Detection of GTP-binding proteins in purified derivatives of rough endoplasmic reticulum

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As a first step in determining the molecular mechanism of membrane fusion stimulated by GTP in rough endoplasmic reticulum (RER), we have looked for GTP-binding proteins. Rough microsomes from rat liver were treated for the release of ribosomes, and the membrane proteins were separated by SDS/polyacrylamide-gel electrophoresis. The polypeptides were then blotted on to nitrocellulose sheets and incubated with [α - 32 P]GTP [Bhullar & Haslam (1987) *Biochem. J.* **245**, 617–620]. A doublet of polypeptides (23 and 24 kDa) was detected in the presence of 2 μ M-MgCl₂. Binding of [α - 32 P]GTP was blocked by 1–5 mM-EDTA, 10–10 000 nM-GTP or 10 μ M-GDP. Either guanosine 5'-[γ -thio]triphosphate or guanosine 5'-[β -imido]triphosphate at 100 nM completely inhibited binding, but ATP, CTP or UTP at 10 μ M did not. Pretreatment of microsomes by mild trypsin treatment (0.5–10 μ g of trypsin/ml, concentrations known not to affect microsomal permeability) led to inhibition of [α - 32 P]GTP binding, suggesting a cytosolic membrane orientation for the GTP-binding proteins. Two-dimensional gel-electrophoretic analysis revealed the 23 and 24 kDa [α - 32 P]GTP-binding proteins to have similar acid isoelectric points. [α - 32 P]GTP binding occurred to similar proteins of rough microsomes from rat liver, rat prostate and dog pancreas, as well as to a 23 kDa protein of rough microsomes from frog liver, but occurred to distinctly different proteins in a rat liver plasma-membrane-enriched fraction. Thus [α - 32 P]GTP binding has been demonstrated to two low-molecular-mass (approx. 21 kDa) proteins in the rough endoplasmic reticulum of several varied cell types.

INTRODUCTION

Several GTP-binding proteins have been described in association with the secretion apparatus of the cell. The studies by Barrowman *et al.* (1986) on the effects of GTP on secretion led Gomperts (1986) to propose that GTP-binding proteins in the secretion-vesicle membrane or plasma membrane could be involved in exocytosis. Indeed, the presence of GTP-binding proteins necessary for secretion has been described on the cytoplasmic face of both the plasma membrane and the secretory vesicles in yeast (Goud *et al.*, 1988; Salminen & Novick, 1987). Segev *et al.* (1988) suggested that GTP-binding proteins may be present at other stages of the secretion pathway in mammalian cells as well as in yeast. Consistent with this suggestion are data indicative of similar proteins in the Golgi apparatus (Melançon *et al.*, 1987; Segev *et al.*, 1988), in transition vesicles (Beckers & Balch, 1989) and rough endoplasmic reticulum (RER) (Godelaine & Beaufay, 1987; Robinson & Austen, 1987; Audigier *et al.*, 1988).

In the endoplasmic reticulum (ER) a variety of GTP-dependent functions have been described, but it is not known whether these functions are subserved by the same GTP-binding protein or by a family of GTP-binding proteins. After the initial demonstration of GTP-stimulated core glycosylation in rough microsomes (microsomal fractions) by Godelaine and her Belgian colleagues (Godelaine *et al.*, 1977), GTP was shown to activate CDP-diacylglycerol formation (Sribney *et al.*,

1977), to stimulate membrane fusion (Paiement *et al.*, 1980; Comerford & Dawson, 1988), to induce membrane permeability changes (Godelaine *et al.*, 1983; Nicchitta *et al.*, 1987), to promote calcium release (Dawson, 1985; Gill *et al.*, 1986; Henne *et al.*, 1987; Nicchitta *et al.*, 1987; Comerford & Dawson, 1988) and to assist in the integration of nascent proteins into RER (Wilson *et al.*, 1988). We have looked for GTP-binding proteins in RER with [α - 32 P]GTP on nitrocellulose blots of rough-microsomal proteins separated by one- and two-dimensional polyacrylamide-gel-electrophoretic methods. We here describe the presence of two highly active [α - 32 P]-GTP-binding proteins of 23 and 24 kDa in purified derivatives of RER from rat liver and other tissues.

MATERIALS AND METHODS

Preparation of subcellular fractions

Rough microsomes were prepared from rat liver homogenates as previously described (Paiement & Bergeron, 1983). The microsomes were stripped of ribosomes by using 5 mM-sodium pyrophosphate, and washed with 3 mM-imidazole buffer, pH 7.4, containing 0.25 M-sucrose (Paiement & Bergeron, 1983). Stripped rough microsomes (SRM) from rat prostate and from *Xenopus laevis* liver were prepared by fractionation as for the rat liver microsomes (see above). SRM from dog pancreas were prepared by the method of Shields & Blobel (1977). These were generously supplied by Dr. D. Shields (Albert

Abbreviations used: ER, endoplasmic reticulum; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; RER, rough endoplasmic reticulum; SRM, stripped rough microsomes.

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Einstein College of Medicine, Bronx, NY, U.S.A.). Purified derivatives of rat liver plasma membranes were obtained by the method of Hubbard *et al.* (1983), with the following modifications. Perfusion was not carried out before homogenization. Homogenization of 10–15 g of liver was done with 20 up-and-down strokes with a Dounce-type glass homogenizer, and the flotation step was carried out in a Beckman SW41 rotor with slow brake.

Morphological procedures

Membrane subfractions were fixed with 2.5% (v/v) ice-cold glutaraldehyde in 50 mM-phosphate buffer (pH 7.4). Fractions were recovered by filtration on to Millipore membranes (0.45 μ m pore size; Millipore Corp, Bedford, MA, U.S.A.) by the Baudhuin *et al.* (1967) procedure. After post-fixation in reduced OsO₄ (Karnovsky, 1971), the membrane pellicles were processed for electron-microscopic analysis (Paiement *et al.*, 1980).

Enzyme assays

Glucose-6-phosphatase was assayed as outlined by Nordlie & Arion (1966), and liberated P_i was detected by the method of Ames & Dubin (1960). Reactions were carried out for 10 min at 30 °C and were stopped by addition of cold (4 °C) trichloroacetic acid. 5'-Nucleotidase activity was determined exactly as described by Hubbard *et al.* (1983), and was based on the procedure of Widnell & Unkeless (1968). Liberated P_i was detected as for the glucose-6-phosphatase assay. Enzyme activity was expressed initially as μ mol of P_i released/h per mg of protein, then converted into a ratio to the initial homogenate value. Duplicate incubations were done routinely and averaged. Three separate membrane preparations were assayed, and results were averaged.

Immunological reagents

Polyclonal antibodies to SRM were produced in rabbits by an immunization protocol identical with that described by Louvard *et al.* (1982), and were previously characterized by immunoblot analysis (Paiement & Roy, 1988).

Electrophoretic blotting procedure and immunodetection

Proteins were first subjected to electrophoresis in the presence of SDS on gradient polyacrylamide gels as described previously (Paiement, 1984). The proteins were then transferred to nitrocellulose sheets by the method of Towbin *et al.* (1979). Immunological detection of proteins on nitrocellulose was done by the procedure of Towbin *et al.* (1979). Molecular masses of GTP-binding peptides in protein blots were estimated by comparing their relative mobilities with those of India-Ink-stained molecular-mass markers on nitrocellulose blots as described by Hancock & Tsang (1983). The molecular-mass markers were from Bio-Rad (Richmond, CA, U.S.A.) and included phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (29 kDa), chymotrypsinogen (25 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The chymotrypsinogen marker was from Boehringer (Mannheim, W. Germany).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed essentially by the method of O'Farrell (1975), with the following modifications. Isoelectric focusing was carried out in 12.5 cm gels (3.5 mm diameter) by using the following solution to pour four gels: 1 ml of acrylamide (28.38% acrylamide, 1.62% *NN'*-methylenebisacrylamide), 150 μ l of 10% Nonidet P40, 6.50 ml of urea (8.25 g dissolved in 6 ml of water), 375 μ l of Pharmalyte ampholyte (pH 2.5–5, 150 μ l; pH 5–8, 150 μ l; pH 3–10, 75 μ l). Catalysts used were 15 μ l of 10% (w/v) ammonium persulphate and 10 μ l of *NNN'*-tetramethylethylenediamine. After polymerization, membrane proteins were solubilized by the procedure of Ames & Nikaido (1976), and were loaded on to the gels and subjected to isoelectric focusing in a Bio-Rad high-voltage electrophoresis unit. Electrophoresis was carried out with a voltage- and current-regulated d.c. power supply (Buchler Instruments, Fort Lee, NJ, U.S.A.). Electrode buffers were 0.01 M-H₃PO₄ (anode) and 0.02 M-NaOH (cathode). Electrophoresis was carried out at 300 V for 18 h and 500 V for 1.5 h. The pH gradient established was measured by cutting rods run with sample buffer alone into 0.5 cm segments, eluting each segment for 3 h with 2 ml of water, and measuring the pH of the eluate.

The second dimension was run by the method of O'Farrell (1975), by using the Laemmli (1970) system and gradient polyacrylamide gels described previously (Paiement, 1984). Two isoelectric-focusing gels were run simultaneously on one continuous gradient slab gel, of 14 cm $\times$ 16 cm dimensions. Individual isoelectric-focusing gels were anchored into place with a 1% agarose solution containing 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 2.3% (w/v) SDS, 0.0625 M-Tris/HCl, pH 6.8, and 0.00125% Bromophenol Blue tracking dye. After electrophoresis at 14 mA for 20 h, the proteins in the gels were either stained with Coomassie Blue R (see below) or transferred on to nitrocellulose sheets by the method of Towbin *et al.* (1979). Protein staining of acrylamide gels was done overnight in 0.025% Coomassie Blue R in 10% (v/v) acetic acid/25% (v/v) propan-2-ol. Optimal destaining was achieved by incubation for 2 h in a solution containing 10% acetic acid, 10% propan-2-ol and 0.0025% Coomassie Blue R. Molecular-mass values of any given proteins were determined by comparing their mobilities with those of standards (same standards used for single-dimension gel electrophoresis described above) run in the same gel in one dimension.

GTP binding to membrane proteins

GTP binding to proteins on nitrocellulose sheets was carried out essentially as described by Bhullar & Haslam (1987). After electrophoretic transfer on to nitrocellulose sheets, the protein blots were washed briefly in buffer containing 50 mM-Tris/HCl, pH 7.5, 0.3% Tween 20 and 2 μ M-MgCl₂. Unless otherwise stated in the text, the protein blots were then incubated for 30 min at ambient temperature in the same buffer containing 1 nM or 1.0 μ Ci of [α -³²P]GTP/ml ([α -³²P]GTP of sp. radioactivity 3000 Ci/mmol, obtained from ICN Radiochemicals, Irvine, CA, U.S.A.). Unless otherwise indicated, 10 μ M-ATP (type A5394; Sigma Chemical Co., St. Louis, MO, U.S.A.) was included in the incubation medium to decrease non-specific binding. After washing several

times in the same buffer without [α - 32 P]GTP, blots were air-dried, and bound 32 P was detected by radioautography (12–90 h at -80°C , by using a Cronex Hi-Plus intensifying screen, from Picker International, Montréal, Québec, Canada).

RESULTS

Rough microsomes from rat liver were treated for the release of ribosomes, and the membrane proteins subjected to separation by SDS/polyacrylamide-gel electrophoresis. The polypeptides were then blotted on to nitrocellulose sheets and incubated with [α - 32 P]GTP as described in the Materials and methods section. Under such conditions we consistently observed [α - 32 P]GTP binding to a group of peptides. A doublet of peptides having approximate molecular masses of 23 and 24 kDa was particularly evident (Fig. 1). The doublet could be discerned when other nucleotides (i.e. ATP, CTP or UTP at $10\ \mu\text{M}$) were included during incubation of the blots in the presence of [α - 32 P]GTP, but could not be observed when blots were incubated in the presence of [α - 32 P]GTP plus $10\ \mu\text{M}$ -GDP, $100\ \text{nM}$ -p[NH]ppG or $100\ \text{nM}$ -GTP[S] (Fig. 1). GTP at concentrations of 100 – $10\,000\ \text{nM}$ completely inhibited binding of [α - 32 P]GTP to the doublet of peptides (Fig. 2). [α - 32 P]GTP binding to proteins other than the doublet of peptides in rough microsomes was considered to be of low specificity, since: (a) it was less sensitive to changing concentrations of unlabelled GTP (results not shown), (b) it mimicked [α - 32 P]CTP binding (results not shown) and (c) it was refractory to the effects of EDTA (results not shown).

We next examined the effect of Mg^{2+} on [α - 32 P]GTP binding. Whereas incubation of blots with $2\ \text{mM}$ - Mg^{2+}

Fig. 1. Detection and specificity of nucleotide binding in rat liver rough microsomes

Samples of SRM ($240\ \mu\text{g}$) were electrophoresed and blotted on to a nitrocellulose sheet. Each nitrocellulose strip was incubated with $1\ \mu\text{Ci}$ of [α - 32 P]GTP/ml, $2\ \mu\text{M}$ - MgCl_2 and in the absence or presence of a designated unlabelled nucleotide as indicated: (a) none; (b) $10\ \mu\text{M}$ -ATP; (c) $10\ \mu\text{M}$ -CTP; (d) $10\ \mu\text{M}$ -UTP; (e) $10\ \mu\text{M}$ -GDP; (f) $0.1\ \mu\text{M}$ -p[NH]ppG; (g) $0.1\ \mu\text{M}$ -GTP[S]. The molecular masses (kDa) of the labelled proteins were calculated from standard curves calibrated by using molecular-mass markers shown on the right.

Fig. 2. Sensitivity of [α - 32 P]GTP binding in rat liver rough microsomes

Samples of SRM ($240\ \mu\text{g}$) were electrophoresed and blotted on to a nitrocellulose sheet. Each nitrocellulose strip was incubated with $1\ \mu\text{Ci}$ of [α - 32 P]GTP/ml and $2\ \mu\text{M}$ - MgCl_2 in the absence of ATP and in the presence of increasing concentrations of unlabelled GTP: (a) 0; (b) $1\ \text{nM}$; (c) $10\ \text{nM}$; (d) $100\ \text{nM}$; (e) $1000\ \text{nM}$; (f) $10\,000\ \text{nM}$.

did not significantly alter [α - 32 P]GTP binding, incubation in the presence of $1\ \text{mM}$ - and $5\ \text{mM}$ -EDTA decreased binding considerably (Fig. 3). This suggests a cation requirement for [α - 32 P]GTP binding by the doublet of peptides.

To examine the non-covalent nature of the interaction between [α - 32 P]GTP and RER proteins, we incubated labelled blots with buffer containing 2% (w/v) SDS. Such treatment led to removal of bound ^{32}P as determined by radioautography of labelled blots, but yet led to negligible protein loss as determined by immunodetection of proteins in similarly treated blots by using polyclonal anti-(rat RER) antibodies (results not shown).

Fig. 3. Effect of Mg^{2+} on [α - 32 P]GTP binding in rat liver rough microsomes

Samples of SRM ($380\ \mu\text{g}$) were electrophoresed and blotted on to a nitrocellulose sheet. The strips were then incubated with $1\ \mu\text{Ci}$ of [α - 32 P]GTP/ml plus one of the following: $2\ \mu\text{M}$ - MgCl_2 (lane a), $2\ \text{mM}$ - MgCl_2 (lane b), $1\ \text{mM}$ -EDTA (lane c) or $5\ \text{mM}$ -EDTA (lane d).

Fig. 4. Detection of [α - 32 P]GTP-binding proteins in different RER preparations

Samples of different SRM were electrophoresed and blotted on to a nitrocellulose sheet. Each nitrocellulose strip containing proteins from different SRM preparations was incubated with $1\ \mu\text{Ci}$ of [α - 32 P]GTP/ml and $2\ \mu\text{M}$ - MgCl_2 : (a) $280\ \mu\text{g}$, rat liver; (b) $325\ \mu\text{g}$, dog pancreas; (c) $280\ \mu\text{g}$, rat prostate; (d) $200\ \mu\text{g}$, frog liver.

Fig. 5. Effect of trypsin on $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding

Samples of SRM (280 μg) were preincubated with various trypsin concentrations (a, 0; b, 0.1 $\mu\text{g}/\text{ml}$; c, 0.5 $\mu\text{g}/\text{ml}$; d, 1 $\mu\text{g}/\text{ml}$; e, 10 $\mu\text{g}/\text{ml}$; f, 100 $\mu\text{g}/\text{ml}$) at 10 $^{\circ}\text{C}$ for 30 min. Immediately after, the samples were boiled for 3 min in the presence of dissociation buffer, and were then subjected to electrophoresis. The gel was then blotted on to a nitrocellulose sheet. The blot was incubated with 1 μCi of $[\alpha\text{-}^{32}\text{P}]\text{GTP}/\text{ml}$ and 2 μM - MgCl_2 as described in Materials and methods.

Membrane specificity for $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding was also examined. A doublet of polypeptides of 23 and 24 kDa was observed to bind $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in protein blots of RER membranes from either dog pancreas or rat prostate (Fig. 4). Protein blots of RER membranes from frog liver revealed $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding to one main polypeptide of 23 kDa (Fig. 4).

Since GTP-binding proteins are generally considered

to be membrane proteins which are oriented towards the cell cytoplasm, we wanted to explore the topography of the main $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ -binding proteins in SRM, using trypsin digestion. Pretreatment of rat liver SRM with 0.1 μg of trypsin/ml led to increased $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding to the 23 and 24 kDa peptides and the appearance of a new peptide of 22 kDa capable of binding this same labelled nucleotide (Fig. 5). Pretreatment with 0.5 μg of trypsin/ml (a concentration known not to affect membrane permeability in rough microsomes; Paiement *et al.*, 1987) considerably decreased the $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding to the doublet and led to increased binding to the 22 kDa constituent (Fig. 5). Pretreatment of SRM with higher concentrations of trypsin (1–100 $\mu\text{g}/\text{ml}$) led to a marked decrease in the binding to the doublet of peptides, but did not prevent binding to the 22 kDa constituent (Fig. 5).

In order to determine whether the $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ -binding proteins detected in our rough-microsomal preparations were not due to the presence of contaminating derivatives of plasma membrane, we decided to compare $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding to proteins in purified rat liver SRM and those in purified rat liver plasma membrane. First we compared the morphological and biochemical properties of these two membrane subfractions and then examined $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ binding to proteins in Western blots after

Fig. 6. Morphological appearance of rough-microsomal and plasma-membrane fractions

Electron micrographs of rat liver rough-microsomal fraction (a) and plasma-membrane fraction (b) are shown. The rough-microsomal fraction shown in (a) consists of a very homogeneous population of small vesicle profiles. At higher magnification (inset) the membranes of the vesicle profiles are limited by ribosomal particles (arrowheads). The plasma-membrane fraction shown in (b) contains mostly sheets of membrane which exhibit three morphologically distinct regions. The bile-canalicular region (BC) is made up of vesicle profiles contained within a single membrane. The lateral surface region (LS) consists of straight electron-dense segments in continuity with the membranes which surround the bile canaliculae. The sinusoidal front (SF) region consists of membrane segments which extend from the lateral surface region and are associated with numerous irregular vesicle profiles. In (a) and (b): bar, 2 μm ; magnification $\times 8900$. Inset: bar, 0.2 μm ; magnification $\times 42000$.

Table 1. Enzymic activity of rough-microsomal and plasma-membrane fractions

Conditions used to isolate the fractions and enzyme assays are described in the Materials and methods section. Values for enzyme activity are given as means ($\pm$ S.D.) for membrane subfractions from three separate experiments. Specific activity is expressed as μ mol of P_i liberated/h per mg of protein.

Enzyme	SRM		Plasma membranes	
	Sp. activity	Purification over homogenate (fold)	Sp. activity	Purification over homogenate (fold)
Glucose-6-phosphatase	29.85 ± 3.42	6.07 ± 0.84	5.38 ± 1.35	1.09 ± 0.16
5'-Nucleotidase	1.55 ± 0.33	0.36 ± 0.01	45.50 ± 10.25	12.94 ± 1.52

electrophoresis on pH-Ampholine (pH 2.5–5, pH 5–8 and pH 3–10 in the proportions 2:2:1, by vol.)-gradient two-dimensional gels.

The relative enrichment of each fraction was analysed by electron microscopy and by biochemistry. By morphology the rough-microsomal fraction was highly enriched with ribosome-studded vesicles (Fig. 6a), and the plasma-membrane fraction contained mainly large double-membrane structures characterized by three sub-compartments, bile canaliculae, lateral surface regions and sinusoidal front regions (Fig. 6b). By biochemistry the rough-microsomal fraction was enriched with the enzyme marker glucose-6-phosphatase but decreased in

amount of 5'-nucleotidase, the enzyme marker for plasma membranes (Table 1). The plasma-membrane fraction contained about the same amount of glucose-6-phosphatase activity as was in the original homogenate, but was enriched with the enzyme 5'-nucleotidase (Table 1).

Radioautograms of two-dimensional gel Western blots of rough-microsomal proteins incubated with [α - 32 P]GTP revealed the presence of a number of [α - 32 P]GTP-binding proteins that differed in both molecular mass and isoelectric point (Fig. 7b). Three groups of proteins of 24, 23 and 22 kDa were made up of constituents that had similar pI values (estimated at 7.3, 7.0 and 6.8) (Fig. 7b).

Fig. 7. Comparison of rough-microsomal and plasma-membrane proteins from rat liver by using two-dimensional gels

Samples of rat liver SRM (99.7 μ g) and plasma membrane (100 μ g) were separated by isoelectric focusing, followed by SDS/polyacrylamide-gel electrophoresis in duplicate. One gel was stained with Coomassie Blue [SRM (a) and plasma membrane (c)], and the other was blotted on to a nitrocellulose sheet and subsequently incubated for detection of [α - 32 P]GTP binding as described in the Materials and methods section [SRM (b) and plasma membrane (d)]. The arrow in (c) indicates a protein with molecular mass and isoelectric point similar to those of actin (Hubbard *et al.*, 1983). The short lines on the right-hand side of each photograph represent, from top to bottom, the position of the following molecular-mass markers: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (29 kDa), chymotrypsinogen (25 kDa) and soybean trypsin inhibitor (21.5 kDa).

The group of 22 kDa proteins contained, in addition, constituents with pI values estimated at 6.6, 6.4, 6.1 and 5.4 (Fig. 7b). The 22 kDa proteins were not evident in blots produced after one-dimensional SDS/polyacrylamide-gel electrophoresis, probably because they were masked by the high labelling of the 23 kDa proteins, and because the resolution was not as good as that obtained with two-dimensional SDS/polyacrylamide-gel electrophoresis. Coomassie Blue staining revealed few proteins in this region of the gel (Fig. 7a). Radioautograms of two-dimensional gel Western blots of plasma-membrane proteins incubated with [α - 32 P]GTP revealed the presence of [α - 32 P]GTP-binding proteins that differed in both molecular mass and isoelectric point (Fig. 7d). Two groups of proteins of 23.5 and 22 kDa were made up of constituents that had dissimilar pI values. The 23.5 kDa proteins were resolved into one discrete constituent (pI $\sim$ 7.0) and a diffuse constituent with estimated pI values extending between 6.7 and 6.2. The 22 kDa proteins were resolved into two diffuse constituents with estimated pI values extending between 7.4 and 6.8 and between 6.7 and 5.6 (Fig. 7d). Coomassie Blue staining did not reveal many proteins in this part of the gel (Fig. 7c).

DISCUSSION

We have documented the identification of two major GTP-binding proteins from purified derivatives of RER. This study also supports the usefulness of [α - 32 P]GTP binding to nitrocellulose blots of proteins of subcellular fractions separated by SDS/polyacrylamide-gel electrophoresis (Bhullar & Haslam, 1987).

The doublet of peptides of 23 kDa and 24 kDa was evident by its ability to bind [α - 32 P]GTP specifically with high affinity. Binding was of very high sensitivity, and was routinely detected with as little as 1 nM-[α - 32 P]GTP. The nucleotide specificity was remarkable in that 1 nM-[α - 32 P]GTP could be detected even in the presence of 1 mM-CTP or 1 mM-ATP (results not shown). The non-hydrolysable analogues of GTP, GTP[S] and p[NH]ppG, inhibited [α - 32 P]GTP binding. Incubations in the presence of 1 mM- and 5 mM-EDTA also inhibited binding, suggesting a Mg^{2+} -dependence for GTP binding. Studies of [α - 32 P]GTP binding using rough-microsomal preparations from different tissue sources suggest that similar GTP-binding proteins may exist in the RER of many cell types.

We used two-dimensional gel electrophoresis to compare [α - 32 P]GTP binding to proteins in rough microsomes with that to proteins in plasma membranes from rat liver. These fractions were shown to be highly enriched in their respective morphological and enzyme markers (Fig. 6 and Table 1). [α - 32 P]GTP-binding studies with Western blots of both types of membrane fractions revealed both similarities and differences. Both membrane fractions contained 22 kDa GTP-binding proteins with similar isoelectric points, including constituents with pI values of 7.3, 7.0, 6.6, 6.4 and 6.1. Although we cannot exclude the possibility that the 22 kDa GTP-binding proteins that we detect in rough microsomes are due to contamination by plasma-membrane derivatives, we consider this unlikely, because of the low amounts of plasma-membrane marker enzyme in this preparation (see Table 1) and because the specific 23.5 kDa GTP-binding proteins with pI values extending from 6.7 to 6.2 found in the

plasma-membrane fraction are absent from the rough-microsomal fraction. The 22 kDa GTP-binding proteins that we detect in rough microsomes may correspond to the 22 kDa ADP-ribosylated protein previously described in purified RER membranes by Robinson & Austen (1987). Purified rat liver rough microsomes contain two specific groups of GTP-binding proteins. Although these have different molecular masses, 23 and 24 kDa, they contain proteins with virtually identical pI values (i.e. 7.3, 7.0 and 6.8). By contrast, the plasma-membrane preparation contains a group of GTP-binding proteins of 23.5 kDa which can be resolved into two constituents with pI values ranging between 7.4 and 6.8, and between 6.7 and 6.2. We conclude that the 23 and 24 kDa GTP-binding proteins detected in the rough-microsomal preparation are different from the 23.5 kDa GTP-binding proteins detected in the plasma-membrane preparation. Nevertheless, the close similarities in molecular mass and isoelectric charge properties of the GTP-binding proteins in these two membrane subfractions provoke questions about the possible relationship between these proteins and those previously described in other cytoplasmic membranes (Segev *et al.*, 1988; Goud *et al.*, 1988; Comerford & Dawson, 1989).

Western blotting of plasma-membrane proteins and incubation with [α - 32 P]GTP did not permit detection of the traditional GTP-binding proteins (G_s or G_i) (Fig. 7). Furthermore, by a similar technique we were unable to detect the 41 kDa GTP-binding protein described in purified RER membranes by Audigier *et al.* (1988). We have carried out electrophoresis under less denaturing conditions (i.e. in the MZE 3328.IV system; Moos *et al.*, 1988), and have transferred proteins on to Immobilon polyvinylidene difluoride membranes and obtained similar results, i.e. we detected low-molecular-mass ($\sim$ 21 kDa) GTP-binding proteins, but not ones of higher molecular mass ($\sim$ 40 kDa) (results not shown). We concur with Bhullar & Haslam (1987), who suggest that high-molecular-mass GTP-binding proteins may be more susceptible to irreversible denaturation during electrophoresis.

The site of location of the 23 and 24 kDa GTP-binding proteins in rough microsomes is the cytosolic surface of the microsomes, on the basis of controlled protease digestion (Fig. 5). Trypsin concentrations known to inhibit RER membrane fusion (a GTP-dependent function) and to maintain enzyme latency to mannose-6-phosphate (Paiement *et al.*, 1987) were observed to alter [α - 32 P]GTP binding. Comerford & Dawson (1989) obtained similar effects with low concentrations of trypsin and a total rat liver microsomal preparation. They suggested that trypsin-sensitive GTP-binding proteins were responsible for membrane fusion, and furthermore that such fusion provoked coincidental Ca^{2+} efflux (Comerford & Dawson, 1989). Direct involvement of a specific GTP-binding protein in membrane fusion, or any of the other GTP-dependent RER functions, has yet to be demonstrated both *in vitro* and *in vivo*. The ER is a highly dynamic membrane system capable of undergoing extensive structural alterations *in vivo* (Lee & Chen, 1988; Paiement *et al.*, 1988); it will be of interest to determine the molecular mechanism responsible for such alterations and whether biochemical changes such as phospholipid synthesis, core glycosylation or Ca^{2+} release occur coincidentally.

The apparent conversion of the labelled proteins into

a protein of 22 kDa by trypsin (Fig. 5) is of interest, especially in light of an observed increase in [α - 32 P]GTP binding after treatment with low concentrations of this protease. We have recently shown that this 22 kDa GTP-binding protein is a soluble protein that can be separated from the rough microsomes by high-speed sedimentation and that it consists mainly of one molecular constituent of pI 7.4 (J. Lanoix, L. Roy & J. Paiement, unpublished work). Thus the conversion of the 23 and 24 kDa GTP-binding proteins into a single 22 kDa GTP-binding protein probably occurs by release of a common peptide fragment which is acidic. When associated with the original GTP-binding proteins, such a fragment may induce conformational constraints such that GTP binding is decreased. *In vivo* the GTP-binding proteins may exist in both membrane-bound and soluble forms. Relocation on to the RER membrane may occur in response to an internal stimulus (via acylation?), and this could have consequences for GTP-binding activity and/or GTPase activity, as was shown for H-*ras* p21 (Calés *et al.*, 1988). The many GTP-dependent events previously described in ER derivatives may represent regulatory processes initiated by the same, or several, GTP-binding proteins. The identity of the proteins involved in each event and the exact mechanism by which such events are stimulated remain to be determined.

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