Phospholipid, cholesterol, polypeptide and glycoprotein composition of hepatic endosome subfractions

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1. The composition of hepatic endosome subfractions was compared directly with that of plasma membranes and Golgi-apparatus fractions. 2. The neutral lipid and phospholipid composition of two endosome subfractions separated on Nycodenz gradients from a parent endosome fraction was similar to that of plasma membranes. The phospholipid/cholesterol ratios and the sphingomyelin contents were high, as in plasma membranes. However, the phosphatidylserine content was low. 3. Endosomal subfractions contained a simpler polypeptide profile than did plasma membranes. However, a large number of glycoproteins were common to both fractions. Two endosome-specific glycoproteins, of M_r 59000 and 38000, were identified. Sialic acid was present at concentrations higher than in plasma membranes. 4. The results indicate that endosomal membranes have a similar composition to plasma membranes that probably reflects their functional interaction during endocytosis and receptor recycling.

INTRODUCTION

Hepatic subcellular membrane vesicles of low density, where various radioiodinated asialoglycoproteins and polypeptide hormones are located after endocytosis (endosomes), have been purified and analysed on Nycodenz density gradients and by free-flow electrophoresis (Evans & Flint, 1985). The ligand-transporting vesicles originate from the cell's endocytic compartment, and they have been subjected to enzymic and morphological characterization in liver (Debanne *et al.*, 1982; Hadjiivanova *et al.*, 1984; Quintart *et al.*, 1984) and cultured cells (Dickson *et al.*, 1983). The hepatic endosome fractions, originally identified on the basis of their content of intact radioiodinated ligands, were shown to be highly enriched in a monensin-activated Mg^{2+} -ATPase (Saermark *et al.*, 1985) that is thought to function as the proton pump accounting for the generation of a low pH in the endocytic networks (Yamashiro & Maxfield, 1984). In the present paper, we analyse the lipid, protein and glycoprotein compositions of the endosome subfractions and compare these with those of plasma membranes, the Golgi apparatus and coated vesicles analysed simultaneously or with the values reported for these organelles in the literature. The results, in conjunction with those on the enzymic composition, lead to a more complete description of the biochemical properties of endosomal membranes and establish in greater detail the identity of this new subcellular organelle.

EXPERIMENTAL

Preparation of subcellular fractions

The endosome fractions were prepared from the livers of male Sprague–Dawley rats (150–200 g) as described previously (Debanne *et al.*, 1982; Evans & Flint, 1985; Saermark *et al.*, 1985). The parent endosome D fraction prepared by fractionation on sucrose gradients (sucrose

density range 1.095–1.117 g/cm³) of post-mitochondrial lysosomal supernatants was then fractionated in iso-osmotic Nycodenz gradients (13.8–27.6%, w/v) to yield two ligand-containing fractions collected within the refractive-index ranges 1.355–1.364, peak density 1.090 g/cm³ (DN-1) and 1.365–1.375, peak density 1.115 g/cm³ (DN-2); a fraction resting on a 70% (w/v) sucrose cushion (D-R) was also collected. Liver plasma membranes enriched in bile-canalicular profiles were prepared as described by Wisher & Evans (1975). Endosome-depleted Golgi fractions were prepared as follows. A loose overlay of the pellet obtained after centrifuging the post-nuclear supernatant at 33000 g for 8 min at speed (Beckman type 30 rotor) was collected by gently swirling in 72% (w/v) sucrose. After dispersion in a loose-fitting Dounce homogenizer, the material was adjusted to 39% (w/v) sucrose and overlaid with equal volumes of 29.5%, 20.5% and 8% (w/v) sucrose. After centrifugation at 97000 g for 3 h (Beckman SW.28 rotor), subfractions, corresponding to Golgi heavy, intermediate and light fractions (Bergeron, 1979; Higgins, 1984) and with a similar morphology and galactosyltransferase content (Evans, 1985; Saermark *et al.*, 1985) were collected.

Lipid analyses

Membrane fractions were suspended in 1.3 ml of 8% sucrose, and protein was determined by the method of Lowry *et al.* (1951). Fractions were then extracted with 10 vol. of chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). The lower layer was collected, washed with 0.2 vol. of water, evaporated to dryness under N₂ and stored at –20 °C in ethanol containing 0.01% butylhydroxytoluene. Recovery of phospholipids by this procedure was estimated by addition of 1-palmitoyl-2-linoleoyl-¹⁴C-glycerophosphocholine to the parent endosome fraction before extraction; the recovery in the ethanol was 96.3 ± 7.6% (S.D.; $n = 12$). Total lipid phosphorus was measured as described by Rouser *et al.* (1970), and

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cholesterol by g.l.c. of the cholesterol trimethylsilyl ethers as described by Hardison & Apter (1972). Phospholipid classes were measured by h.p.l.c. (Jungalwala *et al.*, 1976; James *et al.*, 1981) in a 250 mm × 4 mm Hibar column packed with Lichrospher Si 100 (10 µm) (E. Merck, Darmstadt, Germany), monitored with a variable-wavelength detector set at 205 nm. Sphingomyelin, phosphatidylcholine and a solvent front containing phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine were separated by using acetonitrile/methanol/water (65:21:14, by vol.) pumped at 1 ml/min. The solvent front was evaporated to dryness under N₂ and re-injected into the column by using a dual liquid phase of hexane/propan-2-ol/water [7:8:0.75, by vol. (phase A) and 7:8:1, by vol. (phase B)]. The chromatograph was run (1.5 ml/min) with phase A for 12 min followed by phase B, allowing separation of peaks of solvent front, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. The chromatographic peaks were collected, evaporated to dryness and lipid phosphorus was determined (Rouser *et al.*, 1970). The solvent front contained less than 2% of the total phospholipid, and the components were not identified with phosphatidic acid or lysophosphatidylcholine.

Polypeptide and glycoprotein analyses

Membrane fractions were solubilized in 2% (w/v) sodium dodecyl sulphate/1% mercaptoethanol/50 mM-Tris/HCl, pH 7.4, and electrophoresed in 10% -polyacrylamide gels, as described by Laemmli (1970). Proteins were stained with Coomassie Blue and the glycoproteins were detected by autoradiography after binding to ¹²⁵I-concanavalin A (Gurd & Evans, 1976).

RESULTS AND DISCUSSION

Subcellular-fractionation studies of mammalian tissues, especially liver, have shown that membranes derived from the various subcellular organelles and compartments possess characteristic enzymic markers and polypeptide

profiles; however, differences in lipid composition are less marked (Evans, 1980). Two low-density hepatic endosome fractions in which various radioiodinated ligands were concentrated at short intervals after internalization, and that lacked marker enzymes for other characterized subcellular organelles (Evans & Flint, 1985; Saermark *et al.*, 1985), were analysed for their protein, glycoprotein and lipid compositions.

Lipid composition

The subfractions of highest purity recovered in Nycodenz density gradients, where the internalized ligands were located 1–2 min (DN-2) and 10–15 min (DN-1) after internalization, had a high cholesterol content (Table 1). The cholesterol content of these endosome subfractions was even higher than that of plasma membranes; the cholesterol content of a Golgi fraction examined was low. Analysis of the phospholipids in the fractions indicated that the sphingomyelin content of the endosome fractions DN-1 and DN-2 was similar to that of bile-canalicular plasma membranes (Kremmer *et al.*, 1976; Meier *et al.*, 1984). A further notable feature was the low phosphatidylserine content of the endosome fractions compared with plasma membranes; similar values have been recorded for a rat liver microsomal fraction (Kawamoto *et al.*, 1980) and a rat liver sinusoidal-domain plasma-membrane fraction, that, according to its method of isolation (Wisher & Evans, 1975), would also contain the endosomal membranes now characterized. A rat liver small-vesicle fraction containing latent 5'-nucleotidase activity and probably originating from the endocytic networks also had a high sphingomyelin content (Luzio & Stanley, 1983), and Dickson *et al.* (1983), who analysed endosomes (receptosomes) isolated from KB cells also reported that they had a high cholesterol/phospholipid ratio.

The membranes of the endocytic compartment are in functional continuity with the sinusoidal region of the hepatocyte's plasma membrane, and especially the clathrin-coated pit domains, during receptor-mediated

Table 1. Lipid and sialic acid composition of rat liver endosome subfractions and comparison with plasma membrane and Golgi fractions

For full details of the preparation of the various subcellular fractions, and the methods of lipid analyses, see the Experimental section. A Golgi 'intermediate' subfraction was analysed. Values are means ± s.d. for the numbers of determinations in parentheses. Abbreviation: ND, not determined.

Constituent	Fraction				
	Endosome D	Endosome DN-1	Endosome DN-2	Plasma membranes	Golgi
Phospholipid (µmol/mg of protein)	0.520 ± 0.130 (5)	0.513 ± 0.020 (3)	0.385 ± 0.037 (3)	0.58 ± 0.19 (4)	0.77 ± 0.36 (6)
Cholesterol (mg/mg of protein)	0.036 ± 0.010 (5)	0.137 ± 0.020 (3)	0.119 ± 0.032 (3)	0.10 ± 0.09 (4)	0.067 ± 0.016 (6)
Cholesterol (mg/µmol of phospholipid)	0.072 ± 0.026 (5)	0.267 ± 0.037 (3)	0.308 ± 0.064 (3)	0.16 ± 0.04 (4)	0.097 ± 0.027 (6)
Cholesterol/phospholipid molar ratio	0.19	0.69	0.80	0.42	0.25
Sialic acid (µmol/mg of protein)	63.4 ± 14.4 (5)	92 (1)	69 (1)	37.3 ± 1.7 (3)	ND
Phospholipids (%)					
Solvent front	1.7 ± 1.60 (3)	0.5 ± 0.17 (3)	1.13 ± 0.68 (3)	1.63 ± 0.68 (3)	1.80 ± 1.17 (3)
Phosphatidylethanolamine	19.2 ± 1.12 (3)	21.4 ± 3.46 (3)	22.0 ± 1.9 (3)	19.1 ± 1.4 (3)	20.10 ± 4.37 (3)
Phosphatidylinositol	1.0 ± 0.46 (3)	0.90 ± 0.67 (3)	1.13 ± 0.15 (3)	0.87 ± 0.10 (3)	1.30 ± 1.67 (3)
Phosphatidylserine	2.9 ± 1.33 (3)	3.10 ± 0.55 (3)	3.03 ± 0.55 (3)	7.20 ± 1.95 (3)	3.00 ± 0.91 (3)
Phosphatidylcholine	61.6 ± 2.82 (3)	52.3 ± 8.8 (3)	54.3 ± 3.8 (3)	54.5 ± 3.8 (3)	63.0 ± 10.5 (3)
Sphingomyelin	13.6 ± 1.46 (3)	21.8 ± 5.4 (3)	18.5 ± 0.9 (3)	16.7 ± 2.2 (3)	9.7 ± 6.9 (3)

Fig. 1. Polyacrylamide-gel-electrophoretic separation of the polypeptides in (a) fraction D-R, (b) plasma membranes, (c) endosome fraction DN-1 and (d) endosome fraction DN-2

Gels were stained with Coomassie Blue. For further details see the text.

endocytosis, thus prompting a comparison of the composition of the endosome fractions with those reported for various coated-vesicle fractions. This comparison is based on the assumption that the major source of the coated vesicles is the plasma-membrane coated pits, although they may also be generated from coated membrane regions in the Golgi apparatus (Hanover *et al.*, 1984). Coated vesicles isolated from bovine brain contained a high cholesterol/phospholipid ratio, e.g. 0.57 (Alfsen *et al.*, 1984) or 0.34 (Alstiel & Branton, 1983). Rat liver coated vesicles were also reported to contain a similar cholesterol/phospholipid ratio to that of plasma membranes (Steer *et al.*, 1984). The present results lead to the conclusion that the lipid compositions of endosome membranes, plasma membranes and coated vesicles are similar.

In the purification of endosomes from rat liver, glycosyltransferase activities, generally used as biochemical markers for the Golgi apparatus, were absent from subfraction DN-1, but persisted at a low value in subfraction DN-2 (Evans & Flint, 1985). The lipid analysis of these fractions indicated that the endosome fractions were similar but that they differed substantially from the neutral and phospholipid composition of Golgi now reported or the values determined by others (Zambrano *et al.*, 1976; Howell & Palade, 1982; Higgins, 1984). The present lipid analyses are in accord with fluidity determinations on liver endosome fractions,

Fig. 2. Autoradiograph of the glycoproteins present in (a) endosome fraction DN-2, (b) endosome fraction DN-1 and (c) plasma membranes separated by polyacrylamide-gel electrophoresis, and identified after binding ^{125}I -concanavalin A

For details see the text.

showing that the endosome fractions were closer in lipid fluidity to plasma membranes than to Golgi-apparatus fractions (Whetton *et al.*, 1983). A stringent comparison is not possible because Golgi-apparatus fractions isolated from liver are contaminated by endocytic membranes (Kay *et al.*, 1984; Saermark *et al.*, 1985).

Polypeptide and glycoprotein composition

Comparison of the polypeptide compositions of two highly purified low-density endosome fractions with a rat liver plasma-membrane fraction indicated that the overall profiles differed (Fig. 1). Subfractions DN-1 and DN-2 contained a similar polypeptide composition, but a further, heavier, subfraction (D-R) separated at the interface of Nycodenz gradients and a sucrose cushion, where receptors for asialoglycoproteins and insulin were present at high amounts (Evans & Flint, 1985), contained a different polypeptide composition. In spite of the overall differences in polypeptide profiles between plasma-membrane and endosome fractions, there were many polypeptides common to the fractions, based on electrophoretic mobility. To investigate whether these polypeptides were endocytosed glycoproteins and thus candidates for recycling between the intracellular and cell-surface membranes, polyacrylamide gels were exposed to ^{125}I -concanavalin A. Fig. 2 indicates that a number of glycoproteins, mainly of high M_r , were common to the endosome and plasma-membrane fractions. Fig. 2 also indicates that glycoproteins of M_r 38 000 and 59 000 were present in the endosome fractions but absent from plasma

membranes. These proteins therefore qualify as endosome-specific proteins, albeit of unknown function. Transferrin receptors (M_r 90000) and asialoglycoprotein receptors (M_r 45000) have been identified in the endosomal compartment of human cell lines (Watts, 1984) and rat liver (Debanne *et al.*, 1984).

The sialic acid content of the endosome fractions was also investigated (Table 1). The results show that the endosomes contained, on a protein basis, a higher content of sialic acid than do plasma membranes, further emphasizing the structural similarities between endosomal and plasma membranes, a probable consequence of their functional interaction during endocytosis and receptor recycling. The results agree with analytical studies of rat liver microsomal fractions that show that protein-associated sialic acid was present in low-density cholesterol-containing components separated from endoplasmic-reticulum markers (Amar-Costesec, 1981). Coated vesicles isolated from brain tissue also contain gangliosides and sialoglycoproteins (Gravotta & Maccioni, 1985).

Conclusions

The protein, glycoprotein and lipid compositions of endosome membranes resembled those of the plasma membrane more closely than those of other subcellular organelles. Endosome membranes also resembled coated vesicles in lipid composition, but they lacked the major clathrin heavy chain (M_r 180000) (Rubenstein *et al.*, 1981), as would be expected from their morphology. The results are in accord with a selective internalization and recycling of glycoproteins between the plasma membrane and the endocytic networks. A similar conclusion was reached by labelling after endocytosis the non-cytoplasmic endosomal proteins of a human cell line by using asialoglycoprotein-lactoperoxidase conjugates (Watts, 1984). The transfer of lipid between the networks appears less selective, but this has to be seen against the fact that the lipid compositions of subcellular membranes are less variable than their protein and enzymic compositions. Nevertheless, the relatively high cholesterol and sphingomyelin content of the endosome fraction is highly suggestive of a functional interaction that may extend to an anatomical continuity between the plasma membrane and the membranes comprising the endocytic compartment. The results make plausible the concept that the membranes comprising the endocytic networks are a further 'intracellular' domain of the plasma membrane where the cell is better able to regulate its 'external' environment by controlling the acidity, ionic concentration etc., thereby enabling the selective entry of receptor-bound hormones, serum proteins, neurotransmitters, antibodies or viruses across the endocytic membrane to various regions of the cell.

The work was supported in part by grant AM 28446, National Institutes of Health, Bethesda, MD, U.S.A. and by the U.S. Veterans' Administration Research Service (W.G.H.).

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