Identification and distribution of proteins in isolated endosomal fractions of rat liver: involvement in endocytosis, recycling and transcytosis

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1. The polypeptides of the three endosomal fractions isolated from livers of oestradiol-treated rats were analysed by two-dimensional gel electrophoresis. Silver-stained gels revealed that although the three endosomal fractions shared a generally similar pattern of approx. 120 components, qualitative and quantitative differences between the three endocytic fractions could be demonstrated.

2. The 'early' endosomes [compartment of uncoupling of receptors and ligands (CURL)] comprised the most complex fraction and contained most of the polypeptides found in the 'late' endosomes [multivesicular bodies (MVBs)] and the receptor recycling compartment (RRC). When CURL was analysed by two-dimensional gel electrophoresis after partition with Triton X-114, it showed the largest number of integral membrane polypeptides.

3. Some of the major receptors (polymeric immunoglobulin receptor, transferrin receptor, low-density lipoprotein receptor, asialoglycoprotein receptor, β_1 -integrin, mannose 6-phosphate receptor, epidermal growth factor receptor and AGp110) and internalized ligands (IgA, IgG, albumin, haptoglobin, transferrin and α_2 -macroglobulin) were further studied by Western blotting.

4. The distribution of the identified receptors and ligands among the three endosomal fractions was in agreement with their expected functionalities.

5. The polypeptide composition of the bile was also examined and compared with ligands and proteins identified in the different endocytic fractions.

6. Finally, an electron microscopy study confirms the distinctive physical and ultrastructural features of the three isolated endosomal fractions.

INTRODUCTION

Receptor-mediated endocytosis is a mechanism that has evolved to permit transport into the cell of a widely varied group of compounds, ranging from relatively small hormones to intact virus particles [1–3]. The endocytic compartment is the cellular structure responsible for this complex and regulated function; it consists of a series of vesicles and vesiculotubular structures that sort and transport each internalized molecule towards its final destination. In the hepatocyte the sinusoidal plasma membrane (basolateral membrane) is the specialized region where the receptors that initiate the process are located [4,5].

Recent work suggests that the epithelial cells contain at least three closely related receptor-mediated endocytic pathways: lysosomal, recycling and transcellular. Although the initial step in each is the binding of receptors to their specific ligands, now believed to occur in different specialized regions of the plasma membrane, the internalized complexes soon meet in the first endocytic station, the 'early' endosomes, where sorting is accomplished. From then on, the delivery to 'late' endosomes and lysosomes, recycling back to the plasma membrane or transcytosis, will be performed through a series of regulated events, most of them delimited by the information contained on the cytoplasmic region of receptors [6,7].

A number of methods have been developed for the isolation of hepatic endosomes largely free of contamination by other intracellular membranes [8–14]. In this study we used the method developed by Belcher et al. [14], which distinguishes three endosomal fractions on the basis of their morphologies and also on the basis of their association with, or absence of, various radioligands at different time points with 'early' [compartment of uncoupling receptors and ligands (CURL)] and 'late' [multi-

vesicular body (MVB)] endosomes as well as a third endosomal fraction, the receptor-enriched compartment (RRC), representing the recycling and transcytotic structures [15]. This procedure is based on the shift density that endocytic structures undergo after loading with low-density lipoprotein (LDL) in oestradiol-treated rats. Previous work with this method was devoted mainly to the morphological and kinetic characterization of the isolated fractions but very few proteins were identified.

We used one- and two-dimensional gel electrophoresis and Western blotting to investigate the protein compositions of the three isolated endosome fractions. By the use of a variety of antibodies some of the major receptors and ligands in the hepatic cell were identified. In addition, the proteins of endosomes were compared with those of the bile to achieve insights into the transcytotic pathway. The results indicated that the rat liver endocytic compartment consists of specific and defined stations, with the CURL the central and therefore the most important location for sorting, and RRC being involved in recycling and transcytosis.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats weighing 200–250 g were kept under a controlled lighting schedule with a 12 h dark period. All animals received human care in compliance with institutional guidelines. Food and water were available *ad libitum*; before experiments rats were fasted overnight. All surgical procedures were performed under light diethyl ether anaesthesia.

Bile was collected via cannula (PE-10 tubing; Clay Adams, Parsippany, NJ, U.S.A.) inserted into the common bile duct; it

Abbreviations used: CURL, compartment of uncoupling receptors and ligands; GPI, glycosylphosphatidylinositol; LDL, low-density lipoprotein; MVB, multivesicular body; plgA, polymeric IgA; plgR, polymeric immunoglobulin receptor; RRC, recycling receptor compartment; SC, secretory component.

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Table 1 Antibodies used

Hosts are abbreviated as: m, mouse; g, goat; r, rabbit; s, sheep. Specificities are abbreviated as: m, monoclonal; p, polyclonal. Abbreviations: ASGP, asialoglycoprotein receptor; EGF, epidermal growth factor; M6P receptor, cation-dependent mannose 6-phosphate receptor.

Target	Host	Specificity	Source	Code
plgA α -chain	m	m	Zymed	03-9400
plgR	m	m	K. E. Mostov (San Francisco, CA, U.S.A.)	—
Secretory component	s	p	K. E. Mostov (San Francisco, CA, U.S.A.)	—
Transferrin	g	p	Nordic Immunology	42-285
Transferrin receptor	m	m	K. E. Mostov (San Francisco, CA, U.S.A.)	—
LDL Receptor	m	m	R. J. Havel (San Francisco, CA, U.S.A.)	—
ASGP Receptor	r	p	C. Enrich (Barcelona, Spain)	—
Haptoglobin	r	p	Sigma	H8636
α_2 -Macroglobulin	r	p	Sigma	M1893
β_1 -Integrin	r	p	C. Pujades, C. Enrich (Barcelona, Spain)	—
Albumin	g	p	Nordic Immunology	18-284
M6P receptor	r	p	A. Hille-Rehfeld (Göttingen, Germany)	—
AGp110	r	p	S. C. Stamatoglou (London, U.K.)	—
GPI	r	p	W. H. Evans (Cardiff, Wales, U.K.)	—
Connexin 32	r	p	W. H. Evans (Cardiff, Wales, U.K.)	gap9
EGF Receptor	m	m	Sigma	E2760

was harvested in tubes containing a mixture of protease inhibitors (1 mM PMSF, 100 μ M leupeptin and 150 μ M aprotinin) and stored in aliquots at -80°C . Before the isolation of endosomes, animals were treated with 17- α -ethynyl-estradiol (1 mg/ml in propylene glycol) for 3 days as described to induce the expression of low-density lipoproteins receptors [16].

Antibodies

Table 1 shows the antibodies used. Anti-glycosylphosphatidylinositol (GPI) antibody was prepared from the proteins released from bile canalicular plasma membranes by dissolving the membrane fraction in 1% (w/v) Triton X-114 and performing a two-phase separation as described below. The phosphatidylinositol-specific phospholipase C treatment of the proteins separating into the detergent phase and their release into the aqueous phase is also described below. 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. The antibody was kindly provided by Dr. W. H. Evans (University of Wales, Cardiff, U.K.) and the proteins recognized by this antiserum were characterized previously [17].

Isolation of endocytic and plasma membrane fractions from rat liver

After 3 days of 17- α -ethynyl-estradiol treatment, rats were anaesthetized with diethyl ether, and human LDL [18] (5 mg of protein) was injected into the femoral vein. The method used for the isolation of three endosomal fractions from rat liver has been described in detail by Belcher et al. [14]. Briefly, the livers were removed 15 min later and homogenized in 0.25 M sucrose with protease inhibitors (1 mM PMSF, 100 μ M leupeptin, 150 μ M aprotinin and 1 μ M pepstatin). The homogenate was centrifuged

for 10 min at 2000 rev./min (600 g_{av}) in an SW28 rotor (Beckman). The supernatant was centrifuged at 5100 rev./min (3500 g_{av}) for 20 min in an SW28 rotor (Beckman). Finally, the supernatant was centrifuged at 11900 rev./min (12200 g_{av}) for 20 min in a Type 30 rotor (Beckman). A 64 ml portion of the last supernatant fraction was diluted with 30 ml of Percoll solution (27 ml of Percoll and 3 ml of 2.5 M sucrose at pH 7.4), layered in tubes each containing a marker density of 1.062 g/ml (Pharmacia, Uppsala, Sweden) and centrifuged for 45 min at 17600 rev./min (26700 g_{av}) in a Type 30 rotor (Beckman). The fraction over the marker density was collected, 2 vol. of 0.9% NaCl was added, and the fraction was then layered on 2 ml of 2.5 M sucrose and centrifuged at 11600 rev./min (17800 g_{av}) for 45 min in an SW28 rotor (Beckman). The white crude endosome band was harvested and 2.5 M sucrose was added to a final density of 1.15 g/ml. The 1.15 g/ml portion was loaded at the bottom of the tubes containing a discontinuous sucrose gradient of densities 1.032, 1.074, 1.11 and 1.139 g/ml. Tubes were centrifuged for 170 min at 28000 rev./min (104000 g_{av}) in an SW28 rotor (Beckman). Three distinct populations were obtained at the density interfaces: MVB at 1.032/1.074 g/ml, CURL at 1.074/1.110 g/ml and RRC at 1.110/1.130 g/ml. Each fraction was collected and ice-cold water was added to make isotonic fractions. Finally, the isotonic fractions were pelleted by centrifugation at 20800 rev./min (52000 g_{av}) for 30 min in a 50 Ti rotor (Beckman), resuspended in 0.9% NaCl and stored at -80°C .

Electron microscopy

Pellets from isolated endosome fractions were fixed in 2.5% (v/v) glutaraldehyde/1% (w/v) paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, and prepared for electron microscopy [19].

Gel electrophoresis, Western blotting and analytical procedures

Two-dimensional gel electrophoresis was performed by the method of O'Farrell et al. [20], slightly modified, described in detail by Enrich et al. [21], using a Miniprotean II 2-D CELL from Bio-Rad Laboratories. SDS/PAGE of proteins (1 μ g per lane) was performed in 10% (w/v) polyacrylamide as described by Laemmli [22]. Polypeptides were then stained by the method of Blumm et al. [23] or transferred electrophoretically at 70 V for 90 min at 4°C to Immobilon-P Transfer Membranes (Millipore) and antigens were identified by using different antibodies (see Table 1) diluted in PBS containing 0.5% powdered skimmed milk, and the reaction product was detected by the enhanced chemiluminescence system (Amersham). Image analysis of Western blots and band quantification were performed with a Bio-Image imaging system. The intensities reported in the tables were calculated densitometrically with the Bio-Image (Millipore) program that reads and calculates band intensity and band area. The intensity of each band (one-dimensional gels) is expressed as a percentage of the total intensity (of the three fractions MVB, CURL and RRC); the intensity of MVB, for example, is $100 \times (\text{intensity of MVB}) / (\text{total intensity})$. Only bands in the same gel were compared, but the final percentage corresponds to the mean of at least three different experiments.

For the identification of proteins on the two-dimensional gels, we always ran two gels in parallel: one for silver staining and the other for Western blotting.

5'-Nucleotidase specific activity was assayed by the method of Michell and Hawthorne [24]; the specific activity of sialyl-transferase was measured [25] for the assessment of Golgi

contamination. The protein content of the samples was measured by the method of Bradford [26] with BSA as standard.

Separation of soluble and membrane proteins

Endosomes were solubilized at 4 °C in 10 mM Tris/HCl (pH 7.4)/150 mM NaCl containing 2 % (w/v) pre-condensed Triton X-114 [27]. After clarification by centrifugation for 10 min at 30000 g_{av} the samples were centrifuged at 20 °C for 3 min at 8200 g_{av} . The centrifugation step was repeated for the upper (aqueous) phase. For two-dimensional SDS/PAGE analysis, the lower (detergent) phase was resuspended in 10 mM Tris/HCl/1 % SDS/2 % Nonidet P40.

RESULTS

Ultrastructural characterization of rat liver endosomes

Figure 1(a) and 1(b) and 1(c) show thin sections of representative fields of the three endosomal fractions. MVBs, 'late' endosomes (Figure 1a), form the most homogeneous fraction, containing multivesicular structures with an average diameter of 500 nm. This corresponds to the lowest density fraction and contained injected LDL as electron-dense particles of approx. 22 nm in diameter [in addition to very-low-density lipoprotein (VLDL), β -

VLDL or chylomicrons], as can be observed at high magnification (Figure 1a, right panel). In addition, individual MVBs can contain a variable number of membranous structures attached to the vesicular body. At least two different kinds of membranous appendage can be observed: (1) finger-like appendages containing lipoprotein particles and (2) large membranous appendages lacking lipoproteins. Also, small protrusions, possibly fusion and/or budding events, can often be observed [28].

Figure 1(b) shows the CURL fraction. This is the intermediate-density fraction and is more heterogeneous than MVB. It is composed of tubulo-vesicular structures with vesicular bodies slightly smaller (average 350 nm diameter) than MVB. CURL is believed to be the 'early' endosomal compartment involved in sorting, and the fraction that originates MVBs and RRC. Numerous large membranous appendages similar to those described for MVBs were frequently attached to vacuolar bodies, which are less regular in shape (less spherical) than MVBs. Also, as described for MVBs, numerous (more abundant) small vesicles were attached to the vacuolar bodies resembling budding and/or fusion events (see Figure 1b, right panel). This fraction also contained tubular-membranous structures (diameter approx. 70 nm) that seemed not to be attached to the vacuolar bodies.

Figure 1(c) shows the RRC fraction. This is the highest density fraction and was completely different from MBVs and CURL in

Figure 1 Thin sections of representative fields of the three endosomal fractions from the liver of oestradiol-treated rats injected with human LDL

(a) MVB fraction; the arrow shows the membranous appendage and the arrowhead a finger-like appendage in MVBs. Note the internal LDL particles. Small white arrows in the enlarged MVB indicate an internal membrane bilayer. (b) CURL fraction; small arrows show the budding and/or fusion events emanating from the vacuolar bodies; arrowheads show the membrane extensions. (c) RRC fraction (large membrane extensions without lipoprotein-containing vesicles); arrowheads show the arc-shaped membranes and small arrows show different populations of vesicles. The large open arrow shows a ring-like vesicle. Scale bar in (a), representative of (a) and (b) left panels, is 0.25 μ m; scale bar in top right panel, representative of (c) and the right panels, is 0.125 μ m.

Figure 2 Two-dimensional gel electrophoresis of the three isolated rat liver endosomal fractions

(a) MVB fraction; (b) CURL fraction; (c) RRC fraction. Two-dimensional gels were stained with silver and proteins numbered. In white are the identified proteins (see the text and Tables 2 and 4 for details). The arrows at the top indicate the running direction of isoelectric focusing (pI 4.0–8.7, horizontal) and the PAGE (molecular masses 220–17 kDa, vertical).

appearance, mainly owing to the absence of lipoprotein-containing vesicles. It was a heterogeneous fraction that contained tubules, ring-like structures and vesicles of different sizes. This is the receptor-enriched fraction characterized by its composition in recycling and transcytotic markers [15]. Havel and colleagues considered that RRC had its origin in the membranous appendages emanating from CURL [14]. Two different kinds of tubular elements can be observed: (1) those containing lipoprotein-like material and (2) more electron-dense arc-shaped ones with a

bilayered membrane structure, often with terminal swellings without any detectable coats.

Identification of polypeptides of the three endosomal fractions

Despite their different morphologies, the three rat liver endosomal fractions share a 'similar' population of approx. 120 polypeptides. Figure 2(a) and 2(b) and 2(c) show representative two-dimensional gels of the three endosomal fractions, where

Table 2 Receptors; identified in isolated rat liver endosomal fractions

Abbreviations: ASGP, asialoglycoprotein receptor; EGF, epidermal growth factor; M6P receptor, cation-dependent mannose 6-phosphate receptor; n.d. not detected.

Protein	Spot number		Molecular mass (kDa)	pI	Protein distribution (% of total)		
	Bile	Endosomes			MVB	CURL	RRC
pIg Receptor	—	14	116	4.6	8	27	65
Transferrin receptor	—	4	91	8.5	18	38	44
LDL receptor	—	3, 9, 11	140	3.8–7.5	16	44	40
ASGP receptor	—	68	40	5–6	22	30	48
M6P receptor	—	54	46	4.4	30	34	36
β_1 -Integrin	—	10	130	4.2	20	33	47
AGp110	—	n.d.	110	—	18	46	36
EGF receptor	—	n.d.	170	—	22	34	44

separated silver-stained polypeptides have been numbered (white numbers correspond to known proteins). Quantitative and qualitative differences between the polypeptides in the three isolated fractions have now been observed and a total of 15 specific proteins were identified. Although there was a wide range in the molecular mass and pI distributions of proteins in the three isolated endosome fractions, in general the highest number and pattern complexity corresponded to the low-molecular-mass proteins (15–45 kDa), containing approx. 60 % of the total. In addition, whereas MVB and CURL shared a number of acidic proteins (low-molecular-mass region and pI 4.0–5.5; for example spots numbered 78, 103 and 114), CURL and RRC were the most complex and contained more common basic proteins (pI > 6.5; for example spots numbered 61 and 62) in the low-molecular-mass region.

Table 3 Membrane proteins in endosomal fractions

Results for 5'-nucleotidase are based enzyme activities measured in μ g of AMP hydrolysed/min per μ g of protein with the method of Michell and Hawthorne [24], and are expressed as percentages of the total activity. The 5'-nucleotidase spot number was 24 [15,21].

Protein	Molecular mass (kDa)	Protein distribution (% of total)		
		MVB	CURL	RRC
GPI-110	110	7	13	80
Connexin 32	32	70	26	4
5'-Nucleotidase	69	27	28	45

Table 4 Ligands and bile proteins

Abbreviation: n.d., not detected.

Protein	Spot number		Molecular mass (kDa)	pI	Protein distribution (% of total)		
	Bile	Endosomes			MVB	CURL	RRC
IgA heavy chain	6	46	55	4.8	14	26	60
Secretory component	2	—	80	4.6	—	—	—
Transferrin	3	5	80	8.7	26	36	38
Haptoglobin	7	72, 73, 74, 75	36	5–6.2	61	30	9
α_2 -Macroglobulin	1	7	180	4.8–5.3	54	40	6
Albumin	4	27	68	6.4	19	52	29
IgG heavy chain	5	n.d.	55	8.9	—	—	—
IgA light chain (α)	10	112	28	4.8	7	26	67
IgG light chain (λ)	8	n.d.	28	8.9	—	—	—
IgG light chain (κ)	9	n.d.	27	8.9	—	—	—

Figure 3 Representative Western blots of some of the proteins identified after (top) two- and (bottom) one-dimensional gel electrophoresis

Numbers indicate the spot numbers of the corresponding proteins in Figure 2 (* indicates a spot number in bile). Abbreviations: ASGPR, asialoglycoprotein receptor; GPI, GPI; LDLR, LDL receptor.

Figure 4 Two-dimensional gel electrophoresis of endosomal proteins after partition with Triton X-114

Aqueous (soluble) and integral proteins in (a) MVBs, (b) CURL and (c) RRC. Numbers in circles (soluble) or rectangles (integral) indicate the major proteins of interest (see Table 5 and the text for details).

The degree of contamination from Golgi membranes was measured by the specific activity of sialyltransferase. The activity (nmol/h per mg) in the homogenate was 5.1 ± 0.8 ; in Golgi, 120.3 ± 16.5 ; MVB, 7.1 ± 1.2 ; CURL, 15.7 ± 5.3 ; RRC, 11.9 ± 3.6 (values are means $\pm$ S.D. for two separate experiments). This indicates that the contribution of the Golgi to the isolated endosomal fractions could be neglected.

Receptors and membrane proteins

Using specific antibodies (Table 1) we have identified eight

receptors in the endosome fractions: the polymeric immunoglobulin receptor, transferrin receptor, LDL receptor, asialoglycoprotein receptor, β_1 -integrin, mannose 6-phosphate receptor, AGp110 (a non-integrin fibronectin receptor [29]) and epidermal-growth-factor receptor (Table 2). Figure 3 (top and bottom) shows representative two- and one-dimensional Western blots. The presence of the β -subunit of insulin receptor and LDL-related protein was demonstrated recently [30]. Five of these receptors [polymeric immunoglobulin receptor (pIgR), transferrin receptor, asialoglycoprotein receptor, epidermal growth factor receptor and β_1 -integrin] were enriched in the

Table 5 Unidentified endosomal proteins

Spot number	Molecular mass (kDa)	pI	Localization	Enriched fraction
1	300	8.5	Soluble	CURL/MVB
34, 35	46, 48	8.9	Integral	CURL/RRC
78	33	5.2	Integral	CURL/MVB
103, 104	27	5.8	Soluble	CURL/MVB
110	23	5.7	Integral	CURL/MVB
113, 114, 84	28, 30, 32	4.5	Soluble	CURL/MVB
118	17	4.8	Integral	CURL/RRC

Figure 5 Two-dimensional gel electrophoresis of bile proteins

A two-dimensional gel was stained with silver and the identified proteins were labelled in white (see Table 4, for details). The arrows at the top indicate the running direction of isoelectric focusing (pI 4.0–8.7, horizontal) and the PAGE (molecular masses 220–17 kDa, vertical).

RRC and slightly less so in the CURL (Table 2). LDL receptor and AGp110 were more abundant in the CURL; finally, the cation-dependent mannose 6-phosphate receptor was equally distributed between the three endocytic fractions, in accord with its known shuttling function between the *trans*-Golgi network, plasma membrane, late endosomes and lysosomes.

Also identified in the endocytic fractions (one-dimensional gels) were a group of three membrane proteins with interesting location and trafficking routes (see the Discussion section): connexin 32, 5'-nucleotidase and GPI-110.

5'-Nucleotidase and GPI-110 are GPI-anchored proteins located at the bile canalicular plasma membrane (apical). Connexin 32 is a protein of the gap junctions and therefore is a resident protein of the lateral plasma membrane. Their patterns of distribution in the three endosomal fractions are shown in Table 3.

Ligands and peripheral endosomal proteins

We have analysed the contents of endocytic structures by two different approaches: Western blotting with antibodies of known hepatic ligands or transported proteins (IgA, transferrin, haptoglobin, α_2 -macroglobulin and albumin) (Table 4) and by the use

of Triton X-114 to separate the integral and the peripheral membrane proteins (Figure 4 and Table 5).

Except for albumin, which was found to be enriched in the CURL fraction, the results (Table 4) show that proteins were more abundant in RRC (IgA) or in the MVB (haptoglobin and α_2 -macroglobulin).

The analysis of the integral and peripheral/soluble proteins of the three endosomal fractions, by using the phase partition method with Triton X-114 (Figure 4), revealed that MVB was very simple in terms of membrane proteins and therefore most of its polypeptide constituents corresponded to molecules transported, most probably, to lysosomes (haptoglobin, α_2 -macroglobulin and the proteins encircled in Figure 4). In contrast, CURL and RRC contained a more complex pattern of membrane proteins (rectangles in Figure 4), most probably in accordance with their role in sorting and recycling. Thus, to emphasize qualitative and quantitative differences between the three endosome fractions, a group of unidentified proteins is shown in Table 5 and Figure 4. CURL shares most of the proteins with MVB or RRC; all proteins reach CURL and then are diverted to RRC or to MVB; therefore MVB and RRC do not share those proteins not present in CURL. Work is in progress to identify these different proteins.

Protein composition of the bile: comparison with endocytic fractions

Bile was collected by cannulation of the bile duct and subjected to electrophoresis as described in the Experimental section. The analysis of proteins secreted into the bile and a comparison of their protein composition with those of the endosomal fractions allowed us to study the transcytotic pathway. Figure 5 shows a two-dimensional map of the major proteins of the bile; of a population of approx. 30 major proteins, 10 have been identified by Western blotting on the two-dimensional gels (Table 4); most of the bile proteins are in the acidic range (pI between 4.0 and 6.5).

Six of the identified proteins were also detected in endosomes: IgA (heavy and light chains) was enriched in RRC; haptoglobin and α_2 -macroglobulin were enriched in MVBs, and albumin was more abundant in CURL. The secretory component, the extracellular portion of the pIgR, is one of the major glycoproteins of the bile.

DISCUSSION

The method used for the isolation of endosomes from rat liver relies on the shift density that occurs after loading the endocytic structures with LDL throughout the different stations transporting lipoproteins in the hepatic cell. Thus the separation of endosomes from other organelles is based on the low density of the lipoprotein-filled endosomal vesicles. The purity, kinetics of internalization through the different compartments, and the morphology of the fractions had been established previously [31]. In addition, stereological approaches have shown that although non-parenchymal cells can contribute up to 23 % of the plasma membrane surface area and up to 40 % of the pinocytic surface area [32], the routine use of filtered homogenates has been shown to exclude from liver tissue homogenates a high proportion of non-parenchymal cells [33]. Also, the overall similarity of domain-identified plasma membrane and endosomes prepared from liver tissue and from isolated hepatocytes [12,34] adds confidence to the view that one is dealing with predominantly hepatocyte membranes. Finally, the 30 kDa mannose-binding protein receptor, an endothelial marker, was not identified in the

Figure 6 Major intracellular trafficking routes in the hepatocyte

1, Lysosomal pathway through 'early' and 'late' endocytic compartments; 2, recycling pathway either through the 'early' or more deeply through the 'late' endocytic compartment; 3, 4, transcytotic pathway; 5, apical targeting of GPI-anchored proteins; 6, suggested pathway of connexin 32 to MVBs, from the lateral plasma membrane. Abbreviations: bc, bile canaliculus; tj, tight junction.

two-dimensional gels by lectin-binding studies with ^{125}I -concanavalin A.

Because conditions in different laboratories can give rise to differences in the subcellular fractions isolated, we first analysed, by electron microscopy, the endosomal fractions (MVB, CURL and RRC) obtained from oestradiol-treated rats injected with LDL. Thin sections of the isolated endosomes revealed their unique features and their great similarity to those obtained by Belcher et al. [14] and Jäckle et al. [31], who developed this isolation procedure.

Previous experiments with these highly purified endosome fractions were devoted to kinetic studies of different ligands and the trafficking of lipoproteins to define and characterize the different endocytic compartments. The aim of the present work was the identification of proteins and the acquisition of insights into their distribution between the three endosomal fractions.

The distribution of identified receptors in the different fractions was in accordance with their expected functions in sorting, recycling and/or the transport to the bile of their respective ligands. The receptors involved in recycling and transcytosis or those proteins destined for the canalicular plasma membrane (apical) were more abundant in the RRC fraction (pIgR). Those receptors (as well as most of the proteins in endosomes) can also be detected in CURL, the 'sorting station'. Finally, those proteins on the way to degradation (haptoglobin and α_2 -macroglobulin) were more abundant in MVB.

Transcellular transport in the hepatocyte has two main implications: the secretion into the bile of those ligands destined for the external secretions (for example immunoglobulins and albumin) and the targeting of bile canalicular plasma membrane proteins (apical membrane of the hepatocyte). One group of proteins has a specific targeting signal for apical insertion: the GPI-anchored signal. A priority targeting has been demonstrated for a series of proteins in Madin-Darby canine kidney and CaCo-2 cells, for example: 5'-nucleotidase and alkaline phosphatase. Although a direct route (*trans*-Golgi network $\rightarrow$ late endosomes $\rightarrow$ canalicular membrane) has been suggested [17], Schell et al. [35] demonstrated for 5'-nucleotidase an indirect route along the transcytotic pathway.

The results of this study show a clear enrichment of polymeric IgA (pIgA) (60%), pIgR (65%) and GPI-anchored protein of

110 kDa (80%) in the RRC fraction. For AGp110, the non-integrin fibronectin receptor [29], the distribution is somewhat different and is distributed between CURL and RRC (46%/36%), perhaps indicating that this apical receptor recycles along an 'early' apical endocytic compartment ([36], and D. Ortega and C. Enrich, unpublished work). Whereas β_1 -integrin behaves as a recycling receptor, AGp110 (perhaps owing to its strictly apical location) is more abundant in the CURL fraction. In fact, very little is known about the intracellular trafficking of the ECM receptors.

Six of the identified proteins can be taken as a paradigm of the different routes in the liver (Figure 6).

1. Haptoglobin: this plasma glycoprotein (spot number 72–75/7*; asterisk indicates the spot number of a bile protein), with a molecular mass of 36 kDa, is taken up by the liver by receptor-mediated endocytosis as a haptoglobin-haemoglobin complex, and is degraded in the lysosomes [37]. Haptoglobin was found in the aqueous fractions of CURL and MVB but not in RRC. However, and interestingly, it was also present in the bile (Table 2), which could indicate that the haptoglobin found in the bile either comes from the hepatocyte (endogenous) or takes a different route from pIgA. Thus two possible routes for bile secretion can be envisaged: a direct route involving CURL and RRC (IgA) and an indirect route through the late endosomes (haptoglobin, α_2 -macroglobulin, taken up as a complex with protease) and perhaps the lysosomes [38].

2. TrfR: a recycling receptor (spot number 4) involved in the transferrin transport and the delivery of iron into the hepatocyte [9]; it has a molecular mass of 91 kDa and was never detected in bile. This receptor is more abundant in CURL and RRC, and a small amount appears in MVBs.

3. pIgR: the receptor for pIgA and IgM has a molecular mass of 116 kDa in the endosomes (spot number 14) and is transported from the sinusoidal plasma membrane to the canalicular membrane, where it is proteolytically cleaved to a 80 kDa polypeptide (spot number 2*), the secretory component. We detected this protein in the integral fraction of CURL and RRC and the secretory component in the bile. TrfR or pIgR detected in MVBs might be the result of mis-sorting or the fact that these receptors, after several rounds of recycling, are transported towards their degradation.

4. pIgA: a cargo protein (spot number 46/6*) with molecular masses of 55 kDa (heavy chain) and 28 kDa (light chain), it follows the transcytotic route; it has been detected in the aqueous fractions of CURL, RRC and intact in the bile. A very small amount of this protein was detected in the MVB fraction.

5. GPI-anchored proteins: although the protein recognized by this antibody is unknown [17], these are a class of proteins with a very well defined apical targeting signal and a precise location. The results shown a clearcut distribution between the three endosomal fractions that agrees with the idea that the delivery to the apical (canalicular) surface is through the transcytotic pathway [31].

6. Connexin 32: also interesting and intriguing is the enrichment of connexin 32 in the MVB fraction. Connexin 32 is the major component of rat liver gap junctions in the lateral plasma membrane domain of hepatocytes; in fact, as shown in Table 3, this is the only membrane protein that was more abundant in the MVBs.

Connexins are a family of polypeptides that are involved in the formation of gap junctions; very little is known about their intracellular trafficking and insertion into the lateral plasma membrane [39]. They have a very rapid turnover with a half life of 2–6 h. At least two pools of connexins are believed to exist in liver and other cells [40]. The plaques, which most probably are

transferred to lysosomes for degradation, and the intracellular stores of connexon half channels found in the Golgi-ER region, which are most probably broken down in the proteosomes [41], are the intracellular stores of connexins that can rapidly replenish the cell surface gap junction plaques (W. H. Evans, personal communication).

In conclusion it seems that small differences in the biochemical compositions of endosomes can be translated into significant morphological and functional differences, and the study of structural proteins (annexins, cytoskeleton-related and Rabs) of the endocytic compartment will contribute to the understanding of the complex regulation of intracellular protein trafficking.

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