Removal of Rab GTP-binding Proteins from Golgi Membranes by GDP Dissociation Inhibitor Inhibits Inter-cisternal Transport in the Golgi Stacks*

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Rab proteins are a family of Ras-like GTPases involved in intracellular membrane traffic. Rab GDI, a cytosolic protein which inhibits the dissociation of GDP from various Rab proteins, is required to maintain a pool of Rab proteins in the cytosol. We describe the purification of a cytosolic factor from bovine liver that inhibits intra-cisternal transport between the Golgi stacks. We identify this factor as a Rab GDI. Half-maximal inhibition of transport was observed in the presence of the same concentration of GDI that is required for removal of Rab proteins from the Golgi.

Intracellular transport of proteins through the secretory pathway of eukaryotic cells (1, 2) involve multiple small GTP-binding proteins including members of the Rab, SAR, and ADP ribosylation factor subfamilies of Ras-related proteins. ADP ribosylation factor is required for coatmer binding to Golgi membranes (3, 4), and for coated vesicle formation and vesicular transport (6, 7). SAR is required for budding of transport vesicles from ER (7–11). The Rab family includes more than 30 related members, which have been postulated to serve as key regulators of vesicular transport (for reviews, see Refs. 1, 2, and 12). Although the exact role of Rab proteins is unclear, they are needed for vesicle attachment (13). In mammalian cells, recent studies have additionally indicated that Rab1b is involved in endoplasmic reticulum to Golgi and cis- to medial Golgi transport (14), Rab3a in regulated exocytosis (15), Rab4 in early sorting events along the endocytic pathway (16), Rab5 in early endosome fusion (17), and Rab9 in transport between late endosomes and the trans-Golgi network (18). Cell-free systems that are dependent on Rab proteins have been, and will continue to be, helpful in elucidating the exact mechanism of action of these proteins. Here we describe such a system, discovered serendipitously.

Ras-like GTP-binding proteins are found in at least two distinct conformations; one exhibits high affinity for GTP and the other high affinity for GDP (19). The activity of these small GTP-binding proteins is controlled by at least three classes of proteins: GTPase-activating protein (GAP), GDP dissociation stimulating protein (GDS), and GDP dissociation inhibitory protein (GDI). There is a family of GDI proteins, each specific for one or a few closely related small, GTP-binding proteins. GAP proteins specific for Rab (20) and Ypt1p (21) have been partially purified, and Ypt6p cDNA have been recently cloned (22). GDS proteins that act on Ras (23) and Rab3a (24) have also been isolated.

A GDI that acts on Rab3a has been recently isolated and its gene cloned (25, 26). The Rab3a GDI interacts with the dimethylated lysozyme C terminus of Rab3a (27). This GDI has two mechanistically related activities in a cell-free system; one is to inhibit the GDP/GTP exchange on Rab3a, and the other is to regulate the reversible binding of Rab3a to membranes by removing the GDP-bound form of the Rab protein from membranes. Rab3a GDI appears to interact not only with Rab3a but also with the yeast Sec4p (28), Rab11 (29), Rab9 (30), and various other members of the Rab subfamily (31, 32), which led to the proposal to rename it Rab GDI.

We have used the well characterized cell-free system that reconstitutes transport of the membrane glycoprotein (G protein) of vesicular stomatitis virus (VSV) from the cis compartment of the Golgi to a medial compartment (33) to isolate an inhibitory factor from bovine liver cytosol. Amino acid sequence analysis, as well as the use of specific antibodies against GDI, indicates that this inhibitory factor is identical to the Rab GDI. We show here that the inhibitory effect of Rab GDI on Golgi vesicular transport correlates with the removal of two different Rab proteins from Golgi membranes.

EXPERIMENTAL PROCEDURES

Materials

ATP, UTP, palmitoyl-cytochrome A, and DTT were from Boehringer Mannheim. Creatine phosphokinase, creatine phosphate, nucleotide monophosphate kinase, and protease inhibitors were obtained from Sigma. CM-Sepharose fast flow and Mono S HR 5/5 were from Pharmacia LKB Biotechnology Inc. Donor and acceptor membrane fractions were prepared from VSV-infected CHO 15B and wild type CHO cells, respectively, as described previously (31). Membranes were stored at −80 °C in 1 M sucrose at a protein concentration of 0.6–0.7 mg/ml. Polyclonal antibodies against Rab GDI were a generous gift from Dr. Marino Zerial (EMBL, Heidelberg, Germany).

Cis-to-Medial-Golgi Transport Assay

The standard assay mixture (50 μl) contained 0.5 μCi of UDP-[3H]acetylglucosamine (DuPont NEN), 1 μl of a 1:1 mixture of donor and acceptor Golgi membranes as described (33). Before assaying for inhibition of transport, all column fractions were dialyzed against 25 mM Tris-HCl, pH 7.4, 50 mM KCl, and 1 mM DTT. For the experiment described in Fig. 4, pure GDI (Mono S pool) (20 μg) was incubated with 10 μg of Rab proteins purified according to Kikuchi et al. (34) in a final volume of 80 μl for 10 min at 37 °C, followed by detergent extraction (batchwise) with 100 μg of SM2 Bio-Beads (Bio-Rad). The mixture was then tested for its effect on transport.

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1 The abbreviations used are: GAP, GTPase-activating protein; BFA, brefeldin A; GDI, GDP dissociation inhibitory protein; GDS, GDP dissociation stimulating protein; VSV, vesicular stomatitis virus; DTT, dithiothreitol; CHO, Chinese hamster ovary; GTP-γ-S, guanosine 5′-O-(thiotriphosphate).
Rub-GDI Inhibits Protein Transport

FIG. 1. Chromatography on CM-Sepharose of a factor inhibiting intra-Golgi transport. Ammonium sulfate precipitate (40–90%) obtained from bovine liver cytosol was loaded on a CM-Sepharose column equilibrated with 5 mM potassium phosphate, pH 7.8 and 10 mM β-mercaptoethanol. Proteins were eluted stepwise from the column with 25 mM potassium phosphate, pH 7.8, 10 mM β-mercaptoethanol, and 50 mM KCl (first arrow), and 150 mM KCl (second arrow). Samples (5 µl) were analyzed for transport inhibitory activity (●) and protein concentration (A_{280} nm). Purification of Transport Inhibitory Factor Preparation of Bovine Liver Cytosol—Bovine liver was obtained immediately after slaughter, cut into small pieces, and washed with ice-cold 25 mM Tris-HCl, pH 7.4, and 540 mM sucrose. A 30% (w/v) homogenate was prepared in 25 mM Tris-HCl, pH 7.4, 500 mM KCl, 250 mM sucrose, 2 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.5 µM 1,10-phenanthroline, 2 µM pepstatin A, 2 µg/ml aprotinin, and 0.5 µg/ml leupeptin with a glass Waring blender. The homogenate was centrifuged at 7,500 g for 1 h at 4 °C. The supernatant was decanted, pooled, and centrifuged at 120,000 g for 30 min at 4 °C in a Beckman GS34 rotor (Beckman Instruments, Inc., Palo Alto, CA) rotor for 1 h at 4 °C. The supernatant was decanted and dialyzed against 25 mM Tris-HCl, pH 7.4, 50 mM KCl, and 1 mM DTT. The dialyzed material was collected and clarified by centrifugation at 7,500 g for 1 h at 4 °C. The supernatant was decanted and dialyzed against 25 mM Tris-HCl, pH 7.4, 50 mM KCl, and 150 mM KCl. The column was washed with the equilibration buffer. The fraction containing the inhibitory activity was eluted at 7,500 x g in a Sorvall GS34 rotor for 1 h at 4 °C. This material, termed bovine liver cytosol, had a protein concentration of 40 mg/ml and was stored at –80 °C.

Ammonium Sulfate Precipitation—The frozen cytosol (250 ml, 10,000 mg of protein) was thawed rapidly, and ammonium sulfate was added slowly while stirring to a final concentration of 40% (w/w) at 4 °C. The solution was stirred for 1 h at 4 °C and then centrifuged in a Sorvall GS3 rotor at 7,500 x g for 1 h. The supernatant was decanted and saturated to 90% ammonium sulfate (w/w). The solution was stirred at 4 °C for 1 h and then centrifuged for 30 min at 7,500 x g. The precipitated protein was resuspended in 120 ml of 5 mM potassium phosphate, pH 7.8, and 10 mM β-mercaptoethanol, and dialyzed against this buffer. Insoluble material was removed by centrifugation at 120,000 x g in a Beckman 45Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) rotor for 1 h at 4 °C. This material, termed ammonium sulfate precipitate, had a protein concentration of 40 mg/ml and was stored at –80 °C.

CM-Sepharose Chromatography—The dialyzed solution was loaded on a 2 ml/min on a Mono S HR 5/5 and eluted with a linear gradient of 0–0.5 M KC1 in the dialysis buffer. The fraction containing the inhibitory activity was eluted at about 150 mM KC1 and contained a single 51-kDa protein as determined by electrophoresis and Coomassie staining (Fig. 2).

Amino Acid Sequencing of the Pure Inhibitory Protein Tryptic digestion, peptide separation, and peptide sequencing were performed as described previously (35).

Preparation of Monoclonal Antibodies against Rab1b and Rab6 Monoclonal antibodies were raised against peptides derived from the hypervariable region of rat Rab1b (amino acids 179–208) (36) or human Rab6 (amino acids 176–206) (37). The peptides were coupled to Super carrier (Pierce Chemical Co.), emulsified with Freund’s complete adju vant, and injected intraperitoneally into mice. Hybridomas were cloned by limited dilution and were screened by enzyme-linked immunosorbent chromatography with the peptides and the native Rab proteins.

Western Blot Analysis Proteins were fractionated on SDS-PAGE and electrophoretted on to nitrocellulose. Rab proteins were detected using monoclonal antibodies M1E7 to detect Rab1b and M5B10 to detect Rab6 using peroxidase-conjugated anti-mouse IgG (diluted 1:2000). GDI was detected using polyclonal antibodies raised against recombinant GDI and peroxidase-conjugated anti-Rabbit IgG (diluted 1:2000). Peroxidase labeling was detected by chemiluminescence using the ECL reagent (Amersham Corp.). Rab signals were quantitated by imaging with a Scan Jet Plus scanner (Hewlett Packard) and integration of images using the &a& analysis software from BioSoft (Cambridge, United Kingdom).

RESULTS Purification of Factor That Inhibits Transport of Proteins between Golgi Cisternae—Cytosolic factors required to reconstitute protein transport between Golgi cisternae have been recently characterized (6, 38); however, little is known about factors that may inhibit this process. We have used the transport assay to examine different cytotoxic fractions for such an inhibitory activity. Cytosolic proteins precipitated by 40–90% ammonium sulfate were separated by chromatography on a CM-Sepharose cation exchanger, and fractions were tested for transport inhibitory activity (Fig. 1). A sharp single peak of inhibitory activity was eluted with 50 mM KCl. We further purified this inhibitory activity by gel filtration on Ultrogel AAc54 and FPLC Mono S cation exchange chromatography.

FIG. 2. Protein profiles of fractions through the purification of transport inhibitory factor from bovine cytosol. Proteins were separated by SDS-PAGE (10% gel), and visualized with Coomassie Blue (lanes 1–5) or by immunoblotting with anti-GDI specific antibodies (lane 6). Lanes contained 20 µg of cytosol, 20 µg of ammonium sulfate precipitate, 7 µg of CM-Sepharose pool, 4 µg of Ultrogel AAc54 pool, and 2 µg of Mono S pool (both lanes 5 and 6). Molecular weight markers (Bio-Rad) are as follows: phosphorylase b, 97,400; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500.
Rab-GDI Inhibits Protein Transport

**Table 1**

| Fraction                        | Protein | Total activity* | Specific activity | Purification | Yield |
|--------------------------------|---------|-----------------|-------------------|--------------|-------|
| Differential centrifugation     | mg      | units           | units/mg          | -fold       | %     |
| Ammonium sulfate precipitation  | 7,500   | 12.6 x 10^6     | 1.87 x 10^4       | (1)         | (100) |
| CM-Sepharose                    | 1,200   | 3.0 x 10^6      | 2.5 x 10^3        | 1.3          | 22    |
| Ultrogel AA 54                  | 6       | 3.8 x 10^6      | 0.6 x 10^4        | 330          | 26    |
| Mono S FPLC                    | 2.5     | 2.5 x 10^6      | 1.0 x 10^4        | 530          | 18    |
|                                | 0.9     | 1.4 x 10^6      | 1.6 x 10^4        | 857          | 10    |

* One unit is defined as the amount of factor giving 50% inhibition of [3H]GlcNAc incorporation in a standard transport assay. Specific activities were estimated from the slope (cpm/mg protein) in the linear region of the curve.

Protein profiles of the pools from each purification step are shown in Fig. 2, and quantification of the purification is shown in Table 1. A typical purification results in about 1 mg of a single 51-kDa protein with an ~500-fold increase in specific activity and a 10% yield. The 51-kDa protein is a monomer based on its elution from gel filtration (not shown).

The Purified Inhibitory Protein Is Identical to Rab3 GDI—To identify the 51-kDa protein, we separated the pure fraction on SDS-PAGE and blotted it onto nitrocellulose. The band was then excised from the blot and digested with trypsin. Peptides were purified by high pressure liquid chromatography and microsequenced. The amino acid sequences of three peptides obtained from the pure protein were compared to proteins in the NBRF-PIR release 34 data base. All three peptides are found (with 98% identity) in Rab GDI from bovine brain (Fig. 3). Of the 48 amino acids sequenced, only one difference was found: the threonine at position 124 of GDI was sequenced as an alanine. To confirm the identity the purified 51-kDa protein was probed with antibodies directed against Rab3a GDI. Fig. 2 shows that our purified protein is recognized by the anti-GDI antibodies. These results imply that the inhibitory factor isolated is Rab GDI free of any associated proteins.

As shown in Fig. 4A (open circles), addition of increasing amounts of the purified GDI to the transport assay is progressively inhibitory. Half-maximal inhibition of transport was observed in the presence of ~0.8 µg of GDI. Complete inhibition was observed at 2–5 µg of GDI. Virtually the same inhibition curve was observed when GDI was added in the presence of brefeldin A (Fig. 4A, closed circles), a component that un couples fusion from budding in the cell-free Golgi transport assay (6, 39). Preincubation of GDI with a mixture of Rab proteins purified from bovine brain membranes (34) overcame the inhibitory effect of GDI (Fig. 4A, triangles). Adding the Rab protein fraction to the transport reaction without GDI had no effect (not shown).

The obvious mechanism for the inhibition of transport by Rab GDI would be the extraction of any Rab proteins required for transport from the membranes (as a group). We therefore tested this possibility by Western blotting using monoclonal antibodies to Rab1b and Rab6. As shown in Fig. 4B, addition of increasing concentrations of GDI under conditions of the transport assay resulted in the removal of most of the Rab1b and Rab6 from the Golgi membranes. This effect of GDI on Golgi-bound Rab protein correlates well with its effect on transport (Fig. 4A), with 50% removal of Rab1b and Rab6 accruing at approximately 1.15 and 0.85 µg of GDI, respectively, compared with half-inhibition of transport at 0.8 µg of GDI.

**DISCUSSION**

Here we report that addition of purified Rab GDI to an in vitro Golgi protein transport assay inhibits transport. Since GDI is known to specifically interact with Rab proteins, we propose that the inhibition of transport is due to removal of Rab proteins from the Golgi membrane. We confirm that at least two Rab proteins known to localized to Golgi, Rab1b and Rab6 (14, 40), are removed from the membranes during inhibition.

The assay signal in the presence of BFA is due to direct (uncoupled) fusion between Golgi cisternae (6, 39). The inhibition of this signal by GDI implies that Rab proteins are involved in the membrane fusion steps of transport in the Golgi stack. Whether Rab proteins are also needed for COP-coated vesicle budding cannot be determined from the present data. The fact that uncoupled fusion is resistant to GTPyS (6) would mean either that the relevant Rab protein(s) acts in fusion in its GTP-bound form or that an adequate endogenous supply of the GTP-bound form (existing exchange with added GTPyS) is available for fusion in the cell-free system.

The identification of the inhibitory activity that we purified from bovine liver cytosol as the Rab3 GDI protein, originally discovered by Takai and co-workers (25, 26) was based on amino acid sequence analysis, Western blotting, and coincidence in molecular weight. We found only one amino acid difference between the published sequence of Rab3 GDI and the sequence of the pure inhibitory factor from bovine liver are shown (single-letter code) above the amino acid sequence of Rab3a GDI from bovine brain. The sequence data for Rab GDI were taken from Mateu et al. (26).

**FIG. 3. Comparison of amino acid sequences of the peptides of the pure inhibitory factor with those of Rab3a GDI.** Peptide sequences of the pure inhibitory factor from bovine liver are shown above the amino acid sequence of Rab3a GDI from bovine brain. The sequence data for Rab GDI were taken from Mateu et al. (26).
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It should be noted that maximal inhibition of transport as well as maximal removal of Rab proteins from membranes is observed when GDI is added in excess of endogenous Rab proteins. Most of the Rab proteins in cytosol are in a complex with GDI (32), so that excess free GDI is needed to extract Rab-GDP from membranes by mass action, preventing Rab proteins from binding to membranes and participating in transport.

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FIG. 4. A, inhibition of intra-Golgi transport by purified GDI. Purified GDI (Mono S pool) was added to the cis-to medial Golgi transport assay in the absence (○) or the presence (●) of 150 μg brefeldin A. To prevent the inhibition of transport, GDI was pretreated with purified Rab proteins (●) (see "Experimental Procedures"). Incorporation of [3H]GlcNAC into VSV-G protein was determined after 1 h incubation at 37 °C. B, purified Rab GDI removes Rab proteins from Golgi membranes. Purified Rab GDI was added to the in vitro transport assay as described above and the mixture was incubated for 30 min at 37 °C. The amount of Rab1B (●) and Rab6 (○) remaining on the Golgi membranes was determined by Western blotting (inset) using specific anti-Rab antibodies.