of VSV-G transported from the ER to the Golgi measured by the processing of the N-linked oligosaccharides from the endoglycosidase H sensitive to the endoglycosidase H-resistant forms as described under “Experimental Procedures.”

FIG. 7. Effect of mutation of GDI on transport of VSV-G from the ER to the Golgi. VSV-infected semi-intact cells were incubated in the presence of the indicated mutant (1 μg) as described in the legend to Fig. 6. Values are reported as % of control value in which GDI was absent from the incubation (−80% of total). Standard error of the mean for duplicate determinations was <5% of the reported value.

FIG. 8. The R70E mutant binds more efficiently to membranes than wild-type GDI. VSV-infected semi-intact cells were incubated in the absence or presence of the indicated levels of mutant or wild-type GDI (1–3 μg). The amount of GDI bound to membranes was determined as described under “Experimental Procedures.” Values are reported as nanograms of GDI bound based on quantitative immunoblotting using GDI standards. Recombinant GDI (20 ng) is shown in the right lane.

7). These results demonstrate that extraction of Rab1A (20) or other Rab proteins (10, 19, 41) by incubation in the presence of excess uncomplexed GDI in vitro can be a major determinant in the observed inhibition of vesicular transport.

While most mutants showed a close correlation between their ability to bind Rab1A (Fig. 4), extract Rab1 from membranes (Fig. 5), and inhibit VSV-G transport (Fig. 7), several of the mutants failed to meet this expectation. In particular, the R68E (insert region), R70E (insert region), and N252V (β-strand a3) mutants showed a similar phenotype. They had only limited ability to extract Rab1A from membranes (Fig. 5, lanes i, j, and o, respectively), yet these mutants inhibited transport very efficiently (80–85%) (Fig. 7, lanes b–d, respectively). Indeed, these mutants were more potent in their ability to block VSV-G transport than wild-type GDI (Fig. 7, lane a) using identical levels of protein. One possible explanation for these results was that these substitutions may not block vesicular transport by removing Rab1A from membranes, rather they may exert their negative effect by binding to and blocking the activity of membrane-associated receptors normally involved in the recruitment of wild-type GDI and/or GDI-Rab complexes which are essential for transport. In this regard, the R55D mutation found in the insert region (Fig. 3) was interesting in that it bound Rab normally (Fig. 4, lane b), yet failed to extract Rab1 (Fig. 5, lane h) and had no effect on transport (Fig. 7, lane j), suggesting a more specific role for the insert region in the retrieval of membrane-bound Rab.

To pursue the above possibility, increasing levels of either wild-type GDI or the R70E mutant were added to the in vitro reaction. Following incubation for 30 min, semi-intact cells were pelleted, washed, and the amount of GDI bound to membranes quantitated by immunoblotting. As shown in Fig. 8, whereas addition of wild-type GDI showed only a modest increase in the level of membrane-associated GDI over background, the R70E mutant notably accumulated on membranes. This was particularly evident at the lowest concentration added (0.25 μM) where a 10–20-fold higher level of R70E could be detected over that of wild-type. At this concentration, transport was potently inhibited (Fig. 7, lane c). Importantly, binding of both proteins was saturable. These results suggest that the R70E mutant may bind to a receptor involved in either the delivery or extraction of Rab from membranes, and thereby block transport through inhibition of normal Rab1 cycling.

DISCUSSION

Residues Involved in Rab Recognition—We have provided for the first time functional evidence that conserved residues in SCRs 1B and 3B found at the apex of GDI play a key role in regulating the function of Rab1A in transport of cargo from the ER to the Golgi in vitro. Based on the structure of GDI, mutation of these surface residues would not be expected to grossly alter protein folding, a conclusion consistent with the fact each of the mutants can be readily isolated as soluble proteins following expression in E. coli. These results confirm our prediction (28) that the Rab-binding region is crucial for GDI function.

The GDI mutants analyzed in the present study could be organized into 3 groups based on their ability to bind Rab1A using an assay which measures the ability of GDI to block the intrinsic rate of mGDP dissociation from either prenylated or
unprenylated recombinant Rab. Whereas category I mutants retained normal binding to Rab1A, category II mutants showed partial binding, and category III mutants were largely defective in binding. Despite the more limited dynamic range of the mGDP based assay, binding of Rab3A to the various GDI mutants correlated directly with binding measured using the Q-Sepharose assay (28, 38), validating the utility of the mGDP dissociation assay for comparing the interaction of GDI and GDI mutants with a range of different Rab proteins.

In most, but not all cases, we found that GDI mutants defective in binding Rab1A were also deficient in binding Rab3A. Given the sequence divergence between Rab1 and Rab3 isoforms (−35% identity), this result corroborates the observation that different GDI isoforms can serve as a common cytosolic carrier for multiple Rab proteins (10, 11). Moreover, these results reinforce our previous suggestion that conserved residues found in helix I (Gln-236 and Arg-240) and the β-strand a3 (T248) (Fig. 3) (29) play a functional role in the platform found at the apex of GDI that is involved in Rab binding (28).

Two exceptions to the idea that conserved residues in the platform region play a general role in recognizing all Rabbs were Tyr-39 and Gln-233. Mutation of these residues led to differential effects on Rab1A and 3A binding. Whereas each of these mutants were previously shown to be completely defective in Rab3A binding (28), they had only a partial effect on binding to Rab1A. These results raise the possibility that while one group of residues are utilized to build a consensus binding pocket recognizing residues conserved within the Rab family, other residues in this pocket play a role in modulating this interaction based on sequence information unique to different Rab family members. This feature might be elaborated upon in different GDI isoforms to facilitate recycling of specific Rab species.

The molecular interactions mediating GDI-Rab complex formation are currently unknown. We have proposed that Rab binding by GDI is likely to involve residues in the switch I and switch II effector domains found in all members of the Ras superfamily (2). Mutagenesis of selected residues in Rab effector domains disrupts transport function in vivo (42) and prenylation by REP/GGTase II (43, 44). Moreover, we have found that mutation of conserved residues in the switch 1 region of Rab markedly reduce binding to GDI.3 A further understanding of the residues involved in the interaction between Rab and GDI will require solution of the x-ray structure of the Rab-GDI complex, studies that are currently in progress.

Residues Involved in Membrane Recognition—The proposed role for GDI in vesicular transport is to recycle Rab proteins between membrane-bound and cytosolic pools. Recent studies (14, 16, 45) have suggested that the delivery of Rab9 to membranes involves a “GDI-displacement factor” to release Rab9 from the GDI-Rab9 complex during recruitment to endosomes. In addition, we have recently found that membrane-associated factors may be required for the retrieval of Rab from membranes at the vesicle fusion step.4 Consistent with the need for accessory factors in Rab cycling, we found that mutation of the conserved residue Arg-70 located in the insert region flanking one side of the Rab-binding pocket containing Gln-236 and Arg-240 (Fig. 3) was unable to extract Rab1A from membranes, yet showed potent inhibition of VSV-G transport. A similar effect was observed with the nearby R68E substitution.

Because the R68E and R70E mutants are unlikely to inhibit transport by depleting the membrane-associated pool of Rab1A (as proposed for wild-type GDI), they raise the important possibility that they interfere by binding to a membrane-associated factor. Indeed, we found that the recruitment of the R70E mutant under normal transport conditions was augmented to a level of 10–20-fold over that observed for wild-type GDI. One interpretation is that the factor responsible for membrane binding is Rab itself. In this case, one could envision that while the R70E mutant could bind membrane-associated Rab1(GDP), it would be unable to extract it due to defective interaction with the prenyl group. We consider this unlikely for two reasons. From a functional point of view, we have shown that recycling of membrane-bound Rab1 is unnecessary for transport in vitro (20). Thus, if this were the only interaction defective in mutant transport, function should not be inhibited. Moreover, it is unlikely that the R70E mutant fails to recognize the prenyl group. The binding of the R70E mutant to prenylated versus unprenylated forms of Rab shows a comparable difference to that observed for wild-type GDI.2 A second and favored interpretation is that the R68E and R70E mutants interfere with transport by competing with either the endogenous (wild-type) GDI-Rab1 complex for a membrane component such as GDI replacement factor involved during Rab delivery, or for a factor required for removal of Rab-GDP forms at the vesicle fusion step. Experiments are currently in progress to explore these two possibilities and identify the putative target protein(s).

In general, the requirement for residues present in the Rab binding platform and flanking regions provides new insight into the role of the conserved face of GDI in directing Rab to and from membranes. These residues are likely to function in concert with those of the hypervariable COOH terminus of Rab in mediating the specific targeting of Rabs to distinct subcellular compartments.
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