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Low molecular mass GTP-binding proteins of adrenal chromaffin cells are present on the secretory granule

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Adrenal medullary homogenates and chromaffin granule membranes were separated by SDS-polyacrylamide gel electrophoresis and GTP-binding proteins detected using [α-^{32}P]GTP binding to nitrocellulose blots. Four GTP-binding polypeptides of 24, 22, 20 and 18 kDa were routinely found in medullary homogenates and all were also found in isolated chromaffin granule membranes. The GTP-binding polypeptides co-sedimented with granule membrane markers following separation on sucrose gradients. On the basis of trypsin sensitivity and resistance to extraction, the GTP-binding proteins appeared to be tightly bound to the cytoplasmic surface of the granules. One or more of the secretory granule GTP-binding proteins could be involved in exocytosis in adrenal chromaffin cells.

Secretory granule; GTP-binding protein; Chromaffin cell; Secretion; Exocytosis

1. INTRODUCTION

It is well known that G-proteins play key roles in signal transduction at the plasma membrane [1]. A number of other GTP-binding proteins, that as yet have no clearly defined function, have been identified in mammalian cells. These include the *ras* protooncogenes and related proteins which are believed to have important roles in the control of cell growth [2] and in some aspects of signal transduction [3]. The *ras* proteins are able to bind GTP after transfer to nitrocellulose [4] and several low molecular mass (20–25 kDa) GTP-binding proteins have been identified in studies using GTP-binding to nitrocellulose [5–11]. In some cases these proteins have been purified [8,9] and sequence data obtained [10]. It is clear that certain of these low molecular mass GTP-binding proteins are part of a family related to but distinct from the *ras* protooncogenes.

Studies on a variety of secretory cell types have demonstrated the possible involvement of exocytosis of one or more GTP-binding proteins activatable in permeabilized cells by non-hydrolyzable analogues of GTP [12–17]. It appears that calcium-independent exocytosis can be stimulated through a GTP-binding protein distinct from those involved in established signal transduction pathways [12,13,17]. The suggestion has been made that this putative protein, named G_E, could be present on secretory vesicles [13].

Further insight into the involvement of GTP-binding proteins in exocytosis has come from the study of yeast secretory mutants. In two such mutants, the *ypt1.1* and the SEC4 mutants, the defect lies within genes coding for GTP-binding proteins homologous to the mammalian *ras* proteins [18–20]. In the case of the SEC4 mutant, secretion appears to be blocked at the level of secretory vesicle fusion with the plasma membrane and the Sec4 protein is found on the accumulated secretory vesicles [21]. These findings suggest that the Sec4 protein plays an essential role in exocytotic membrane fusion. It is not known whether constitutive or regulated secretion in mammalian cells involves Sec4-like proteins [22]. If such proteins are involved in regulated secretion then it would be expected that these low molecular mass GTP-

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binding proteins would be present on the secretory vesicle. We have examined this possibility and report that low molecular mass GTP-binding proteins of the bovine adrenal chromaffin cell are indeed present on isolated secretory granule membranes.

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]GTP (3000 Ci/mmol), anti-mouse-biotin, streptavidin-peroxidase and Hyperfilm β-max were obtained from Amersham International (Amersham, England). Guanyl-5'-yl imidodiphosphate (GppNHp) and guanosine 5'-[thio]triphosphate (GTP[S]) were from Boehringer Mannheim (Lewes, England). Anti-p65 (Ab48) was a gift from Dr L. Reichardt (University of California, San Francisco, CA).

2.2. *Preparation of chromaffin granules*

Bovine adrenal medullas were homogenised in 0.3 M sucrose, 1 mM EGTA, 5 mM Hepes, pH 7.3 (buffer A), filtered through muslin and centrifuged at 800 × *g* for 15 min. The supernatant was centrifuged at 17 000 × *g* for 20 min to sediment the large granule fraction. Mitochondria were washed from the surface of the pellet, and the remaining pellet was resuspended in buffer A and washed once by centrifugation at 17 000 × *g* for 20 min. The pellet was again resuspended in buffer A and in some experiments this fraction was used as a crude granule fraction. Alternatively, the material was overlaid on a cushion of 1.7 M sucrose, 1 mM EGTA, 1 mM $MgSO_4$, 5 mM Hepes (pH 7.3) and centrifuged at 100 000 × *g* for 60 min. The pellet was resuspended in 1 mM $MgSO_4$, 20 mM Hepes (pH 7.3) and freeze-thawed to lyse the secretory granules. The granule membranes were sedimented by centrifugation at 100 000 × *g* for 60 min and resuspended for use as the purified granule membrane fraction or for further fractionation by resuspension in buffer A, application to a continuous 13–29% sucrose gradient and centrifugation at 115 000 × *g* for 90 min. During isolation, fractions were maintained at 0–4°C.

2.3. *Trypsin treatment of intact granules*

Aliquots of the crude granule fraction were incubated for 35 min at room temperature with no additions, with 50 μg/ml trypsin or with 50 μg/ml trypsin and 100 μg/ml soybean trypsin inhibitor. Soybean trypsin inhibitor (100 μg/ml) was added to the first two incubation mixtures for the last 5 min. The granules were centrifuged at 16 000 × *g* for 1 min in an MSE microcentaur, solubilised in SDS-solubilisation buffer and boiled immediately.

2.4. *Extraction of granule membranes*

Purified granule membranes were incubated in 1 mM $MgSO_4$, 20 mM Hepes (pH 7.3) with no additions, with 1 M NaCl, 6 M urea or 1% Triton X-100 for 10 min on ice. The membranes were centrifuged at 16 000 × *g* for 4 min and the pellet resuspended to its original volume. The pellet and supernatant samples were mixed with SDS-solubilisation buffer.

2.5. *[α-^{32}P]GTP binding*

Samples of adrenal medullary homogenate, isolated granules or granule membranes were solubilised in SDS-solubilisation buffer (1.25% SDS, 2 mM EDTA, 10% sucrose, 10% glycerol, 1% 2-mercaptoethanol, 125 mM Tris-HCl, pH 6.8), boiled immediately for 2 min and separated by SDS-polyacrylamide gel electrophoresis on 10% minigels. The polypeptides were transferred to nitrocellulose by transverse electroblotting and stained using ponceau S. Binding of [α-^{32}P]GTP was carried out essentially as in [5] except that the nitrocellulose was first incubated with buffer containing 0.3% BSA. The blots were then incubated in a buffer containing 0.3% Tween 20, 2 μM $MgCl_2$, 50 mM Tris-HCl (pH 7.5) and 1 μCi [α-^{32}P]GTP. In some experiments, 100 μM GTP[S] or 100 μM GppNHp were included in the incubation. The blots were washed, air-dried and autoradiography carried out by exposure to Hyperfilm β-max for 1–5 days.

2.6. *Immunoblotting*

Nitrocellulose blots were incubated with 3% BSA, 0.2% Triton X-100 in PBS for 30 min, anti-p65 (Ab48, 1:1000) for 18 h, anti-mouse-biotin (1:300) for 1 h, streptavidin-peroxidase (1:300) for 30 min and the reaction developed with H_2O_2 and diaminobenzidine.

3. RESULTS AND DISCUSSION

Nitrocellulose blots of separated polypeptides of adrenal medullary homogenate or chromaffin granule membranes were incubated with [α-^{32}P]GTP. Four GTP-binding polypeptides were routinely detected in both the homogenate and granule membrane: two major components of 24 and 20 kDa and two minor components of 22 and 18 kDa (fig.1). The binding of [α-^{32}P]GTP to nitrocellulose blots was specific since it was completely abolished in the presence of 10 μM GTP[S] or 10 μM GppNHp. From a comparison of the level of [α-^{32}P]GTP binding to granule membranes and homogenate run at the same protein concentration it is clear that the GTP-binding proteins are enriched in the granule membrane fraction compared to the whole medullary homogenate.

In order to verify the association of the GTP-binding polypeptides with secretory granule membranes, the membranes were fractionated further on continuous sucrose gradients. Chromaffin granules were initially separated from other organelles and other membranes including plasma membranes due to their high density. Following lysis of the granules the granule membranes can subsequently be separated from contaminating membranes due to the fact that they now have a lower density than plasma membranes, mitochon-

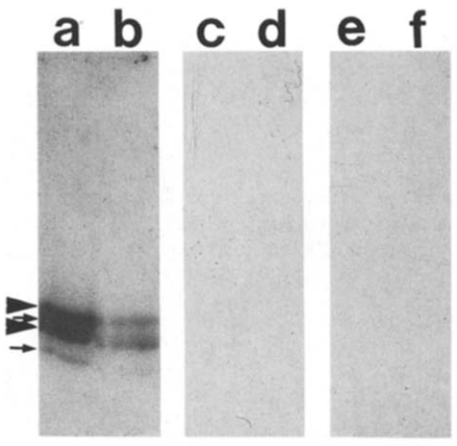

Fig.1. GTP-binding proteins in adrenal medullary homogenate cells and chromaffin granule membranes. Following electrophoresis polypeptides were transferred to nitrocellulose and GTP-binding polypeptides detected by binding of [α-^{32}P]GTP and autoradiography. Binding of [α-^{32}P]GTP to polypeptides of chromaffin granule membranes (a,c,e) and adrenal medullary homogenate (b,d,f) with no additions (a,b) or in the presence of 10 μM GTP[S] (c,d) or 10 μM GppNHp (e,f). In each case 15 μg protein was run per track. The arrowheads indicate the major 24 and 20 kDa GTP-binding polypeptides and the small arrows the minor 22 and 18 kDa GTP-binding polypeptides.

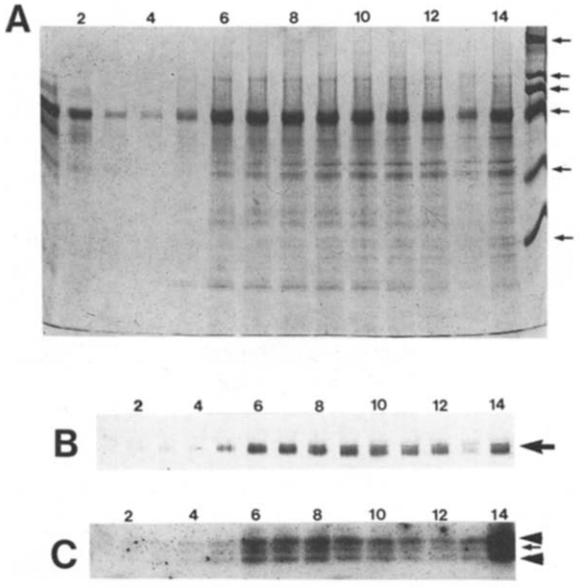

Fig.2. GTP-binding proteins in granule membranes separated on continuous sucrose gradients. The purified granule membrane fraction was further separated on a 13–29% sucrose gradient. Gradient fractions were separated by SDS-polyacrylamide gel electrophoresis for staining with Coomassie blue (A) or after transfer to nitrocellulose staining with anti-p65 (B) or detection of GTP-binding proteins (C). The arrows in A indicate molecular mass markers (from top to bottom 200, 116, 94, 67, 42 and 29 kDa). The arrow in B indicates p65. In C the arrowheads indicate GTP-binding polypeptides of 24 and 20 kDa, the arrow denoting the 22 kDa polypeptide. Fractions are numbered from the top of the gradient; fraction 14 is the pelleted fraction. The GTP-binding polypeptides co-sedimented with the granule membrane polypeptide p65.

drial and microsomal membranes [23,24]. Granule membranes were fractionated on 13–29% sucrose gradients and the distribution of the granule membranes determined by immunoblotting of gradient fractions with an antibody against the integral granule membrane polypeptide p65 [25,26]. Granule membranes were found in the region of the gradient expected from previous studies [25,26] with the highest amounts of p65 in fractions 6–9 (fig.2). The GTP-binding polypeptides co-distributed in the gradient with p65 being at their highest levels in fractions 6–9. These results provide further evidence that the GTP-binding polypeptides are associated with the secretory granule membranes.

To determine whether the GTP-binding polypeptides were associated with the cytoplasmic or intragranular surface of the granule membrane, the effect of trypsin treatment of intact granules on binding of [α-^{32}P]GTP was examined. As shown in fig.3 treatment with 50 μg/ml trypsin almost completely abolished binding of [α-^{32}P]GTP to the granule membranes. The effect of trypsin was largely prevented by soybean trypsin inhibitor. The trypsin sensitivity of [α-^{32}P]GTP binding demonstrated that the GTP-binding polypeptides are on the cytoplasmic surface of the secretory granules.

One possibility is that the GTP-binding polypeptides became associated with the granule membranes following redistribution during homogenisation. If this were the case then the polypeptides would be readily extractable from the granule membranes. A series of extractions was carried out to examine this point. As shown in fig.4 extraction with 1 M NaCl which should extract loosely bound and extrinsic membrane proteins had little effect on the amount of GTP-binding polypeptides associated with the granule membranes. Even extraction with 6 M urea (which reduced the overall level of GTP binding recovered) did not remove all

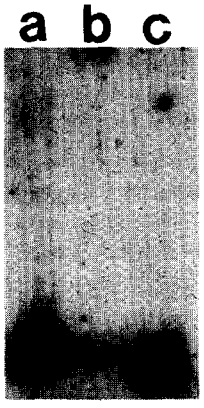

Fig.3. Effect of trypsin treatment of intact granules on GTP-binding proteins. Intact granules (crude granule fraction) were incubated with no additions (a), with 50 μg/ml trypsin (b) or with 50 μg/ml trypsin and 100 μg/ml soybean trypsin inhibitor (c) separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The binding of [α-^{32}P]GTP was almost completely abolished by trypsin treatment.

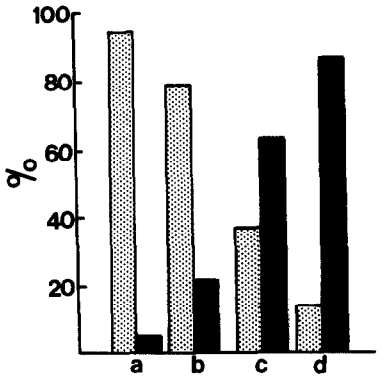

Fig.4. Effect of various extraction conditions on [α-^{32}P]GTP binding to chromaffin granule membranes. Chromaffin granule membranes were incubated under control conditions (a), or extracted with 1 M NaCl (b), 6 M urea (c) or 1% Triton X-100 (d) and separated into pellet and supernatant fractions. The polypeptides were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The histogram shows quantitation of [α-^{32}P]GTP binding to the 24 kDa GTP-binding polypeptide derived from densitometry. Data are the means of two determinations. For each condition the amount of binding in each fraction was calculated as a percentage of the total binding recovered. Similar results were obtained for all of the GTP-binding polypeptides. Stippled bars, pellet; filled bars, supernatant.

of the GTP-binding polypeptides from the membranes. Polypeptides that bound [α-^{32}P]GTP could only be completely solubilised by 1% Triton X-100. These results suggest that the GTP-binding polypeptides were tightly bound to the granule membranes and a proportion of them behaved like integral membrane proteins, arguing against association with the granule membrane being a homogenisation artefact.

Previous work on the *ras* proteins and the Sec4 protein indicated that they can exist as soluble or membrane-associated forms. The membrane-bound form of *ras* and the yeast protein YPT1 behave like integral membrane proteins following fatty acid acylation [27].

The results show that low molecular mass GTP-binding proteins are tightly associated with the cytoplasmic surface of the secretory granules of adrenal chromaffin cells. The GTP-binding proteins are unlikely to be exclusively associated with contaminating membranes, since the GTP-binding proteins co-sedimented with the integral granule membrane polypeptide p65 on continuous sucrose gradients and on a protein basis the GTP-binding proteins were enriched in the granule membrane fraction compared to the homogenate.

Recently, three α-subunits of members of the signal transducing G-protein family (G_o and two other pertussis toxin substrates) have been detected on membranes of chromaffin granule membranes [28]. In addition, G_i has been found in the specific granule fraction of neutrophils [29]. This G-protein is unlikely to have a direct essential role in exocytosis, since it was not detectable on the azurophilic granules from the same cell. The presence of formyl peptide receptors in addition to G_i in the specific granule fraction has resulted in the suggestion that the secretory granule G-protein and coupled receptor may form a pool that can be rapidly inserted into the plasma membrane by exocytosis to modify cell sensitivity to peptide [29]. The functions of the low molecular mass GTP-binding proteins found in mammalian cells are unknown. However, the finding that several of these proteins are localised on secretory granule membranes is of interest considering that the yeast low molecular mass GTP-binding proteins SEC4 and YPT1 both play essential roles in the secretory pathway [19–21]. YPT1 is localised on the Golgi [19] but the SEC4 protein is essential for exocytosis itself and, in the mutant, the protein is found on the accumulated secretory vesicles. It is tempting to speculate that one or more of the GTP-binding

proteins that we have detected on the chromaffin granule membrane could be related to the SEC4 protein. If any of the chromaffin granule GTP-binding proteins are SEC4-like proteins then they may have an essential role in regulated exocytosis in the adrenal chromaffin cell.

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