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Isolation and characterization of three endosomal fractions from the liver of estradiol-treated rats

(endosomes/receptors/Golgi/low density lipoprotein)

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ABSTRACT **Three distinct endosomal fractions were isolated in high purity from livers of estradiol-treated rats. Each fraction had characteristic physical and ultrastructural properties, but the lipid composition and major proteins of their membranes were similar and differed from those derived from the Golgi apparatus. Injected radioiodinated low density lipoproteins accumulated first in the fraction of intermediate density and later in the low density fraction. The latter was composed almost exclusively of lipoprotein-filled multivesicular bodies, most of which had a single membranous appendage. The fraction of intermediate density was composed of lipoprotein-filled vesicles that were smaller than multivesicular bodies and also had membranous appendages. The high density fraction was composed of membranes resembling the appendages of the two vesicular fractions. All three fractions were enriched in receptors for low density lipoproteins and asialoglycoproteins, but receptor concentrations were considerably reduced in multivesicular bodies. The fraction of intermediate density may represent the compartment of uncoupling of receptor and ligand (CURL) described by Geuze *et al.* [Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Lodish, H. F. & Schwartz, A. L. (1983) *Cell* 32, 277–287]. CURL vesicles may lose some of their appendages as multivesicular bodies are formed. The high density fraction then may represent a receptor-recycling compartment.**

The pathway of receptor-mediated endocytosis of macromolecules in rat liver has been studied extensively (1–11). We have used estradiol-treated rats, in which low density lipoprotein (LDL) receptors are expressed at a high level, to define the LDL pathway in rat hepatocytes and have shown that a substantial fraction of LDL accumulates in multivesicular bodies (MVBs) within 15 min of intravenous injection (1). We have isolated MVBs from these cells (7) and have provided evidence that they fuse with primary lysosomes in the bile canalicular pole of the cell and are subsequently converted to secondary lysosomes (8). Earlier steps of the pathway and the site from which LDL receptors are recycled have remained less well defined. In the current research, we have modified our method for isolation to obtain a more highly purified MVB fraction, together with two additional fractions whose membranes have similar lipid and protein compositions.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats fed standard chow (Ralston Purina, St. Louis, MO) were treated with 17-α-ethinyl estradiol to increase the number of hepatic LDL receptors, as described (12, 13).

^{125}I-Labeled LDL. Human LDL (ρ = 1.025–1.050 g/ml) were isolated from blood serum of normolipidemic adults (14) and labeled with ^{125}I to a specific activity of ≈135 μCi/mg (1 Ci = 37 GBq) by a modification (15) of the method of McFarlane (16).

Isolation of Organelles. Endosomes were isolated from a low density fraction of liver homogenates by modifying the method for isolation of MVBs from the liver of estradiol-treated rats (7). Routinely, three rats were anesthetized with diethyl ether, and ^{125}I-labeled LDL (^{125}I-LDL) (3–5 mg of protein) was injected into the femoral vein. At specified times thereafter, the livers were removed and homogenized in 0.25 M sucrose as described (7). The following protease inhibitors (Sigma) were present in the homogenizing solution: 110 μM antipain, 40 μM pepstatin, 1.9 mM benzamidine, together with 0.8 mg of bacitracin per ml. Sixty-four milliliters of the third supernatant fraction (7) was diluted with 30 ml of isotonic Percoll (pH 7.4) (Percoll/2.5 M sucrose, 9:1, vol/vol) and centrifuged in a Beckman 50.3 Ti rotor for 45 min at 29,900 × g_{av}. The gradient was harvested down to marker beads of density 1.062 g/ml. Two volumes of ice-cold 0.15 M NaCl was added, and the fraction was layered onto 2 ml of 2.5 M sucrose. The tubes were centrifuged at 17,800 × g_{av} in Beckman SW 41 rotors for 45 min. The white endosome bands were removed from the sucrose cushions in a volume of 1 ml or less per tube and the density was raised to ≈1.15 g/ml with 0.38 ml of 2.5 M sucrose per ml. Four discontinuous sucrose gradients were prepared by successively layering 2.0 ml each of sucrose solutions with densities of 1.033, 1.074, 1.11, and 1.13 g/ml. Portions of the endosome fraction (2–4 ml) were layered at the bottom of each tube. These were centrifuged in a Beckman SW 41 rotor at 197,500 × g_{av} for 90 min. Three distinct pure white fluffy bands were obtained at the interfaces. Each fraction was harvested, its sucrose concentration was measured with a refractometer and then made isotonic by addition of water and pelleted by centrifugation in a Beckman 50.3 Ti rotor at 28,800 × g_{av} for 30 min, and finally it was resuspended in a total volume of ≈0.5 ml of 0.15 M NaCl.

Golgi-rich fractions (7) and plasma membranes (17) were isolated from livers of estradiol-treated rats as described.

Isolation of Membranes. To separate the membranes of endosomes and the Golgi fraction from their lipoprotein contents, each fraction was diluted to 4 ml with 1 mM suramin (final concentration) (18) and passed twice through a French pressure cell (SLM Aminco, Urbana, IL) at 16,000 psi (1 psi = 6.89 kPa). The membranes were sedimented at 120,000 ×

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Abbreviations: LDL, low density lipoproteins; ^{125}I-LDL, ^{125}I-labeled LDL; CURL, compartment of uncoupling of receptor and ligand; MVBs, multivesicular bodies.

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g_{av} for 2 hr in a Beckman SW 41 rotor. The supernatant contained 85–95% of the lipoprotein contents. The membranes were resuspended in 1 ml of 20 mM NaCl/50 mM Tris·HCl/0.63 mM $CaCl_2$, pH 7.5, by repeated aspiration through 22- and 25-gauge needles and recentrifuged as described above. The membranes were then resuspended as described above and sonicated (model W 185, Branson Ultrasonics, Plainview, NY) on ice at 50 W three times at 1-min intervals for 15 sec.

Assay of LDL Receptors. Total and specific binding of ^{125}I-LDL to endosome and Golgi membranes was measured at 4°C (13). Nonspecific binding, routinely measured in the presence of 10 mM EDTA, was found to be comparable to binding in the presence of a 20-fold excess of unlabeled LDL. LDL receptor content was estimated by measuring maximum specific binding (B_{max}) with a saturating concentration of ^{125}I-LDL (200 μg/ml).

Immunoblotting Analysis of Receptors. LDL receptor was purified from crude membrane preparations from livers of estradiol-treated rats as described (19). Antiserum against the LDL receptor was raised in a rabbit. Immunoblotting was carried out with this antiserum (20) and an antiserum to asialoglycoprotein receptor from rat liver (4).

Analytical Procedures. Protein was measured by the method of Petersen (21). Phospholipids (22), cholesterol (23), 5′-nucleotidase (24), and sialyltransferase (25) were measured in membrane fractions. Membrane phospholipids were extracted into chloroform (26) and separated by thin-layer chromatography (27). Components were eluted with chloroform/methanol/H_2O/glacial acetic acid (65:35:4.5:0.5) for analysis of lipid P (22). Membrane proteins were separated by NaDodSO$_4$/PAGE (28). ^{125}I was measured by γ scintillation spectrometry.

Electron Microscropy. Pellets of intact endosome fractions were prepared for thin sectioning and negative staining (7).

RESULTS

As described below, the endosomè fraction of lowest density is composed of highly purified MVBs. To determine the functional relationship between MVBs and the two fractions of higher density, we isolated these fractions from livers obtained 2.5, 7.5, and 15 min after injection of ^{125}I-LDL (at these times, the mean content of injected ^{125}I in the livers was 10.1%, 15.2%, and 25.2%, respectively). To increase the mass of endosomes, we injected a large amount of LDL (3–5 mg of protein). The recovered mass of MVB membranes increased from 0.19 ± 0.13 mg of protein (mean ± SD) in livers of uninjected animals to 0.34 ± 0.11 mg 2.5 min after injection, and further to 0.55 ± 0.16 mg and 0.52 ± 0.14 mg after 7.5 and 15 min. By contrast, the protein mass of the two other fractions increased only slightly.

The fraction of lowest density contained almost exclusively MVBs in both negatively stained and in thin-sectioned preparations. Intact MVBs were 0.5–0.6 μm in diameter, and most MVBs in negative stains contained a single attached large appendage as reported (7). The large appendages were seen in thin sections to be double bilayer structures in several different configurations (Fig. 1 *Upper*, arrows). MVBs were filled with lipoprotein particles the size of remnants of chylomicrons and very low density lipoproteins (diameter, 250–1000 Å) and of injected LDL (≈220 Å).

The fraction of intermediate density was characterized by smaller lipoprotein-containing vesicles (0.3–0.4 μm) which were often elongated (Fig. 1 *Middle*, arrowheads). The content lipoproteins were the same as for MVBs. As with MVBs (7), internal bilayer vesicles were often present, as determined by negative staining (not shown). Membranous appendages were attached to the smaller lipoprotein-containing vesicles (Fig. 1 *Middle*, arrows), but some also appeared to be unattached in negative stains, unlike the MVB fraction (not shown). These membranous appendages were more heterogeneous than those of MVBs. For reasons that will be

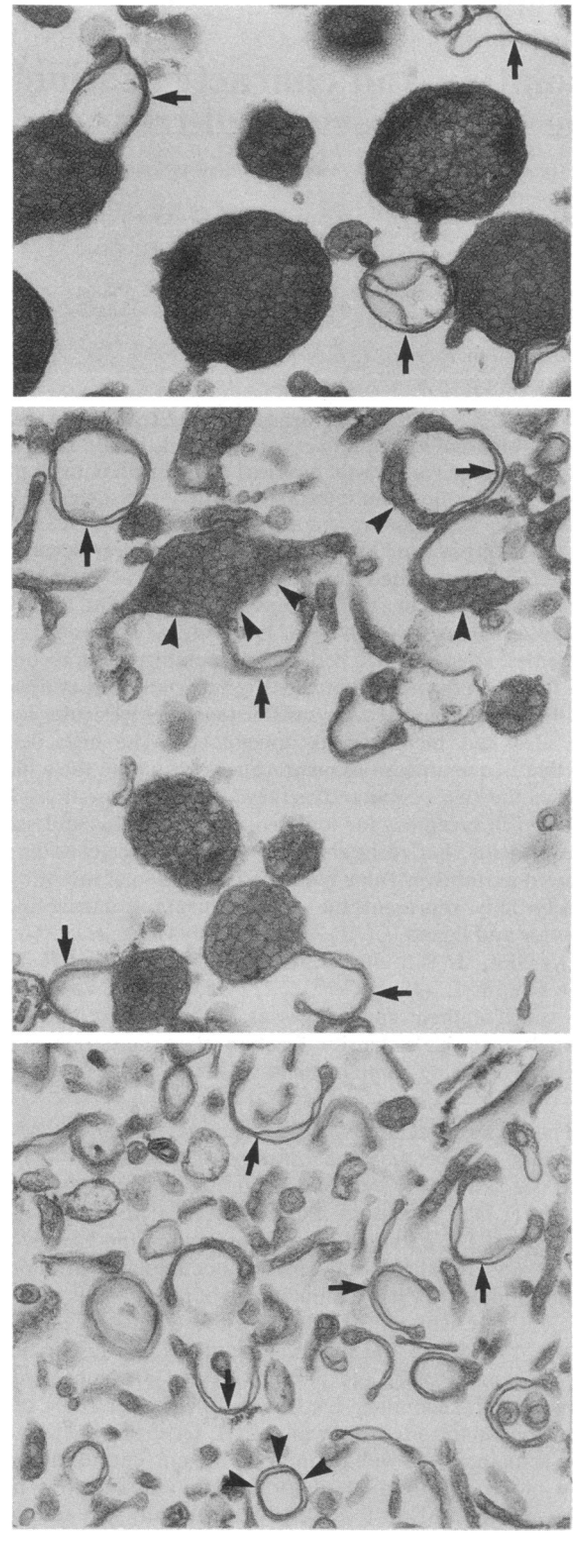

FIG. 1. (*Upper*) MVB; (*Middle*) CURL; (*Lower*) receptor-rich membranes. MVBs (0.5–0.6 μm) contain an attached membranous appendage (arrows). CURL fraction contains smaller (0.3–0.4 μm) lipoprotein-containing vesicles, often elongate (arrowheads), with similar appendages (arrows). The receptor-rich fraction contains double bilayer structures (arrows and arrowheads) that resemble the MVB and CURL appendages.

evident later, we refer to this intermediate fraction as "CURL" (compartment of uncoupling of receptor and ligand).

The fraction of highest density was characterized by bow or arc-shaped double bilayer membranous structures, often with terminal swellings (Fig. 1 *Lower*, arrows). These structures also appeared as rings (Fig. 1 *Lower*, arrowheads). Many of the double bilayer structures of this fraction resemble the attached membranous appendages of the MVB and the CURL fractions.

At 2.5 min after injection of ^{125}I-LDL, the concentration of ^{125}I was 58% higher in the fraction of intermediate density than in MVBs (Table 1). However, between 2.5 and 15 min, the concentration of ^{125}I increased 11-fold in MVBs and only 2-fold in the intermediate fraction. The concentration of ^{125}I in the fraction of high density remained at considerably lower levels. Very little ^{125}I-LDL was found in the Golgi fraction. The amount of ^{125}I recovered in MVBs increased 18-fold after 15 min (Table 1), concomitant with an increase in MVB mass. These results suggest that the intermediate fraction is a precursor of MVBs.

The apparent affinity of LDL receptors for LDL in the suramin-treated membranes of the endosome fractions (≈25 μg of LDL per ml) was comparable to that found previously in crude membrane fractions (29) (data not shown). The specific binding of LDL at saturation was determined on the membranes isolated from each fraction as a measure of LDL receptor number. Specific binding did not change demonstrably in any of the endosome fractions after injection of LDL (Table 1). Therefore, the average concentration of receptors in each fraction was calculated for all experiments (n = 12). Receptor concentration was 3- to 4-fold higher in membranes from the fractions of intermediate and high density (72 ± 12 and 88 ± 32 pmol per mg of membrane protein, respectively) than in MVB membranes (22 ± 7 pmol/mg). Although the apparent concentration of LDL receptors in MVBs remained constant in LDL-injected animals, the total number of LDL receptors that accumulated in MVBs increased ≈3-fold (Table 1).

Fifteen minutes after injection of ^{125}I-LDL, about three-quarters of endosomal LDL was in MVBs and this fraction contained many more LDL molecules than receptors (Table 1). The lower concentration of LDL receptors in MVB membranes was also evident in immunoblots (Fig. 2*A*). Golgi, liver homogenate, and plasma membrane all appeared to contain much lower receptor concentrations. Similar results for the three endosome fractions were obtained with immunoblots developed with specific antiserum to the asialoglycoprotein receptor (Fig. 2*B*).

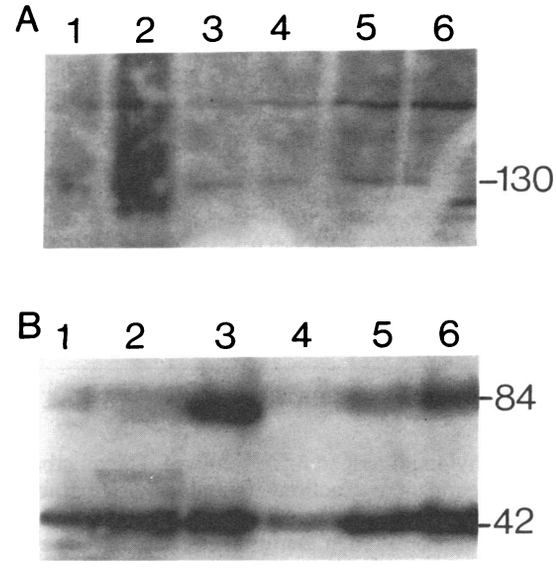

FIG. 2. Immunoblots of LDL receptors (*A*) and asialoglycoprotein receptors (*B*) with liver membranes. Lanes: 1, liver homogenate (300 μg of protein); 2, Golgi membranes (300 μg); 3, plasma membranes (113 μg); 4, MVB membranes (10 μg); 5, CURL membranes (10 μg); 6, receptor-rich membranes (10 μg). Only the receptor-containing regions of each gel are shown. Membranes were separated under nonreducing (*A*) or reducing (*B*) conditions by $NaDodSO_4$ gradient (3–20%) gel electrophoresis. Proteins, transferred to a nitrocellulose membrane, were incubated with polyclonal rabbit IgG against rat LDL receptor (*A*) or rabbit antiserum against rat asialoglycoprotein receptor (*B*). Receptor-bound antibodies were visualized with ^{125}I-labeled protein A. The LDL receptor had an apparent M_r of 130,000. A higher molecular weight band related to the LDL receptor is also seen. The asialoglycoprotein receptor had a M_r of 42,000 and an apparent dimer at M_r 84,000. Numbers on right represent $M_r \times 10^{-3}$.

Consistent with the ultrastructural characteristics of the three endosome fractions, none of them was appreciably contaminated by trans-Golgi elements, as indicated by the low activity of sialyltransferase (Table 2). However, each fraction was enriched in 5′-nucleotidase, a plasma membrane marker. All three endosome fractions exhibited active electrogenic ATP-dependent acidification that was abolished by

Table 1. Quantitation of LDL and LDL receptors in membranes of endosome and Golgi fractions after injection of ^{125}I-LDL

Fraction	Time after injection, min	^{125}I-LDL		LDL receptors		^{125}I-LDL/LDL receptors
		pmol/mg	pmol	pmol/mg	pmol	
Intermediate density (CURL)	0	—	—	67.4 ± 10.2	22.8 ± 17.0	—
	2.5	111.6 ± 41.3	32.5 ± 14.5	58.2 ± 7.2	19.6 ± 15.5	1.7
	7.5	142.7 ± 81.1	74.1 ± 51.6	84.8 ± 23.2	37.4 ± 4.1	2.0
	15	227.3 ± 165.2	89.7 ± 66.5	76.8 ± 9.0	30.1 ± 2.9	3.0
Low density (MVB)	0	—	—	23.0 ± 8.8	3.8 ± 1.8	—
	2.5	70.5 ± 14.6	22.9 ± 3.6	21.8 ± 7.2	7.0 ± 1.7	3.3
	7.5	437.0 ± 232.8	266.8 ± 177.8	21.8 ± 7.0	12.2 ± 5.9	21.9
	15	797.7 ± 350.1	401.2 ± 169.8	23.0 ± 6.2	11.6 ± 2.7	34.6
High density (receptor-rich)	0	—	—	115.8 ± 19.1	53.1 ± 25.8	—
	2.5	18.7 ± 11.6	9.5 ± 6.3	70.5 ± 17.8	34.3 ± 31.0	0.3
	7.5	14.4 ± 6.9	8.8 ± 5.6	73.0 ± 48.4	33.2 ± 6.9	0.3
	15	54.4 ± 27.8	23.4 ± 1.5	92.2 ± 3.1	46.0 ± 25.0	0.5
Golgi	15	3.8 ± 0.3	19.9 ± 6.3	0.9 ± 0.0	4.4 ± 1.2	4.5

Mass (pmol) of ^{125}I-LDL was calculated by dividing the radioactivity in each fraction by the specific activity of the injected ^{125}I-LDL (M_r of LDL protein, 512,000). LDL receptors were assumed to bind LDL monovalently and to be saturated at the concentration of ^{125}I-LDL protein used in the binding assay (200 μg/ml). Specific activity is calculated per mg of membrane protein. Time 0 represents fractions from noninjected animals. Numbers are means ± SD for three experiments.

Table 2. Enzymatic activity and composition of membranes

Fraction	Glucose 6-phosphatase, $\mu g \cdot min^{-1} \cdot mg^{-1}$	Sialyltransferase, $nmol \cdot hr^{-1} \cdot mg^{-1}$	5'-nucleotidase, $\mu g \cdot min^{-1} \cdot mg^{-1}$	Phospholipids/protein, mg/mg	Cholesterol/phospholipids, mg/mg	Sphingomyelin/phosphatidylcholine, mol/mol
Homogenate	4.2 ± 1.5 (3)	4.5 (1)	1.1 ± 0.2 (7)	—	—	—
Intermediate density endosomes (CURL)	0.5 ± 0.9 (3)	10.5 ± 2.2 (3)	7.5 ± 2.6 (6)	1.3 ± 0.2 (3)	0.30 ± 0.04 (3)	0.60 ± 0.08 (3)
Low density endosomes (MVB)	1.9 ± 0.3 (3)	5.8 ± 2.0 (3)	10.5 ± 3.8 (6)	0.8 ± 0.2 (3)	0.33 ± 0.03 (3)	0.90 ± 0.27 (3)
High density endosomes (receptor-rich)	0.0 ± 0.0 (3)	6.5 ± 2.6 (3)	6.4 ± 1.4 (6)	1.0 ± 0.1 (3)	0.25 ± 0.04 (3)	0.74 ± 0.10 (3)
Golgi	—	108.0 ± 8.1 (3)	3.8 ± 2.5 (3)	0.9*	0.082*	0.083 ± 0.02 (3)
Plasma membranes	2.6 (1)	—	21.0 ± 12.2 (2)	—	—	0.34 ± 0.14 (3)

Values are means ± SD. Number of experiments is indicated in parentheses.
*From ref. 7.

1 mM *N*-ethylmaleimide (data not shown). The membranes of the three endosome fractions contained comparable high concentrations of cholesterol and sphingomyelin, as compared with those of Golgi membranes (Table 2). The protein components of the three endosome membranes were strikingly similar and distinct from those of Golgi membranes, as shown by the protein patterns seen in $NaDodSO_4$ gel electrophoretograms (Fig. 3).

DISCUSSION

In the current research, we have separated three morphologically distinct endosomal structures from livers of estradiol-treated rats that have strikingly similar membrane compositions. Given the low content of sialyltransferase, none of the fractions appears to contain appreciable trans-Golgi elements. We have previously shown that MVBs have low activities of arylsulfatase and glucose 6-phosphatase (7), indicating little contamination with secondary lysosomes and endoplasmic reticulum, respectively. The paucity of contamination by nonendosomal organelles is supported by the ultrastructural characteristics of the three fractions. The fraction of lowest density consists almost entirely of MVBs, the purity of which is greater than that reported previously (7), as judged from the enrichment of ^{125}I-LDL 15 min after intravenous injection (222-fold as compared with 107-fold over liver homogenate on a protein basis). The MVB and intermediate density fractions differ primarily in the size and shape of the lipoprotein-filled vesicles: MVBs are larger and more spherical. The vesicles in both fractions have membranous appendages, the structure of which closely resembles the high density fraction. Injected ^{125}I-LDL appear first in the intermediate density fraction and later in MVBs and the former fraction is considerably more enriched in LDL receptors as well as asialoglycoprotein receptors (Table 1; Fig. 2). Thus, many of these receptors seem to leave the receptor-rich vesicular fraction by separation of the membranous appendages as MVBs are formed, presumably with continuing endosome fusion. The receptor-rich fraction of highest density is almost certainly derived from the membranous appendages of the other two fractions.

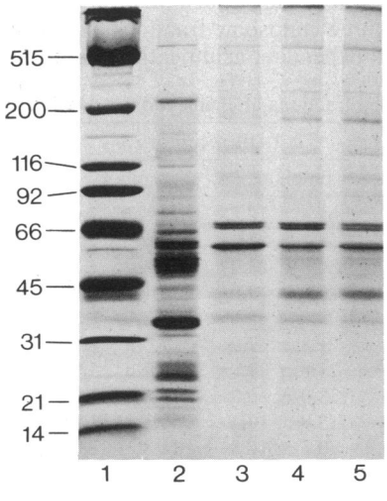

FIG. 3. $NaDodSO_4$ gradient (3–20%) gel electrophoretogram showing proteins of endosomal and Golgi membranes (Coomassie blue staining). Lanes: 1, molecular weight standards; 2, Golgi membranes (50 μg); 3, MVB membranes (50 μg); 4, CURL membranes (50 μg); 5, receptor-rich membranes (50 μg). Numbers on left represent $M_r \times 10^{-3}$.

Because MVBs evidently contain the lowest fraction of appendageal membrane relative to vesicular membrane, our results are consistent with the hypothesis that the receptors are concentrated in the appendages. It is therefore reasonable to suggest that the receptor-rich vesicular fraction corresponds to the endosomal compartment defined as CURL by Geuze and his associates, in which receptors are thought to be segregated in membranous extensions of endosomal vesicles (2). Whether the receptor-rich membranes found in the fraction of highest density exist as such in the cell and represent a receptor-recycling compartment is uncertain because they could have become dissociated from the endosomal vesicles during isolation. Even if receptors do concentrate in the appendageal membranes, it is evident that the most prevalent proteins of the endosomal membrane distribute more or less equally between the appendages and vesicles (Fig. 3).

Each of our three endosomal fractions contains an active proton translocase with the characteristics of the proton ATPase described previously in coated vesicles and endosomes, including MVBs (30). Recently, the three-dimensional structure of an acidic endocytic compartment in cultured baby hamster kidney cells has been constructed from images of serial thin sections (31). It was shown that this endosomal compartment is composed of vesicles, each associated with several tubules, which do not anastomose to form a reticulum. It was estimated that the tubules contain about two-thirds of the membrane surface area of the endosomes. These observations fit very well with the ultrastructural characteristics of our vesicular fractions, especially the intermediate density fraction, and lead us to suggest that the separate vesicular structures that we have isolated do not result from rupture of membranes that interconnect the lipoprotein-filled vesicles.

Each of our endosomal populations seems to be distinct from the trans-Golgi reticulum as described by several groups, particularly by Roth *et al.*, who found that immunoreactive sialyltransferase in rat liver is concentrated in trans-cisternae and the associated trans-tubular network (32).

Not only do our endosomal fractions contain little sialyltransferase (Table 2), they are also much richer in cholesterol and sphingomyelin than Golgi fractions (ref. 33; Table 3).

A number of investigators have separated ligand-containing endosomal fractions from rat liver. Consistent with our observations, the lipid composition of these fractions has resembled that of plasma membranes (3, 6), and, where examined, protein patterns have been found to differ from and to be more restricted than those of plasma membranes (3, 6, 10, 11). In some cases (4, 5, 9, 10), receptor-rich and receptor-poor populations have been separated and, with one exception (10), the receptor-rich fraction has been found to have a higher density. The receptor-rich fraction has been shown to acquire ligand earlier than the receptor-poor fraction. The observations of two groups of investigators are of particular relevance to ours.

Evans and Flint (5) used Nycodenz gradients to obtain three endosomal fractions, which resemble ours in a number of respects, although each fraction appeared contaminated by nonendosomal organelles and lacked unique ultrastructural characteristics. As in the current study, their fraction of intermediate density was receptor-rich and appeared to give rise to a lighter receptor-poor fraction. The lipid composition of both resembled that of plasma membranes and they contained similar protein and glycoprotein arrays, distinct from those of plasma membrane (6). A third, most dense, fraction was receptor-rich but, unlike our most dense fraction, it differed from the other two in protein composition.

Mueller and Hubbard (9) have also isolated from rat liver both an "early" endosomal fraction rich in receptors for asialoorosomucoid, and a "late" endosomal fraction lacking receptors. The former fraction was composed mainly of small irregular vesicles and the latter was rich in larger vesicles identified as MVBs. It is important to note that the MVBs lacked membranous appendages, which were assumed to have been dissociated during the isolation procedure. The two fractions otherwise resemble ours in general appearance and lipoprotein content, but their purity was not assessed. Based on data indicating that endosomes representing the receptor-rich fraction are present both near the plasma membrane (peripheral endosomes) and near the bile canalicular pole of hepatocytes (internal endosomes) (4), they suggested that asialoglycoprotein receptors are not completely segregated at the periphery of the cell. This interpretation is consistent with the fact that MVBs in situ (near the canalicular pole) do contain membranous appendages (1) and with our observation that isolated MVB fractions, in which appendages persist, retain some receptors.

The fractions that we have isolated and partially characterized in the current study can be placed within the context of the endocytic pathway in hepatocytes and other mammalian cells, as it is now understood (34, 35). Our results are consistent with the concept that receptor-rich endosomes exist as discrete organelles; that they fuse and migrate to the Golgi–lysosome region of polarized cells and in the process sequester ligands within enlarging structures that become MVBs; that MVBs are an immediate precursor of secondary lysosomes; and that receptors are segregated in tubular appendages from the enlarging vesicular compartment containing ligands. The availability of highly purified endosomal fractions, which have distinct chemical and structural characteristics, should facilitate more detailed studies of their maturation and analysis of the mechanism by which receptors are segregated and dissociated.

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