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Cytosolic RAB3D Is Associated With RAB Escort Protein (REP), Not RAB-GDP Dissociation Inhibitor (GDI), in Dispersed Chief Cells From Guinea Pig Stomach

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Abstract Rab3D, a low-molecular-weight GTP-binding protein believed to be involved with regulated exocytosis, is associated with secretory granules in gastric chief cells. Although Rab3D is predominantly membrane associated, a significant fraction is cytosolic. Rab proteins are geranylgeranylated on their C-terminal cysteine motifs by geranylgeranyltransferase (GGTase). Rab escort protein (REP) is required to present Rab proteins to GGTase and may accompany newly modified Rab proteins to their target membrane. In most tissues, cytosolic Rab proteins are complexed with rab-GDP dissociation inhibitor (rab-GDI). In the present study, we examined the interactions of Rab3D with cytosolic proteins in dispersed chief cells. Two REP isoforms and at least two GDI isoforms are present in chief cell and brain cytosol. When chief cell cytosol was fractionated by gel filtration chromatography, Rab3D eluted with REP at >150 kDa, whereas rab-GDI eluted as a separate 65-kDa peak, suggesting that Rab3D exists as a complex with REP, but not with rab-GDI. In addition, a small fraction of Rab3D eluted as a monomer at 29 kDa. As has been demonstrated previously, in brain cytosol, Rab3 proteins co-elute with rab-GDI at approx. 90 kDa, suggesting that Rab3 proteins undergo active cycling between membrane and cytosolic compartments in this tissue. In vitro experiments revealed that Rab3D remains associated with REP after geranylgeranylation. Our findings suggest that, in gastric chief cells, Rab3D remains associated with REP after geranylgeranylation until it is presented to its target membrane. J. Cell. Biochem. 72:540–548, 1999.

Key words: Rab proteins; geranylgeranylation; membrane targeting; secretion

Low-molecular weight GTP-binding proteins belonging to the rab subfamily of ras-like proteins play an important role in vesicular transport and regulated exocytosis in mammalian cells [Novick and Brenwald, 1993; Pfeffer, 1994]. Each Rab protein is associated with a distinct membrane compartment and involved with a particular transport step. For example, Rab3 proteins (isoforms A, B, C, and D) are localized to secretory vesicles and are believed to be involved in regulated exocytosis [Lledo et al., 1993, 1994; Ferro-Novick and Novick, 1993]. Inhibition of Rab3B expression in pituitary cells using antisense oligonucleotides results in a significant decrease in calcium-dependent exocytosis, indicating that Rab3B facilitates secretion [Lledo et al., 1993]. By contrast, overexpression of Rab3A inhibits calcium-induced secretion in PC12 cells, suggesting an inhibitory role for this isoform [Johannes et al., 1994; Weber et al., 1996]. Hence, although highly homologous, Rab3 isoforms appear to have distinct roles with respect to secretion.

Binding of guanine nucleotides to Rab proteins is regulated by their low intrinsic GTPase activity and by accessory proteins [Novick and Brenwald, 1993]. A 54-kDa protein that inhibits GDP dissociation from rab3A was purified from bovine brain cytosol and has been termed rab GDP-Dissociation Inhibitor, or rab-GDI [Sasaki et al., 1990]. Rab-GDI also inhibits GTP binding to GDP-bound Rab3A and removes Rab proteins from membranes [see Pfeffer, 1995, for review]. Moreover, most cytosolic

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Rab proteins exists as a complex with rab-GDI and these complexes may represent a cytosolic pool of Rab proteins that are eventually presented to their target membranes [Regazzi et al., 1992; Yang et al., 1994]. Two rab-GDI isoforms have been cloned from bovine brain [Nishimura et al., 1994] and one from mouse skeletal muscle [Shisheva et al., 1994]. Although rab-GDI was first identified in brain, it is now apparent that most tissues express at least one rab-GDI isoform. Gastric chief cells express two rab-GDI isoforms that differ with respect to molecular mass and subcellular localization [Raffaniello et al., 1996a]. Moreover, rab-GDI inhibits GTPγS-induced pepsinogen secretion from chief cells [Raffaniello et al., 1996a]. These observations indicate a role for rab-GDI and Rab proteins in exocytosis in these cells.

Geranylgeranylation of Rab proteins on their C-terminal cysteine motifs is essential for their membrane association and function. This post-translational modification occurs via geranylgeranyl transferase II (GGTase), a heterodimer composed of α- and β-subunits of 60 and 38 kDa, respectively [Seabra et al., 1992a,b]. An accessory factor, Rab escort protein (REP), is also required and presents Rab proteins to GGTase [Cremers et al., 1994]. Like rab-GDI, REP is able to form stable complexes with Rab proteins, inhibit GDP dissociation and solubilize membrane-associated Rab proteins [Alexandrov et al., 1994]. Recent studies indicate that REP, not rab-GDI, may accompany newly geranylgeranylated Rab proteins to their target membranes, whereas rab-GDI is required solely for recycling of Rab proteins [Alexandrov et al., 1994; Wilson et al., 1996]. In contrast to these findings, newly synthesized Rab5 exists as a complex with rab-GDI [Sanford et al., 1995].

Dispersed chief cells secrete pepsinogen in response to various agonists [for review, see Raufman, 1992]. We and others have demonstrated that Rab3D is expressed in guinea pig gastric chief cells and is associated with secretory granules [Tang et al., 1994; Raffaniello et al., 1996b]. Rab3D is believed to be involved in regulated exocytosis in many cell types including pancreatic and parotid acinar cells [Valentijin et al., 1996; Ohnishi et al., 1996; Raffaniello et al., 1996c] and mast cells [Oberhauser et al., 1994]. It was recently demonstrated that overexpression of Rab3D in pancreatic acini in transgenic mice enhances GTPγS- and calcium-induced amylase release, indicating that Rab3D plays a stimulatory role in agonist-induced amylase release [Ohnishi et al., 1997]. In gastric chief cells, the interactions between Rab3D and accessory proteins like rab-GDI and REP-1 have not been examined. As these interactions are important with respect to Rab protein localization and function, we investigated Rab3D interactions with rab-GDI and REP in cytosol prepared from gastric chief cells.

MATERIALS AND METHODS

Materials

Male Hartley guinea pigs (150–200 g) were obtained from CAMM Research Lab Animals (Wayne, NJ); gel filtration media, collagenase (type I), bovine serum albumin (fraction V) (BSA), EGTA, leupeptin, Triton X-114, and adenosine triphosphate (ATP) from Sigma Chemical Co. (St. Louis, MO); basal medium (Eagle) amino acids and essential vitamin solution from Grand Island Biological (Grand Island, NY); Percoll and prestained molecular-weight markers from Pharmacia (Piscataway, NJ); geranylgeranyl pyrophosphate from Biomol (Plymouth Meeting, PA).

Antibodies and Recombinant Proteins

His-tagged-Rab3D was prepared in *Escherichia coli* and purified by affinity chromatography. Antiserum to Rab3D was raised in rabbits using recombinant Rab3D as an immunogen. The Rab-GDI-specific antibody was obtained from Zymed Laboratories (San Francisco, CA). This antibody was raised against a 20-amino acid sequence from bovine brain Rab-GDI and reacts with recombinant GDIα, β, and 2. Monoclonal antibodies specific for Rab5 and Rab3 (clone 42.1) isoforms were provided by Dr. Reinhard Jahn. Recombinant GGTase, REP-1, and antibodies to REP proteins (J905) were kindly provided by Dr. Miguel Seabra.

Tissue Preparation

A suspension consisting of >90% chief cells was prepared from guinea pig stomach, as previously described [Raufman et al., 1984].

Preparation of Subcellular Fractions

Dispersed chief cells were washed with cold phosphate-buffered saline (PBS) and resuspended in sonication solution (20 mM Tris-HCl [pH 7.0], 0.3 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM

EGTA). The cells were sonicated (3 × 10-s bursts) and centrifuged at 120,000*g* for 40 min. The supernatant represented the cytosolic fraction. The pellet was resuspended in an equal volume of sonication solution and represented the membrane fraction. Aliquots were separated on sodium dodecyl sulfate (SDS)/12% polyacrylamide gels and electroblotted to nitrocellulose membranes (Schleicher and Schuel, Keene, NH).

Immunoblotting of Cellular and Gel Filtration Fractions

Subcellular fractions or gel filtration fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membranes. The blots were incubated with 1% bovine serum albumin (BSA) in TBST (50 mM Tris [pH 7.5], 0.15 M NaCl, 0.05% Tween-20) for >2 h to block nonspecific binding, and then incubated overnight with TBST + BSA containing the primary antibody. Blots were washed 5 × 10 min with TBST + BSA and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG. After extensive washing with TBST, bands were visualized using the ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Band intensity was quantitated by densitometry using NIH Image 1.60 software.

Gel Filtration Chromatography

Aliquots of chief cell or brain cytosol (2–4 mg protein) were applied to a 40-ml Sephacryl S200 column equilibrated with column buffer (64 mM Tris-HCl [pH 8.0], 100 mM NaCl, 8 mM $MgCl_2$, 2 mM EDTA, 0.2 mM dithiothreitol, 10 μM GDP, and 1 mM PMSF), and 0.5-ml fractions were collected. Rab proteins, rab-GDI, and REP were detected in alternate column fractions by immunoblotting and densitometry. Molecular masses were determined using protein standards.

Triton X-114 Extraction

Triton X-114 extraction was performed as described by Bordier (1981). Samples (300 μl) were adjusted to 1% Triton X-114 and incubated at 4°C for 30 min. The samples were then layered over a solution of 6% sucrose, 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.06% Triton X-114 and incubated at 30°C for 3 min. After centrifugation (300*g*, 3 min), the aqueous and detergent phases were collected and analyzed for Rab3D as described above.

In Vitro Isoprenylation of Recombinant Rab3D

Recombinant His-tagged Rab3D (1 μM) was incubated in reaction buffer (50 mM HEPES [pH 7.2], 78 mM KCl, 4 mM $MgCl_2$, 8 mM $CaCl_2$, 1 mM dithiothreitol, 10 mM EGTA, 10 μM GDP, 60 μM geranylgeranyl pyrophosphate) in the presence or absence of REP-1 (1 μM) and/or GGTase (1 μM). After incubation for 30 min at 30°C, the reaction mixture (final volume, 200 μl) was either extracted with Triton X-114 to detect lipid-modified Rab3D or applied to a S-200 gel filtration column.

Immunoprecipitation of Rab3D

His-Rab3D was incubated with recombinant REP-1 in the presence or absence of GGTase for 30 min as described above (reaction volume, 50 μl). The reaction was terminated by the addition of cold TBS (150 μl). Anti-Rab3D antiserum conjugated to protein A-agarose (40 μl) was added to each reaction and the samples were incubated overnight. The pellets were washed 3× with ice-cold TBS, and bound proteins were eluted from the agarose beads with Immunopure IgG Elution buffer (pH 2.8) (Pierce, Rockford, IL). Eluted proteins were separated by 10% PAGE and immunoblotted for Rab3D and REP.

RESULTS

Immunoblotting of Rab3D in Subcellular Fractions Prepared from Gastric Chief Cells

Rab3D-specific antiserum was prepared in rabbits using recombinant Rab3D as antigen. Immunoblotting revealed that the Rab3D antiserum reacts with recombinant Rab3D, but not recombinant Rab3A (Fig. 1A, middle). Recombinant Rab3D runs as a higher-molecular-mass protein (~33 kDa) than endogenous Rab3D (Fig. 1B,C) because it is a His-tagged protein. Rab3 isoforms A, B, and C, but not D, are expressed in brain [Matsui et al., 1988] and the Rab3D antiserum did not react with Rab3 isoforms in brain lysate (Fig. 1A, middle). A monoclonal antibody that reacts with all four Rab3 isoforms detected recombinant Rab3D and Rab3A, as well as a diffuse band in the brain lysate (Fig. 1A, right). No bands were observed when preimmune rabbit serum was used (Fig. 1A,

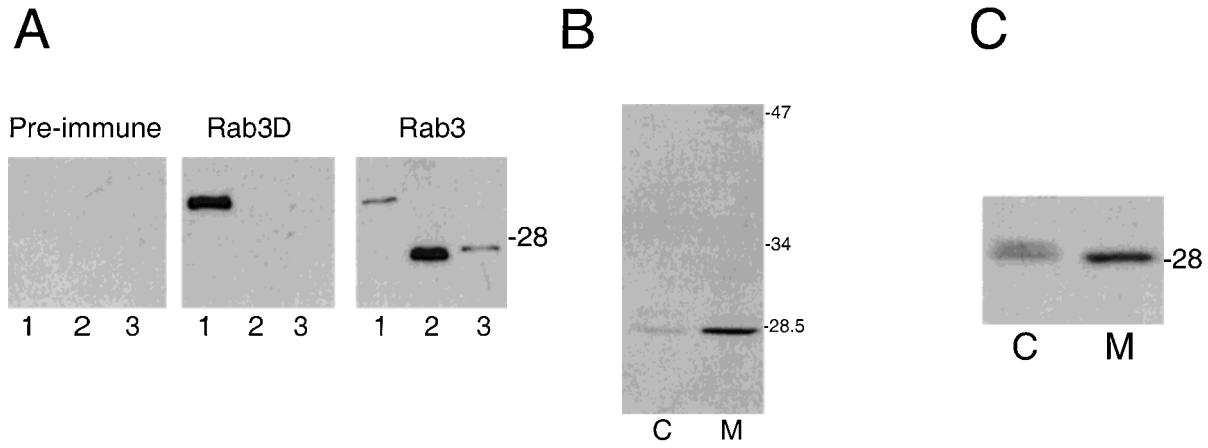

Fig. 1. Immunoblotting of chief cell subcellular fractions for Rab3D. **A:** Recombinant Rab3D (**lane 1**), guinea pig brain lysate (**lane 2**), and recombinant Rab3A (**lane 3**) were immunoblotted with preimmune antiserum (left), Rab3D-specific antiserum (middle) or a monoclonal antibody that recognizes all Rab3 isoforms (right). **B,C:** Cytosolic (C) and membrane (M) fractions prepared from two separate chief cell preparations were immunoblotted with Rab3D-specific antiserum. Molecular-mass markers (kDa) are indicated on right.

left). These results indicate that the Rab3D antiserum is specific for the Rab3D isoform.

Cytosolic and membrane fractions prepared from chief cells were immunoblotted with the Rab3D-specific antiserum. A 28-kDa band was detected in chief cell membrane and cytosolic fractions, although staining was more intense in the membrane fraction (Fig. 1B). In some samples, an additional band at approximately 29 kDa was observed in the cytosol (Fig. 1C). Based on what has been observed with other Rab proteins, this band most likely represents nongeranylgeranylated Rab3D. These results indicate that although Rab3D is predominantly membrane-associated in chief cells, a significant fraction (10–40%) is present in the cytosol.

Rab-GDI and REP Expression in Guinea Pig Chief Cell and Brain Cytosol

Next, we examined the relative levels of REP and rab-GDI isoforms in chief cell and brain cytosol. Equal amounts of cytosolic protein from these tissues were immunoblotted with REP- and rab-GDI-specific antisera. The REP-specific antiserum (J905) recognized two REP isoforms in both tissues (Fig. 2). The higher molecular mass band is most likely REP-1, whereas the lower band represents REP-2. Most tissues express both REP isoforms to varying degrees [Desnoyers et al., 1996]. REP-1 levels were slightly higher in guinea pig brain cytosol compared with chief cell cytosol. Quantitative immunoblotting showed that REP-1 levels were approximately twofold higher in brain. In contrast, relative levels of REP-2 were similar in chief cell and brain cytosol. Moreover, the REP-2 band in brain cytosol was somewhat diffuse, suggesting that it may be modified in this tissue.

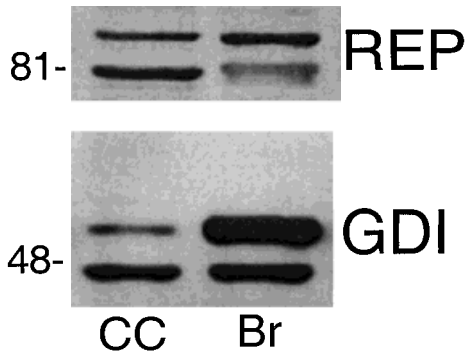

Fig. 2. Relative levels of REP and rab-GDI in chief cell and brain cytosol. Equal amounts of chief cell (CC) and brain (Br) cytosol (35 µg protein) were separated on 10% SDS-PAGE gels and immunoblotted with antiserum J905, which recognizes both REP isoforms, or antiserum specific for rab-GDI isoforms. Molecular-mass markers (kDa) are indicated on left.

In both tissues, two GDI isoforms were detected in the cytosol using an antiserum which recognizes all known GDI isoforms (Fig. 2). Based on its molecular mass and subcellular localization, the top band (approximately 56 kDa) most likely represents GDIα, whereas the lower band may represent a GDIβ/2-like isoform. Relative levels of the lower GDI band were similar in brain and chief cell cytosol, whereas GDIα levels were at least fivefold higher in brain.

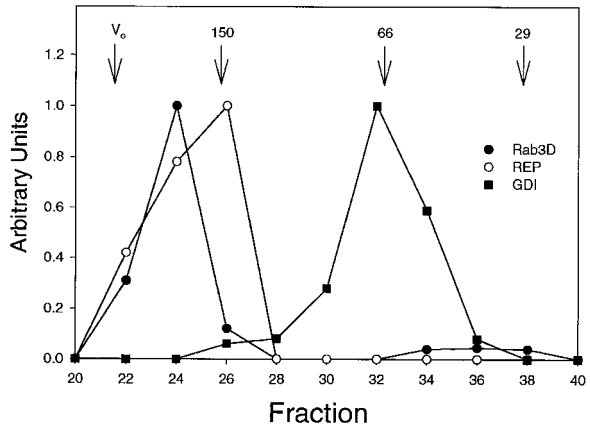

Fig. 3. S-200 chromatography of chief cell cytosolic proteins. Chief cell cytosol was fractionated by S200 gel filtration chromatography and alternate fractions were immunoblotted for Rab3D, REP, and rab-GDI. Relative levels of Rab3D, REP, and rab-GDI in the fractions were determined by densitometry. Alcohol dehydrogenase, bovine serum albumin and carbonic anhydrase were used as 150-, 66-, and 29-kDa molecular mass standards, respectively.

Gel Filtration Chromatography of Gastric Chief Cell Cytosolic Proteins

To determine whether cytosolic Rab3D exists as a complex with rab accessory proteins, chief cell cytosol was fractionated by S-200 gel filtration chromatography, and fractions were immunoblotted for Rab3D, rab-GDI, and REP. As shown in Figure 3, Rab3D eluted as two peaks—a major peak near the void volume and a minor, low-molecular-weight peak at approximately 29 kDa—which most likely represents monomeric Rab3D. The major Rab3D peak eluted with REP in fractions 22–26, suggesting that Rab3D and REP are complexed in chief cell cytosol. Although the predicted molecular mass of REP is 73 kDa, REP-1 not complexed to any Rab protein elutes at approximately 100–140 kDa when applied to a gel filtration column [Seabra et al., 1992b] (see Fig. 5). Hence, Rab3D and REP would be expected to form a complex eluting near the void volume, as was observed in these experiments. Moreover, it has been suggested that Rab protein:REP complexes may exist with a 2:2 or 2:1 stoichiometry after prenylation [Shen and Seabra, 1994], which would result in formation of a complex with a molecular mass of 170–200 kDa. However, we were unable to determine the stoichiometry of the REP:Rab3D complex in chief cell cytosol with the methods employed in the present study. Although Rab3D and REP elute in the same fractions, it is evident that the Rab3D and REP peaks do not directly overlap one another (Fig. 3). One must keep in mind that the REP peak most likely represents REP complexed to Rab3D, as well as other Rab proteins of similar but varying molecular masses.

Interestingly, Rab3D and rab-GDI did not co-elute in any of the cytosol preparations examined. However, our data do not exclude the possibility that low levels of Rab3D or other Rab proteins are complexed with rab-GDI in chief cells.

In addition to these observations, immunostaining of Rab3D in the high molecular mass fractions revealed a 28-kDa band, whereas immunostaining of Rab3D in the lower molecular mass fractions revealed a 29-kDa band (not shown). As discussed above, the differences in Rab3D mobility on SDS/PAGE suggest that Rab3D present in the high-molecular-mass fractions is geranylgeranylated, whereas monomeric Rab3D in the low-molecular-mass fractions is not (see Fig. 5A). Moreover, Rab3D in the low-molecular-mass fractions extracted with Triton X-114 partitioned exclusively into the aqueous fraction, indicating that it is not lipid modified (not shown).

Gel Filtration Chromatography of Guinea Pig Brain Cytosolic Proteins

In contrast to what we observed in chief cell cytosol, others have demonstrated that cytosolic Rab proteins co-elute with rab-GDI in brain [Yang et al., 1994]. We applied guinea pig brain cytosol to our S-200 column and immunoblotted alternate samples for Rab3 proteins, Rab5, and rab-GDI. Consistent with what others have demonstrated, rab-GDI eluted as a broad band indicating that it is complexed to other proteins (Fig. 4). Rab3 proteins (Fig. 4) and Rab5 (not shown) co-eluted with rab-GDI at a molecular mass of 95 kDa, suggesting that Rab proteins are complexed with rab-GDI in guinea pig brain cytosol. In fact, in brain cytosol, Rab proteins did not elute at <30 kDa, indicating that all Rab proteins exist as complexes with rab-GDI or other proteins. Moreover, rab-GDI eluted at a higher molecular mass in brain than in chief cell cytosol (cf. Figs. 3 and 4). These data confirm previous findings [Yang et al., 1994] and verify our ability to detect Rab3 protein:rab-GDI complexes by S-200 gel filtration chromatography.

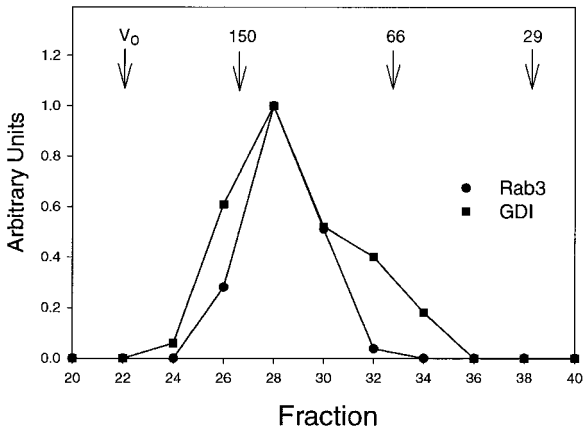

Fig. 4. S-200 chromatography of guinea pig brain cytosolic proteins. Guinea pig brain cytosol was fractionated by S-200 gel filtration chromatography and alternate fractions were immunoblotted for Rab3 proteins and rab-GDI. Relative levels of Rab3 proteins and rab-GDI in the fractions were determined by densitometry. Standards are as in Figure 3.

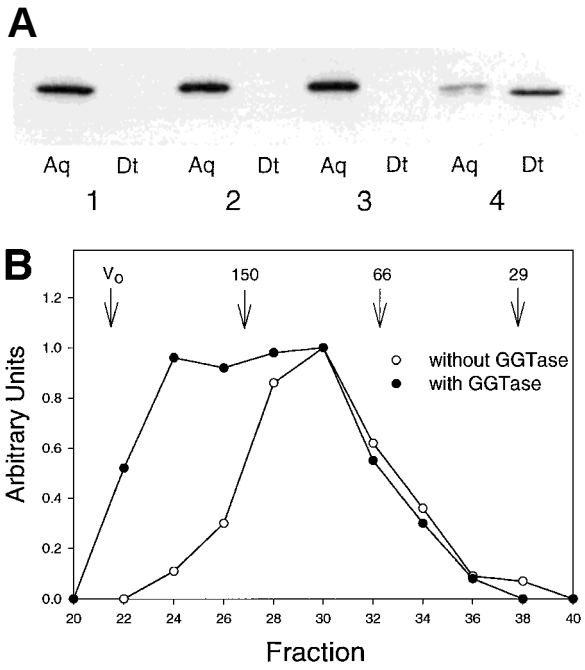

Fig. 5. **A:** In vitro geranylgeranylation of recombinant Rab3D. Recombinant His-Rab3D was incubated in the presence of recombinant REP (1), GGTase (2), no additions (3), or REP and GGTase (4) for 30 min at 30°C. The reactions were extracted with Triton X-114, and aqueous (aq) and detergent (dt) phases were immunoblotted for Rab3D. **B:** S-200 chromatography of REP and REP:Rab3D complexes formed in vitro. Recombinant REP-1 was incubated with His-Rab3D in the presence (●) or absence (○) of GGTase for 30 min at 30°C. The reactions were fractionated by S-200 gel filtration chromatography and alternate fractions were immunoblotted for REP.

Rab3D Remains Bound to REP-1 After Isoprenylation In Vitro

To examine more directly the interactions between Rab3D and REP, Rab3D was geranylgeranylated in vitro. As shown in Figure 5A (lanes 1–3), recombinant Rab3D partitions exclusively into the aqueous phase when incubated in the absence of REP-1 and GGTase, or when REP-1 or GGTase are added to the reaction individually. When both GGTase and REP-1 were added to the reaction, Rab3D partitioned into the detergent phase and displayed a higher mobility, indicating that it was geranylgeranylated (Fig. 5A, lane 4).

Recombinant Rab3D and REP-1 were incubated in the presence or absence of GGTase and the reactions were fractionated by S200 gel filtration chromatography. As shown in Figure 5B (closed circles), when GGTase is present, REP-1 eluted as a broad peak from the void volume to 100 kDa. Rab3D co-eluted with REP-1 in the high-molecular-mass fractions under these conditions (not shown). However, when the reaction is performed in the absence of GGTase, REP-1 elutes as a more narrow peak at a lower molecular mass (Fig. 5B, open circles), indicating a lower level of complex formation between REP-1 and nongeranylgeranylated Rab3D.

The formation of a stable Rab3D:REP complex was further examined by incubating His-Rab3D with recombinant REP-1 in the presence or absence of GGTase and then immunoprecipitating Rab3D from the reactions under native conditions. As shown, in Figure 6, His-Rab3D was immunoprecipitated from both samples. However, His-Rab3D immunoprecipitated from samples containing GGTase displayed a higher mobility, indicating that His-Rab3D was geranylgeranylated. Although REP-1 co-immunoprecipitated with Rab3D in the presence or absence of GGTase (Fig. 6), a greater amount of REP-1 co-immunoprecipitated with Rab3D when GGTase was present, suggesting that REP forms a more stable complex with geranylgeranylated Rab3D.

In contrast to the in vitro geranylgeranylation experiments in which high levels of REP:Rab3D complexes are formed, we were unable to co-immunoprecipitate Rab3D and REP from chief cell fractions. This is most likely due to the relatively low levels of these proteins in chief cell cytosol. Similarly, in NRK cells overexpressing Rab5, co-immunoprecipitation of Rab5 and REP-1 was observed, while endogenous

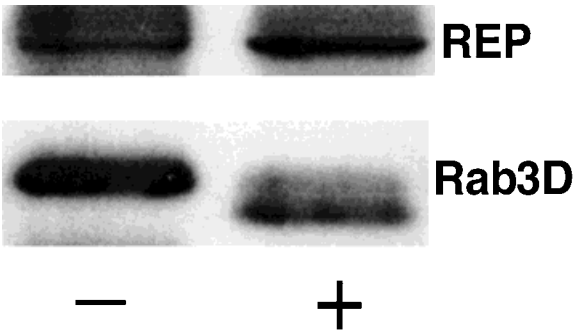

Fig. 6. Co-immunoprecipitation of recombinant REP-1 with Rab3D. His-Rab3D was incubated with recombinant REP-1 in the presence (+) or absence (−) of GGTase for 30 min at 30°C as described under Materials and Methods. Rab3D was immunoprecipitated from the reactions with anti-Rab3D antiserum in the absence of detergents. Immunoprecipitates were immunoblotted for Rab3D and REP. Note that Rab3D incubated in the presence of GGTase displayed a higher mobility, indicating that it was geranylgeranylated.

REP-1:Rab5 complexes did not co-immunoprecipitate in cells not overexpressing Rab5 [Alexadrov et al., 1994]. Moreover, antibody-specific epitopes may be masked by the formation of REP:Rab3D complexes, interfering with the immunoprecipitation of these proteins.

DISCUSSION

Rab3D is expressed in chief cells and believed to be involved with regulated exocytosis [Tang et al., 1994; Raffaniello et al., 1996a]. Rab protein interactions with accessory proteins like rab-GDI and REP are important since these interactions regulate Rab protein localization and function. In the present study, we examined the interactions between Rab3D and these accessory proteins in chief cell cytosol. In many cells, including neurons, hepatocytes and insulin-secreting cell lines, cytosolic Rab proteins exist as complexes with the rab-GDI isoforms expressed in these tissues [Yang et al., 1994; Soldati et al., 1993; Shisheva and Czech, 1997]. In Chinese hamster ovary (CHO) cells overexpressing Rab9, prenylated Rab9 co-eluted with rab-GDI as an 80-kDa complex [Soldati et al., 1993]. Similarly, Rab5 synthesized and geranylgeranylated in vitro in the presence of reticulocyte lysate forms a complex with a 45-kDa reticulocyte rab-GDI [Sanford et al., 1995]. On the basis of these findings, it has been suggested that immediately after geranylgeranylatation, Rab proteins dissociate from REP and form complexes with rab-GDI. Hence, we were surprised to find that cytosolic Rab3D is not associated with rab-GDI in chief cells.

In contrast to the studies described above, other investigators have demonstrated that while rab-GDI is required for recycling, REP is sufficient for the initial membrane targeting of Rab proteins [Alexandrov et al., 1994; Wilson et al., 1996]. Our results support a role for REP in the initial targeting of Rab proteins and indicate that following geranylgeranylation, Rab3D remains associated with REP in chief cell cytosol. We have also fractionated cytosol from rat parotid acinar cells by S200 gel filtration chromatography. As in gastric chief cells, Rab3D in parotid acinar cell cytosol elutes with REP, not GDI (unpublished observations). Hence, we propose that in gastric chief cells and parotid acinar cells, nascent Rab3D molecules bind REP, undergo prenylation and remain bound to REP until they are delivered to the secretory granule membrane.

It has previously been observed that REP-1 complexed with mono- or di-geranylgeranylated Rab proteins is more stable than REP-1 complexed with nongeranylgeranylated Rab proteins [Shen and Seabra, 1996]. Consistent with these observations, in chief cells cytosols fractionated by gel filtration chromatography, Rab3D eluting with REP is predominantly geranylgeranylated, while Rab3D in the low-molecular-mass fractions is apparently not modified and does not elute with REP. Moreover, recombinant Rab3D geranylgeranylated in vitro elutes with and co-immunoprecipitates with REP-1 to a greater degree than nongeranylgeranylated Rab3D. These observations suggest that cytosolic Rab3D proteins form a complex with REP, are immediately geranylgeranylated and remain associated with REP. In contrast to our findings, others have observed that nongeranylgeranylated Rab proteins do form stable complexes with REP in vitro [Alexandrov et al., 1994]. Taken together, these observations indicate that the ability REP and nonmodified Rab proteins to form complexes may vary among different Rab proteins. Further studies are required to examine the different affinities of mono- and di-geranylgeranylated Rab3D, as well as nonmodified Rab3D, for REP and/or geranylgeranyl transferase.

A small pool of Rab3D remains unmodified and unbound to REP in chief cell cytosol. This may be due to the limited amount of unbound REP in the cytosol. In the in vitro geranylgeran-

ylation experiments, the broad REP peak observed in Figure 5B probably represents REP bound to Rab3D as well as noncomplexed REP. When compared with the more narrow REP peak observed in the high molecular mass fractions in Figure 3, it appears that little, if any, noncomplexed REP exists in chief cell cytosol. These observations suggest that newly synthesized Rab proteins exist as monomers and remain unmodified until noncomplexed REP becomes available.

In the present study, complexes between rab-GDI and Rab3D could not be detected in chief cell cytosol. As discussed above, this is in contrast to what has been observed with Rab proteins in other tissues. One possible explanation is that in chief cells cycling of Rab3D between the membrane and cytosol does not occur or occurs at a much lower rate. Consistent with this notion is the observation that agonist-induced exocytosis is not accompanied by redistribution of Rab3 proteins to the cytosolic compartment in chief cells [Jena et al., 1994]. Hence, only nascent Rab3D, which has either not been lipid modified or has just undergone geranylgeranylation, is present in the cytosol. Yang et al. [1992] suggested that Rab proteins will complex with the most abundant rab-GDI isoform in the cytosol. Although cytosolic levels of GDIα and REP-1 were both higher in brain when compared with chief cells, the difference in GDIα levels was more pronounced (fivefold higher in brain). REP proteins are considered to belong to the superfamily of GDI-like proteins, as REP and GDI family members demonstrate highly conserved regions of primary sequence [Wu et al., 1996]. Hence, in chief cells, REP may be the most abundant "rab-GDI."

Although rab-GDI and REP proteins are similar in many ways, they are functionally distinct. Rab-GDI cannot substitute for REP in the geranylgeranylation reaction [Alexandrov et al., 1994], whereas rab-GDI, not REP, is required to recycle membrane-associated Rab proteins [Wilson et al., 1996]. Our findings indicate that Rab3D does not interact with rab-GDI. Further studies are required to determine the functions of rab-GDI isoforms expressed in gastric chief cells. However, the results from the present study suggest a role for REP in the prenylation and initial membrane targeting of Rab3D to the secretory granule membrane.

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