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Rab-GDI Presents Functional Rab9 to the Intracellular Transport Machinery and Contributes Selectivity to Rab9 Membrane Recruitment*

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Rab proteins occur in the cytosol bound to Rab-GDP dissociation inhibitor (GDI). We demonstrate here that cytosolic complexes of Rab9 bound to GDI represent a functional pool of Rab9 protein that can be utilized for transport from late endosomes to the trans Golgi network *in vitro*. Immunodepletion of GDI and Rab proteins bound to GDI led to the loss of cytosol activity; readdition of pure Rab9-GDI complexes fully restored cytosol activity. Delipidated serum albumin could solubilize prenylated Rab9 protein, but unlike Rab9-GDI complexes, Rab9-serum albumin complexes led to indiscriminate membrane association of Rab9 protein. Rab9 delivered to membranes by serum albumin was functional, but GDI increased the efficiency of Rab9 utilization, presumably because it suppressed Rab9 protein mistargeting. Finally, GDI inhibited transport of proteins from late endosomes to the trans Golgi network, likely because of its capacity to inhibit the membrane recruitment of cytosolic Rab9. These experiments show that GDI contributes to the selectivity of Rab9 membrane recruitment and presents functional Rab9 to the endosome-trans Golgi network transport machinery.

Rab GTPases are key regulators of vesicular transport (1–6). A variety of genetic and biochemical experiments suggest that this family of Ras-like GTPases functions in the processes by which membrane-bound transport vesicles identify and/or fuse with their targets. Over 30 different Rab proteins have been identified to date, and most organelles of the secretory and endocytic pathways bear distinct sets of Rab GTPases on their surfaces. The unique localizations of Rab proteins require specific structural determinants (7–9), as well as covalent attachment of polyisoprenoid geranyl-geranyl moieties to carboxyl-terminal cysteine residues (10).

Rab proteins catalyze vesicle targeting in their GTP-bound conformations, after which the bound GTP is hydrolyzed (11). After vesicle fusion, prenylated Rab proteins in their GDP conformations are thought to be retrieved from membranes by an abundant cytosolic protein termed Rab-GDI,[1] or GDP dissociation inhibitor (referred to here as GDI). Takai and co-workers (12, 13) were the first to identify and purify this class of proteins as factors that inhibit the release of GDP, but not GTP, from Rab proteins. GDI interacts preferentially with prenylated Rab proteins in their GDP-bound conformations and can displace a variety of Rab proteins from their membrane targets *in vitro* (13–18). After membrane retrieval, GDI has been proposed to have the capacity to deliver Rab proteins to their membranes of origin, where they become incorporated into the transport machinery in their GTP-bound conformations.

We study the transport of mannose 6-phosphate receptors from late endosomes to the trans Golgi network. We have shown that Rab9 protein stimulates this transport process both in living cells (19) and in an *in vitro* system (20) that reconstitutes this transport event (21). Rab9 protein, purified from an overexpressing *Escherichia coli* strain, can be prenylated *in vitro* and assembles into a complex with cytosolic GDI (15). In addition, GDI can deliver prenylated Rab9 to late endosome membranes by a process that is coupled with GDI displacement and nucleotide exchange (Ref. 22; see also Ref. 23).

If GDI functions as a true Rab-recycling factor, it should deliver prenylated Rab proteins to their corresponding organelles in a functional form. In this report, we show that GDI delivers a functional Rab9 protein that can drive the *in vitro* transport of mannose 6-phosphate receptors from late endosomes to the trans Golgi network. In addition, we show that GDI contributes selectivity to the process by which Rab9 is delivered to late endosome membranes. These experiments provide new insight into the role of GDI and demonstrate that cytosolic pools of Rab proteins bound to GDI represent functional recycling pools of intracellular transport factors.

MATERIALS AND METHODS

GDI was purified from bovine brain according to Sasaki *et al.* (13). Crude cytosol and wild type Golgi fractions were prepared as described (21, 24). Protein was measured according to Bradford (25) using bovine serum albumin as standard.

Preparation of Prenyl-Rab9-GDI and Prenyl-Rab9-BSA Complexes—Prenyl-Rab9-GDI complexes were reconstituted with 95% efficiency by dialyzing purified, prenylated Rab9 with an equimolar amount of purified bovine brain GDI, followed by Sephacryl S100 gel filtration chromatography as described elsewhere.[2] Fractions containing Rab9-GDI complexes were pooled, and aliquots were quickly frozen and stored at −80 °C with 1.0 mg/ml BSA as carrier. Prenylated Rab9 was purified to >90% homogeneity in a three-step purification protocol[2] starting from membranes of Baculovirus-infected insect cells solubilized in 2% CHAPS, followed by chromatography in 1% CHAPS on Sephacryl S100 and Mono Q.

Rab9-BSA complexes were assembled by dialyzing pure prenylated Rab9 in the presence of a 10-fold molar excess of delipidated BSA (Sigma). After ultracentrifugation to remove some residual, non-BSA-complexed (and likely aggregated) Rab9, the approximate mass of the resulting complex was determined by gel filtration chromatography to be ~100 kDa. Quantitation of Coomassie Blue-stained gels indicated

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[1] The abbreviations used are: GDI, GDP dissociation inhibitor; BSA, bovine serum albumin; CHO, Chinese hamster ovary; TGN, trans Golgi network; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

[2] Soldati, T., Shapiro, A. D., and Pfeffer, S. R. (1994) *Methods Enzymol.*, in press.

that Rab9-BSA complexes were comprised of 2–3 mol of prenyl-Rab9/mol of BSA. Aliquots were quick-frozen in nitrogen and stored at −80 °C.

Preparation of Anti-GDI IgG-Affi-Gel 10—IgGs were purified from immune or preimmune rabbit sera on protein A-Superose by fast protein liquid chromatography. Serum samples were diluted into 100 mM Tris, pH 8, 100 mM NaCl for binding to the column. The column was washed subsequently with 100 mM Tris, pH 8, followed by 10 mM Tris, pH 8. IgGs were eluted in 0.8-ml fractions with 100 mM glycine, pH 3, directly into 80 μl of 1 M Tris, pH 8. Purified antibodies were concentrated by a 50% ammonium sulfate precipitation and coupled to Affi-Gel 10 (Bio-Rad) overnight at 4 °C (according to the manufacturer) after dialysis into 0.1 M MOPS, pH 7.5. Anti-GDI IgG resins were prepared at 25 mg of IgG/ml of resin. Resins were recycled with 0.1 M glycine, pH 2.4, followed by washing once with 1 M Tris, pH 8 and twice with cytosol buffer (25 mM Tris, pH 7.5, 50 mM KCl). Resins were stored in cytosol buffer with 0.02% sodium azide.

Depletion of GDI-Rab Complexes from CHO Cytosol—GDI-Rab complexes were immunodepleted from CHO cytosol using anti-GDI IgG-Affi-Gel 10. Alternatively, mock depletion was carried out with preimmune IgG-Affi-Gel. Briefly, 1 ml of cytosol (7 mg/ml) was incubated with 200 μl of anti-GDI antibody resin for three serial 3-h incubations at 4 °C. Immunodepletion was followed by a 20-min incubation with protein A-agarose to remove any free IgG. Depletion levels were measured by quantitative Western blotting. Detection of GDI was carried out using affinity-purified antibodies raised against purified bovine brain GDI as described (15). Secondary antibodies were goat anti-rabbit IgG conjugated to horseradish peroxidase; antigen-antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham Corp.). GDI immunodepletion was monitored by densitometric scanning of autoradiograms. Due to a lack of cross-reactivity of CHO Rab9 with our monoclonal anti-Rab9 antibody (15), the level of Rab9 co-depletion with GDI was measured in HeLa cell cytosol and in cytosol obtained from CHO cells, which overexpress canine Rab9 ~50-fold (15) following the identical anti-GDI immunodepletion protocol.

Determination of Transport from Late Endosomes to Trans Golgi Network in Vitro—Transport was carried out according to Goda and Pfeffer (21) with minor modifications. [^{35}S]Methionine- and cysteine-labeled cells were swollen in hypotonic buffer (10 mM Hepes-KOH pH 7.2, 15 mM KCl) and then scraped into a new homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, pH 7.4, 1 mM EDTA), which yields a transport reaction that is more reproducibly cytosol-dependent. Transport reactions were carried out for 2 h at 37 °C. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, and quantitation was performed using a PhosphorImager (Molecular Dynamics).

Membrane Recruitment of Rab9 Protein in Vitro—Recruitment of prenylated Rab9 onto late endosomes or red blood cell ghosts was detected after pelleting of the membranes and quantitative immunoblotting as described (22). Nucleotide exchange was also measured in parallel (22). Prenylated Rab9 in the form of either a GDI or BSA complex was incubated for 40 min at 37 °C with an endosome-enriched membrane fraction or lysed red blood cell ghosts in the presence of [^{35}S]GTPγS. Binding of nucleotide was monitored by a rapid filtration assay. To obtain values for the membrane-triggered [^{35}S]GTPγS binding to Rab9 protein, background values obtained from experiments with the membranes alone and the Rab9-GDI complex alone were subtracted.

RESULTS

Rab9 occurs in the cytosol as a complex with GDI (15). To determine if this complex provided functional Rab9 protein to the transport machinery, we tested whether depletion of GDI and Rab9-GDI complexes from CHO cytosol reduced the ability of CHO cytosol to support endosome-to-TGN transport *in vitro* (21). Polyclonal anti-bovine GDI IgGs were coupled to Affi-Gel, and cytosol was incubated with this matrix to deplete both GDI and Rab proteins bound to GDI. Immunoblot analyses of the resulting cytosol confirmed that immunodepletion led to removal of up to 90% of the GDI present in CHO cytosol. Moreover, in parallel experiments, Rab9 was co-immunodepleted with the same efficiency as GDI (data not shown).

As shown in Fig. 1, when compared with complete or mock-depleted CHO cytosol, depletion of 85% of cytosolic GDI led to the loss of ~85% of the cytosol's activity. Readdition of purified bovine GDI alone had no effect; however, addition of purified, prenylated Rab9-GDI complexes fully restored the activity of

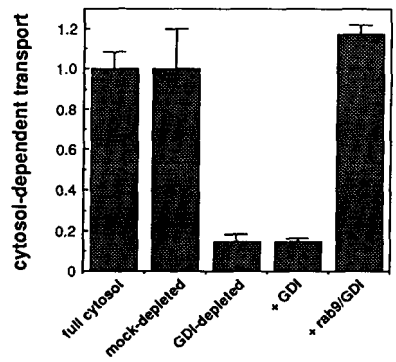

FIG. 1. **GDI presents functional Rab9 to the transport machinery.** Endosome-TGN transport was performed with CHO cytosol, mock depleted cytosol, or GDI-depleted cytosol at 0.9 mg/ml. Where indicated, GDI was added to a final concentration of 0.5 μg/ml either alone or complexed with prenylated Rab9, in the presence of GDI-depleted cytosol. Transport in the absence of cytosol was subtracted to yield cytosol-dependent transport. Cytosol stimulated transport ~3.5-fold. Values represent the mean ± S.E. of several experiments, each carried out at least in duplicate.

GDI-depleted cytosol. This experiment demonstrated that GDI presents functional Rab9 protein to the endosome-to-TGN transport machinery.

GDI is believed to maintain prenylated Rab proteins in a soluble form, as they recycle between their fusion membrane targets and their organelles of origin. Although purified, prenylated Rab proteins are insoluble in the absence of detergent, we found that they could be solubilized when dialyzed out of detergent in the presence of delipidated BSA. Under these conditions, Rab9 occurred as a ~100-kDa species that likely represents 1 mol of BSA bound to roughly 2 mol of prenyl-Rab9.

We have shown previously that GDI delivers Rab9 protein with high efficiency to late endosome membranes, with strong preference for these membranes compared with endoplasmic reticulum membranes or lysed red blood cell ghosts (22). If GDI functions only as a solubilizing factor, BSA should be capable of delivering prenylated Rab9 to membranes with a similar specificity for late endosomes. As we have reported previously, Rab9-GDI complexes were delivered with high efficiency to late endosome membranes; significantly less Rab9 protein became associated with lysed red blood cell ghosts (Fig. 2*A*, *left columns*). In addition, membrane association correlated with an almost equimolar exchange of bound GDP for GTP (Fig. 2*B*, *left columns*). However, if Rab9 was presented to membranes in the form of a complex with BSA, it was delivered to red blood cell ghosts almost as well as to late endosome membranes (Fig. 2*A*, *right columns*). In the presence of either membrane, bound GDP was exchanged for GTP (Fig. 2*B*, *right columns*), due to the spontaneous rate of nucleotide exchange onto free or BSA-bound Rab9 protein under the experimental conditions employed. These experiments confirm the ability of GDI to inhibit GDP release from Rab9 protein. More importantly, they demonstrate that tight binding of GDI to prenylated Rab proteins decreases promiscuous interaction of otherwise hydrophobic Rab proteins with inappropriate membrane targets.

Rab9 was functional if presented to membranes by BSA, despite a higher level of mistargeting (Fig. 3). However, GDI complexes were significantly more potent in terms of their ability to stimulate transport at all concentrations tested. These findings are consistent with a model in which tight binding of prenyl Rab9 to GDI favors selective interaction with proteins specifically localized to the surface of late endosomes. As a weaker BSA complex, Rab9 likely partitions into a variety of other membranes, decreasing its available active concentration on late endosomes.

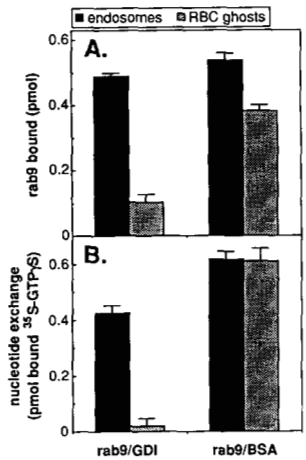

FIG. 2. **GDI contributes to the selectivity of Rab9 recruitment.** Membrane recruitment and nucleotide exchange were determined using an endosome-enriched membrane fraction (*dark bars*) or lysed red blood cell ghosts (*light bars*) as potential membrane targets for Rab9 recruitment. Prenylated Rab9 protein was presented to the membranes in the form of a complex with GDI or BSA. Values represent the mean (± S.E.) of several experiments, each carried out at least in duplicate.

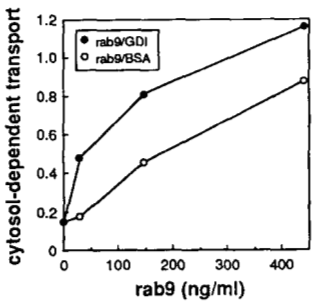

FIG. 3. **GDI increases the efficiency of Rab9 utilization for endosome-TGN transport.** Endosome-TGN transport was measured in the presence of GDI-depleted cytosol (0.9 mg/ml); reactions were supplemented with either Rab9-GDI (●) or Rab9-BSA complexes (○) at the indicated concentrations. Cytosol-dependent transport was obtained by subtracting transport measured in the absence of cytosol. Transport was stimulated approximately 3.5-fold by untreated cytosol. Values shown represent the mean of duplicate determinations derived from one or two experiments; standard error was less than 10% for all values shown.

CHO cytosol contains ~100 ng/mg GDI and ~8 ng/mg Rab9. In our reconstitution experiments, addition of 5 ng of Rab9, as a complex with GDI, led to significant stimulation of transport in reactions depleted of Rab9-GDI complexes. Addition of ~50 ng yielded the same amount of transport as full cytosol. This indicates that the preparation of purified, reconstituted, prenyl-Rab9-GDI complexes was ~20–30% active, a value that matches well with nucleotide binding estimations carried out in parallel on prenylated Rab9 protein prepared in this manner.

We have recently shown that unoccupied GDI inhibits the GDI-mediated delivery of Rab9 onto late endosome membranes (22). These observations predict that GDI should inhibit transport in reactions that require recruitment of prenylated Rab9 from cytosolic GDI complexes. As shown in Fig. 4*A*, unoccupied GDI inhibited transport carried out in the presence of normally saturating concentrations of CHO cytosol. These results support the conclusion that cytosolic Rab proteins are first recruited onto membranes by a GDI-inhibitable process; after nucleotide exchange, the Rab protein would be incorporated into nascent transport vesicles. This matches the GDI-mediated inhibition of intra-Golgi transport reported by Rothman and colleagues (26).

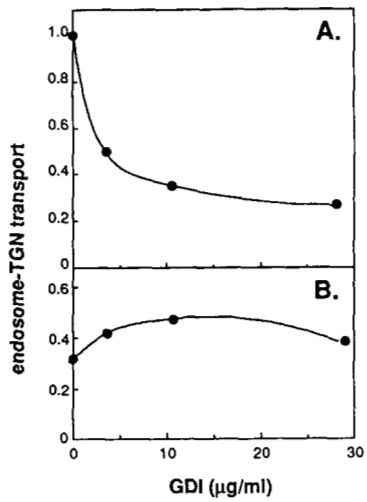

FIG. 4. **Effect of GDI on endosome-TGN transport.** Reactions were performed in the presence (*A*) or absence (*B*) of CHO cytosol (1 mg/ml). Results shown represent total transport rather than cytosol-dependent transport; values shown are the mean of duplicates from two separate experiments.

When transport reactions were carried out in the absence of exogenous cytosol, the same levels of GDI yielded a slight stimulation of endosome-to-TGN transport (Fig. 4*B*). Under these conditions, the added GDI may retrieve some Rab9-GDP from the endogenous membranes and make it available for additional transport events.

DISCUSSION

We have shown that removal of GDI and Rab proteins bound to GDI, by an immunodepletion protocol, eliminates the ability of cytosol to stimulate the transport of mannose 6-phosphate receptors from late endosomes to the trans Golgi network *in vitro*. The loss of cytosol activity was not due to the depletion of GDI, because readdition of GDI alone was not sufficient to restore cytosol activity. In contrast, addition of purified Rab9-GDI complexes fully restored activity to the Rab protein-GDI complex-depleted cytosol. This demonstrates that cytosolic Rab9, bound to GDI, is an essential cytosolic factor for endosome-TGN transport *in vitro*. It also appears that Rab9 is the only Rab protein required for this transport step, since readdition of Rab9, in its GDI- or BSA-bound form, was sufficient to restore maximal *in vitro* transport.

Rab9 protein can stimulate endosome-to-TGN transport when added exogenously in an unprenylated form, when added together with the prenylation precursor, geranylgeranyl phosphate (20), or in its prenylated form, complexed with purified bovine brain GDI (22). Nevertheless, those experiments did not address the functional significance of the cytosolic pool of GDI-bound Rab proteins. The experiments presented here show that GDI contributes to the selectivity of Rab protein membrane recruitment. Perhaps by binding tightly to geranylgeranyl groups, GDI suppresses nonspecific membrane association of hydrophobic Rab proteins and thereby enhances the recognition of Rab protein structural determinants by the organelle-specific Rab recruitment machinery.

Garrett *et al.* (17) have recently shown that GDI is an essential yeast gene product. In addition, cells depleted of GDI display multiple defects in protein transport and a loss in the soluble pool of Sec4p, a secretory granule-localized, Rab protein homolog (17). These findings support a model in which GDI functions to recycle Rab proteins from membranes back into the soluble pool. Accordingly, a block in Rab protein recycling would be expected to interfere with multiple steps in membrane trafficking.

Our biochemical experiments complement those of Novick and co-workers and confirm that GDI functions to recycle Rab proteins in a functional form and to facilitate their accurate delivery to the appropriate membrane-bound compartment.

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