Priority targeting of glycosyl-phosphatidylinositol-anchored proteins to the bile-canalicular (apical) plasma membrane of hepatocytes

Involvement of 'late' endosomes

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1. Liver plasma membranes originating from the sinusoidal, lateral and canalicular surface domains of hepatocytes were covalently labelled with sulpho-*N*-hydroxysuccinamide-biotin. After solubilization in Triton X-114, treatment with a phosphatidylinositol-specific phospholipase C (PI-PLC), two-phase partitioning and ¹²⁵I-streptavidin labelling of the proteins resolved by PAGE, six major polypeptides (molecular masses 110, 85, 70, 55, 38 and 35 kDa) were shown to be anchored in bile canalicular membrane vesicles by a glycosyl-phosphatidylinositol (G-PI) 'tail'. 2. Permeabilized 'early' and 'late' endocytic vesicles isolated from liver were also examined. Two polypeptides (110 and 35 kDa) were shown to be anchored by a G-PI tail in 'late' endocytic vesicles. 3. Analysis of marker enzymes in bile-canalicular vesicles treated with PI-PLC showed that 5'-nucleotidase and alkaline phosphatase, but not leucine aminopeptidase and ecto-Ca²⁺-ATPase activities were released from the membrane. A low release and recovery of alkaline phosphodiesterase activity was noted. The cleavage from the membrane of 5'-nucleotidase as a 70 kDa polypeptide was confirmed by Western blotting using an antibody to this enzyme. 4. Antibodies raised to proteins released from bile-canalicular vesicles by PI-PLC treatment, and purified by partitioning in aqueous and Triton X-114 phases, localized to the bile canaliculi in thin liver sections. Antibodies to proteins not hydrolysed by this treatment stained by immunofluorescence the sinusoidal and canalicular surface regions of hepatocytes. 5. Antibodies generated to proteins cleaved by PI-PLC treatment of canalicular vesicles were shown to identify, by Western blotting, a major 110 kDa polypeptide in these vesicles. Two polypeptides (55 and 38 kDa) were detected in MDCK and HepG-2 cultured cells. 6. Since two of the six G-PI-anchored proteins targeted to the bile-canalicular plasma membrane were also detected in 'late' endocytic vesicles, the results suggest that a junction where exocytic and endocytic traffic routes meet occurs in a 'late' endocytic compartment.

INTRODUCTION

Many proteins are anchored at their C-terminus into the plasma membrane by a glycosyl-phosphatidylinositol (G-PI) tail (Ferguson & Williams, 1988; Low & Saltiel, 1988; Low, 1989; Turner & Hooper, 1989). The G-PI tail is added at the C-terminus of newly synthesized proteins in the endoplasmic reticulum after removal of a hydrophobic sequence from the C-terminus (Caras & Weddell, 1989). It was shown recently, by using kidney and intestinal cell lines, that proteins anchored into the cell surface in this manner were found exclusively at the apical domain, suggesting that the way in which a protein is anchored in the membrane of epithelial cells is a signal that can influence its intracellular targeting to a specific surface area (Lisanti *et al.*, 1988, 1989; Brown *et al.*, 1989).

We describe here an extension of previous work on the generation and biochemical properties of the plasma membrane domains of hepatocytes (Evans, 1980, 1981; Evans *et al.*, 1980; Evans & Enrich, 1989). Using domain-identified liver plasma membranes, we show that six polypeptides, attached to the membrane by a G-PI anchor, were confined predominantly to the apical bile-canalicular plasma membrane region. Two of these G-PI-anchored polypeptides were also detected in 'late' endocytic vesicles. The results demonstrate that a direct biogenetic pathway to the bile-canalicular plasma membrane domain exists for proteins attached in the membrane by a G-PI tail. Two of the proteins released by phospholipase C treatment were

shown to be canalicular enzymes used as subcellular markers for this plasma-membrane domain. Antibodies generated to the proteins released by the enzymic treatment of bile-canalicular vesicles were also shown to locate to the bile-canalicular zone in liver sections, thereby confirming the chemical labelling experiments. These antibodies also identified similar antigens in MDCK and HepG-2 cells.

EXPERIMENTAL

Materials

Phosphatidylinositol-specific phospholipase C (PI-PLC) (from *Bacillus cereus*) (50 units/100 µl) and proteinase inhibitors were from Boehringer Mannheim, Mannheim, Germany. Sulpho-*N*-hydroxysuccinamide (NHS)-biotin and streptavidin were from Pierce Chemical Co., Rockford, IL, U.S.A. Nycodenz was from Nycomed U.K., Sheldon, Birmingham, U.K. Other materials were from Sigma Chemical Co., Poole, Dorset, U.K.

Preparation of subcellular fractions

The preparation of sinusoidal plasma membranes was carried out as described by Wisher & Evans (1975). A modified procedure was used to prepare highly purified canalicular and lateral plasma membranes [the preceding paper (Ali & Evans, 1990)]. In summary, a bile-canalicular plasma-membrane subfraction collected from the 8/37 %-(w/v)-sucrose-gradient interface (Wisher

Abbreviations used: G-PI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; NHS, *N*-hydroxysuccinamide.

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& Evans, 1975) was sonicated and resolved further in iso-osmotic 10–30% (w/v)-Nycodenz gradients made in 8% (w/v) sucrose. A highly purified (the specific activity of leucine aminopeptidase was increased 115-fold over the homogenate) vesicular bile-canalicular fraction was collected at a density of 1.055 g/ml. A 'lateral' plasma-membrane fraction [(Na⁺ + K⁺)-dependent ATPase specific activity was increased 37-fold over the homogenate] was prepared by sonication of the plasma-membrane fraction located at the 37/43% sucrose interface, and separation in the same Nycodenz gradients at a density of 1.175 g/ml. Endosomes were prepared and characterized as described previously (Evans & Flint, 1985; Evans, 1988) and were designated kinetically as 'late' or 'early' according to the positions on the Nycodenz gradients of various radioiodinated ligands within the vesicles and that were taken up by liver 10–15 min or 2–5 min respectively after injection into the portal vein. A 'heavy' ligand-free endosome fraction was collected at higher density on the Nycodenz gradients as described by Evans & Flint (1985).

Covalent labelling and PI-PLC treatment of subcellular fractions

The procedure described by Lisanti *et al.* (1988) for biotinylation of cultured epithelial cells was adapted for chemical labelling of subcellular fractions. Plasma-membrane fractions (200 µg of protein) were incubated, with shaking, in sulpho-NHS-biotin (0.25 mg) in 500 µl of a phosphate-buffered (10 mM-sodium phosphate/0.15 M-NaCl) saline, pH 7.4, containing 1 mM-MgCl₂ and 0.1 mM-CaCl₂ for 30 min at 4 °C. After centrifugation (13000 g for 10 min; Eppendorf Microfuge), the membrane pellets were washed twice by resuspension and re-centrifugation in the same buffer (500 µl). The pellets were dissolved by suspension in 500 µl of 1% Triton X-114 (pre-condensed using butylated hydroxytoluene)/10 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl/1 mM-EDTA for 1 h at 4 °C. Approx. 90% of the membrane protein was solubilized by this method. After generation of two phases at 30 °C by further incubation for 5 min, followed by centrifugation at 600 g for 2 min (Bordier, 1981), the detergent (lower) phase was collected and washed twice with 500 µl of 10 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl/1 mM-EDTA. The final detergent phase (100 µl) was diluted to 500 µl by the addition of 100 mM-Tris/HCl (pH 7.4)/50 mM-NaCl/1 mM-EDTA, and half was used for PI-PLC treatment, with the remainder serving as a control. To 250 µl of the detergent phase were added 30 µl of PI-PLC (1.75 units) and the reaction allowed to continue for 1 h at 37 °C with constant mixing (Eppendorf mixer 5432); 30 µl of water were added to controls. All these steps were carried out in the presence of the proteinase inhibitors pepstatin, chymostatin, antipain and leupeptin (each 5 µg/ml). After two-phase separation (at 30 °C), the upper (aqueous) phase was collected and washed by a further round of two-phase separations. The final upper phase was freeze-dried and then dissolved in 75 mM-Tris/HCl (pH 6.8)/2% (w/v) SDS/0.55 M-β-mercaptoethanol/0.003% Bromophenol Blue/10% (w/v) glycerol for analysis of polypeptides by PAGE (see below).

With the endosome fractions (200 µg of protein) biotinylation was carried out for 30 min at 4 °C in 500 µl of phosphate-buffered saline (pH 7.4)/1 mM-MgCl₂/0.1 mM-CaCl₂ in the presence of saponin (0.2 mg/ml) to open up vesicles (Shears *et al.*, 1988).

Electrophoresis, streptavidin overlay and immunoblotting

Polypeptides of subcellular fractions were analysed by electrophoresis in 10%-polyacrylamide gels (Laemmli, 1970). Cells (MDCK and HepG-2 grown to confluency) were suspended in 10 ml of standard Eagle's medium supplemented with foetal-

calf serum and were then pelleted by centrifugation (1000 g for 10 min) and the pellet washed twice by resuspension and centrifugation in 1 mM-NaHCO₃. Cells were homogenized and then centrifuged at 13000 g for 10 min (Eppendorf Microfuge). After resuspension of the pellet in 0.5 ml of phosphate-buffered saline, pH 7.0, 50 µg (of protein) was used for electrophoretic analysis.

Polypeptides were transferred electrophoretically from polyacrylamide gels to nitrocellulose sheets (0.1 µm pore size; Schleicher und Schüll, Dassel, Germany) as described by Burnette (1981). Streptavidin (150 µg) was radioiodinated (1 mCi of Na¹²⁵I; Amersham International) using chloramine-T, and separated from free iodine on Sephadex G-25M columns (Pharmacia PD-10; bed volume 9 ml) to yield streptavidin of specific radioactivity 5 µCi/µg. For the streptavidin overlay, nitrocellulose sheets were washed for 10 min in phosphate-buffered saline, pH 7.4, and blocked by washing with 3% (w/v) BSA/1% (w/v) skimmed milk/0.5% Tween 20/10% (v/v) glycerol/1 M-glucose (in phosphate-buffered saline, pH 7.4) for 1 h at room temperature. Nitrocellulose sheets were exposed to 40 µl of a stock solution of ¹²⁵I-streptavidin added to 20 ml of buffer (see above for comparison). Sheets were then washed (three times for 10 min) with 0.5% Tween 20 in phosphate-buffered saline, pH 7.4, dried in air, and subjected to autoradiography (Sargiacomo *et al.*, 1989).

For Western blotting, nitrocellulose sheets were exposed to the antibodies to proteins released from bile-canalicular plasma-membrane vesicles and purified as described above. A 1:50 dilution of the antiserum was used, as described by Ali *et al.* (1989).

Enzyme analysis

Bile-canalicular vesicles (5–20 µg of protein) were suspended in 25 µl of 10 mM-Tris/HCl, pH 7.4, containing 1 µl of PI-PLC and proteinase inhibitors (see above) for 45 min at 37 °C. Controls were run under the same conditions in the absence of enzyme. The PI-PLC was dialysed extensively against 10 mM-Tris/HCl, pH 7.4, before use. After centrifugation (13000 g for 15 min), enzyme activity in the supernatant and the pellet were determined as follows: 5'-nucleotidase (EC 3.1.3.5) (Michell & Hawthorne, 1965); alkaline phosphatase (EC 3.1.3.1) (Pekarth *et al.*, 1972); alkaline phosphodiesterase I (EC 3.1.4.1) (Razzel, 1963); leucine aminopeptidase (EC 3.4.11.1) (Goldberg & Rutenberg, 1958). Ca²⁺-dependent ATPase was measured by using 0.2 µM-Mg²⁺ and 0.5 µM-Ca²⁺ (free ion concentrations) as described by Birch-Machin & Dawson (1985). Protein was measured as described by Bradford (1976), with BSA as standard.

Preparation of antisera

Antibodies were prepared to the proteins released from bile-canalicular vesicles by dissolving membranes (400 µg of protein) in 1% Triton X-114 and carrying out a two-phase separation as described above. The PI-PLC treatment of the proteins separating into the detergent phase and their release into the aqueous phase were also carried out as described above. The enzymically released protein fraction as well as proteins remaining in the detergent phase were mixed with Freund's complete adjuvant and administered intramuscularly to rabbits. After 6 weeks, enzymically released and unreleased proteins deriving from 200 µg of canalicular plasma membranes and purified by two-phase separation were mixed with Freund's incomplete adjuvant and administered subcutaneously. Rabbits were bled 10 days later at twice-weekly intervals.

Antibodies raised in rabbits to rat liver 5'-nucleotidase were a gift from Dr. Paul Luzio, Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K.

Fig. 1. Analysis by PAGE of G-PI-anchored polypeptides

Liver plasma membranes (a) and permeabilized endosomes (b) were covalently labelled with sulfo-NHS-biotin and solubilized in Triton X-114. The polypeptides released by treatment with PI-PLC were purified by two-phase separation and resolved by gel electrophoresis. After transfer to nitrocellulose sheets, overlaying with ^{125}I -labelled streptavidin and autoradiography, the enzyme-treated (+) and control (–) fractions were compared. Polypeptides completely released by PI-PLC are indicated by arrows. Lanes: 1, bile-canalicular plasma membranes; 2, lateral plasma membranes; 3, sinusoidal plasma membranes; 4, 'late' endosomes; 5, 'early' endosomes.

Immunofluorescence microscopy

Sections of frozen rat liver (6–10 μm thick) on gelatin-coated glass slides, after immersion in chloroform/acetone (1:1, v/v) for 3 min, were dried and then incubated in antibodies diluted 50-fold. After 30 min at room temperature, sections were stained by a fluorescein isothiocyanate-labelled goat anti-rabbit Ig for 1 h and observed under a fluorescence microscope.

RESULTS

Three liver plasma-membrane fractions, shown to originate from the canalicular, sinusoidal and lateral surface domains, were used for the vectorial labelling experiments. The canalicular and sinusoidal plasma membranes were isolated as predominantly right-side-out vesicles (Evans, 1980; Shears *et al.*, 1988). These plasma-membrane fractions were covalently labelled using sulfo-NHS-biotin, solubilized in Triton X-114, and the integral proteins that partitioned into the detergent phase of a two-phase separation were treated with PI-PLC. Biotin-labelled

polypeptides released by the enzyme treatment were partitioned into the aqueous phase and analysed by PAGE were recognized by the binding of radioiodinated streptavidin by comparison with a control experiment in which no enzymic treatment was involved. Fig. 1(a) shows that, in the bile-canalicular plasma-membrane fraction, six major polypeptides (110, 85, 70, 55, 38 and 35 kDa) were released by PI-PLC treatment, whereas, in lateral and sinusoidal plasma membranes, no additional polypeptides were identified by streptavidin–biotin binding in the presence or absence of enzymic treatment. In lateral plasma membranes treated with PI-PLC, there was an increase in labelling of 68, 65, 60 and 35 kDa polypeptides. Two of the G-PI-anchored polypeptides identified in bile-canalicular vesicles (110 and 35 kDa) were also present in 'late', but not in 'early', endosomes (Fig. 1b).

Experiments were carried out to identify the membrane constituents attached in the membrane by a G-PI anchor. Attention was focused on ectoenzymes used as marker constituents for the bile-canalicular plasma membranes. Highly purified bile-canalicular vesicles were incubated with PI-PLC, and the distribution of the various enzyme activities between the pellet and the supernatant was determined. Table 1 shows that there was almost complete release into the supernatant of 5'-nucleotidase and alkaline phosphatase activities from the bile-canalicular vesicles. In contrast, leucine aminopeptidase and Ca^{2+} -ATPase activities were retained in the pellet. The result obtained with alkaline phosphodiesterase was less clear-cut, with about one-third of this enzyme activity being released. However, overall recoveries were low.

Antibodies were raised to the detergent-solubilized integral membrane proteins of bile-canalicular plasma membranes that were released by PI-PLC and that partitioned into an aqueous phase. These antibodies were shown to react at the bile-canalicular pole of hepatocytes in liver sections (Fig. 2a). In contrast, antibodies raised to the proteins retained in the detergent phase after PI-PLC treatment, i.e. non-G-PI-anchored integral proteins, stained both the bile and blood sinusoidal surface areas of the hepatocyte (Fig. 2b).

The antibodies to the G-PI-anchored glycoprotein were also used to study, by immunoblotting, the distribution of polypeptides in the liver subcellular fractions and in cultured cells. Fig. 3 shows that these antibodies identified in canalicular plasma membrane a major 110 kDa and a minor 55 kDa polypeptide that probably corresponded to those identified by streptavidin–biotin labelling (Fig. 1). A labelled 110 kDa polypeptide was also seen in 'late' endosomes, thus confirming the streptavidin–biotin-labelling results. The endosomal membranes also contained other polypeptides of lower molecular mass (90 and 30 kDa) that did not correspond to those identified when

Table 1. Release of bile-canalicular plasma-membrane marker enzymes by G-PI-specific PLC treatment

Recoveries are expressed as a percentage of total activity in the bile-canalicular plasma-membrane fraction used.

Enzyme activity	Activity recovered (%)			
	Control		Enzyme treatment	
	Sediment	Supernatant	Sediment	Supernatant
5'-Nucleotidase	87 $\pm$ 5 (3)	11 $\pm$ 5 (3)	15 $\pm$ 8 (4)	116 $\pm$ 19 (4)
Alkaline phosphatase	91 $\pm$ 5 (3)	7 $\pm$ 3 (3)	7 $\pm$ 2 (3)	87 $\pm$ 4 (3)
Alkaline phosphodiesterase	89 (2)	6 (2)	20 (2)	36 (2)
Leucine aminopeptidase	85 (2)	10 (2)	83 (2)	15 (2)
Ecto- Ca^{2+} -ATPase	90 (2)	7 (2)	87 (2)	6 (2)

Fig. 2. Immunofluorescence staining of liver sections with antibodies to G-PI-anchored and non-anchored polypeptides

In (a) sections were stained with antibodies to the polypeptides released from Triton X-114-solubilized bile-canalicular vesicles by PI-PLC treatment, and then purified by two-phase partitioning. In (b) sections were stained with antibodies to the lower Triton X-114 phase after release into an aqueous phase of the G-PI-anchored polypeptides. Arrowheads show stained bile canaliculi; arrows point to sinusoidal surfaces of hepatocytes stained in (b). Magnifications: (a) $\times 700$; (b) $\times 500$.

streptavidin-biotin-labelled bile-canalicular plasma-membrane vesicles were examined. Fig. 4 compares the polypeptides recognized by the antiserum in HepG-2 and MDCK cells. The antibodies recognized, in the cell lines, polypeptides of 55 and 38 kDa and, in MDCK cells, a minor 110 kDa polypeptide that corresponded in electrophoretic mobility to those identified by covalent labelling of bile-canalicular vesicles.

Finally, the nature of some of the G-PI-anchored proteins was examined by the use of antibodies. After treatment of bile-canalicular vesicles with PI-PLC, an antibody to 5'-nucleotidase showed that a high proportion of a 70 kDa component corresponding to the known molecular mass of this enzyme (Misumi *et al.*, 1990) was released into the supernatant in comparison with a control in which no enzyme was added (Fig. 5b). By using the antibodies generated to the G-PI-anchored proteins it was shown (Fig. 5a) that about 30% of the unidentified 110 kDa polypeptide was released into the supernatant. The antibodies therefore recognized a 110 kDa polypeptide that was released by treatment of canalicular vesicles with PI-PLC.

Fig. 3. Western blotting of various liver subcellular fractions

The fractions were resolved by PAGE transferred to nitrocellulose sheets and stained with antibodies to G-PI-anchored bile-canalicular proteins. Lanes (50 μ g of protein each): 1, canalicular plasma membranes; 2, lateral plasma membranes; 3, sinusoidal plasma membranes; 4, 'late' endosomes; 5, 'early' endosomes; 6, 'heavy' endosomes. The molecular masses of the major polypeptides are indicated.

Fig. 4. Comparison by Western blotting of G-PI-anchored polypeptides in plasma membranes and cell lines

The antibodies raised to the G-PI-anchored proteins of liver bile-canalicular plasma membranes were used to stain the electrophoretically resolved polypeptides of: 1, canalicular plasma membranes; 2, sinusoidal plasma membranes; 3, HepG-2 homogenates; 4, MDCK homogenates. The molecular masses of the major polypeptides are indicated.

DISCUSSION

The present work shows, using highly purified liver plasma-membrane domain-identified subfractions, that six major proteins are priority-targeted to the hepatocyte's apical cell-surface domain, i.e. the bile-canalicular plasma membrane. Although various proteins, including enzymes, have been shown to be segregated at the sinusoidal, lateral and canalicular plasma

Fig. 5. Identification of G-PI-anchored proteins released by PI-PLC treatment from bile-canalicular plasma membranes

After PI-PLC treatment of bile-canalicular plasma membranes, the supernatants and membrane pellets were examined by PAGE, transferred to nitrocellulose sheets and stained by (a) antibodies to the purified bile-canalicular G-PI proteins and (b) antibodies to 5'-nucleotidase. 1, Sediment; 2, supernatant. Identical amounts of protein (50 µg) were used in each experiment. +, Experimental; -, control (i.e. no enzymic treatment). The molecular masses of a major bile-canalicular PI-anchored polypeptide (110 kDa) and 5'-nucleotidase (70 kDa) are indicated.

membrane domains (Evans, 1980; Evans & Enrich, 1989), the present work shows that a specific category of membrane proteins, anchored into the membrane by a G-PI anchor, are priority-targeted to the bile-canalicular surface area. The adoption of this approach to study vectorial targeting to the cell surface, in contrast with studies using intact cultured cells (Rodriguez-Boulán & Salas, 1989), depends on the development of methods for dissecting the hepatocyte's plasma membrane into its constitutive domains (Wisher & Evans, 1974; Evans, 1980; Shears *et al.*, 1988; Ali *et al.*, 1989). We have taken advantage of the development of further major improvements in methods using Nycodenz gradients for preparing bile-canalicular vesicles and lateral plasma membranes (Ali *et al.*, 1989; Ali & Evans, 1990). For example, five bile-canalicular plasma-membrane marker enzymes: leucine aminopeptidase, 5'-nucleotidase, alkaline phosphodiesterase, alkaline phosphatase and Ca^{2+} -ATPase were enriched 115–153-fold in the canalicular vesicles relative to the liver tissue homogenate. No $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity, a basolateral marker enzyme, was detected. The lateral plasma-membrane fraction used in the present work showed a 37-fold enrichment of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity relative to the tissue homogenate.

The polypeptides shown to be G-PI-anchored and to be priority-targeted to the bile-canalicular plasma membrane of the hepatocyte (110, 85, 70, 55, 38 and 35 kDa) were of similar molecular masses to those identified by Lisanti *et al.* (1988) at the apical surface of a renal epithelial (MDCK) cell line. An antibody raised to the G-PI-anchored proteins of liver bile-canalicular vesicles also cross-reacted with polypeptides of 110, 55 and 38 kDa in MDCK and HepG-2 cultured cell lines, suggesting that there may be a general mechanism operational in polarized epithelial cells for directing subsets of membrane polypeptides to the apical surface domain.

In the present work it was possible to identify some of the proteins at the bile-canalicular plasma-membrane domain. Enzymic analysis of PI-PLC-treated bile-canalicular vesicles showed that a virtually complete release of 5'-nucleotidase and alkaline phosphatase activities occurred. The release of 5'-nucleotidase by phospholipase treatment was also confirmed by using a specific antibody. These two glycoproteins have been

shown to be membrane-anchored by a G-PI tail in a variety of cells (Ferguson & Williams, 1988; Turner & Hooper, 1989). For example, alkaline phosphatase was shown to be anchored in this manner to kidney microsomes (Low & Zilversmit, 1980) and placenta (Ognata *et al.*, 1988; Takami *et al.*, 1988) and 5'-nucleotidase to liver membranes (Baylies *et al.*, 1990; Misumi *et al.*, 1990). The present data do not permit a firm conclusion regarding the mechanism of anchoring of alkaline phosphodiesterase I activity, for it was only partially released by PI-PLC treatment of bile-canalicular vesicles, and enzyme recoveries were low. Low & Finean (1978) and Nakabayashi & Ikezawa (1986) showed that alkaline phosphodiesterase activity was not released from liver components by PI-PLC treatment. A number of polypeptides located at the liver's plasma membrane, including the alkaline phosphodiesterase I (Evans, 1974; Elovson, 1980), have a molecular mass of about 110 kDa. Of the many polypeptides located at the hepatocyte's bile-canalicular pole, the present work indicates that the 110 kDa polypeptide is unlikely to correspond to the Ca^{2+} -ATPase nor to leucine aminopeptidase for these ectoenzymes were not released by treatment of canalicular vesicles with PI-PLC. One can also exclude a 110 kDa glycoprotein that may be involved in the transport of organic anions into bile (Hong & Doyle, 1987) and a dipeptidyl peptidase (Ogata *et al.*, 1989), for they contain a hydrophobic trans-membrane amino acid sequence and thus a different mode of attachment in the membrane. Thus the identity of the G-PI-anchored 110 kDa polypeptide present in bile-canalicular vesicles remains to be clarified.

The results obtained using antibodies to the proteins released by treatment with PI-PLC of detergent-solubilized bile-canalicular vesicles and purified by partitioning between aqueous and Triton X-114 phases reinforced the apical localization of this category of membrane protein on hepatocytes. In thin liver sections, the antibodies raised to the enzymically released G-PI-anchored proteins localized to the bile canaliculi. The antibodies were also used to demonstrate, by immunoblotting, that a major 110 kDa polypeptide was absent, or present in extremely low amounts, in lateral and sinusoidal plasma membranes and in 'early' endosomes. The detection of low amounts of the 110 kDa polypeptide by the antibodies in other fractions is probably accounted for by considerations of fraction purity (Evans, 1988).

A caveat applying to the present approach for identifying G-PI-anchored membrane proteins in liver tissues as well as in cultured epithelial cells (Lisanti *et al.*, 1988, 1989) is that the use of Triton X-114 in the solubilization and temperature-controlled two-phase partitioning steps may select for subclasses of membrane polypeptides preferentially soluble in this detergent. Consequently, this method may fail to identify membrane constituents that behave anomalously when these methods of extraction and purification are used. Although under the conditions adopted a high proportion of the membrane proteins were solubilized, the anomalous behaviour of some membrane and membrane-associated proteins, e.g. acetylcholine receptor subunits (Maher & Singer, 1985) and fibronectin isoforms in liver plasma membranes (Enrich *et al.*, 1988), in two-phase partitioning systems has been reported. This tendency of categories of membrane proteins of intermediate hydrophobicity to partition anomalously is due in part to the presence of 0.7 mM-Triton X-114 in the aqueous phase (Pryde, 1986). However, the application of hydrophobic resins, e.g. phenyl-Sepharose, to remove residual Triton X-114 in the aqueous phase has recently supported further the apical distribution of G-PI proteins cultured in epithelial cells (Lisanti *et al.*, 1990). In the lateral plasma membranes, although no major biotin-labelled polypeptides were identified as being exclusively G-PI-anchored on the basis of their complete release, there was an increase in the intensity of

labelling of at least four polypeptides after PI-PLC treatment. Although the reasons underlying the results with this fraction containing membrane sheets as well as vesicles were not clear, one explanation is that certain polypeptides may be anchored in the membrane by a short transmembrane amino acid sequence as well as by a G-PI anchor (Dustin *et al.*, 1987; Ferguson & Williams, 1988). The adoption of methods that circumvent these methodological drawbacks may lead to the identification of a wider range of G-PI-anchored proteins on epithelial-cell surfaces and provide more comprehensive information on the fidelity of transfer of proteins to the apical-basolateral surface domains of epithelial cells.

Finally, the present work identifies a more direct route than hitherto described for transferring from the Golgi to the bile-canalicular plasma membrane a class of glycoproteins identified on the basis of direct covalent labelling and immunological evidence. Previous studies (Evans, 1981; Bartles *et al.*, 1987) have shown that, in liver tissue, many newly synthesized glycoproteins take an indirect route to the bile-canalicular plasma membrane involving a detour to the sinusoidal plasma membrane. The present results indicate that the route followed by two G-PI-anchored polypeptides, presumably from the Golgi, joins at the 'late' endocytic compartment with the major indirect route to the bile-canalicular plasma membrane. The antibodies generated to bile-canalicular non-G-PI-anchored proteins localized to both the sinusoidal and canalicular plasma membranes. The transcellular stage of this indirect route to the bile-canalicular domain of the plasma membrane is believed to involve endocytic vesicles. The implications of these results are shown in Scheme 1, indicating that 'late' endocytic vesicles are mainly involved in the direct targeting of specific G-PI-anchored proteins to the bile-canalicular plasma membrane. The results provides a further distinction, besides enzymic (Evans & Flint, 1985; Shears *et al.*, 1988), between the 'early' and 'late' hepatic endosomes. Furthermore, the present results show that 'late', but not 'early', endosomes are involved in the regulation of

aspects of intracellular transport to specific surface domains of hepatocytes and in the linkage of exocytic and endocytic pathways. Antibodies raised to endosomal integral membrane proteins have located a major endocytic compartment to a region surrounding and underlying the bile-canalicular plasma membrane of hepatocytes in liver sections (Enrich & Evans, 1989). Clearly, the antibodies raised to the G-PI-anchored proteins of bile-canalicular plasma membranes can provide the means for studying the kinetics of transfer of this class of membrane protein to the hepatocyte's apical plasma membrane, and in dissecting the role of the subcompartments of endocytic apparatus in generating cell-surface polarity in epithelial cells.

N.A. thanks the Wellcome Trust for a Fellowship. We thank Ms. Rosa Aligue (University of Barcelona, Barcelona, Spain) for the immunocytochemical-localization studies, and Mrs. Lydia Pearson for preparing the manuscript.

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Scheme 1. Routes of insertion of glycoproteins into the bile-canalicular plasma membrane

In route A, glycoproteins traffic indirectly from the Golgi to the bile-canalicular plasma membrane via the sinusoidal plasma membrane. Routes B are more direct routes taken by six G-PI-anchored glycoproteins to the plasma membrane; of these, two bile-canalicular glycoproteins were identified in 'late' endosomes.

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Received 12 January 1990/24 April 1990; accepted 1 May 1990