

# Distribution of G-proteins in rat liver plasma-membrane domains and endocytic pathways

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1. The distribution of the  $\alpha$ - and  $\beta$ -subunits of nucleotide-binding G-proteins among rat liver sinusoidal, lateral and canalicular plasma membranes, endosomes, Golgi membranes and lysosomes was investigated. 2. Pertussis-toxin-catalysed ADP-ribosylation identified a 41 kDa inhibitory  $\alpha$ -subunit in all liver plasma-membrane functional domains as well as in endosomes. An antibody to a synthetic peptide corresponding to a C-terminal sequence of the inhibitory  $\alpha$ -subunit also identified the 41 kDa polypeptide in all plasma-membrane domains, in 'early' and 'late' endosomes and in Golgi membranes; this polypeptide was not detected in lysosomes. The antibody-binding studies showed that bile-canalicular plasma membranes had the highest content of the inhibitory  $\alpha$ -subunit. 3. Immunofluorescent microscopy confirmed the presence of the inhibitory  $\alpha$ -subunit in all regions of the hepatocyte's cell surface. 4. An antibody recognizing the  $\beta$ -subunit showed that a 36 kDa polypeptide was present in all plasma membranes and in 'early' and 'late' endosomes; it was not detected in lysosomes. The relative distribution among the fractions of this polypeptide was similar to the distribution of the inhibitory  $\alpha$ -subunit. 5. The presence of high levels of the G-protein inhibitory  $\alpha$ -subunit in bile-canalicular plasma membranes was confirmed by demonstration of its co-fractionation with marker enzymes in Nycodenz gradients and by free-flow electrophoresis. The significance of this location is discussed.

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## INTRODUCTION

G-proteins are a family of nucleotide-binding regulatory proteins that feature in the coupling of ligand-stimulated cell-surface receptors to effector enzymes and ion channels (see reviews by Stryer & Bourne, 1986; Gilman, 1987). G-proteins are homologous heterotrimeric entities composed of a 39–52 kDa GTP-binding  $\alpha$ -subunit, a 35–36 kDa  $\beta$ -subunit and an approx 9 kDa  $\gamma$ -subunit. The  $\alpha$ -subunits of various G-proteins display a lower degree of similarity to each other than do  $\beta$ - and  $\gamma$ -subunits, for they link a variety of cell-surface receptors to various effector systems. The  $\alpha$ -subunit is also involved in the binding and hydrolysis of GTP, and it associates reversibly with the  $\beta$ - and  $\gamma$ -subunits (Lochrie & Simon, 1988). G-proteins have been characterized in terms of their ability to be ADP-ribosylated by cholera or pertussis toxin, to bind and hydrolyse GTP, and to be activated by non-hydrolysable GTP analogues. They have also been identified by using antibodies raised to the purified subunits or to peptide sequences (Milligan *et al.*, 1986, 1987; Milligan, 1988). A number of G-proteins have been purified and characterized, including  $G_s$  (Northrup *et al.*, 1983) and  $G_i$  (Katada *et al.*, 1987; Luetje *et al.*, 1987), which stimulate or inhibit the adenylate cyclase respectively,  $G_t$  (transducin), which couples rhodopsin to cyclic GMP phosphodiesterase in rod outer segment (Gilman, 1987),  $G_o$ , of unknown function and present in high abundance in brain (Stryer & Bourne, 1986),  $G_k$ , the activating protein postulated to regulate  $K^+$  channels (Yatani *et al.*, 1987), and  $G_n$ , postulated to transduce signals in neutrophils from chemotactic-factor receptors to a phospholipase C (Gierschick *et al.*, 1987).

It has been generally accepted that G-proteins function mainly in cell-surface membranes, where receptors for various ligands and ion channels are located. The presence in hepatocytes of specialized plasma-membrane domains (sinusoidal, lateral and canalicular), and the role played by the endocytic compartment in the uptake and transcytosis of ligands bound to receptors, raise the question of whether G-proteins are confined to the sinusoidal plasma membrane or are more widely distributed in all regions of the plasma membrane as well as in certain intracellular membranes. The availability of established subcellular-fractionation techniques (Evans, 1987) and methods for identification of G-proteins (Milligan, 1988) can now allow their distribution in various plasma and intracellular membrane fractions to be investigated. In the present work, we identify two G-protein subunits in liver plasma-membrane fractions arising from the major functional domains and in endocytic membranes. The G-proteins were found at the highest levels in the bile-canalicular plasma membranes, and their likely functions here are discussed.

## MATERIALS AND METHODS

### Materials

Antiserum SGI was produced in rabbits against a decapeptide corresponding to the C-terminal amino acid sequence of the  $\alpha$ -subunit of purified inhibitory G-protein as described by Goldsmith *et al.* (1987). Antiserum RV6 (Gierschick *et al.*, 1986) was raised against a mixture of holomeric pertussis-toxin-sensitive G-protein purified

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Abbreviation used: PBS: phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.4).

from bovine brain and contained antibodies specifically recognizing  $\beta$ -subunit on Western blots (Milligan & Klee, 1985). [ $^{32}$ P]NAD<sup>+</sup> (sp. radioactivity 25–50 Ci/mmol) was obtained from New England Nuclear, Dreieich, Germany. Islet-activating protein, the pertussis toxin, was obtained from Porton Products, Salisbury, U.K. Nycodenz was purchased from Nycomed U.K., Sheldon, Birmingham, U.K. Other materials were obtained from either Sigma or BDH.

#### Preparation of plasma membranes, endosomes and other subcellular fractions

Membrane subfractions originating from the sinusoidal, lateral and canalicular plasma-membrane domains were prepared from the livers of Sprague-Dawley male rats (150–200 g) and characterized, as described by Wisner & Evans (1975), and modified by Evans *et al.* (1980). Livers were first perfused with ice-cold 0.9% NaCl and then homogenized. A canalicular (CAN) and two lateral (Lat<sub>A</sub> and Lat<sub>B</sub>) plasma-membrane fractions from the nuclear pellet and a sinusoidal plasma-membrane fraction (SIN) from post-nuclear supernatant were collected. All fractions were suspended in 0.25 M-sucrose and kept at  $-70^{\circ}\text{C}$ .

Endosome fractions were isolated as previously described from sucrose gradients, followed by equilibrium centrifugation in shallow Nycodenz gradients (Evans & Flint, 1985; Saermark *et al.*, 1985). 'Early' endosomes (DN-2) corresponded to fractions equilibrating in the Nycodenz gradient within the refractive-index range 1.365–1.375 (peak density of various internalized ligands 2–3 min after uptake by liver was 1.115 g/ml), and 'late' endosomes (DN-1) corresponded to those within the refractive-index range 1.355–1.364 (peak density of internalized ligands 10–15 min after uptake by liver was 1.090 g/ml). A further endosome fraction (D-R), shown to contain high levels of insulin- and asialoglycoprotein-binding sites (Evans & Flint, 1985), was collected at the interface of the Nycodenz gradient and the sucrose cushion. Fractions were diluted in 10 mM-Tris/HCl, pH 7.4, pelleted by centrifugation at 100 000 *g* for 30 min and stored in the same buffer at  $-70^{\circ}\text{C}$ . Endosome-depleted Golgi fractions of low (L), intermediate (I) and heavy (H) density in sucrose gradients were prepared as described by Evans (1985). Lysosomes were isolated by the method of Wattiaux *et al.* (1978). Bile was collected by cannulation of the biliary duct.

#### Analysis of canalicular plasma membranes on Nycodenz gradient

The plasma-membrane fraction prepared as described above from the rat liver nuclear pellet was suspended in 20 ml of 8% (w/v) sucrose by using 20 strokes of a tight-fitting Dounce homogenizer (Blaessig, Rochester, NY, U.S.A.). This suspension was overlaid on to a continuous gradient made by mixing 12 ml each of 35% and 55% (w/v) sucrose; the gradient rested on 5 ml of a 60% (w/v) sucrose cushion. After centrifugation at 100 000 *g* for 4 h (Beckman SW28 rotor), 30  $\times$  1.2 ml fractions were collected. Fractions containing bile-canalicular plasma membranes (density range 1.048–1.150 g/ml) were pooled, and the membranes were sedimented by centrifugation at 100 000 *g* for 30 min and resuspended in 6% (w/v) sucrose by using a tight-fitting homogenizer. The membranes were loaded on to a continuous gradient made from 12 ml each of 10% and 30% (w/v) Nycodenz

dissolved in 8% sucrose. After centrifugation at 100 000 *g* for 4 h, the gradients were unloaded and 30  $\times$  1.2 ml fractions collected. Refractive indices were recorded and enzymic activities determined in the fractions.

#### Analysis of canalicular plasma membranes by free-flow electrophoresis

The canalicular plasma-membrane fraction prepared as described above was also analysed in a VAP-22 free-flow electrophoresis apparatus (Bender and Hobein, Munich, Germany). The electrode buffer was 100 mM-acetic acid, adjusted to pH 7.2, and the buffer in the separating chamber was 10 mM-triethanolamine/10 mM-acetic acid, pH 7.2, made iso-osmolar with 0.27 M-sorbitol. The electrophoretic conditions used were 1000 V, 130 mA and  $4^{\circ}\text{C}$ . The chamber-buffer flow rate was approx. 2 ml/h per fraction. Approx. 80  $\times$  4 ml fractions were collected; their  $A_{280}$  was recorded and various enzyme activities were determined.

#### Enzyme assays

Alkaline phosphodiesterase I (EC 3.1.4.1) was assayed as described by Razzel (1963), with thymidine *p*-nitrophenylphosphate as a standard. Leucyl  $\beta$ -naphthylamidase (EC 3.4.11.1) was assayed as described by Goldberg & Rutenburg (1958). 5'-Nucleotidase (EC 3.1.3.5) activity was measured as described by Michell & Hawthorne (1965). Ca<sup>2+</sup>-ATPase activity was measured by a lactate dehydrogenase/pyruvate kinase coupled assay as described by Warren *et al.* (1974). The incubation medium (1 ml) contained 50 mM-Tris/HCl, pH 7.5, 100 mM-KCl, 5  $\mu\text{M}$ -MgCl<sub>2</sub>, 2.4 mM-ATP, 0.15 mM-NADH, 0.4 mM-phosphoenolpyruvate, 10 units of lactate dehydrogenase, 100 units of pyruvate kinase, 50  $\mu\text{M}$ -CaCl<sub>2</sub> and approx. 25  $\mu\text{g}$  of membrane protein. The change in  $A_{340}$  was measured by titrating free Ca<sup>2+</sup> with EGTA in the presence of 1  $\mu\text{M}$  of the calcium ionophore A23187. Ca<sup>2+</sup>-dependent ATPase activity was considered as the activity abolished by addition of 4 mM-EGTA in the above reaction mixture. Protein was measured by the method of Bradford (1976), with bovine serum albumin as standard.

#### Pertussis-toxin-catalysed ADP-ribosylation

To label membranes with [ $^{32}$ P]NAD<sup>+</sup> in the presence of pertussis toxin, various membrane fractions (50  $\mu\text{g}$  of protein) were suspended in 0.1% Lubrol and incubated at  $32^{\circ}\text{C}$  with 25 mM-Tris/HCl, pH 7.5, 10 mM-thymidine, 1 mM-EDTA, 5 mM-dithiothreitol, 5 mM-ATP, 100  $\mu\text{M}$ -GTP, 22  $\mu\text{g}$  each of leupeptin and antipain, 0.6  $\mu\text{g}$  activated pertussis toxin and 10  $\mu\text{M}$ -[ $^{32}$ P]NAD<sup>+</sup> (30 Ci/ml) in a volume of 60  $\mu\text{l}$ . The reaction was started by sequential addition of toxin and [ $^{32}$ P]NAD<sup>+</sup>. Pertussis toxin was first activated at  $37^{\circ}\text{C}$  by incubation of 9  $\mu\text{g}$  of pertussis toxin/100  $\mu\text{l}$  containing 25 mM-dithiothreitol in a capped Eppendorf tube for 30 min, followed by addition of 50  $\mu\text{l}$  of 75 mM-Tris/HCl buffer, pH 7.5, containing 0.1% bovine serum albumin (fraction V). The activated toxin was used immediately. After 45 min, the ADP-ribosylation reaction was stopped by adding 1 ml of ice-cold 25 mM-Tris/HCl buffer (pH 7.5)/10 mM-MgCl<sub>2</sub> and centrifuged (Minifuge) for 15 min at  $4^{\circ}\text{C}$ . The pellet was washed once with the same buffer. The final sediment was dissolved in Laemmli (1970) buffer containing 75 mM-Tris/HCl, pH 6.8, 2% SDS, 0.55 M- $\beta$ -

mercaptoethanol, 0.003% Bromophenol Blue and 10% (w/v) glycerol, boiled for 2–3 min and analysed by polyacrylamide-gel electrophoresis.

### Electrophoresis and Western blotting

Membrane fractions (50  $\mu$ g of protein) were solubilized in Laemmli buffer and resolved by polyacrylamide-gel electrophoresis in SDS-containing buffers, by using either 10% or 7.5–15.0% gradient gels (Laemmli, 1970). After electrophoresis, proteins were transferred to nitrocellulose papers (0.1  $\mu$ m; Schleicher and Schuell) by electrophoresis for 16 h at 12 V (Burnette, 1981). The nitrocellulose papers were washed for 2  $\times$  10 min with PBS (phosphate-buffered saline, pH 7.4) at room temperature, and non-specific binding was blocked by washing in 5% (w/v) skimmed milk dissolved in PBS, pH 7.4. Nitrocellulose papers were incubated for 2–3 h at 37  $^{\circ}$ C in the first antibody (diluted 1:200) in 1% skimmed milk dissolved in PBS. After several washes with PBS, the nitrocellulose papers were incubated for 45 min at room temperature with 20  $\mu$ Ci of  $^{125}$ I-Protein A in 20 ml of 3% skimmed milk dissolved in PBS. Finally, the nitrocellulose paper was extensively washed with 0.1% Triton X-100 in PBS, dried and exposed for autoradiography using Kodak X-AR films.

Autoradiographs were scanned with a Zeineh soft-laser scanning densitometer (T. and J. Crump Scientific Instruments, Rayleigh, Essex, U.K.) to quantify the labelling intensity of  $\alpha_1$  (inhibitory  $\alpha$ )- and  $\beta$ -subunits of G-proteins in relative terms. The amounts of  $\alpha_1$  and  $\beta$ -subunits were calculated as the percentage of the total intensity in all the plasma-membrane, endosome and Golgi fractions prepared. To demonstrate the linearity between the amount of membrane protein loaded on the gel and the increase in densitometric signal of anti-serum-G-protein complex, various amounts of a liver bile-canalicular plasma-membrane fraction polypeptides resolved by polyacrylamide-gel electrophoresis and transferred to nitrocellulose paper were analysed by staining with the G-protein  $\alpha_1$  antibody.

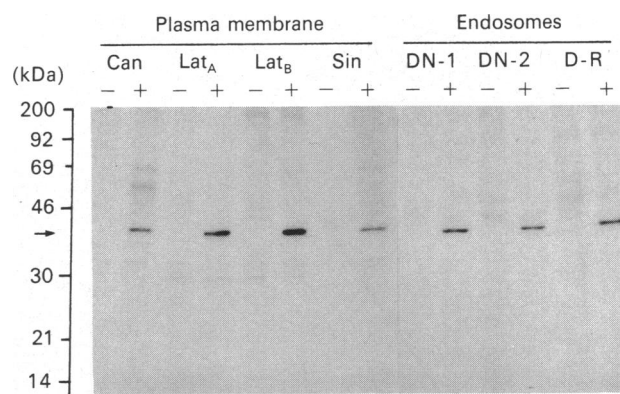
### Immunolocalization by light microscopy

Frozen sections (8–10  $\mu$ m thickness) of rat liver were prepared and mounted on gelatin-coated microscope slides, dipped in acetone at  $-20^{\circ}$ C for 5 min or immersed in chloroform/acetone (1:1, v/v) for 3 min and then stored at  $-20^{\circ}$ C. For staining, the sections were equilibrated at room temperature, immersed in PBS and then incubated with the first antibody to the  $\alpha$ -subunit of the G-protein. After several washes with PBS, the second antibody, a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody diluted 1:50 in PBS/1% bovine serum albumin, was applied in the dark for 1 h at room temperature. After staining, all samples were mounted with PBS/glycerol (1:9, v/v) and 1 mg of *p*-phenylenediamine/ml for light-microscopic observation.

## RESULTS

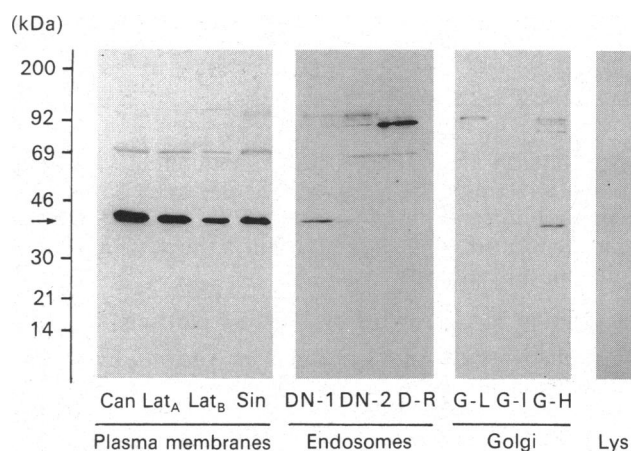
### Localization of G-protein by ADP-ribosylation

Islet-activating protein (pertussis toxin), which catalysed ADP-ribosylation of the  $\alpha$ -subunit of inhibitory G-protein ( $\alpha_1$ ) was used to investigate the G-protein distribution in various plasma-membrane and endosome fractions. Liver subcellular fractions incubated with [ $^{32}$ P]NAD $^{+}$  were analysed by SDS/polyacrylamide-gel



**Fig. 1. Pertussis-toxin-catalysed ADP-ribosylation of the  $\alpha$ -subunit of the inhibitory G-protein**

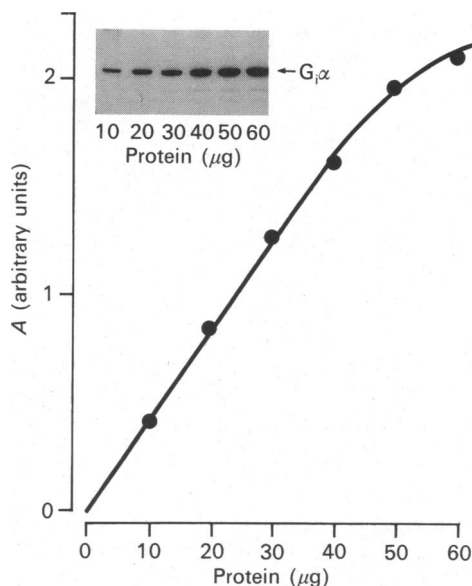
Membrane fractions (50  $\mu$ g) were incubated with [ $^{32}$ P]NAD $^{+}$  in the presence (+) or absence (-) of activated pertussis toxin as described in the Materials and methods section. Proteins were resolved in 10%-polyacrylamide gels, and autoradiography was carried out on the dried gels. Arrow points to 41 kDa polypeptide labelled in all the plasma-membrane and endosome fractions. Abbreviations: Can, canalicular plasma membranes; Lat $_A$ , lateral-A plasma membranes; Lat $_B$ , lateral-B plasma membranes; Sin, sinusoidal plasma membranes; DN-1, 'late' endosomes; DN-2, 'early' endosomes; D-R, receptor-enriched endosomes.



**Fig. 2. Identification of the  $\alpha$ -subunit of the inhibitory G-protein by Western blotting**

Membrane fractions (50  $\mu$ g) were resolved in 7.5–15.0%-polyacrylamide gradient gels, electrophoretically transferred to nitrocellulose paper and stained with the anti-peptide serum. Arrow points to the position of the 41 kDa polypeptide present in the fractions. For abbreviations, see Fig. 1 legend; also: Lys, lysosomes; G-L, G-I, G-H, Golgi light, intermediate and heavy.

electrophoresis and autoradiography. The results showed that pertussis toxin specifically catalysed the incorporation of the [ $^{32}$ P]ADP-ribose moiety of [ $^{32}$ P]NAD $^{+}$  into a 41 kDa polypeptide that was present in all plasma-membrane and endosome fractions (Fig. 1). No labelling of polypeptides was detected in control-treated membrane fractions in the absence of pertussis toxin. Comparative observation of the extent of labelling of the  $\alpha_1$  subunit by ADP-ribosylation revealed that lateral



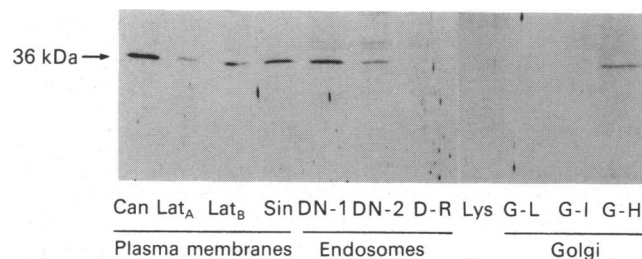
**Fig. 3. Quantification by densitometry of the amounts of  $\alpha$ -subunit of inhibitory G-protein ( $G_i\alpha$ ) in the bile-canalicular plasma membrane**

Various amounts of plasma membranes were resolved in 10%-acrylamide gels, electrophoretically transferred to nitrocellulose paper and stained with the  $\alpha_i$  antibody. Densitometric scanning of the autoradiograph of the immunoblot shown in the inset was performed as described in the Materials and methods section. The Figure shows the linearity between the densitometric signal detecting 41 kDa polypeptide and membrane protein up to approx. 50  $\mu\text{g}$ .

plasma-membrane fractions had higher contents of the  $\alpha_i$  subunit as compared with canalicular and sinusoidal plasma-membrane domains. In the endosome fractions the incorporation of radioactivity into the  $\alpha_i$  subunit was similar in all fractions.

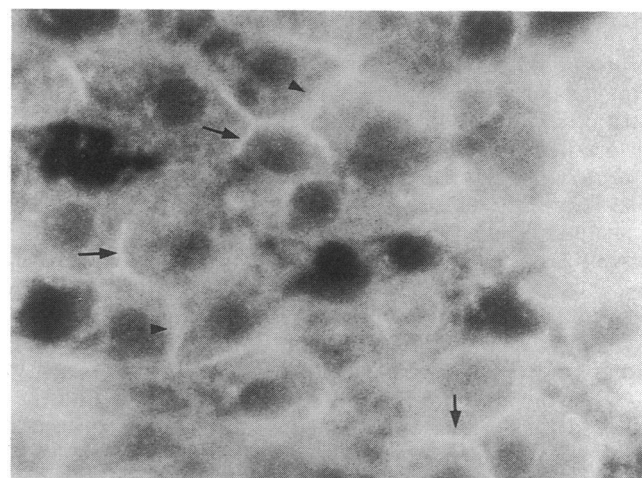
#### Localization of G-proteins by Western blotting

The distribution of G-proteins in various liver sub-cellular fractions was also examined by Western blotting. First, an antibody raised against a synthetic peptide corresponding to the C-terminal 10 amino acid residues of the  $\alpha_i$  subunit was used. This anti-peptide antibody detected a 41 kDa polypeptide in all the plasma-membrane fractions as well as in 'early' (DN-2) and 'late' (DN-1) endosomes (Fig. 2). In the liver plasma-membrane fractions, a linear increase within the range used of the intensity of staining by the antibody to the  $\alpha$ -subunit of the 41 kDa polypeptide was demonstrated (Fig. 3), in agreement with previous work with the same antibodies to  $\alpha$  or  $\beta$  subunits using different membranes or purified G-proteins. Comparison of the relative distribution of intensity ratio [expressed as %,  $\pm$  S.E.M. ( $n = 3$ ) of the total intensity found in all fractions of autoradiographic staining of the labelled 41 kDa polypeptide among the plasma membrane, endosome and Golgi fractions] showed that canalicular plasma membranes had by far the highest contents of the  $\alpha_i$  subunit; these values were canalicular plasma membrane  $32 \pm 9$ , lateral  $H_A$  plasma membrane  $10 \pm 3$ , lateral  $H_B$  plasma membrane  $12 \pm 5$ , sinusoidal plasma membrane  $13 \pm 4$ , 'late' endosomes  $14 \pm 7$ , 'early' endosomes  $4 \pm 1$ , and Golgi



**Fig. 4. Identification of the  $\beta$ -subunit of G-proteins by Western blotting**

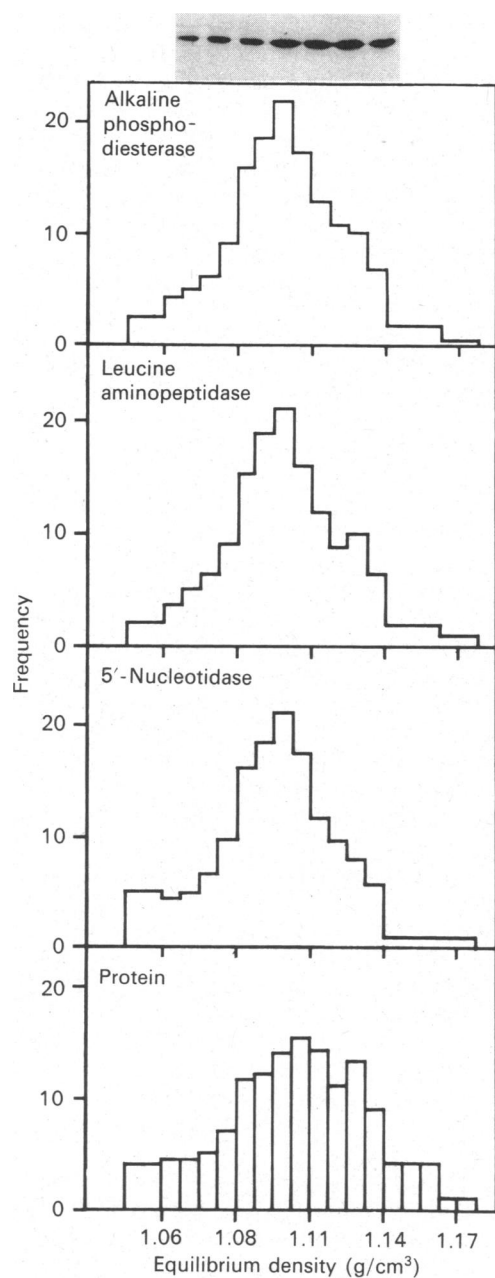
Membrane fractions (50  $\mu\text{g}$ ) were resolved in 10%-polyacrylamide gels, electrophoretically transferred to nitrocellulose paper and stained with an antiserum raised to the  $\beta$ -subunit of G-proteins. The position of the 36 kDa polypeptide present in the various fractions is shown. For abbreviations see legends to Figs. 1 and 2.



**Fig. 5. Immunofluorescent-microscopic localization of the  $\alpha$ -subunit of the inhibitory G-protein**

Thin liver sections were stained by antibodies to the  $\alpha$ -subunit of the inhibitory-subunit G-protein, followed by a fluorescein isothiocyanate-conjugated antibody. Labelling is localized at the hepatocyte cell surface, especially at the lateral (arrow) and canalicular (arrowhead) regions. Magnification  $\times 700$ .

fractions  $17 \pm 2$ . In contrast with the results obtained by ADP-ribosylation, this antiserum did not detect the 41 kDa polypeptide in receptor-rich endosomes, but it was noted that an intense labelling of a 90 kDa polypeptide occurred in this fraction. Subcellular fractions originating from the Golgi apparatus also had low contents of the  $\alpha_i$  subunit, with the Golgi fractions collected at the highest density in the sucrose gradients showing higher contents of the  $\alpha_i$  subunit, followed by the intermediate- and low-density Golgi fraction (Fig. 2). Lysosomes were devoid of any detectable amounts of the  $\alpha_i$  subunit (Fig. 2). Thus the overall distribution of the  $\alpha$ -subunit of inhibitory G-protein indicated that, in addition to its location in fractions originating from the blood-facing sinusoidal and lateral regions of the plasma membrane, it was also present at higher levels in canalicular plasma membranes. This led us to investigate whether G-proteins were released from the bile-canalicular membrane into bile. However, analysis of

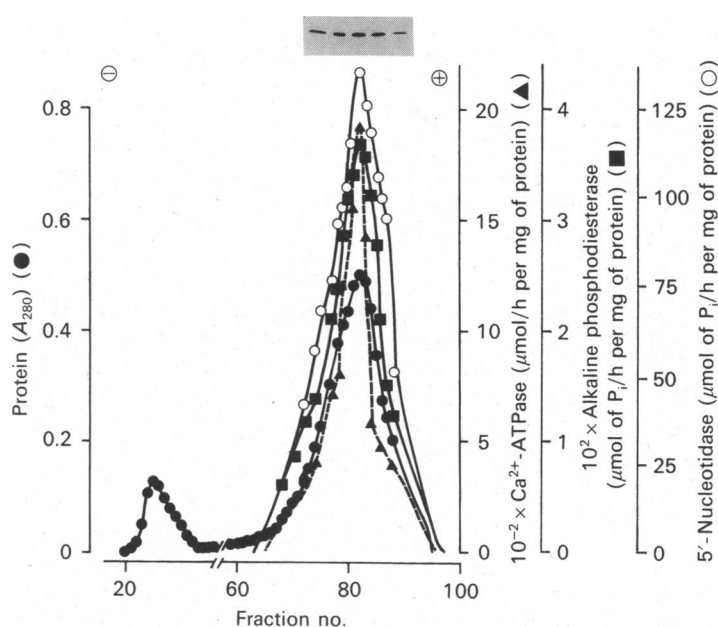


**Fig. 6. Resolution of a bile-canalicular plasma-membrane fraction in Nycodez gradients**

The frequency distribution of protein and three marker enzymes in the gradient is shown. The top panel shows an autoradiograph of the gradient fractions electrophoresed in a 10% polyacrylamide gel and electrophoretically transferred to nitrocellulose for staining with the antibody that recognized the 41 kDa  $\alpha$ -subunit. Equal amounts of proteins (50  $\mu$ g) were applied to each channel.

bile clearly indicated that the  $\alpha_i$  subunit was undetectable in bile (results not shown).

An antiserum raised to the  $\beta$ -subunit of G-proteins detected a 36 kDa polypeptide in all the plasma-membrane fractions as well as in the endosome fractions. From Fig. 4, an estimate of the relative distribution pattern of the  $\beta$ -subunit in the various fractions was made, and the distribution was found to be similar to



**Fig. 7. Resolution of a bile-canalicular plasma-membrane fraction by free-flow electrophoresis**

The activities of three marker enzymes and protein across the electrophoretic chamber are shown. The upper part of the Figure shows an autoradiograph of fractions electrophoresed in a 10% polyacrylamide gel and transferred to nitrocellulose for staining with antibody that recognized the 41 kDa  $\alpha$ -subunit. Equal amounts of proteins (50  $\mu$ g) were applied to each channel.

that observed with the  $\alpha$ -subunit, with the intensity of the labelled 36 kDa polypeptide (expressed as %) in one experiment being: canalicular plasma membrane 40%, lateral  $H_A$  plasma membrane 6%, lateral  $H_B$  plasma membrane 9%, sinusoidal plasma membrane 13%, 'late' endosomes 17%, 'early' endosomes 5%, and Golgi fractions 10%. This antibody did not detect the  $\beta$ -subunit in lysosomal fractions.

#### Localization of G-proteins in liver sections by immunofluorescence microscopy

Immunofluorescence microscopy of liver sections using the antibody to the  $\alpha_i$  subunit of G-proteins was carried out. In tissue sections incubated with antibodies to the  $\alpha_i$  subunit the fluorescent labelling was observed all around the hepatocyte, with the more intense labelling being located at the bile-canalicular regions (Fig. 5). Low-intensity intracellular fluorescence observed may correspond to the location of G-proteins in the endocytic compartment, as indicated by the biochemical data.

#### Localization of G-proteins in canalicular plasma membranes after subfractionation

In view of recent reports that intracellular vesicles with inositol phosphate-activated calcium-storage properties are present at close proximity to the liver plasma membrane (Guillemette *et al.*, 1988), which could result in a subpopulation of vesicles co-purifying along with the canalicular plasma membranes, further fractionation of the canalicular plasma-membrane fraction was carried

out to investigate the distribution of G-proteins and marker enzymes. Canalicular plasma membranes were fractionated in shallow isopycnic Nycodenz gradients (Fig. 6) or by free-flow electrophoresis (Fig. 7). Fractionation of the canalicular plasma membranes on Nycodenz gradients yielded a single peak of protein. Frequency distribution profiles calculated for the protein and three bile-canalicular plasma-membrane marker enzymes (Evans, 1980) indicated a co-distribution of 5'-nucleotidase, leucine aminopeptidase and alkaline phosphodiesterase activities (Fig. 6). Equal amounts of proteins from fractions corresponding to the peak frequency range of canalicular membranes were analysed by Western blotting using the antibody to the  $\alpha_1$  subunit. The autoradiograph (Fig. 7, top panel) shows the intensity of the 41 kDa protein to correspond to the position of the canalicular plasma-membrane marker enzymes.

Fractionation of canalicular plasma membranes by free-flow electrophoresis also yielded a major protein peak containing the 5'-nucleotidase, alkaline phosphodiesterase and  $\text{Ca}^{2+}$ -activated ATPase activities (Fig. 7). A minor peak of protein located at the cathode was devoid of these canalicular plasma-membrane marker enzymes. Western blotting showed that the  $\alpha_1$  subunit was distributed in the fractions containing the canalicular plasma-membrane marker enzymes. G-protein was not detected in the minor protein peak located near the anode (results not shown).

## DISCUSSION

Nucleotide-binding G-proteins are widely implicated in signal transduction across the plasma membrane. In view of the regionalization of the plasma membrane of polarized cells, and the endocytic uptake by cells of receptor-ligand complexes, it is appropriate to examine the extent to which G-proteins are present in the various regions of the plasma membrane and in intracellular membranes, especially in those originating from the endocytic compartment and lysosomes. This information can allow the subcellular compartment of G-proteins and associated components of the signalling apparatus to be mapped. In the present work the availability of membrane fractions originating from hepatocyte surface domains, the endocytic compartment, lysosomes and the Golgi apparatus has allowed the subcellular distribution of G-protein  $\alpha$ - and  $\beta$ -subunits to be determined. This assessment of the subcellular distribution of G-proteins is carried out against the background that hormone- and neurotransmitter-activated adenylate cyclase activities were confined mainly to the sinusoidal plasma membrane (Wisher & Evans, 1975; Hadjiivanova *et al.*, 1984) and, in contrast, that the D-*myo*-inositol 1,3,4-trisphosphatase and 1,3,4,5-tetrakisphosphatase activities were more widely distributed, with substantial enzyme activities being found in all three plasma-membrane domains and in endocytic membranes (Shears *et al.*, 1988).

Two biochemical approaches in combination with an immunolocalization study attested to the wide distribution of G-proteins in hepatocyte membrane fractions. Pertussis-toxin-mediated ADP-ribosylation, shown to identify at least three  $\alpha$ -subunit polypeptides (39, 40 and 41 kDa) (Hescheler *et al.*, 1987; McKenzie *et al.*, 1988; Toutant *et al.*, 1988), identified a 41 kDa subunit in all

three plasma-membrane domains and in endosomes. Other pertussis-toxin substrates detected in, e.g., muscle (Toutant *et al.*, 1988) and neutrophils (Bokoch *et al.*, 1988) were not identified in the present work. The widespread presence in the subcellular fraction of the inhibitory  $\alpha$ -subunit was confirmed by using an antibody raised to a synthetic peptide corresponding to a sequence at its C-terminus, which identified a 41 kDa polypeptide in the fractions. However, some minor differences were noted between the toxin-mediated ribosylation and antibody techniques. The antibodies detected only extremely low amounts of the 41 and 36 kDa polypeptides in one of the endosome subfractions, and the relative intensity of the autoradiographic staining of these protein subunits obtained by the two techniques varied between the fractions examined. These differences in the semi-quantitative assessment of G-protein levels obtained by the antibody and ribosylation techniques were also experienced by other workers (Scherer *et al.*, 1987). An explanation for this apparent discrepancy may be that the pertussis-toxin substrate in fraction D-R is structurally different from the 41 kDa protein present in the other fraction. It is therefore considered likely that the values for the relative distribution of the two subunits obtained by the use of antibodies are more likely to be correct. Indeed, there was a close correspondence in the plasma-membrane fractions between the relative intensities obtained with both antibodies, suggesting that both  $\alpha$ - and  $\beta$ -subunits were associated with the various membranes examined. The distribution of the G-protein subunits between the fractions assessed by antibody binding makes it unlikely that the values reflect the non-specific association of cytosolic G-proteins with the membranes, for the 41 kDa  $\alpha$ -subunit was shown, by partitioning between aqueous and Triton X-114 phases, to be predominantly a hydrophobic protein in plasma-membrane and endosome fractions (N. Ali, G. Milligan & W. H. Evans, unpublished work). Staining by immunofluorescence of liver sections confirmed independently the presence of the  $\alpha$ -subunit of the G-protein at surface areas of the hepatocyte.

The present results showed that the highest level of both G-protein subunits was in bile-canalicular plasma membranes. A more thorough biochemical analysis of the bile-canalicular plasma membranes resolved by equilibrium Nycodenz-gradient centrifugation and by free-flow electrophoresis showed clearly that the  $\alpha$ -subunit co-fractionated with four marker-enzyme activities. The presence of higher relative amounts of the G-protein subunits at the bile-canalicular face of the hepatocyte than at the blood-bathed sinusoidal and lateral surface raises two questions. First, the functions of G-proteins at the bile-canalicular membrane of the hepatocyte require explanation. In view of the high  $\text{Ca}^{2+}$ -ATPase activities in bile-canalicular membranes (Bachs *et al.*, 1985), one possibility is that the G-proteins now identified are linked in some way in controlling  $\text{Ca}^{2+}$  movements across these membranes. The presence of high levels of enzymes hydrolysing inositol tris- and tetrakis-phosphate in liver bile-canalicular plasma membranes (Shears *et al.*, 1988) would also support the notion that a high level of metabolic regulation occurs in the membranes comprising the bile-canalicular front of hepatocytes. Second, a route or routes than can account for the transport of G-proteins to the bile-canalicular region of the cell must exist. G-proteins, present in the lateral plasma membrane



of the hepatocyte, are unlikely to diffuse into the bile-canalicular plasma membrane, for tight junctions do not allow transfer of inner-bilayer lipids and probably any associated proteins (Gumbiner, 1987). More likely is the possibility that the trafficking of endocytic membranes results in the formation of a vesicular conduit connecting the sinusoidal and canalicular plasma-membrane domains. This transcellular route in hepatocytes is postulated to explain the receptor-mediated transfer of polymeric IgA from blood to bile (Mostov & Simister, 1985; Schiff *et al.*, 1986). The detection of the G-proteins in 'early' and 'late' endosomes would support a similar mechanism for their transfer from the sinusoidal to the bile-canalicular membrane. The trafficking of G-proteins between plasma-membrane domains via the endocytic pathway must, however, be regulated, for the  $\alpha$ - and  $\beta$ -subunit were not detected in lysosomes. A third possibility is the direct transfer of G-proteins to the bile-canalicular region from the Golgi apparatus (Evans, 1980). A role for G-proteins, especially an inhibitory subunit featuring in secretory processes, has been advocated (Melancom *et al.*, 1987; Bourne, 1988), and low levels of the G-protein  $\alpha$ -subunit were detected in the endosome-depleted Golgi fractions examined in the present work. Since these fractions may in addition contain low amounts of plasma membranes (e.g. in the Golgi subfraction) or endosomes (e.g. in Golgi I and L subfractions), it is not possible to distinguish between G-proteins being intrinsic functional proteins in the Golgi membranes isolated, or whether they are contaminants. Nevertheless, one overall conclusion emerging is that G-proteins may play a wider role in membrane trafficking, being involved in endocytic membrane routing in addition to their proposed role in the secretory pathway.

The present work, showing that G-proteins are widely distributed in apical and basolateral liver plasma-membrane domains, as well as in endocytic membranes, suggests that cells may regulate receptor activity, and thus responsiveness to circulating ligands, by direct transfer from the plasma membrane of crucial components of the signalling apparatus. Thus the confinement of adenylate cyclase activity to the plasma membrane (Hadjivanova *et al.*, 1984), whereas at least the  $\alpha$ - and the  $\beta$ -subunits of G-proteins and the enzymes hydrolysing inositol tris- and tetrakis-phosphate are found more widely distributed intracellularly, suggests a complex processing and compartmentation of these components involved in signal transduction. Support for this idea comes from studies on the distribution of G-protein subunits in neutrophils (Bokoch *et al.*, 1988; Rotrosen *et al.*, 1988), showing that G-proteins are also located in granule-enriched fractions and the cytosol. The present work, using a tissue composed predominantly of polarized cells, adds new information on the distribution of G-proteins and points to the involvement of the endocytic compartment in mobilizing the G-proteins between various compartments. Thus the endocytic compartment assumes a further function that could be a part of its general role in the co-ordination of the processing and sorting of receptor-ligand complexes and the G-protein constituents associated with these complexes.

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