Dominant Negative Rab3D Mutants Reduce GTP-bound Endogenous Rab3D in Pancreatic Acini*

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Two dominant negative mutants of Rab3D, N135I and T36N were recently reported to inhibit the early phase of regulated amylase secretion from mouse pancreatic acini (Chen, X., Edwards, J. A., Logsdon, C. D., Ernst, S. A., and Williams, J. A. (2002) J. Biol. Chem. 277, 18002–18009). Immunocytochemical studies showed that while the wild-type Rab3D localized to zymogen granules, the two dominant negative mutants did not localize to granules and were primarily in the basolateral regions of the cell. The present study, therefore, evaluated the potential mechanisms by which the dominant negative mutants might act. An affinity precipitation assay based on the property of the Rab3 effector Rim1 to interact only with GTP-bound Rab3D was developed. 78.9 ± 4.5% of total endogenous Rab3D was found in the GTP-bound form. The expression of dominant negative Rab3D, and its Q26N, N135I, and T36N mutants had no effect on the total amount of endogenous Rab3D. However, the dominant negative mutants, T36N and N135I, reduced GTP-bound endogenous Rab3D by 70.0 ± 3.5% and 72.7 ± 1.2%, respectively, while the wild-type Rab3D and Q81L mutant had no effect. Triton X-114 phase separation and cell fractionation studies showed that dominant negative Rab3D mutants did not alter isoprenylation or membrane association of endogenous Rab3D. The dominant negative Rab3D did not affect the amount of endogenous Rab3D on purified zymogen granules as assessed by either Western blotting or immunocytochemistry, but reduced the GTP-bound form by 78.6 ± 3.3%. The two dominant negative Rab3D mutants, therefore, interfere with endogenous Rab3D function by blocking the GDP/GTP exchange but not zymogen granule targeting of endogenous Rab3D.

Small GTPases of the Rab/Ypt family form the largest branch of the Ras-related small G-protein superfamily and are recognized as key protein components involved in vesicular trafficking and membrane fusion in eukaryotic cells (1, 2). Rab proteins act as molecular switches, which cycle between the GDP-bound inactive and GTP-bound active forms. The conversion from the GDP-bound form to the GTP-bound form is stimulated by a Rab GEF (guanine nucleotide exchange factor), and the conversion of the GTP-bound form to the GDP-bound form is catalyzed by a Rab GAP (GTPase-activating protein) (3). A characteristic of Rab proteins is that a cycle of association with and dissociation from membranes is superimposed onto their GDP/GTP cycle. These two types of cycling are essential for Rab function in vesicle trafficking and fusion. At steady state, a portion of a Rab protein is detected in the cytosol. This pool is maintained in the GTP-bound form through interaction with another important regulator of Rab cycling, Rab GDI (GDP dissociation inhibitor) (1).

A model of Rab GDP/GTP and membrane/cytosol cycling had been developed based on pioneering studies of Rab9 and Rab5 (4, 5), and modified according to more recent studies (6–10). In this model, GDI delivers Rab to the target membranes and itself is released into the cytosol. In a consecutive but separate step, the membrane-associated, inactive Rab proteins are converted into their active forms by compartment-specific GEFs (11–13). The Rab, now in its GTP-bound form, recruits its effector(s) to the vesicle. Following fusion with the acceptor compartment, a GAP stimulates the Rab protein to hydrolyze its bound GTP and the resulting GDP-bound form is recognized and retrieved from acceptor compartment by Rab GDI, possibly facilitated by other factors.

Pancreatic acinar cells are the functional unit of digestive enzyme secretion and have long been used as a model to study the packaging and secretion of secretory proteins and its control by neurotransmitters and hormones (14–16). In acinar cells, secretory proteins are transferred by vesicular fusion through a series of compartments to the mature zymogen granule, which upon cellular stimulation fuses with the apical plasma membrane to release its contents into the lumen. The Rab3 proteins are the Rab species associated with synaptic or secretory vesicles in neurons, neuroendocrine, endocrine, and exocrine cells and are thought to play an important role in regulated exocytosis (17–21). Recent work in our laboratory and others (22, 23) has demonstrated that Rab3D is the only detectable Rab3 isoform in rodent pancreatic acini and is localized on zymogen granules. More recently, we reported (24) that dominant negative mutants of Rab3D, N135I and T36N, introduced by adenoviral vector expression, inhibited regulated amylase secretion by mouse pancreatic acini, while the wild-type and active mutant Q81L had no effect. Localization of adenoviral expressed Rab protein showed that wild-type Rab3D lo-
calized to zymogen granules. The two dominant negative mutants did not localize to granules and were primarily in the basolateral cytoplasmic region of the cell. These observations raise the question of how the cytosolic dominant negative Rab3D mutants interfere with the zymogen granules (20)-associated endogenous Rab3D and inhibit its function in acinar secretion.

To evaluate the potential mechanisms by which the dominant negative mutants might act, it is necessary to directly monitor active, GTP-bound Rab3D in acinar cells. Recently, affinity precipitation assays for detecting cellular GTP bound members of the Rho family have been developed (25–28). This method takes advantage of the fact that the effector proteins interact only with GTP-bound G-protein and that binding of G-protein to the effector protein inhibits the GTPase activity of the G-protein. In the present study, we developed an affinity precipitation assay to monitor the active state of endogenous Rab3D in pancreatic acinar cells based on the property of the Rab3 effector Rim to interact only with GTP bound Rab3 (29). A high percentage (~80%) of endogenous Rab3D was found to be in GTP-bound conformation. In acini infected with adenoviruses expressing either two of the dominant negative Rab3D mutants, N135I and T36N, the GTP-bound endogenous Rab3D was reduced to only 30% of that of control acini. Furthermore, Western blot together with immunocytochemistry demonstrated that the dominant negative Rab3D mutants dramatically reduced the active state, but not the total amount, of endogenous Rab3D on the zymogen granules.

EXPERIMENTAL PROCEDURES

Materials—The plasmid encoding GST (glutathione S-transferase)-Rim (Rab3-interacting molecule) (amino acids 1–399) fusion protein was obtained from Dr. Ronald W. Hof, University of Michigan. Anti-Rab3D antisera was a gift from Dr. Mark McNiven (Mayo Clinic, Rochester, MN). Glutathione-Sepharose 4B beads and Percoll were purchased from Amersham Biosciences; Rat monoclonal anti-HA antibody (gift from Dr. S. London, Rochester, MN). Glutathione-Sepharose 4B beads and Percoll were purchased from Amersham Biosciences; Rat monoclonal anti-HA antibody (gift from Dr. S. London, Rochester, MN). Glutathione-Sepharose 4B beads and Percoll were purchased from Amersham Biosciences; Rat monoclonal anti-HA antibody (gift from Dr. S. London, Rochester, MN). Glutathione-Sepharose 4B beads and Percoll were purchased from Amersham Biosciences; Rat monoclonal anti-HA antibody (gift from Dr. S. London, Rochester, MN).

Isolation, Short Term Culture, and Adenoviral Infection of Pancreatic Acini—As previously described (24), pancreatic acini were isolated from male ICR mice by collagenase digestion. Isolated acini from 2–3 pancreas were resuspended in Dulbecco's modified Eagle's medium (DMEM) and divided into 4 or 5 150-mm Petri dishes each containing 30 ml of Dulbecco's modified Eagle's medium enriched with 0.5% fetal bovine serum, 0.02% soybean trypsin inhibitor and antibiotics, and incubated at 37 °C overnight. The HA-tagged wild-type and mutant mouse Rab3D adenoviral constructs were made using AdEASY system as described previously (24). The shuttle vector pAdTrack-CMV also encodes EGFP driven by a separate CMV promoter. An adenovirus expressing bacterial β-galactosidase and EGFP, each under the control of a separate CMV promoter was used as the control virus. In the adenoviral infection experiments, either control β-galactosidase or various HA-tagged Rab3D adeno virus (10⁶ pfu/ml) were added, to the culture medium at the beginning of the incubation. Under this condition, over 95% of acini were infected as indicated by their EGFP signal. After 24 h of incubation, either control β-galactosidase or EGFP-expressing adenoviruses (10⁶ pfu/ml) were added, to the mixture, lyzed by means of a French Press in buffer containing 20% sucrose, 10% glycerol, 50 mM Tris (pH 8.0), 0.2 mM Na₂S₂O₃, 2 mM MgCl₂, 2 mM EDTA, and 1% Triton X-100. GST-Rim was purified by incubation with glutathione-Sepharose 4B beads (Amersham Biosciences) by 1 h at 4 °C. The beads were washed three times with lysis buffer and stored at −20 °C in storage buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 50% glycerol.

Preparation of Zymogen Granules—In vitro GDP or GTP-γ-S protein loading was used for negative and positive controls (26, 29). 50 μl of 0.2 mM EDTA and 2 μl of 100 mM GTP-γ-S or 10 μl of 100 mM GDP were added to 1 ml of acinar lysate (0.5 mg protein concentration), and the mixtures were incubated at 30 °C for 30 min. The reaction was terminated by adding 60 μl of 1 x MgCl₂ and placing the samples on ice.

Densitometric analysis of appropriately exposed film was performed and quantified using BioRad Multi-Analyzer software. The amount of GST-Rim bound Rab3D was compared among different groups of samples and expressed as percentage of that of the control group.

Immunocytochemistry and Confocal Microscopy—Purified zymogen granules prepared from β-galactosidase (control) and Rab3D T36N adenovirus-infected acini were resuspended in a small volume of PBS and transferred onto microscope slides (Fisher Superfrost Plus). The granules were allowed to bind to slides for 10 min at room temperature in humid box, and then the PBS solution was replaced with 4% paraformaldehyde in PBS for 1 h at room temperature. After fixation, samples were rinsed with PBS, blocked with 5% normal goat serum in PBS for 30 min at room temperature, and then incubated with rabbit anti-Rab3D antibody diluted 1:200 or rat anti-HA antibody diluted 1:500 in PBS for 1.5 h at room temperature. After rinsing with PBS, and blocking with 2% normal goat serum in PBS, samples were incubated with Cy3-conjugated donkey anti-rabbit or anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in 2% normal goat serum in PBS. Slides were viewed with a Zeiss LSM 510 confocal microscope and digitized images were processed using Photoshop 6.0 software (Adobe Systems Inc., Mountain View, CA). For quantification of immunofluorescence, digital images from three microscopic fields for each viral construct were chosen at random from each of three independent experiments and analyzed using Metamorph 6.0.4 software (Universal Imaging, Inc., Malvern, PA). After background and any saturated pixels were excluded, the mean intensities from the separate fields were averaged. Results were expressed as percent of control (β-galactosidase) mean pixel intensity for the three experiments.

Subcellular Fractionation and Triton X-114 Phase Separation—Isolated pancreatic acini were resuspended in lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 2% glycerol supplemented with 0.2 mM Na₂S₂O₃ and 1 μM leupeptin and aprotinin and 1 mM phenylmethylsulfonlfluoride. The suspension was sonicated briefly and a PBS prepared by centrifugation for 10 min at 800 × g at 4 °C. High speed supernatants and membrane pellets, designated as cytosolic (C) and membrane (M) were obtained by centrifugation of the PBS at 100,000 × g for 45 min at 4 °C in a Beckman ultracentrifuge using a TL-100 4.5 rotor. The membrane pellet was dissolved in lysis buffer containing 0.5% Triton X-100 and centrifuged again at 20,000 × g to remove insoluble proteins.

To examine Rab3D isoprenylation, a Triton X-114 partitioning method was used as described previously (22, 31). Briefly, isolated acini
were resuspended in 1 ml of lysis buffer containing 25 mM Tris, 150 mM NaCl, 1% Triton X-114, 5 mM EDTA, and supplemented with the proteinase inhibitors and sonicated. The acinar lysate was then incubated at 37 °C for 2 min and the resulting turbid solution was centrifuged at 1000 × g for 2 min at room temperature. The upper clear aqueous phase was separated from the bottom turbid detergent phase. The two phases were then both brought to a 1-ml volume with 1% Triton X-114. Both phases were kept on ice until the solutions became clear. The above process was repeated two more times to wash away contaminants.

**RESULTS**

**GST-RIM Interaction with GTP-bound Rab3D in Acinar Lysate Can Be Used to Assess the GTP Binding Status of Endogenous Rab3D in Vivo**—To determine how the guanine nucleotide state of Rab3D influenced its interaction with Rim, different amounts (250, 500μg) of acinar lysate were treated to load all small G-proteins with GDP or GTPΓS as negative or positive controls, respectively, and then incubated with GST-Rim (1–399) in a “pulldown” protocol. Similar to prior studies with Rab3A (29), GST-Rim pulled down Rab3D when liganded with GTP but not with GDP (Fig. 1). This demonstrated that GST-RIM associated exclusively with the GTP-bound Rab3D and that the pull-down assay was able to detect the GTP-bound endogenous Rab3D. Moreover, the pull-down signals were proportional to the amount of total lysate. On the basis of these results, we compared the pull-down signal from the same amount of acinar lysate with or without GTPΓS loading. Assuming that GTPΓS activated all endogenous Rab3D and the signal can be used as 100%, we estimated that about 80% of the endogenous Rab3D was in active, GTP-bound conformation (Fig. 1). In four experiments, quantitative densitometry showed 78.9 ± 4.5% to be GTP-ligated (Table I). In separate subcellular fractionation experiments (Fig. 2A), we found that about 80% of total Rab3D was in the membrane fraction. The similarity of the percentage of GTP-bound and that of membrane-bound Rab3D led us to hypothesize that membrane-bound Rab3D was all GTP-bound while cytosolic Rab3D was GDP-bound. This hypothesis was tested by performing pull-down assays of Rab3D on the membrane and cytosolic fraction separately (Fig. 2, B and C). About 90% of membrane associated Rab3D was found to be GTP-ligated while only 2% of soluble Rab3D was GTP-ligated (Table I).

**Dominant Negative Rab3D Mutants Reduce GTP-bound Endogenous Rab3D in Acinar Cells**—We have reported that the dominant negative Rab3D mutants, Rab3D N135I and T36N, preferentially inhibited acinar amylase secretion (24). To address the mechanism by which dominant negative Rab3D mutants act, we examined the effect of the overexpressed wild-type and mutant Rab3D on the active GTP-bound state of endogenous Rab3D by use of the GST-RIM pulldown assay. The representative Western blot results are shown in Fig. 3. Because of the higher molecular weight introduced by the HA tag, anti-Rab3D antibody revealed two bands in each lane: the top band is overexpressed HA-tagged Rab3D, which may also be visualized by immunoblotting with anti-HA antibody, and the bottom is Rab3D, which does not react with anti-HA. None of the overexpressed Rab3D constructs affected the total amount of endogenous Rab3D (Fig. 3A). When the amount of GTP-bound Rab3D was evaluated using the GST-Rim pull-down, expression of wild-type Rab3D and the Q81L mutant had no effect, but both dominant negative Rab3D mutants, T36N and N135I greatly reduced GTP bound endogenous Rab3D (Fig. 3B). Quantitative data showed that the reduction was 70.0 ± 3.5% and 72.7 ± 1.2% respectively (Fig. 3C). One unexpected difference between T36N and N135I was that N135I interacted with GST-Rim as indicated in the Western blot following pull-down (Fig. 3B). This pull-down of N135I was independent of the presence of GDP or GTPΓS and appeared to result from guanine nucleotide-independent interaction of Rab3D N135I and Rim (data not shown).

**Dominant Negative Rab3D Mutants Did Not Affect Isoprenylation and Membrane Association of Endogenous Rab3D**—Early studies (4, 5) and our current results (Fig. 2) indicate that membrane association and GDP/GTP exchange of Rab proteins are closely coupled. We therefore tested whether or not the reduction of active Rab3D by dominant negative Rab3D mutants was due to the reduction of either isoprenylation or membrane association of endogenous Rab3D. The Triton X-114 partitioning method was used to address this issue since the...
isoprenylated Rab3D was expected to be in the detergent phase. As shown in Fig. 4A, in control acini, endogenous Rab3D was exclusively in the detergent phase consistent with a complete isoprenylation of Rab3D. In the acini expressing HA-tagged Rab3D constructs, neither the wild type nor the T36N mutant had an effect on the isoprenylation of endogenous Rab3D. Dominant negative Rab3D mutants did not affect isoprenylation and membrane association of endogenous Rab3D. A, to examine Rab3D isoprenylation, the Triton X-114 partitioning method was used. Acini infected with control, wild-type, or Rab3D T36N mutant virus were lysed, and the upper clear aqueous phase (A) was separated from the bottom turbid detergent phase (D). Both phases were brought to equal volumes, run on SDS-PAGE and blotted with anti-Rab3D antibody. B, high speed supernatants and membrane pellets were obtained by centrifugation. The resulting membrane pellets were then solubilized. The total amount of endogenous Rab3D in the supernatant or membrane pellet was then compared separately between acini infected with control virus and with Rab3D T36N virus on Western blot. Results shown are representative of at least three independent experiments. Statistical significance was calculated using Student’s t test with **p < 0.01** representing significance indicated by *.

Fig. 3. Dominant negative Rab3D mutants reduced GTP-bound endogenous Rab3D in acinar cells. Isolated acini were incubated with 10⁶ pfu/ml control β-galactosidase (cont), wild-type Rab3D, or mutant Rab3D adenoviruses for 16 h. Acini were then lysed and divided into two aliquots. One-half was used as a total lysate for analysis on 12% SDS-polyacrylamide gels (A), and the other half was used for the GST-RIM pull-down assay (B). In Western blot experiments, anti-Rab3D antibody revealed two bands in each lane: the top corresponding to overexpressed HA-tagged Rab3D and the bottom to endogenous Rab3D. C, averages of GTP-bound endogenous Rab3D in pancreatic acinar cells infected with different Rab3D constructs were compared with that of control and expressed as percentage of control. The results shown are means and S.E. for four independent experiments. Statistical significance was calculated using Student’s t test with **p < 0.01** representing significance indicated by *.

Fig. 4. Dominant negative Rab3D mutants did not affect isoprenylation and membrane association of endogenous Rab3D. A, to examine Rab3D isoprenylation, the Triton X-114 partitioning method was used. Acini infected with control, wild-type, or Rab3D T36N mutant virus were lysed, and the upper clear aqueous phase (A) was separated from the bottom turbid detergent phase (D). Both phases were brought to equal volumes, run on SDS-PAGE and blotted with anti-Rab3D antibody. B, high speed supernatants and membrane pellets were obtained by centrifugation. The resulting membrane pellets were then solubilized. The total amount of endogenous Rab3D in the supernatant or membrane pellet was then compared separately between acini infected with control virus and with Rab3D T36N virus on Western blot. Results shown are representative of at least three independent experiments.
Rab3D. However, a significant portion of overexpressed Rab3D from both wild-type and T36N were in the aqueous phase. Similar partitioning of Rab3D N135I mutant was also seen (data not shown). This could be due to a limited capacity for isoprenylation, or more likely the isoprenyl group was not as well protected in the cytosol as on the membrane. In either case, this result demonstrated that dominant negative Rab3D did not interfere with the isoprenylation of endogenous Rab3D.

We then examine the effect of dominant negative Rab3D on the membrane association of endogenous Rab3D using a subcellular fractionation method. As shown in Fig. 4B, the amount of membrane associated endogenous Rab3D was the same in control and T36N virus infected acini. On the other hand, T36N itself was largely cytosolic, which is consistent with the basolateral cytosolic localization demonstrated in our previous immunocytochemistry studies (24). These data demonstrated that dominant negative Rab3D mutant did not affect membrane association of endogenous Rab3D.

**Dominant Negative Rab3D Significantly Reduced the Active State but Not the Total Amount of Endogenous Rab3D on Purified ZGs**—The precise localization of Rab proteins to the correct organelle or membrane compartment is essential for their functions in regulating vesicular trafficking. Previous studies from our laboratory and others had shown that endogenous Rab3D was highly enriched on zymogen granule membrane in acinar cells (22, 32). We therefore examined the effect of dominant negative Rab3D mutants on the ZG targeting of endogenous Rab3D. In these experiments purified ZG were prepared from mouse acini by Percoll gradient ultracentrifugation. The ZGs were then lysed and divided with one-half used to determine the total relative amount of Rab3D on ZGs, and the other half used for the GST-RIM pull-down assay to measure the amount of active Rab3D on ZGs. Compared with control, dominant negative Rab3D mutant T36N did not significantly reduce the total amount of endogenous Rab3D on ZGs, but dramatically reduced the amount of GTP-bound endogenous Rab3D by about 70% (Fig. 5). It is worth noting that compared with the total acinar lysate where the amount of HA-tagged Rab3D T36N was 2–3-fold that of endogenous Rab3D (left panel), the T36N was almost undetectable on purified ZGs, which is in agreement with its deficiency in ZG targeting. In a limited number of experiments similar results showing a reduction in active Rab3D without a change in total Rab3D present on ZGs was seen after expression of the N135I mutant (data not shown).

To address the amount of Rab3D on ZGs by an alternative and more direct way, we used an immunocytochemical approach on purified ZGs. Immunostaining of ZGs with anti-HA antibody (Fig. 6, A and C) confirmed that wild-type HA-tagged Rab3D targeted to the granules, whereas there was essentially no granule staining for HA when acini were infected with Rab3D T36N. There were comparable numbers of ZGs in both wild-type and T36N groups as indicated in Normarski images (Fig. 6, B and D). We next immunostained the purified ZGs for endogenous Rab3D, and then compared the immunofluorescence of ZGs from control and T36N adenovirus infected acini by confocal microscopy (Fig. 7, A and C). The purity of these ZG preparations was indicated by the corresponding Nomarski images (Fig. 7, B and D), while the fluorescence images showed that the majority of ZGs were immunoreactive for Rab3D. As expected from the previous study with intact acini (24), Rab3D was localized to ZG membranes (Fig. 7, A and C). Compared with ZG from β-galactosidase controls (Fig. 7A), there was no obvious reduction in Rab3D staining of ZGs from T36N-infected acini (Fig. 7C). The average immunofluorescence pixel intensity in confocal images from β-galactosidase and T36N-infected acini were then quantified (see “Experimental Procedures”), and these results together with those from biochemical studies on the effect of dominant negative Rab3D are summarized in Table II. While the total cellular and ZG Rab3D were essentially unchanged (about 95% of control), the GTP-bound Rab3D on ZGs in T36N-infected acini was only 21.4% ± 3.3% of

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**Fig. 5.** Dominant negative Rab3D significantly reduced the active state but not the total amount of endogenous Rab3D on purified ZGs. Isolated pancreatic acini were infected overnight with control β-galactosidase or Rab3D T36N virus. A, total lysates compared by Western blotting using anti-Rab3D antibody. B, ZG were purified using a Percoll gradient and lysed. One-half was used as total ZG lysates to run directly on 12% SDS-polyacrylamide gels (ZG-total) and the other half was used for the GST-RIM pull-down assay (ZG-pull-down). Results shown are representative of at least three independent experiments.

**Fig. 6.** Confocal immunofluorescence staining of HA-tagged Rab3D on purified ZGs. Purified ZGs from wild-type or T36N Rab3D adenovirus-infected acini were stained with anti-HA antibody to detect the overexpressed Rab3D (A and C). Corresponding Nomarski images are also shown (B and D). The wild-type Rab3D group showed strong staining (A), whereas there was no detectable signal in Rab3D T36N virus-infected acini (C).
control. Taking all these results together, it can be concluded that dominant negative Rab3D mutants blocked the activation but not the ZG targeting of endogenous Rab3D. This results in mainly the GDP bound, inactive form of Rab3D on ZGs, thereby providing a mechanism by which the dominant negative Rab3D mutants inhibited the acinar secretion.  

Table II  
The effect of dominant negative Rab3D T36N on total and active endogenous Rab3D on ZGs

|                      | % control |
|----------------------|-----------|
| Total cellular Rab3D (Western blot) | 95.9 ± 4.5 |
| Rab3D on ZGs (Western blot)          | 84.3 ± 9.1 |
| Rab3D on ZGs (immunostaining)        | 94.5 ± 5.9 |
| GTP-bound Rab3D on ZGs               | 21.4 ± 3.3* |

DISCUSSION  

In this study, we developed a GST-Rim affinity precipitation assay for active Rab3D to examine the effect of dominant negative Rab3D mutants on the active state of endogenous Rab3D. We verified this newly developed assay to demonstrate that the GST-Rim construct associated exclusively with the GTP-bound Rab3D and that the pull-down assay was able to detect the GTP-bound endogenous Rab3D. GST-Rim only pulled down Rab3D from GTP-bound endogenous Rab3D on ZGs, whereas membrane- and cytosolic Rab3D was predominantly GDP-bound, whereas ZG-associated Rab3D was ~50% GTP-bound (36). Our results are in general agreement with the results from these studies. Two major conclusions thus can be derived from these studies and might be generalized to fit other Rab’s in other systems: 1) a high percentage of Rab proteins are maintained in the active GTP-ligated state; 2) all of the active Rab proteins are associated with membrane, whereas the cytosolic pool of Rab’s are inactive.

In a previous study, we reported that both Rab3D mutants, N135I and T36N, functioned as dominant negative mutants and inhibited regulated exocytosis in pancreatic acinar cells (10). This result is consistent with the findings using equivalent mutants in many other Rab proteins including Rab1 (38), Rab5 (39), Rab7 (40), Rab9 (41), Rab11 (42), Rab27a (43), and yeast Rab homologues SEC4p (44) and Ypt1 (45). According to the observations in our current study, we developed a tentative model on how the dominant negative Rab3D mutants might act in acinar cells. Due to their reduced nucleotide binding and thus higher tendency to attain the guanine nucleotide-free conformation, the two dominant negative Rab3D mutants compete for binding to a yet unidentified Rab3 GEF with much greater affinity than the endogenous Rab3D. Because their basolateral localization, they sequester the GEF in the cytoplasmic region and therefore prevent the activation of endogenous Rab3D and result in inactive Rab3D on ZGs, which then cannot carry out their normal function in acinar secretion.

In the case of Ras, a consensus has been reached that the dominant-inhibitory mutants work in cells by competing with normal Ras for binding to RasGEFs in their nucleotide-free conformations. The “dead-end” complexes thus prevent the activation of endogenous Ras by RasGEFs (46). The dominant negative Rab mutants are believed to act similarly (40, 43, 45, 47, 48), and two studies have shown that dominant negative Rab3A (49) and Ypt1 (45) inhibited GEF stimulated guanine nucleotide exchange on corresponding wild-type Rab protein using in vitro GDP release and GTP uptake assays. In the present study, we demonstrated that two dominant negative mutants of a small G-protein, Rab3D, dramatically reduced (about 70%) the GTP-bound endogenous Rab3D in pancreatic acinar cells, and thus provided the most direct in vivo evidence supporting the above hypothesis on how dominant negative Rab mutants function in general. It is worth noting that on one hand, N135I reduced active endogenous Rab3D, on the other hand, it can still interact with potential effectors such as Rim, although in a guanine nucleotide independent manner. However, because of its mislocalization in the cell, this mutant cannot substitute for the normal function of endogenous Rab3D. Whether this could result in sequestering the corresponding Rab3D effectors away from ZGs as an additional inhibitory mechanism remains to be determined.

According to the current model of Rab GDP/GTP and mem-
brane/cytosol cycling, the target membrane association and GDP/GTP exchange of Rab proteins are closely coupled but distinguishable steps and the GDP/GTP exchange reaction does not seem to be a prerequisite for the Rab protein binding to the target membranes. This is supported by the findings that the GDP-bound form can be transiently detected in the membranes after delivery of Rab protein-Rab GDI complexes to membranes (4, 5). Consistent with this model, both Western blot and immunocytochemistry methods showed that dominant negative Rab3D significantly reduced the active state but not the total amount of endogenous Rab3D on purified ZGs. This result indicates that in acinar cells overexpressing dominant negative Rab3D the majority of endogenous Rab3D on ZGs are indeed in GDP-bound form. Accordingly, under the circumstance in our study, the target membrane association and GDP/GTP exchange of Rab3D were uncoupled, possibly because the dominant negative mutants sequestered corresponding GEF in the cytosol and resulted in the deficiency in GEF activity and thus Rab3D activation on ZGs. These results are also consistent with two relevant studies on GEF deficiency. In one study, it was found that Sec2p was a GEF for Sec4p, and Sec4p was still on secretory vesicles in sec2 mutants (50). In the other, no difference in Rab3A distribution was observed between Rab3 GEF knockout and wild type embryos (51). Since it is commonly believed that GDP-bound Rab3s are the target for rabGDI retrieval, it is not clear why the GDP-bound Rab3D could be stably present on ZGs. As proposed by Luan (10), this might be because distinct Rab3s are differentially sensitive to GDI or because another recycling factor required for GDP Rab extraction is not present on ZGs, but rather present on the acceptor membrane, in this case, the apical plasma membrane.

We have shown here that GDP-bound Rab3D can still localize to ZGs but T36N cannot. Currently we do not know the exact reason for its mislocalization, but a study of equivalent Sec4p mutants provides some insight (48). It was shown in this study that neither Sec4p T34N nor N133I interacted with the Sec4p mutants provides some insight (48). It was shown in this study that neither Sec4p T34N nor N133I interacted with the Sec4p mutants. In this report, we have shown that dominant negative Rab3D mutants can interact with Rab GDI isoform expressed in pancreatic acinar cells and rather acts as a regulator in acinar cells. However, it is not yet known at which step, namely tethering, docking or formation of the SNARE complex, Rab3D exerts its regulatory function. Recently, evidence has accumulated for a link between Rab and secretory vesicle positioning near the targeting membrane. In AtT-20 cells expressing dominant negative Rab3D, the majority of dense core granules are scattered in the cytoplasm (20). Similarly, in Rab3 GEP−/− embryos, most of the synaptic vesicles were located apart from the presynaptic plasma membrane, indicating that they did not readily undergo exocytosis (51). However, dominant negative Rab3D had little effect on the ZG distribution in acinar cells as the gross distribution of ZGs was not altered in semi-thin sections stained with toluidine blue. Further studies at the EM level will be needed to examine the positioning of ZGs in the vicinity of the apical plasma membrane.

With regard to the mechanism by which Rab proteins might mediate secretory vesicle positioning, recent studies have demonstrated that the localization of myosin Va on melanosomes was Rab27a-dependent, and myosin Va and Rab27a interacted indirectly in a GTP-dependent manner (43, 53) through melanosin (54), a Rab27a effector with homology to the Rab3a effector Rabphilin. In close parallel with Rab27a, recent investigations suggested that GTP-bound Sec4p was an essential component of the secretory vesicle receptor for Myo2p, and formed a complex with Myo2p on vesicle membranes (55). Although this may not be a general model for Rab3 function in the post-Golgi transport step, the function of Rab27 in melanocytes provides a clue for the potential mechanism by which Rab3D may act in acinar cells. Whether the localization of any of these myosins on ZGs is dependent on Rab3D, or whether Rab3D can interact with any myosin motor in a GTP-dependent manner, is an interesting future direction for investigation.

In conclusion, in the current study, we addressed the mechanism by which the dominant negative Rab3D mutants interfere with endogenous Rab3D. By using the GST-RIM pull-down assay, we found that Rab3D existed on zymogen granules in GDP-bound form in normal acinar cells. When the dominant negative Rab3D mutants were overexpressed, the GTP-bound endogenous Rab3D was dramatically reduced. Dominant negative Rab3D mutants did not affect the ZG association of Rab3D and thus left the inactive endogenous Rab3D on ZGs, which could not exert their normal function in acinar secretion.

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