Redistribution of 23 kDa tubulovesicle-associated GTP-binding proteins during parietal cell stimulation

Marc D. BASSON,*† James R. GOLDENRING,*‡§ Laura H. TANG,*† Jonathan J. LEWIS,*† Philip PADFIELD,† James D. JAMIESON† and Irvin M. MODLIN*†

*Gastrointestinal Surgical Research Unit, Department of Surgery, †Department of Cell Biology, Yale University School of Medicine, and the ‡Department of Veterans Affairs Medical Center, New Haven, CT 06510, U.S.A.

Small GTP-binding proteins are important regulators of intracellular traffic. The presence of several small GTP-binding proteins was documented in subfractions of rabbit parietal cells. Upon maximal stimulation of the cells with a combination of histamine and forskolin, one 23 kDa GTP-binding band was observed to decrease in a 50 000 *g* membrane fraction while increasing in 4000 *g* membranes. The 23 kDa band resolved into one major and two minor species on two-dimensional gels. GTP-binding species of 23 kDa, 24 kDa and 25 kDa were present in purified preparations of tubulovesicles. The three isoelectric species of the 23 kDa proteins observed in parietal cell 50 000 *g* microsomes were enriched in tubulovesicle preparations. None of the tubulovesicle-associated GTP-binding proteins were substrates for ADP-ribosylation by a preparation of botulinum D toxin. These results indicate that tubulovesicles contain discrete small GTP-binding proteins which redistribute during parietal cell stimulation.

INTRODUCTION

A novel class of 20–30 kDa small GTP-binding protein has been described in a number of cell systems [1–6]. Structurally dissimilar from classical membrane-bound GTP-binding proteins such as G_s and G_i , the small GTP-binding proteins appear to represent a family of homologous regulatory proteins [5,7]. Several investigations have suggested a critical role for these small GTP-binding proteins in the regulation of intracellular vesicular trafficking [3,8–13] as well as exocytosis [14–17].

The role of classical GTP-binding proteins in parietal cell secretion is well established. G_s and G_i respectively mediate the stimulation and inhibition of adenylate cyclase. Parietal cells amplify their apical secretory surface through the formation of a secretory canaliculus from the fusion of tubulovesicles containing H^+/K^+ -ATPase [18,19]. Although this reversible vesicle fusion is now well established, the mechanisms responsible for both the fusion of tubulovesicles with the apical canaliculus and recycling out of the secretory surface remain obscure. The roles of small GTP-binding proteins in these events have been studied. We now report the localization on parietal cell tubulovesicles of particular GTP-binding proteins. As in other systems, these small GTP-binding proteins may play a critical role in the trafficking of membrane vesicles.

MATERIALS AND METHODS

Materials

[α - ^{32}P]GTP was purchased from Amersham. ^{125}I -labelled goat anti-mouse IgG was purchased from ICN. Nitrocellulose (0.2 μ m pore size) was obtained from S&S (Keane, NH, U.S.A.). Botulinum A and D toxin preparations were from Wako Chemicals (Richmond, VA, U.S.A.). Pertussis and cholera toxins were obtained from List Biochemical (San Francisco, CA, U.S.A.). Ampholytes (pH 3–10) were from Pharmacia. Prestained molecular-mass markers (low range) were purchased from Bio-Rad. Unstained molecular-mass markers were obtained from Pharmacia. Nycodenz solution was purchased from Accurate

Chemicals (Westbury, NY, U.S.A.). Gelbond PAG film and Acrylaide cross-linker were purchased from FMC Bioproducts (Rockland, ME, U.S.A.). All other chemicals were routinely obtained from Sigma and were of the highest purity available. Monoclonal antibodies against H^+/K^+ -ATPase (HK 111) were provided by Dr. Adam Smolka (Medical University of South Carolina).

Preparation of rabbit parietal cells

Rabbit parietal cells were prepared from rabbit fundic mucosa by collagenase and Pronase digestion, followed by Nycodenz-gradient enrichment, by the method of Mazzeo *et al.* [20] as modified by Lewis *et al.* [21]. Parietal cell preparations contained $85 \pm 5\%$ pure parietal cells, as assessed by haematoxylin and eosin staining. Parietal cells showed greater than 95% viability for 4 h after isolation, as assessed by Trypan Blue exclusion.

Preparation of tubulovesicles

Isolated gastric glands were prepared from rabbit fundic gastric mucosa by a modification of the method of Berglinth [22]. Tubulovesicles were prepared from gastric glands homogenized in 5 vol. of Buffer A (113 mM-mannitol, 37 mM-sucrose, 0.4 mM-EDTA, 4 mM-Mes, pH 6.7, 1 mM-benzamidine, 1 μ g of leupeptin/ml, 1 μ g of aprotinin/ml) with 15 strokes of a Potter homogenizer at 900 rev./min [23]. The resulting homogenate was centrifuged at 4000 *g* for 10 min. The supernatant was then centrifuged at 14 500 *g* for 10 min, and finally the 14 500 *g* supernatant was centrifuged for 60 min at 50 000 *g* (giving crude microsomes; CM). The CM pellet was resuspended in Buffer B (300 mM-sucrose, 0.2 mM-EDTA, 5 mM-Tris/HCl, pH 7.4) and then fractionated over a cushion of 20% (w/v) sucrose in 5 mM-Hepes, pH 7.4, at 135 000 *g* for 60 min in a SW-24 rotor. The first sucrose gradient separated the CM into a microsome pellet (PM) and an interface fraction. The interface fraction was resuspended in Buffer B and layered over a second sucrose cushion and centrifuged as in the first spin. The interface fraction was collected as the purified tubulovesicle (TV) preparation and, after 5-fold dilution with Buffer B, tubulovesicles

§ To whom all correspondence should be addressed at: Department of Surgery, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, U.S.A.

were pelleted at 100 000 *g* for 90 min. During this preparation, over 50 % of the total H⁺/K⁺-ATPase immunoreactivity was lost into the pelleted microsomes. However, Coomassie Blue staining of the tubulovesicle preparation revealed that over 90 % of the protein staining was accounted for by the α and β subunits of the H⁺/K⁺-ATPase.

Preparation of subcellular fractions

Subcellular fractions were prepared from stimulated and unstimulated parietal cells by a modification of the methods of Forte and colleagues [24] originally developed for the fractionation of gastric glands. Isolated cells (5×10^6 /ml) were incubated in Buffer C (133 mM-NaCl, 5.4 mM-KCl, 5 mM-Na₂HPO₄, 1 mM-NaH₂PO₄, 1.2 M-MgSO₄, 1 mM-CaCl₂, 0.5 mM-dithiothreitol, 1 mM-pyruvate, 10 mM-Hepes, pH 7.4, 2 mg of BSA/ml, 10 mM-dextrose, 10 mg of Phenol Red/l) under a constant stream of humidified 100 % O₂ for a recovery period of 10 min at 37 °C. Under the standard protocol, the cells were then incubated for a further 45 min in the presence of 100 μ M-cimetidine (unstimulated) or 100 μ M-histamine plus 10 μ M-forskolin (maximally stimulated). After 45 min, the cells were pelleted at 40 *g* for 3 min at 4 °C. The cell pellets were then homogenized on ice at 10×10^6 cells/ml of Buffer A with 15 strokes of a Potter homogenizer at 900 rev./min. The homogenates were then centrifuged at 4 °C for 10 min at 40 *g* (P0). The resulting supernatant was then centrifuged at 4000 *g* for 10 min (P1). The 4000 *g* supernatant was then centrifuged at 14 500 *g* for 15 min (P2), and a final membrane pellet was prepared by centrifugation at 50 000 *g* for 60 min (P3). The final high-speed supernatant (S3) was utilized as the 'cytosolic' fraction. Each pellet was resuspended in homogenization buffer and re-centrifuged to provide washed pellets. Each fraction was then resuspended in Buffer B to a concentration of approx. 2 mg of protein/ml. Protein concentrations in each fraction were determined by the method of Bradford [25], with BSA as a standard.

GTP-binding protein resolution

Subcellular fractions were resolved on SDS/PAGE gels (15 % acrylamide) [26]. After transfer to nitrocellulose [27], the resulting electrophoretic transfers were incubated with [α -³²P]GTP by the method of Bhullar & Haslam [28]. GTP-binding bands were detected by autoradiography (Kodak XRP-1 or XAR-5 film). Prestained and unstained molecular-mass markers were utilized to determine apparent molecular masses. For two-dimensional blots, proteins were resolved in two-dimensional gels by the method of O'Farrell [29] with pH 3–10 ampholytes.

Competition studies were performed by preincubating nitrocellulose blots for 20 min in GTP-binding buffer in the presence of competing agents including GTP (0.1 nM–1 μ M), GDP (0.1 nM–1 μ M), cyclic GMP (1 μ M) or ATP (1 μ M). In studies on unstimulated and stimulated cells, samples of P1 and P3 membrane were evaluated for GTP binding as well as for the presence of H⁺/K⁺-ATPase. For immunoblotting, proteins were resolved on SDS/PAGE gels and electrophoretically transferred to Immobilon P. H⁺/K⁺-ATPase was then detected on the blot with antibody HK 111 at 1:2000 dilution. Autoradiographs of both one-dimensional immunoblots and two-dimensional GTP-binding blots were quantified with a Bioimage digitizing scanner.

ADP-ribosylation studies

Botulinum A and D toxin preparations were activated by incubation with 10 mM-dithiothreitol for 10 min at 30 °C. Subcellular extracts were incubated at 37 °C with 10 μ g of

toxin/ml and 2 μ M-[³²P]NAD⁺ in 50 mM-triethanolamine buffer (pH 7.5), 10 mM-thymidine, 0.1 mM-GTP, 1 mM-ATP, 1 mM-EDTA, 1.5 mM-MgCl₂ and 10 mM-dithiothreitol. Reactions were terminated with 10 % (w/v) trichloroacetic acid, and precipitates were resolved by SDS/PAGE (15 %-acrylamide gels) by using Gelbond PAG film and a 30:1 acrylamide/Acrylaide cross-linker ratio. ADP-ribosylated bands were detected by autoradiography.

Pertussis and cholera toxins were activated by incubation for 30 min at 37 °C with 50 mM-dithiothreitol. Subcellular extracts were incubated with pertussis toxin (75 μ g/ml) and 2 μ M-[³²P]NAD⁺ in 10 mM-Tris/HCl (pH 7.5) with 10 mM-thymidine, 0.1 mM-GTP, 1 mM-ATP, 1 mM-EDTA and 1 mM-dithiothreitol. For cholera-toxin ADP-ribosylation, extracts were incubated together with toxin (75 μ g/ml) and 2 μ M-[³²P]NAD⁺ in 100 mM-potassium phosphate (pH 7.5), with the same co-reactants as for pertussis-toxin ribosylation with the addition of 1 mM-MgCl₂. Reactions were terminated and proteins were resolved as above for botulinum toxin.

RESULTS AND DISCUSSION

GTP-binding proteins in parietal cell subfractions

The presence of small GTP-binding proteins was evaluated in subfractions from isolated rabbit parietal cells. Fig. 1 demonstrates that several species of GTP-binding proteins could be resolved according to their apparent molecular mass, with varying distribution throughout the fractions. Six major species were identified, with apparent molecular masses of 25, 24, 23, 22.5, 21 and 16 kDa. The 23 kDa protein was most prominent in the P3 membrane fraction, whereas the 24 and 25 kDa proteins were more prominent in the P0 and P1 membrane fractions. The cytosolic S3 fraction possessed much less GTP-binding activity. All binding to these proteins was abolished in the presence of

Fig. 1. GTP-binding proteins in parietal-cell subfractions

Parietal-cell subfractions were investigated on blots of proteins separated by SDS/PAGE (15 %-acrylamide gels) for GTP-binding activity. Six significant bands were consistently observed with varying relative concentrations in the specific fractions, including major binding bands at 25, 24, 23 and 22.5 kDa and two minor bands at 21 and 16 kDa. The positions of molecular-mass markers (kDa) are shown on the left.

Fig. 2. Redistribution of GTP-binding proteins and H⁺/K⁺-ATPase during parietal cell stimulation

(a) GTP-binding proteins in P1 (50 μ g) and P3 (10 μ g) membrane from unstimulated (U) and stimulated (S) parietal cells separated on 15%-acrylamide gels. With stimulation, there was a consistent increase in GTP-binding to a 23 kDa band in P1, whereas GTP binding to the 23 kDa band decreased in P3. (b) Immunoreactivity for H⁺/K⁺-ATPase in P1 and P3 membrane from unstimulated (U) and stimulated (S) cells separated on 8%-acrylamide gels. Similar to the GTP binding, with stimulation there was an increase in proton-pump immunoreactivity in P1, whereas immunoreactivity decreased in P3.

unlabelled GTP or GDP in concentrations greater than 100 nM. In contrast, cyclic GMP or ATP at concentrations of 1 μ M had no effect on the binding of GTP. Binding of GTP to all of the proteins also required Mg²⁺, and was abolished in the presence of 5 mM-EDTA.

Redistribution of GTP-binding proteins during stimulation

Since the small GTP-binding proteins are associated with membrane trafficking in a number of systems, we investigated the distribution of these proteins in membrane fractions from unstimulated and stimulated cells. Comparison of P1 and P3 membrane has been utilized previously to examine the translocation of the H⁺/K⁺-ATPase during stimulated fusion of tubulovesicles with the apical canalculus [24]. Fig. 2(a) demonstrates the GTP-binding pattern for P1 and P3 membrane from unstimulated and stimulated cells. A decrease in the labelling of the 23 kDa protein in P3 was observed with stimulation. At the same time, an increase in 23 kDa-protein labelling was noted in the P1 fraction. Although the alterations in 23 kDa labelling were consistent, exact quantifications were not possible in P1 because of the close proximity of other GTP-binding bands. In parallel samples (Fig. 2b), as previously described [24], H⁺/K⁺-ATPase immunoreactivity increased in P1 upon stimulation ($240 \pm 41\%$ compared with UP1; $n = 3$) while decreasing in SP3 ($61 \pm 7\%$ compared to UP3; $n = 3$).

Since precise quantification of the alterations in 23 kDa-protein GTP binding in P1 membrane was not possible in one-dimensional blots, because of the adjacent GTP-binding band, the redistribution of GTP-binding proteins in P1 and P3 membranes was assessed in blots of proteins from subcellular fractions resolved in two dimensions. A major 23 kDa GTP-binding protein resolved as multiple isoelectric species (Fig. 3). Under these conditions, the other GTP-binding bands did not focus, thus allowing the better observation of the 23 kDa GTP-binding protein in P1 membrane. In the P3 fraction, the 23 kDa band resolved into one major and two minor species. Upon stimulation, binding to all three of these spots decreased

Fig. 3. Redistribution of GTP-binding proteins observed in two dimensions

Proteins (20 μ g) from P1 and P3 membrane fractions from unstimulated (U) and stimulated (S) parietal cells were resolved in two dimensions and then transferred to nitrocellulose for GTP binding. Three GTP-binding protein species were observed in UP3 membrane, all with molecular masses of 23 kDa. SP3 membrane demonstrated a consistent decrease in all three species. In comparison with UP1 membrane, SP1 membrane demonstrated an increase in the major 23 kDa GTP-binding species.

proportionally. The major GTP-binding species decreased in SP3 to $37 \pm 7\%$ ($n = 3$) of the level seen in UP3. In contrast, in the SP1 membrane, there was a marked increase ($324 \pm 23\%$ compared with UP1; $n = 3$) in the binding to a 23 kDa species that co-migrated with the major species in the unstimulated P3. We have been unable to resolve the other GTP-binding species in the P1 membrane. Lanoix *et al.* [30] have previously resolved platelet GTP-binding proteins into a number of isoelectric species in two-dimensional gels. In the present study, it is unclear whether the other GTP-binding proteins failed to focus or whether particular GTP-binding proteins did not renature fully after isolation by isoelectric focusing and urea denaturation. Nevertheless, the present results do indicate that discrete GTP-binding proteins are relocating upon stimulation. The 23 kDa species, however, must be considered to represent only a minimum number of candidate tubulovesicle-associated proteins.

Association of GTP-binding proteins with tubulovesicles

The above data suggested that particular GTP-binding proteins redistributed during stimulation in a pattern similar to that seen for the H⁺/K⁺-ATPase. Such redistribution is consistent with the fusion of tubulovesicles with the apical surface to form the secretory canalculus. We therefore investigated the association of GTP-binding proteins with tubulovesicles, using standard preparations of highly enriched vesicles from rabbit gastric glands. Fig. 4 demonstrates that, although crude 50000 g membranes showed a major enrichment for GTP-binding proteins over the homogenate, both pelleted microsomes and enriched tubulovesicles contained substantial levels of GTP-binding proteins. The 23 kDa, 24 kDa and 25 kDa species were consistently present in both pelleted microsomes and tubulovesicle fractions. These results indicate that a number of

Fig. 4. Presence of GTP-binding protein in tubulovesicles

GTP binding was investigated in proteins (20 μ g) from homogenate (H), crude 50000 *g* membranes (CM) from isolated gastric glands, purified tubulovesicles (TV) and a pelleted microsomal fraction (PM). Three GTP-binding bands of 23, 24 and 25 kDa were observed in all three microsomal membrane fractions. The positions of molecular-mass markers (kDa) are indicated at the left.

Fig. 5. Resolution of GTP-binding proteins in two dimensions

Proteins (20 μ g) from crude microsomes (CM), pelleted microsomes (PM) and purified tubulovesicles (TV) were resolved in two dimensions and transferred to nitrocellulose for GTP binding. Five major species, all with apparent molecular masses of 23 kDa, were resolved (1–5). The major spot (1) was enriched in the TV preparation relative to the PM. Species (2) and (5) were also enriched in TV, whereas (3) and (4) were enriched in PM.

small GTP-binding proteins may be associated with tubulovesicles.

In order to compare tubulovesicle GTP-binding proteins with GTP-binding proteins demonstrated in subcellular fractions, we resolved the microsomal proteins in two dimensions. Once again, only the 23 kDa species were resolved in two-dimensional gels. Fig. 5 shows that the crude light microsomes (CM) displayed one major and four minor species. The pelleted microsomes (PM) demonstrated a relative decrease in the amount of the major species and two of the minor species, but was enriched for two of the other minor species. Finally, the tubulovesicle preparation was enriched for the major GTP-binding species and two of the minor species, and demonstrated little of the two species that were prominent in the pelleted microsomes. The major and two minor species in the tubulovesicle preparation co-migrated with the three isoelectric species observed in the parietal-cell P3 fraction. These results suggest that GTP-binding proteins which redistributed during stimulation were present on tubulovesicles along with the proton pump.

Toxin sensitivity of GTP-binding proteins

ADP-ribosylation by botulinum toxin has been recognized as an important characteristic of some small GTP-binding proteins [31–33]. The enzymic activity responsible for the ADP-

ribosylation is botulinum C3 toxin, which is present as a major contaminant of botulinum D toxin preparations. We therefore investigated the ability of a botulinum D toxin preparation to ADP-ribosylate microsomal proteins (Fig. 6). Although a 23 kDa substrate for botulinum toxin was present in both the crude microsomes and the pelleted microsomes, no botulinum-toxin-mediated ADP-ribosylation was observed in the tubulovesicle fraction. No labelling of any low-molecular-mass species in any of the microsomal fractions was observed with botulinum A, pertussis or cholera toxins. These data demonstrate that the tubulovesicle-associated GTP-binding proteins are not substrates for toxin ADP-ribosylation. Furthermore, the data demonstrate that specific small GTP-binding proteins are likely to be distributed in discrete membrane fractions.

The small GTP-binding proteins represent a family of multifunctional regulatory proteins. A number of GTP-binding proteins of between 20 and 28 kDa have been described, with varying similarity to the viral *ras* oncogene products [7]. Several GTP-binding proteins (SEC4 and YPT) have been described in yeast which regulate trafficking of membrane vesicles between compartments [3,8]. In mammalian tissues, small GTP-binding proteins are highly enriched in brain [5,12,34–38]. A family of homologous GTP-binding proteins of 23–25 kDa has been

Fig. 6. Botulinum-toxin ADP-ribosylation of microsomal membrane proteins

Samples identical with those used for GTP binding in Fig. 4 were ADP-ribosylated in the presence of a botulinum D toxin preparation. Although a 23 kDa ADP-ribosylated band was observed in both CM and PM membrane, no ADP-ribosylation was observed in the TV preparation. On two-dimensional gels, the ADP-ribosylated species focused as a single species (results not shown). Positions of molecular-mass markers are indicated at the left.

described in the brain [5]. These *rab* proteins possess from 37% to 75% similarity to yeast YPT protein. The *rab3* protein localizes in brain tissue exclusively to small synaptic vesicles [11], as well as in adrenal chromaffin granules [39]. Recent investigations have demonstrated the association of *rab2* protein with an intermediate compartment between endoplasmic reticulum and the Golgi apparatus in MDCK cells [40], suggesting a role for the protein in processing along the exocytotic pathway. The *rab6* protein has been localized to the Golgi apparatus in several cell systems [41]. In contrast, *rab5* and *rab7* proteins have been localized to endosomal pathways in MDCK cells [40]. These results suggest that the *rab* proteins, as well as other members of small GTP-binding protein families, are likely to play diverse roles in intracellular membrane trafficking.

The apical membrane of the parietal cell undergoes a dynamic extension during stimulation. The reversible vesicle fusion involves at least (1) the directed movement of tubulovesicles toward the apical membrane, (2) the fusion between plasma membrane and tubulovesicles and (3) the recycling of tubulovesicles [18]. Similar to other cell systems, parietal cells possess a broad array of small GTP-binding proteins with varying subcellular distributions. The results reported here indicate that a discrete group of GTP-binding proteins is associated with tubulovesicles in gastric parietal cells. The experimental observation of GTP-binding protein redistribution during stimulation is dependent partly on the exclusive partitioning of proteins into specific vesicle membrane compartments. Given the possible diverse utility that these proteins may display, one cannot at present predict how overall

cellular distribution will reflect functional roles. Thus, although the three 23 kDa GTP-binding protein species detailed here represent only a minimum number of possible vesicle-associated species, it is clear that discrete GTP-binding proteins are associated with tubulovesicles and are translocated during stimulation. Future investigations will be required to identify tubulovesicle-associated GTP-binding protein as members of specific *ras*-related gene families involved in membrane vesicle processing.

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