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Identification and purification from bovine brain of a guanine-nucleotide-binding protein distinct from G_s, G_i and G_o

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A guanine-nucleotide-binding protein (G-protein) was purified from cholate extracts of bovine brain membranes by sequential DEAE-Sephacel, Ultrogel AcA-34, heptylamine–Sepharose and Sephadex G-150 chromatography. Guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[^{35}S])-binding activity copurified with a 25000 Da peptide and a 35000–36000 Da protein doublet. Neither pertussis toxin nor cholera toxin catalysed the ADP-ribosylation of a protein associated with the GTP[^{35}S]-binding activity. Photoaffinity labelling of the purified protein with 8-azido[γ-^{32}P]GTP indicated that the GTP-binding site resides on the 25000 Da protein. The 35000–36000 Da protein doublet was electrophoretically indistinguishable from the β-subunits of other GTP-binding proteins, and the 36000 Da protein was recognized by antiserum to oligomeric G_t. The purified protein specifically bound 17.2 nmol of GTP[^{35}S]/mg of protein. The K_d of the binding site for radioligand was approx. 15 nM. The brain GTP-binding protein co-migrated during SDS/polyacrylamide-gel electrophoresis with a GTP-binding protein, named G_p, purified from human placenta [Evans, Brown, Fraser & Northup (1986) J. Biol. Chem. **261**, 7052–7059], and cross-reacted with antiserum raised against the placental protein, but not with antiserum raised to brain G_o. SDS/polyacrylamide-gel electrophoresis of the brain and placental GTP-binding proteins in the presence of *Staphylococcus aureus* V8 protease yielded identical peptide maps.

INTRODUCTION

A family of structurally and functionally homologous, membrane-associated, guanine-nucleotide-binding regulatory proteins (G-proteins) subserves an obligatory function in a variety of transmembrane signalling processes (Gilman, 1984). G_s, G_i and G_t are the functionally recognized members of this family in that the enzymes subject to modulation have been identified, i.e. hormone-sensitive adenylate cyclase and retinal cyclic GMP phosphodiesterase. G-proteins also have been implicated as transducing elements in the hormone–receptor-mediated regulation of phospholipid metabolism (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985) and ion channels (Pfaffinger *et al.*, 1985; Breitwieser & Szabo, 1985; Andrade *et al.*, 1986; Hescheler *et al.*, 1987; Sasaki & Sato, 1987). The identity of the G-proteins involved in these signalling systems has not been established.

The probability that many of the multifarious processes of communication in the central nervous system involve signalling mechanisms unrelated to the regulation of adenylate cyclase has prompted interest in the identification and purification of novel G-proteins from brain. For example, several laboratories have reported the purification of a G-protein called 'G_o' that apparently possesses the characteristic $\alpha\beta\gamma$ oligomeric subunit structure of the G-proteins of known function, and that, like G_i and G_t, is a substrate for pertussis-toxin-catalysed ADP-ribosylation (Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985; Katada *et al.*, 1986). We now report the identification and purification of a 'new' GTP-binding activity from bovine brain membranes. The GTP-binding protein has a molecular mass of 25000 Da, co-purifies with a characteristic 35000–36000 Da doublet, which most likely represents a β-subunit, and is electrophoretically, structurally and immunologically identical with a GTP-binding protein called 'G_p' recently purified from placenta.§

Abbreviations used: G-protein, any GTP-binding protein that resembles a family of homologous proteins, composed to date of G_s, G_i, G_o and G_t; G_s and G_i, the identified regulatory components of adenylate cyclase that mediate stimulation and inhibition respectively; G_t, transducin from retinal rod outer segments; G_o, a GTP-binding protein originally purified from membranes of bovine brain; G_p, a GTP-binding protein originally purified from placenta membranes; ARF, the protein factor necessary for cholera-toxin-catalysed ADP-ribosylation of G_s; GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppA, 5′-adenylyl imidodiphosphate; PAGE, polyacrylamide gel electrophoresis.

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§ A protein called 'G_p' has been purified previously from human placenta and platelet (Evans *et al.*, 1986). To date there is no evidence that this protein is equivalent to the putative G-protein 'G_p' implicated in receptor-mediated regulation of phosphoinositide hydrolysis. In the present work a protein was purified from bovine brain that is electrophoretically, immunologically and structurally indistinguishable from the protein previously purified from placenta. Owing to the recently widespread acceptance of the term 'G_p' in the phosphoinositide-signalling field, it is probably less confusing to refer to the protein studied here as '$G_{\alpha\text{-}25000}$' until a physiological role for the brain protein can be established.

EXPERIMENTAL

Membrane preparation

Fresh bovine brains were placed on ice and transported to the laboratory, where all subsequent procedures were performed at 4 °C. The cerebral cortices were dissected free from cerebellum and brain stem, and placed in 10% (w/v) sucrose/20 mM-Tris (pH 7.5)/0.1 mM-phenylmethanesulphonyl fluoride/0.1 mM-benzamidine. Meninges, large blood vessels and blood clots were removed, and the tissue was minced with scissors and homogenized in 4 vol. of buffer with a Waring blender and a Polytron. The resulting suspension was filtered through gauze, and membranes were collected by centrifugation for 20 min at 38000 *g*. The membrane pellets were washed twice by resuspension in 4 vol. of buffer with a Polytron, followed by centrifugation for 20 min at 38000 *g*. The final pellet was resuspended to a protein concentration of approx. 10 mg/ml and stored at −80 °C.

G-protein purification

Membranes (7–10 g of protein) were thawed rapidly in a 35 °C water bath and collected by centrifugation at 38000 *g* for 30 min at 4 °C. The pelleted membranes were washed once with TED buffer (20 mM-Tris, pH 8.0, 1 mM-EDTA, 1 mM-dithiothreitol and 0.1 mM-benzamidine). The washed membranes were resuspended to 2 litres with TED buffer containing a final concentration of 1% sodium cholate and incubated with constant stirring at 4 °C for 60 min. The bulk of the extracted membranes was removed by centrifugation at 38000 *g* for 20 min. The supernatant was collected, and the remaining membranes were removed by centrifugation at 200000 *g* for 60 min. The supernatant (1660 ml) was collected as the soluble extract.

The extracted material was applied (flow rate 0.75 ml/min) to a 430 ml (2.5 cm × 88 cm) column of DEAE-Sephacel (Pharmacia) equilibrated with 2 litres of TED/1% sodium cholate. The column was washed with 1 bed volume of equilibration buffer, and the proteins were eluted with a 900 ml linear gradient of 0–175 mM-NaCl in TED/1% sodium cholate; 10 ml fractions were collected. The column then was washed sequentially with 200 ml of 175 mM-NaCl, 400 ml of 250 mM-NaCl and 500 ml of 500 mM-NaCl, all in TED/1% sodium cholate.

The trailing shoulder of the large peak of GTP[^{35}S]-binding activity (fractions 85–125; 380 ml) that was eluted from the DEAE-Sephacel column (Fig. 1) was pooled and concentrated to approx. 36 ml by pressure filtration through an Amicon PM-10 membrane. The material was applied to a 422 ml column (2.5 cm × 86 cm) of Ultrogel AcA-34 (LKB) at a flow rate of 0.3 ml/min and eluted with TED/1% sodium cholate/100 mM-NaCl; 3.2 ml fractions were collected (Fig. 2).

The second peak of GTP[^{35}S]-binding activity eluted from Ultrogel AcA-34 (fractions 110–128; 59 ml) was pooled and diluted with 177 ml of TED/100 mM-NaCl. This diluted pool was applied (flow rate 0.3 ml/min) to an 81 ml (1.5 cm × 46 cm) column of heptylamine–Sepharose previously equilibrated with TED/0.25% sodium cholate/100 mM-NaCl. The column was washed with 40 ml of TED/0.25% sodium cholate/100 mM-NaCl, followed by 50 ml of TED/0.25% sodium cholate/300 mM-NaCl. The GTP[^{35}S]-binding activity was then eluted with a 320 ml linear gradient of TED/0.25% sodium cholate/200 mM-NaCl to TED/1.3% sodium cholate/50 mM-NaCl; 2.8 ml fractions were collected in polypropylene tubes. The column was washed with an additional 80 ml of TED/1.3% sodium cholate/50 mM-NaCl and regenerated with TED/1% sodium cholate/500 mM-NaCl/7 M urea.

The single peak of GTP[^{35}S]-binding activity (fractions 80–105; 70 ml) from the heptylamine–Sepharose column (Fig. 3) was pooled and concentrated by pressure filtration through an Amicon PM-10 membrane to a volume of 5 ml. This material was applied to a 127 ml (1.5 cm × 72 cm) column of Sephadex G-150 (superfine grade) and eluted with TED/1% sodium cholate/100 mM-NaCl (flow rate 0.074 ml/min); 0.9 ml fractions were collected in polypropylene conical microfuge tubes. Two independent pools were taken; the first (fractions 70–86; 15 ml) contained one major contaminant, of 47000 Da, in addition to 25000 Da and 35000–36000 Da proteins, and a second pool (fractions 87–94; 6.8 ml) consisted of the 25000 Da and 35000 Da proteins. These two pools were concentrated by pressure filtration through an Amicon PM-10 membrane to final volumes of 0.75 and 1.12 ml respectively. The proteins were stored frozen at −80 °C.

Assays

Binding of GTP[^{35}S] to G-proteins was determined by methods previously described (Northup *et al.*, 1982), with minor modifications. In the standard assay, 10 μl of sample was diluted with 90 μl of a solution containing 20 mM-Hepes, pH 8.0, 1 mM-EDTA, 1 mM-dithiothreitol and 0.1% Lubrol. The binding reaction was initiated by the additon of 100 μl of a solution containing 50 mM-Hepes, pH 8.0, 1 mM-EDTA, 100 mM-$MgCl_2$, 200 mM-NaCl, 4 μM-GTP[S] and GTP[^{35}S] (10^5 c.p.m.). The assays were incubated at 30 °C for 60 min, and the reaction was terminated by diluting the samples with 4 ml of ice-cold wash buffer [20 mM-Tris (pH 8.0)/25 mM-$MgCl_2$/100 mM-NaCl] followed by rapid filtration through BA85 nitrocellulose filters (Schleicher and Schuell). The filters were washed with 4 × 4 ml of cold wash buffer, dried, placed in 4 ml of scintillation fluid, and the retained radioactivity was measured in a scintillation counter. Scatchard analysis of the binding of GTP[^{35}S] to the purified protein (second pool from the Sephadex G-150 column) was accomplished by varying the concentration of non-radioactive GTP[S] from 1 to 100 nM in the presence of a constant amount of GTP[^{35}S]. Specific activities were calculated for each ligand concentration, and these activities were used to calculate the mol of bound ligand at a respective free ligand concentration. Non-specific binding (in the presence of 100 μM-GTP[S]) was less than 5% of the total radioactivity retained by the filter.

Pertussis-toxin-catalysed ADP-ribosylation of the 41000 and 39000 Da α-subunits of G_i and G_o was carried out essentially as described by Bokoch *et al.* (1984). The ribosylation reaction was initiated by addition of 5 μl of a column fraction to 95 μl of a reaction mixture such that the final concentrations of reagents were: 10 mM-thymidine, 1 mM-ATP, 0.2 mM-GTP, 2.5 mM-$MgCl_2$, 1 mM-EDTA, 10 mM-dithiothreitol, 100 mM-Tris, pH 8.0, 1 mg of phosphatidylcholine (egg yolk)/ml, 10 μM-NAD^+, 10^7 c.p.m. of

[^{32}P]NAD$^+$ and 13.5 μg of pertussis toxin/ml. Incubation was for 90 min at 30 °C, and the reaction was terminated by the addition of 500 μl of 3% SDS containing 10 μg of bovine serum albumin/ml. Protein was precipitated by addition of 500 μl of cold 30% (v/v) trichloroacetic acid. The precipitated samples were rapidly filtered through BA85 nitrocellulose filters and washed with 5 × 4 ml of cold 6% trichloroacetic acid, dried, placed in 4 ml of scintillation fluid, and radioactivity was quantified in a scintillation counter.

Photoaffinity labelling of the purified guanine-nucleotide-binding protein with 8-azido[γ-^{32}P]GTP was performed as described elsewhere (Evans *et al.*, 1986). Briefly, 5 μl of purified protein (~ 2.75 μg) was incubated on ice for 10 min in a final reaction volume of 100 μl. The reaction contained the following reagents (final concns.): 50 mM-Hepes, pH 8.0, 1 mM-EDTA, 100 mM-NaCl, 20 mM-$MgCl_2$, 0.1% Lubrol, 50 μM-dithiothreitol and 5 μM-8-azido[γ-^{32}P]GTP (sp. radioactivity 12.7 Ci/mmol). The samples were irradiated at a distance of 2.5 cm for 5 min with a Mineralite UVG-54 lamp (254 nm). Additional purified protein (10 μg) was added as a carrier, and the samples were precipitated overnight at 4 °C with trichloroacetic acid in the presence of 2% SDS. The samples were prepared for electrophoresis as described below and subjected to SDS/PAGE on a linear gradient gel of 11–14% acrylamide.

Immunoblots

Purified G-proteins (5 μg/lane) were loaded on a linear gradient gel (11–14% acrylamide) and subjected to SDS/PAGE. Upon completion of electrophoresis, the gel was removed and soaked for 20 min in a solution containing 25 mM-Tris, pH 8.3, 192 mM-glycine, 20% (v/v) methanol and 0.1% SDS. The proteins were electrophoretically transferred (30 V for 12–14 h at 4 °C) from the gel to a nitrocellulose sheet. The blot was washed twice with 20 mM-Tris (pH 7.5)/500 mM-NaCl, and the remaining protein-binding sites were blocked by incubating the nitrocellulose sheet for 30 min in 3% bovine serum albumin/20 mM-Tris (pH 7.5)/500 mM-NaCl. The immunoblots were incubated overnight at room temperature with a polyclonal antiserum raised against the α-subunit of bovine brain G_o, the α-subunit of human platelet G_p, or oligomeric G_t. The primary antibody was removed and the blot washed twice with 20 mM-Tris (pH 7.5)/500 mM-NaCl/0.05% Tween 20, and once with 20 mM-Tris (pH 7.5)/500 mM-NaCl. The nitrocellulose sheet was then incubated with a 1:1000 dilution of anti-rabbit IgG–alkaline phosphatase conjugate (Sigma Chemical Co.) in 20 mM-Tris (pH 7.5)/500 mM-NaCl/1% albumin for 1–2 h. The blot was washed three times with 20 mM-Tris (pH 7.5)/500 mM-NaCl/0.05% Tween 20, and developed with 0.5 mg of 5-bromo-4-chloroindol-3-yl phosphate/ml in 1 M-Tris, pH 8.8.

SDS/PAGE

Electrophoresis of polypeptides through linear gradient gels of 11–14% polyacrylamide was accomplished with the discontinuous system described by Laemmli (1970). Purified G_p from human placenta and the purified G-protein from bovine brain also were electrophoresed through single-concentration gels of 10, 12, 14 and 16% acrylamide. Ferguson plots were constructed (Neville, 1971; Ferguson, 1964) and used to determine the apparent molecular mass of the α-subunit. Except for samples labelled with 8-azido[α-^{32}P]GTP, all samples were alkylated with *N*-ethylmaleimide before electrophoresis as described by Sternweis & Robishaw (1984). This alkylation procedure enhanced the clarity and resolution of polypeptide bands. All gels were run at 6 °C. Proteins were detected with Coomassie Blue or silver as described previously (Evans *et al.*, 1986). Autoradiography of photoaffinity-labelled samples was accomplished by exposing the dried gels to Kodak XAR-5 film in DuPont Cronex Hi-Plus intensifying screens at −80 °C. Molecular-mass standards were obtained from Bio-Rad and LKB. *Staphylococcus aureus* V8 protease (Boehringer–Mannheim) digestion of the purified G-proteins was accomplished by the method of Lam & Kasper (1980).

Protein assay

Proteins were measured by the method of Lowry *et al.* (1951), and the method described by Schaffner & Weissman (1973), using Amido Black. Bovine serum albumin was used as the standard for both assays.

Materials

Sodium cholate (Sigma) was re-purified by the method described by Ross & Schatz (1978). [^{32}P]P_i and 8-azido[γ-^{32}P]GTP (12.7 Ci/mmol) were obtained from ICN Radiochemicals, and GTP[^{35}S] (1022 Ci/mmol) was from New England Nuclear. GTP[S] and p[NH]ppA were from Boehringer–Mannheim. Heptylamine–Sepharose was prepared from monoaminoheptane by the method of Shaltiel (1974) with modifications (Northup *et al.*, 1980). The method of Cassel & Pfeuffer (1978) was used to prepare [^{32}P]NAD$^+$, and pertussis toxin was purified from cultures of *Bordetella pertussis* as described previously (Hughes *et al.*, 1984).

RESULTS

Soluble extract from bovine brain membranes was bound to DEAE-Sephacel and eluted with a gradient of NaCl (Fig. 1). Approx. 40% of the GTP[^{35}S]-binding activity in the cholate extract was recovered from DEAE-Sephacel. GTP[^{35}S]-binding activity was eluted as one major broad, asymmetrical, peak of relatively high specific activity and two smaller peaks of relatively low specific activity. The asymmetry of this main peak was observed in seven independent preparations under several elution conditions. In one preparation, two distinct peaks of GTP[^{35}S]-binding activity were resolved at these concentrations of NaCl.

Since at least the α-subunits of G_i and G_o are substrates for ADP-ribosylation by pertussis toxin, the elution pattern of proteins labelled in the presence of [^{32}P]NAD$^+$ and pertussis toxin was determined. A symmetrical peak (fractions 65–90) of ^{32}P-labelled substrate was eluted with the first half of the major peak of GTP[^{35}S]-binding activity (Fig. 1). Pertussis toxin catalysed the [^{32}P]ADP-ribosylation of 39000 and 41000 Da proteins in these fractions, as determined by autoradiography of samples subjected to SDS/PAGE (G. L. Waldo, unpublished work). In contrast, the trailing edge of the major peak of GTP[^{35}S]-binding activity contained very little pertussis-toxin substrate. A 35000 and 36000 Da protein doublet could be detected across the entire peak of GTP[^{35}S]-binding activity, and the

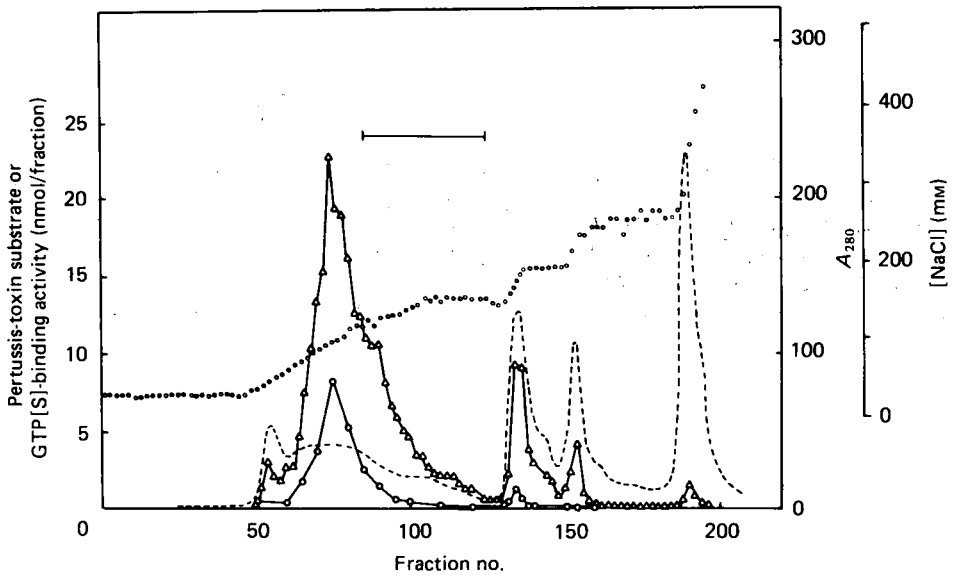

Fig. 1. DEAE-Sephacel chromatography of bovine brain cholate extract

Proteins were eluted from the column with a 900 ml linear gradient of 0–175 mM-NaCl; 10 ml fractions were collected. The NaCl concentration (o o o) of the eluate was determined by measuring the conductivity of each fraction. The elution of protein from the column, expressed as A_{280} (----), was monitored with an absorbance detector attached to the column outlet. GTP[^{35}S]-binding activity (△) and pertussis-toxin substrate (○) were measured by filtration assay. Fractions 85–125 were pooled (horizontal bar) and concentrated as described in the Experimental section.

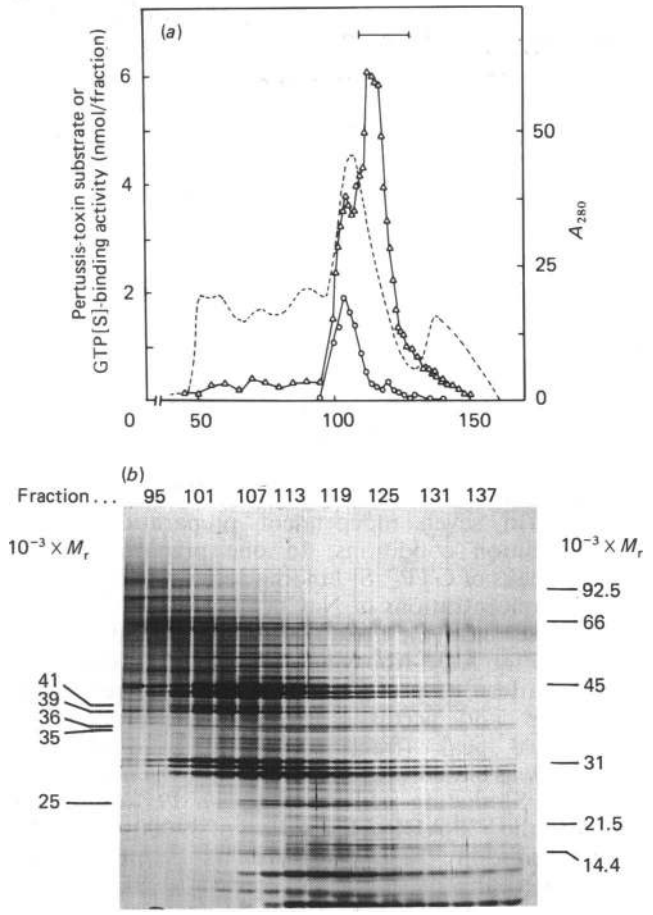

Fig. 2. Ultrogel AcA-34 chromatography of the 25000 Da-protein-enriched pool from DEAE-Sephacel

(*a*) Elution profiles of GTP[^{35}S]-binding activity (△), pertussis-toxin substrate (○) and A_{280} (----) are shown; 3.2 ml fractions were collected. Horizontal bar indicates fractions pooled for next step. (*b*) SDS/PAGE of fractions obtained from Ultrogel AcA-34. Samples of the indicated fractions were alkylated with *N*-ethylmaleimide and dissolved in sample buffer. Lanes of a linear gradient gel consisting of 11–14% acrylamide were loaded with 20 μl of prepared sample containing 5 μl of a column fraction. Proteins were detected with silver stain. Protein standards are indicated on the right.

distribution of a protein of 25000 Da corresponded well to the trailing shoulder (fractions 85–120) of GTP[^{35}S]-binding activity. These fractions were pooled, concentrated, and chromatographed through Ultrogel AcA-34 (Fig. 2*a*). The GTP[^{35}S]-binding activity was eluted as two peaks. The first peak apparently contained G_o and G_i, as indicated by pertussis-toxin-catalysed [^{32}P]ADP-ribosylation. The second peak comprised most of the GTP-binding protein, and contained very little pertussis-toxin substrate. As with the shoulder of GTP[^{35}S]-binding activity that was eluted from DEAE-Sephacel, the prominent GTP[^{35}S]-binding activity from AcA-34 co-eluted with a protein of 25000 Da (Fig. 2*b*). Thus selective pooling of the broad peak of GTP[^{35}S]-binding activity that was eluted from DEAE-Sephacel allows the clear resolution of two peaks of activity on elution of that material from AcA-34.

AcA-34 fractions 110–128 were pooled, diluted to a final cholate concentration of 0.25%, and chromatographed through heptylamine–Sepharose. The GTP[^{35}S]-binding activity was eluted as a single peak (Fig. 3*a*). A small amount of material labelled in the presence of [^{32}P]NAD$^+$ and pertussis toxin was eluted before that peak. The major protein bands associated with the GTP[^{35}S]-binding activity were a 35000–36000 Da protein doublet, a 25000 Da protein and a 47000 Da protein (Fig. 3*b*).

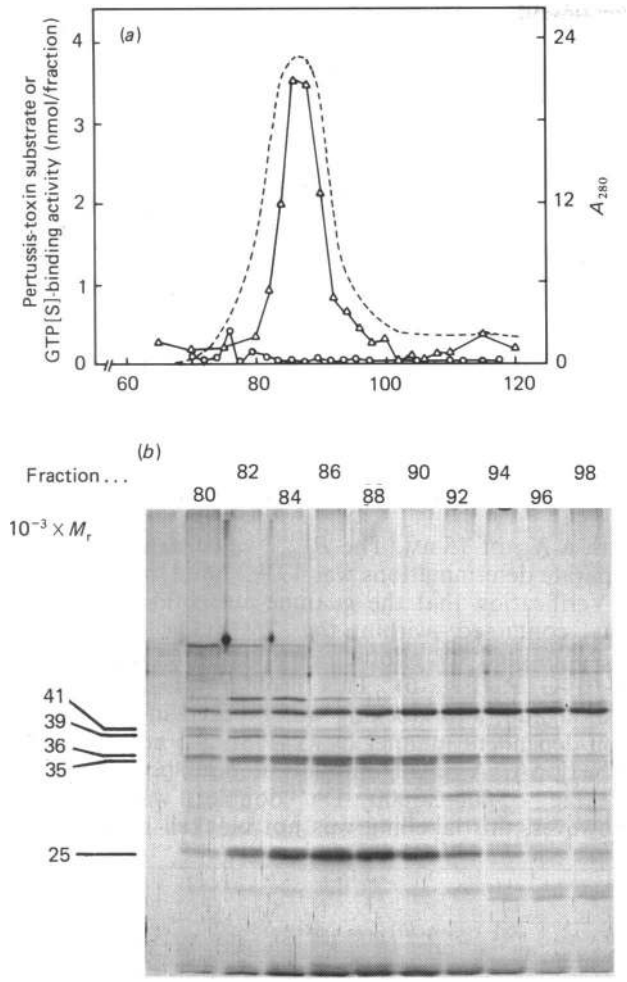

Fig. 3. Heptylamine–Sepharose chromatography of the 25000 Da-protein-enriched pool from Ultrogel AcA-34

(*a*) Elution profiles of GTP[^{35}S]-binding activity (△), pertussis-toxin substrate (○) and A_{280} (----) are shown; 2.8 ml fractions were collected. (*b*) SDS/PAGE of fractions obtained from heptylamine–Sepharose. Samples were prepared and loaded as described in Fig. 2. Proteins were detected with silver stain.

The pool (fractions 80–105) of GTP[^{35}S]-binding activity from heptylamine–Sepharose was concentrated and applied to a Sephadex G-150 (superfine) gel-filtration column (Fig. 4*a*). Although no pertussis-toxin substrate could be detected by filtration assay of fractions from this column, autoradiography of SDS/PAGE gels with long exposure in intensifying screens revealed very low amounts of [^{32}P]ADP-ribosylated proteins of approx. 39000 and 41000 Da in fractions 70–80 (G. L. Waldo, unpublished work). Relatively small amounts of proteins of 39000 and 41000 Da also were silver-stained on SDS/PAGE gels of these fractions (Fig. 4*b*). The major silver-stained proteins were of 25000, 35000–36000 and 47000 Da. In contrast, on the basis of both silver staining of SDS/PAGE gels and autoradiography of samples labelled in the presence of pertussis toxin and [^{32}P]NAD$^+$, no G_i or G_o could be detected in fractions 87–94. The major protein bands in these fractions were the 35000–36000 Da doublet and the 25000 Da protein. Densitometry of gels indicated that 88 and 97% of the protein stained by silver and Coomassie Blue, respectively, was associated with these proteins.

A summary of the quantification of the GTP[^{35}S]-binding activity co-purifying with the 25000 Da protein is presented in Table 1. It should be noted that GTP[^{35}S]-binding assays were carried out at a single concentration of radioligand. Thus the data do not represent true specific activities of the protein(s). Also, it was not possible to quantify that amount of GTP[^{35}S]-binding activity in the original cholate extract that represented the 25000 Da-associated GTP[^{35}S]-binding activity. Thus the values for recovery presented in Table 1 are relative to the total GTP[^{35}S]-binding

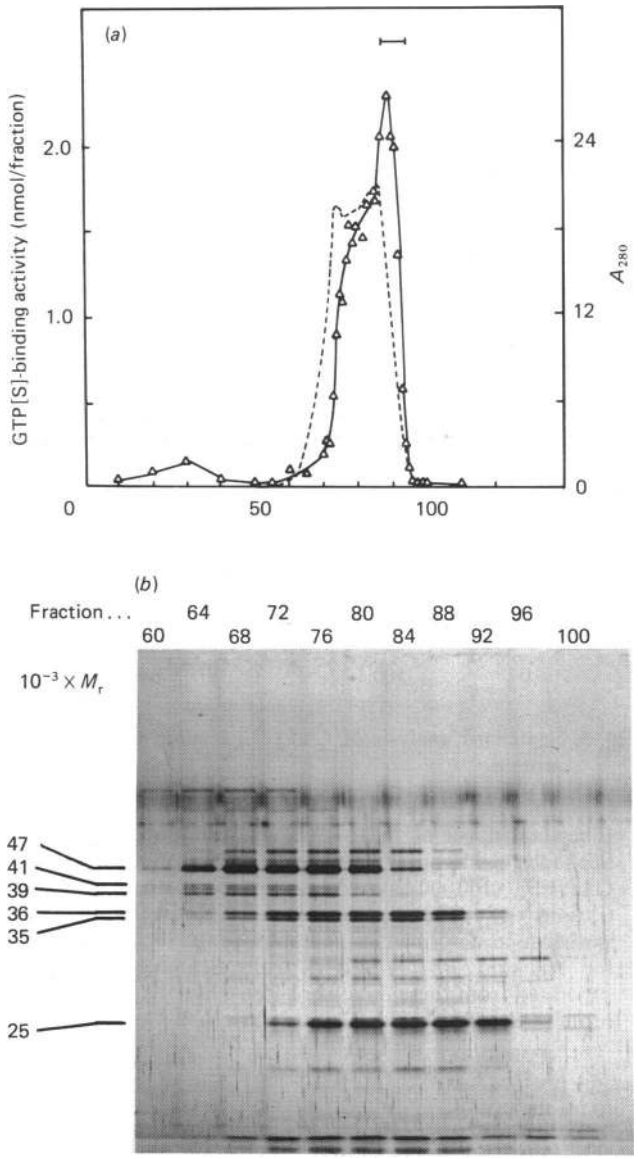

Fig. 4. Sephadex G-150 (superfine) chromatography of the 25000 Da-protein-enriched pool from heptylamine–Sepharose

(*a*) Elution profiles of GTP[^{35}S]-binding activity (△) and A_{280} (----) are shown; 0.9 ml fractions were collected. (*b*) SDS/PAGE of fractions obtained from Sephadex G-150. Samples were prepared and loaded as described in Fig. 2. Proteins were detected with silver stain.

Table 1. Purification of a G-protein containing a 25000 Da GTP-binding subunit

Preparation of membranes, detergent extraction, and the steps of purification are described in detail in the Experimental and Results sections. Recovery values represent the percentage of total GTP[S] binding in brain extract that was recovered in the 25 Da protein pool.

Step	Fractions pooled	Volume (ml)	Protein (mg)	GTP[S] binding (nmol)	GTP[S] binding (nmol/mg)	Recovery (%)
Membranes	–	1800	7420	3360	0.45	–
Cholate extract	–	1660	2010	1420	0.71	100
DEAE-Sephacel	85–125	380	110	162	1.47	11.4
Ultrogel AcA-34	110–128	59	24.2	75.9	3.14	5.35
Heptylamine–Sepharose	80–105	70	5.53	31.7	5.73	2.23
Sephadex G-150	70–86	15	3.28	8.65	2.64	0.61
	87–94	6.8	0.62	5.55	8.89	0.39

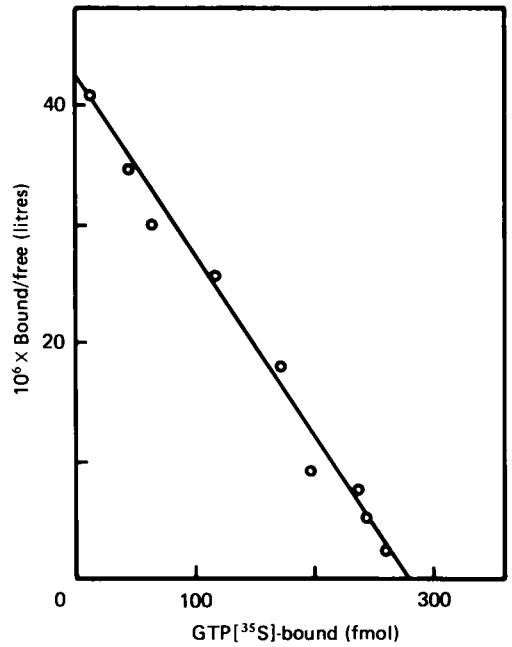

Fig. 5. Scatchard analysis of GTP[^{35}S] binding to the purified protein

Purified protein (see Fig. 7*a*, lane 3, for Coomassie-Blue-stained gel of the purified protein pool) from the Sephadex G-150 pool (fractions 87–94) was incubated with GTP[^{35}S] (160000 c.p.m./assay) and various concentrations of GTP[S] to generate saturation-binding isotherms as described in the Experimental section. Values for K_D and $B_{max.}$ derived from Scatchard analysis generated from these data were 15 nM and 17 pmol/μg of protein respectively.

activity in the extract, not to the starting amount of 25000 Da-protein-associated binding activity. In general, fractions were pooled to obtain the highest purity and not necessarily the best yield. Values in Table 1 also do not account for removal of samples for various assays during the purification.

Saturation-binding isotherms for GTP[^{35}S] were generated with the purified protein. Transformation of these data by the method of Scatchard is presented in Fig. 5. The binding of GTP[^{35}S] was to a single high-affinity site with a K_D of 15 nM. The $B_{max.}$ value measured in five separate determinations was 17 ± 2 nmol/mg of protein.

Verification that the guanine-nucleotide-binding site was contained within the 25000 Da protein was accomplished by photoaffinity labelling with 8-azido-[γ-^{32}P]GTP (Fig. 6). This protein was labelled with radioligand, and labelling was blocked in a concentration-dependent manner by GTP[S], but not by 100 μM-p[NH]ppA. A small amount of non-specific labelling of the 35000–36000 Da doublet was observed. However, this labelling was not blocked by GTP[S] or

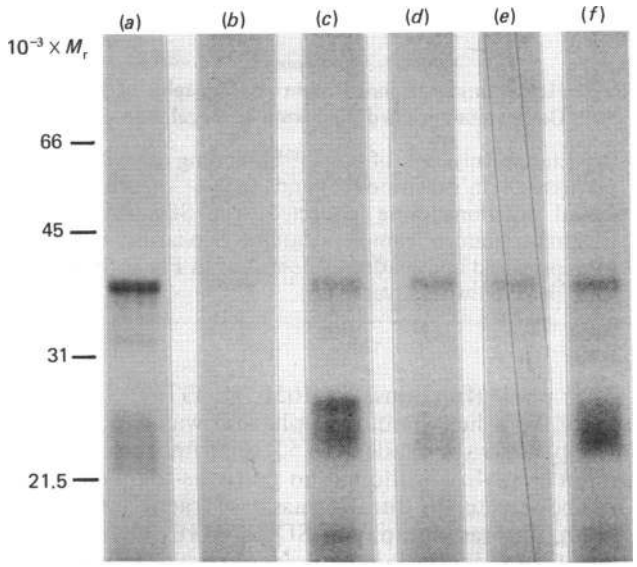

Fig. 6. Photoaffinity labelling of the purified protein with 8-azido[γ-^{32}P]GTP

Photoaffinity labelling of the purified protein was accomplished as described in the Experimental section. The labelled proteins were electrophoresed on an 11–14% SDS/polyacrylamide gel and stained with Coomassie Blue (lane *a*). The gel was dried and used to develop an autoradiogram (lanes *b*–*f*). Lanes (*b*)–(*f*) were loaded with 10 μg of protein previously incubated with 8-azido-[γ-^{32}P]GTP under the following conditions: (*b*) no u.v. irradiation; (*c*) u.v. irradiation; (*d*) u.v. irradiation and 3 μM-GTP[S]; (*e*) u.v. irradiation and 30 μM-GTP[S]; (*f*) u.v. irradiation and 100 μM-p[NH]ppA.

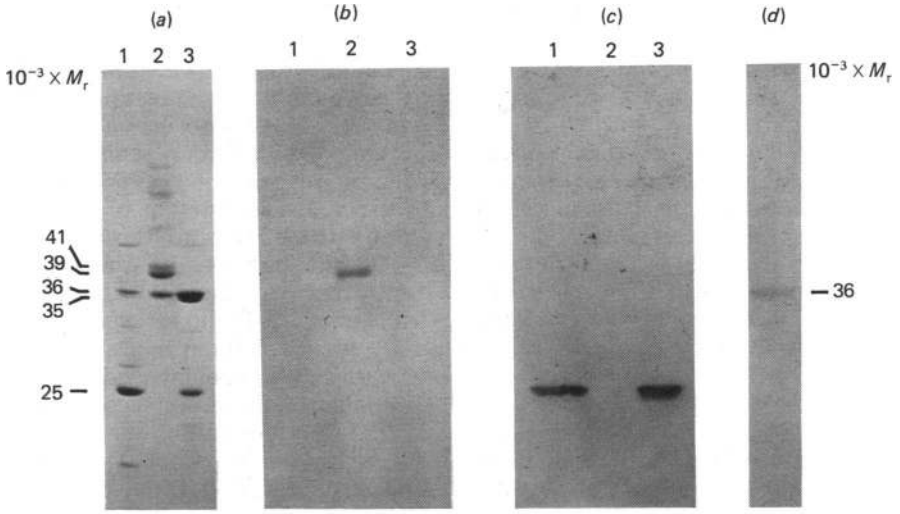

Fig. 7. Comparative immunoblot analysis of the 25000 Da GTP[S]-binding protein from bovine brain, Gp from human placenta and G_o/G_i from bovine brain

Portions (10 μg/lane for Coomassie Blue staining, panel *a*; 5 μg/lane for immunoblots, panels *b–d*) of the indicated samples were alkylated, electrophoresed, transblotted, and staining was developed as described in the Experimental section. Placental G_p (lane 1, panels *a*, *b* and *c*) was purified as previously described. Bovine brain G_o/G_i (lane 2, panels *a*, *b* and *c*) was purified through Ultrogel AcA-34 and heptylamine–Sepharose from DEAE-Sephacel fractions 60–84 (see Fig. 1). The 25000 Da GTP[S]-binding protein from bovine brain (lane 3, panels *a*, *b* and *c*, and panel *d*) was obtained from the Sephadex G-150 pool (see Fig. 4; fractions 87–94). The transblotted proteins (panels *b–d*) were incubated with antisera raised against the α-subunit of bovine brain G_o (panel *b*), the α-subunit of human platelet G_p (panel *c*) or oligomeric G_t (panel *d*).

p[NH]ppA. A small amount of specific binding of 8-azido[γ-^{32}P]GTP to a protein smaller than the 25000 Da protein was also observed. This labelling was not prominent in two other experiments.

The electrophoretic similarity of the 25000 Da protein to the GTP-binding protein, G_p, previously purified from human placenta and platelets by Evans *et al.* (1986) was assessed. G_p purified from human placenta and the protein purified from bovine brain were electrophoresed on a series of single-concentration (10%, 12%, 14% and 16%) SDS/polyacrylamide gels. The proteins from both brain and placenta co-migrated on these gels, as well as on 11–14%-acrylamide gradient gels. Plots of log M_r versus R_F for each single concentration gel consistently yielded values for the apparent molecular mass of the α-subunit of G_p (and the protein from brain) of 24000–25000 Da. Ferguson plots were constructed from these data, and retardation coefficients (K_R) were obtained (G. L. Waldo, unpublished work). From a plot of log K_R versus log M_r, a molecular mass of 25000 Da was obtained for the two proteins.

The immunological similarities of the two proteins were also examined by immunoblot analysis (Fig. 7). The 25000 Da proteins from bovine brain and human placenta were both recognized by polyclonal antiserum raised against the G_p protein purified from human platelets (lanes 1 and 3, Fig. 7*c*). This antiserum did not recognize the α-subunits of either G_i or G_o (lane 2, Fig. 7*c*). Conversely, antiserum raised against bovine brain G_o (Fig. 7*b*) recognized the α-subunit of G_o, but not the α-subunit of G_i (lane 2) or the 25000 Da protein from brain or placenta (lanes 1 and 3). Antiserum raised against oligomeric G_t recognized the 36000 Da protein,

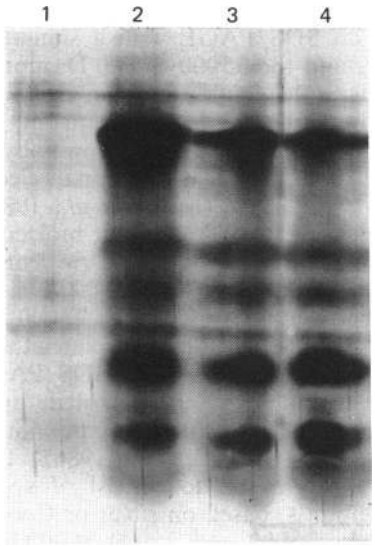

Fig. 8. *S. aureus* V8 protease digestion of bovine brain, human placental and human platelet 25000 Da GTP-binding proteins

Approx. 2 μg of the brain (lane 2), placental (lane 3) and platelet (lane 4) proteins were purified by preparative gel electrophoresis on a 0.75 mm-thick slab of 11% acrylamide. The 25000 Da proteins were re-electrophoresed through a 1.5 mm-thick slab of 15% acrylamide in the presence of 1 μg of *S. aureus* V8 protease as described in the Experimental section. Proteins were detected by silver staining. Lane 1 represents 1 μg of the protease alone.

which co-purified with the brain protein (25000 Da; Fig. 7(*d*)) and which co-purified with brain G_o/G_i and placental G_p (G. L. Waldo, unpublished work). Coomassie Blue staining of the purified G-proteins is presented in Fig. 7(*a*).

Finally, the structural similarity of the proteins purified from bovine brain, human placenta and human platelets was assessed by *S. aureus* V8 protease digestion of the electrophoretically purified proteins (Fig. 8). Approx. 2 μg of each protein was electrophoresed through a 15%-polyacrylamide gel in the presence of 1 μg of the protease. The three preparations yielded identical peptides on proteolysis.

DISCUSSION

It is now widely acknowledged that membrane preparations from the bovine and rat central nervous system contain the G-proteins, G_s, G_i and G_o (Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Milligan & Klee, 1985; Katada *et al.*, 1986). In this paper we have demonstrated that membranes from bovine brain also contain another GTP-binding protein that binds GTP[^{35}S] with high affinity and is not a substrate for either pertussis toxin or cholera toxin.

The GTP[^{35}S]-binding activity purified from bovine brain always was associated with a 25000 Da protein and a 35000–36000 Da protein doublet. The 25000 Da protein was photoaffinity labelled by 8-azido[γ-^{32}P]GTP and cross-reacted with antiserum to the putative α-subunit of a GTP-binding protein called G_p, recently purified from human placenta. The 36000 Da protein was recognized by antibody raised against oligomeric G_t. The 25000 Da protein from bovine brain always co-migrated on SDS/PAGE with a similar subunit of placental G_p, and the 35000–36000 Da doublet always co-migrated with the β-subunits from G_o, G_i, G_p and G_t from several sources. The α-subunit of the protein purified from human placenta was initially assigned a molecular mass of 21000 Da, based on its electrophoretic co-migration with H-ras (Evans *et al.*, 1986). A more rigorous estimation of the apparent molecular mass of the protein was carried out in the present study, and resulted in a value of approx. 25000 Da. Finally, *S. aureus* V8 protease digestion of the 25000 Da proteins purified from bovine brain, human platelet and placenta yielded identical peptide maps on SDS/PAGE. On the basis of these data, the brain protein apparently is equivalent to the protein called G_p, previously purified from placenta by Evans *et al.* (1986). The purified brain protein was found to consist of approximately equivalent amounts, based on silver or Coomassie Blue staining, of the 25000 and 35000–36000 Da proteins. The presence of a potential γ-subunit co-purifying with the GTP[^{35}S]-binding activity was not established in that SDS/PAGE was not routinely carried out under conditions where relatively small peptides could be identified. Nonetheless, a protein(s) of 10000 Da or less was often observed during SDS/PAGE of the purified brain protein. Taken together, the co-purification of the 25000 and 35000/36000 Da proteins, with the precedence established with other members of the G-protein family, suggest that a heterotrimeric protein has been purified. This idea is also consistent with the fact that the purified protein bound 1 mol of GTP[^{35}S]/mol of 58000 Da protein. Unequivocal proof that this protein, like other GTP-binding proteins, is a functional heterotrimer must await further studies, including examination of the hydrodynamic properties of the protein under activating conditions.

Sternweis & Robishaw (1984) have reported that G_s is eluted from DEAE-Sephacel as a trailing shoulder of the major peak of GTP[^{35}S]-binding activity. Thus, at least at the initial step of purification, G_s has elution properties similar to those of $G_{\alpha\text{-}25000}$. However, the GTP-binding protein purified from bovine brain apparently is not contaminated with G_s. No cholera-toxin substrate was found associated with this protein over the last several column steps. However, this may not be completely compelling evidence for absence of G_s, since optimal conditions for successful cholera-toxin-catalysed [^{32}P]ADP-ribosylation were not established (see below). Nevertheless, no 45000 Da protein was found in the purified fractions from bovine brain. It should be pointed out that the protein of approx. 47000 Da that co-purified with the GTP[^{35}S]-binding activity through much of the purification scheme did not co-migrate with the α-subunit of purified G_s under several conditions of SDS/PAGE.

Kahn & Gilman (1984, 1986) have reported that the protein cofactor, ARF, necessary for cholera-toxin-catalysed ADP-ribosylation of the α-subunit of G_s is itself a GTP-binding protein. This activity is associated with a doublet of proteins with reported molecular masses of approx. 21000 Da (Kahn & Gilman, 1984). Antiserum raised against the protein purified from placenta does not recognize bovine brain ARF and anti-ARF antiserum does not recognize the placental protein (T. Evans, unpublished work). Such observations, coupled with the failure of brain and liver ARF to bind guanine nucleotides in the presence of detergent and the requirement for high ionic strength for such binding activity (Kahn & Gilman, 1986), make it highly unlikely that $G_{\alpha\text{-}25000}$ and ARF activity represent the same gene product.

Owing to the large amounts of G_o and G_i, it was difficult to arrive at reliable quantification of the amount of individual G-proteins in the starting bovine brain membrane fraction; however, conservative estimates indicate that 2.5–5% of the GTP[^{35}S]-binding activity in the cholate extract of bovine brain is associated with the protein purified here. This would correspond to approx. 5–10 pmol of this G-protein/mg of membrane protein, and is clearly a relevant concentration of protein when compared with the specific activity of receptors and ion channels in the brain. Demonstration of functional interaction of this protein with receptors and/or ion channels is a crucial next step in confirmation of its potential physiological importance in brain.

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