Biochem. J. (1989) 257, 759–768 (Printed in Great Britain)

Cellular location of the cleavage event of the polymeric immunoglobulin receptor and fate of its anchoring domain in the rat hepatocyte

Roberto SOLARI,*‡ Esther SCHAERER,* Corinne TALLICHET,* Lelita T. BRAITERMAN,† Ann L. HUBBARD† and Jean-Pierre KRAEHENBUHL*

*Swiss Institute for Experimental Cancer Research and the Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland, and †Department of Cell Biology, Johns Hopkins University, Baltimore, MD 21205, U.S.A.

Transcytosis of polymeric immunoglobulin (pIg) across glandular and mucosal epithelia is mediated by a member of the immunoglobulin supergene family, the pIg receptor. During transcellular routing, the receptor is cleaved and its ectoplasmic domain, known as secretory component (SC), is released into secretions bound to pIg. Using receptor-domain-specific antibodies, we have combined cell fractionation and immunoblotting of rat liver to examine the cellular routing of the receptor, the cellular location of the cleavage event and the fate of the anchor domain. Cleavage is a late event in receptor processing. It appears to occur at the canalicular plasma membrane, since intact receptor is present in this membrane domain and no SC is detected in whole liver homogenate or in cell fractions. The membrane anchor remaining after cleavage can be recovered in bile, as well as in a low-density fraction obtained after equilibrium centrifugation of liver (microsomal fractions) on sucrose density gradients. These data suggest that the membrane-anchor domain may be internalized as well as secreted together with SC into bile.

INTRODUCTION

The polymeric immunoglobulin (pIg) receptor [1], which mediates transport of polymeric IgA antibodies across mucosal or glandular epithelia [2], is synthesized as a transmembrane glycoprotein precursor [3,4]. The receptor is cleaved during transcytosis with the concomitant release of the ectoplasmic domain [5,6] tightly bound to the pIg antibodies into secretions. Examination of pIg-receptor biosynthesis in the rabbit mammary gland [6] and rat liver [7–9] has established a working hypothesis for receptor routing in the cell which has largely been borne out by more recent studies *in vitro* in which the pIg receptor cDNA was expressed in fibroblasts [10] or dog kidney epithelial (MDCK) cells [11,12]. These observations demonstrate that the receptor is synthesized as a core-glycosylated precursor on the rough endoplasmic reticulum (rER) and further processed in the Golgi complex. It is assumed, although it has not been proven, that the receptor is sorted and directed first to the basolateral membrane [11], which corresponds to the sinusoidal membrane in the hepatocyte. The receptor is subsequently endocytosed [13] together with other membrane receptors [14]. At some stage in the endocytic pathway, the pIg receptor is directed to the apical plasma membrane, the canalicular membrane in the hepatocyte [15]. Consequently the receptor must be sorted from other co-endocytosed receptors, which are either recycled back to the basolateral plasma membrane or degraded in lysosomes [13,14]. The final stage in the pIg receptor's transcellular journey is the cleavage of its membrane-anchoring domain and the secretion from the apical pole of the cell of the ectodomain (SC), either free or bound to polymeric IgA antibodies [16].

In the present study we made use of antibodies directed against either the membrane-anchoring or the ectoplasmic domains of the pIg receptor [17] in order to investigate the cellular location of the cleavage event and to determine the fate of the membrane-anchoring domain after cleavage. Using cell fractionation and immunoblotting procedures, we have determined in the rat liver that cleavage is a late event in receptor processing and most likely occurs at the canalicular membrane. Upon cleavage both the ectoplasmic and the membrane-anchoring domains are released into bile. The anchoring domain is also recovered in a fraction enriched in multivesicular bodies, suggesting that a portion of the receptor's anchor is internalized and degraded in the lysosomal system.

MATERIALS AND METHODS

Reagents

$Na^{125}I$ (carrier-free) and protein A–horseradish peroxidase were obtained from Amersham International (Amersham, Bucks., U.K.); leupeptin, pepstatin A, antipain and 4-chloro-1-naphthol were from Sigma (St. Louis, MO, U.S.A.); phenylmethanesulphonyl fluoride (PMSF) and acrylamide were from Serva (D-6900 Heidelberg, Germany); SDS, Triton X-100, deoxycholate

Abbreviations used: pIg, polymeric immunoglobulin; SC, secretory component; (r)ER, (rough) endoplasmic reticulum; PMSF, phenylmethanesulphonyl fluoride; Gal-trans, galactosyltransferase; G-6-P, glucose-6-phosphatase; β-Gal, β-galactosidase; β-NAG, β-*N*-acetylglucosaminidase; APDE, alkaline phosphodiesterase; 5′-NUC, 5′-nucleotidase; BSA, bovine serum albumin.

‡ Present address and address for correspondence and reprint requests: Glaxo Group Research Ltd., Greenford Road, Greenford, Middx. UB6 OHE, U.K.

and Nonidet P40 were from Fluka (Buchs, Switzerland); soybean trypsin inhibitor was from Worthington (Freehold, NJ, U.S.A.); XAR-5 autoradiography film was from Kodak (Eastman Kodak, Rochester, NY, U.S.A.); rabbit anti-mouse IgG conjugated to horseradish peroxidase was from Dakopatts a/s (DK 2600, Glostrop, Denmark) and nitrocellulose (pore size 0.45 μm) was from Millipore (Bedford, MA, U.S.A.)

Biological materials

Sprague–Dawley or OFA rats of either sex weighing between 150 and 250 g were obtained from IFFA CREDO (L'Arbresle, France).

Tissue fractionation

Separation of microsomal components. OFA rats, which had been previously fasted for at least 16 h, were killed by cervical dislocation, the livers surgically removed and immediately perfused with ice-cold 0.15 M-NaCl/10 mM-phosphate buffer, pH 7.4. The livers were subsequently fractionated by a modification of the method of Dunn & Hubbard [18]. Livers were weighed and minced in 5 vol. of ice-cold 0.25 M-sucrose in 3 mM-imidazole buffer, pH 7.4, containing 1 mM-PMSF, pepstatin A (2 μg/ml), leupeptin and antipain, as well as 1 mM-soybean trypsin inhibitor (SI buffer). The tissue was homogenized in a Potter-type homogenizer (Bellco) by seven up-and-down strokes, and the homogenate was filtered through four layers of gauze. The homogenate was centrifuged at 1000 $g_{av.}$ for 10 min to remove nuclei and cell debris. The supernatant was centrifuged 10 min at 12000 $g_{av.}$ and the resulting pellet (12 K pellet) was saved for further analysis. The supernatant was centrifuged at 145000 $g_{av.}$ for 90 min (70 Ti rotor), resulting in a microsomal pellet and a supernatant equivalent to a cytosolic fraction. The microsomal pellet was resuspended in SI buffer by three strokes of a loose-fitting Dounce homogenizer, and 4 ml (65–85 mg of protein) was layered on to a 32 ml linear 1.11–1.25 g/ml sucrose gradient in 3 mM-imidazole buffer, pH 7.4. After centrifugation for 16 h at 83000 $g_{av.}$ (SW 27 rotor, Beckman Instruments), 18 2-ml fractions were collected from the bottom of the gradient.

Enzyme assays were performed on each liver fraction and each fraction from the sucrose density gradient to establish the distribution of various organelles, as previously described [18,19]. The microsomal pellet contained approx. 19% of the total protein, 35% of the Golgi marker galactosyltransferase (Gal-trans) activity, 38% of the ER marker glucose-6-phosphatase (G-6-P) activity, 16% and 18% respectively of the lysosomal marker enzymes β-galactosidase (β-Gal) and β-*N*-acetylglucosaminidase (β-NAG) and 23 and 31% respectively of the plasma-membrane marker enzymes alkaline phosphodiesterase (APDE) and 5′-nucleotidase (5′-NUC).

Separation of plasma-membrane domains. Hepatocyte plasma-membrane sheets were prepared, vesiculated by sonication and resolved in 1.06–1.17 g/ml sucrose density gradients as previously described [20,21].

Analytical procedures

Iodination. Proteins were radioiodinated with $Na^{125}I$ by the chloramine-T method [22].

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed as described by Maizel [23] with a 75:2 weight ratio of acrylamide to bisacrylamide. Vertical slab gels (1.5 mm thick) with 5–13% (w/v) polyacrylamide gradients were used for analytical purposes. Samples were concentrated for electrophoresis by precipitation in 10% (w/v) trichloroacetic acid in the presence of 0.125% sodium deoxycholate. The resulting pellet was extracted in 90% (v/v) acetone/0.1 M-HCl at −20 °C. The final precipitate was dissolved in electrophoresis sample buffer containing 1% (v/v) 2-mercaptoethanol.

Two-dimensional peptide-map analysis. The intact pIg receptor and the 34 kDa protein [band 2 in Fig. 3 (below), lane 1] were excised from stained polyacrylamide gels and radioiodinated. The proteins were hydrolysed for 24 h at 37 °C with chymotrypsin (50 $\mu g \cdot ml^{-1}$) in 50 mM-NH_4HCO_3, pH 8.0, containing BSA (2.5 $mg \cdot ml^{-1}$) as a carrier. The peptides generated were separated on cellulose plates by electrophoresis in the first dimension and chromatography in the second and analysed by autoradiography as described [24].

Antibodies. The monoclonal antibody directed against the cytoplasmic tail (anti-tail antibody) was prepared as described [17]. The polyclonal antibody directed against the ectoplasmic domain (SC) of the receptor (anti-SC antibody) was raised in rabbits by multiple-site intradermal injections of purified pIg receptor in complete (first injection) and incomplete (second injection) Freund's adjuvant. The polyclonal antibody against rat serum albumin was raised in rabbits by an identical protocol. The monoclonal antibody designated HA4 was obtained and iodinated as described in [20]. The polyclonal antibody against the asialoglycoprotein receptor was raised in rabbits as described [25].

Immunoblots. Proteins separated on polyacrylamide gels were transferred to nitrocellulose by the method of Towbin *et al.* [26]. All immunolabelling procedures were performed in the Blotto incubation cocktail [27]. The pIg receptor was detected with the monoclonal antibody directed against the cytoplasmic tail of the pIg receptor [17] as a first stage, and with a rabbit and anti-mouse IgG coupled to horseradish peroxidase as a second stage. The receptor was also detected with a rabbit polyclonal antibody directed against the ectodomain of the pIg receptor which recognizes both the receptor and SC, followed by Protein A coupled to horseradish peroxidase. Rat serum albumin was detected by incubation with a rabbit polyconal antibody followed by Protein A–horseradish peroxidase or ^{125}I-labelled Protein A. The asialoglycoprotein receptor was detected with a rabbit polyclonal antibody followed by ^{125}I-Protein A, whereas HA4 was detected with a directly radioiodinated anti-HA4 monoclonal antibody.

Pulse–chase experiments. Rat livers were pulsed with 500 μCi of [^{35}S]cysteine by injection into the portal vein. At 15 min intervals, bile samples were collected through a canula placed in the bile duct.

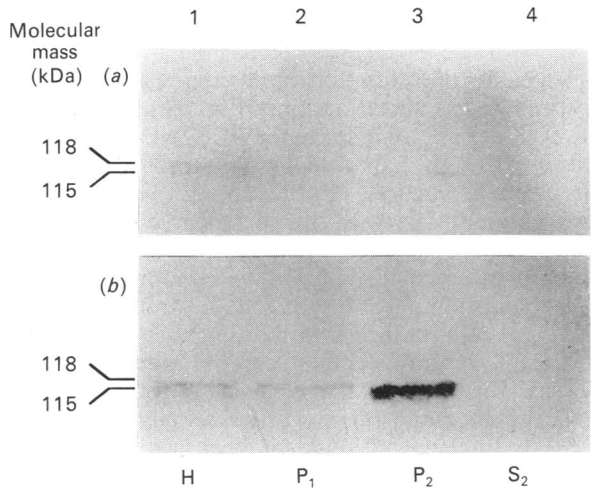

Fig. 1. Recovery and enrichment of pIg receptor in rat liver fractions

(*a*) The 12000 *g* (12 K) nuclear/lysosomal/mitochondrial pellet (P_1), the 100000 *g* (100 K) microsomal pellet (P_2) and the cytosolic supernatant (S_2) were adjusted to the original volume of the homogenate (H) and equal volumes were loaded on an SDS/5–13%-(w/v)-polyacrylamide gel, transferred on to nitrocellulose and immunoblotted with the anti-tail antibody. (*b*) Equal amounts of proteins from the homogenate (H), the 12 K pellet (P_1), the 100 K microsomal pellet (P_2) and the cytosolic supernatant (S_2) were loaded on an SDS/polyacrylamide gel and processed as in (*a*). Two polypeptides with molecular masses of 118 and 115 kDa were detected in the homogenate (lane 1). The 118 kDa form was recovered in the P_1 pellet (lane 2), and the 115 kDa form was enriched 6-fold in the P_2 pellet (lane 3); no receptor was found in the cytosolic fraction (lane 4).

RESULTS

Subcellular distribution of the pIg receptor, SC and the membrane-anchoring domain

The initial aim was to determine the intracellular pools of the pIg receptor in rat liver using antibodies specific for different domains of the receptor. To this end, a liver homogenate was separated into 12000 *g* pellet (12 K) and 100000 *g* microsomal and cytosolic fractions. The recovery and enrichment of pIg receptor in each fraction was determined by immunoblotting experiments using the monoclonal antibody directed against the cytoplasmic tail. Two bands of equal intensity with molecular masses of 118 and 115 kDa were detected in the homogenate (Fig. 1, lane 1). The 118 kDa polypeptide was almost entirely recovered from the homogenate in the 12 K pellet (Fig. 1*a*, lane 2), a fraction significantly enriched in plasma-membrane-enzyme activity (results not shown). The 115 kDa polypeptide was recovered from the homogenate in the microsomal fraction with a 6-fold enrichment as determined by densitometry (Fig. 1, lane 3). There was no receptor present in the cytosolic fraction (Fig. 1, lane 4). The crude microsomal pellet was subsequently separated by centrifugation on a linear sucrose density gradient. The distribution of selected marker-enzyme activities in the fractions recovered from linear sucrose density gradients is presented in Fig. 2(*d*). This method clearly does not result in the purification of intracellular organelles, but rather resolves them into characteristic profiles against which the distribution of the pIg receptor can be compared. Such comparisons can be used to surmise the intracellular location of the receptor with some degree of confidence. There are four broad zones in the gradient which are of particular interest. The lysosomal-marker-enzyme activity shows a bimodal distribution, with a main peak between 1.18 and 1.22 g/ml (fractions 11–15: zone IV) and a smaller peak between 1.08 and 1.13 g/ml (fractions 2 and 3, zone I). The Golgi elements, represented by the *trans* marker Gal-trans, equilibrate at a density between 1.11 and 1.14 g/ml (fractions 4 and 5, zone II), whereas the rER and plasma-membrane co-equilibrate between 1.15 and 1.21 g/ml (fractions 7–12, zone III). In such a fractionation scheme, endosomes, as defined by a 10–20 min pulse at 37 °C with ^{125}I-labelled epidermal growth factor, equilibrate at a density of 1.08–1.13 g/ml, which is slightly less than that of the Golgi [18]. Consequently the Golgi fraction would clearly be contaminated with such endosomes.

When the distribution of the pIg receptor was determined on such a gradient using immunoblot analysis with the anti-tail antibody, the labelling pattern revealed that the core-glycosylated endoglycosidase H-sensitive form of the receptor (M_r 105000) is concentrated in zone III corresponding to the peak of rER-marker-enzyme activity (fractions 4–16). The terminally glycosylated forms (M_r 115000 and 118000) are associated with the peak of Golgi-marker-enzyme activity (fractions 4–5) (Fig. 2*a*). These observations were confirmed by biosynthetic labelling experiments and endoglycosidase H-sensitivity studies [28]. The most significant pool of the receptor is in the Golgi/endosome fractions (zone II), whereas there is relatively little of the terminally glycosylated receptor coincident with the main plasma-membrane peak in zone III (fractions 8–12).

In addition to the core- and terminally glycosylated forms of the receptor, the anti-tail antibody also reveals a doublet of polypeptides with M_r values of 34000 and 32000, which correspond to the expected M_r of the membrane-anchoring domain of the receptor generated after cleavage. Binding of anti-tail antibody to the 34–32 kDa doublet is competed for by addition of purified pIg receptor (Fig. 3), but not by purified SC (results not shown), thus confirming its identity as the membrane anchor of the receptor. The identity of the 34 kDa polypeptide as the membrane-anchor domain of the pIg receptor was further established by two-dimensional peptide-map analysis (Fig. 4). The proteins from fraction 3 of the microsomal sucrose gradient were loaded on a SDS/polyacrylamide slab gel, electrophoresed, and stained with Coomassie Blue. The bands corresponding to the M_r of the receptor or its membrane-anchor domain were cut out of the gel (Fig. 3, lane 1), re-electrophoresed and immunoblotted to identify them as the intact receptor or the membrane anchor. Those bands which were positive on immunoblots were radioiodinated, subjected to partial proteolysis and the resulting peptides analysed by two-dimensional gel electrophoresis. The corresponding peptide maps indicate that the major peptides generated by proteolysis of the 34 kDa protein are also present upon proteolysis of the intact receptor (Fig. 4).

Immunoblotting of the microsomal sucrose gradient was repeated using the anti-SC antibody. The antibody recognizes both the intact receptor and its secreted form

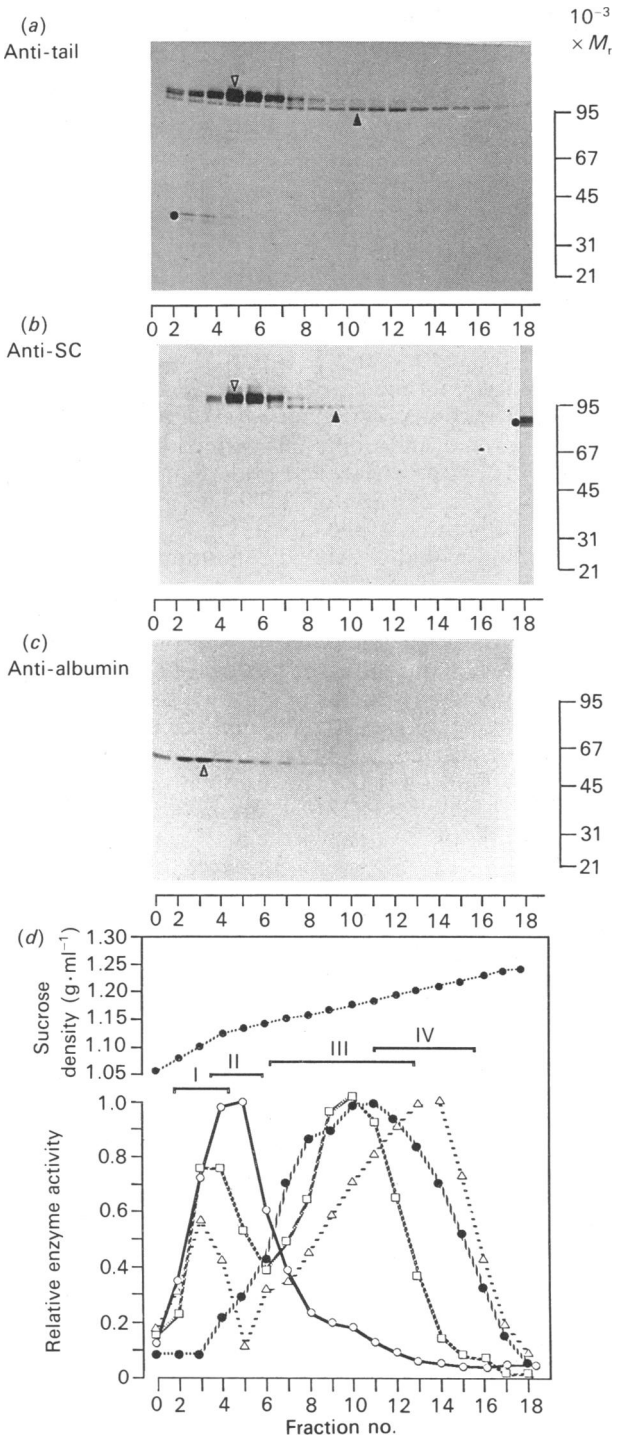

Fig. 2. Localization of the pIg receptor and serum albumin rat liver microsomes separated on sucrose density gradients

A portion (50 μg) of proteins of fractions 1–18 from sucrose gradients were electrophoresed on a 5–13 %-(w/v)-polyacrylamide gel under reducing conditions and transferred on to nitrocellulose. (*a*) Immunoblotting with the anti-tail monoclonal antibody followed by horseradish peroxidase-conjugated rabbit anti-mouse Ig. The antibody reveals the core-glycosylated receptor (M_r 105000) (▲) co-equilibrating in the gradient with G-6-P activity, and the terminally glycosylated receptor (△) (M_r 115000–118000) co-equilibrating in the gradient with the Gal-trans activity. This method reveals relatively little mature receptor co-equilibrating with plasma-membrane markers in fractions 9–11. The antibody recognizes the membrane-anchoring domain of the receptor, which has an M_r of 34000–32000 and which equilibrates in fractions 2 and 3 (●). (*b*) Immunoblotting with the anti-SC antibody followed by horseradish peroxidase-conjugated Protein A. The antibody reveals the core-glycosylated receptor (M_r 105000) (▲) co-equilibrating in the gradient with the G-6-P activity, and the terminally glycosylated receptor (M_r 115000–118000) (▽) co-equilibrating in the gradient with the Gal-trans activity. This antibody reveals relatively little mature receptor co-equilibrating with plasma-membrane markers in fractions 9–11. In addition, the antibody does not reveal the presence of SC in any microsomal compartment. A sample of rat bile is included in lane 18 to show the M_r of SC, the cleaved form of the receptor (●). (*c*) Immunoblotting with rabbit anti-rat albumin serum followed by horseradish peroxidase-conjugated Protein A. This antiserum reveals the highest concentration of albumin to be in fractions 2 and 3 (△). (*d*) Distribution of marker-enzyme activities after equilibration of rat liver microsomes on linear 1.11–1.25 g/ml sucrose density gradients. The top panel indicates sucrose density (●). The relative enzyme activities have been calculated assuming the maximal activity to equal unity: □, APDE I (plasma membrane); △, β-NAG (lysosomes); ○, Gal-trans (Golgi); ● G-6-P (rER).

(SC) found in bile. As expected, the antibody reveals the large pool of terminally glycosylated receptor in the Golgi/endosome fraction, and the core-glycosylated receptor in the rER fractions (Fig. 2*b*). The antibody, however, does not recognize the 34–32 kDa polypeptides, and interestingly fails to detect SC in any region of the microsomal fraction. For comparative purposes, a sample of rat bile was run in place of fraction 18 to demonstrate both the M_r of SC and the fact that the antibody can recognize SC in bile. By immunoblotting of the whole homogenate, the 12 K and the cytosolic fractions with anti-SC antibody, we were unable to detect SC in any cellular compartment (results not shown). This suggests that cleavage of the receptor in an intracellular compartment with subsequent accumulation of SC is unlikely, although it does not rule out rapid exocytosis after intracellular cleavage. Failure to detect SC in liver homogenates, even though the concentration of SC in bile is high, is probably due to the negligible contribution of canalicular fluid to liver volume [29].

The subcellular distribution of albumin, a major secretory product of the hepatocyte, was compared with that of the pIg receptor, on microsomal sucrose gradient fractions (Fig. 2*c*). The results demonstrate that serum albumin is most abundant in vesicles, equilibrating at a density of 1.07–1.10 g/ml (fractions 2 and 3). On morphological grounds [19], the most abundant components of this fraction appear to be lipoprotein-loaded secretory vesicles with a diameter between 200 and 300 nm, in addition to small vesicles with a diameter of approx. 100 nm and multivesicular bodies which have been shown to equilibrate at similar low densities [30]. Consequently, there is coincidence between the biochemical localization of the intracellular albumin pool and the characteristic secretory-vesicle morphology of fraction 3.

The pIg receptor and the membrane-anchoring domain are associated with distinct vesicular carriers

We next determined whether the 34–32 kDa membrane anchor and the intact receptor are associated with the

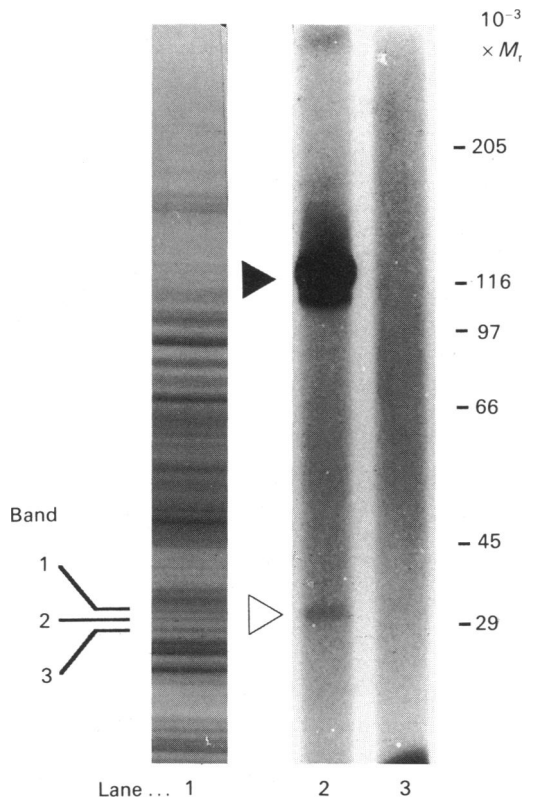

Fig. 3. Competition by intact pIg receptor for binding of the anti-tail monoclonal antibody to the 34 kDa protein

Samples of fraction 3 taken from a liver microsomal pellet equilibrated on 1.11–1.25 g/ml sucrose gradients were electrophoresed on SDS/5–13 %-polyacrylamide gels stained with Coomassie Blue (lane 1) or transferred to nitrocellulose (lanes 2 and 3). The nitrocellulose strips were incubated with ^{125}I-labelled anti-tail antibody (lane 2) or ^{125}I-labelled anti-tail antibody following a preincubation with 30 μg of purified pIg receptor (lane 3). The blots were exposed to preflashed Kodak XAR film at −70 °C with an intensifying screen. The antibody recognizes both the pIg receptor (▶) and the 34 kDa protein (▷) present in this fraction (lane 2), and binding to both proteins is blocked by the addition of pIg receptor (lane 3). Three discrete bands (1, 2 and 3) on the Coomassie Blue staining pattern correspond to the M_r of the membrane anchor. Each band was excised from the gel and re-electrophoresed, transferred to nitrocellulose and immunoblotted. Only band 2 reacts with the anti-tail antibody.

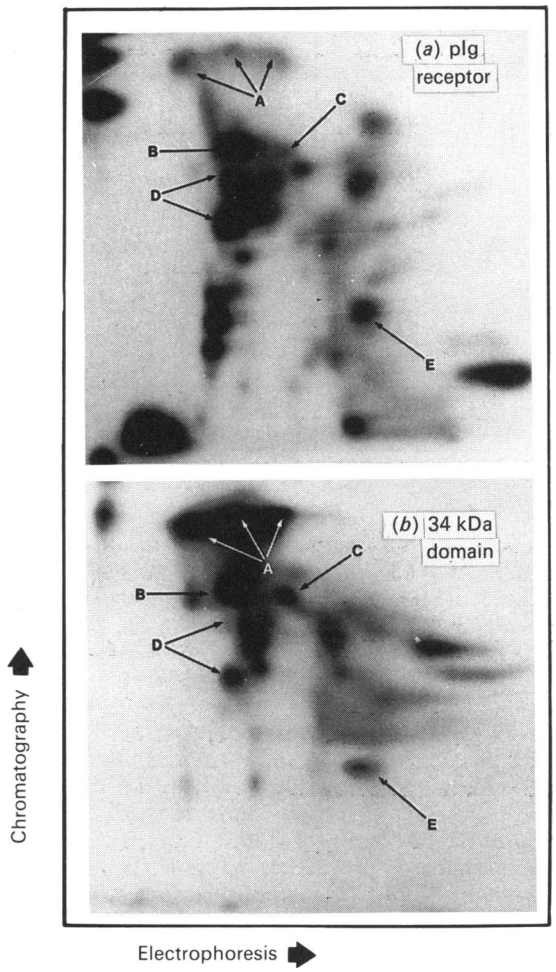

Fig. 4. Two-dimensional peptide map of the pIg receptor (*a*) and the 34 kDa membrane-anchoring domain (*b*)

The band 2 (Fig. 3, lane 1) from fraction 3 of a microsomal pellet and the band corresponding to the intact receptor were excised from the gel. The proteins were radioiodinated and hydrolysed for 24 h at 37 °C with chymotrypsin (50 μg·ml^{-1}). The peptides generated were analysed on cellulose sheets by electrophoresis in the first dimension and chromatography in the second. The peptides were subsequently revealed by autoradiography. The pattern of peptides produced on the proteolysis of the 34 kDa protein (band 2) overlaps with the peptide map of the pIg receptor (peptides A–E).

same or different vesicular carriers in the light fraction (fraction 3 in Fig. 2). Fraction 3 was collected and centrifuged for 90 min at 100000 $g_{av.}$ in the presence or absence of 0.1 % Triton X-100. Equal amounts of protein from the pellets and supernatants were separated by SDS/PAGE and analysed by immunoblotting using either the anti-albumin or the anti-tail antibody (Fig. 5). In the absence of detergent, the albumin is most concentrated in the pellet, whereas in the presence of Triton X-100 it is completely non-sedimentable. The intact pIg receptor is almost completely sedimented in the absence of detergent, whereas the 34 kDa protein remains exclusively in the supernatant. Addition of 0.1 % Triton X-100 to fraction 3 before re-centrifugation results in the recovery of both the receptor and the 34 kDa polypeptide in the supernatant. These findings, summarized in Table 1, suggest that the 34 kDa protein is not associated with the same membranes as the intact receptor. To test whether the 34 kDa membrane anchor is membrane-associated, the supernatant from the re-centrifugation of the fraction 3 was centrifuged at 300000 $g_{av.}$ for 1 h, and the distribution of the 34 kDa protein and rat albumin was analysed in the 300 K (300000 g) pellet and supernatant. The 32–34 kDa protein was consistently and exclusively detected in the pellet, indicating that it is membrane-associated (results not shown). Analysis of bile by Western blotting with the anti-tail antibody reveals a 32–30 kDa doublet [Fig. 7*b* below, lane 4] that is not sedimentable at 300000 $g_{av.}$ for 1 h. The reason for the membrane-anchoring domain appearing as a 32–34 kDa doublet in the microsomal light fraction 3, and as a 32–30 kDa doublet in bile, is

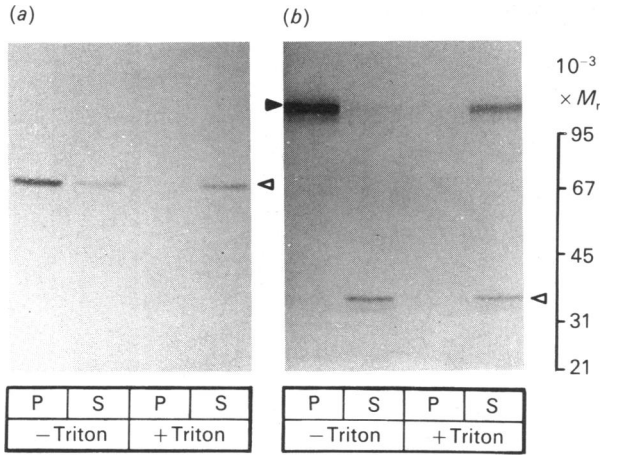

Fig. 5. Sedimentability of the pIg receptor and albumin in fraction 3

Fraction 3, collected from rat liver microsomes separated on 1.11–1.25 g/ml sucrose density gradients, was centrifuged at 100000 $g_{av.}$ for 90 min in the presence or absence of 0.1% Triton X-100. Samples (75 μg) of the pellets (P_1) and supernatants (S_1) were concentrated by deoxycholate/trichloroacetic acid precipitation and electrophoresed on SDS/5–13%-polyacrylamide gels. The proteins were transferred to nitrocellulose and immunoblotted either with the rabbit anti-rat albumin serum (*a*) or the anti-tail monoclonal antibody (*b*). In the absence of detergent, the albumin is most concentrated in the pellet, whereas in the presence of Triton X-100 it is completely non-sedimentable (◁). The pIg receptor (▶) is almost totally sedimented in the absence of detergent and non-sedimentable in its presence. However, the 34 kDa membrane-anchoring domain (◁) is non-sedimentable even in the absence of detergent (*b*).

unclear. Indeed, the intensity of the bands varies from preparation to preparation, suggesting that these may represent a series of proteolytic fragments.

The intact pIg receptor is associated with both the sinusoidal and the canalicular plasma membranes

In order to effect the vectorial transport of polymeric Ig from the circulation to the bile, the pIg receptor must shuttle from the sinusoidal plasma membrane to the canalicular plasma membrane. Release of the ligand into the bile requires cleavage of the receptor's membrane-anchoring domain. If cleavage occurs before arrival at the canalicular plasma membrane, one would not expect to find intact receptor in this membrane domain. Conversely, if cleavage occurs after delivery of the receptor to the canalicular plasma membrane, then one would predict that intact receptor could be detectable in this domain.

To test these hypotheses, we separated plasma-membrane sheets into fractions enriched in either canalicular or sinusoidal marker proteins by using well-established techniques [20,21]. Plasma-membrane sheets were vesiculated by sonication and subsequently the two membrane domains were separated by ultracentrifugation on linear sucrose density gradients. The gradients were fractionated and analysed by immunoblotting using the anti-tail antibody, as well as antibodies to the asialoglycoprotein receptor and HA4, which have respectively been shown to label exclusively the sinusoidal

Table 1. Sedimentability of the pIg receptor, the membrane anchor and selected marker-enzyme activities in the light microsomal fraction 3

Fraction 3 collected from rat liver microsomes separated on a continuous sucrose density gradient was centrifuged at 100000 $g_{av.}$ for 90 min in the presence or absence of 0.1% Triton X-100. The distribution of protein, serum albumin and marker-enzyme activities in the pellets represent the means determined from two separate experiments. Serum albumin was quantified by dot-blot analysis using rabbit anti-albumin antibodies followed by ^{125}I-protein A, or by scanning the immunoblots (Fig. 5*a*). The pIg receptor and the 34–32 kDa anchor domain were quantified by scanning immunoblots (Fig. 5*b*).

	Sedimentability (%)	
	−Triton	+Triton
Total protein	21	4
Serum albumin	85	2
β-Gal	53	0.5
APDE	89	1.5
5′-NUC	74	3
pIg receptor	95	< 0.5
32 kDa membrane anchor	0.5	< 0.1

or canalicular plasma-membrane domains [20,31]. The main organellar contaminant of these fractions is the rER. From our biosynthetic studies [28], however, we known that in the rER the pIg receptor is present as the 105 kDa core-glycosylated form and consequently is easily distinguishable from the mature 115/118 kDa doublet present in the plasma membrane. The distribution of the pIg receptor does not parallel either the distribution of HA4 or of the asialoglycoprotein receptor, but is overlapping, thus indicating that the pIg receptor is present in both membrane domains (Fig. 6).

Both the ectoplasmic and anchoring domains of the pIg receptor are recovered in bile

As shown by pulse–chase experiments, biosynthetically labelled SC, which is resolved as an 84 kDa band, first appears in bile 30–45 min after the intravenous injection of [^{35}S]cysteine, and peak levels are reached between 90 and 105 min (Fig. 7*a*). Analysis of whole bile samples by SDS/polyacrylamide-gel electrophoresis and fluorography reveals that SC is the major protein to be synthesized and secreted by the liver into bile over this time course. Also seen in Fig. 7(*a*) is the presence of a 32/30 kDa doublet, which appears in bile slightly later than SC. We were unable to immunoprecipitate satisfactorily the biosynthetically labelled 84 kDa and the 30 kDa proteins with the domain-specific antibodies, probably because of the high content of bile salts. In order to identify the 84 kDa protein as SC and the 32/30 kDa doublet as the receptor's anchor, bile samples were resolved by SDS/polyacrylamide-gel electrophoresis, the proteins were transferred on to nitrocellulose, and the sheets incubated with anti-SC or anti-tail antibodies. As shown in Fig. 7(*b*), the anti-SC antibody reacts with the major 84 kDa protein, which is not recognized by the anti-tail antibody. Conversely, the anti-tail antibody recognizes the 32/30 kDa doublet, but fails to interact with the 84 kDa protein. The M_r of the putative receptor's

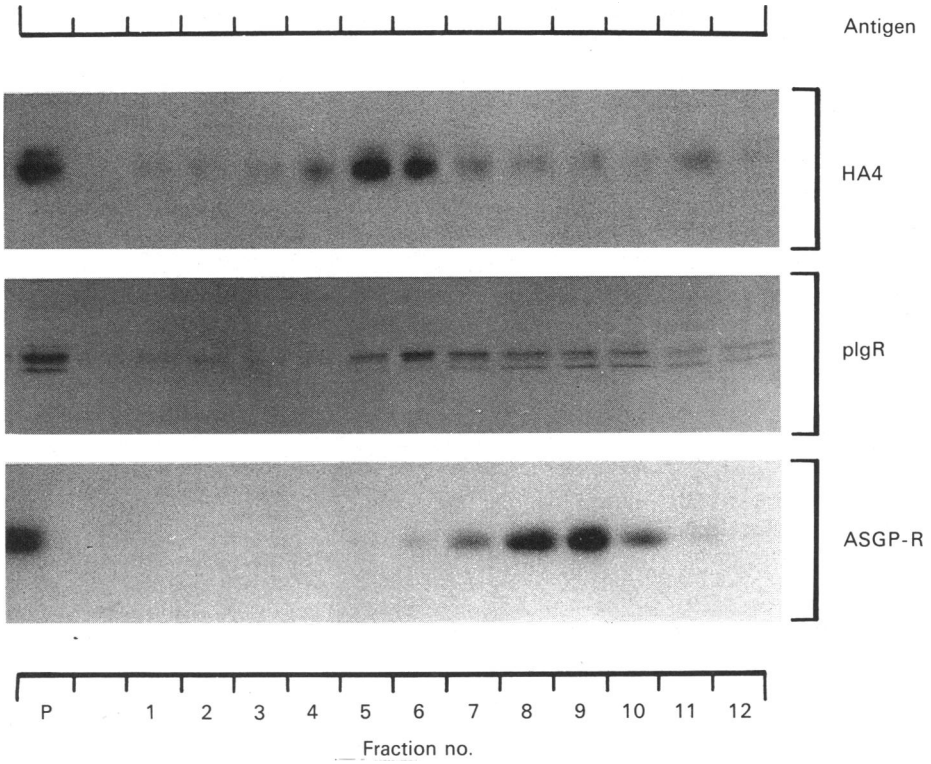

Fig. 6. Detection of the pIg-receptor (pIgR) in sinusoidal- and canalicular-plasma-membrane domains

Hepatocyte plasma-membrane sheets were prepared and vesiculated by sonication. The sinusoidal and canalicular domains were separated by ultracentrifugation on linear sucrose density gradients. Samples of each fraction from the gradient and an aliquot of the total plasma-membrane preparation (P) were concentrated by deoxycholate/trichloroacetic acid precipitation and electrophoresed on SDS/5–13%-polyacrylamide gels. The proteins were transferred to nitrocellulose and immunoblotted either with ^{125}I-labelled monoclonal anti-HA4 or with rabbit anti-asialoglycoprotein receptor (ASGP-R) followed by ^{125}I-Protein A. The distribution of the antigens was revealed by autoradiography of the nitrocellulose. The pIg receptor was detected using the anti-tail antibody followed by a rabbit anti-mouse IgG–horseradish peroxidase conjugate. Equilibration of the canalicular plasma membrane shows a peak in fractions 5–6 as revealed by the presence of the HA4 antigen, whereas the sinusoidal plasma membrane as revealed by the asialoglycoprotein receptor equilibrates at a higher density, peaking in fractions 8–9. Distribution of the pIg receptor is somewhat intermediate between the distribution of HA4 and the ASGP–receptor, suggesting that the pIg receptor is present in both canalicular- and sinusoidal-plasma-membrane domains.

anchor in bile is slightly lower than that recovered in the microsomal light fraction 3 (Figs. 2*a* and Fig 7*b*, lane 6), suggesting that further processing occurs upon cleavage.

DISCUSSION

Studies on the biosynthesis of the pIg receptor in a variety of cell types have demonstrated the kinetics of receptor synthesis, its subsequent processing in the Golgi complex and finally the arrival of SC, the ectodomain of the receptor, in secretions after receptor cleavage [5–9, 32].

In the present investigation we have used a combination of cell fractionation coupled to immunodetection techniques with antibodies directed against different domains of the receptor to determine the subcellular location of the receptor cleavage event and the fate of the membrane-anchoring domain in the rat hepatocyte. The experimental procedures are based upon a liver fractionation protocol adapted from previously published methods [18]. We have chosen to separate the components of a microsomal fraction on sucrose gradients to examine the trends in pIg-receptor distribution, rather than attempt quantification in enriched fractions of subcellular organelles. Even though the yield of each organelle in the original microsomal fraction is not identical, the only components significantly under-represented in the sucrose gradients are the plasma membrane and the lysosomes, which sediment preferentially in the 12 K fraction. The bimodal distribution of lysosomes is similar to previous observations made in cultured human fibroblasts [33] and in the rat liver [34]. Examination of fraction 3 by electron microscopy [19] reveals a complex mixture of vesicular structures comprising lipoprotein-containing secretory vesicles, multivesicular bodies and small vesicles with a diameter of approx. 100 nm. Multivesicular bodies have also been shown to equilibrate at low densities [30].

Using these techniques coupled to biosynthetic labelling and immunoprecipitation, we have demonstrated that the receptor is synthesized as a 105 kDa core-glycosylated protein on the rough endoplasmic reticulum (rER), and that transport to the Golgi, where terminal glycosylation results in the formation of a 115 and 118 kDa doublet, has a half time of approx. 30 min [28]. The vesicular carriers involved in the transport of the receptor from the Golgi to the sinusoidal plasma membrane have yet to be unequivocally identified. It has been suggested that the receptor is present in *trans*-Golgi cisternae, but is absent from the large lipoprotein-loaded

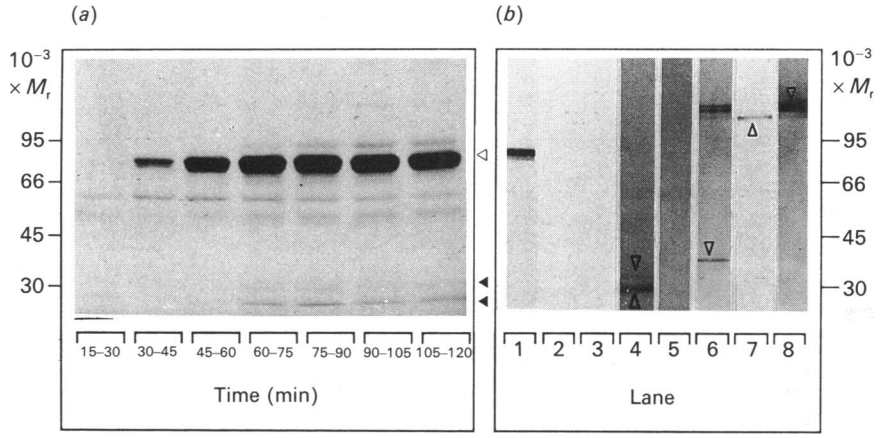

Fig. 7. Secretion of SC and shedding of the pIg receptor's membrane anchor into bile

Rat livers were pulsed as described in the Materials and methods section. Portions (30 ml) of bile collected at 15 min intervals were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions followed by fluorography (*a*). SC, the secreted ectodomain of the receptor, is the major biosynthetically labelled protein to be secreted into the bile (◁). It was identified by immunoblotting of a bile sample with the anti-SC antibody (*b*, lane 1). In the presence of 25 μg of purified pIg receptor (lane 2) or in the presence of 25 μg of purified SC (lane 3), the immunodetection of the 84 kDa protein is abolished. With the anti-tail antibody a 32/30 kDa doublet is detected in bile (lane 4) (▽△). The immunodetection of the 32/30 kDa doublet can be competed by purified receptor (lane 5). The protein detected with the anti-tail antibody co-migrates with the 32/30 kDa doublet, which is secreted into bile from biosynthetically labelled liver (*a*, 60–75 min) (◀). The membrane anchor detected in bile is shorter than the membrane anchor detected in fraction 3 from sucrose density gradient of rat liver microsomes (▽, lane 6). The 32–34 kDa polypeptide is not detected in the rER-enriched fraction 9. In this fraction, only the core-glycosylated receptor is detected (△, lane 7). In the Golgi-enriched fraction 5, only the terminally glycosylated form of the intact receptor can be detected (▽, lane 8).

secretory vesicles derived from these cisternae [9]. These authors [37] propose separate carriers for transporting secretory plasma-membrane proteins and the pIg receptor from the Golgi to the sinusoidal plasma membrane, a mechanism which has been demonstrated for membrane and regulated secretory proteins in pituitary-tumour cells [35]. In the present study we detected large amounts of receptor in a fraction highly enriched in lipoprotein-containing secretory vesicles (fraction 3). This suggests that the receptor is indeed associated with the vesicular carrier transporting albumin and other secretory products from the Golgi complex to the sinusoidal membrane. This is further supported by previous studies in which we have been able to immunoadsorb the lipoprotein-containing secretory vesicles on to *Staphylococcus aureus* coated with the anti-tail monoclonal antibody [17]. It is not known, however, whether membrane and secretory proteins are further sorted into separate vesicles before their arrival at the sinusoidal plasma membrane. The distribution of the receptor on the plasma membrane of the hepatocyte, as determined by immunocytochemistry, is quite distinct from other cells which transport Ig [36]. In the hepatocyte the receptor is not diffusely distributed over the sinusoidal plasma membrane, but is concentrated in pits [13]. The endocytic pathway followed by the receptor–ligand complex involves internalization in coated pits and vesicles [13] and transport across the cell to the canalicular membrane in smooth-surfaced vesicles [13,15,16,37–39]. If cleavage of the membrane-anchoring domain of the receptor occurs during transcytosis from sinusoidal to canalicular plasma membrane, one would expect to find the cleaved form of the receptor (SC) (84 kDa) in the endosomal transport vesicles. Using the anti-SC antibody, we have been unable to detect the SC 84 kDa in any microsomal component or in samples of whole liver homogenate or cytosolic fractions. The abundance of the cleaved form and the complete absence of the intact receptor in bile suggests that cleavage occurs at the canalicular plasma membrane. Recent studies using rat hepatocyte cultures have clearly shown that cleavage of the pIg receptor to SC is a cell-surface event and appears to be mediated by a membrane-bound thiol proteinase [40]. In order to strengthen further the proposal that cleavage occurs at the canalicular plasma membrane, we have separated plasma-membrane sheets into sinusoidal- and canalicular-enriched fractions and examined the distribution of the receptor. In contrast with previous observations [41], we were able to detect the pIg receptor in both the canalicular and sinusoidal domains, so confirming that receptor cleavage does not precede delivery to the canalicular plasma membrane. This is also supported by immunocytochemical studies, which indicated that the receptor is still bound to its ligand on the canalicular plasma membrane [14,36].

What is the fate of the membrane-anchoring domain after cleavage of the receptor at the canalicular plasma membrane and the release of the SC–pIg complex into the bile? Since we have a monoclonal antibody directed against the cytoplasmic tail of the receptor's membrane-anchoring domain, we have been able to demonstrate its presence both in bile and in a microsomal fraction with a density of ~1.09 mg/ml. We believe the 34 and 32 kDa polypeptides are recognized by the antibody to be the membrane anchor of the receptor, because this antibody is a monoclonal known to recognize this domain [17], and the size of these polypeptides corresponds to the difference in size between the intact receptor (M_r 118000; 115000) and its secreted form (M_r 84000). Furthermore, binding of the antibody to the 34–30 kDa polypeptides is

blocked by addition of purified intact receptor, but not by SC. Finally, the peptide map of the 34 kDa protein overlaps with that of the intact receptor. The fact that the 34 kDa membrane anchor which is present in fraction 3 is no longer sedimentable after equilibration on sucrose gradients suggests that it may be associated with the small vesicles of the multivesicular bodies. Disruption of the secretory vesicles and multivesicular bodies after equilibration on the sucrose gradient is reflected by the recovery in the supernatant of about 50% of the β-galactosidase and 80% of the total protein (Table 1). Examination of the non-sedimentable component of fraction 3 by election microscopy reveals the presence of lipoprotein particles and vesicles with diameters in the 100–150 nm range. The fact that the 34–32 kDa protein is sedimentable at 300000 *g* suggests that the receptor's anchor is membrane-associated. Another possibility for the loss of sedimentability of the membrane anchor is that it is present in small vesicles which are abundant in the vicinity of the bile canaliculus [13]. These small vesicles may initially sediment in the microsomal pellet associated with the plasma membrane, but are sheared off or ruptured once equilibrated in sucrose gradients. However, the precise nature of the intracellular location of the 34–32 kDa membrane anchor remains unclear, owing to the heterogeneity of the components which co-equilibrate in fraction 3. Consequently, these techniques do not permit us to define the final routing of the anchoring domain after receptor cleavage. Nevertheless, taken together, these observations would suggest that, after receptor cleavage at the canalicular plasma membrane, the membrane anchor undergoes a series of further modifications which permit its shedding into bile. Whether membrane shedding at the canalicular plasma membrane is facilitated by the presence of bile salts remains to be determined. The reason for difference in size of the receptor anchor in the light fraction and bile is presently not understood. It is conceivable that the receptor undergoes a series of proteolytic cleavages when it moves from the canalicular membrane into bile. The presence of high concentration of bile salts might expose sites which then become available for proteolysis.

In conclusion, we have investigated the intracellular routing of the pIg receptor in the rat hepatocyte and demonstrated the presence of the core-glycosylated form in the rER, the accumulation of the terminally glycosylated form in Golgi-endosome fractions and its association with both the sinusoidal and canalicular plasma membranes. The absence of SC in intracellular organelles suggests that cleavage does not occur intracellularly, but at the canalicular membrane. The presence of a 30 kDa protein corresponding to the receptor membrane anchor in bile suggests that, upon cleavage, the anchor is released into the canalicular lumen, possibly by membrane shedding. One cannot rule out, however, that a portion of the receptor's anchor is internalized by endocytosis, transferred to multivesicular bodies, as reflected by its presence in a fraction enriched in these organelles, and finally degraded in lysosomes.

This work was supported financially by the Swiss National Science Foundation (grant no. 3.398.086). We thank Dr. Jean M. Wilson and Dr. Thomas Kleyman for critically revising this manuscript before its submission.

REFERENCES

1. Mostov, K. E., Friedlander, M. & Blobel, G. (1984) Nature (London) **308**, 37–43
2. Brandtzaeg, P. (1981) Clin. Exp. Immunol. **44**, 221–232
3. Kuhn, L. C. & Kraehenbuhl, J.-P. (1981) J. Biol. Chem. **256**, 12490–12495
4. Mostov, K. E., Kraehenbuhl, J.-P. & Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 7257–7261
5. Mostov, K. E. & Blobel, G. (1982) J. Biol. Chem. **257**, 11816–11821
6. Solari, R. & Kraehenbuhl, J.-P. (1985) Immunology Today **6**, 17–20
7. Sztul, E. S., Howell, K. E. & Palade, G. E. (1983) J. Cell Biol. **97**, 1582–1591
8. Sztul, E. S., Howell, K. E. & Palade, G. E. (1985) J. Cell Biol. **100**, 1248–1254
9. Sztul, E. S., Howell, K. E. & Palade, G. E. (1985) J. Cell Biol. **100**, 1255–1261
10. Deitcher, D. L., Neutra, M. R. & Mostov, K. E. (1986) J. Cell. Biol. **102**, 911–919
11. Mostov, K. E. & Deitcher, D. L. (1986) Cell (Cambridge, Mass.) **46**, 613–621
12. Mostov, K. E., de Bruyn Kops, A. & Deitcher, D. L. (1986) Cell (Cambridge, Mass.) **47**, 359–364
13. Hoppe, C. A., Connolly, T. P. & Hubbard, A. L. (1985) J. Cell Biol. **101**, 2113–2123
14. Geuze, H. J., Slot, J. W., Ger, J. A., Strous, A. M., Peppard, J., Von Figura, K., Hasilik, A. & Schwartz, A. L. (1984) Cell (Cambridge, Mass) **37**, 195–204
15. Mullock, B. M., Hinton, R. H., Dobrota, M., Peppard, J. & Orlans, E. (1979) Biochem. Biophys. Acta **587**, 381–391
16. Mullock, B. M., Jones, R. S. & Hinton, R. H. (1980) FEBS Lett. **113**, 201–205
17. Solari, R., Kuhn, L. & Kraehenbuhl, J.-P. (1985) J. Biol. Chem. **260**, 1141–1145
18. Dunn, W. A. & Hubbard, A. L. (1984) J. Cell. Biol. **98**, 2148–2159
19. Solari, R., Racine, L., Tallichet, C. & Kraehenbuhl, J.-P. (1986) J. Histochem. Cytochem. **34**, 17–23
20. Bartles, J. R., Braitherman, L. T. & Hubbard, A. L. (1985) J. Cell Biol. **100**, 1126–1138
21. Bartles, J. R., Feracci, H. M., Stieger, B. & Hubbard, A. L. (1987) J. Cell Biol. **105**, 1241–1251
22. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) Biochem. J. **89**, 114–123
23. Maizel, J. V. (1971) Methods Virol. **5**, 179–246
24. Speicher, D. W., Morrow, J. S., Knowles, W. J. & Marchesi, V. T. (1982) J. Biol. Chem. **257**, 9093–9101
25. Wall, D. A. & Hubbard, A. L. (1985) J. Cell Biol. **101**, 2104–2112
26. Towbin, A., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **86**, 435–454
27. Johnson, D. A., Gautsch, J. W., Sportsman, J. R. & Elder, J. (1984) Gene Technol. **1**, 3–8
28. Solari, R. & Kraehenbuhl, J.-P. (1987) in The Mammary Gland: Development, Regulation and Function (Neville, M. & Daniel, C., eds.), pp. 269–298, Plenum Press, New York and London
29. Weibel, E. R., Staubli, W. & Gaugi, H. R. (1969) J. Cell Biol. **42**, 68–91
30. Hornick, C. A., Hamilton, R. L., Spaziani, E., Enders, G. H. & Havel, R. J. (1985) J. Cell Biol. **100**, 1558–1569
31. Hubbard, A. L., Bartles, J. R. & Braitherman, L. T. (1985) J. Cell Biol. **100**, 1115–1125
32. Solari, R. & Kraehenbuhl, J.-P. (1984) Cell (Cambridge, Mass.) **36**, 61–71
33. Rome, L. H., Garvin, A. J., Allietta, M. M. & Neufeld, E. F. (1979) Cell (Cambridge, Mass.) **17**, 143–153

34. Pertoft, H., Warmgard, B. & Hook, M. (1978) Biochem. J. **174**, 309–317
35. Gumbiner, B. & Kelley, R. B. (1982) Cell (Cambridge, Mass.) **28**, 51–59
36. Takahashi, I., Nakane, P. K. & Brown, W. R. (1982) J. Immunol. **128**, 1181–1187
37. Birbeck, M. S. C., Cartwright, P., Hall, J. G., Orlans, E. & Peppard, J. (1979) Immunology **37**, 477–484
38. Mullock, B. M., Hinton, R. H., Dobrota, M., Peppard, J. & Orlans, E. (1980) Biochem. J. **190**, 819–826
39. Mullock, B. M., Luzio, J. P. & Hinton, R. H. (1983) Biochem. J. **214**, 823–827
40. Musil, L. S. & Baenziger, J. U. (1987) J. Cell Biol. **104**, 1725–1733
41. Meier, P. J., Sztul, E. S., Rueben, A., Boyer, J. L. (1984) J. Cell Biol. **98**, 991–1000

Received 19 May 1988/21 July 1988; accepted 25 July 1988