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Rab GDI: A Solubilizing and Recycling Factor for rab9 Protein

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Rab proteins are thought to function in the processes by which transport vesicles identify and/or fuse with their respective target membranes. The bulk of these proteins are membrane associated, but a measurable fraction can be found in the cytosol. The cytosolic forms of rab3A, rab11, and Sec4 occur as equimolar complexes with a class of proteins termed "GDIs," or "GDP dissociation inhibitors." We show here that the cytosolic form of rab9, a protein required for transport between late endosomes and the *trans* Golgi network, also occurs as a complex with a GDI-like protein, with an apparent mass of ~80 kD. Complex formation could be reconstituted in vitro using recombinant rab9 protein, cytosol, ATP, and geranylgeranyl diphosphate, and was shown to require an intact rab9 carboxy terminus, as well as rab9 geranylgeranylation. Monoprenylation was sufficient for complex formation because a mutant rab9 protein bearing the carboxy terminal sequence, CLLL, was prenylated in vitro by geranylgeranyl transferase I and was efficiently incorporated into 80-kD complexes. Purified, prenylated rab9 could also assemble into 80-kD complexes by addition of purified, rab3A GDI. Finally, rab3A-GDI had the capacity to solubilize $rab9_{GDP}$, but not $rab9_{GTP}$, from cytoplasmic membranes. These findings support the proposal that GDI proteins serve to recycle rab proteins from their target membranes after completion of a rab protein-mediated, catalytic cycle. Thus GDI proteins have the potential to regulate the availability of specific intracellular transport factors.

INTRODUCTION

Rab proteins represent a family of ras-like GTPases that play an essential role in the transport of proteins between membrane-bound compartments (see Balch, 1990; Bourne *et al.*, 1991; Goud and McCaffrey, 1991; Hall, 1992; Pfeffer, 1992 for review). The first rab family member to be identified, Sec4p, was identified as a yeast gene, which when mutated, led to the accumulation of secretory vesicles at the nonpermissive temperature (Salminen and Novick, 1987; Goud *et al.*, 1988). Since that time, as many as 30 rab proteins have been identified, each localized to the surface of distinct membrane bound compartments. Rab proteins are thought to function in vesicle targeting and/or fusion events, because antibodies directed against rab1 (or its yeast homolog, *YPT1*) inhibit endoplasmic reticulum-derived transport vesicle fusion (Plutner *et al.*, 1991; Rexach and Schekman, 1991; Segev, 1991), anti-rab5 antibodies block early endosome fusion (Gorvel *et al.*, 1991), and as described above, *SEC4* mutant yeast strains accumulate secretory vesicles (Novick *et al.*, 1980). It is widely believed that rab proteins cycle between specific target membranes and the surfaces of newly forming transport vesicles, to facilitate a new round of vesicle transport.

Rab proteins acquire one or two, 20-carbon long, geranylgeranyl (GG) moieties, which are attached in thioether linkage to cysteine residues located at or near rab protein carboxy-termini (reviewed in Magee and Newman, 1992). The presence of such prenyl groups is essential for both rab localization and function. Yet the hydrophobic nature of prenylated rab proteins strongly suggests that they cannot dissociate spontaneously from membranes. Thus recycling of rab proteins from target membranes is likely to require additional factors.

Proteins termed "GDIs", for GDP-dissociation inhibitors, are excellent candidates for rab recycling factors. Takai and coworkers were the first to identify and purify this class of proteins as factors that inhibited the release of GDP, but not GTP, from rab3A (also termed smg p25A; Matsui *et al.*, 1990; Sasaki *et al.*, 1990) and rab11 (also termed 24K G; Ueda *et al.*, 1991). Rab3A-GDI can interact with rab3A, rab11, and Sec4p, how-

ever, rab11-GDI appears not to interact with rab3A (Sasaki *et al.*, 1991; Ueda *et al.*, 1991). Recent results suggest that the majority of small GTP binding proteins in the cytosol appear to be complexed with a GDI-like protein (Regazzi *et al.*, 1992).

Araki *et al.* (1990) have shown that rab3A-GDI can dissociate prenylated rab3A from synaptic plasma membranes and vesicles. Similarly, Regazzi *et al.* (1992) showed that rab3A-GDI can dissociate a variety of unidentified, small GTP binding proteins from cellular membranes. In both cases, dissociation was only observed for rab proteins bearing bound GDP. These characteristics make GDI proteins well suited to retrieve prenylated rab proteins from target membranes after they have completed a functional catalytic cycle.

We have recently shown that rab9 plays a key role in the transport of proteins between late endosomes and the *trans* Golgi network (Lombardi *et al.*, 1993). Purified, recombinant, rab9 protein stimulated transport in a cell free system that reconstitutes this event (Goda and Pfeffer, 1988). Using gel filtration chromatography, we show here that like other small GTP binding proteins, cytosolic rab9 occurs as an 80-kD complex with a GDI-like protein. Purified, recombinant rab9 protein could also be assembled into 80-kD complexes in vitro, as long as the protein possessed an intact carboxy-terminus and had been incubated under conditions which favor prenylation. Moreover, prenylated rab9 could be shown to form an equimolar complex with authentic rab3A-GDI. Finally, rab3A-GDI had the capacity to dissociate the GDP form of prenylated rab9 protein from cellular membranes. These findings support a role for GDI proteins in the solubilization and recycling of rabs from their target membranes, at the end of a rab protein-mediated catalytic cycle.

MATERIALS AND METHODS

Crude cytosol was prepared as described (Goda and Pfeffer, 1991) from Chinese hamster ovary (CHO) cell lines stably overexpressing rab9 at 10- (clone 3) or 50- (clone 2) fold higher levels than untransfected cells. A complete description of these cell lines will be presented elsewhere (Riederer and Pfeffer, unpublished data). Identical results were obtained in untransfected cells, but the analyses were much more difficult due to the low levels of rab9 protein present. Rab9 protein was purified from an *Escherichia coli*-overproducing strain as described (Shapiro *et al.*, 1993). To prepare ^{35}S-methionine labeled rab9, a cloned rab9 cDNA (Lombardi *et al.*, 1993) was transcribed in vitro by using T7 RNA polymerase (Pharmacia, Piscataway, NJ); rab9-encoding RNA was used as a template for in vitro translation in a reticulocyte lysate (Promega, Madison, WI). The mutant protein, rab9-CLLL was engineered with the use of polymerase chain reaction (PCR) with pGEM-rab9 (Lombardi *et al.*, 1993) as template. The 5′ sense oligonucleotide was the standard T7 primer, which anneals to the T7 RNA polymerase site of the vector. The 39 base long, 3′-antisense primer, 5′GGCAGCTGTCATGACAGCAGGCAAGATGAGCTAGGCTTG3′, which anneals to the 3′-most rab9 cDNA coding sequence, was designed to replace the -CC carboxyterminus with a -CLLL amino acid sequence, to conserve the TGA stop codon, and to introduce a 3′ *Pvu*II restriction site. This 0.6 kb PCR product was then digested with *Nco* I (which cleaves at the AUG start codon) and *Pvu*II, and ligated in frame into a pET8c vector, precleaved with *Nco* I and *Bam*HI (blunted). Recombinants were screened by restriction digestion and desired mutants identified by double stranded DNA sequencing. Rab9-CLLL was then purified from an *E. coli*-overproducing strain essentially as described for wild-type rab9 (Shapiro *et al.*, 1993). Identity of the protein was confirmed by anti-rab9 immunoblotting and functional GTP binding capacity was checked by α-^{32}P-GTP overlay.

Rat liver endosomes were purified by sucrose gradient flotation and were enriched in the rat liver Golgi membrane fractions obtained as described (Goda and Pfeffer, 1991). Protein was determined with the use of a BioRad (Richmond, CA) kit with bovine serum albumin as standard.

Gel Filtration Chromatography and Fraction Analysis

Samples were analyzed on a 50 ml Sephacryl S100 (Pharmacia) column equilibrated and eluted in S100 buffer (64 mM tris(hydroxymethyl)aminomethane [Tris]/HCl pH 8; 100 mM NaCl; 8 mM $MgCl_2$; 2 mM EDTA; 0.2 mM dithiothreitol [DTT]; 10 μM GDP and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Forty 0.4-ml fractions were collected; alternate fractions were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and conventional immunoblotting (Burnette, 1981). Rab9 protein was detected using rabbit or mouse antibodies raised against native, recombinant rab9 protein; antibodies were used in the form of an IgG fraction of rabbit anti-rab9 antiserum or an ascites fluid containing mouse anti-rab9 IgG. Detection of GDI was carried out using affinity purified antibodies raised against purified rab3A-GDI (see below). Secondary antibodies were either goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (BioRad). All antibodies were used at 1:1000 dilution; antigen-antibody complexes were detected by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL). α-^{32}P-GTP overlays were carried out as described (Serafini *et al.*, 1991). Quantitation of both ECL and α-^{32}P-GTP overlay signals on X-ray films (Kodak, Rochester, NY) was carried out with the use of a densitometric scanner (Model 300 A, Molecular Dynamics Sunnyvale, CA). In some cases, quantitation was done with the use of a PhosphorImager system (Molecular Dynamics).

In Vitro Prenylation

One microgram of purified rab9 (100 nM) was prenylated in the presence of 5.6 mg/ml of CHO cytosol (Balch *et al.*, 1984) and either 10 μM geranylgeranyl pyrophosphate (GGPP, American Radiolabeled Chemicals, St Louis, MO) or 1 μM GGPP and 0.1 μM ^{3}H-GGPP, by incubation for 1 h at 37°C. The buffer conditions were similar to those used for in vitro endosome-to-TGN transport (Goda and Pfeffer, 1988): 22 mM *N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid (HEPES)/KOH pH 7.2, 20 mM Tris/HCl, 116 mM KCl, 4.3 mM Mg(OAc + Cl_2), 2 mM DTT, 0.2 mM GDP, plus a protease inhibitor cocktail and an ATP regenerating system. The in vitro prenylation reaction was clarified by ultracentrifugation at 100,000 *g* for 10 min in a TLA100.2 rotor (Beckman, Fullerton, CA) and analyzed by gel filtration chromatography.

GDI Purification

Rab3A-GDI was purified from bovine brain precisely as described by Takai and coworkers (Sasaki *et al.*, 1990). In certain experiments, rab3A-GDI was further purified and separated from the cholate used in the purification by Sephacryl S100 chromatography in S100 buffer. Rab3A-GDI was also used as an immunogen to raise antibodies in rabbits; the antibodies were affinity purified by binding to the antigen, immobilized on Affigel 10 matrix (BioRad), and eluted with 0.1 M glycine/HCl pH 2.4 followed by immediate neutralization with 0.1 volume of 1 M KPO_4 pH 7.2.

Rab9-GDI Complex Reconstitution

In vitro prenylation was carried out by using a mixture of purified rab9 (1 μg) and ^{35}S-rab9 (50 μl of a standard in vitro translation reaction). Reaction mixtures were then chromatographed as described above using Sephacryl S100; fractions containing prenylated rab9 were pooled (2.8 ml), diluted to 50 mM Tris/HCl pH 8, 40 mM NaCl, (total volume, 7 ml), and the prenylated rab9-GDI complexes were disrupted by addition of cholate to 1%. Prenylated rab9 was then purified away from bulk cytosolic proteins (and GDI, as detected by immunoblotting with anti-rab3A-GDI serum) by binding to Q-Sepharose (Pharmacia, 200 μl) in 50 mM Tris/HCl pH 8, 40 mM NaCl, 1% cholate, followed by salt elution in the same buffer plus 500 mM NaCl. Fractions enriched in prenylated rab9 were incubated in the presence or absence of 8 μg of purified rab3A-GDI, and complex formation was allowed to take place by reducing the cholate concentration by dialysis for 12 h at 4°C against S100 buffer, with or without 0.2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS). Before S100 chromatography, any sedimentable material was removed by ultracentrifugation at 100,000 *g* in a TLA 100.2 rotor. The pellet and resulting fractions were analyzed by anti-rab9 immunoblotting and autoradiography to obtain precise quantitation of the different rab9 forms.

Immunoprecipitation of rab9 Complexes Under Native Conditions

Crude cytosol was prepared from clone 2 CHO cells, which were metabolically labeled with ^{35}S-methionine as described (Goda and Pfeffer, 1991), using the same buffer as for in vitro prenylation (see above). Immunoprecipitations of the cytosol (250 μg) were carried out directly in this buffer using either preimmune IgG (1 μg), affinity purified anti-rab9 antibodies (1 μg), or affinity purified anti-rab3A-GDI antibodies (0.1 μg). After a 3-h incubation at 4°C, Protein A agarose (Sigma, 5 μl) was added for 1 h, and immune complexes were collected by pelleting and washing six times in S100 buffer + 0.1% of gelatin. One-half of each immunoprecipitated material was analyzed by SDS-PAGE and immunoblotting with anti-rab9 or anti-GDI antibodies. For experiments analyzing rab9-CLLL, recombinant protein was prenylated in vitro as described above, and the sample was then subjected to S100 gel filtration chromatography. Fractions eluting in the region of ~80 kD were then immunoprecipitated as described above.

Effect of GDI on Membrane-Associated rab9

Rab9-GDP was dissociated from membranes essentially as described (Araki *et al.*, 1990; Sasaki *et al.*, 1991; Regazzi *et al.*, 1992). Briefly, membranes were purified from one confluent, 10-cm dish of clone 2 CHO cells by collecting the 2.0/0.5 M interphase from the recommended sucrose step gradient. Membranes (150 μg) were incubated in the same buffer as for in vitro prenylation for 2 h at 30°C in the presence of either GTPγS or GDP (1 mM). This incubation was followed by addition of 6 μg of purified rab3A-GDI for 5 min at 30°C. Samples were then separated into membrane and soluble fractions by centrifugation through a sucrose step gradient and analyzed by SDS-PAGE and immunoblotting with the monoclonal anti-rab9 IgG.

RESULTS

Cytosolic rab9 Occurs as an 80-kD Complex

Rab proteins are found primarily in association with membranes, however, a measurable fraction can be detected in the cytosol. Others have shown that the cytosolic forms of the majority of small GTP binding proteins occur as an ~80-kD complex, as detected by sucrose gradient sedimentation (Regazzi *et al.*, 1992). This complex is believed to represent rab proteins bound to GDI. CHO cells contain on the order of 10 ng rab9/mg protein, ~80–90% of which is membrane-bound (Lombardi *et al.*, 1993). Given the low abundance of rab9, we used stably transfected CHO cell lines that overexpress rab9 ~10-fold, to study the cytosolic form of rab9 protein.

As shown in Figure 1, Sephacryl S100 gel filtration chromatography revealed that cytosolic rab9 was found in a complex of ~80 kD (B). In addition, the 80-kD complex cochromatographed with the bulk of cytosolic small GTP binding proteins of ~25–30 kD (Figure 1C), as detected by ^{32}P-GTP overlay of the fractions of CHO cytosol. A small amount of the 25–30 kD GTP binding proteins chromatographed in the void volume of the S100 column (Figure 1C, fractions 1–4); only a few small GTP binding proteins of ~20 kD chromatographed as monomers (Figure 1C, fractions 15–17) and probably represent members of the ADP ribosylation factor family of proteins (Regazzi *et al.*, 1992). Thus like other rab

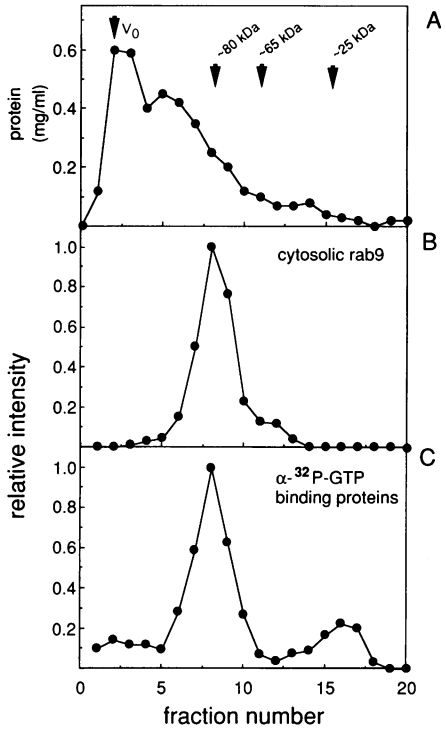

Figure 1. Rab9 co-chromatographs with the majority of small GTP binding proteins present in cytosol. A crude cytosol fraction (2.8 mg) from CHO clone 3 cells overexpressing rab9 was subjected to Sephacryl S100 gel filtration chromatography. (A) Total protein distribution. V_0 indicates the void volume as determined by the elution of blue dextran (MW ~2000 kD); hemoglobin was used as a ~65-kD size marker, and rab9ΔC was used as the 25-kD marker (see Figure 2). (B) Distribution of rab9 as determined by immunoblotting. In some experiments, a minor fraction of rab9 eluted in the void volume. (C) Distribution of total, small cytosolic GTP binding proteins detected by ^{32}P-GTP overlay.

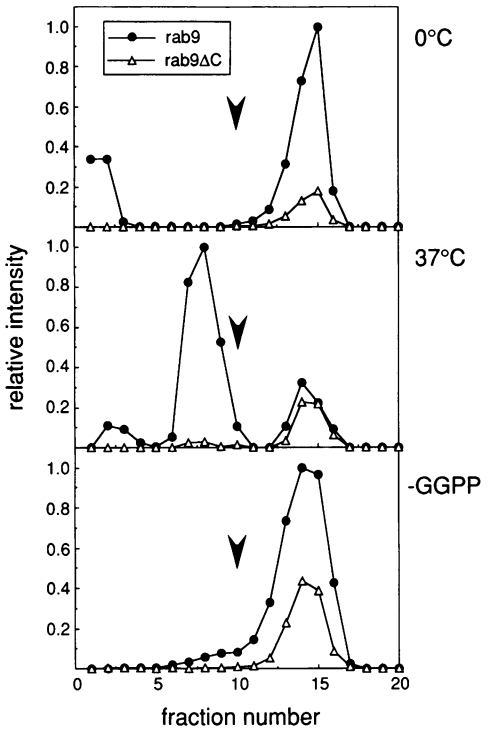

Figure 2. Reconstitution of rab9 cytosolic complexes in vitro. Recombinant rab9 (1 μg) was incubated in the presence of CHO cytosol, ATP, an ATP regenerating system, in the presence or absence of GGPP. Samples were then analyzed by S100 gel filtration chromatography followed by immunoblotting to detect rab9. The signals were quantified by laser scanning densitometry. The closed circles represent the distribution of rab9; open triangles represent rab9ΔC. The arrowheads denote the position of the hemoglobin marker (~65 kD).

proteins, cytosolic rab9 is likely to occur in association with a GDI-like protein.

Reconstitution of Cytoplasmic Complexes In Vitro

The availability of recombinant rab9 protein (Lombardi *et al.*, 1993; Shapiro *et al.*, 1993) enabled us to initiate an analysis of the requirements for assembly of rab9 into complexes with GDI-like proteins. Purified rab9 was incubated under a variety of assembly conditions and then analyzed by gel filtration chromatography. The potential assembly of rab9 into complexes with cytosolic proteins was then monitored by immunoblot analysis of column fractions.

When recombinant rab9 was incubated for 1 h on ice in the presence of cytosol, an ATP regenerating system, and GGPP, the bulk of the rab9 protein chromatographed as a monomeric species, at ~25 kD (Figure 2, top). In contrast, when the mixture was warmed to 37°C for the same time interval, the majority of rab9 shifted its chromatographic properties and eluted at ~80 kD (Figure 2, middle). However, simple omission of the prenylation precursor, GGPP, resulted in only very low levels of complex formation (Figure 2, bottom). These results implied that the pool of endogenous GGPP in our desalted CHO cytosol preparation was too low to yield significant rab9 prenylation, as judged by the ability of rab9 to form a complex with one or more cytosolic components. Finally, a carboxy-terminally truncated rab9 protein (rab9ΔC) produced during isolation of the wild-type protein (Lombardi *et al.*, 1993) chromatographed at ~25 kD under all conditions. These results strongly suggested that recombinant rab9 was prenylated in vitro. In addition, this modification appeared to be required for assembly into an 80-kD complex that was indistinguishable from that containing cellular, cytosolic rab9 protein (Figure 1).

A small, but reproducible amount of rab9 was found to chromatograph in the void volume of the S100 column (cf. Figure 2, middle, fractions 2 and 3). Moreover, after incubation at 37°C in the absence of an ATP regenerating system, the bulk of rab9 was found in a sedimentable aggregate, as has been reported previously for rab5 by others (Kurzchalia *et al.*, 1992). The significance and composition of these larger complexes is unclear at present and will require further analysis.

Only Prenylated rab9 Is Assembled into Complexes

Direct examination of the electrophoretic patterns of chromatographically resolved rab9 complexes provided additional information regarding the modification state of rab9 present in the complexes. Figure 3 shows the electrophoretic mobilities of rab9 protein before and after prenylation. Rab9 produced in *E. coli* (Figure 3, lane 1) and in a reticulocyte lysate (not shown), displayed the same apparent molecular weights upon SDS-PAGE, and electrophoresed more slowly than rab9 obtained from tissue sources (Figure 3, lane 3: rat liver endosomes) or cultured cells (not shown). The mobility of rab9 produced in *E. coli* represents unprenylated rab9 protein. After a 1 h, 37°C incubation with cytosolic proteins and GGPP, the electrophoretic mobility of a large fraction of recombinant rab9 shifted to that of the rab9 present in rat liver endosome membranes (Figure

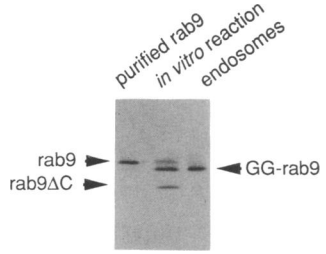

Figure 3. In vitro prenylated rab9 coelectrophoreses with rab9 found in vivo. Recombinant rab9 purified from *E. coli* cells (10 ng), the product of the in vitro prenylation and complex formation reaction (1/40 of the total sample), and 100 μg of rat liver endosomes were analyzed for rab9 protein by 12% SDS-PAGE and immunoblotting.

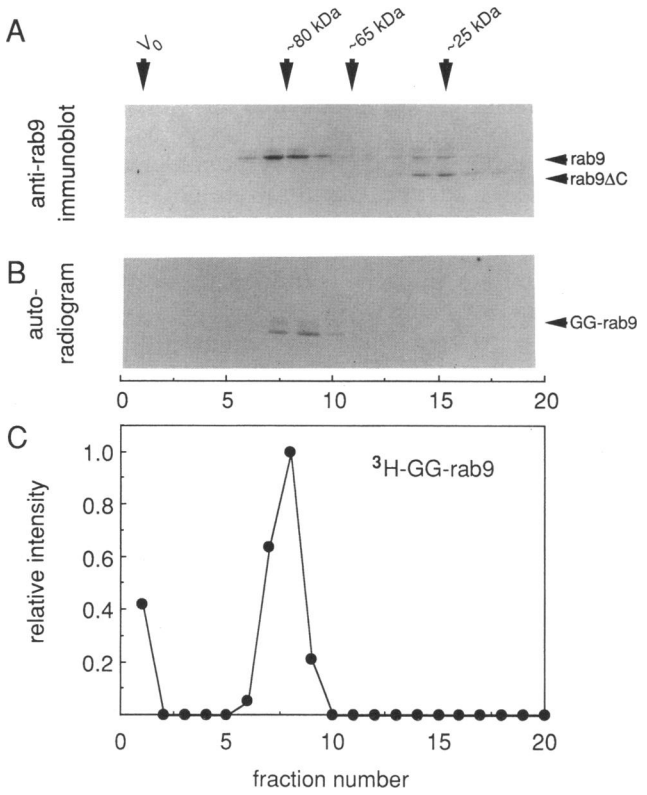

Figure 4. In vitro prenylated rab9 is recruited quantitatively into an 80-kD complex. An in vitro prenylation reaction carried out in the presence of 1 μCi ^{3}H-GGPP was analyzed by gel filtration chromatography, SDS-PAGE, and anti-rab9 immunoblotting (A), and autoradiography (B). Quantitative analysis of the chromatographic profile shown in (B) is presented in panel (C). Markers were the same as in Figure 1.

3, lane 2). We have shown elsewhere that the intermediate-mobility species obtained after in vitro prenylation incorporates ^{3}H-GG when reactions are carried out in the presence of ^{3}H-GGPP (Lombardi *et al.*, 1993). The fastest migrating species obtained after in vitro incubation represented rab9ΔC, which was present at low levels in the purified rab9 preparation (Figure 3, lane 1), and increased in abundance after incubation at 37°C.

Figure 4A presents the immunoblot patterns of S100 column fractions obtained after complex formation with purified rab9. As shown in Figure 2, all of the rab9ΔC chromatographed at ~25 kD. Fractions in the 80-kD region contained two rab9 polypeptide species: one which coelectrophoresed with membrane-associated, cellular rab9, and another that migrated slightly more slowly. When the prenylation reaction was carried out in the presence of ^{3}H-GGPP, autoradiography of the immunoblot membrane revealed that ^{3}H-prenylated rab9 cochromatographed precisely and exclusively with rab9 in the 80-kD complex fractions (Figure 4B). ^{3}H was incorporated into both of the rab9 species detected in the 80-kD fractions, suggesting that the forms may represent mono- and diprenylated rab9. The appearance of the ^{3}H-rab9 was strictly dependent upon the addition of purified rab9 to the reaction and therefore did not represent additional prenylation of endogenous rab proteins present in the cytosol fraction.

Analogous results were obtained when purified rab7 protein was incubated under these conditions; the protein was prenylated and all of the prenylated form chromatographed at ~80 kD. Together these results confirm that rab9 is geranylgeranylated in vitro. Prenylation appeared to be an absolute prerequisite for complex formation, because both an intact carboxy terminus and supplemental GGPP were required to detect complex formation in vitro. In addition, all prenylated rab9 was found in the form of a cytoplasmic complex.

Monoprenylation Is Sufficient for Complex Formation

To investigate further the importance of prenylation in rab9 complex formation, we used site-directed mutagenesis to generate a rab9 mutant protein bearing at its carboxy terminus the sequence, CLLL, rather than CC. This form of rab protein is predicted to be a substrate for geranylgeranyl transferase I (Moores *et al.*, 1991), which would generate monoprenylated rab9. Rab9-CLLL was expressed in *E. coli*, purified to homogeneity, and tested for its prenylation and ability to form 80-kD complexes upon incubation with cytosol, ATP, and GGPP. As shown in Figure 5, gel filtration analysis of rab9-CLLL-containing, complex formation reactions revealed that a significant fraction of rab9-CLLL migrated at 80 kD (Figure 5A). Like wild-type rab9 protein,

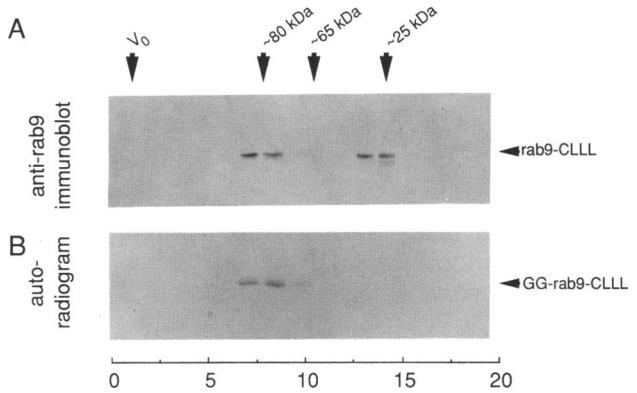

Figure 5. Mono-prenylated rab9 assembles into an 80-kD complex. Purified, recombinant mutant rab9-CLLL (~2 μg) was prenylated in vitro, and subjected to gel filtration chromatography, followed by SDS-PAGE and anti-rab9 immunoblotting (A). (B) Autoradiography of column fractions obtained from a reaction carried out in the presence of 1 μCi ^{3}H-GGPP. Markers were as in Figure 1. It is important to note that this reaction contained excess rab9-CLLL, which is likely to explain the apparent difference in the efficiency of complex formation, relative to reactions containing wild-type rab9 protein.

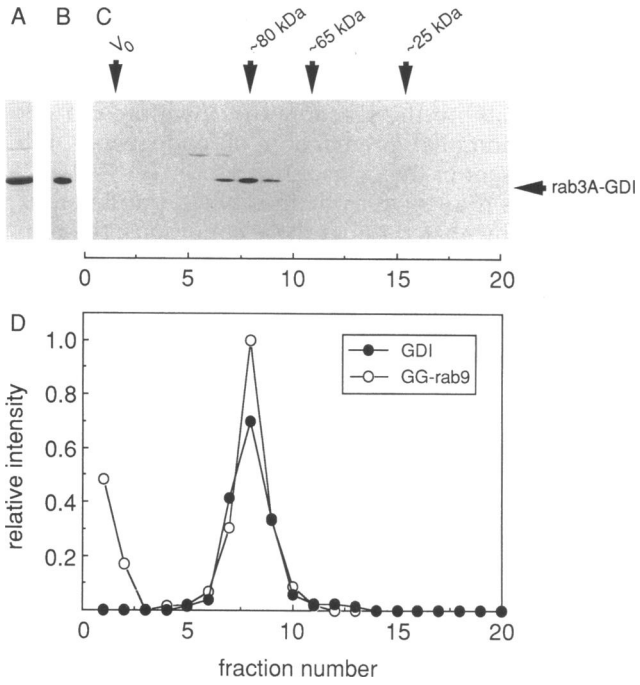

Figure 6. A GDI-like protein cofractionates with the 80-kD rab9 complex. (A) Coomassie blue stained, 6% SDS-PAGE of purified Rab3A-GDI (2.5 μg). (B) Immunoblot of purified GDI (10 ng) with affinity purified rabbit anti-rab3A GDI antibodies. (C) Gel filtration chromatography of cytosol (1 mg) from cells overexpressing rab9 followed by immunoblotting with affinity purified anti-rab3A-GDI antibodies. (D) Quantitative results obtained after reprobing the identical nitrocellulose filter with anti-rab9 antibodies.

rab9-CLLL was prenylated in vitro, as determined by monitoring its incorporation of ^{3}H-GG from ^{3}H-GGPP (Figure 5B). All monoprenylated rab9-CLLL chromatographed at the size expected for the 80-kD complex. These data strongly suggest that monoprenylation is sufficient to permit rab9 assembly with a GDI-like protein. Immunoprecipitation experiments described below confirm this conclusion.

It is important to note that the experiment presented in Figure 5 was carried out using excess rab9-CLLL. Thus we do not believe that monoprenylated rab9 is incorporated significantly less efficiently than wild-type rab9 into 80-kD complexes. Additional experiments will be needed to confirm this point unequivocally.

A GDI-like Protein Cofractionates with the 80-kD rab9 Complex

We next determined the distribution of GDI-like proteins in fractionated cytosol by immunoblotting with affinity purified, anti-rab3A-GDI antibodies. For these experiments, rab3A GDI was purified to ≥90% homogeneity (Figure 6, lane A) and used to raise antibodies in rabbits, which recognized the purified protein by immunoblotting (Figure 6B). As shown in Figure 6C, anti-rab3A-GDI antibodies detected a polypeptide in CHO cytosol that had the same electrophoretic mobility as purified rab3A GDI. This polypeptide cochromatographed precisely with prenylated rab9 in fractions containing the 80-kD complex (Figure 6D), as determined by reprobing the identical immunoblot with anti-rab9 antibodies. Identical results were obtained when the products of in vitro prenylation reactions were analyzed. Anti-rab3A-GDI antibodies failed to react with any polypeptides present in the fractions containing monomeric rab9 (Figure 6C).

In addition to the GDI-like protein electrophoresing at ~55 kD (Figure 6C), the affinity purified anti-rab3A-GDI antibody displayed some reactivity with a 65-kD polypeptide present in the CHO cytosol (Figure 6, A and C). This protein chromatographed ahead of GDI upon gel filtration (Figure 6C). Only the protein of ~55 kD that electrophoresed with the same mobility as rab3A-GDI purified from bovine brain (Figure 6, A and B) cofractionated with rab9 and the 80-kD complex (Figure 6, C and D). It is important to note that under the conditions of these experiments, purified (monomeric) rab3A-GDI chromatographed at ~65 kD (at the position of the hemoglobin marker), a position distinct from the endogenous, cytosolic GDI complexes. Thus a GDI-like protein copurifies with prenylated rab9 upon S100 gel filtration chromatography.

Mono- and Di-Prenylated rab9 Are Associated with a GDI-like Protein

Although rab9 copurified with a GDI-like protein, the above experiment did not establish whether the two proteins were in fact, physically associated. To test this directly, we carried out immunoprecipitation experiments under native conditions to isolate potential rab9-GDI complexes. CHO cytosol from clone 2 cells was incubated with either preimmune serum, affinity purified anti-GDI antibodies, or affinity purified anti-rab9

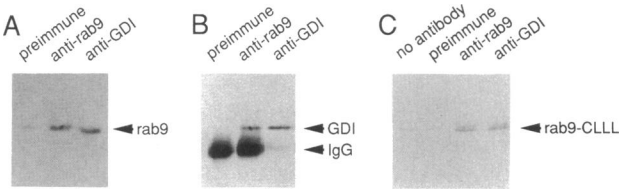

Figure 7. Rab9 and GDI can be coimmunoprecipitated. Immunoprecipitations were carried out under native conditions using cytosolic extracts (250 μg) of rab9 overexpressing cells (CHO clone 2). Precipitations were carried out with preimmune IgG (lane 1), affinity purified anti-rab9 (lane 2), or anti-rab3A-GDI (lane 3) antibodies. The presence of rab9 and GDI in the precipitates was detected using monoclonal anti-rab9 (A) or affinity purified anti-rab3A-GDI antibodies (B). The precipitation of GDI in panel B was ~30% as efficient as that shown in panel A, as determined by analysis of immunoprecipitated, ^{35}S-labeled proteins. (C) Precipitation of 80-kD complexes assembled in vitro with rab9-CLLL.

antibodies, and the immunoprecipitates were then analyzed for the presence of rab9 by immunoblotting. As shown in Figure 7A, rab9 was efficiently precipitated by either anti-rab9 antibodies (lane 2) or anti-GDI-antibodies (lane 3) but failed to be precipitated by preimmune serum (lane 1). Parallel analyses of rab9 immunoprecipitates for the presence of GDI confirmed the association of rab9 and GDI (Figure 7B). Immunoblot analysis revealed that samples immunoprecipitated with anti-rab9 antibodies (lane 2) contained a polypeptide, which comigrated perfectly with GDI immunoprecipitated from the same cytosol fraction (lane 3). Quantitation of this experiment indicated that ~30% of the GDI that was precipitable with anti-GDI antibodies was present in the anti-rab9 immunoprecipitates. Given that the cytosol used in this experiment contained higher than normal levels of rab9 protein, this value is in good agreement with our estimates of the abundance of rab9 and GDI in this cytosol preparation (see below).

As described above, rab9-CLLL assembled into ~80-kD complexes after in vitro prenylation (Figure 5). To confirm that these complexes represented rab9 bound to GDI, we subjected the complexes to immunoprecipitation with antibodies directed against either rab9 protein or rab3A-GDI. As shown in Figure 7C, rab9-CLLL was efficiently precipitated with either affinity purified anti-GDI antibodies or anti-rab9 antibodies. This experiment indicates that monoprenylation is sufficient for the interaction of rab9 with GDI. Although the abundance of monoprenylated rab proteins in living cells is not known at present, these observations provide baseline information regarding the minimum requirements for rab protein-GDI interactions.

It is interesting to note that neither the anti-rab9 antibodies nor the anti-GDI antibodies precipitated their corresponding antigens as efficiently under the native conditions of this experiment as compared with immunoprecipitations carried out in a detergent solution containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS (RIPA buffer). This suggests that complex formation masks specific epitopes that are recognized by the two protein-specific antibodies.

In summary, these experiments demonstrate that rab9 and a GDI-like protein are found as a complex in CHO cytosol. In addition, as shown above, these results substantiate our conclusion that essentially all prenylated rab9 occurs in association with a GDI-like protein.

Prenylated rab9 Assembles with Purified rab3A-GDI

To further confirm the physical association of rab9 with a GDI-like protein, we tested whether prenylated rab9 could interact directly with purified rab3A-GDI. A fraction highly enriched in prenylated rab9 was obtained as follows. A mixture of purified, recombinant rab9 and in vitro translated, ^{35}S-rab9, was prenylated in vitro and subjected to S100 chromatography. Fractions containing the prenylated rab9 (at ~80 kD) were pooled and the complex disrupted by addition of 1% cholate. Although the complex was stable in 1% CHAPS, it was disrupted by 1% cholate, as confirmed by gel filtration chromatography. Prenylated rab9 was then purified away from most of the other proteins present in the fraction (including proteins recognized by the anti-rab3A-GDI antibody) by binding to a Q-Sepharose matrix followed by salt elution.

Rab3A-GDI was then added to prenylated ^{35}S-rab9, and the components were allowed to associate while cholate was removed by dialysis. A mock reaction lacking rab3A-GDI was carried out in parallel. The potential association of detergent-solubilized, prenylated rab9 with purified GDI was then assessed by gel filtration of the dialyzed samples followed by autoradiography (or immunoblotting) to monitor rab9 distribution.

As shown in Figure 8 (○), when prenylated rab9 was incubated alone during dialysis and then gel filtered, most of the protein chromatographed in the void fractions, which indicated that it had formed an aggregate ≥100 kD. Similar aggregates have been detected for rab3A after detergent removal (Araki *et al.*, 1990). In contrast, when dialysis was carried out in the presence of rab3A-GDI (●) rab9 was converted almost quantitatively to the 80-kD form.

In the absence of GDI, a fraction of prenylated rab9 was also found in the form of a sedimentable aggregate (≫100 kD), probably due to self-association. In addition, when CHAPS was omitted from the dialysis buffer during the complex reconstitution, most prenylated rab9 was also found in a sedimentable aggregate. However, such aggregates were not observed if GDI was present under these conditions. Thus prenylated rab9 can be

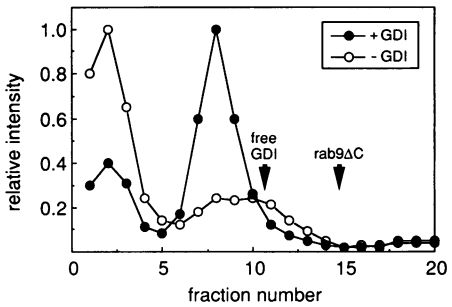

Figure 8. Purified rab3A-GDI forms a complex with prenylated rab9 in vitro. Purified rab3A-GDI and in vitro prenylated, ^{35}S-rab9 were incubated together in S100 buffer containing 1% cholate. The proteins were allowed to reassociate during dialysis to remove cholate (●, +GDI). A mock incubation omitting rab3A-GDI from the dialysis (○, −GDI) was carried out in parallel. Reactions were then analyzed by gel filtration chromatography followed by SDS-PAGE, transfer to nitrocellulose, autoradiography (shown) or anti-rab9 immunoblotting. Both detection methods gave identical results, and the quantitation of the autoradiographic signal using PhosphorImager technology is presented.

converted to an 80-kD form by addition of GDI protein. Together, these data provide independent confirmation of the notion that GDI has the capacity to solubilize prenylated rab9 and that prenylated rab9 and GDI are capable of direct molecular association.

Rab3A-GDI Dissociates rab9-GDP from Membranes

Rab3A-GDI has been shown to dissociate the GDP- but not GTP-bound forms of rab3A (Araki *et al.*, 1990) and other unidentified small GTPases (Regazzi *et al.*, 1992) from membranes. We tested whether purified rab3A-GDI had the capacity to solubilize membrane-associated rab9-GDP. Total cellular membranes were preincubated for 2 h in the presence of an excess of either GTPγS or GDP to permit a quantitative exchange of these nucleotides for already-bound nucleotides. Rab3A-GDI was then added for 5 min, and the reactions were fractionated to obtain membrane and soluble fractions. As shown in Figure 9, SDS-PAGE and immunoblotting of the membrane and soluble fractions revealed that rab9 was efficiently extracted from cellular membranes upon addition of rab3A-GDI. In the presence of GDP, rab3A-GDI led to the transfer of almost 85% of rab9 from the membranes to the soluble fraction. The small amount of rab9 remaining membrane-associated under these conditions may have been due to an incomplete exchange of bound GTP for the added GDP. In contrast, after preincubation with GTPγS, only ~12% of rab9 was found in the supernatant, probably due to a low level of rab9-GDP generated as a consequence of hydrolysis of either bound GTP or GTPγS. Importantly, in the absence of GDI, both GDP- and GTPγS-bound forms of rab9 remained quantitatively membrane-associated. Thus rab3A-GDI can dissociate rab9-GDP, but not rab9-GTPγS, from CHO cell membranes.

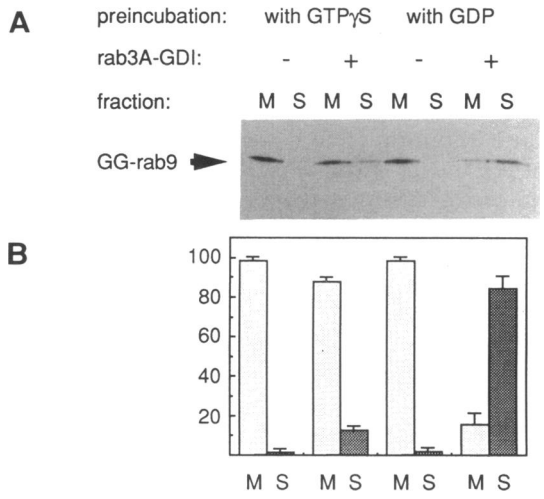

Figure 9. Purified rab3A-GDI dissociates rab9-GDP, but not rab9-GTP, from cellular membranes. A crude membrane fraction (150 μg) from clone 2 cells was preincubated for 2 h at 30°C in the presence of 1 mM GTPγS or GDP. The incubation was continued for 5 min in the presence (+) or absence (−) of purified rab3A-GDI (6 μg). Finally, membrane (M) and soluble fractions (S) were obtained and analyzed by SDS-PAGE and immunoblotting using a monoclonal anti-rab9 antibody. (A) A representative immunoblot analysis of 1/3 of each reaction; (B) quantitation of 3 experiments (±SD).

DISCUSSION

We have shown here that cytosolic rab9 occurs as an 80-kD complex. Two lines of evidence suggest that these cytosolic complexes contain one 25-kD rab9 molecule bound to one 55 kD, GDI-like molecule. First, an anti-GDI immunoreactive protein of 55-kD cochromatographs with the 80-kD complex upon gel filtration. In addition, rab9 and this GDI-like protein can be coimmunoprecipitated using either anti-rab3A-GDI antibodies or anti-rab9 antibodies. Thus, like rab3A, rab9 associates with a GDI-like protein when present in the cytosol.

By using recombinant rab9 protein, we were able to reconstitute the formation of cytosolic complexes that were indistinguishable in their physical properties from those present in CHO cytosol. In vitro complex formation required an intact rab9 carboxy terminus. In addition, prenylation seemed to be required, because complex formation was only observed in reactions supplemented with GGPP. All of the prenylated rab9 was detected in the form of 80-kD complexes, supporting the proposal that GDI has the capacity to efficiently solubilize an otherwise, extremely hydrophobic protein.

The first step in complex formation in vitro appears to be rab9 prenylation, a reaction that is likely to be catalyzed by rab geranylgeranyl transferase II. Prenylation was efficient, in that perhaps as much as 0.5 μg rab9 was prenylated in reactions supplemented with GGPP after 1 h at 37°C. The product of the reaction is likely to represent di-geranylgeranylated rab9, because reactions containing either an excess of rab9 or limiting amounts of GGPP yielded two electrophoretic forms of prenylated rab9. Direct quantitation confirmed the incorporation of >1 mol ^{3}H-GG/mol of rab9. In addition, a rab9 mutant protein bearing the C-terminal sequence, CLLL, was a substrate for monoprenylation by geranylgeranyl transferase I and displayed a single electrophoretic mobility after prenylation in the presence of ^{3}H-GGPP. Monoprenylated rab9 also formed a cytosolic complex with a GDI-like molecule, which demonstrated that monoprenylation is sufficient for stable rab protein-GDI interaction.

The efficient conversion of prenylated rab9 into 80-kD complexes indicates that free GDI is not a limiting component in CHO cytosol. Indeed, as much as 50-fold overexpression of rab9 did not reduce the fraction of cytosolic rab9 protein that was present in an 80-kD complex, nor did it lead to the mislocalization of the protein (Lombardi *et al.*, 1993). Our minimum estimates

suggest that 1 mg of CHO cytosol contains ~100 ng of GDI and ~1 ng rab9. If one assumes that a given cell may contain on the order of 20 rab proteins at levels comparable to rab9, GDI would indeed be present in the cytosol in excess of rab proteins. However, upon 50-fold overexpression of rab9 protein, this might no longer be the case. It is possible that GDI levels increase in parallel under conditions of rab protein overexpression. Alternatively, overexpression of a specific rab protein could alter the balance of all rab proteins between membrane and cytosol fractions, making available additional GDI molecules. Further experiments will be needed to distinguish between these two possibilities.

To date, two rab-specific GDIs have been purified: one from rat liver cytosol ("rab11-GDI"; Ueda *et al.*, 1991) and another from bovine brain ("rab3A-GDI"; Sasaki *et al.*, 1990). The purified proteins differ in terms of the specific rab proteins they are capable of interacting with. Nevertheless, the proteins share identical molecular weights, isoelectric points, and immunoreactivities, and contain highly homologous amino acid sequences (Ueda *et al.*, 1991). It is not yet clear if the distinct characteristics of the two proteins are due to species differences, or instead, to functional differences between members of a closely related family of GDI proteins.

The GDI-like protein that we have shown to be associated with rab9 in CHO cytosol has the same electrophoretic mobility as bovine brain rab3A-GDI. In addition, the protein is recognized by affinity purified, anti-rab3A-GDI antibodies. These data strongly suggest that the protein is indeed, a GDI. However, more work will be needed to determine if the GDI present in CHO cytosol is more closely related in its functional properties to rab3A or rab11 GDI. Whatever its true identity, the GDI present in CHO cytosol shares with rab3A-GDI the ability to interact with prenylated rab9. Both proteins formed equimolar complexes with prenylated rab9, which displayed identical chromatographic properties and stabilities to disruption by detergents such as CHAPS and cholate. Moreover, rab3A-GDI efficiently displaced rab9 from cytoplasmic membranes, when present in its GDP conformation. These results confirm the broad specificity of rab3A-GDI for multiple rab proteins (Ueda *et al.*, 1991; Regazzi *et al.*, 1992) and support the proposal that GDIs are general transport factors, which appear to be capable of solubilizing newly prenylated rab proteins, recycling rab proteins from membrane targets after GTP hydrolysis, and potentially also, of regulating the availability of transport factors under different physiological conditions.

We have recently shown that purified, recombinant rab9 can facilitate the transport of mannose 6-phosphate receptors between late endosomes and the *trans* Golgi network in vitro (Lombardi *et al.*, 1993). Prenylation was essential for rab9 activity in vitro. The results presented here demonstrate that after synthesis and prenylation, rab9 interacts strongly with a GDI-like protein. These data suggest further that rab9 is incorporated into the transport machinery from a rab9-GDI complex. Presumably, it is this complex that is recognized by a nucleotide exchange factor that may be associated with the donor, late endosome membranes. A nucleotide exchange factor (or GDS) may serve to catalyze the specific accumulation of rab9 on the surface of late endosome membranes (Pfeffer, 1992). Our ability to reconstitute rab9-GDI complexes from purified components should enable us to investigate the mechanism by which rab proteins are recruited onto specific cytoplasmic organelles, to initiate their catalytic cycle within the context of vesicle targeting reactions.

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