# Guanine Nucleotide Dissociation Inhibitor Is Essential for Rab1 Function in Budding from the Endoplasmic Reticulum and Transport through the Golgi Stack

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*Abstract.* The small GTPase Rab1 is required for vesicular traffic from the ER to the *cis*-Golgi compartment, and for transport between the *cis* and medial compartments of the Golgi stack. In the present study, we examine the role of guanine nucleotide dissociation inhibitor (GDI) in regulating the function of Rab1 in the transport of vesicular stomatitis virus glycoprotein (VSV-G) in vitro. Incubation in the presence of excess GDI rapidly ($t_{1/2}$ < 30 s) extracted Rab1 from membranes, inhibiting vesicle budding from the ER and sequential transport between the *cis*-, medial-, and *trans*-Golgi cisternae. These results demonstrate a direct role for GDI in the recycling of Rab proteins. Analysis of rat liver cytosol by gel filtration revealed that a major pool of Rab1 fractionates with a molecular mass of ~80 kD in the form of a GDI-Rab1 complex. When the GDI-Rab1 complex was depleted from cytosol by use of a Rab1-specific antibody, VSV-G failed to exit the ER. However, supplementation of depleted cytosol with a GDI-Rab1 complex prepared in vitro from recombinant forms of Rab1 and GDI efficiently restored export from the ER, and transport through the Golgi stack. These results provide evidence that a cytosolic GDI-Rab1 complex is required for the formation of non-clathrin-coated vesicles mediating transport through the secretory pathway.

THE *Rab/YPT/SEC4* gene family encodes a ubiquitous group of small GTPases which are recognized to regulate traffic in yeast and mammalian cells (for review see Pryer et al., 1992; Ferro-Novick and Novick, 1993; Novick and Brennwald, 1993; Nuoffer and Balch, 1994). The mammalian Rab protein family encompasses greater than 30 related members (for review see Simons and Zerial, 1993; Zerial and Stenmark, 1993). Rab proteins appear to be essential for the formation of functional carrier vesicles, and are likely to play a key role in regulating vesicle targeting and/or fusion at distinct stages in the endocytic and exocytic pathways. However, the specific function(s) of individual Rab proteins in these diverse trafficking events are presently unknown.

Similar to other members of the ras superfamily, Rab proteins undergo a series of conformational changes associated with the binding and hydrolysis of guanine nucleotides. These are believed to control the vectorial assembly and/or disassembly of multi-subunit protein complexes involved in vesicle formation, targeting, and/or fusion. The intrinsic rates of guanine nucleotide exchange and hydrolysis are low for most Rab proteins examined to date. Therefore, Rab protein function is thought to require a series of accessory factors which modulate progression through the GTPase cycle. In general, these include (*a*) guanine nucleotide exchange proteins (GEPs)[1], which promote exchange of GDP for GTP, (*b*) GTPase-activating proteins (GAPs), which accelerate GTP hydrolysis, and (*c*) guanine nucleotide dissociation inhibitors (GDIs), which form soluble complexes with a wide range of Rab proteins and thereby prevent guanine nucleotide exchange. These factors are likely to be required to regulate the function or recycling of individual Rab proteins during a single round of vesicle formation, targeting, and fusion.

GDI was first isolated from bovine brain as a factor which prevented the dissociation of GDP from Rab3a (Sasaki et al., 1990). Rab3a-GDI was subsequently found to be active on a broad spectrum of Rab proteins (Sasaki et al., 1991; Regazzi et al., 1992; Garrett et al., 1993; Soldati et al., 1993) and renamed "Rab-GDI" (Ullrich et al., 1993). The cytosolic forms of Rab3, Rab5, and Rab9 have been shown to exist in the form of ~80 kD GDI-Rab complexes (Regazzi et al., 1992; Soldati et al., 1993; Ullrich et al., 1993). The

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1. *Abbreviations used in this paper*: endo H, endoglycosidase H; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEP, guanine nucleotide exchange protein; GTPγS, guanosine-5′-O-(3-thiotriphosphate); NTA, nitrilotriacetic acid; VSV-G, vesicular stomatitis virus glycoprotein; VTC, vesicular-tubular clusters.

GDP- but not the GTP-bound form of Rab proteins interacts with GDI. Moreover, complex formation between Rab proteins and GDI required posttranslational modification of carboxyl-terminal cysteine residue(s) with geranylgeranyl lipids (Musha et al., 1992; Garrett et al., 1993; Soldati et al., 1993; Ullrich et al., 1993). GDI also has the interesting property of being able to extract the GDP-bound form of Rab proteins from intracellular membranes, a feature highlighting its potential role in recycling (Ullrich et al., 1993). In keeping with its solubilizing activity, GDI was recently identified as a factor present in bovine brain cytosol which inhibits vesicular transport between early Golgi compartments in vitro (Elazar et al., 1994). These results are consistent with the requirement for Rab1 (and possibly other Rab proteins) in transport between the *cis*- and medial-Golgi compartments in vivo (Tisdale et al., 1992; Wilson et al., 1994) and in vitro (Plutner et al., 1991; Schwaninger et al., 1991; Davidson and Balch, 1993). Two isoforms of Rab-GDI, $\alpha$ and $\beta$, have been identified in rat, human, and *Drosophila* (Sasaki et al., 1990; Ueda et al., 1991; Nishimura et al., 1994), suggesting the existence of a GDI family. Recently, a yeast homologue to bovine brain GDI (GDI1) has been cloned (Garrett et al., 1994). Depletion of Gdi1p revealed a role of GDI in multiple steps of the secretory pathway in yeast (Garrett et al., 1994).

Evidence supporting a role for GDI in membrane transport has also come from studies on vesicular traffic through the endocytic pathway. A GDI-Rab9 complex has been demonstrated to stimulate transport between the late endosome and the *trans*-Golgi network (TGN) in vitro (Soldati et al., 1993). In addition, incubation of perforated cells with a GDI-Rab5 complex promotes endosome–endosome fusion (Ullrich et al., 1994). Delivery of both Rab5 and Rab9 to membranes via the respective GDI-Rab complex in vitro is accompanied by guanine nucleotide exchange (Soldati et al., 1994; Ullrich et al., 1994).

In the present study, we examine the role of GDI and a GDI-Rab1 complex in regulating vesicular transport between the ER and Golgi compartments. We find that the addition of excess GDI to an assay which reconstitutes the transport of vesicular stomatitis virus glycoprotein (VSV-G) through the early secretory pathway in perforated cells (Plutner et al., 1992; Davidson and Balch, 1993) inhibits export of VSV-G from the ER, and blocks transport between both early and late Golgi cisternae, providing biochemical evidence for its role as a recycling factor. Consistent with a role for GDI in recycling, gel filtration of rat liver cytosol showed Rab1 to fractionate with GDI as an ~80-kD complex. Cytosol immunodepleted of the GDI-Rab1 complex failed to support export from the ER or transport through the Golgi stack. Transport could be restored by supplementing depleted cytosol with a GDI-Rab1 complex generated in vitro. These data demonstrate that the GDI-Rab1 complex is an essential, soluble precursor for the formation of non-clathrin-coated vesicles mediating the transport of cargo between early compartments of the secretory pathway.

## Materials and Methods

### Materials

Reagents used to measure transport of VSV-G were obtained as described (Plutner et al., 1992; Davidson and Balch, 1993). Antibodies specific for Rab1 (m4D3c, m5C6b, and p68) were prepared as described (Plutner et al., 1991). A plasmid encoding a glutathione-S-transferase (GST)-GDI fusion protein (pGEX-2T-GDI) was generously provided by Y. Takai (Kobe University, Kobe, Japan). A polyclonal serum recognizing GDI was generated from rabbits as described (Plutner et al., 1991) using the GST-GDI fusion protein purified by affinity chromatography on glutathione-Sepharose. GDI was purified from bovine brain as described (Sasaki et al., 1990). A monoclonal antibody (M3A5) specific for $\beta$-COP was generously provided by T. Kreis (University of Geneva, Switzerland) (Duden et al., 1991; Pepperkok et al., 1993). A polyclonal serum recognizing $\alpha$-1,2-mannosidase II (anti-Man II) was kindly provided by M. Farquhar (University of California, San Diego, CA) (Velasco et al., 1993). A monoclonal antibody specific for the carboxyl terminus of VSV-G (P5D4) was kindly provided by T. Kreis (Kreis, 1986). Rat liver cytosol immunodepleted of Rab1 was prepared as described (Peter et al., 1993).

### Transport Assays

Transport of VSV-G between the *cis*- and medial-Golgi compartments in vitro using enriched Golgi membranes was measured as described (Balch et al., 1984). Transport of VSV-G between the ER and Golgi compartments in vitro using mechanically perforated NRK cells (in a 40-$\mu$l reaction volume) was assessed as described (Plutner et al., 1992; Davidson and Balch, 1993). VSV-G processing intermediates were separated by SDS-PAGE and quantitated as described (Davidson and Balch, 1993) using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Measurement of transport using indirect immunofluorescence was performed using digitonin-permeabilized NRK cells as described (Plutner et al., 1992). Negatives were scanned into a computer using a UMAX UC1260 digital scanner at 1200 dpi resolution. The number of VSV-G containing punctate, pre-Golgi intermediates (VTCs) per cell (Plutner et al., 1992), was quantitated using the software package NIH Image (version 1.55a).

### Preparation of Recombinant GDI

The bacterial expression construct containing the $NH_2$ terminally $His_6$-tagged version of bovine GDI was generated by digesting pGEX-2T-GDI with NcoI and Bam HI, and inserting the GDI sequence into pET-11d in a three-piece ligation with an NdeI-$His_6$-NcoI linker. Protein was expressed in *E. coli* BL21 (DE3) as described previously (Nuoffer et al., 1994). $His_6$-GDI was purified by metal-chelate affinity chromatography on $Ni^{2+}$-nitrilotriacetic acid (NTA)–agarose (Quiagen, Chatsworth, CA) as described (Nuoffer et al., 1994), followed by fractionation on Mono Q™ (Pharmacia LKB Biotechnology, Piscataway, NJ) as described (Sasaki et al., 1990). Purified, $His_6$-GDI was dialyzed against 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid (Hepes)/KOH (pH 7.2) and 125 mM K-acetate (25/125) and stored at −70°C.

### Purification of Recombinant Prenylated Rab1b

A DNA fragment encoding a $NH_2$ terminally $His_6$-tagged form of Rab1b flanked by appropriate restriction sites was generated by PCR and inserted into a pBlueBac baculovirus transfer vector (Invitrogen, San Diego, CA) for expression under control of the polyhedrin promoter. Recombinant baculovirus was generated according to the manufacturer's instructions. *Spodoptera frugiperda* (Sf9) cells were grown in Ex-Cell 400™ (JRH Bioscience, Lenexa, KS) to a density of ~2 × $10^6$ cells per ml and infected at a multiplicity of infection of ~10 for 70 h. Cells were harvested, washed, and resuspended in 2 vol of 25 mM Hepes/KOH (pH 7.2), 1 mM $MgCl_2$, frozen in liquid $N_2$ and stored at −70°C. Cells were thawed, diluted with an additional 2 vol of buffer supplemented with 300 mM NaCl and protease inhibitor cocktail, and broken by $N_2$ cavitation (25 min, 500 psi). Homogenates were centrifuged for 5 min at 900 *g* and the supernatant was centrifuged for 1 h at 100,000 *g*. Membrane pellets were resuspended in homogenization buffer, centrifuged as above and $His_6$-Rab1bGG was extracted from the membranes using homogenization buffer supplemented with 0.6% 3-(3-cholamidopropyl)dimethylammonio-1 propanesulfonate (CHAPS). Extracts were clarified by centrifugation as above and $His_6$-Rab1bGG was purified by chromatography on $Ni^{2+}$-NTA agarose and MonoQ columns in the presence of 0.6% CHAPS as described above. $His_6$-Rab1bGG–containing fractions were pooled, concentrated, dialyzed against 25/125 containing 0.6% CHAPS and stored at −70°C. Preparations were contaminated (5–10%) with non-prenylated $His_6$-Rab1b, most likely reflecting variability in the efficiency of cell homogenization.

### *Membrane Extraction of Rab Proteins with GDI*

Membranes were treated with purified bovine brain GDI or recombinant $His_6$-GDI as described (Sasaki et al., 1991; Regazzi et al., 1992; Soldati et al., 1993). Briefly, 300 $\mu$g of perforated cells or 50 $\mu$g of enriched Golgi membranes were incubated for 2 h at 30°C with either 1 mM GDP or 1 mM GTP$\gamma$S in a buffer containing 22 mM Hepes/KOH, 20 mM Tris/HCl, 116 mM KCl, 4.3 mM Mg-acetate, 2 mM DTT and a protease inhibitor cocktail (final pH 7.2). Subsequently, 5 $\mu$g of bovine brain GDI or $His_6$-GDI were added and the incubation was continued for 5 min at 30°C. The samples were centrifuged for 10 min at 16,000 *g* and the pellets were washed once. The soluble and membrane-containing fractions were analyzed by 10% SDS-PAGE and Western blotting using GDI-specific and Rab1b-specific (p68) antibodies (Plutner et al., 1992). The blots were developed using an ECL™ kit (Pharmacia LKB Biotechnology). All quantitation was based on standard curves using purified recombinant $His_6$-Rab1b or $His_6$-GDI.

### *Fractionation of Rat Liver Cytosol*

0.5 ml of rat liver cytosol (Davidson and Balch, 1993) was gel filtered using Superose 6™ (24 ml bed volume) (Pharmacia LKB Biotechnology) equilibrated with 25/125. The excluded "void" volume containing the high molecular mass complex form of Rab1 (Peter et al., 1993) was discarded. The included fractions were pooled and concentrated to 0.5 ml using a micro-concentrator 10 (Amicon Division, W. R. Grace and Co., Beverly, MA), and gel filtered using a Superdex 75™ column (24-ml bed volume) (Pharmacia LKB Biotechnology) equilibrated with 25/125. Fractions (0.5 ml) were collected and analyzed by 10% SDS-PAGE and Western blotting using antibodies directed against Rab1b and GDI.

### *GDI-Rab1 Complex Formation In Vitro*

A fourfold molar excess of $His_6$-Rab1bGG was mixed with $His_6$-GDI in the presence of 1 mM $MgCl_2$, 10 mM GDP, 0.6% CHAPS for 10 min on ice and dialyzed against 25 mM $NaPO_4$ (pH 7.2), 1 mM $MgCl_2$, 0.18% CHAPS for 2 h at room temperature. The sample was then centrifuged for 10 min at 16,000 *g* to remove precipitated $His_6$-Rab1bGG. Complex formation was routinely 70% efficient (with respect to GDI) under these conditions. The complex was then separated from residual free $His_6$-Rab1b/$His_6$-Rab1bGG and GDI by gel filtration over a Superdex 75™ column in 25/125. Where indicated, the GDI-Rab1bGG complex containing pool was loaded on a Mono Q column equilibrated with 20 mM Tris-HCl (pH 7.2), 1 mM $MgCl_2$ and developed with a 0–500 mM NaCl gradient. Rab1 eluted at ~125 mM NaCl; the GDI-Rab1bGG complex eluted at ~220 mM NaCl.

### *Electron Microscopy*

Incubation of cells for electron microscopy and immunogold labeling of VSV-G was performed as described (Balch et al., 1994). Images generated on a JEOL 1200EX electron microscope at a magnification of 20K were scanned into a computer using a UMAX UC1260 digital scanner at 500 dpi resolution. The mean linear density of gold particles per $\mu$m membrane length in the ER and vesicular-tubular clusters (VTCs) was quantitated using the software package NIH Image (version 1.55a).

## *Results*

### *Excess GDI Inhibits ER to Golgi Transport In Vitro*

The small GTPase Rab1 is essential for protein export from the ER in mammalian cells (Plutner et al., 1990, 1991; Schwaninger et al., 1992; Tisdale et al., 1992; Nuoffer et al., 1994; Pind et al., 1994). Given the recent observation that GDI is able to extract multiple members of the Rab protein family from membranes in vitro (Ullrich et al., 1993), we examined whether GDI could be used as a tool to study Rab1 function in the transport of VSV-G, a type I transmembrane protein, between the ER and the Golgi stack.

To analyze the effects of GDI on transport in vitro, we used semi-intact cells, a population of cells whose plasma membrane has been selectively disrupted to expose intact, functional ER and Golgi compartments (Beckers et al., 1987; Plutner et al., 1992; Davidson and Balch, 1993). Export of VSV-G from the ER can be synchronized through use of a temperature-sensitive variant of VSV-G (tsO45) which is retained in the ER when cells are incubated at the restrictive temperature (39.5°C) due to a thermoreversible-folding defect (Lafay, 1974). During cotranslational insertion into the ER, VSV-G acquires two N-linked high mannose oligosaccharides which are sensitive to digestion with endoglycosidase H (endo H). After perforation on ice, the cells are incubated at the permissive temperature (32°C) in the presence of cytosol and ATP to initiate transport. The first 15–20 min of incubation encompass a "lag" period during which vesicles bud from the ER and are targeted to the Golgi region (Beckers et al., 1990; Plutner et al., 1992; Balch et al., 1994; Pind et al., 1994). This is followed by the fusion of transport vesicles to the *cis*/medial-Golgi compartments where VSV-G is rapidly processed to transient endo H–resistant "$R_I$" forms (Schwaninger et al., 1991; Davidson and Balch, 1993) by resident mannosidases and glycosyl transferases. Subsequently, VSV-G is transported to *trans*-Golgi compartments (Davidson and Balch, 1993; Balch et al., 1994) where it is processed to the mature $R_T$ form containing terminal sialic acids (Plutner et al., 1992; Davidson and Balch, 1993). These carbohydrate processing intermediates can be resolved by their unique mobilities using SDS-PAGE (Laemmli, 1970) and provide quantitative measures of ER to Golgi and intra-Golgi transport.

When $His_6$-tagged GDI (prepared from *E. coli*) or native GDI (purified from bovine brain [Sasaki et al., 1990]) were incubated with perforated cells or with a Golgi membrane fraction prepared by sucrose density centrifugation (to be referred to as "enriched Golgi membranes") (Balch et al., 1984), ~50% of the total Rab1 pool was rapidly extracted from the membranes (data not shown), consistent with previous reports which have demonstrated the ability of GDI to extract multiple Rab proteins from membranes (Garrett et al., 1993; Soldati et al., 1993; Ullrich et al., 1993). Preequilibration of the membranes with the nonhydrolyzable analog of GTP, GTP$\gamma$S, inhibited solubilization of Rab1 (data not shown), consistent with the view that GDI interacts with the GDP- but not the GTP-bound form of Rab proteins (Sasaki et al., 1990).

To examine the effect of GDI on ER to Golgi transport, perforated cells were incubated with increasing concentrations of $His_6$-GDI in the presence of cytosol, ATP, and UDP-GlcNAc (to promote processing of VSV-G to $R_I$ forms). As shown in Fig. 1 *A*, GDI inhibited the transport of VSV-G from the ER to the *cis*/medial-Golgi compartments. Half-maximal inhibition ($IC_{50}$) was observed with the addition of 2–3 pmol of GDI. Complete inhibition was achieved in the presence of 10 pmol (250 nM final concentration) (Fig. 1 *A*) which corresponds to an approximate 10–20-fold molar excess over the endogenous Rab1 pool. Inhibition could be neutralized by preincubating $His_6$-GDI with a 2–3-fold molar excess (10 pmol) of recombinant $His_6$-prenylated Rab1b (containing two geranylgeranyl groups [Rab1GG]) before addition to the assay (Fig. 1 *B*, *closed squares*). The inhibition observed at higher concentrations of $His_6$-Rab1GG (Fig. 1 *B*, >10 pmol, *closed squares*) is due to nonspecific effects of the CHAPS detergent used to solubilize $His_6$-Rab1GG. In contrast, the effect of $His_6$-GDI on transport

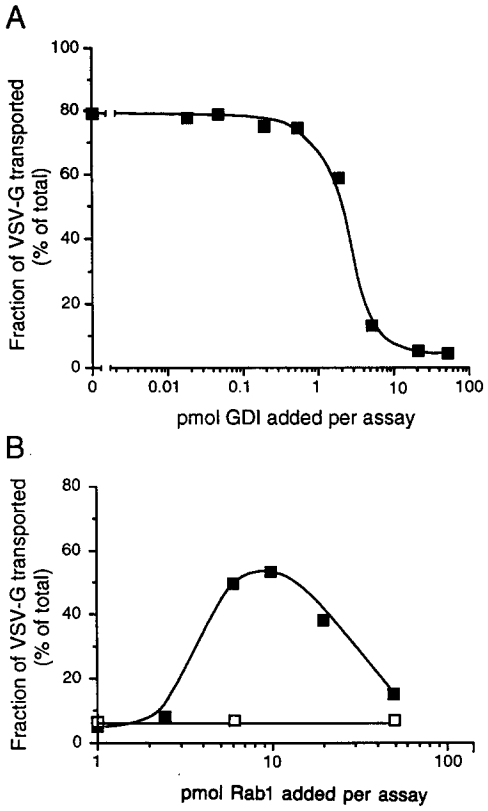


*Figure 1.* GDI inhibits ER to Golgi transport in vitro. Perforated NRK cells were incubated in the presence of cytosol and ATP as described in Materials and Methods. (*A*) The indicated amounts of GDI were added to each assay mix and the samples were incubated for 90 min at 32°C. (*B*) Reactions were supplemented with 10 pmol of GDI and the indicated amount of prenylated Rablb (RablGG) (*closed squares*) or unprenylated Rablb (*open squares*). The samples were preincubated for 30 min on ice and subsequently shifted to 32°C for 90 min. The inhibition observed in the presence of >10 pmol of RablGG reflects non-specific effects of the detergent included in the RablGG buffer. The fraction of VSV-G processed to the $R_1$ form was determined as described in the Materials and Methods.

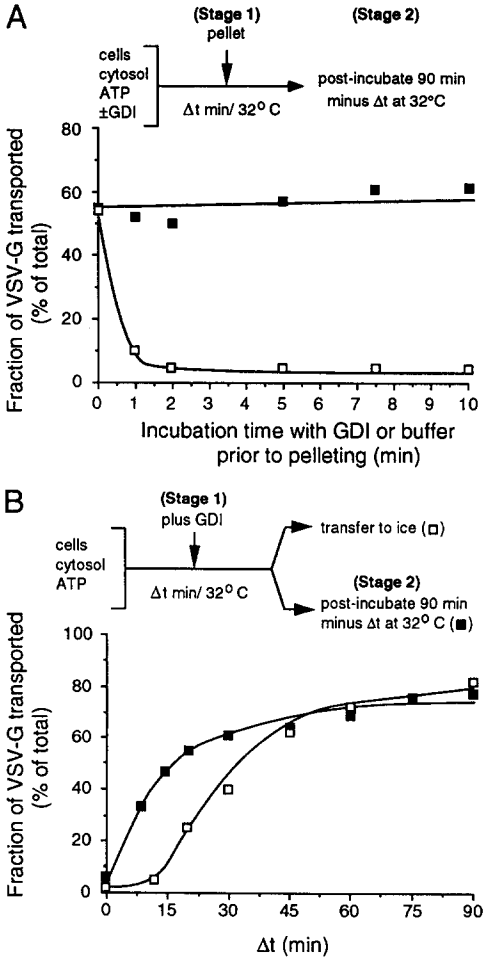


*Figure 2.* GDI inhibits an early step in ER to Golgi transport in perforated cells. (*A*) Perforated cells, cytosol, and ATP were incubated with 20 pmol of GDI (*open squares*) or buffer (*closed squares*) for the indicated time at 32°C (*Stage 1*). Cells were pelleted, the supernatant discarded, and the membranes resuspended in fresh assay mix containing cytosol and ATP, and the incubation continued for a total of 90 min at 32°C (*Stage 2*). (*B*) (*open squares*) Perforated cells were incubated for increasing time at 32°C in the presence of cytosol and ATP before transfer to ice. (*Closed squares*) Perforated cells were incubated for increasing time at 32°C in the presence of cytosol and ATP before transfer to ice (*Stage 1*). Each reaction was then supplemented with 20 pmol of GDI and the samples were incubated for a total of 90 min at 32°C (*Stage 2*). Transport was terminated by transfer to ice and the fraction of VSV-G processed to the $R_1$ form was determined as described in Materials and Methods.

could not be neutralized by preincubation with a recombinant Rablb lacking the prenyl groups (Fig. 1 *B*, *open squares*), emphasizing the importance of the geranylgeranyl lipids for the physiological association of GDI with Rabl. This observation is consistent with our previous results demonstrating that prenylation is essential for Rabl function in vivo (Tisdale et al., 1992) and in vitro (Nuoffer et al., 1994). Identical results were obtained using native GDI purified from bovine brain (data not shown), demonstrating that the $NH_2$-terminal $His_6$-tag does not interfere with the function of GDI. All subsequent experiments were performed using recombinant $His_6$-GDI.

### *GDI Inhibits Vesicle Budding from the ER*

To examine the kinetics of inhibition, perforated cells were incubated for increasing time at 32°C in the presence of 10 pmol GDI (Fig. 2 *A*, *Stage 1*), pelleted to remove >80–90% of the excess GDI (based on Western blotting) and reincubated in the presence of fresh cytosol and ATP for 90 min (Fig. 2 *A*, *Stage 2*). No effect was observed after preincubating perforated cells with GDI for 20 min on ice (data not shown), which illustrates the efficiency of the washing step. In contrast, inhibition was complete after only a 2-min exposure to GDI at 32°C ($t_{1/2}$ = ~30 s). Inhibition was irreversible as incubation for an additional 90 min in the presence of fresh cytosol and ATP failed to restore transport (Fig. 2 *A*, *open squares*). Consistent with the rapid kinetics of inhibition, a 2-min incubation at 32°C in the presence of GDI was sufficient (data not shown) to extract ~50% of Rabl from membranes based on Western blotting. These results demonstrate that GDI acts rapidly, providing a useful

tool to identify the step(s) in ER to Golgi transport which are sensitive to depletion of Rab protein(s).

To temporally define the site of action of GDI, perforated cells were first preincubated for increasing time at 32°C in the absence of the inhibitor (Fig. 2 *B*, *Stage 1*). Subsequently, GDI was added and the cells were further incubated for a total time of 90 min at 32°C (Fig. 2 *B*, *Stage 2*) to allow any VSV-G which had migrated past the GDI-sensitive step in the first stage to be delivered to and be processed in the Golgi stack. As shown in Fig. 2 *B*, VSV-G rapidly ($t_{1/2}$ of ~15 min) migrated past the GDI-sensitive step. Under these conditions, no lag period was detected emphasizing that transit through the GDI-sensitive step occurs much earlier than delivery of VSV-G to the *cis*-Golgi compartment ($t_{1/2}$ = ~30 min) (Fig. 2 *B*, *compare closed squares to open squares*). These results establish three important points. First, since GDI was present throughout the second stage of incubation, the results exclude the possibility that GDI interferes with posttranslational processing of VSV-G oligosaccharides after the fusion of carrier vesicles with the Golgi stack. Second, the inhibitory effect of GDI is restricted to a very early step in transport, possibly vesicle budding. Finally, we have previously demonstrated that Rab1 is required for a late, vesicle targeting and/or fusion step (Pind et al., 1994). Thus, the ability of GDI to inhibit only at early timepoints strongly suggests that newly recruited Rab1 rapidly acquires a conformation which is insensitive to extraction by GDI. One possibility, among others, is that it is converted to the GTP-bound form during coat assembly or vesicle budding through the activity of GEP.

To morphologically characterize the GDI-sensitive step in transport, we examined the migration of VSV-G from the ER to the Golgi in cells permeabilized with digitonin, a technique which allows us to follow transport in vitro using indirect immunofluorescence or immunoelectron microscopy (Plutner et al., 1992; Balch et al., 1994). Incubation of the cells for 30 min in the presence of cytosol and ATP promotes transport of ~30–50% VSV-G to punctate, pre-Golgi intermediates (Fig. 3 *B*, *arrows*) and the peri-nuclear Golgi stack (Fig. 3 *B*, *arrowhead*) as indicated by colocalization with α-1,2 mannosidase II (data not shown) (Plutner et al., 1992; Balch et al., 1994). At high resolution, pre-Golgi intermediates are composed of clusters of 60–80 nm carrier vesicles and small tubular elements (referred to as VTCs) (Balch et al., 1994). After a standard 30-min incubation, the number of VTCs per cell (based on indirect immunofluorescence) ranges from 17 to 69 with an average value of 43 (Table I) (85 cells examined). In contrast, incubation in the presence of excess GDI largely prevented the export of VSV-G from the ER (Fig. 3 *C*). After 30 min of incubation, VSV-G was retained in a reticular ER pattern, similar to its distribution before incubation (compare Fig. 3 *C* and 3 *A*). In this case, the number of VTCs per cell ranges from 0 to 10 with an average value of 5.6 (62 cells examined) (Table I). The small fraction of VSV-G detected in typical pre-Golgi intermediates in the presence of GDI most likely reflects variability in cell permeabilization and consequently accessibility of the ER to GDI in different cells. In contrast to the striking effects of GDI on the transport of VSV-G, under these conditions GDI had no detectable effects on the morphological integrity of either the ER or the Golgi stack based on indirect immunofluorescence (data not shown).

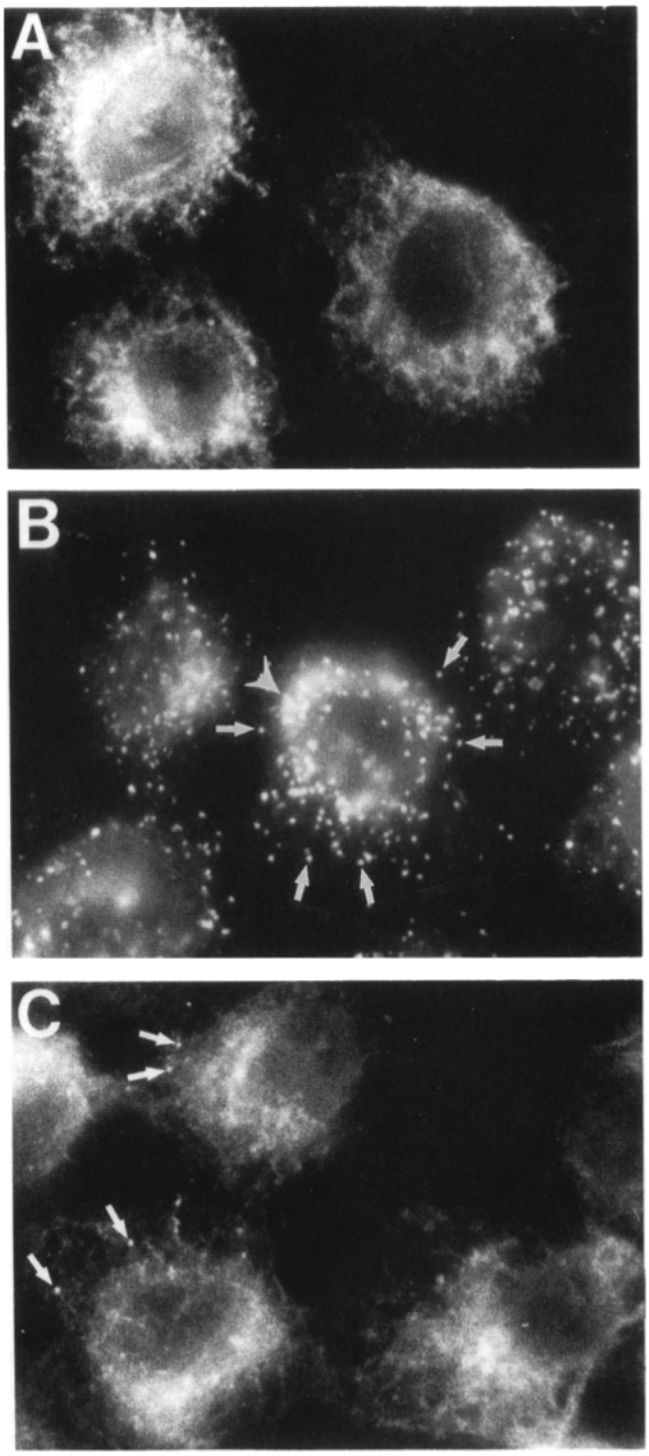


*Figure 3.* Incubation in vitro in the presence of GDI prevents export of VSV-G protein from the ER. NRK cells were permeabilized with digitonin and incubated in a complete cocktail containing cytosol and ATP for 45 min on ice (*A*), at 32°C in the absence (*B*) or presence (*C*) of 20 pmol GDI. The distribution of VSV-G was determined using indirect immunofluorescence as described previously (Plutner et al., 1992). In *B*, the exposure has been adjusted to highlight the distribution of VSV-G to punctate pre-Golgi intermediates over background VSV-G retained in the ER (~50% [Balch et al., 1994]).

*Table I. Distribution of VSV-G Using Indirect Immunofluorescence or Immunoelectron Microscopy in the Presence or Absence of GDI*

I. Indirect immunofluorescence

| Condition | Number of cells examined | Number of punctate elements (VTCs) per cell (±SD) |
|---|---|---|
| Minus GDI | 62 | 43 ± 26 |
| Plus GDI | 85 | 5.6 ± 5.0 |

II. Immunoelectron microscopy

| Condition | Number of cells examined | Fraction of cells examined containing VTCs | Number of VTCs detected | Density of VSV-G in the ER (gold particles per μm membrane length ±SD) | Density of VSV-G in VTCs (gold particles per μm membrane length ±SD) |
|---|---|---|---|---|---|
| Minus GDI | 20 | 0.86 | 38 | 5.8 ± 2.6 [694] | 30.2 ± 10.2 [712] |
| Plus GDI | 20 | 0.15 | 4 | 5.6 ± 3.2 [582] | 35.2 ± 10.6 [70] |

Digitonin permeabilized cells were incubated in vitro as described (Plutner et al., 1992; Balch et al., 1994) in the presence or absence of 500 nM GDI for 20 min at 32°C. The distribution of VSV-G to VTCs was quantitated as described in the Materials and Methods. The number of gold particles counted for each density determination is shown in brackets ([ ]).

While the distribution of VSV-G revealed by indirect immunofluorescence was entirely consistent with the possibility that GDI inhibited vesicle budding, we used immunoelectron microscopy to verify this point. For this purpose, permeabilized cells incubated in the presence or absence of GDI were analyzed for the appearance of VSV-G containing VTCs by immunogold labeling (Balch et al., 1994; Pind et al., 1994). We have previously demonstrated (Balch et al., 1994) that incubation of permeabilized cells for 20 min in vitro results in a partial, transient appearance of VSV-G in VTCs where it is concentrated nearly 5–10-fold over its bulk distribution in the ER (Balch et al., 1994). As shown in Table I, in 86% of the control cells (incubated in the absence of GDI) VSV-G could be readily detected in VTCs, consistent with the abundance of punctate pre-Golgi intermediates detected by indirect immunofluorescence (Fig. 3 *B*; Table I). In contrast, in the parallel incubation in the presence of GDI, only 15% of the cells at high resolution contained VTCs labeling with VSV-G (Table I). We also examined the density of VSV-G (gold particles per μm membrane length) in the ER and VTCs in the presence or absence of GDI. At this early time point, the amount of VSV-G exported from the ER to VTCs (which is generally 20–40% of the total when averaged over several experiments [Balch et al., 1994] ) was not readily apparent given the standard error of the mean (Table I). However, the density of VSV-G in the few VTCs detected in cells incubated in the presence of GDI was approximately sixfold over that in the ER, a value which indicates a degree of concentration similar to that in control cells (Table I) (Balch et al., 1994). These results suggest that a reduction in Rab protein per se does not lead to an alteration in the ability of cells to concentrate VSV-G during export.

The combined results demonstrate that the presence of excess GDI inhibits vesicle budding from the ER in vitro, presumably by either preventing efficient recruitment of Rabl (and possibly other Rab proteins) from the cytosol or by depleting a critical Rab protein pool present on the ER membrane in the GDP-bound form.

### *Rab Protein(s) Are Required for Transport of VSV-G between the Cis/Medial- and Trans-Golgi Compartments in Perforated Cells*

We previously reported the Rabl is required for transport of VSV-G between the *cis*- and medial-Golgi compartments, but not between the medial- and *trans*-Golgi compartments in perforated cells (Plutner et al., 1991; Davidson and Balch, 1993). In support of this observation, a factor isolated from bovine brain cytosol which inhibits transport between the *cis* and medial compartments of enriched Golgi membranes was shown to be Rab-GDI (Elazar et al., 1994).

To establish whether Rab proteins are essential for transport between all Golgi cisternae, we examined whether vesicular traffic between the medial- and *trans*-Golgi compartments was sensitive to GDI by following its effects on the processing of VSV-G from the $R_I$ (*cis*/medial) to the $R_T$ (*trans*) forms in perforated cells. In this case, cells were incubated in the presence of the sugar nucleotide precursors UDP-GlcNAc, UDP-Gal, and CMP-Sia to promote processing of VSV-G to the $R_T$ form (Davidson and Balch, 1993).

Perforated cells were first incubated for increasing time in the absence of GDI (Fig. 4, *Stage 1*). Subsequently, GDI was added and the cells were further incubated for a total of 150 min (Fig. 4, *Stage 2*) to allow transport of VSV-G to the *trans*-Golgi compartment. As shown in Fig. 4, processing of VSV-G to the $R_T$ form (Fig. 4, *solid line, closed squares*) occurs with a substantial lag period compared to the appearance of the transient $R_I$ form (Fig. 4, *dashed line, closed circles*) as transport through the Golgi stack requires additional rounds of vesicle budding and fusion between successive cisternae (Plutner et al., 1991; Schwaninger et al., 1991; Davidson and Balch, 1993). After migration through the GDI-sensitive step involved in ER to *cis*/medial-Golgi transport (Fig. 4, *dotted line, open circles*; $t_{½} \sim 15$ min), VSV-G encountered an additional GDI-sensitive step preceding the appearance of the terminal $R_T$ form with a $t_{½}$ of ~60 min (Fig. 4, *solid line, open squares*). Both GDI-sensitive steps

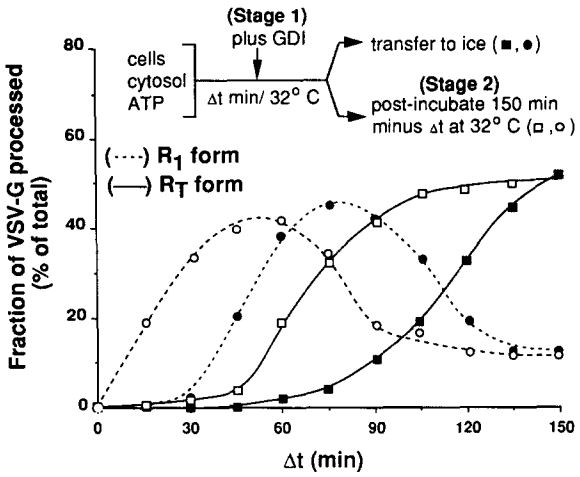


*Figure 4.* Transport of VSV-G between the medial- and *trans*-Golgi compartments requires a Rab protein. (*Closed circles and squares*) Perforated cells were incubated at 32°C for the indicated times in an assay mix supplemented with UDP-GlcNAc, UDP-Gal, and CMP-Sia before transfer to ice. The fraction of VSV-G processed to the transient $R_I$ (*dotted line, closed circles*) and complex $R_T$ (*solid line, closed squares*) forms was determined as described in Materials and Methods. (*open circles and squares*) Perforated cells were incubated at 32°C for the indicated times (*Stage 1*), transferred to ice, and supplemented with 20 pmol of GDI. The samples were then incubated at 32°C for a total of 150 min (*Stage 2*). The fraction of VSV-G processed to the transient $R_I$ (*dotted line, open circles*) and complex $R_T$ (*solid line, open squares*) forms was determined as described in the Materials and Methods.

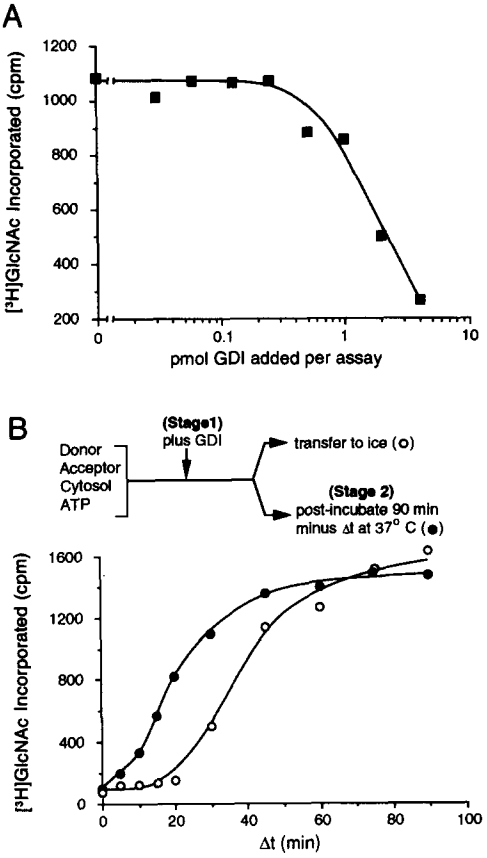


*Figure 5.* GDI inhibits intercisternal transport between enriched Golgi fractions. (*A*) Donor and acceptor Golgi membranes were incubated in the presence of bovine brain cytosol, ATP, and the indicated amounts of GDI for 90 min at 37°C in the presence of [$^3$H]GlcNAc as described in Materials and Methods. (*B*) (*open circles*) Donor and acceptor Golgi membranes, cytosol, and ATP were incubated for the indicated time at 37°C, and transferred to ice to terminate transport. (*Closed circles*) Donor and acceptor Golgi membranes, cytosol, and ATP were incubated for the indicated time at 37°C, transferred to ice and each reaction supplemented with 2.5 pmol GDI (*Stage 1*). Subsequently, the samples were incubated for a total of 90 min at 37°C (*Stage 2*). Transport was terminated by transfer to ice and the amount of [$^3$H]GlcNAc incorporated into VSV-G determined as described in Materials and Methods.

significantly preceded processing of VSV-G to the $R_I$ or $R_T$ forms (Fig. 4, *dotted lines, compare open to closed circles* ($R_I$ form); *solid lines, compare open to closed squares* ([$R_T$ form]). Similar results are obtained when perforated cells are incubated in the presence of GTPγS, a reagent which prevents the uncoating of both ER to Golgi and intra-Golgi vesicular carriers in perforated cells (Beckers and Balch, 1989; Schwaninger et al., 1991; Davidson and Balch, 1993). The data not only support the interpretation that a presently unknown Rab protein(s) is required for transport between late Golgi cisternae, but also provide biochemical evidence for a role of GDI as a recycling factor for Rab proteins involved in intra-Golgi transport, and by inference, transport between the ER and the Golgi, and other steps of the exocytic pathway.

### *An Early Step in Intercisternal Transport between Golgi Membranes Is Sensitive to GDI*

To examine the inhibitory effect of GDI on transport between Golgi compartments in more detail, we used an assay which reconstitutes the transport of VSV-G between *cis*-(donor) and medial-(acceptor) Golgi cisternae in vitro using enriched Golgi membranes prepared by sucrose gradient density centrifugation (Balch et al., 1984). In this assay, we follow the transport-coupled incorporation of [$^3$H]*N*-acetylglucosamine ([$^3$H]GlcNAc) into VSV-G upon fusion of carrier vesicles derived from a donor Golgi compartment (deficient in GlcNAc transferase ([GlcNAc Tr I]) to an acceptor Golgi compartment prepared from wild-type cells (containing GlcNAc Tr I) (Balch et al., 1984).

As shown in Fig. 5 *A*, recombinant GDI, like native GDI (Elazar et al., 1994), inhibited transport in a dose-dependent manner ($IC_{50}$ = 1 pmol). Similar to the results observed for ER to Golgi transport in perforated cells (Fig. 2 *A*), inhibition was neutralized by preincubation of GDI with RablGG before addition to the assay (data not shown). Preincubation of Golgi membranes with a 2–3-fold molar excess of GDI over the endogenous pool of Rab1 irreversibly inhibited transport with a $t_{1/2}$ of 3–5 min on ice, or <15 s at 37°C (data not shown), indicating that enriched Golgi membranes are exquisitely sensitive to GDI. Employing the two stage approach described above in which GDI is added after increasing time of incubation, we found that VSV-G was transported through the GDI-sensitive step without a significant

lag and with a $t_{1/2}$ of ~15–20-min (Fig. 5 *B*, *closed circles*), distinctively earlier than the half-time (~20–30 min) for its delivery to the acceptor Golgi compartment (Fig. 5 *B*, *open circles*).

To determine which of the two compartments, the donor or the acceptor, required a Rab protein(s) for function, each was separately preincubated with GDI for 5 min on ice before incubation with the reciprocal untreated membrane fraction at 37°C in the presence of cytosol and ATP. As anticipated from the above results, the donor function was completely inhibited by preincubation with GDI (Fig. 6 *b*). Interestingly, the acceptor function was also sensitive to treatment with GDI (Fig. 6 *c*). Given our recent observation that Rabl is essential to maintain the structural integrity of the Golgi stack in vivo (Nuoffer et al., 1994; Wilson et al., 1994), the inhibitory effect of GDI on acceptor function may be indirect. When examined using electron microscopy, Golgi membranes incubated in the absence of GDI in vitro retain their stacked structure and show numerous budding and vesicular profiles (Balch et al., 1984; Balch and Rothman, 1985). In contrast, incubation in the presence of GDI for 90 min resulted in the complete loss of recognizable Golgi stacks (data not shown). Thus, while GDI rapidly blocks intra-Golgi transport by presumably inhibiting vesicle budding, prolonged exposure of Golgi membranes to GDI in vitro may cause disruption of the stack.

### *Rabl from Rat Liver Cytosol Fractionates as a GDI-Rabl Complex*

GDI forms soluble complexes with a number of Rab proteins in vivo (Sasaki et al., 1990, 1991; Garrett et al., 1993; Soldati et al., 1993; Ullrich et al., 1993). We examined whether Rabl was present as a GDI-Rabl complex in rat liver cytosol. Gel filtration of rat liver cytosol using Superose 6 resolves Rabl into two functional pools (Peter et al., 1993). One pool (Pl) elutes in the void fraction as part of a high molecular mass complex (S value of 18–19 S). This fraction contains ~10–20% of the total Rabl and supports vesicle budding from the ER in vitro (Peter et al., 1993). The remaining Rabl (80–90% of total) elutes as a lower molecular mass species (Peter et al., 1993). When the latter pool (P2) was further fractionated using Superdex 75 (Fig. 7 *A*), a 20–25-kD monomeric form of Rabl could not be detected. Instead, Rabl eluted with a molecular mass of ~80 kD, comigrating with an ~55-kD protein which was recognized by our Rab-GDI specific antibody (Fig. 7 *B*), suggesting that Rabl is present as a GDI-Rabl complex in rat liver cytosol. Consistent with this interpretation, a portion of the GDI detected in these fractions could be immunoprecipitated by the Rabl-specific antibody (data not shown). Since prenylated forms of Rabl are insoluble in the absence of detergent, it is likely that GDI acts as a solubilizing factor to escort cytosolic Rabl during recycling for use in multiple rounds of vesicle budding and fusion.

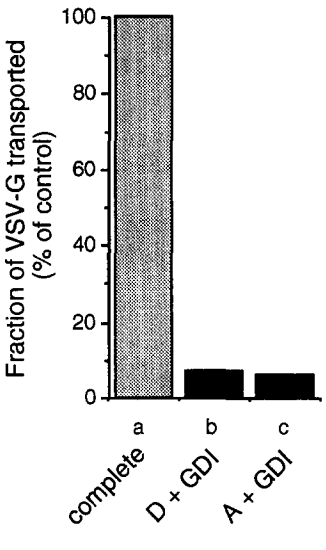


*Figure 6.* Both the donor and acceptor Golgi compartments require Rab proteins for function. *a*) Donor (*D*), acceptor (*A*), cytosol, and ATP were preincubated on ice for 5 min before incubation for 90 min at 37°C. (*b* and *c*) Donor (*b*) or acceptor (*c*) membranes were preincubated in the presence of 2.5 pmol GDI for 5 min on ice. Membranes were pelleted to remove GDI and resuspended in a fresh assay mix containing untreated acceptor (*b*) or donor (*c*). Subsequently, the reactions were incubated for a total of 90 min at 37°C. Transport was terminated by transfer to ice and the amount of [$^3$H]GlcNAc incorporated into VSV-G determined as described in Materials and Methods. The data are expressed in terms of % of transport observed in the mock control lacking GDI.

### *The GDI-Rabl Complex Serves as a Functional Source of Rabl In Vitro*

To examine the biological role of the GDI-Rabl complex found in rat liver cytosol, we incubated purified recombinant

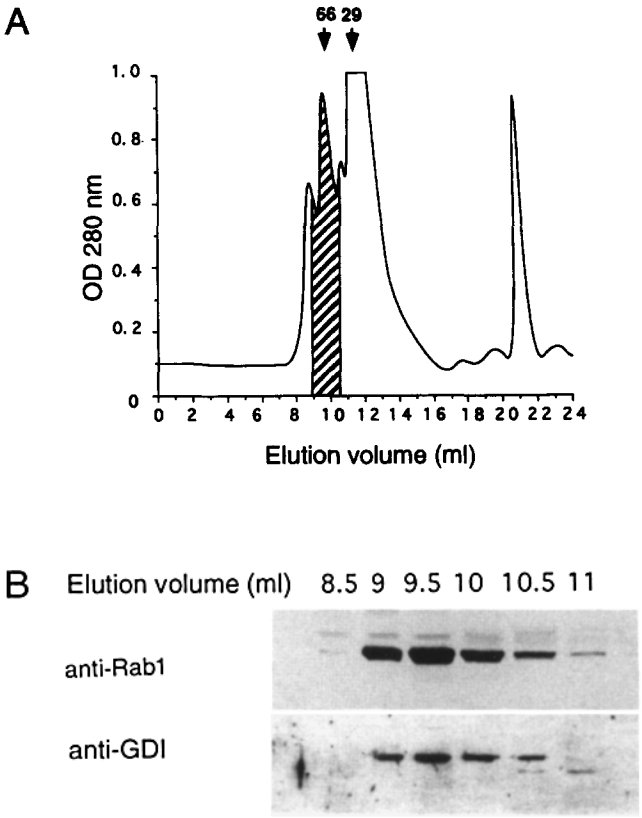


*Figure 7.* Rabl present in rat liver cytosol fractionates as an ~80-kD GDI-Rabl complex. (*A*) 1 ml of rat liver cytosol prepared as described in Materials and Methods was gel filtered over Superdex 75™ (24-ml bed volume). The shaded area shows the part of the elution profile that contains Rabl and GDI as shown by Western blotting in *B*. The indicated molecular mass standards represent BSA (66 kD) and carbonic anhydrase (29 kD). (*B*) Fractions of 0.5 ml were collected, aliquots analyzed by SDS-PAGE, and immunoblotting for Rabl and GDI as described in Materials and Methods.

$His_6$-RablGG in the absence or presence of purified recombinant $His_6$-GDI at a molar ratio 4:1 for 10 min on ice in the presence of 0.6% CHAPS, and subsequently dialyzed the mixture for 2 h against buffer containing 0.18% CHAPS to promote complex formation, but prevent aggregation of free RablGG. Under these conditions, > 60–70% of GDI formed a complex with RablGG. When the products were resolved using gel filtration in the absence of detergent (Fig. 8), most of the free RablGG failed to enter the column (due to aggregation), while a soluble form of RablGG eluted as an ~80-kD GDI-RablGG complex (Fig. 8, fractions *17* and *18*). This peak partially overlapped with the distribution of a small amount of residual free GDI (Fig. 8, fractions *18* and *19*).

To demonstrate that the GDI-RablGG complex eluting from the gel filtration column contained a functional form of Rabl, we analyzed the ability of individual fractions to restore transport activity to Rabl-depleted cytosol (Peter et al., 1993). For this purpose, the P2 pool was immune depleted of >95% of the GDI-Rabl complex (Peter et al., 1993). As shown previously (Peter et al., 1993), the mock-depleted P2 pool contains all of the components required to support a full round of ER to Golgi transport in perforated cells (Fig. 8 *c*).

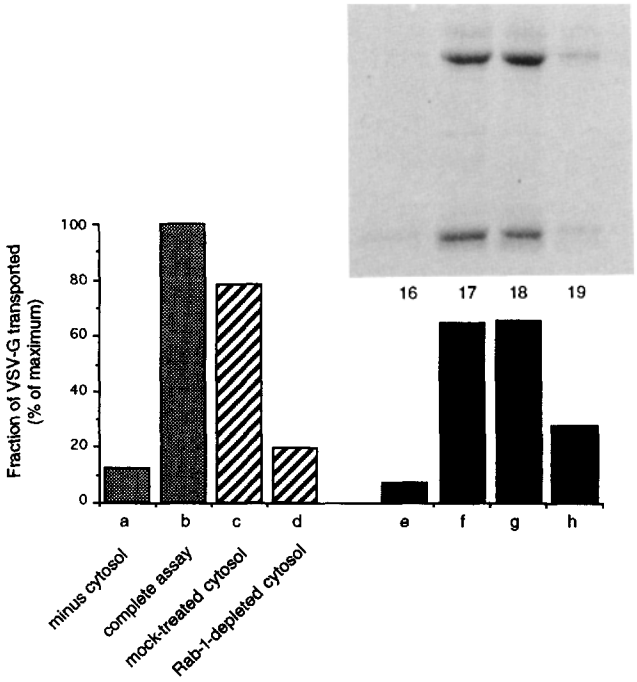


*Figure 8.* Recombinant $His_6$-tagged RablGG and $His_6$-tagged GDI form a complex in vitro. Perforated cells were either incubated in the absence (*a*) or presence (*b*) of cytosol for 90 min at 32°C, or with 75 μg of a cytosol fraction mock-depleted (*c*) or depleted (*d*) of Rabl as described in Materials and Methods. In *e–i*, perforated cells were incubated in the presence of Rabl-depleted cytosol and 10 μl of the fraction from the gel filtration column shown in the inset. The fraction of VSV-G processed to the $R_1$ form is reported as the % of maximum transport observed in the complete assay (*b*). (*inset*) $His_6$-RablGG and $His_6$-GDI were incubated in vitro as described in Materials and Methods and gel filtered using Superdex 75™. The fractions containing Rabl, GDI, or the GDI-Rabl complex were analyzed by SDS-PAGE and staining with Coomassie blue R.

In contrast, VSV-G was not processed to the $R_1$ form in cells incubated in the presence of the Rabl-depleted P2 pool (Fig. 8 *d*). No stimulation of transport was observed when Rabl-depleted cytosol was supplemented with gel filtration fractions lacking the GDI-RablGG complex (e.g., fraction *16*) (Fig. 8 *e*), while supplementation with fractions *17* and *18* (which contained the GDI-RablGG complex), efficiently (>80–90%) restored transport and processing of VSV-G to the $R_1$ form (Fig. 8, *f* and *g*). Compared to the activity found in fraction *19* (Fig. 8 *h*), fractions *17* and *18* are likely to contribute saturating levels of the GDI-RablGG complex to the assay. When a transport cocktail containing Rabl-depleted cytosol supplemented with the GDI-RablGG complex was incubated in the presence of all three sugar nucleotide precursors (UDP-GlcNAc, UDP-Gal, and CMP-Sia), processing of VSV-G to the *trans*-Golgi $R_T$ form was observed (data not shown). These results demonstrate that the GDI-RablGG complex is both necessary and sufficient to restore full transport activity to Rabl-depleted cytosol.

When the fractions containing the GDI-RablGG complex were further analyzed using ion exchange chromatography, the GDI-Rabl complex eluted as a peak at 220 mM with a stoichiometry of 1:1 (Fig. 9, *inset*). Analysis of the GDI-RablGG complex by isoelectric focusing revealed that the complex migrated as a novel species with a $pK_i$ of 5.8, distinctly different from RablGG ($pK_i$ = 6.2) or GDI ($pK_i$ = 5.6; [Sasaki et al., 1990]). The amount of the GDI-RablGG complex required to restore 50% of the total transport activity to Rabl-depleted cytosol was found to be ~3–5 pmol (Fig. 9). This represents an approximately threefold molar excess over the endogenous GDI-Rabl present in cytosol required for a similar level of transport. No transport was ob-

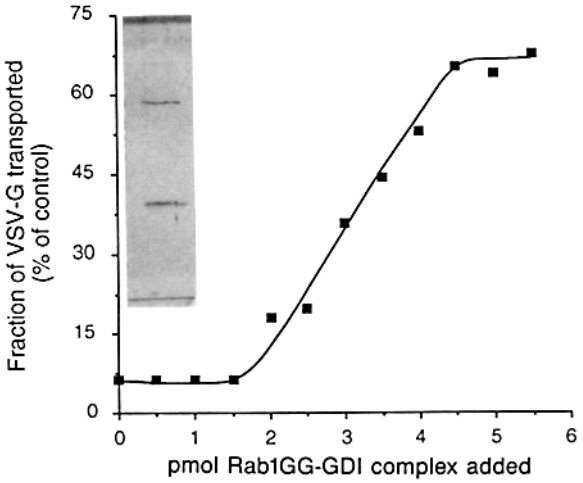


*Figure 9.* A Rabl-GDI complex generated in vitro is both necessary and sufficient to restore transport activity to Rabl-depleted cytosol. Active fractions from the Superdex 75™ were resolved on Mono Q as described in Materials and Methods, and the peak fraction was analyzed by SDS-PAGE and staining with Coomassie blue R (*inset*). Activity of the purified complex was determined by incubating perforated cells in the presence of ATP, Rabl-depleted cytosol and the indicated amount of GDI-RablGG complex. The fraction of VSV-G processed to the $R_1$ form is reported as the % of maximum transport observed in the control containing mock-depleted cytosol.

served when the Rabl-depleted cytosol was supplemented with equivalent amounts of $His_6$-RablGG or $His_6$-GDI (data not shown), attesting to the need to deliver Rabl in the form of a GDI-RablGG complex. Interestingly, the GDI-RablGG complex was unable to restore transport after pretreatment of either perforated cells or enriched Golgi membranes with GDI, suggesting a requirement for additional components, possibly including other Rab protein(s).

### *A Low Molecular Mass Form of β-COP Is Required for Vesicle Budding from the ER*

We previously demonstrated that ER to Golgi transport depends on β-COP and that β-COP appears to be an essential component of a Rabl/β-COP containing high molecular complex active in vesicle budding from the ER (Peter et al., 1993). To address the possibility that this complex is assembled from lower molecular mass precursors, we examined whether the Rabl-depleted P2 pool lacking the 18–19 S complex, when supplemented with recombinant GDI-RablGG complex, retained the essential requirement for β-COP. As shown in Fig. 10, addition of a monoclonal antibody specific for β-COP to the reaction potently inhibited transport. These results are consistent with the possibility that β-COP, possibly in the form of coatomer (Waters et al., 1991), along with GDI-Rabl complex may represent precursors for the assembly of a higher molecular mass complex active in vesicle budding from the ER (Peter et al., 1993).

## *Discussion*

We have demonstrated an essential role for GDI and a GDI-Rabl complex in ER to Golgi traffic and intercisternal transport through the Golgi stack. These results provide a new line of evidence that Rabl and possibly other Rab proteins are essential for vesicle budding from the ER and for transport between all compartments of the early secretory pathway.

### *Effect of GDI on Vesicular Transport*

Incubation of either perforated cells or enriched Golgi membranes with GDI rapidly extracted a pool of Rabl (and presumably other Rab proteins) from membranes resulting in inhibition of transport. An ~10–20-fold molar excess of GDI over the endogenous Rabl was required to elicit rapid and complete inhibition. GDI has been shown to interact with a broad range of Rab proteins (Sasaki et al., 1991; Regazzi et al., 1992; Garrett et al., 1993; Soldati et al., 1993; Ullrich et al., 1993) (for review see Simons and Zerial, 1993; Zerial and Stenmark, 1993; Nuoffer and Balch, 1994). Therefore, it is evident that the actual molar excess of GDI over Rabl is considerably lower. This point is illustrated by the reduced molar excess of GDI (2–3-fold relative to Rabl) required to inhibit transport between Golgi membranes which are highly enriched in Rabl (Plutner et al., 1991). The inhibitory effect of excess GDI on the transport of VSV-G between the ER and Golgi compartments is consistent with the recent observation that depletion of Gdilp, the yeast homologue of bovine GDI, leads to partial inhibition of transport throughout the exocytic pathway (Garrett et al., 1994). However, not all steps involved in vesicle formation are sensitive to Rab-GDI. Using an in vitro assay which reconstitutes clathrin-coated vesicle formation from the cell surface (Carter et al., 1993), the internalization of EGF and transferrin receptor were totally insensitive to GDI (Schmid, S., personal communication).

Although it remains to be elucidated how the presence of excess GDI inhibits the normal recycling of Rab proteins in vitro, the combined results indicate that a large pool of free GDI is unlikely to be present in intact cells. One possible explanation for the irreversible effect of GDI on transport in vitro is that a budding reaction which is initiated in the absence of Rab proteins subsequently aborts—thereby indirectly blocking further rounds of vesicle budding. Alternatively, the irreversibility could reflect the possibility that rat liver cytosol may not contain an adequate pool of all Rab proteins depleted during preincubation with excess GDI. In addition to Rabl, Rab2 is an attractive candidate for a function in ER to Golgi transport given its localization to pre-Golgi intermediates (Chavrier et al., 1990) and our previous studies which demonstrate that transdominant mutants of Rab2 are potent inhibitors of ER to Golgi transport in vivo (Tisdale et al., 1992). Other potential candidates include Rabl2 and Rab24 which have also been localized to the ER and the Golgi stack (Olkkonen et al., 1993).

An important goal of these studies was to provide a test for GDI as a recycling factor for Rab proteins. During preparation of perforated cells and enriched Golgi membranes, conversion of Rab proteins to the GDP-bound form as a consequence of their low, but detectable intrinsic rates of GTP hydrolysis, may be favored. Thus, the sensitivity of transport to GDI when added before incubation in vitro could be explained by extraction of Rab proteins residing in membranes in their GDP-bound form. Evidence that GDI was involved in recycling came from experiments demonstrating that the protein inhibited at least two distinct transport steps, export from the ER as well as transport between early and late Golgi compartments. In the latter case, transport is expected to use Rabl (and/or other Rab proteins) which are being recycled. For example, we have previously demonstrated that a transdominant mutant of Rabl restricted to the GDP form not only interferes with ER to Golgi but also intra-Golgi transport (Schwaninger et al., 1991; Davidson et al., 1992; Plutner et al., 1992; Davidson and Balch, 1993). Thus, Rabl is being recruited at both early and late time points of incuba-

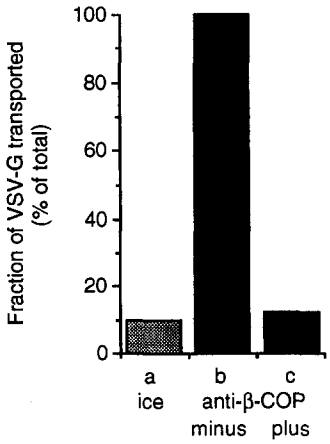


*Figure 10.* Transport requires both β-COP and the GDI-Rabl complex. Perforated cells were incubated in the presence of ATP, Rabl-depleted cytosol, and 1 μg (10 pmol) of purified GDI-RablGG complex for 90 min in the absence (*a–b*) or presence (*c*) of the β-COP specific antibody (M3A5) on ice (*a*) or at 32°C (*b–c*) as described previously. Transport was terminated by transfer to ice and the fraction of VSV-G processed to the $R_1$ form was determined as described in Materials and Methods.

tion in vitro. Moreover, given that the free guanine nucleotide pool is efficiently maintained in the GTP form in vitro (Balch, W. E., unpublished observations), guanine nucleotide exchange, through the activity of GEPs, would be expected to function throughout the time course of incubation in vitro. The ability of GDI to trap Rabl (or other Rab proteins) in their transient GDP-bound forms after initiation of transport in vitro provides biochemical evidence for their accessibility to GDI and its role in recycling. In vivo, the solubilizing (escort) activity of GDI is likely to be restricted to a subpopulation of Rab proteins in the GDP-bound form which undergo recycling after GTP hydrolysis (Sasaki et al., 1990). Although we focused our attention on resolving a GDI-sensitive step preceding the processing of VSV-G to the terminally sialylated complex oligosaccharide form, additional steps are likely to be detected for transport between the *cis*- and *trans*-Golgi compartments pending a more detailed analysis of processing intermediates (Plutner et al., 1991; Schwaninger et al., 1992; Davidson and Balch, 1993). These studies provide the first direct measure for the physiological function of GDI as a recycling factor for Rab proteins and are consistent with the interpretation that Gdilp is required for ER to Golgi and intra-Golgi transport in yeast (Garrett et al., 1994).

### *Role of the GDI-Rabl Complex in ER to Golgi Transport*

The kinetic properties of inhibition by GDI are diagnostic of an early block in export from the ER. In particular, migration of VSV-G through the GDI-sensitive step occurred without a lag and preceded vesicle fusion by 10–15 min. These results eliminate the trivial possibility that GDI was in some unknown fashion inhibiting the processing of VSV-G oligosaccharides in the Golgi stack. Moreover, morphological analysis using indirect immunofluorescence clearly demonstrated that GDI prevented the exit of VSV-G from the ER, providing direct evidence for inhibition at an early formation step. This interpretation was confirmed using immunoelectron microscopy. We suggest that a GDP-bound form of Rabl (and possibly other Rab protein[s]) is critical for initiating the formation of functional carrier vesicles from the ER. The progression of VSV-G past the GDI-sensitive step may provide an indirect measure of the conversion of Rabl from the GDP- to the GTP-bound form.

Consistent with the possibility that GDI serves as a recycling factor for Rabl in ER to Golgi transport, we found that a major pool of Rabl in rat liver cytosol fractionates as a GDI-Rabl complex. Similar complexes have been detected between GDI and Rab3, Rab5, and Rab9 (Regazzi et al., 1992; Garrett et al., 1993; Soldati et al., 1993; Ullrich et al. 1993). The GDI-RablGG complex generated in vitro was both necessary and sufficient to restore transport activity to Rabl-depleted cytosol. The amount of complex required to restore transport was ~3–5-fold over the endogenous Rabl required to support an equivalent level of transport. The requirement for an excess of recombinant complex could reflect several factors. One possibility is that the complex generated in vitro is not fully active. In fact, recent observations suggest an important role for phosphorylation in regulating the function of GDI (Steele-Mortimer et al., 1993; Zahner and Cheney, 1993). Alternatively, the endogenous Rabl pool may be associated with a distinct isoform of GDI, one which is more efficient in the recycling of Rabl. Two highly related isoforms of Rab-GDI, $\alpha$ and $\beta$, have been detected in rat. These are 99 and 86%, respectively, identical to bovine Rab-GDI (Sasaki et al., 1990; Ueda et al., 1991; Nishimura et al., 1994). The bovine Rab-GDI ($\alpha$-isoform) used in the present studies not only has the ability to interact with a wide range of Rab proteins (Ullrich et al., 1993), but also has a broad tissue distribution. The $\alpha$-isoform in rat is more abundant in brain, whereas the $\beta$-isoform has a more ubiquitous distribution (Nonaka et al., 1991). Consistent with the role of Rab-GDI in Rabl function in the present studies, ~50% of the Rabl found in rat liver cytosol has been found to be complexed to the $\alpha$-isoform. The remainder is associated with the $\beta$-isoform (Goud, B., personal communication).

In addition to GDI-Rabl complex(es), which comprise ~80–90% of the total Rabl pool present in rat liver cytosol (Peter et al., 1993), ~10–15% of cytosolic Rabl resides in a larger protein complex (S-value of 18–19 S) (Peter et al., 1993). This complex is required for vesicle budding from the ER (Peter et al., 1993). Components of this complex include $\beta$-COP (Peter et al., 1993), a protein abundant in nonclathrin coats found on ER to Golgi and intra-Golgi vesicular carriers (Duden et al., 1991; Serafini et al., 1991; Pepperkok et al., 1993; Pind et al., 1994) and it contains a mammalian homologue of yeast Sec23p, a GAP specific for the small GTPase Sarl, which is essential for vesicle budding from the ER in both yeast and mammalian cells (Hicke and Schekman, 1989; Orci et al., 1991; Hicke et al., 1992; Kuge et al., 1993*a*; Salama et al., 1993). Rabl-depleted cytosol supplemented with the recombinant GDI-RablGG complex still required $\beta$-COP to promote transport. These results suggest that the high molecular mass complex may be assembled from precursors such as GDI-Rabl, coatomer (Waters et al., 1991; Kuge et al., 1993*b*; Orci et al., 1993; Hara-Kuge et al., 1994), and components involved in Sarl function (Hicke and Schekman, 1989; Nakano and Muramatsu, 1989; Hicke et al., 1992; Kuge et al., 1993; Salama et al., 1993; Oka and Nakano, 1994). Given this possibility, it will be important to determine whether Rabl and other proteins are delivered to membranes directly via GDI as suggested in the case of Rab5 and Rab9 (Soldati et al., 1994; Ullrich et al., 1994) or in the context of additional cytosolic factors.

These studies confirm a growing body of evidence indicating that Rabl may be required for vesicle budding from the ER, at least in mammalian cells. We have previously demonstrated that both monoclonal and polyclonal antibodies specific for Rabl inhibit export from the ER in vitro (Plutner et al., 1991; Schwaninger et al., 1992). In support of this observation, Rabl is an abundant protein on VTCs (Pind et al., 1994) and a transdominant mutant predicted to restrict Rabl to the GDP-bound form inhibits vesicle formation in vivo and in vitro (Nuoffer et al., 1994). Similar results were obtained with the equivalent Sarl mutant (Kuge et al., 1993*a*). Since Sarl is required for vesicle budding in yeast and mammalian cells (Salama et al., 1993; Kuge et al., 1993*a*), these results suggest an important role for both Rabl and Sarl specific exchange factors (GEPs) in our assay (Kuge et al., 1993; Nuoffer et al., 1994). In contrast to the apparent role of Rabl in vesicle budding, Yptl, the yeast Rabl homologue (Haubruck et al., 1989), is not required for vesicle formation

using an assay which reconstitutes ER to Golgi transport from yeast membranes as functional ER to Golgi carrier vesicles can be readily generated in its absence (Salama et al., 1993). However, Yptlp is essential for a late targeting and/or fusion step (Bacon et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990; Becker et al., 1991; Segev, 1991), a result compatible with its presence on isolated carrier vesicles (Lian and Ferro-Novick, 1993). The latter results are consistent with the effect on transport of a transdominant mutant of Rabl which is defective for guanine nucleotide binding and likely to mimic a constitutively activated form (Pind et al., 1994). In the presence of this mutant, VSV-G is exported from the ER but accumulates in prominent VTCs which fail to fuse with the Golgi stack (Pind et al., 1994). Thus, the requirement for Rabl in mammalian cells can be divided into two steps, a recruitment step, which appears to be coupled to vesicle formation, and a later step in which Rabl is likely to regulate the assembly/disassembly of a protein complex involved in vesicle targeting/fusion (Nuoffer et al., 1994; Pind et al., 1994).

There are several possibilities to explain the apparent discrepancies between Rabl and Yptlp function in vesicle budding. First, it is conceivable that a deficiency in Rabl function could indirectly affect vesicle budding by interfering with the recycling of other components critical for this step in mammalian cells. For example, depletion of Gdilp (Garret et al., 1994) or yeast mutants defective for Yptlp function accumulate a relatively small pool of transport vesicles (compared to the late-acting sec4 mutants) and elaborate an extensive ER reticulum (for review see Pryer et al., 1992). This is similar to defects in the SEC18 gene product (NSF), a protein which is now recognized to be involved in vesicle fusion (for review see Rothman and Orci, 1992) (Kaiser and Schekman, 1990; Söllner et al., 1993). Thus, defects in vivo at a late targeting/fusion step can lead to depletion of components required for vesicle budding. A limitation to this interpretation is the clear dependence of vesicle formation in vitro on the presence of the GDI-Rabl complex, similar to the requirements for Sarl (Salama et al., 1993; Kuge et al., 1993; Balch, W. E., unpublished observations). A second potential explanation is that VSV-G, a transmembrane protein used to follow ER to Golgi transport in mammalian cells and prepro-$\alpha$-factor, a soluble protein used to study transport in yeast, differ mechanistically (at least in part) in their sorting and recruitment into carrier vesicles. A third possibility is that mammalian cells and yeast may differ in the order and/or stringency with which transport components are recruited during vesicle formation. For example, Yptlp may associate with the transport machinery at a post-budding step, whereas Rabl is recruited in the context of a large protein complex functional in budding as suggested previously (Peter et al., 1993). Further experiments are in progress to explore these and other possibilities.

### *Role of the GDI-Rabl Complex in Intercisternal Transport through the Golgi Stack*

In addition to establishing a requirement for Rabl and GDI in the export of VSV-G from the ER, we explored the role of both proteins in intra-Golgi transport. GDI was found to be a potent inhibitor of transport not only between the *cis*/medial compartments as reported previously (Elazar et al., 1994), but also for transport of VSV-G between the medial and *trans* compartments. Thus, Rab proteins are likely to play a critical role in transport at all levels of the Golgi stack. Among the known Rab proteins, a possible candidate for function in transport between late Golgi compartments is Rab6 (Antony et al., 1992; Tixier-Vidal et al., 1993) given its localization to the medial/*trans*-Golgi compartments (Jones et al., 1993).

Golgi membranes prepared by density gradient centrifugation were found to be very sensitive to inhibition by GDI. The effects of GDI are consistent with our molecular analysis of Rabl function in transport between the *cis*- and medial-Golgi compartments (Davidson and Balch, 1993), as well as the ability of a polyclonal antibody specific for Rabl (Plutner et al., 1991) to inhibit transport between enriched Golgi membranes (Peter, F., and W. E. Balch, unpublished observations). While the kinetic properties of inhibition of GDI were consistent with an essential role of Rab proteins in vesicle budding from the donor cisternae, this point remains difficult to establish due to the inhibitory effect of GDI on the acceptor Golgi fraction and the absence of recognizable Golgi stacks after prolonged exposure of Golgi membranes to GDI in vitro. However, the latter correlates with our recent observations that Rabl is critical for the morphological integrity of the Golgi complex in vivo (Nuoffer et al., 1994; Wilson et al., 1994). Microinjection of cells with various transdominant Rabl mutants triggers the complete disassembly of the Golgi stack into 60-nm carrier vesicles and 100–300-nm Golgi remnants (Nuoffer et al., 1994; Wilson et al., 1994). Thus, extraction of Rab proteins from the acceptor membrane may indirectly render the compartment incompetent for fusion.

An understanding of the role of Rabl in the vesicle budding/fusion cycle in intra-Golgi as well as ER to Golgi transport will clearly require the identification of other upstream/downstream effectors either regulating or being regulated by Rabl, an approach we are currently pursuing.

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