Purification and characterization of predominant G-protein from bovine lung membranes

Biochemical and immunochemical comparison with G_{i1} and G_o purified from brain

Yasunori KANAHO, Stanley T. CROOKE and Jeffrey M. STADEL*

Department of Molecular Pharmacology, Smith Kline & French Laboratories, Philadelphia, PA 19101, U.S.A.

The predominant guanine nucleotide-binding protein (G-protein) of bovine lung membranes, termed G_L , has been purified and compared biochemically, immunochemically and functionally with G_i and G_o purified from rabbit brain. The purified G_L appeared to have a similar subunit structure to G_i and G_o , being composed of α , β and possibly γ subunits. On Coomassie Blue-stained SDS/polyacrylamide gels and immunoblots, the α subunit of G_L ($G_{L\alpha}$) displayed an intermediate mobility (40 kDa) between those of G_i and G_o ($G_{i\alpha}$ and $G_{o\alpha}$). $G_{L\alpha}$ was [32 P]ADP-ribosylated in the presence of pertussis toxin and [32 P]NAD⁺. Analysis of [32 P]ADP-ribosylated α subunits by SDS/polyacrylamide-gel electrophoresis and isoelectric focusing showed that $G_{L\alpha}$ was distinct from $G_{i\alpha}$ and $G_{o\alpha}$, but very similar to the predominant G-protein in neutrophil membranes. Immunochemical characterization also revealed that G_L was distinct from G_i and G_o , but was indistinguishable from the G-protein of neutrophils, which has been tentatively identified as G_{i2} [Goldsmith, Gierschik, Milligan, Unson, Vinitsky, Maleck & Spiegel (1987) *J. Biol. Chem.* **262**, 14683–14688]. In functional studies, higher Mg^{2+} concentrations were required for guanosine 5'-[γ - 35 S]thio]triphosphate (GTP[35 S]) binding to G_L than were required for nucleotide binding to G_o , whereas G_i showed a Mg^{2+} -dependence similar to that of G_L . The kinetics of GTP[35 S] binding to G_L was quite different from those of G_i and G_o ; $t_{1/2}$ values of maximal binding were 30, 15 and 5 min respectively. In contrast, the rate of hydrolysis of [γ - 32 P]GTP by G_L ($t_{1/2} \sim 1$ min) was approx. 4 times faster than that by G_i or G_o . These results indicated that the predominant G-protein purified from lung is structurally and functionally distinct from G_i and G_o of brain, but structurally indistinguishable from G_{i2} of neutrophils.

INTRODUCTION

Two guanine nucleotide-binding proteins (G-proteins), G_i and G_o , which are substrates for pertussis-toxin-catalysed ADP-ribosylation, have been purified from brain and well characterized (Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Katada *et al.*, 1986; Gilman, 1987). They are heterotrimers, consisting of distinct α subunits and indistinguishable $\beta\gamma$ subunits. Both the α subunits of G_i ($G_{i\alpha}$) and G_o ($G_{o\alpha}$) possess a high-affinity binding site for GTP and an ADP-ribosylation site, but they are structurally different, as determined by their molecular masses (41 kDa and 39 kDa respectively) (Sternweis & Robishaw, 1984; Neer *et al.*, 1984; Katada *et al.*, 1986; Gilman, 1987) and by their unique immunological specificities (Mumby *et al.*, 1986). G_i couples its specific receptors to the inhibition of adenylate cyclase activity [see Gilman (1987) for review]. The function of G_o is still not clearly defined, but it may regulate the opening of Ca^{2+} channels (Hoscheler *et al.*, 1987).

A third pertussis-toxin substrate has been purified from neutrophils (Gierschik *et al.*, 1987) and differentiated HL60 myeloid cells (Oinuma *et al.*, 1987). This

new G-protein appears to have the same basic trimeric subunit structure as G_i and G_o . Its β subunits, consisting of both 36 kDa and 35 kDa isoforms, were found to be indistinguishable from those of G_i and G_o (Oinuma *et al.*, 1987). However, the α subunit of the purified protein was shown to be distinct from $G_{i\alpha}$ and $G_{o\alpha}$ (Gierschik *et al.*, 1987; Oinuma *et al.*, 1987). [32 P]ADP-ribosylated α subunit of the G-protein purified from neutrophils and differentiated HL60 cells migrated intermediately between labelled $G_{i\alpha}$ and $G_{o\alpha}$ on SDS/PAGE, with an apparent molecular mass of 40 kDa. Antisera specific to $G_{i\alpha}$ and $G_{o\alpha}$ failed to cross-react with the 40 kDa α subunit.

Additionally, three $G_{i\alpha}$ subtypes, termed $G_{i1\alpha}$, $G_{i2\alpha}$ and $G_{i3\alpha}$, have been identified by sequencing of cDNA clones (Jones & Reed, 1987). An amino acid sequence predicted by $G_{i1\alpha}$ cDNA corresponds to that determined from $G_{i\alpha}$ purified from brain (Nukada *et al.*, 1986); therefore we will refer to brain G_i as G_{i1} . Goldsmith *et al.* (1987) have developed an antiserum raised against a synthetic decapeptide predicted by the $G_{i2\alpha}$ cDNA sequence. This antiserum strongly reacted with the α subunit of the G-protein purified from neutrophil membranes, but did not

Abbreviations used: G-proteins, guanine nucleotide-binding proteins; G_i , the G-protein of adenylate cyclase that mediates inhibition; G_o , another G-protein of unknown function; G_{i2} , the predominant G-protein of neutrophil membranes; G_L , the predominant G-protein purified from bovine lung membranes; T, transducin from bovine rod outer segments; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppA, adenosine 5'-[β -imido]triphosphate; DTT, dithiothreitol; TED, 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/1 mM-DDT; HED, 50 mM-sodium Hepes (pH 8.0)/0.1 mM-EDTA/0.1 mM-DDT; PAGE, polyacrylamide-gel electrophoresis.

* To whom reprint requests should be addressed.

cross-react with $G_{i1\alpha}$ or $G_{o\alpha}$ purified from brain. Thus the G-protein of neutrophils was tentatively identified as G_{i2} . The predominant pertussis-toxin-sensitive G-protein purified from human erythrocytes, which promotes opening of K^+ channels, has been identified as G_{i3} (Codina *et al.*, 1988).

It appears that G_{i2} can couple chemotactic receptors to polyphosphoinositide-specific phospholipase C in neutrophils, but not to adenylate cyclase (Smith *et al.*, 1985; Verghese *et al.*, 1985). Similarly in lung, leukotriene D_4 induces the formation of inositol trisphosphate, but fails to inhibit adenylate cyclase (Mong *et al.*, 1987). These findings permitted us to speculate that lung might be a source of G_{i2} . In the present report, we have purified the predominant G-protein from bovine lung and characterized it in comparison with G_{i1} and G_o purified from brain, and also with the predominant pertussis-toxin substrate in neutrophil membranes, G_{i2} . Our results indicate that the G-protein from lung is both structurally and functionally different from brain G_{i1} and G_o , but is structurally similar to, or the same as, neutrophil G_{i2} .

MATERIALS AND METHODS

Materials

GTP[35 S], [α - 32 P]NAD $^{+}$, 125 I-labelled goat anti-rabbit IgG F(ab') $_2$ and [γ - 32 P]GTP were purchased from New England Nuclear; reagents for isoelectric focusing and SDS/PAGE were from Bio-Rad; and all other chemicals were from Sigma. Cholate was purified as previously reported (Ross & Schatz, 1976), and Lubrol PX was deionized before use.

Preparation of tissue membranes and purification of proteins

Crude membranes from rabbit cerebral cortex (Sternweis & Robishaw, 1984) and bovine lung (Mong *et al.*, 1986) were prepared as previously reported. Neutrophils were harvested from the rabbit peritoneum and plasma membranes were prepared by the method of Matsumoto *et al.* (1987). These membranes were suspended in 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/1 mM-DTT (TED) and stored at -70°C .

Rhodopsin was purified under dim red light from rod outer-segment membranes as described by Hong & Hubbell (1973), except that membranes were not freeze-dried. Liposomes of egg-yolk phosphatidylcholine (3 mg/ml) with rhodopsin (1.5 mg/ml) in 15 mM-sodium phosphate, pH 7.5, were prepared by dialysis and stored at -70°C .

Transducin (T) was extracted from rod outer segments of fresh bovine retinas with 100 μM -GTP as described by Kühn (1980). $T_{\beta\gamma}$ subunits were separated from T_{α} by using Blue Sepharose CL-6B (Shinozawa *et al.*, 1980). The purified $T_{\beta\gamma}$ (~ 250 μg of protein/ml) was stored at -70°C in 20 mM-Tris/HCl (pH 7.5)/0.5 mM-MgCl $_2$ /1 mM-DTT/50 μM -EGTA/0.1 mM-phenylmethanesulphonyl fluoride.

G_{i1} and G_o from brain were purified as described by Katada *et al.* (1986). Briefly, crude membranes prepared from rabbit cerebral cortex were extracted with TED containing 1% sodium cholate and aprotinin by incubating for 1 h at 4°C . The concentration of aprotinin in this and the following solutions was always 50 kallikrein-inhibitory units/ml. After centrifugation at 100 000 g

for 1 h, the clear supernatant was applied to a DEAE-Sephacel column (5 cm $\times$ 30 cm). Fractions containing GTP[35 S]-binding activity were pooled and sequentially chromatographed on Sephacryl S-200 (2.5 cm $\times$ 90 cm) and hydroxyapatite (2.5 cm $\times$ 10 cm) columns. Fractions from the hydroxyapatite column having GTP[35 S]-binding activity contained both G_{i1} and G_o . These fractions were applied to a DEAE-Toyopearl column (2.5 cm $\times$ 10 cm) which had been equilibrated with TED/aprotinin containing 0.5% Lubrol, and then the column was eluted with a linear gradient of NaCl (25–200 mM) in TED/aprotinin containing 0.5% Lubrol. At this step, G_{i1} and G_o were separated. The separated G_{i1} and G_o were concentrated by a second hydroxyapatite column (1.5 cm $\times$ 6 cm) and then chromatographed on a Sephadex G-50 column (1.5 cm $\times$ 5 cm) in 50 mM-sodium Hepes (pH 8.0)/0.1 mM-EDTA/0.1 mM-DTT (HED) containing 0.1% Lubrol. Purities of G_{i1} and G_o were over 90% and 80% respectively, as assessed by Coomassie Blue staining on SDS/polyacrylamide gels.

G_L from bovine lung membranes was purified by the method of Oinuma *et al.* (1987) with some modifications. The extract of lung membranes in TED/aprotinin containing 1% Lubrol was applied to a DEAE-Sephacel column (5 cm $\times$ 30 cm) which had been equilibrated with TED/aprotinin containing 0.5% Lubrol. Fractions containing GTP[35 S]-binding activity were sequentially chromatographed on hydroxyapatite (2.5 cm $\times$ 15 cm), Sephacryl S-200 (2.5 cm $\times$ 90 cm), DEAE-Toyopearl (2.5 cm $\times$ 10 cm) and second hydroxyapatite (1.5 cm $\times$ 6 cm) columns in TED/aprotinin containing 0.5% Lubrol. At the DEAE-Toyopearl column-chromatography step, only a single peak of GTP[35 S]-binding activity was observed. After the second hydroxyapatite column, G_L was further purified by h.p.l.c. on a Mono Q column in the same buffer. The purified G_L was stored in HED containing 0.1% Lubrol at -70°C after filtration through a Sephadex G-50 column (1.5 cm $\times$ 5 cm) in the same buffer. Purity of G_L was greater than 70% as assessed by Coomassie Blue staining on SDS/polyacrylamide gels. Substantial activities of the purified G-proteins were retained after 2 months of storage at -70°C , although the activity gradually decreased during storage.

[32 P]ADP-ribosylation of G-proteins

Pertussis toxin was activated by incubating for 10 min at 30°C in 50 mM-glycine (pH 8.0), 20 mM-DTT and 1 mg of ovalbumin/ml. Purified G_{i1} , G_o and G_L and neutrophil membranes at the concentration cited were incubated with the activated pertussis toxin (4 μg /ml) and 5 μM -[32 P]NAD $^{+}$ (~ 5 μCi /assay) at 30°C for 1 h as previously reported (Matsumoto *et al.*, 1987), except that Lubrol PX was added to give a final concentration of 0.05% (total volume 100 μl).

SDS/PAGE and isoelectric focusing

Native or [32 P]ADP-ribosylated proteins were precipitated with cold 10% trichloroacetic acid. For SDS/PAGE, precipitated proteins were solubilized with SDS sample buffer and subjected to electrophoresis on SDS/polyacrylamide gels by the method of Laemmli (1970). For isoelectric focusing, precipitated proteins were washed once with diethyl ether, and then solubilized in 100 μl of 9 M-urea/4% Nonidet P-40/2% β -mercaptoethanol/2% Bio-Lyte (pH 3–10) at room temperature.

Isoelectric focusing in the pH range 4–7 was performed by the method of O'Farrell (1975) on slab gels.

Antibodies

T_β antiserum was raised against $T_{\beta\gamma}$ purified from bovine retinal rod outer segments. A New Zealand White rabbit was injected subcutaneously with 100 μ g of $T_{\beta\gamma}$ in complete Freund's adjuvant. Then 3 weeks after the initial injection, the rabbit was injected with 100 μ g of $T_{\beta\gamma}$ in incomplete Freund's adjuvant, and antiserum was collected 1 week after the second injection. This antiserum detected only 36 kDa β subunit, but not the γ subunit of G_{i1} , G_o and T , and therefore was termed T_β antiserum.

G_{α} and $G_{\alpha,common}$ antisera were generously given by Dr. D. R. Manning of the University of Pennsylvania School of Medicine. $G_{i\alpha,common}$ and $G_{i2\alpha}$ antisera were generously given by Dr. A. Spiegel of the National Institutes of Health. Briefly, G_{α} and $G_{\alpha,common}$ antisera were raised against purified G_{α} from bovine brain and a synthetic peptide common to T_α and G_{α} , respectively (Mumby *et al.*, 1986). $G_{i\alpha,common}$ and $G_{i2\alpha}$ antisera were raised against synthetic decapeptides corresponding to the C-terminus of T_α and the internal sequence of $G_{i2\alpha}$ predicted by a cDNA clone, respectively (Goldsmith *et al.*, 1987). Antisera obtained were stored at -70°C , and used without further purification.

Immunoblotting

Proteins were transferred on to nitrocellulose sheets from SDS/polyacrylamide gels. The nitrocellulose sheets were incubated in 50 mM-Tris/HCl (pH 8.0) containing 2 mM- CaCl_2 , 80 mM-NaCl, 5% (w/v) non-fat dried milk and 0.2% Nonidet P-40 (Buffer A) at 37°C for 1 h to block non-specific protein binding (Harris *et al.*, 1985). The blots were then incubated at room temperature for 2 h with a 1:250 dilution of each rabbit antiserum. After three 10 min washes with Buffer A, the blots were incubated at room temperature for 1 h with radiolabeled goat anti-rabbit IgG F(ab')₂ (10^6 c.p.m./ml) in 50 mM-Tris/HCl (pH 8.0) containing 2 mM- CaCl_2 , 0.2% SDS and 2% Nonidet P-40. The sheets were washed for 3×10 min with Buffer A and then with 50 mM-Tris/HCl (pH 8.0)/2 mM- CaCl_2 /80 mM-NaCl, and then dried and exposed to Kodak X-Omat films.

GTP[^{35}S] binding

G-proteins were identified by their ability to bind GTP[^{35}S]. In the standard assay for fractions from columns, samples were diluted (1:10) with TED/0.1% Lubrol. The diluted samples (50 μ l) were mixed with 50 μ l of a solution to give (final concns.) 50 mM-Hepes (pH 8.0), 20 mM- MgCl_2 , 1 mM-EDTA, 1 mM-DTT, 100 mM-NaCl, 0.1% Lubrol, 0.5 mM-p[NH]ppA and 2.5 μ M-GTP[^{35}S] ($\sim 4 \times 10^5$ c.p.m.). After incubation of samples at 30°C for 1 h, GTP[^{35}S] bound to protein was determined by dilution and filtration as described previously (Sternweis & Robishaw, 1984). For the GTP[^{35}S] binding studies with purified proteins, alterations are indicated in the Figure legends.

Rate of hydrolysis of bound [γ - ^{32}P]GTP

The single-turnover rate of bound [γ - ^{32}P]GTP was determined as described by Navon & Fung (1984). Each of the purified G-proteins, at the concentration cited, was incubated with 1 μ M-[γ - ^{32}P]GTP ($\sim 8 \times 10^5$ c.p.m.) in the presence of photolysed rhodopsin (0.5 μ g) in

50 mM-Hepes (pH 8.0), 20 mM- MgCl_2 , 1 mM-EDTA, 1 mM-DTT, 100 mM-NaCl, 0.1% Lubrol and 0.5 mM-p[NH]ppA at 4°C for 10 min (total volume 100 μ l). After incubation, samples were transferred to Millipore filters, which were washed with 2×2.5 ml of ice-cold 20 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/25 mM- MgCl_2 . Filters were kept at 4°C , and at the indicated time thereafter were washed again with four successive additions (each 2.5 ml) of the same buffer to remove [^{32}P]P_i released before transfer to vials for radioassays.

RESULTS

Biochemical comparison of G_L , G_{i1} , G_o and G_{i2}

The predominant G-protein of bovine lung membranes, G_L , was purified about 35-fold with approx. 2% recovery, as described in Table 1. The specific activity of purified G_L was approx. 3 nmol/mg of protein, as determined by GTP[^{35}S] binding. Assuming the molecular mass of G_L to be 85 kDa, GTP[^{35}S] binding was approx. 0.3 mol/mol of purified protein. The lower specific activity of G_L compared with that of purified G_{i1} or G_o (~ 6 –7 nmol/mg of protein) is probably due to inactivation of G_L during purification.

Fig. 1(a) shows purified G_L in comparison with purified G_{i1} and G_o from rabbit brain, on a Coomassie Blue-stained SDS/polyacrylamide gel. Although the G_L preparation contained two prominent contaminants with molecular masses of ~ 67 kDa and ~ 110 kDa, it appears that G_L has a similar subunit structure to G_{i1} and G_o , with α , β and possibly γ subunits. The α and β subunits of each G-protein were identified on immunoblots probed with $G_{\alpha,common}$ and T_β antiserum respectively (Figs. 1b and 1c). On Coomassie Blue-stained SDS/polyacrylamide gels, $G_{L\alpha}$ displayed an intermediate mobility between $G_{i1\alpha}$ and $G_{o\alpha}$, and this was also true on immunoblots probed with $G_{\alpha,common}$ antiserum. It has been reported that the β subunits of G_{i1} and G_o can be

Fig. 1. Analysis of purified G_L in comparison with purified brain G_{i1} and G_o on Coomassie Blue-stained SDS/polyacrylamide gels and immunoblots

A 10 μ g sample of each purified G-protein was resolved on SDS/12%-polyacrylamide gels and stained with Coomassie Blue (a). For immunoblots, 0.1 μ g of each purified G-protein was resolved on SDS/12%-polyacrylamide gels, transferred to nitrocellulose sheets and then probed with $G_{\alpha,common}$ (b) or T_β (c) antiserum as described in the Materials and methods section. Lane 1, G_{i1} ; lane 2, G_o ; lane 3, G_L . The results shown are representative of three experiments. Positions of M_r standards are shown on the right.

Table 1. Purification of G_L from bovine lung membranes

Step	Volume (ml)	Protein (mg)	GTP[35 S] binding		Recovery (%)
			(nmol)	(nmol/mg of protein)	
Extract	650	2964	269	0.09	100
DEAE-Sephacel	260	393	106	0.27	39.4
Hydroxyapatite	30	83	56	0.67	20.8
Sephacryl S-200	42	32	48	1.50	17.8
DEAE-Toyopearl	15	12.3	35	2.83	13.0
Second hydroxyapatite	6	5.4	16	2.96	5.9
Mono Q	3	1.5	4.6	3.11	1.7

resolved into a 36 kDa/35 kDa doublet (Sternweis & Robishaw, 1984). A similar doublet was observed for G_L , as well as G_{i1} and G_o , on Coomassie Blue-stained SDS/polyacrylamide gels (Fig. 1a). However, only a single band (36 kDa) was detected on immunoblots probed with T_β antiserum (Fig. 1c), consistent with previous reports (Mumby *et al.*, 1986). This appears to be because only a single band, of 36 kDa, was found for T_β (Fong *et al.*, 1986). Thus G_L appears to share a common subunit structure with other known G-proteins.

$G_{L\alpha}$ was found to be a substrate for pertussis-toxin-catalysed ADP-ribosylation. [32 P]ADP-ribosylated $G_{L\alpha}$, $G_{i1\alpha}$, $G_{o\alpha}$ and $G_{i2\alpha}$ of neutrophil membranes were compared by SDS/PAGE (Fig. 2). Again, labelled $G_{L\alpha}$ displayed an intermediate mobility between labelled $G_{i1\alpha}$ and $G_{o\alpha}$, and co-migrated with labelled $G_{i2\alpha}$ of neutrophil membranes.

Fig. 2. SDS/PAGE of [32 P]ADP-ribosylated G_{i1} , G_o , G_L and G_{i2}

Each of the purified G-proteins and membranes of rabbit neutrophils were [32 P]ADP-ribosylated by activated pertussis toxin and [32 P]NAD $^{+}$ as described in the Materials and methods section, and resolved on SDS/12%-polyacrylamide gels. The dried gels were exposed to Kodak X-Omat films. The proteins employed for [32 P]ADP-ribosylation to obtain comparable extent of labelling were 50 ng of G_{i1} (lane 1), 50 ng of G_o (lane 2), 200 ng of G_L (lane 3) and 4 μ g of neutrophil membranes (lane 4). The results shown are representative of three experiments.

Fig. 3. Isoelectric focusing of [32 P]ADP-ribosylated G_{i1} , G_o , G_L and G_{i2}

Each of the purified G-proteins and the G-protein of neutrophil membranes were analysed by isoelectric focusing after [32 P]ADP-ribosylation by activated pertussis toxin and [32 P]NAD $^{+}$ as described in the Materials and methods section. The dried gels were exposed to Kodak X-Omat films. Proteins used were: 50 ng of G_{i1} (lane 1), 50 ng of G_o (lane 2), 200 ng of G_L (lane 3) and 4 μ g of neutrophil membranes (lane 4). The results shown are representative of four experiments.

Fig. 4. Immunoblots of G_{i1} , G_o , G_L and G_{i2}

The purified G-proteins and the G-protein of neutrophil membranes were probed with $G_{L, common}$ (a), $G_{o\alpha}$ (b) and $G_{i2\alpha}$ (c) antisera after separation on SDS/polyacrylamide gels and transfer to nitrocellulose sheets as described in the Materials and methods section. Lane 1, 0.1 μ g of G_{i1} ; lane 2, 0.1 μ g of G_o ; lane 3, 0.1 μ g of G_L ; lane 4, 4 μ g of neutrophil membranes. The data shown are representative of four experiments.

Fig. 5. Mg^{2+} requirement for $GTP[^{35}S]$ binding to purified G_{i1} , G_o and G_L

$GTP[^{35}S]$ binding to each G-protein was assayed as described in the Materials and methods section after incubation for 1 h at 25 °C in the presence of 1 mM-EDTA and the indicated concentrations of Mg^{2+} in a total volume of 100 μ l. Proteins used were: 5 μ g of G_{i1} /ml ($\bullet$), 5 μ g of G_o /ml ($\blacktriangle$) and 15 μ g of G_L /ml ($\blacksquare$). Maximal $GTP[^{35}S]$ binding to G_{i1} , G_o and G_L was 6.42, 6.40 and 2.43 nmol/mg of protein respectively. The results shown are representative of three independent experiments.

Structural differences between $G_{L\alpha}$ and both $G_{i1\alpha}$ and $G_{o\alpha}$, as well as similarities between $G_{L\alpha}$ and $G_{i2\alpha}$, were much more apparent on isoelectric-focusing gels, although multiple bands for each [^{32}P]ADP-ribosylated α subunit were observed (Fig. 3). The pI values of the predominant doublet of labelled $G_{i1\alpha}$ were 5.75 and 5.80, whereas labelled $G_{o\alpha}$ showed a poorly resolved doublet with apparent pI values of about 5.60. [^{32}P]ADP-ribosylated $G_{L\alpha}$ yielded a pattern distinct from that for either $G_{i1\alpha}$ or $G_{o\alpha}$, showing four bands, a doublet with pI values of about 5.60, a prominent band of pI $\approx$ 5.70 and a minor band of pI $\approx$ 5.75. The pI values of the $G_{L\alpha}$ doublet were very similar to, but slightly and reproducibly different from, those of $G_{o\alpha}$. Although two minor bands of $G_{L\alpha}$ (a band in the doublet with lower pI value and a band with pI $\approx$ 5.75) were not observed in the labelled $G_{i2\alpha}$ of neutrophil membranes, $G_{L\alpha}$ was found to share two major bands (pI $\approx$ 5.60 and $\approx$ 5.70) with $G_{i2\alpha}$.

Immunochemical comparison of G_L , G_{i1} , G_o and G_{i2}

Fig. 4 represents immunoblots of three purified G-proteins and G_{i2} of neutrophil membranes. $G_{i\alpha, common}$ antiserum reacted with all the G-protein preparations, but to a lesser extent with $G_{o\alpha}$, suggesting that G_L is a member of the G_i family (Fig. 4a). The immunoblots probed with $G_{o\alpha}$ and $G_{i2\alpha}$ antiserum (Figs. 4b and 4c respectively) clearly discriminated $G_{L\alpha}$ and $G_{i2\alpha}$ from $G_{i1\alpha}$ and $G_{o\alpha}$. $G_{o\alpha}$ antiserum reacted with $G_{o\alpha}$ and strongly cross-reacted with $G_{i1\alpha}$, but did not react with $G_{L\alpha}$ or $G_{i2\alpha}$. In contrast, $G_{i2\alpha}$ antiserum reacted strongly with both $G_{L\alpha}$ and $G_{i2\alpha}$, but not with $G_{i1\alpha}$ or $G_{o\alpha}$.

Fig. 6. Kinetics of $GTP[^{35}S]$ binding to G_{i1} , G_o or G_L

Experiment and symbols are as in Fig. 5, except that incubation was carried out for the indicated times and Mg^{2+} was added to give a final concentration of 25 mM in the presence of 1 mM-EDTA. B_{∞} and B_t are the amounts of $GTP[^{35}S]$ bound to each G-protein at times infinity and t respectively. Values of B_{∞} (maximal binding as determined at 2 h) were 5.60, 6.32 and 2.62 nmol/mg of protein for G_{i1} , G_o and G_L respectively. The results shown are representative of three independent experiments.

Functional comparison of purified G_L , G_{i1} and G_o

Fig. 5 shows the Mg^{2+} -dependence of each purified G-protein for $GTP[^{35}S]$ binding. In the absence of added Mg^{2+} , but in the presence of 1 mM-EDTA, $GTP[^{35}S]$ binding to G_o was greater than 50 % of maximum, and attained maximal binding in the presence of 0.1 mM- Mg^{2+} added. On the other hand, the binding of $GTP[^{35}S]$ to G_L and G_{i1} in the absence of added Mg^{2+} was lower (approx. 30 % of maximum), and both G-proteins required millimolar concentrations of added Mg^{2+} to achieve maximal binding.

The kinetics of $GTP[^{35}S]$ binding to each G-protein was quite different (Fig. 6). The binding reaction was carried out in the presence of 1 mM-EDTA and 25 mM- $MgCl_2$, i.e. under conditions where maximal $GTP[^{35}S]$ binding to each G-protein was observed upon incubation for 2 h. The half-times of maximal binding were approx. 5, 15 and 30 min for G_o , G_{i1} and G_L respectively.

It has been reported that G_{i1} and G_o can interact *in vitro* with the photoreceptor, rhodopsin, resulting in the stimulation of their GTPase activities (Kanaho *et al.*, 1984; Tsai *et al.*, 1987). The stimulation of GTPase activity by photolysed rhodopsin was also observed for purified G_L (results not shown). This is probably due to the acceleration of GTP-GDP exchange on α subunits by the association of the G-proteins with rhodopsin. In fact, the presence of photolysed rhodopsin was required for the detection of [γ - ^{32}P]GTP binding to purified G_{i1} , G_o and G_L (results not shown). By taking advantage of rhodopsin stimulation of [γ - ^{32}P]GTP binding, the rate of hydrolysis of bound [γ - ^{32}P]GTP by each G-protein was

Fig. 7. Single turnover of hydrolysis of bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ by G_{11} , G_0 and G_L

The rate of hydrolysis of bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was assayed as described in the Materials and methods section. Symbols are as in Fig. 5. Proteins used were: $5\text{ }\mu\text{g}$ of G_{11}/ml , $5\text{ }\mu\text{g}$ of G_0/ml and $15\text{ }\mu\text{g}$ of G_L/ml . B_t , B_0 and B_∞ are the radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ bound to each G-protein at the times t , 0 and infinity respectively. B_∞ was determined 2 h after transfer of the samples to nitrocellulose membranes, which showed background radioactivity. The results shown are representative of three independent experiments.

determined (Fig. 7). The half-time of hydrolysis of bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ by G_L was approx. 1 min, whereas those for G_{11} and G_0 were both approx. 4 min. Thus G_L could be distinguished from G_{11} and G_0 of brain by both structural and functional criteria.

DISCUSSION

In the present study, we have purified the predominant pertussis-toxin substrate from bovine lung, G_L , and demonstrated that G_L is biochemically and immunochemically distinct from G_{11} and G_0 purified from rabbit brain. Although G_L was purified from bovine tissue and G_{11} and G_0 were from rabbit tissue, it is unlikely that the structural differences observed were solely due to species differences, since corresponding G_α subunits across species share over 98% identity in predicted amino acid sequences (Jones & Reed, 1987). Moreover, the results of isoelectric focusing and immunoblotting for G_L using membranes prepared from both rabbit and bovine lungs were exactly the same as those for purified G_L (results not shown). This observation also provides evidence that structural differences observed between purified G_L and brain G-proteins, G_{11} and G_0 , are not due to artifacts arising from the purification process.

Our data suggest that G_L is structurally very similar to, if not the same as, G_{12} of neutrophil membranes. This conclusion is based on the findings that (1) $[\text{P}^{32}]\text{ADP-ribosylated } G_{12}$ and $G_{12\alpha}$ co-migrated on SDS/poly-

acrylamide gels, (2) both G-proteins showed two major bands in common on isoelectric focusing, and (3) $G_{12\alpha}$ antiserum, which did not cross-react with G_{11} or G_0 , strongly reacted with both G_L and G_{12} of neutrophils. Jones & Reed (1987) reported that $G_{12\alpha}$ mRNA is abundant in lung. Furthermore, Mumby *et al.* (1988) demonstrated that the major G-protein of bovine lung may be G_{12} , as assessed by immunoblots probed with antiserum raised to a synthetic peptide predicted by $G_{12\alpha}$ cDNA clone. Thus several lines of evidence indicate that the predominant pertussis-toxin substrate in lung is the G_{12} subtype.

Isoelectric focusing of $G_{L\alpha}$, however, showed two minor bands which were not observed for $G_{12\alpha}$ of neutrophil membranes, suggesting that the G_L preparation may contain small amounts of other G-proteins. It should be noted that G_L was prepared from a tissue composed of heterologous cell types, which may contribute to G-protein heterogeneity. Another isoform of G_{12} , termed $G_{13\alpha}$, has been identified by cDNA cloning (Jones & Reed, 1987). These results raise the possibility that the minor bands in the G_L preparation on isoelectric focusing may be attributed to G-protein isoforms such as G_{13} .

A multiple banding pattern on isoelectric focusing was observed for each of the G-proteins tested, consistent with previous reports for G_s (Schliefer *et al.*, 1980) and G_{11} (Deery *et al.*, 1987). It is unlikely that this multiplicity is due to different extents of $[\text{P}^{32}]\text{ADP-ribosylation}$, since stoichiometric (1:1) incorporation of ADP-ribose into G_{11} and G_0 has been reported (Katada *et al.*, 1986). An alternative explanation may be other forms of post-translational modifications. It has been demonstrated that purified brain G_{11} and the 41 kDa pertussis-toxin substrate in platelets can be phosphorylated by protein kinase C (Katada *et al.*, 1985). It appears that neutrophil G_{12} may also be a substrate for protein kinase C (Matsumoto *et al.*, 1986). These results suggest that phosphorylation may contribute to the multiband patterns of G-proteins on isoelectric focusing. However, this point remains to be clarified.

In addition to biochemical and immunochemical characterization of G_L , we have demonstrated that G_L is functionally different from brain G_{11} and G_0 as assessed by the Mg^{2+} -dependence for $\text{GTP}[\text{P}^{35}\text{S}]$ binding, the kinetics of $\text{GTP}[\text{P}^{35}\text{S}]$ binding and the single-turnover rate of hydrolysis of bound $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. Since it is generally accepted that the binding of GTP to α subunits causes their dissociation from $\beta\gamma$ subunits and the individual α subunits then hydrolyse bound GTP to GDP [see Gilman (1987) for review], the difference in single-turnover rates of GTP hydrolysis is characteristic of the α subunit of each G-protein. In the structural studies, $G_{L\beta\gamma}$ was indistinguishable from β subunits of G_{11} and G_0 on Coomassie Blue-stained gels and immunoblots. Oinuma *et al.* (1987) reported that $\beta\gamma$ subunits purified from HL 60 cells, probably $G_{12\beta\gamma}$, are functionally identical with $\beta\gamma$ subunits of G_{11} and G_0 . These findings, taken together with our result that G_L may be G_{12} , support the notion that $\beta\gamma$ subunits among the G_i protein family are identical or very similar. Although we cannot totally rule out functional differences of $\beta\gamma$ subunits, it is probable that the differences among G-proteins in their Mg^{2+} requirements for $\text{GTP}[\text{P}^{35}\text{S}]$ binding and the kinetics of $\text{GTP}[\text{P}^{35}\text{S}]$ binding reflect the distinct properties of each α subunit.

With the advantage of a readily available supply, lung appears to be a very good source for the purification of the G₁₂ isoform. However, this G-protein appeared to be partially inactivated during purification, resulting in a lower specific activity compared with G₁₁ or G_o; to achieve the same maximal extent of GTP[³⁵S] binding or to yield a comparable extent of [³²P]ADP-ribosylation by pertussis toxin, 3–4-fold higher concentrations of G_L than of G₁₁ and G_o were required. Previous reports (Gierschik *et al.*, 1987; Dickey *et al.*, 1987) indicated that G₁₂ of neutrophils was highly unstable after solubilization in cholate solution unless stabilizers such as Al³⁺-F⁻ or ethylene glycol were added. In the present study, we employed the detergent Lubrol without the use of stabilizers throughout the purification. Although we found that G_L was indeed more stable in Lubrol than in cholate, our preparations were contaminated with two proteins, of ~ 67 kDa and ~ 110 kDa (Fig. 1a, lane 3). The problem of contamination may be due, in part, to the choice of detergent. Oinuma *et al.* (1987) reported the purification of a 40 kDa G-protein from HL60 cells in Lubrol, and their final preparation also contained two contaminants of ~ 60 kDa and ~ 65 kDa. In contrast, 40 kDa G-proteins purified in cholate, but in the presence of stabilizers, were nearly homogeneous (Gierschik *et al.*, 1987; Dickey *et al.*, 1987; Morishita *et al.*, 1988). Thus it appears to be difficult to purify G-proteins to homogeneity in Lubrol solution.

In conclusion, G_L is structurally and functionally distinct from brain G₁₁ and G_o, and structurally indistinguishable from neutrophil G₁₂. These structural and functional differences among G-proteins most likely reflect their physiological roles in transmitting ligand-receptor signals to distinct effectors, e.g. enzymes and ion channels. A distinct functional role for G₁₂ in lung has not yet been determined. In the neutrophil, G₁₂ appears to couple chemotactic receptors to polyphosphoinositide hydrolysis, leading eventually to physiological functions such as enzyme release (Snyderman & Verghese, 1987; Polakis *et al.*, 1988). By analogy with the signalling system in neutrophils, it is possible to speculate about postulated functions for G₁₂ in lung. Leukotrienes have been shown to promote contraction of lung parenchymal strips (Lewis *et al.*, 1980) and to stimulate phosphatidylinositol turnover (Mong *et al.*, 1987). Moreover, binding of leukotrienes to lung membranes is regulated by guanine nucleotides (Bruns *et al.*, 1983; Pong & DeHaven, 1983; Mong *et al.*, 1984). However, the sensitivity of these phenomena to pertussis toxin have not yet been reported. Further studies are needed to clarify these questions.

Note added in proof (received 17 February 1989)

During the course of these studies, Morishita *et al.* (1988) reported the purification of a G-protein from lung with a 40 kDa α subunit.

We are very grateful to Dr. D. R. Manning for providing G_o α and G _{α , common} antisera, to Dr. A. Spiegel for providing G₁₂, common and G₁₂ α antisera, and to Dr. T. Katada for his helpful discussion.

REFERENCES

- Bruns, R. F., Thomsen, W. J. & Pugsley, T. A. (1983) *Life Sci.* **33**, 645–653
- Codina, J., Olate, J., Abramowitz, J., Mattera, R., Cook, R. G. & Birnbaumer, L. (1988) *J. Biol. Chem.* **263**, 6748–6750
- Deery, W. J., Rebeiro-Neto, F. & Field, J. B. (1987) *Biochem. Biophys. Res. Commun.* **144**, 536–542
- Dickey, B. F., Pyun, H. Y., Williamson, K. C. & Navarro, J. (1987) *FEBS Lett.* **219**, 289–292
- Fong, H. K., Hurley, J. B., Hopkins, R. S., Miake-Lye, R., Johnson, M. S., Doolittle, R. E. & Simon, M. I. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2162–2166
- Gierschik, P., Sidiropoulos, D., Spiegel, A. & Jakobs, K. H. (1987) *Eur. J. Biochem.* **165**, 185–194
- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- Goldsmith, P., Gierschik, P., Milligan, G., Unson, C. G., Vinitzky, R., Maleck, H. L. & Spiegel, A. (1987) *J. Biol. Chem.* **262**, 14683–14688
- Harris, B. B., Robishaw, J. D., Mumby, S. M. & Gilman, A. G. (1985) *Science* **229**, 1274–1277
- Hong, K. & Hubbell, W. L. (1973) *Biochemistry* **12**, 4517–4523
- Hoscheler, J., Rosenthal, W., Trautwein, W. & Schultz, G. (1987) *Nature (London)* **325**, 445–447
- Jones, D. T. & Reed, R. R. (1987) *J. Biol. Chem.* **262**, 14241–14249
- Kanaho, Y., Tsai, S.-C., Adamik, R., Hewlett, E. L., Moss, J. & Vaughan, M. (1984) *J. Biol. Chem.* **259**, 7378–7381
- Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **151**, 431–437
- Katada, T., Oinuma, M. & Ui, M. (1986) *J. Biol. Chem.* **261**, 8182–8191
- Kühn, H. (1980) *Nature (London)* **283**, 587–589
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lewis, R. A., Austen, K. F., Drazen, J. M., Clark, D. A., Marfat, A. & Corey, E. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3710–3714
- Matsumoto, T., Molski, T. F. P., Volpi, M., Pelz, C., Kanaho, Y., Becker, E. L., Feinstein, M. B., Naccache, P. H. & Sha'afi, R. I. (1986) *FEBS Lett.* **198**, 295–300
- Matsumoto, T., Molski, T. F. P., Kanaho, Y., Becker, E. L. & Sha'afi, R. I. (1987) *Biochem. Biophys. Res. Commun.* **143**, 489–498
- Mong, S., Wu, H.-L., Hogaboom, G. K., Clark, M. A., Stadel, J. M. & Crooke, S. T. (1984) *Eur. J. Pharmacol.* **106**, 241–253
- Mong, S., Wu, H.-L., Stadel, J. M., Clark, M. & Crooke, S. T. (1986) *Mol. Pharmacol.* **29**, 235–243
- Mong, S., Hoffman, K., Wu, H.-L. & Crooke, S. T. (1987) *Mol. Pharmacol.* **31**, 35–41
- Morishita, R., Kato, K. & Asano, T. (1988) *Eur. J. Biochem.* **174**, 87–94
- Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 265–269
- Mumby, S., Pang, I.-H., Gilman, A. G. & Sternweis, P. C. (1988) *J. Biol. Chem.* **263**, 2020–2026
- Navon, S. E. & Fung, B. K.-K. (1984) *J. Biol. Chem.* **259**, 6686–6693
- Neer, E. J., Lok, J. M. & Wolf, L. G. (1984) *J. Biol. Chem.* **259**, 14222–14229
- Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Haga, K., Haga, T., Ichiyama, A., Kangawa, K., Hiranaga, M., Matsumoto, M. & Numa, S. (1986) *FEBS Lett.* **197**, 305–310
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Oinuma, M., Katada, T. & Ui, M. (1987) *J. Biol. Chem.* **262**, 8347–8353
- Polakis, P. G., UHING, R. J. & Snyderman, R. (1988) *J. Biol. Chem.* **263**, 4969–4976
- Pong, S.-S. & DeHaven, R. N. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7415–7419

- Ross, E. & Schatz, G. (1976) *J. Biol. Chem.* **251**, 1991–1996
- Schliefer, L. S., Garrison, J. C., Sternweis, P. C., Northup, J. K. & Gilman, A. G. (1980) *J. Biol. Chem.* **255**, 2641–2644
- Shinozawa, T., Uchida, S., Martin, E., Cafiso, D., Hubbell, W. & Bitensky, M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1408–1411
- Smith, C. D., Lane, B. C., Kusaka, I., Verghese, M. W. & Snyderman, R. (1985) *J. Biol. Chem.* **260**, 5875–5878
- Snyderman, R. & Verghese, M. W. (1987) *Rev. Infect. Dis.* **9**, S562–S569
- Sternweis, P. C. & Robishaw, J. D. (1984) *J. Biol. Chem.* **259**, 13806–13813
- Tsai, S.-C., Adamik, R., Kanaho, Y., Halpern, J. L. & Moss, J. (1987) *Biochemistry* **26**, 4728–4733
- Verghese, M. W., Fox, K., McPhail, L. C. & Snyderman, R. (1985) *J. Biol. Chem.* **260**, 6769–6775

Received 22 August 1988/9 December 1988; accepted 16 December 1988