Redistribution of a Rab3-like GTP-binding Protein from Secretory Granules to the Golgi Complex in Pancreatic Acinar Cells during Regulated Exocytosis

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Abstract. Regulated secretion from pancreatic acinar cells occurs by exocytosis of zymogen granules (ZG) at the apical plasmalemma. ZGs originate from the TGN and undergo prolonged maturation and condensation. After exocytosis, the zymogen granule membrane (ZGM) is retrieved from the plasma membrane and ultimately reaches the TGN. In this study, we analyzed the fate of a low M_r GTP-binding protein during induced exocytosis and membrane retrieval using immunoblots as well as light and electron microscopic immunocytochemistry. This 27-kD protein, identified by a monoclonal antibody that recognizes rab3A and B, may be a novel rab3 isoform. In resting acinar cells, the rab3-like protein was detected primarily on the cytoplasmic face of ZGs, with little labeling of the Golgi complex and no significant labeling of the apical plasmalemma or any other intracellular membranes. Stimulation of pancreatic lobules in vitro by carbamylcholine for 15 min, resulted in massive exocytosis that led to a near doubling of the area of the apical plasma membrane. However, no relocation of the rab3-like protein to the apical plasmalemma was seen. After 3 h of induced exocytosis, during which time ~90% of the ZGs is released, the rab3-like protein appeared to translocate to small vesicles and newly forming secretory granules in the TGN. No significant increase of the rab3-like protein was found in the cytosolic fraction at any time during stimulation. Since the protein is not detected on the apical plasmalemma after stimulation, we conclude that recycling may involve a membrane dissociation-association cycle that accompanies regulated exocytosis.

A great deal of information has recently been obtained concerning the mechanism of exocytosis from eukaryotic cells. All cells, including simpler systems such as yeast, use the "constitutive" pathway where exocytotic vesicles continuously fuse with the plasma membrane. The regulated pathway (as occurs in pancreatic acinar cells) requires that proteins destined for release are temporarily stored in granules whose contents are discharged in response to secretagogues (Burgess and Kelly, 1987). It has become clear that the membrane fusion steps along the secretory pathway and on the returning endocytic loop are regulated by members of the rab family of low M_r GTP-binding proteins (M_r 20-28 kD) which are related to *ras* (Hall, 1990; Novick et al., 1988; Takai et al., 1992; Balch, 1990). Studies in yeast have determined that SEC4 encodes a *ras*-like 23.5 kD GTP-binding protein, Sec4p, that is associated with secretory vesicles, and, along with other interacting proteins, is responsible for membrane targeting and

fusion. Mutations in the *SEC4* gene cause defects in exocytosis that leads to the post-Golgi accumulation of secretory vesicles (Walworth et al., 1989).

Several low M_r GTP-binding proteins belonging to the *ras* superfamily and termed rab proteins have been identified (Touchot et al., 1987; Chavrier et al., 1990b) and are associated with compartments involved in the exocytic and endocytic pathways, where they are presumed to regulate membrane traffic (Chavrier et al., 1990a; Chavrier et al., 1990b; Pfeffer, 1992; Balch, 1990; Takai et al., 1992). Rab3A, a low M_r GTP-binding protein that is preferentially localized to neuronal and neuroendocrine tissues (Zahraoui et al., 1989; Fischer von Mollard et al., 1990; Touchot et al., 1987), has been found by immunoblots and immunoelectron microscopy to be localized selectively to post-Golgi synaptic vesicles (Fischer von Mollard et al., 1990; Matteoli et al., 1991). It is also found in regulated secretory cells, including pancreatic islets and acinar cells (Mizoguchi et al., 1989). Rab3A has been shown to associate with the synaptic vesicle membrane which relocates to the cell surface and not to the Golgi complex during massive stimulation of the frog neuromuscular junction (Matteoli et al., 1991). Finally, studies

using isolated synaptosomes show a decrease in rab3A immunoreactivity on synaptic vesicle membranes as a consequence of neurotransmitter release (Fischer von Mollard et al., 1991), suggesting the involvement of rab3A in regulated exocytosis of neurotransmitters.

Previous studies in our laboratory (Padfield and Jamieson, 1991) and others (Lambert et al., 1990; Schnefel et al., 1992) have demonstrated the association of at least seven low M_r GTP-binding proteins (29–21.5 kD) with zymogen granule membranes (ZGM)[1] by [α-^{32}P]GTP-overlay assays. Subsequent immunoblot analysis of purified ZGMs using different rab antibodies showed that rab3 immunoreactivity was associated with zymogen granules (ZG) and ZGMs ([Schnefel et al., 1992] and the present study). More recently, we (Padfield et al., 1992) have shown that introduction of peptides from the effector domain of rab3A into permeabilized pancreatic acinar cells stimulated amylase release, suggesting the involvement of a rab3-like protein in regulated secretion. To further determine the role of a rab3-like protein in regulated exocytosis, we examined its subcellular distribution in pancreatic acinar cells before and after stimulation of secretion by carbamylcholine. The results of our studies indicate that a rab3-like protein is associated with ZGs in resting acinar cells and appears to undergo relocation to the Golgi complex after near complete exocytosis of ZGs.

Materials and Methods

Reagents

Sucrose was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Hepes and Trasylol were from Boehringer Mannheim (Indianapolis, IN). PMSF, carbamylcholine, NP-40, protein A-Sepharose, and benzamidine were from Sigma Chem. Co. (St. Louis, MO). Soybean trypsin inhibitor (STI) was purchased from Calbiochem Co. (LaJolla, CA). Glycine was from Bio-Rad Labs. (Richmond, CA). Tris and agarose (SeaKem LE) were purchased from American Bioanalytical Co. (Natick, MA). X-OMAT-AR and TMAX films and Triton X-100 were from the Eastman Kodak Co. (Rochester, NY). Cronex Lightning Plus intensifying screens were from E. I. Du Pont De Nemours and Co. (Wilmington, DE). [α-^{32}P]GTP and molecular weight markers were purchased from Amersham Corp. (Arlington Heights, IL). 0.2 μm nitrocellulose sheets were from Schleicher and Schuell Inc. (Keene, NH). Glutaraldehyde, paraformaldehyde, osmium tetroxide, and embedding media were from Electron Microscopy Sciences (Fort Washington, PA). AquaPolymount was from Polysciences (Warrington, PA).

Animals

Sprague-Dawley rats were purchased from Camm Research Institute (Wayne, NJ), and were starved overnight with water given ad libitum before each experiment.

Antibodies

Monoclonal antibodies (clones 42.1 and 42.2), generated against recombinant rab3A, were described earlier (Matteoli et al., 1991). Clone 42.2 was demonstrated to be specific for rab3A while the rab3A/B monoclonal antibody (clone 42.1) recognized recombinant rab3A, rab3B, and rab3C. Polyclonal rabbit antibodies specific for exocrine secretory granule membrane proteins were a gift from Dr. J. David Castle, University of Virginia and Dr. Richard Cameron, Yale University. These antibodies were raised against membrane proteins and from rat parotid secretory granules and recognize granules from parotid, submandibular, and lachrymal glands as well as the exocrine pancreas (Cameron et al., 1986). Affinity purified rab-

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1. *Abbreviations used in this paper:* STI, soybean trypsin inhibitor; ZG, zymogen granules; ZGM, zymogen granule membranes.

bit anti-mouse IgG and Texas red-conjugated goat anti-rabbit IgG was purchased from Cappel Organon Teknika Corporation (West Chester, PA). 5 and 10 nm gold-labeled goat anti-mouse IgG(Fc) and ^{125}I-labeled sheep anti-mouse IgG were purchased from Amersham Corp.

Rabbit anti-mouse IgG in PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.2 mM Na_2HPO_4, pH 7.4) was bound to protein A-Sepharose in PBS for 2 h at room temperature to give a final antibody concentration of 50 μg/ml in a 1:1 slurry. After adsorption, the protein A-Sepharose-antibody complex was washed twice with PBS and resuspended in PBS to make a 1:1 slurry.

Cell Fractionation

Rat pancreatic ZGMs were prepared and their purity determined as described earlier (Cameron et al., 1986; Jena et al., 1991). Homogenates of rat brain were prepared in 25 mM Hepes, pH 7.4 containing 0.3 M sucrose, 0.5 mM $MgCl_2$, 1 mM benzamidine, 0.5 mM PMSF, 0.1% wt/vol. Trasylol and 0.01% STI.

Preparation and Stimulation of Pancreatic Lobules

Pancreatic lobules were prepared according to published methods (Amsterdam et al., 1978). For each experiment, the pancreas of a male Sprague-Dawley rat (100–150 g; starved overnight with water given ad libitum) was used. 25–30 lobules were used for each experimental condition. The incubation medium, saturated with O_2, consisted of 20 mM glucose, 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM Hepes, pH 7.4, 0.1% BSA, and 0.01% STI without or with 1 μM carbamylcholine.

To study the localization of rab3-like immunoreactivity after stimulation of rat pancreatic lobules, the lobules were incubated with or without 1 μM carbamylcholine and processed for light or electron microscopy or homogenized in 5 vol of homogenization buffer for immunoblot analysis. The homogenization buffer contained 25 mM Hepes, pH 6.8, 0.3 M sucrose, 0.5 mM $MgCl_2$, 1.0 mM benzamidine, 0.5 mM PMSF, 0.1% wt/vol. Trasylol and 0.01% STI. Lobules were homogenized using 10 strokes of a chilled 30-ml Brendler size B homogenizer (0.13–0.18 mm clearance; A. H. Thomas, Philadelphia, PA) and centrifuged at 200,000 g for 1 h at 4°C, using a Beckman TLA-100.2 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA).

Immunoprecipitation and GTP-Overlays

Proteins were immunoprecipitated from purified ZGMs, brain homogenates, and from fractions obtained from stimulated or resting pancreatic lobules using the monoclonal antibody against rab3A/B. Fractions in PBS containing 1% SDS were boiled for 5 min, diluted 10-fold in cold PBS, and clarified by centrifugation at 13,000 g for 5 min. The solubilized and clarified material was incubated with the rab 3A/B antibody (1:100 dilution) for 12 h at 4°C with mild agitation. Protein A-Sepharose-rabbit anti-mouse antibody conjugate was then added and incubation continued for 2 h at room temperature. The resultant protein A-Sepharose-antibody-antigen complex was washed twice with 20 vol of 400 mM NaCl, 1% NP-40 in 50 mM Tris-HCl, pH 7.5, and once with 20 vol of 500 mM NaCl, 2 mM EDTA and 10 mM Tris-HCl, pH 7.5. After washing, antigens were eluted from the beads with Laemmli reducing sample preparation buffer (Laemmli, 1970) containing 1% β-mercaptoethanol for 30 min at room temperature. The beads were removed by centrifugation at 13,000 g for 5 min and the supernatant was boiled for 5 min. 25 μl of eluate were analyzed by SDS-12.5% PAGE (Laemmli, 1970) followed by electrotransfer to 0.2 μm nitrocellulose sheets. [α-^{32}P]GTP-overlays were carried out as described before (Padfield and Jamieson, 1991; Lapetina and Reep, 1987) except that 10 μM ATP was present in the binding buffer. [α-^{32}P]GTP-binding was detected by autoradiography of overlays.

Immunoblot Analysis

Pancreatic fractions or brain homogenates boiled in Laemmli reducing sample preparation buffer (Laemmli, 1970) and clarified by centrifugation at 13,000 g for 5 min, were resolved on SDS-12.5% PAGE. Proteins were electrotransferred to 0.2 μm nitrocellulose sheets, incubated for 1 h at room temperature in blocking buffer (5% non-fat milk protein in PBS containing 0.1% Triton X-100 and 0.02% NaN_3), and immunoblotted with the rab3A/B monoclonal antibody at a 1:1,000 dilution in blocking buffer for 4 h at room temperature. ^{125}I-labeled sheep anti-mouse secondary antibody (0.1 μCi/ml) in blocking buffer was used to probe the primary antibody

for 4 h at room temperature. Immunoblots were exposed to X-OMAT-AR film with intensifying screens at −70°C for times indicated in the figure legends.

Processing of Pancreatic Lobules for Light and Confocal Microscopy

After incubation, lobules were rinsed in ice-cold PBS for 30 s and fixed for 2 h in ice-cold fixative (3% paraformaldehyde and 0.25% glutaraldehyde in PBS). The lobules were then washed and stored in ice-cold 50 mM Tris-HCl, pH 7.4, before cryosectioning. Either 8 μm or 25 μm thick frozen sections were prepared using a Reichert-Jung 2800 Frigocut cryostat (Cambridge Instruments, GmbH, Heidelberg, Germany) and placed on chromalum-gelatin-coated slides. The tissue sections were washed with 0.1 M TBS (100 mM NaCl in 100 mM Tris-HCl, pH 7.4) for 2 min, followed by 1% NaBH₄ for 10 min and water rinsed. The sections were then incubated for 1 h with 0.1% BSA in TBS (to block non-specific binding sites) before a 1-h incubation at room temperature with primary antibodies (either rab3A/B or ZGM antibodies) diluted in 0.1% BSA-TBS. The sections were then washed in 0.01% BSA-TBS and incubated with secondary Texas red-conjugated antibodies, diluted in 0.1% BSA-TBS, followed by washing with 0.01% BSA-TBS and coverslipped using AquaPolymount. The 8-μm sections were viewed by epifluorescence microscopy using a Zeiss D7082 Axiophot Photomicroscope (Carl Zeiss, Inc., Thornwood, NY) and photographed on Kodak TMAX film. The 25-μm sections were viewed on a MRC-600 confocal laser scanning microscope (Bio-Rad Microscience Division, Cambridge, MA). Specimens were excited at 488 nm by an argon/krypton laser using a rhodamine filter (680EF-32) and observed with a 63× objective; images of 1.5 μm thick optical sections were stored on an optical disk. Images on the computer monitor were photographed using a Nikon F3 camera on Kodak TMAX film.

Immunoelectron Microscopy

Immunogold labeling of 1% agarose embedded rat pancreatic tissue fragments was performed using a modification of a published technique (De Camilli et al., 1983). After incubation, stimulated and unstimulated lobules were lightly homogenized in 5 vol of homogenization buffer, using two strokes in a 30-ml type B Brendler hand-held homogenizer. The homogenization buffer consisted of 250 mM sucrose, 25 mM KCl, 5 mM MGCl₂, 2 mM EGTA and 10 mM sodium phosphate, pH 6. The homogenized tissue was added to 15 vol of ice-cold isotonic fixative containing 3% paraformaldehyde, 0.25% glutaraldehyde, 5 mM sodium phosphate, pH 6 in 0.3 M sucrose, and kept on ice for 30 min with intermittent mixing before centrifugation at 20,000 g for 45 min at 4°C. The resulting pellet was resuspended in 4 vol of ice-cold, 120 mM NaH₂PO₄, pH 7.4 and embedded in agarose. Primary antibodies, diluted 1:30 in 5% BSA in buffer A (0.5 M NaCl in 20 mM NaH₂PO₄, pH 7.4), were allowed to diffuse into the agarose blocks for 12 h at room temperature. The blocks were washed five times in 10 vol of Buffer A over 1.5 h, and then incubated for 6 h at room temperature in goat anti-mouse IgG coupled to 5 or 10 nm colloidal gold diluted 1:10 in 5% BSA in Buffer A. The agarose-tissue blocks were fixed again with 1% glutaraldehyde, osmicated, dehydrated, and embedded in Epon. Epon blocks were sectioned, stained with uranyl acetate and lead citrate, and observed and photographed on a Philips EM410 transmission electron microscope (Philips Inc., Eindhoven, The Netherlands).

Immunogold Quantification

The relative distribution of immunogold over organelles in acinar cells was determined on micrographs from two separate experiments. Fields were photographed at 7,100× and enlarged to 21,300×. Immunogold distribution was measured on at least five micrographs from each experimental condition. Micrographs were selected for quantification if an acinar lumen, a nuclear profile, and elements of the Golgi complex were visible, thus defining the apical/basolateral axis of the cell. Additionally, micrographs were counted only if the basolateral plasmalemma was visible and morphologically disrupted in order to ensure that absence of labeling was not due to poor access of primary antibody or immunogold. Gold particles were assumed to be associated with a membrane or organelle if they were within 1 mm (=50 nm) of the structures in the print. The following organelles/membranes were analyzed: ZGs, the Golgi complex, the RER, mitochondria, and the plasmalemma, both apical and basolateral. Elements of the Golgi complex were outlined on the micrographs before counting gold particles. In resting acinar cells, the Golgi complex includes stacked cisternae and smooth-surfaced vesicles. In stimulated cells the Golgi complex also includes pre-secretory granules and vesicles on the trans-side of the Golgi, which contain prematurely concentrated secretory proteins. Gold particles that were associated with unidentifiable structures or the cytoplasmic matrix were assigned to an "unknown" category; under all conditions, 3–8% of the gold particles fell into this category. An average of ∼1,600 gold particles were counted in all electron micrographs used for analysis of experimental conditions. Controls consisted of elimination of the primary antibody in the incubations. Gold particles were not associated with specific structures in controls and the number of particles per micrograph was much smaller than present in the least labeled experimental micrographs. Data were expressed as percent distribution of gold particles over cell structures.

Surface Area of Membranes

Morphometry was performed on electron micrographs of tissue samples from different treatments using published methods (Weibel and Bolender, 1973). The same micrographs used for quantitation of immunogold distribution were used for assessment of surface area (S_v) of subcellular membranes. Micrographs were overlaid with a 1 × 1 cm transparent grid that covered the micrograph. Intersections of membrane profiles with all lines in the grid were used to calculate S_v.

Statistical Analysis

Statistical analyses of % gold distribution was performed using descriptive statistics and paired sample t-tests.

Results

Rab3-like GTP-binding Proteins Are Associated with ZGs in Pancreatic Acinar Cells

To determine the distribution of the rab3-like protein in the rat pancreas, we carried out immunofluorescent light microscopy on 8-μm thick cryosections of pancreatic tissue using the rab3A/B and rab3A monoclonal antibodies. As shown in Fig. 1, the rab3A/B antibody stains both exocrine (apical regions of acinar cells) and endocrine tissue (Fig. 1 a) while the rab3A monoclonal antibody stains only islets (Fig. 1 b). These results indicate that the rab3A/B antibody could be used to study the subcellular distribution of the acinar cell rab3-like antigen.

Immunoblots and GTP-Overlays of Immunoprecipitates: Unstimulated Cells

The association of a rab3-like GTP-binding protein with ZGMs in pancreatic acinar cells was examined using immunoblots and GTP-overlays on proteins immunoprecipitated by the rab3A/B monoclonal antibody. Immunoblots of pancreatic homogenates and ZGMs, and of brain homogenates, revealed two bands, one at ∼26 kD in brain homogenates and another at ∼27 kD in pancreatic ZGMs (Fig. 2 a). The 27-kD protein in ZGMs was also detected in pancreatic homogenates but at greatly reduced levels. Immunoprecipitation of ZGMs using the rab3A/B monoclonal antibody followed by GTP-overlays detected a 27-kD GTP-binding protein (Fig. 2 b) which was enriched relative to the pancreatic homogenate. GTP-overlays of proteins immunoprecipitated from the brain with the rab3A/B antibody detected a 26-kD GTP-binding protein. Immunoblots of brain and pancreatic homogenates and of ZGMs using the rab3A monoclonal antibody revealed a band of ∼26 kD in the brain but did not detect immunoreactive material in the pancreas (data not shown). Immunoblots of rat pancreatic islets and brain homogenates probed with the rab3A and rab3A/B antibodies,
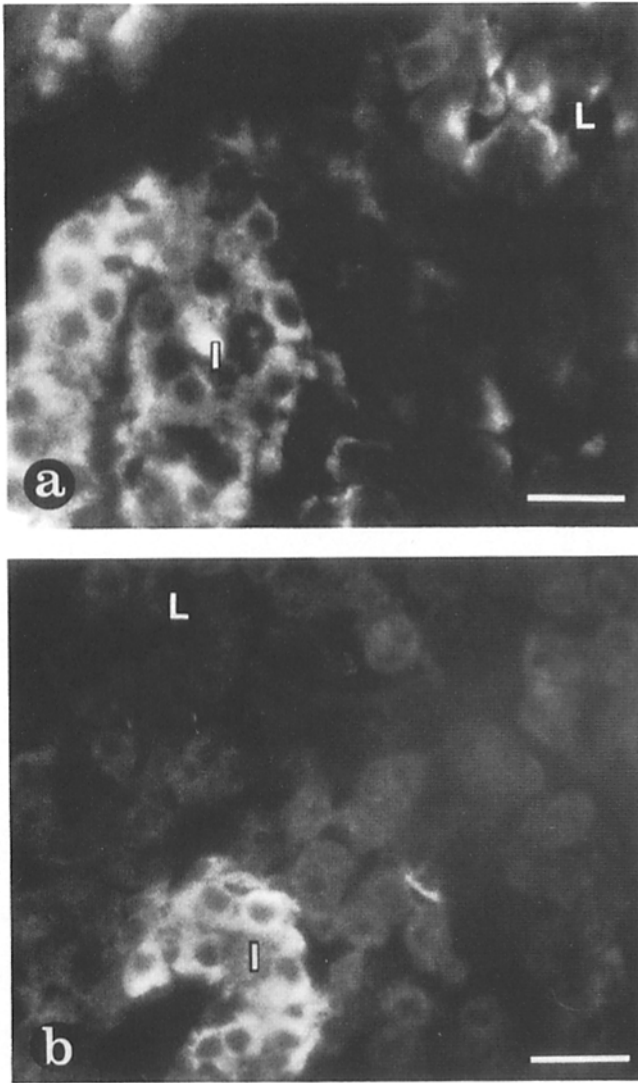
Figure 1. Immunofluorescent localization of rab3 isoforms in rat pancreas on eight μm thick frozen sections of rat pancreas. (*a*) Sections stained with the rab3A/B antibody. Note staining with this antibody of pancreatic islets (*I*) and the apical regions of acinar cells adjacent to the acinar lumen (*L*). (*b*) Sections stained with the rab3A monoclonal antibody show exclusive localization to islet cells (*I*). Bar, 10 μm.

showed that both antibodies recognized proteins of identical M_r (∼26 kD) in brain homogenates and islets (data not shown). Since we do not know the nature of the 27-kD GTP-binding protein that is immunoprecipitated by the rab3A/B monoclonal antibody, we will refer to it in this paper as a rab3-like protein.

Light and Electron Microscopic Immunocytochemistry: Unstimulated Cells

Light and EM immunocytochemistry were carried out on unstimulated pancreatic lobules in order to determine the distribution of rab3-like proteins in resting acinar cells. Fig. 3 *a* shows the distribution of rab3-like antigen in unstimulated pancreatic lobules using confocal immunofluorescence light microscopy. The apical to basolateral axis of several of the cells can be identified by the orientation of the negative nu-
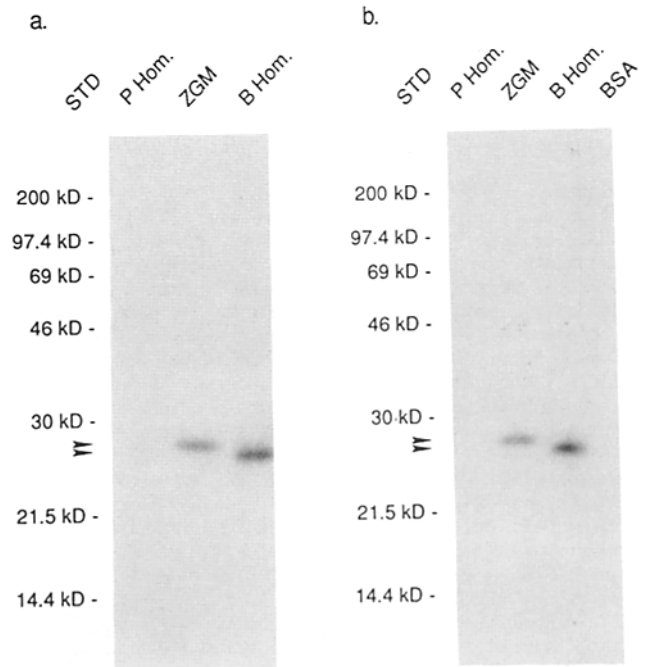

Figure 2. Identification of the rab3A/B immunoreactive antigen in exocrine pancreas as a rab3-like, GTP-binding protein. (*a*) Immunoblot analysis. Five μg each of pancreatic homogenates (*P Hom.*), ZGMs (*ZGM*), and brain homogenates (*B Hom.*) were subjected to immunoblot analysis using the rab3A/B monoclonal antibody. (*b*) [α-^{32}P]GTP-overlays on proteins immunoprecipitated from *P Hom.*, *ZGM*, and *B. Hom.* Immunoprecipitates were generated by the rab3A/B monoclonal antibody using 50 μg of protein for each immunoprecipitation. Immunoblots and GTP-overlays were exposed for 2.5 d and 2 d, respectively. In *a*, a 27-kD rab3A/B immunoreactive band is seen in *ZGMs* and comigrates with a barely detectable band in *P Hom.* In *B Hom.*, a 26-kD immunoreactive band is seen. Control immunoblots using only ^{125}I-sheep anti-mouse secondary antibody revealed no immunoreactive bands with any of the samples (not shown). *b* shows a 27-kD [α-^{32}P]GTP-binding protein immunoprecipitated from *ZGMs* using the rab3A/B antibody. GTP-binding is not seen in the *P Hom.* lane in this overlay but is detectable when larger amounts of pancreatic homogenate are used in the immunoprecipitations (e.g., Fig. 6 *b*). A 26-kD [α-^{32}P]GTP-binding protein is immunoprecipitated from *B Hom.* As a control, 50 μg of *B Hom.* and 50 μg of BSA were incubated without primary antibody before exposure to protein A-Sepharose beads carrying adsorbed rabbit anti-mouse IgG. No [α-^{32}P]GTP-binding was observed in these control immunoprecipitates (data not shown). The positions of M_r markers ranging from 200 to 14.4 kD are indicated; arrowheads point to the extrapolated positions at 26 and 27 kD. The data are representative of at least two separate experiments.

clear profile and the unstained branching acinar lumen. Note that the rab3-like immunoreactivity is intensely localized to the apical region of resting acinar cells. Immunostaining was not detected in a linear pattern on either the apical plasmalemma or on the basolateral plasmalemma. No patterns typical of the supranuclear Golgi complex were seen. To demonstrate that the rab3-like staining pattern corresponded to ZGs in the apical pole of the cell, we carried out parallel localizations using a polyclonal antibody that recognizes rat pancreatic ZGM proteins. As seen in Fig. 3 *d*, the staining pattern with the granule membrane protein antibody is nearly
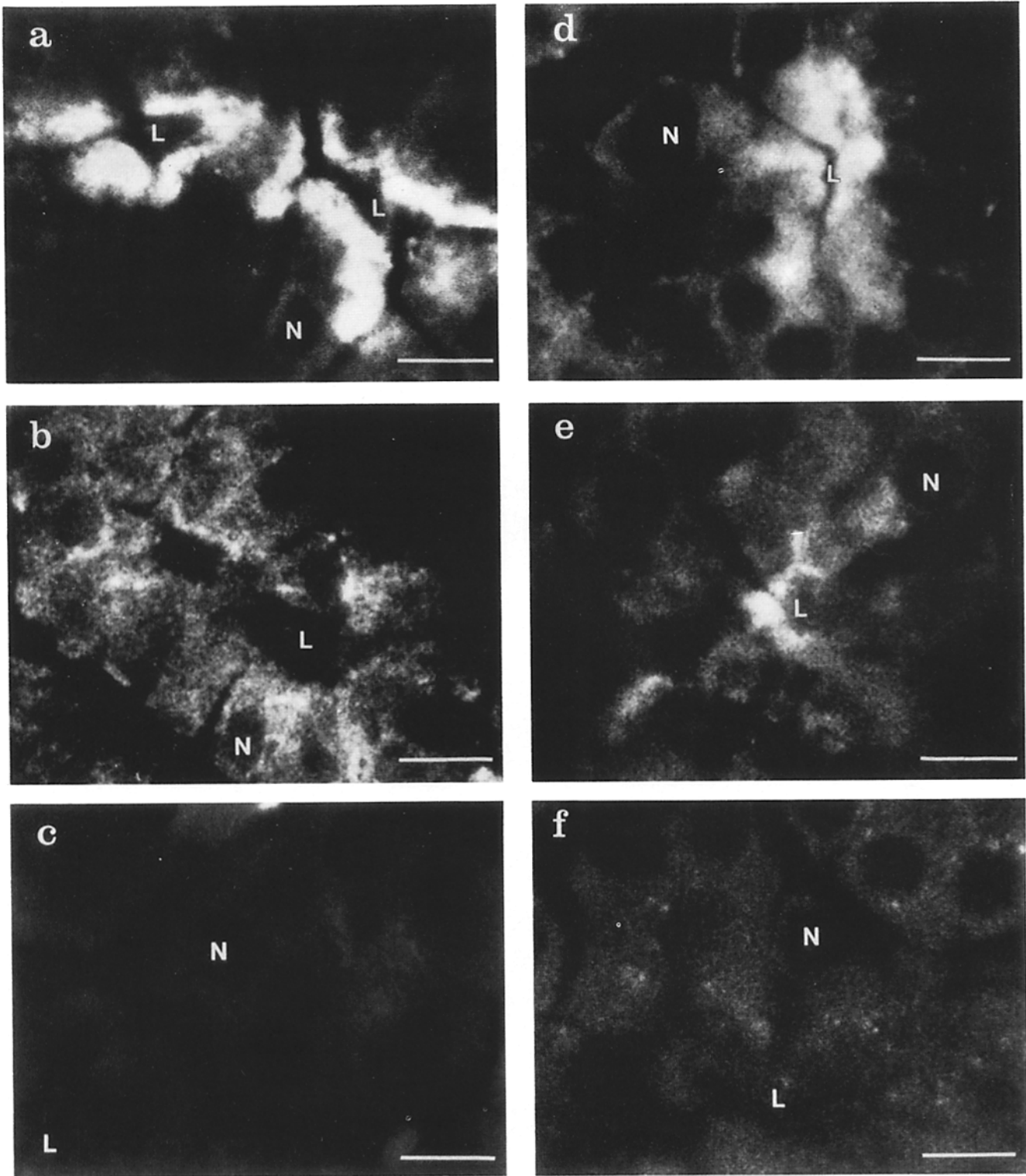
Figure 3. Immunolocalization of rab3A/B and ZGM antibodies on sections of rat pancreatic lobules as detected by confocal microscopy. The lobules were incubated in vitro for 3 h without (*a*, *d*, *c*, and *f*) or with 1 μM carbamylcholine (*b* and *e*). The localizations of rab3A/B and ZGM antibodies on unstimulated acinar cells are shown in *a* and *d*, respectively. Note the intense staining with the rab3A/B antibody in the apical regions of acinar cells (*a*) adjacent to acinar lumina (*L*) which corresponds to the distribution of the ZGM antibody labeling (*d*). Basally located nuclei are indicated (*N*). After 3 h of stimulation, rab3A/B immunostaining is located throughout the acinar cells in a punctate form (*b*) with focal accumulations in the supranuclear region of some cells. After carbamylcholine treatment, ZGM antibody staining (*e*) is markedly reduced from the apical regions of all but a few of the acinar cells with a suggestion of supranuclear localization in some cells. *c* and *f* are controls in which only the secondary antibodies were applied. Lobules were prepared for confocal laser scanning immunofluorescence microscopy as described and 1.5 μm thick optical sections were collected from the interiors of 25 μm frozen sections. Micrographs shown are representative of four separate experiments. Bar, 10 μm.
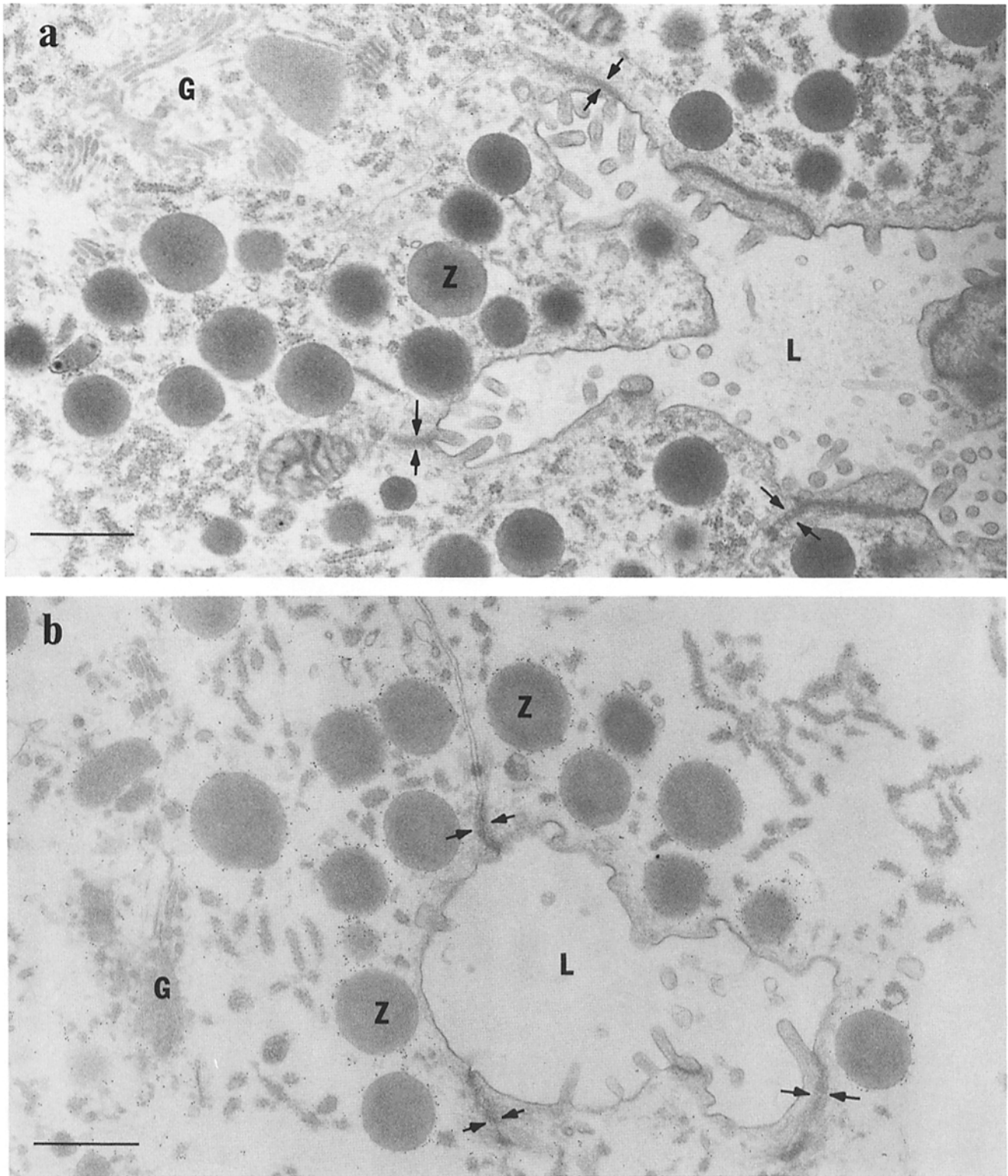
Figure 4. Electron microscopic immunogold localization using the rab3A/B monoclonal antibody on pancreatic lobules. Lobules were incubated in vitro without (*a* and *b*) or with (*c* and *d*) 1 μM carbamylcholine. Fig. 4 *a* is a control in which only goat anti–mouse IgG coupled to 5 nm gold was applied to unstimulated lobules incubated for 3 h in vitro. No specific gold labeling is seen. Fig. 4 *b* illustrates the apical regions of three acinar cells in unstimulated lobules incubated in vitro for 3 h and stained with the rab3A/B antibody followed by 5 nm gold. Note the intact apical plasma membrane surrounding an acinar lumen (*L*); adjacent acinar cells are associated with each other via junctional complexes (*apposed arrows*). Immunogold staining is evident on the cytoplasmic face of ZGs. The apical and basolateral plasma membranes are devoid of labeling. Labeling of the RER and of the Golgi complex (*G*) is relatively light. After 15 min stimulation by 1 μM carbamylcholine (Fig. 4 *c*), the apical plasmalemma is markedly increased in area due to insertion of secretory granule membranes
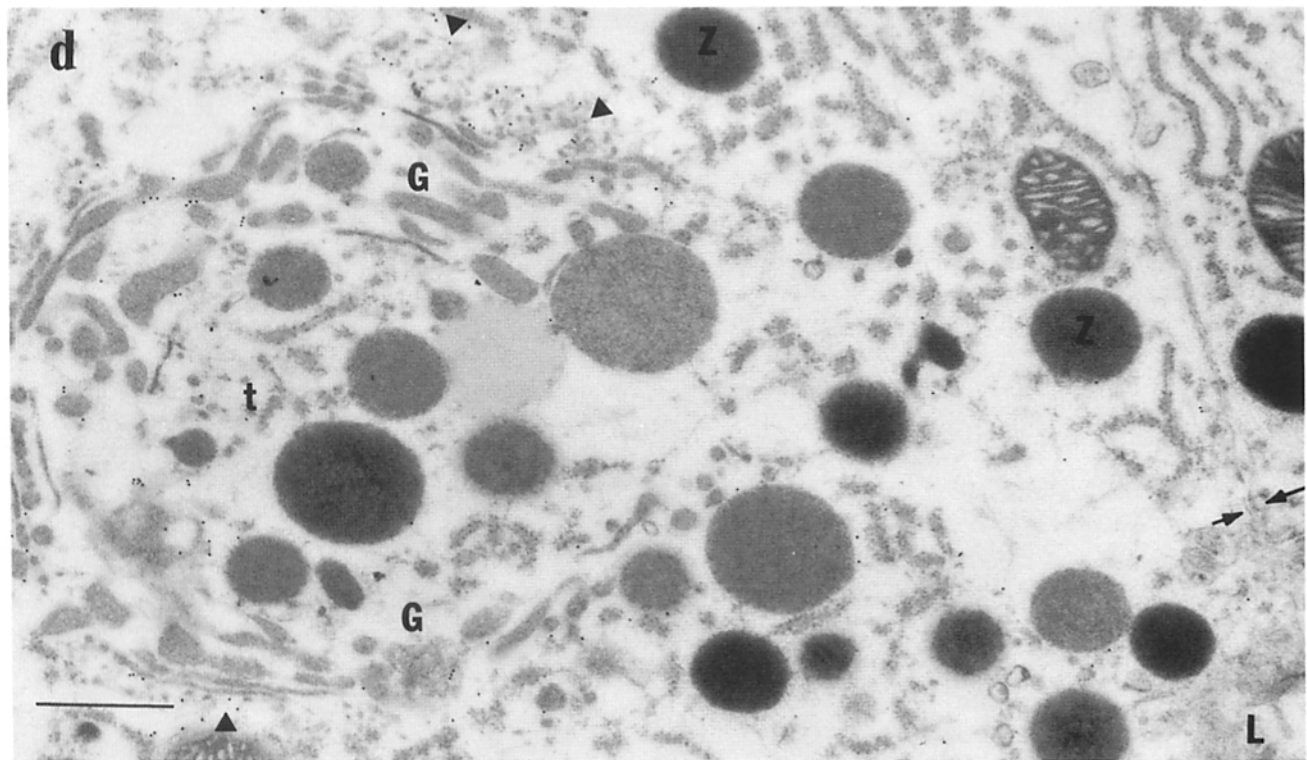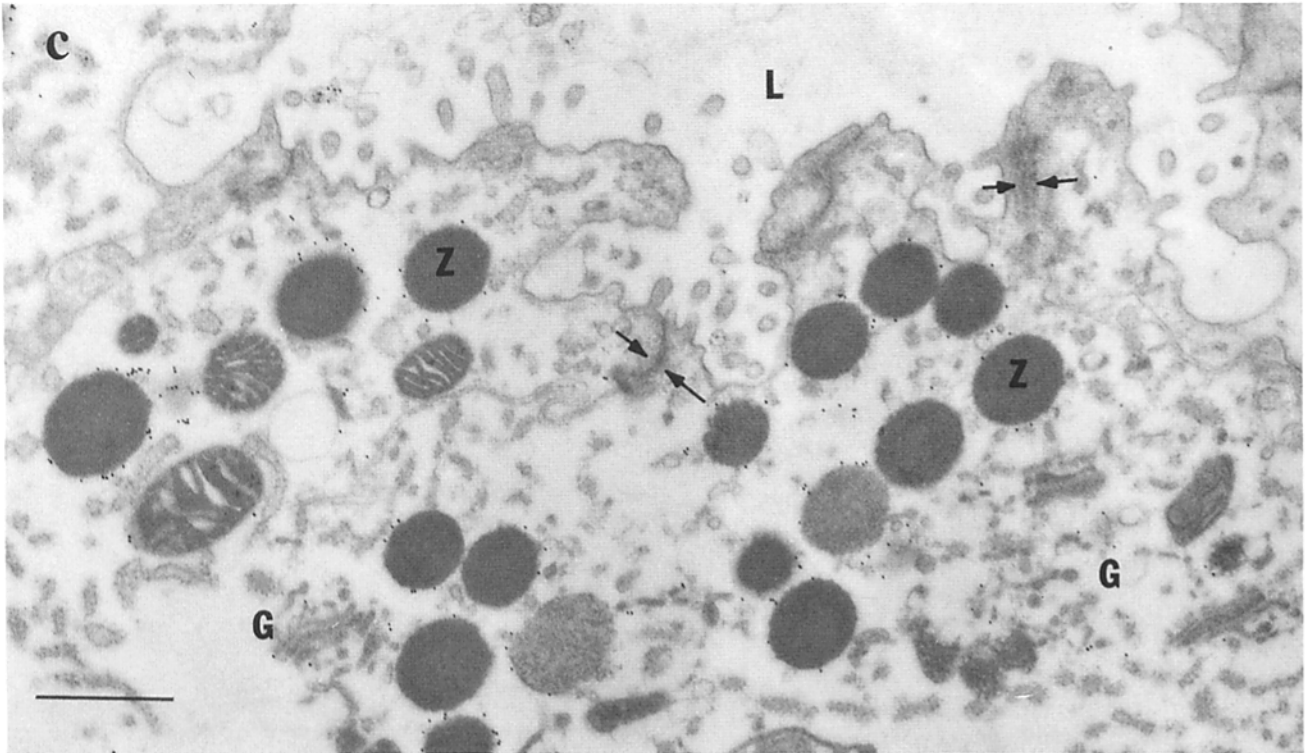
as indicated by the numerous omega-shaped invaginations which correspond to exocytotic profiles. Residual ZGs at this time are labeled with 10 nm immunogold whereas the apical and basolateral plasma membranes are unlabeled. Labeling of the three Golgi complexes (G) in the field is not different from that seen in unstimulated acinar cells. After stimulation of lobules for 3 h (Fig. 4 d), the Golgi complex (G) is markedly enlarged compared to unstimulated acinar cells (Fig. 4 b). Cisternal elements of the Golgi complex are filled with electron dense material which is particularly apparent in the TGN (t) where small, newly forming secretory granules are seen. At this time, the apical plasma membrane area (L) is no longer amplified. Immunogold now labels elements of the Golgi complex (G) including small smooth-surfaced vesicles (arrowheads). Apposed arrows indicate a junctional complex between acinar cells. Bar, 0.5 μm.

identical to that obtained with the rab3A/B antibody (Fig. 3 a). This staining pattern is also similar to that obtained after immunostaining for exocrine secretory proteins (Cornell-Bell et al., 1993).

To localize the rab3-like proteins more precisely, immunogold EM was carried out on agarose-embedded pancreatic lobules. Fig. 4 b shows the distribution of rab3A/B immunoreactivity in the supranuclear region of unstimulated acinar cells where the acinar lumen (L) is delineated by junctional complexes. Note the excellent morphology with this technique including preservation of the polarized distribution of organelles in the basolateral-apical axis. In resting cells, the majority of the gold particles outlines the cytoplasmic faces of mature ZGs located in the apical cytoplasm. Little label is associated with either the apical or basolateral plasmalemma. Labeling of the Golgi complex is sparse, as is that of the RER. Analysis of the relative distribution of gold particles among organelles in resting cells (see Fig. 5) shows that labeling of granules is 5–6-fold greater than that of the Golgi complex (and several fold greater than the plasmalemma) despite the fact that the granule membrane area is only ~2.5-fold greater than that of the Golgi complex (Table I).

Rab3-Like Immunoreactivity Redistributes from ZGs to the Golgi Complex in Stimulated Pancreatic Acinar Cells

Previous studies (Fischer von Mollard et al., 1991) indicated that during synaptic vesicle exocytosis and membrane reinternalization, rab3A appears to dissociate from the synaptic vesicle membrane. This may or may not be the case for the rab3-like protein in acinar cells where the cycle of membrane insertion and retrieval is much slower than synaptic vesicle release. Since a large area (up to ~30× of the apical plasmalemma) of zymogen granule membrane is inserted into the apical plasmalemma during stimulated exocytosis, the pancreatic acinar cell represents a good system in which to study membrane recycling after secretion (Jamieson and Palade, 1971b). Lobules were stimulated in vitro with an optimal (1 μM) concentration of carbamylcholine, which leads to ~60% net discharge of cellular amylase during 3 h of incubation (data not shown).

Light and Electron Microscopic Immunocytochemistry: Stimulated Cells

Redistribution of the rab3-like protein was marked after 3 h stimulation when the majority of the rab3A/B immunoreactivity distributed throughout the cell in a particulate pattern
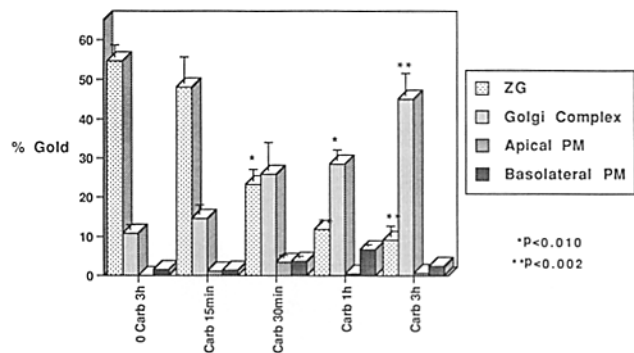

Figure 5. Quantitation of immunogold distribution in electron micrographs of unstimulated and carbamylcholine-stimulated acinar cells. Data from two separate experiments are expressed as percent total gold particles counted over cell structures and are the means ± SEM. Probability values are from paired sample t-tests in which the experimental time points were compared to data from lobules incubated for 3 h without carbamylcholine.

(Fig. 3 b) as visualized by light microscopic immunocytochemistry. Note especially that the apical plasmalemma, whose limits are defined by the negatively staining acinar lumen, does not exhibit a linear staining pattern. After 3 h of stimulation, the acinar lumen is no longer enlarged (Fig. 3, b and e), in contrast to that seen at earlier times. Immunostaining with a ZGM antibody (Fig. 3 e) shows a marked reduction in apical labeling after 3 h of stimulation. The ZGM staining that remains in the apical pole of some of the acinar cells in this micrograph is presumably due to unreleased ZGs. Fig. 3, c and f are controls in which the secondary antibodies alone have been applied.

After 15 min of stimulation, the acinar lumen is markedly enlarged due to the transient increase in the apical plasmalemmal area. At the EM level, the majority of the immunogold label is still associated with ZGs (Fig. 4 c) with no quantitative increase in immunogold label associated with the apical or basolateral plasmalemma or with the Golgi complex (Fig. 5). However, analysis of membrane areas after 15 min of stimulation showed a ~twofold increase in the area of the apical plasmalemma and a ~twofold decrease in the area of the ZGMs (Table I). Other intracellular membrane areas showed no change.

Prolonged stimulation of pancreatic lobules over 1 and 3 h is accompanied by a striking increase (3–4-fold) in the percent immunogold associated with the Golgi complex (Fig. 5). The % gold labeling of the Golgi reached a maximum

Table I. Relative Membrane Surface Areas (Sᵥ) of Subcellular Organelles from Control and Stimulated Pancreatic Acinar Cells

Time/treatment	ZG	G	RER	Ap	Bl
0 Carb 3 h	19.3 ± 4.1	7.7 ± 1.9	59.8 ± 4.6	2.7 ± 0.7	5.0 ± 0.3
1 μM Carb 15 min	10.4 ± 1.2	6.2 ± 1.1	63.6 ± 3.1	5.3 ± 1.1	4.6 ± 1.0
1 μM Carb 30 min	6.9 ± 1.5	7.8 ± 1.9	71.5 ± 1.9	2.1 ± 0.8	6.8 ± 1.1
1 μM Carb 1 h	11.1 ± 2.0	9.3 ± 0.8	65.1 ± 2.7	1.9 ± 0.1	6.0 ± 1.7
1 μM Carb 3 h	8.8 ± 3.9	14.7 ± 3.3	65.4 ± 6.0	1.5 ± 0.6	3.5 ± 0.9

Values represent means of % Sᵥ ± SEM. ZG, zymogen granules; G, Golgi complex; RER, rough surfaced endoplasmic reticulum; Ap, apical plasmalemma; and Bl, basolateral plasmalemma.

at the longest time examined, at which time the area of Golgi membranes had increased only approximately twofold, suggesting that the number of rab3-like immunoreactive sites in the Golgi region had increased. After 3 h of stimulation (Fig. 4 d), the Golgi complex in many of the acinar cells is amplified and is characterized by concentrated secretory proteins in all of its vesicles and cisternae. Small newly forming secretory granules are common on the trans side of the Golgi, characteristic of hyperstimulated acinar cells of other species (Jamieson and Palade, 1971a). These newly forming secretory granules, as well as small dense vesicles located in the TGN, are labeled by immunogold.

It is of interest to note that in 3 h stimulated cells, the residual undischarged granules appear largely unlabeled. In fact, ∼10% of acinar cells respond only partly or not at all to carbamylcholine and in these cells, only ∼20% of the granules are labeled (>5 gold particles/granule). In unstimulated cells, ∼73% of ZGs label with immunogold. A possible explanation for this observation is that in the unstimulated state, the majority of the granules is secretion competent, i.e., they possess the necessary components for exocytosis including the rab3-like protein. After 3 h of stimulation, when ∼60% of cellular amylase has been discharged, most of the secretion competent ZGs will have been released. The residual ZGs which are now mainly unlabeled (∼80%) may correspond to a secretion incompetent population.

No increase in specific labeling of apical or basolateral plasmalemma was seen by immunogold EM throughout the time course of stimulation, consistent with the light microscopic immunocytochemistry (Fig. 3). The relative distribution of rab3-like immunogold over the RER and the mitochondria (not shown) remained constant during the course of stimulation as did the relative surface area of the RER (Table I). The percent unassignable gold particles in the acinar cells remained low (3–8%) throughout stimulation. Fig. 4 a shows an immunogold control in which no primary antibody was applied; it indicates the very low levels of non-specific labeling with this technique.

Immunoblots and GTP-Overlays of Immunoprecipitates: Stimulated Cells

As the rab3-like protein does not appear to relocate to the plasma membrane during stimulation (Figs. 3 and 4), it was of interest to see whether a portion of the rab3-like immunoreactivity found in the acinar cell redistributes from membranes to the cytosol during regulated exocytosis. After in vitro stimulation of lobules for 3 h, experimental and control tissues were separated into sedimentable and soluble fractions and analyzed by immunoblotting using the rab3A/B monoclonal antibody. GTP-overlays were performed on proteins immunoprecipitated by the rab3A/B antibody in order to determine if changes in the distribution of this GTP-binding protein took place during regulated exocytosis. As shown in Fig. 6 a, the same 27 kD immunoreactive band is present in all fractions. Significantly, 3 h of stimulation did not result in changes in distribution of rab3A/B immunoreactivity between the particulate and cytosolic fractions compared to lobules incubated for 3 h without secretagogue. [α-³²P]GTP-overlay assays of rab3A/B immunoprecipitated proteins from the same samples used for immunoblots independently showed no increase in the amount of the GTP-binding protein in the cytosolic fractions from stimulated
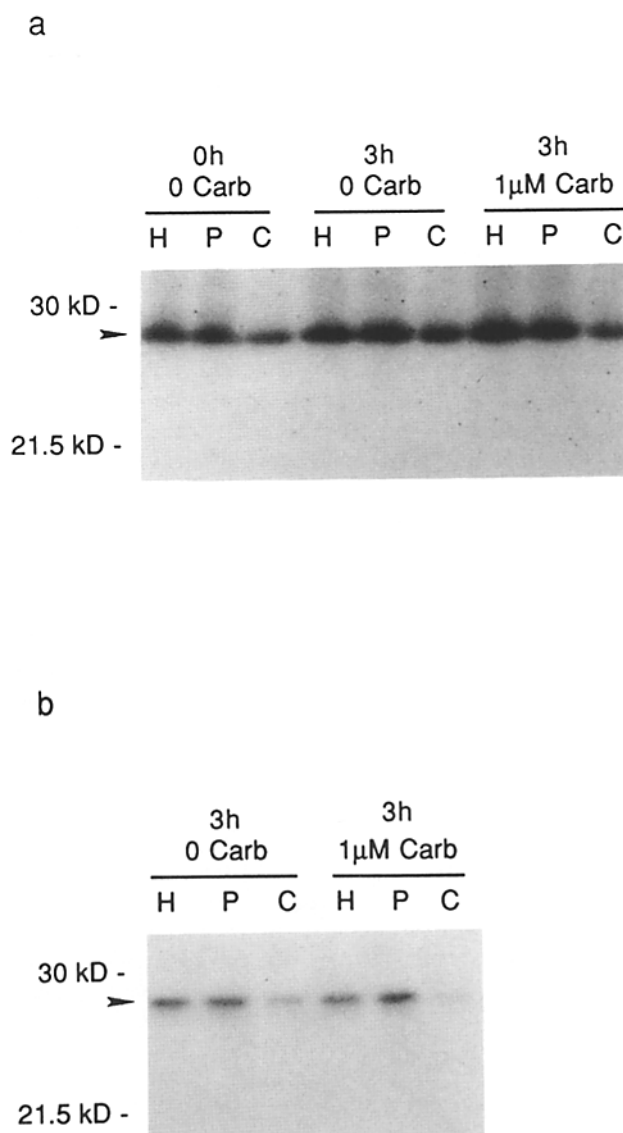
a

b

Figure 6. Immunoblots and [α-³²P]GTP-overlays of immunoprecipitated rat pancreatic fractions from stimulated and unstimulated pancreatic lobules. (a) 25 μg of proteins from the pancreatic homogenates (H), 200,000 g particulate fractions (P) and the remaining cytosol (C) from unstimulated (0 Carb) and stimulated lobules (1 μM Carb) were subjected to SDS-PAGE and immunoblotting, using the rab3A/B monoclonal antibody. Immunoblots were exposed for 4.5 d. (b) 2 mg proteins in H, P, or C fractions derived from unstimulated and stimulated lobules were immunoprecipitated using the rab3A/B monoclonal antibody, followed by GTP-overlay assays. GTP-overlays were exposed for 2 d. Positions of M_r markers at 30 kD (carbonic anhydrase) and 21.5 kD (STI) are indicated; arrowheads indicate the extrapolated position at 27 kD. Data are representative of three separate experiments.

lobules (Fig. 6 b). Similar studies carried out on lobules stimulated in vitro for 30 min showed no increase in the cytosolic level of rab3A/B immunoreactivity compared to unstimulated lobules (data not shown).

Discussion

In the present study, we have analyzed how stimulation of

secretion from pancreatic acinar cells affects the distribution of a low M_r GTP-binding protein that is specifically associated with the membranes of ZGs in the resting acinar cell. Our data show that prolonged stimulation leads to a massive relocation of this rab3-like protein from the granule membrane to membranes of the Golgi complex. No significant labeling of the apical plasmalemma or of any other organelle was observed at any of the times examined during carbamylcholine stimulation, even when the area of the apical plasmalemma had doubled due to the exocytotic incorporation of ZGM. This suggests that the rab3-like protein follows a route that is different from that of the bulk ZGM after regulated exocytosis.

The identity of the rab3-like protein remains to be determined. The protein immunoprecipitated by the rab3A/B antibody binds GTP. It is not identical with rab3A since in parallel incubations, a rab3A-specific monoclonal antibody did not react with this protein. In contrast, rab3A was readily detectable in islets with this antibody, both by immunocytochemistry (Fig. 1) and by immunoblotting (E. M. Konieczko, and J. D. Jamieson, unpublished observations). Furthermore, polyclonal antibodies specific for rab3A, rab3B, or rab3C failed to react with the protein in ZGMs (unpublished observations). Finally, it is unlikely that the rab3A/B antibody recognizes more distantly related rab proteins since it reacts with only a single band in immunoblots (compare immunoblots and GTP-overlays in Fig. 2) and was not reactive with several other rab proteins (e.g., rabs 4–7; unpublished observations). We cannot rule out the possibility that the protein is related to rab3D (Baldini et al., 1992); it is also conceivable that it represents a new isoform of rab3 whose primary structure remains to be elucidated.

In resting acinar cells, the majority (50–60%) of the rab3-like GTP-binding antigen was localized to the cytoplasmic face of ZG membranes in the apical region as detected by immunoelectron microscopy. The highest density of labeling was observed on ZGs closest to the apical plasmalemma and may represent granules that are secretion competent. Condensing vacuoles near the TGN showed a consistently lower density of immunogold staining. About 10% of the label was associated with elements of the Golgi complex, with a labeling density approximately twofold lower than that on apically located ZGs. Neither the apical nor basolateral membranes were significantly labeled. This observation agrees with the absence of linear staining seen by immunofluorescent microscopy.

Upon stimulation of acinar cells, ZGs in the apical region undergo exocytosis that nears completion within 3 h. This leads to a transient enlargement of the apical plasmalemma indicating that endocytic membrane removal is temporarily outpaced by exocytotic membrane incorporation during the first 15–30 min of stimulation. Subsequently, the area of the Golgi complex increases (approximately twofold after 3 h of stimulation in our experiments) which is probably due to the influx of endocytosed membrane from the apical surface of the cell. Quantitation of immunogold labeling of the rab3A/B antigen after stimulation revealed a redistribution that did not passively follow the presumed pathway of membrane flow. Immunogold label associated with the ZGM was reduced approximately fivefold over 3 h and the labeling density also decreased more than twofold. In parallel, labeling of the Golgi complex surpassed the relative increase in its membrane area (3–4 fold increase in total labeling, approxi-

mately twofold increase in labeling density). Most of the immunogold labeling in the Golgi region was focally associated with small smooth-surfaced vesicles and newly forming small secretory granules in the TGN. At no time during the course of in vitro stimulation did we detect any increase in immunolabeling of the apical plasmalemma. If rab3-like proteins had remained associated with the ZGM components during or after exocytosis, labeling of the apical plasmalemma should have been visible both by light as well as by EM.

Unlike the nerve terminal (Fischer von Mollard et al., 1991), stimulation of pancreatic acini did not result in an obvious decrease of sedimentable rab3A/B immunoreactivity, suggesting that the steady state equilibrium between membrane-bound and cytosolic rab3-like protein did not undergo major changes during the induction of massive exocytosis and membrane recycling.

How can these findings be integrated into a coherent picture of rab3 recycling? Although many points remain to be clarified, our data support the following hypotheses:

After regulated exocytosis, the net translocation of rab3-like protein to small vesicles in the Golgi complex suggests that membrane reassociation of the protein may occur at a step in the membrane cycle that precedes ZG formation. Since the pathway of membrane recycling to the Golgi in the acinar cell is not clear, it remains to be established whether this translocation involves active sorting or follows the bulk flow of the recycling membrane material. It should be borne in mid, however, that under long-term stimulation, the distribution pattern of the rab3-like protein may be modified by newly synthesized rab3-like GTP-binding proteins.

The selective association of the rab3-like protein with ZGs in resting acini may be achieved by a gradual concentration on the ZGM during the relatively long ZG maturation period (1–2 h). Exportable proteins are sorted into the secretory pathway at the TGN (Tooze et al., 1989) and undergo progressive concentration in newly forming secretory granules (condensing vacuoles) that are finally reduced in size. Concomitant with protein concentration into mature granules is the removal of excess membrane in the form of small vesicles (Arvan and Castle, 1987). Thus, the increased concentration of the rab3-like protein on mature ZGs may reflect the reduction of membrane area during the condensation process. Alternatively, the rab3-like protein could be actively involved in the biogenesis of ZGs.

Our data suggest that the rab3-like protein dissociates from the ZGM during exocytosis, analogous to the dissociation of rab3A from synaptic vesicles in neurons (Fischer von Mollard et al., 1991). This view is supported by the absence of labeling of the apical plasmalemma after stimulation despite insertion of ZGMs into this domain. We suggest that during exocytosis, GTP is hydrolyzed rendering the protein accessible to the action of a GDP-dissociation inhibitor that removes GDP-bound rab proteins from membranes (Araki et al., 1990; Regazzi et al., 1992). It is possible that if the amount of GDI available to interact with the GDP-bound form of the rab3-like protein is limiting, the rab3-like protein may not be detectable at increased levels in the cytosolic pool after regulated exocytosis. Interestingly, it has been demonstrated that induction of exocytosis at the neuromuscular junction causes translocation of rab3A to the plasmalemma (Matteoli et al., 1991). It is conceivable that due to the faster exocytotic response in neurons, rab3A dissociation from the

membrane cannot keep pace with the rate of exocytosis, supporting the view that GDP-dissociation inhibitor mediated solubilization of rab3-like proteins could be a rate limiting step in the rab3 dissociation-association cycle.

As an alternative, we cannot exclude the possibility that the rab3-like protein remains membrane-bound throughout the cycle of ZGM insertion and endocytic retrieval from the apical plasmalemma. This would require a highly efficient and rapid vesicular retrieval mechanism that internalizes membranes enriched in the rab3-like protein in preference to other ZGM components. The rab3-like protein may also be directly involved in vesicular retrieval. However, as we do not see immunoreactive patches on the apical plasmalemma or labeled small smooth-surfaced vesicles in the apical cytoplasm at any time during induced secretion, we favor an interpretation of our data that is based on an association-dissociation cycle of the rab3-like protein.

What is the function of the acinar cell rab3-like protein in regulated exocytosis? By analogy with a model for the function of Sec4p in exocytosis from yeast (Walworth et al., 1989), the acinar cell rab3-like protein may be involved in the final stages of membrane fusion during regulated exocytosis. Whether the rab3-like protein is equivalent to a GTP-binding protein termed G_E (Gomperts, 1990) that has been suggested to be involved in mammalian regulated exocytosis is unknown.

In summary, our data demonstrate that a low M_r GTP-binding protein which is highly selective for the exocytic compartment in the pancreatic acinar cell under resting conditions, undergoes a massive redistribution to the Golgi complex during stimulation. This may involve a membrane dissociation-association cycle or selective retrieval of membrane patches enriched in the rab3-like protein. Since the membrane dynamics in the acinar cell during stimulated exocytosis and compensatory endocytosis are relatively slow, this system should allow us to examine in more detail the route and mechanisms of membrane recycling and the roles for this rab3-like GTP-binding protein in the process.

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