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Detection of low molecular mass GTP-binding proteins in chromaffin granules and other subcellular fractions of chromaffin cells

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A homogenate of purified chromaffin cells was fractionated, after removal of the nuclear fraction, by sucrose density gradient ultracentrifugation. The presence and subcellular localization of low molecular mass GTP-binding proteins was explored by incubation of blots of proteins from different subcellular fractions with [α-^{32}P]GTP in the presence of Mg^{2+}. The fractions enriched in intact chromaffin granule markers, i.e. catecholamines, chromogranin A, chromogranin B and cytochrome *b*-561 were also enriched in labelled GTP-binding proteins. Two major labelled components of 23 and 29 kDa were rapidly detected by autoradiography. Traces of 26 and 27 kDa components were also present. These components were detectable in both plasma and granule membranes. In addition to these components, the cytosolic fraction contained another GTP-binding protein of about 20 kDa. Binding of [α-^{32}P]GTP was specific and dependent on Mg^{2+}. By analogy to the findings reported in non-mammalian systems, the observations described here suggest the involvement of low molecular mass GTP-binding proteins in the chromaffin cell secretory process.

GTP-binding protein; Chromaffin granule; Secretory process; Gene product, ras

1. INTRODUCTION

Several families of GTP-binding proteins have been described [1]: (i) the soluble factors which participate in protein synthesis [2]; (ii) the tubulin subunits [3]; (iii) the well known oligomeric G-proteins [4]; and (iv) the recently found low molecular mass GTP-binding proteins which have been implicated in secretion [5]. The importance of this latter group has been demonstrated in yeast *Saccharomyces cerevisiae* using specific mutations affecting various steps of the secretory pathway [6–8]. A lethal mutation of the YPT1 gene coding for a 23 kDa GTP-binding protein (YPT1p) caused membranes and vesicles to accumulate, disrupted microtubule organization and decreased secretion [6,7]. YPT1p appears to reside in the Golgi apparatus [6]. Mutations in SEC4 gene coding for a 23.5 kDa GTP-binding protein (SEC4p) abolished the late steps of secretion [8]. SEC4p is associated not only with the plasma membrane but also with secretory vesicles [9]. These as well as other proteins are all ras-related proteins [10,11].

Two techniques have been used to detect GTP-binding proteins. The most widely used technique involves ADP-ribosylation by toxins derived from *V. cholerae* (G_s and transducin) and *B. pertussis* (G_i, G_o and transducin) [4]. The second method involves the labelling with [α-^{32}P]GTP on nitrocellulose blots of proteins separated by SDS-PAGE. This latter approach has been used to

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; GTP, guanosine triphosphate; GTP-γ-S, guanosine 5′-(3-*O*-thio)-triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)-triphosphate; DTT, dithiothreitol; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′*N*′-tetraacetic acid

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detect ras p21 proteins [12] as well as other low molecular mass GTP-binding proteins present in mouse brain, lung, kidney and spleen [13], NIH3T3 cells [13], human neutrophils [14] and human platelet membranes [15].

In the present work, we investigate the presence and localization of low molecular mass GTP-binding proteins in purified bovine adrenal chromaffin cells. A preliminary account of the results has been presented elsewhere [16].

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]GTP (3700 Ci/mmol) was purchased from NEN (Du Pont Canada Inc., Mississauga, Ont., Canada). Nitrocellulose membranes (0.2 μm pore size) were obtained from Technical Marketing (Ottawa, Ontario, Canada). ATP, GMP, GDP, GTP and GTP-γ-S were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Protein standards for electrophoresis, alkaline phosphatase conjugated anti-rabbit IgG and the alkaline phosphatase color development reagents were from Bio-Rad Laboratories (Mississauga, Ont., Canada). Rabbit polyclonal antibodies were generous gifts from Dr P.J. Fleming, Georgetown University Medical Center, Washington, DC, USA (anti-cytochrome *b*-561) and Dr H. Winkler, University of Innsbruck, Austria (anti-chromogranin A and anti-chromogranin B).

2.2. *Subcellular fractionation of chromaffin cells*

Bovine chromaffin cells isolated as described previously [17] were further purified on Percoll density gradients [18]. A 50×10^6 cell pellet was resuspended in 1.5 ml of a buffer containing 0.25 M sucrose, 1 mM EGTA, 0.2 mM PMSF and 3 mM Hepes, pH 7.0 (4°C). The cell suspension was then homogenized in a 3 ml Potter-Elvehjem type tissue grinder using 20 up and down strokes at 2700 rpm (4°C). The homogenate was centrifuged at $12000 \times g$ (Beckman microfuge) for 15 s to sediment the nuclear fraction. The supernatant obtained (S1) was then layered over a sucrose gradient formed by twenty-one 0.4 ml layers of sucrose of increasing concentrations (20 to 60%, w/w, at 2% steps, buffered with 1 mM EGTA, 0.2 mM PMSF and 3 mM Hepes, pH 7.0) prepared according to Luthe [19]. The gradient was centrifuged for 15 h at $120000 \times g$ in a Beckman SW28.1 rotor. Nineteen fractions (0.5 ml) were collected from top to bottom and then stored at −80°C until further use.

2.3. *SDS-PAGE, blotting of proteins and immunostaining*

Protein samples were subjected to electrophoresis using a highly porous SDS-PAGE system recently developed in our laboratory [20]. The proteins resolved on gel were transferred to nitrocellulose sheets electrophoretically as described previously [20]. The antibodies (anti-cytochrome *b*-561, anti-chromogranin A and anti-chromogranin B) were used at a dilution of 1:10000 on separate blots (1 h incubation) [21]. Alkaline phosphatase conjugated anti-rabbit IgG (1:3000) was used as second antibody.

2.4. *Incubation of blots with [α-^{32}P]GTP*

Nitrocellulose blots were incubated for 30 min at room temperature in a buffer containing 0.3% (w/v) bovine serum albumin (BSA), 0.25% (w/v) Tween-20, 2 mM DTT, 50 μM $MgCl_2$, 20 mM Tris-HCl, pH 7.7, and 5 μCi of [α-^{32}P]GTP/15 ml (final concentration 1 nM) [12,16]. Exceptions to this procedure are indicated in the figure legends. After washing twice in the same buffer without BSA and [α-^{32}P]GTP, blots were air dried and bound ^{32}P was detected by autoradiography (16–48 h at −80°C).

2.5. *Other methods*

Catecholamines were measured using the trihydroxyindole fluorometric method of Anton and Sayre [22]. The method of Markwell et al. [23] was used to determine protein concentrations.

3. RESULTS AND DISCUSSION

Upon centrifugation of the chromaffin cell S1 supernatant on sucrose density gradients, 19 fractions were collected from top to bottom. Protein and catecholamine contents were determined for each fraction (fig.1A). Catecholamine measurements showed that fractions 14 to 19 contained the bulk of intact chromaffin granules. These fractions also represent the second main protein peak of the gradient corresponding to the denser sucrose concentrations (1.6 to 2.0 M). Free catecholamines released from granules broken during the homogenization procedure were also detected at the top of the gradient (fractions 1–3) within the cytosol fraction.

Fig.1B shows the amido black stained polypeptide pattern of each of the 19 fractions after SDS-PAGE and electrotransfer. Immunoblottings were also carried out to characterize the sucrose gradient fractionation using rabbit polyclonal antibodies raised against various chromaffin granule markers. The corresponding immunoblots obtained with two chromaffin granule soluble protein markers, chromogranin A (80 kDa) and chromogranin B (117.5 kDa) and the granule membrane marker cytochrome *b*-561 (26 kDa) are shown in fig.1C. The smaller molecular mass bands which cross-react with the antibodies against chromogranin B (fig.1C, top) and chromogranin A (fig.1C, middle) are proteolytic derivatives of the precursors of molecular mass 117500 Da (chromogranin B) and 80 kDa (chromogranin A), respectively [24]. The higher molecular mass immunoreactive band observed with the anti-

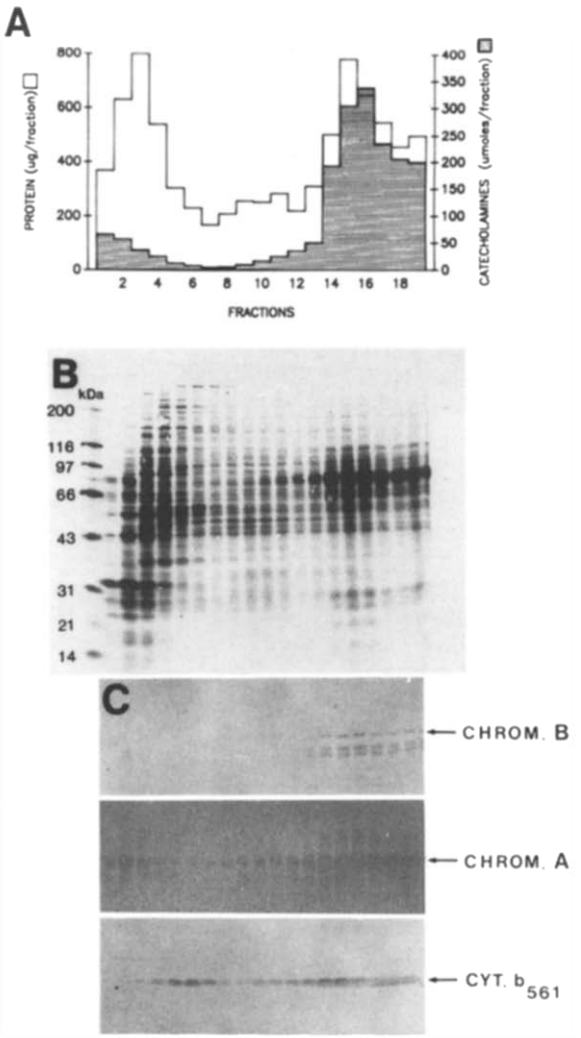

Fig.1. Characterization of the fractions obtained upon sucrose density centrifugation. (A) Catecholamines (■) and protein (□) contents of the 19 fractions collected. Aliquots (25 μl) of the fractions were also separated by SDS-PAGE and transferred on nitrocellulose membranes (B,C). (B) Polypeptide patterns visualized by amido black staining. (C) Immunostainings with antibodies against chromogranin B (117.5 kDa) (top), chromogranin A (80 kDa) (middle) and cytochrome *b*-561 (26 kDa) (bottom). The arrows indicate the position of the marker considered.

chromogranin A antibody (fig.1C, middle) is probably a non-specific staining.

Upon incubation of the blots with [α-^{32}P]GTP under the conditions described in section 2, a family of low molecular mass proteins was detected by autoradiography in chromaffin cell subcellular fractions (fig.2). The fractions 14–19 which were

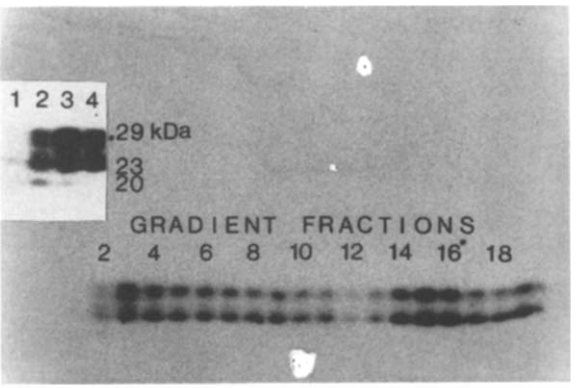

Fig.2. Detection by autoradiography of the low molecular mass GTP-binding proteins on blots of 19 gradient fractions from the same experiment described in fig.1. The 20 kDa component of the cytosolic fraction (fractions 1–3) was observed upon longer exposure (inset).

enriched in intact chromaffin granule markers, i.e. catecholamines, chromogranin A, chromogranin B and cytochrome *b*-561 were also enriched in 23 and 29 kDa GTP-binding proteins (fig.2). Longer exposure of the autoradiogram also reveals the presence of traces of 26 and 27 kDa labelled polypeptides. Thus, at least 4 labelled components (23, 26, 27 and 29 kDa) were detected upon monodimensional electrophoresis. These GTP-binding proteins were also present in fractions 4–7 corresponding to chromaffin granule membranes as indicated by immunostaining with anti-cytochrome *b*-561 (fig.1C, bottom). In this sucrose gradient, the plasma membrane was found to correspond to the fractions 3 and 4. Indeed these fractions contain the α-bungarotoxin-binding component as detected by ^{125}I-α-bungarotoxin overlay technique (Doucet and Trifaró, in preparation). No such labelled binding site is present in the fractions 5 to 19. This observation rules out the possibility of cross contamination for instance of the chromaffin granule fractions (14–19) by the plasma membrane. The GTP-binding proteins described above were also present in chromaffin cell plasma membranes purified on polycationic beads as described by Fournier and Trifaró [21] (data not shown). In addition to these labelled polypeptides, the cytosolic fraction at the top of the gradient (fractions 1–3) which was enriched in free catecholamines and chromogranins A and B, contained another GTP-binding protein of about

20 kDa (fig.2 inset). The 29 kDa GTP-binding protein is likely to correspond to the 27 kDa (G_n 27) GTP-binding protein detected in platelets, brain, adrenal gland and other tissues using 2 μM $MgCl_2$ in the incubation medium [15]. The labelling of these polypeptides was abolished completely in the presence of 5 mM CDTA (data not shown).

Binding of [α-^{32}P]GTP to cytosolic components was specific in that it was unaffected by the addition of 10 μM ATP and only partly blocked by 10 μM GMP (fig.3B,C). However, 10 μM GDP, GTP-γ-S or GTP completely abolished binding (fig.3D–F).

In mast cells [25] and human neutrophils [26] intracellular addition of GTP-γ-S is an effective stimulus to induce exocytosis. In permeabilized cultured cells, Gpp(NH)p, a non-hydrolyzable analogue of GTP, also produces stimulation of the ATP-dependent, Ca^{2+}-independent release of catecholamines [27]. However, in freshly isolated bovine chromaffin cells, GTP-γ-S inhibits Ca^{2+}-dependent exocytosis [28]. According to Bourne's model [5], the hydrolysis-resistant GTP analog GTP-γ-S should block vesicle transport by preventing the vesicle-$X_{GTP\text{-}\gamma\text{-}S}$YZ complex to set the stage for fusion [5]. In the related PC12 cells, microinjection of the 21 kDa ras proto-oncogene product induces NGF-like differentiation [29]. Moreover, microinjection of anti-ras antibodies can block the NGF-induced differentiation [30].

To our knowledge, the study described here is the first demonstration of the presence of low molecular mass GTP-binding proteins (23–29 kDa) in mammalian secretory vesicles. The GTP-binding proteins present in membranes from chromaffin granules and plasma membranes show a very similar subcellular localization as the *Bordetella pertussis* substrates (39, 40 and 41 kDa) recently found in chromaffin cells [31]. These findings and the observations described above indicate that regulation of biological processes by GTP-binding proteins in chromaffin cells is indeed very complex. Among these processes and given the subcellular localization of these proteins, the control of exocytosis is likely to be affected (see [5]).

Whether the products of the ras gene superfamily [6–11] are related to the group of low molecular mass GTP-binding proteins described in these studies remains to be determined.

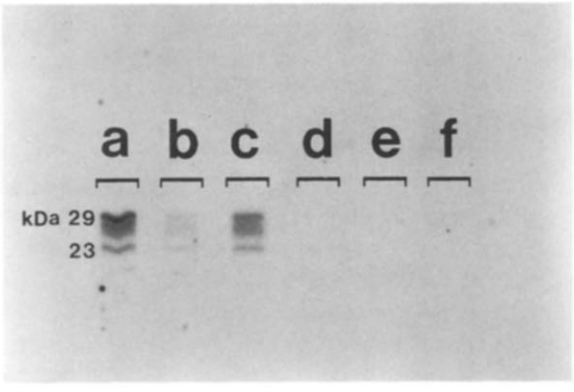

Fig.3. Specificity of nucleotide binding to chromaffin cell soluble proteins. Aliquots (30 μg) of chromaffin cell cytosol fraction were electrophoresed and blotted onto nitrocellulose. Membrane strips were incubated with 1 nM [α-^{32}P]GTP and 50 μM MgCl in the absence or presence of different nucleotides. Autoradiograms were obtained after the following treatments: (a) no nucleotide added; (b) 10 μM GMP; (c) 10 μM ATP; (d) 10 μM GDP; (e) 10 μM GTP-γ-S; (f) 10 μM GTP.

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