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Actions and Expression of RAB-GDP Dissociation Inhibitor in Dispersed Chief Cells from Guinea Pig Stomach

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Rab proteins are a family of ras-like proteins that are involved in intracellular membrane trafficking. Rab-GDP dissociation inhibitor prevents dissociation of GDP from Rab proteins and extracts Rab proteins from cell membranes *in vitro.* In the present study, we examined the effects of recombinant rab-GDI on Rab proteins in gastric chief cells. Rab-GDI extracted GTP-binding proteins, including Rab3 and Rab5, from chief cell membranes in a dose-dependent manner. Maximal concentrations of rab-GDI ($\geqslant$50 μg/ml) removed approx. 40% of membrane-associated Rab3. Moreover, preincubation of permeabilized chief cells with rab-GDI resulted in a 35% decrease in GTPγS(100 μM)-induced pepsinogen secretion, suggesting that membrane-associated Rab proteins are involved in the final stages of secretion. Immunostaining of chief cell cytosolic and membrane fractions with GDI-specific antisera revealed bands at 56 and 48 kDa, respectively, indicating that chief cells express two isoforms of rab-GDI. In gastric chief cells, regulation of Rab proteins by rab-GDI plays an important role in mediating exocytosis.

In mammalian cells, low molecular weight (LMW) GTP-binding proteins belonging to the rab subfamily of ras-like proteins play an important role in vesicular transport and regulated exocytosis (1,2). Like Ras proteins, Rab proteins cycle between GDP- and GTP-bound conformations and are believed to be active when bound to GTP (3). Each Rab protein is associated with a distinct membrane compartment and is involved with a particular transport step. Rab3 proteins are localized to secretory organelles (4–6) and are believed to play a role in regulated exocytosis (see refs. 7 and 8 for review). Four rab3 isoforms have been cloned, three from rat brain (rab3A, B and C) and one from adipocytes (rab3D) (9,10).

Binding of guanine nucleotides to Rab proteins is regulated by their low intrinsic GTPase activity and by accessory proteins (see ref. 11 for review). A 54-kDa protein that inhibits GDP dissociation from rab3A was purified from bovine brain cytosol and has been termed rab-**G**DP-**D**issociation **I**nhibitor or rab-GDI (12). This protein also inhibits GTP binding to GDP-bound Rab3A and removes Rab proteins from membranes (13,14). Moreover, most cytosolic Rab proteins exist as a complex with rab-GDI (15). Two rab-GDI isoforms have been cloned from brain (16) and one from mouse skeletal muscle (17). Although rab-GDI was first identified and characterized in brain, it is now apparent that most tissues express at least one rab-GDI isoform (16,18).

GTPγS stimulates pepsinogen secretion from permeabilized chief cells independent of changes in calcium, cAMP or activation of protein kinase C, suggesting a role for LMW GTP-binding proteins in secretion (19). Recently, we demonstrated that Rab3D is expressed in

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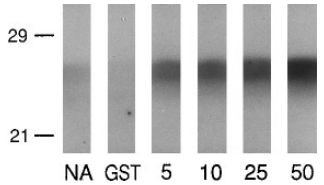

FIG. 1. Effect of recombinant rab-αGDI on membrane-associated LMW GTP-binding proteins in dispersed chief cells. Chief cell membranes were incubated with no additions (NA), GST or increasing concentrations of recombinant rab-αGDI (5–50 μg/ml) for 1 hr at 30°C, then centrifuged at 120,000 $\times$ g for 40 min. LMW GTP-binding proteins in the supernatant were detected by the ^{32}P-GTP overlay assay as described in Methods. Similar results were obtained in three separate experiments.

guinea pig gastric chief cells and is associated with chief cell secretory granules (20). Rab3D was also detected on secretory granules of gastric chief cells prepared from rabbit (21). Since membrane association and GTP binding are essential for Rab protein activation, rab-GDI activity directly affects Rab protein function. In the present study, we used dispersed chief cells to examine the effects of recombinant rab-GDI on membrane-association of Rab proteins and GTPγS-induced pepsinogen secretion.

MATERIALS AND METHODS

Materials. Male Hartley guinea pigs (150–200 g) were obtained from CAMM Research Lab Animals (Wayne, NJ); collagenase (type I), bovine serum albumin (fraction V) (BSA), EGTA, leupeptin, Triton X-100, Nonidet P-40, sodium deoxycholate and adenosine triphosphate (ATP) from Sigma; streptolysin O (SLO), basal medium (Eagle) amino acids and essential vitamin solution from Grand Island Biological, Grand Island, NY; Percoll and prestained molecular weight markers from Pharmacia; [α^{32}P]GTP from New England Nuclear (MA); and ^{125}I-albumin from ICN (CA). Polyclonal antibodies recognizing Rab-GDI were kindly provided by Dr. Olivia Steele-Mortimer (EMBL, Germany). Monoclonal antibodies specific for Rab5 and Rab3 isoforms were provided by Dr. Reinhard Jahn (Yale Univ. Medical Center, CT).

Tissue preparation and permeabilization. A suspension consisting of >90% chief cells was prepared from guinea pig stomach as described previously (22) and kept in standard incubation solution containing Ca^{++} and Mg^{++} for 10 min before proceeding. The cells were washed three times and resuspended in permeabilization solution that consisted of the following: 120 mM KCl; 30 mM NaCl; 1 mM $MgCl_2$; 1 mM K_2HPO_4; 10 mM PIPES (pH 7.0); 1 mM ATP and 1 mg/ml BSA. Calcium-independent GTPγS-induced secretion was measured in permeabilization solution that contained 0.4 mM EGTA. Unless stated otherwise, SLO and agonists were added simultaneously to the cell suspensions.

Pepsinogen secretion. Peptic activity was determined as described previously (23) using ^{125}I-albumin as substrate. Pepsinogen secretion was expressed as the percentage of total cellular pepsinogen at the start of the incubation that was released into the medium during the incubation.

Expression of recombinant αGDI. The plasmid containing the coding region of the rab-αGDI fusion protein (pGEX-2t-rat-rab-GDIα) was kindly provided by Dr.Kimihiko Sano (Kobe University, Japan) and rab-αGDI was produced as a fusion protein with glutathione-S-transferase (GST) as described by Nishimura et al. (16). The recombinant protein was stored at −80°C until use. Affinity purified GST-rab-αGDI fusion protein was examined by SDS-PAGE and Western Blotting. Staining with Commassie blue revealed a band with a molecular mass of approximately 82 kDa that stained with the rab-GDI-specific antibody.

Preparation of subcellular fractions and identification of LMW GTP-binding proteins. Dispersed chief cells were washed with cold 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 $\times$ 10 sec bursts) and centrifuged at 120,000 $\times$ 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 SDS/12% polyacrylamide gels and electroblotted to nitrocellulose membranes (Schleicher and Schuel, NH). GTP-binding proteins were detected on nitrocellulose membranes by the [α-^{32}P]GTP overlay assay as described previously (24). Rab proteins in chief cell fractions were detected by Western blotting as described previously (20). Band intensity was quantitated by densitometry.

Membrane dissociation assay. Chief cell membranes were washed and resuspended in assay buffer (20 mM Tris-HCl [pH 7.0], 250 mM sucrose, 1 mM EGTA, 6 mM $MgCl_2$, 1 mM DTT, 1 μg/ml aprotinin, 1 μg/ml leupeptin and

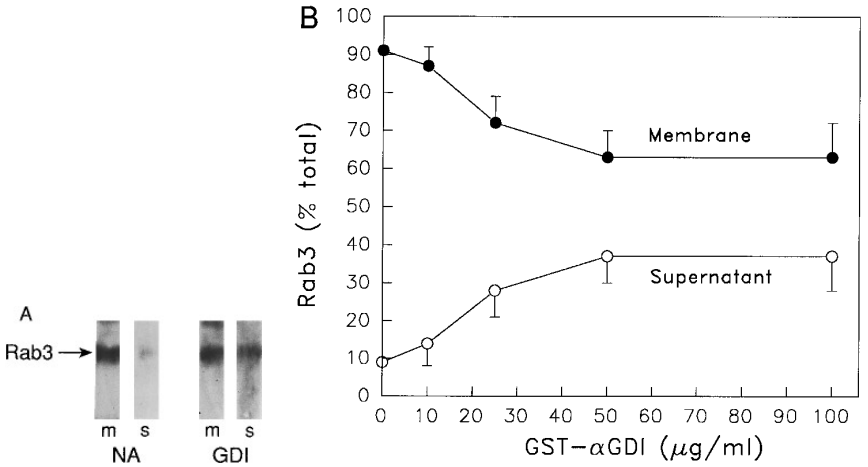

FIG. 2. Effect of recombinant rab-αGDI on membrane-associated Rab3 proteins in dispersed chief cells. **A.** Chief cell membrane were incubated with no additions (NA) or recombinant rab-αGDI (50 μg/ml) (GDI) for 1 hr at 30°C. Following centrifugation at 120,000 $\times$ g for 40 min, membrane (m) and supernatant (s) fractions were immunoblotted for Rab3 proteins as described in Methods. **B.** Chief cell membranes were incubated with no additions or increasing concentrations of recombinant rab-αGDI for 1 hr at 30°C. Following centrifugation at 120,000 $\times$ g for 40 min, Rab3 levels (% total) in the membrane and supernatant fractions were determined by Western blotting and densitometry. Values represent the mean $\pm$ SEM of three experiments.

1 mM PMSF) using a 25-guage needle. Membranes (100 μl) were incubated in the absence or presence of recombinant rab-αGDI for 60 min at 30°C, then centrifuged at 120,000 $\times$ g for 40 min. The supernatant was removed and the membranes were resuspended in sonication buffer. Levels of Rab3, Rab5 and LMW GTP-binding proteins in the membrane and supernatant fractions were determined as described above.

Statistical analysis. Significance between two means was determined by Student's unpaired *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of recombinant rab-αGDI on membrane-associated LMW GTP-binding proteins in dispersed chief cells. Recently, we identified several membrane-associated LMW GTP-binding proteins in chief cells (24). We examined the effects of recombinant rab-αGDI on these proteins by incubating chief cell membranes with no additions, GST or increasing concentrations of recombinant GST-rab-αGDI. In the absence of added rab-αGDI, LMW GTP-binding proteins were not extracted into the supernatant (Fig. 1). However, rab-αGDI removed LMW GTP-binding proteins from chief cell membranes in a dose-dependent manner, whereas GST alone was without effect. It should be noted that, even at the highest concentration of rab-GDI examined (100 μg/ml), a significant portion of LMW GTP-binding proteins remained associated with chief cell membranes (not shown).

We used monoclonal antibodies specific for Rab3 and Rab5 to examine the effects of rab-αGDI on membrane-associated Rab proteins in chief cells. This Rab3 antibody recognizes a 27-kDa band on chief cell membranes, which is most likely Rab3D (20). In the absence of rab-αGDI, Rab3 was detected almost exclusively in the membrane fraction (Fig. 2A). However, following incubation of chief cell membranes with rab-αGDI, Rab3 was detected in the supernatant (Fig. 2A). Rab-αGDI removed membrane-associated Rab3 proteins in a dose-dependent manner (Fig. 2B). Maximal removal of Rab3 from chief cell membranes (approx. 40%) was observed with $\geqslant$50 μg/ml rab-GDI. Similar results were obtained with Rab5 (not shown).

Effect of recombinant rab-αGDI on GTPγS-induced pepsinogen secretion from permeabil-

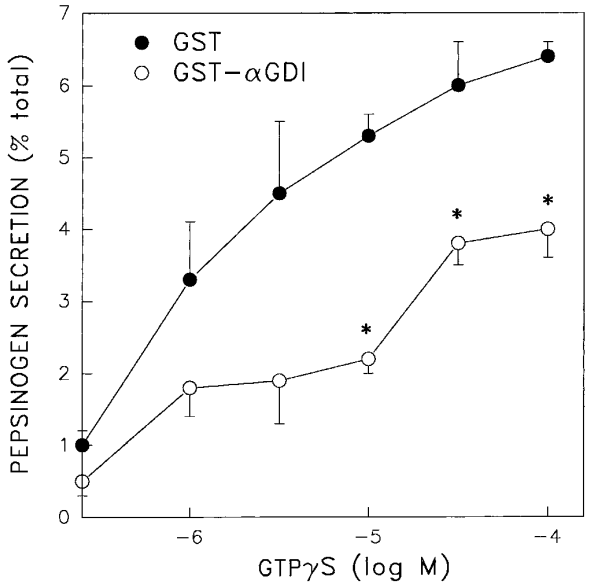

FIG. 3. Effect of recombinant rab-αGDI on GTPγS-induced pepsinogen secretion from permeabilized chief cells. Dispersed chief cells were permeabilized with streptolysin O for 20 min at 37°C in the presence of GST (filled circles) or the GST-rab-αGDI (hollow circles) fusion protein (30 μg/ml). The cells were then incubated with increasing concentrations of GTPγS for an additional 10 min. Pepsinogen release during this 10-min incubation was determined as described in Methods. Values represent the mean ± SEM from four experiments. * Indicates value is significantly less than value obtained with GST ($p < 0.05$, paired t-test).

ized chief cells. Previously, we demonstrated that pre-permeabilization of dispersed chief cells does not alter GTPγS-induced pepsinogen secretion (24). Because excess rab-GDI solubilizes membrane-associated Rab proteins *in vitro*, rab-GDI has been used as a tool to examine the requirement of Rab proteins in intracellular vesicle transport (14,25,26). To examine the effects of rab-αGDI on GTPγS-induced pepsinogen secretion, dispersed chief cells were permeabilized with streptolysin O for 20 min in the presence of GST or the GST-rab-αGDI fusion protein (30 μg/ml). As shown in Fig. 3, rab-αGDI inhibited basal and GTPγS-induced secretion. Secretion with 10 and 100 μM GTPγS was inhibited 60 and 35%, respectively. GST alone did not affect basal or GTPγS-induced secretion (not shown), indicating that the effect of GST-rab-αGDI on secretion is specific. These results indicate that membrane-associated Rab proteins are required for pepsinogen secretion. Rab-GDI may inhibit secretion by removing Rab proteins from chief cell membranes, as well as by inhibiting the release of GDP from membrane-associated Rab proteins.

Expression of rab-GDI in dispersed chief cells. Cytosolic and membrane fractions were prepared from dispersed chief cells and immunoblotted for rab-GDI. An equal amount of guinea pig brain cytosol was also probed and served as a positive control. The rab-GDI antibody detected a 56-kDa band in chief cell (C) and brain (Br) cytosol preparations (Fig.4-upper arrow). The molecular mass of the band recognized by this antibody is similar to that of the αGDI isoform, which is also predominantly a cytosolic protein (13). Staining was more intense in brain compared to chief cell cytosol, indicating that brain expresses higher levels of rab-GDI. A faint 56-kDa band observed in the chief cell membrane fraction may represent a small pool of membrane-associated αGDI.

In addition to the 56-kDa band detected in the chief cell cytosol, the GDI antibody stained

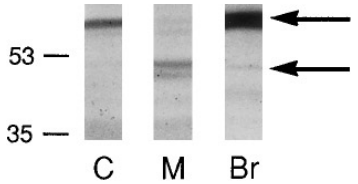

FIG. 4. Expression of rab-GDI in dispersed chief cells. Cytosolic (C) and membrane (M) fractions were prepared from dispersed chief cells and immunoblotted for rab-GDI as described in Methods. An equal amount of guinea pig brain cytosol (Br) was also probed. Values at left indicate molecular weight standards in kilodaltons. See text for description of bands at arrows.

a doublet at about 48 kDa in the chief cell membrane fraction (M) which most likely represents a different GDI isoform (Fig.4-lower arrow). Based on its molecular mass and its association with membranes, this GDI isoform resembles βGDI (17,27). Hence, these results indicate that gastric chief cells express two GDI isoforms which are localized to distinct cellular fractions. Further studies are needed to identify definitively these isoforms and determine their precise cellular localization. Whereas αGDI may be involved in the shuttling of Rab proteins between the cytosol and membrane, βGDI may regulate the activity of membrane-associated Rab proteins. However, separate functions for the two GDI isoforms have not yet been demonstrated.

Because rab-GDI interacts with most Rab proteins, we are unable to determine the specific Rab protein(s) involved with GTPγS-induced pepsinogen secretion. Nevertheless, these data indicate that binding of GTPγS to membrane-associated Rab proteins is an important event in exocytosis. Moreover, since rab-GDI and Rab proteins are both expressed in chief cells, rab-GDI regulation of Rab protein function and localization may also be of great importance in mediating agonist-induced secretion.

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