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Determination of the intracellular distribution and pool sizes of apolipoprotein B in rabbit liver

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We have investigated the intracellular distribution of apolipoprotein B (apo B) in rabbit liver by immunoblotting, radioimmunoassay (r.i.a.) and enzyme-linked immunoassay (e.l.i.s.a.). Apo B100 was detected in total microsomes, rough microsomes, smooth microsomes, *trans*-enriched Golgi and *cis*-enriched Golgi and membrane and cisternal-content subfractions prepared from these fractions. There was also evidence of degradation of apo B100 in the Golgi membrane fractions. The amount of apo B in the subcellular fractions detected by competitive r.i.a. or e.l.i.s.a. ranged from 1.5 μg/mg of protein in the rough endoplasmic reticulum to 13 μg/mg of protein in the *trans*-Golgi fraction. Using internal standards (NADPH–cytochrome *c* reductase for the endoplasmic reticulum and galactosyltransferase for the Golgi membranes) it was calculated that all the apo B of liver is recovered within the secretory compartment, with 63% of the total apo B in the endoplasmic reticulum and the remainder in the Golgi. When the subcellular fractions were separated into membranes and cisternal contents, 60%, 50%, 60% and 30% of the total apo B was recovered in the membrane of the rough microsomes, smooth microsomes, *cis*-Golgi and *trans*-Golgi respectively. Using competitive e.l.i.s.a. we found that the membrane-bound form of the apo B was exposed at the cytosolic surface of the intact subcellular fractions. These observations are consistent with a model for assembly of very-low-density lipoproteins (VLDL) in which newly synthesized apo B is incorporated into a membrane-bound pool and a lumenal pool. The membrane-bound pool not used for VLDL assembly may be degraded, possibly in the Golgi region.

INTRODUCTION

Very-low-density lipoproteins (VLDL) are the vehicle of transport of endogenous triacylglycerol in plasma from the liver and are the precursors of the low-density lipoproteins (LDL). Apolipoprotein B (apo B), the major protein component of VLDL, is essential for the secretion of VLDL and is the ligand for the LDL receptor. We have previously investigated the intracellular events in the assembly of apo B and lipids and secretion of VLDL by rat liver (Higgins, 1984, 1988; Higgins & Hutson, 1984; Higgins & Fieldsend, 1987). Briefly, these studies have suggested that triacylglycerol-rich precursors with low contents of phospholipid, cholesterol and apo B are initially sequestered in the cisternal space of the endoplasmic reticulum. The precursor particles move to the Golgi apparatus, where apo B, phospholipid and cholesterol are added to form completed VLDL for secretion. These results are consistent with the observations of other investigators on HEP-G2 cells and chick liver cells (Siuto-Mangano *et al.*, 1982; Janero & Lane, 1983; Böstrom *et al.*, 1988). There is also evidence that some newly synthesized apo B remains associated with the endoplasmic-reticulum membrane rather than moving into the cisternal space (Borchardt & Davis, 1987; Wong & Pino, 1987; Böstrom *et al.*, 1988; Bamberger & Lane, 1988; Davis *et al.*, 1990; Cartwright & Higgins, 1992).

Most investigations of VLDL metabolism in liver have been on rats. Although many of the details of synthesis, assembly and secretion of VLDL are probably fundamental to all mammalian liver, there are significant differences in hepatic lipoprotein metabolism between the rat and human. Of small laboratory animals the rabbit more closely resembles human. Both apo B100 and apo B48 are secreted by rat liver, whereas only the former is secreted by rabbit and human liver (Gherardi *et al.*, 1988). Atherosclerosis is difficult to produce in laboratory rats, but can be induced in rabbits, and hypercholesterolaemic rabbits have been used as a model for atherosclerosis in human. For these reasons we have now turned to the rabbit as an experimental animal. In the present investigation we have developed a quantitative assay for apo B in the subcellular fractions and used this to determine the intracellular distribution of apo B in rabbit liver.

METHODS AND MATERIALS

Animals

Dwarf lop-ear or small New Zealand White rabbits (approx. 2 kg) were used for all studies. These were bred and housed in the University field laboratories and fed on normal rabbit pellets. For most experiments the animals were used between 09:00 and 10:00 h.

Materials

Sac-cel was purchased from IDS (Washington, Tyne and Wear, U.K.); [^{3}H]leucine and ^{125}I were purchased from Amersham; other chemicals and reagents were from Sigma.

Antibodies

Primary polyclonal antibodies used for these studies were sheep anti-(rabbit apo B) raised against rabbit LDL (fraction *d* 1.019–1.063) (Gherardi *et al.*, 1988) and sheep anti-(human apo B) (Boehringher). Double immunodiffusion and immunoblotting against serum lipoprotein fractions demonstrated that both

Abbreviations used: VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins; apo B, apolipoprotein B; TBS, Tris-buffered saline; r.i.a., radioimmunoassay.

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antisera were specific for apo B. MAC 31, a monoclonal antibody against apo B, was also used in some experiments (Gherardi *et al.*, 1988). Secondary antibodies were donkey anti-(sheep IgG) coupled to alkaline phosphatase for studies with polyclonal antisera, or goat anti-(rat IgG) coupled to alkaline phosphatase for studies with monoclonal antibodies.

Preparation of subcellular fractions

Total microsomes, rough microsomes, smooth microsomes, Golgi *trans*-enriched and Golgi *cis*-enriched fractions were prepared from rabbit liver by the methods previously described for rat liver (Higgins, 1984; Higgins & Hutson, 1984; Higgins & Fieldsend, 1987), with the modification that a cocktail of proteinase inhibitors was added to the homogenate and the subcellular fractions to minimize proteolysis (Cartwright & Higgins, 1992). Each rabbit liver, approx. 60 g in weight, was divided into two parts, 20 g for preparation of microsomal fractions and 40 g for preparation of Golgi fractions. All subsequent steps were as described previously for rat liver. The subcellular fractions were suspended in Tris-buffered saline (TBS) or water and were either used immediately for electrophoresis and immunoblotting or stored at −20 °C for further analysis.

The subcellular fractions which consist of closed vesicles containing VLDL precursors and secretory proteins were subfractionated into membrane and cisternal-content fractions by treatment with 100 mM-Na_2CO_3, pH 11, as described previously (Howell & Palade, 1982; Fujiki *et al*, 1982; Higgins & Hutson, 1984). For electrophoresis, the content fractions were concentrated 10–20-fold by using Centricon 30 micro-concentrator tubes (Amicon). For radioimmunoassay (r.i.a.), the content fractions were dialysed overnight at 4 °C against 150 mM-NaCl/0.24 mM-EDTA, pH 7.4, before concentration.

In some experiments [^{3}H]leucine (100 μCi) or ^{125}I-LDL (see below) in 0.1 ml of 0.14 M-NaCl was injected into the portal vein of rabbits 15–30 min before exsanguination and removal of the liver for subcellular fractionation.

Assay of marker enzymes

NADPH–cytochrome *c* reductase was used as a marker for endoplasmic reticulum, and UDP-galactose galactosyltransferase was used as a marker for Golgi membranes (Higgins, 1976, 1984; Higgins & Fieldsend, 1987). Protein was determined by the method of Lowry *et al.* (1951).

Preparation of lipoprotein standards

Rabbit blood was taken by exsanguination or by bleeding from the ear vein. Phenylmethanesulphonyl fluoride (20 μM), EDTA (100 μM) and NaN_3 (1.5 μM) were added, the red cells were pelleted by centrifugation at 4000 rev./min for 10 min, and lipoprotein fractions were isolated immediately from the plasma. Inhibitors of proteolysis were added after each centrifugation step.

LDL fractions. Three (*d* 1.019–1.063), which were used as standards for electrophoresis or for r.i.a., were isolated by sequential flotation in an ultracentrifuge (24 h at 150000 ***g*** in the SW41 rotor) after adjusting the density of the plasma with KBr (Havel *et al.*, 1955; Radding & Steinberg, 1960). The top 1.0 ml, which contained LDL, was removed and pooled, readjusted to $d = 1.063$ with solid KBr, overlaid with buffered KBr (*d* 1.063) and re-centrifuged. The LDL was removed and dialysed overnight at 4 °C against 150 mM-NaCl/0.24 mM-EDTA, pH 7.4, divided into batches and stored under nitrogen at 7 °C after addition of NaN_3. Electrophoresis or immunoblotting of the LDL preparation showed only one major band corresponding to apo B-100, and a very faint band corresponding to apo E (Gherhardi *et al.*, 1988). The stability of the preparations was poor compared with human or rat LDL; even in the presence of proteinase inhibitors the apo B-100 was degraded slowly after storage for about 2 weeks.

^{125}I-labelled LDL. These were prepared as described by MacFarlane (1958) as modified by Bilheimer *et al.* (1972) and Goldstein *et al.* (1983). Incorporation of the radiolabel into LDL lipid was about 20%, higher than found when either human or rat LDL was radioiodinated. Labelling of the lipid could not be decreased by modification of the methods or by using the lactoperoxidase method. Rabbit LDL apo B was more susceptible to aggregation after radioiodination than was either human or rat LDL apo B, and on SDS/PAGE the apo B formed two bands, one of which ran above the apo B marker. Some 70–80% of the radiolabel incorporated into the LDL was in the two apo B bands, and the remainder was in the lipid which moved to the bottom of the gradient (Phillips & Timko, 1972).

Total plasma lipoproteins. These ($d < 1.21$) were isolated by ultracentrifugation as above. Only apo B100 was detected in total plasma lipoproteins of LDL from normal rabbits. To obtain an apo B48 standard in addition to apo B100, total lipoproteins were prepared from plasma from hyperlipaemic rabbits.

Electrophoresis

After determination of the protein content by the method of Lowry *et al.* (1951) modified by addition of 1% SDS to reagent B (Markwell *et al.*, 1978), lipoproteins, subcellular fractions and membrane and content fractions were mixed with at least an equivalent volume of sample buffer [30 mM-Tris, 3 mM-EDTA, 3% (w/v) SDS, 15% (v/v) glycerol, pH 6.5, with 15% mercaptoethanol added immediatedly before use] and solubilized by incubation at 60 °C for 15 min. Samples containing 50–75 μg of subcellular-fraction protein and 10 μg of LDL or total lipoprotein protein (apo-B standards) were applied to gradient gels (3–20%) of polyacrylamide. The proteins were separated at constant voltage (70 V) for 20 h by using a Hoeffer water-cooled vertical-electrophoresis apparatus. Some gels were subsequently stained with Coomassie Blue or silver stain; the proteins from others were transferred on to nitrocellulose membranes by using a Bio-Rad transblot cell with heat exchanger at 500 mA for 60 min. For electrotransfer the gel was trimmed and washed in blotting buffer (Tris/glycine, pH 7.4, containing 20% methanol) and put into a sandwich made up of filter paper soaked in 0.5% SDS, the gel, nitrocellulose membrane, and filter paper soaked in blotting buffer. The efficiency of the electrotransfer was checked in preliminary experiments by staining the gels after transfer with Coomassie Blue.

Immunostaining of apo B

After transfer of the proteins, the nitrocellulose membrane was blocked by washing overnight in Tris-buffered saline containing 0.5% Tween (TTBS) and 5% (w/v) crystalline BSA, followed by the primary antibody diluted 1:1000 in TTBS containing 0.5% BSA overnight. The membranes were washed for 30 min each with three changes of TTBS. The membranes were then incubated overnight with the secondary antibody complexed to alkaline phosphatase diluted 1:1000 in TTBS containing 0.5% crystalline BSA. The membranes were washed with three changes of buffer as above, followed by a final wash in veronal buffer (150 mM, pH 9.6) for 15 min. The alkaline phosphatase was then detected by colour development in Nitro Blue Tetrazolium (0.1 mg/ml), 5-bromo-4-chloro-3-indolyl phosphate (0.05 mg/ml) and $MgCl_2$ (50 mM) in veronal buffer.

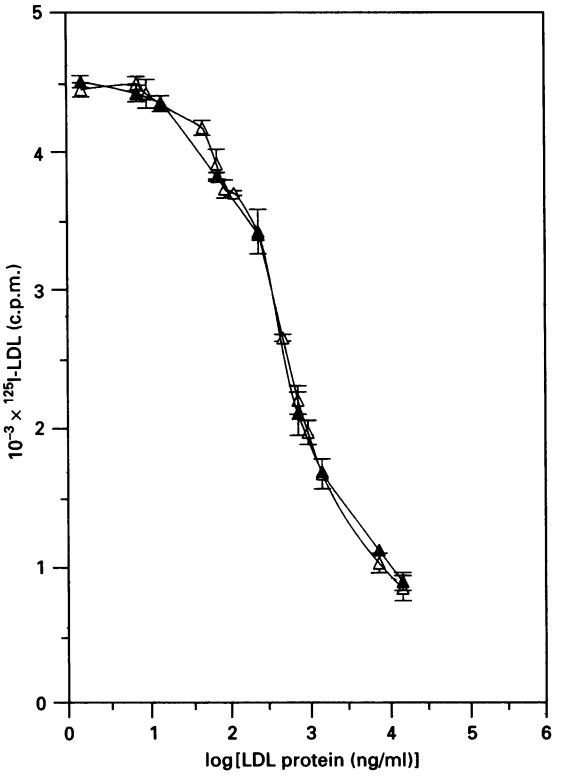

Fig. 1. Competition curve for assay of LDL-apo B by r.i.a.

R.i.a. competition curves were generated as described in the Methods and materials section. ^{125}I-LDL (c.p.m.) precipitated is plotted against the log of LDL protein concentration (ng/ml of LDL preparation). Non-specific binding (radioactivity precipitated in the absence of primary antibody) was about 500 c.p.m. The total radioactivity in the LDL protein was about 8000 c.p.m. The values plotted are means ± S.D.: △, in the absence of detergent; ▲, in the presence of detergent.

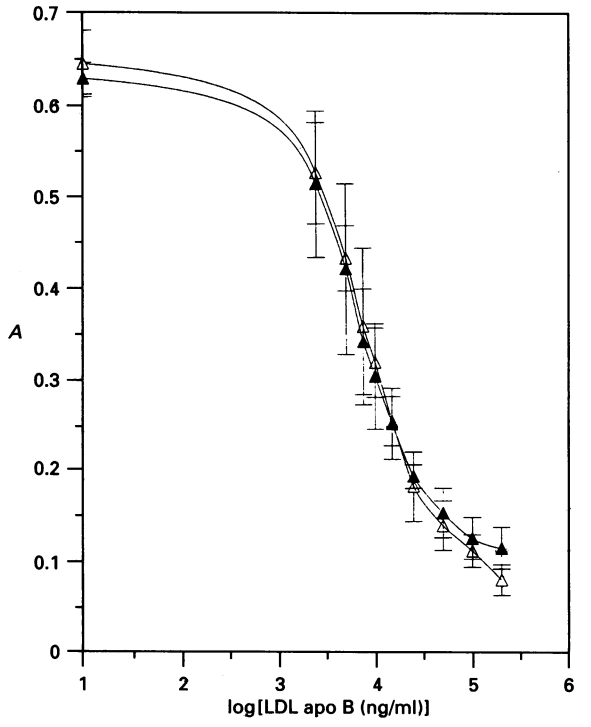

Fig. 2. Competion curve for assay of LDL apo B by e.l.i.s.a.

E.l.i.s.a. competition curves were generated as described in the Methods and materials section. A_{405} is plotted against the log of the concentration of LDL apo B (ng/ml of LDL preparation). Values plotted are means ± S.D.: △, in the presence of detergent; ▲, in the absence of detergent.

Radioimmunoassay

The r.i.a. was based on competition between ^{125}I-LDL and either unlabelled LDL or subcellular fractions for a limited amount of anti-apo B antibody. In all the results reported the antibody used was sheep anti-(rabbit apo B). However, exactly similar results were obtained with the commercial sheep anti-(human apo B) antibody.

Antibody dilution curves. These were prepared for each batch of antibody to establish appropriate concentrations for the assay. ^{125}I-LDL (sp. radioactivity 240 c.p.m./ng) were diluted in r.i.a. buffer (50 mM-barbituric acid/barbitone, pH 8.6, containing 3% r.i.a.-grade BSA, 0.01% EDTA and 0.1% NaN_3) to approx. 10000 c.p.m./100 μl. Then 100 μl of LDL, 50 μl of r.i.a. buffer and 100 μl of antibody (diluted between 1:100 and 1:300000) were mixed and incubated at 4 °C for 18 h in r.i.a. tubes. Next 200 μl of a suspension of Sac-Cel [donkey anti-(sheep IgG) linked to cellulose beads] was added, and the tubes were left for 30 min at room temperature. The Sac-Cel was pelleted by centrifuging at 3000 *g* for 15 min, the supernatant was carefully aspirated and the tubes were counted for radioactivity in a γ-counter.

Competition curves. These were generated in the same way as antibody-dilution curves, by using 100 μl of the appropriate concentration of antibody, 100 μl of ^{125}I-LDL and 50 μl containing LDL at a range of concentrations (0.03–300 μg of protein/ml). Competition curves were also generated in which 0.5% Triton X-100 and 0.5% deoxycholate were added to the r.i.a. buffer. Similar curves were obtained in the presence or absence of detergent (Fig. 1).

R.i.a. of apo B in subcellular fractions. This was performed in the same way, except that r.i.a. buffer containing 0.5% Triton X-100 and 0.5% deoxycholate was used throughout to solubilize the membranes. The assay contained 100 μl of ^{125}I-LDL, 100 μl of antibody and 50 μl of subcellular fraction in r.i.a. buffer. A range of concentrations of subcellular fractions (0.5–3 mg of protein/ml and dilutions prepared from these (up to 20-fold in r.i.a. buffer) were used for each assay to ensure that the apo B content lay on the linear part of the competition curve. LDL apo B competition curves were always generated at the same time as the subcellular fractions were assayed.

E.l.i.s.a.

LDL (0.53 μg of apo B in $Na_2CO_3/NaHCO_3$ buffer, pH 9.6/well) were placed in e.l.i.s.a. plates (Nunc), which were sealed with Clingfilm and refrigerated overnight. The wells were washed four times with wash buffer [TBS (50 mM-Tris/200 mM-NaCl, pH 7.4) containing 0.05% (v/v) Tween 20 and 0.5% (w/v) r.i.a. grade BSA] and blocked for 2 h with TBS containing 3% BSA. The wells were aspirated, and 100 μl of antibody (dilutions up to 1:200000) in TBS containing 1% BSA were added together with 50 μl of TBS or 50 μl of LDL (0.1–150 μg/ml) or 50 μl of microsomes and incubated overnight with shaking. The wells were washed four times with wash buffer, and 150 μl of secondary antibody diluted 1:1000 in TBS containing 1% BSA was added. Plates were incubated for 4 h and washed four times with wash buffer, followed by a wash with 0.1 M-glycine buffer, pH 10.4, containing 1 mM-$MgCl_2$ and 1 mM-$ZnCl_2$. Alkaline phosphatase bound was measured with *p*-nitrophenyl phosphate as substrate; the reaction was terminated by addition of 50 μl of 0.1 M-EDTA

Table 1. Specific activities of marker enzymes of rabbit subcellular fractions

Subcellular fractions were prepared as described in the Methods and materials section. The protein recovery (mg/g of liver) was determined for each fraction, and the specific activities of UDP-galactose galactosyltransferase (nmol of galactose transferred/min per mg of protein) and NADPH–cytochrome *c* reductase (nmol of cytochrome *c* reduced/min per mg of protein) were determined in each fraction. The results are expressed as means ± S.D. The enrichment is the activity in the fraction divided by that of the homogenate; the yield is the total activity recovered in each fraction as a percentage of that in the homogenate.

Fraction	Protein (mg/g of liver)	Galactosyltransferase activity			NADPH–cytochrome *c* reductase		
		Sp. activity	Enrichment	Yield	Sp. activity	Enrichment	Yield
Homogenate	182.14 ± 36.5 (5)	0.017 ± 0.003			2.37 ± 0.031		
Microsomes	11.93 ± 1.91 (9)	0.038 ± 0.014	2.28	14.96	10.22 ± 3.10	4.31	28.30
Rough microsomes	1.42 ± 0.44 (9)	0.043 ± 0.014	2.57	2.01	10.30 ± 3.16	4.34	3.37
Smooth microsomes	5.92 ± 2.03 (9)	0.143 ± 0.037	8.56	27.8	8.23 ± 0.78	3.37	11.31
Golgi-*trans*	0.24 ± 0.09 (8)	0.611 ± 0.120	36.60	7.69	0.70 ± 0.19	0.42	0.04
Golgi-*cis*	2.07 ± 0.70 (8)	0.231 ± 0.059	12.75	14.51	3.12 ± 1.06	1.32	1.49

after 30 min and read at 405 nm in an LKB plate reader. A competition curve using a range of LDL concentrations (0.0015–150 μg/ml) was performed on each plate, together with assays of subcellular fractions. In some experiments 0.4% taurocholate was added to the incubation to open the membrane vesicles (Arion *et al.*, 1976). Exactly similar curves were obtained in the presence and absence of taurocholate (Fig. 2).

RESULTS AND DISCUSSION

Subcellular fractions from rabbit liver

The methods used for preparation of subcellular fractions from rabbit liver were essentially the same as those used previously for rat liver by ourselves and other investigators. The fractionation procedures when applied to rabbit liver behaved in the same way as those applied to rat liver, except that yields of microsomal fractions as mg of protein per g of liver, particularly of the rough microsomes, were lower (Table 1).

The specific activity of the Golgi marker UDP-galactose galactosyltransferase was enriched in the *trans*-Golgi fraction by approx. 40-fold, with a recovery of about 8%. This Golgi fraction contained only 0.04% of the endoplasmic-reticulum marker NADPH–cytochrome *c* reductase (Table 1). The total microsomal fraction contained 15% of the Golgi marker enzyme, although this was only enriched about 2-fold compared with the total homogenate. The quantity of endoplasmic-reticulum membrane in liver cells is at least an order of magnitude greater than the quantity of Golgi membranes. The contribution of Golgi membranes to the endoplasmic-reticulum fractions is therefore small. The microsomal fractions were enriched with the endoplasmic-reticulum marker NADPH–cytochrome *c* reductase approx. 4-fold (Table 1).

In the procedure used for isolation of Golgi membranes, a *cis*-enriched fraction is obtained in addition to the *trans*-enriched fraction. This is a less pure fraction containing 14% of the *trans*-Golgi marker with an enrichment of 12-fold, and contains 1.4% of the endoplasmic-reticulum marker with an enrichment of 1.3-fold. The identification of the *trans*-enriched and *cis*-enriched fractions was made first by Erhrenreich *et al.* (1973) on the basis of morphological appearance, and later on the basis of the function of these fractions in glycosylation of secreted proteins (Bergeron, 1979; Roth & Berger, 1982; Roth *et al.*, 1985). Futterman *et al.* (1990) have confirmed this identification, using immunoblotting with anti-mannosidase II as a *cis*-element marker and sialyltransferase as a *trans*-element marker.

Separation of vesicular subcellular fractions into membranes and cisternal contents

Approx. 50% of the total protein and 60–70% of the secretory proteins labelled by intraportal injection of [^{3}H]leucine (Kreibich *et al.*, 1973) were released into the content fraction by Na_2CO_3 treatment (Higgins & Hutson, 1984; Higgins & Fieldsend, 1987). Similar amounts of labelled protein were also released when the vesicles were treated with 0.4% taurocholate, an alternative and more drastic way of opening membrane vesicles. These results are similar to those obtained when rat liver vesicular fractions were separated into membrane and content fractions (Higgins & Hutson, 1984; Howell & Palade, 1982; Fujiki *et al.*, 1982).

Identification of apo B in subcellular fractions

The major immunostaining band of total microsomes, rough microsomes, smooth microsomes, *trans*-enriched Golgi and *cis*-enriched Golgi and membrane and content fractions was apo B100, with the greatest amount apparent in the *trans*-Golgi fraction (Fig. 3). In addition to apo B100 there was a faint immunostaining band corresponding in mobility to apo B48 in total, membrane and content fractions. Similar results were obtained by using MAC 31, a primary antibody which recognizes an epitope on both apo B48 and apo B100 (Gherardi *et al.*, 1988) (Fig. 4). This band was very faint, but does suggest that apo B48 may be produced in small amounts in rabbit liver, although it is not present in LDL. Membrane and total fractions, particularly of the Golgi fractions, also showed other faint immunoreactive bands of faster mobility than apo B. These were not visible in the content fractions, suggesting that apo B degradation may occur in the membranes of the subcellular fractions.

Quantification of apo B in rabbit subcellular fractions

R.i.a. The apo B contents of the total, rough- and smooth-microsome fractions measured by r.i.a. were 1.8, 1.6 and 2.4 μg of apo B/mg of fraction protein respectively. The Golgi fractions contained more apo B, with 13 μg/mg of protein of the *trans*-Golgi fraction and 3 μg/mg of protein of the *cis*-Golgi fraction (Table 2). By using marker enzymes as internal standards, it can be calculated that there are 66 μg of apo B100 in the endoplasmic reticulum from 1 g of liver (NADPH–cytochrome *c* reductase-containing elements, corrected for Golgi contamination) and 39 μg of apo B100 in the Golgi fraction (UDP-galactose galactosyltransferase-containing elements) from 1 g of liver. The homogenate contains 100 μg of apo B/g of liver. All of the apo

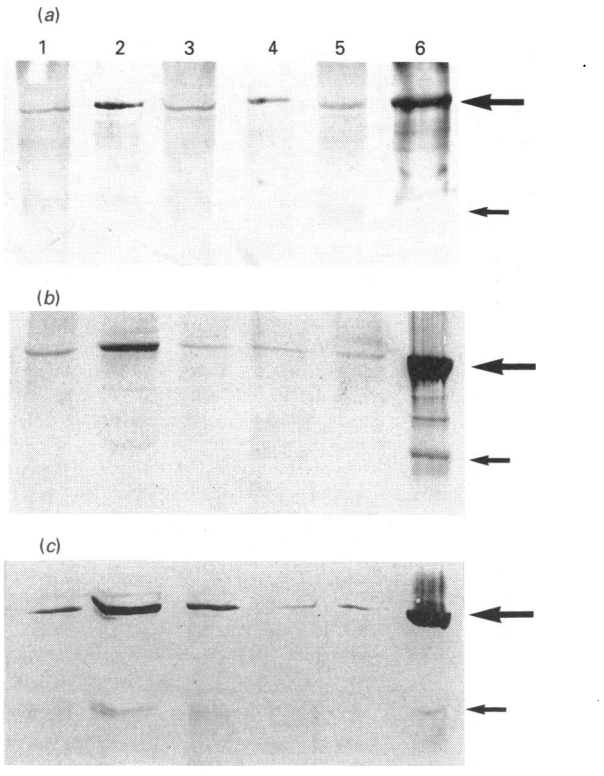

Fig. 3. Detection of apo B in subcellular fractions of rabbit liver using a polyclonal antiserum

Subcellular fractions (total microsomes, rough microsomes, smooth microsomes, *trans*-enriched Golgi and *cis*-enriched Golgi) and membrane and cisternal-content fractions from these were prepared as described in the Methods and materials section. The proteins (75 μg) were separated by SDS/PAGE on 3–20% gradient gels and electrotransferred on to nitrocellulose membranes. Apo B was detected by immunoblotting using sheep anti-(rabbit apo B) as primary antibody as described in the Methods and materials section. (*a*) Total subcellular fractions; (*b*) membrane fractions; (*c*) cisternal-content fractions. Large arrows indicate apo B100 and small arrows apo B48. Lanes: 1, *cis*-enriched Golgi; 2, *trans*-enriched Golgi; 3, smooth microsomes; 4, rough microsomes; 5, total microsomes; 6, total lipoproteins from hypercholesterolaemic-rabbit serum.

Table 2. Apo B content of rabbit liver subcellular fractions

Subcellular fractions were prepared, and the apo B content of each fraction was determined by r.i.a. or e.l.i.s.a., as described in the Methods and materials section. A range of concentrations was assayed to ensure linearity. The results are expressed as means ± S.D. of determinations on separate fractions (no. of determinations).

Fraction	Apo B (μg/mg of protein)	
	R.i.a.	E.l.i.s.a.
Homogenate	0.548 ± 0.04 (6)	0.568 ± 0.06 (8)
Microsomes	1.839 ± 0.35 (9)	2.340 ± 0.23 (8)
Rough microsomes	1.653 ± 0.32 (8)	1.255 ± 0.03 (5)
Smooth microsomes	2.434 ± 0.37 (8)	2.244 ± 0.23 (8)
Golgi-*trans*	12.930 ± 0.64 (18)	11.850 ± 0.94 (5)
Golgi-*cis*	3.000 ± 0.80 (6)	2.767 ± 0.28 (8)

B of the whole liver homogenate is therefore recovered in the two secretory compartments, with approximately two-thirds of the total apo B in the endoplasmic reticulum. The distribution of apo

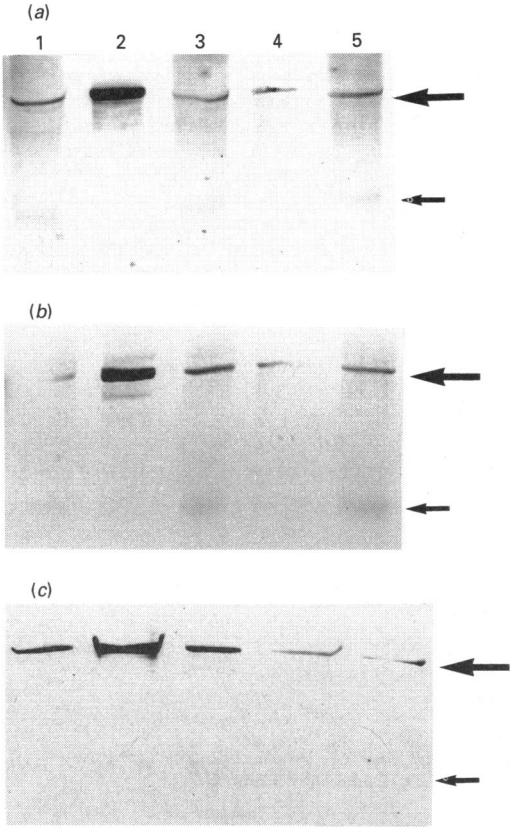

Fig. 4. Detection of apo B in subcellular fractions of rabbit liver by immunoblotting using MAC 31 as a monoclonal antibody against apo B

Subcellular fractions (total microsomes, rough microsomes, smooth microsomes, *trans*-enriched Golgi and *cis*-enriched Golgi) and membrane and cisternal-content fractions from these were prepared as described in the Methods and materials section. The proteins (75 μg) were separated by SDS/PAGE on 3–20% gradient gels and electrotransferred on to nitrocellulose membranes. Apo B was detected by immunoblotting using MAC 31 as primary antibody as described in the Methods and materials section. (*a*) Total subcellular fractions; (*b*) membrane fractions; (*c*) cisternal-content fractions. Long arrows indicate apo B100 and small arrows apo B48. Lanes: 1, *cis*-enriched Golgi; 2, *trans*-enriched Golgi; 3, smooth microsomes; 4, rough microsomes; 5, total microsomes.

B was consistent with the amount of apo B detected by immunoblotting in Figs. 3 and 4.

E.l.i.s.a. The amount of apo B in each subcellular fraction was similar when determined by e.l.i.s.a. (Table 2).

Distribution of apo B between membrane and content fractions of subcellular fractions

When subcellular fractions were opened to release the vesicular contents, approx. 40% of the apo B of the rough microsomes, 50% of the apo B of the smooth microsomes, 40% of the apo B of the *cis*-Golgi and 70% of the apo B of the *trans*-Golgi were released (Table 3). In each fraction therefore a significant amount of apo B remained membrane-bound.

We are not aware of any other studies of the distribution of apo B in rabbit subcellular fractions. However, there have been several previous reports from studies of rat liver and HEP-G2 cells suggesting that newly synthesized apo B may remain associated with the endoplasmic-reticulum membrane (Borchardt & Davis, 1987; Wong & Pino, 1987; Bamberger & Lane, 1988; Böstrom *et al.*, 1988; Cartwright & Higgins, 1992). Our studies

Table 3. Distribution of apo B in membrane and contents subfractions of subcellular fractions

Subcellular fractions were prepared, and the vesicular fractions were opened by treatment with Na_2CO_3 and membrane and contents fractions were prepared, as described in the Methods and materials section. The apo B content of each subfraction was determined by r.i.a. The results are expressed as μg of apo B/mg of the original unopened subcellular fraction, as means ± S.D. (no. of determinations). In column 3 the apo B in the contents fraction as a percentage of that recovered in membranes and contents is given, and in column 4 the apo B recovered in the membrane and contents fractions as a percentage of that in the original unopened fraction is given.

Fraction	Apo B (μg/mg of original fraction)		Percentage in contents	Recovery (%)
	Membrane	Contents		
Total microsomes	0.76 ± 0.21 (9)	0.81 ± 0.14 (7)	51.4	85.5
Rough microsomes	1.03 ± 0.14 (7)	0.71 ± 0.23 (4)	40.9	105.7
Smooth microsomes	1.01 ± 0.28 (8)	1.01 ± 0.34 (6)	50.1	82.8
Golgi-*trans*	2.54 ± 0.24 (14)	7.81 ± 1.33 (18)	68.8	87.8
Golgi-*cis*	1.48 ± 0.36 (12)	1.00 ± 0.58 (5)	40.32	82.6

Table 4. Incorporation of intraperitoneally injected ^{125}I-LDL into subcellular fractions of rabbit liver

^{125}I-LDL (about 500000 d.p.m.) was injected into the portal vein of anaesthetized rabbits 30 min before removal of the liver and preparation of subcellular fractions; 15% of the injected radioactivity was in the whole liver. Subcellular fractions were isolated as described in the Methods and materials section, and were counted for radioactivity with a γ-counter. The results are means ± S.D. of three determinations: 'Percentage recovered' is the radioactivity (d.p.m.) recovered in each subcellular fraction as a percentage of that in the total homogenate. 'Enrichment' is the radioactivity (d.p.m./mg of protein) in each subcellular fraction divided by that in the homogenate.

Fraction	Incorporation (d.p.m./mg of protein)	Percentage recovered in subcellular fraction	Enrichment
Homogenate	4.91 ± 0.2		
Total microsomes	9.19 ± 0.31	18.35 ± 0.61	1.87
Rough microsomes	9.80 ± 1.65	2.88 ± 0.04	2.4
Smooth microsomes	13.31 ± 0.42	11.94 ± 1.4	2.79
trans-Enriched Golgi	48.79 ± 10.76	1.23 ± 0.16	9.94
cis-Enriched Golgi	17.72 ± 2.56	6.65 ± 0.63	3.61

are the first to quantify the pool sizes and to indicate that a relatively large fraction of apo B in the Golgi region is also membrane-bound rather than in the cisternal space. The distribution of apo B 100 differs quantitatively from that in the rat, in which 90% of the apo B in the endoplasmic reticulum and 30% of that in the *trans*-Golgi were membrane-bound (Cartwright & Higgins, 1992).

Investigation of the contamination of subcellular fractions with endocytic vesicles

While this work was in progress, Hamilton *et al.* (1991) reported that Golgi preparations prepared from rat liver by methods based on those of Erhrenreich *et al.* (1973) were grossly contaminated with endocytic vesicles. To determine whether endocytic vesicles contributed to our fractions, we injected ^{125}I-labelled LDL into rabbits before removal of the liver and isolation of the subcellular fractions (Table 4). After 15 min, the peak time for labelling endocytic vesicles (Evans, 1988), the recovery of ^{125}I label in the *trans*-enriched Golgi fraction was about 1% of that in the homogenate, with larger amounts in the *cis*-enriched fraction (6%), the smooth microsomes (11%) and the total microsomes (18%). The enrichment of fractions with ^{125}I was greatest in the *trans*-enriched fraction, at 10-fold. There is therefore some contamination of the *trans*-Golgi fraction, but this is less than one-fifth of that found by Hamilton *et al.* (1991) and Hornick *et al.* (1985). Hamilton *et al.* (1991) also found that more than 90% of the apo B taken up by endocytosis was released when the Golgi vesicles were opened. It is therefore possible that apo B taken up by endocytosis could contribute to the pool of apo B in the Golgi-content fraction, but not to that in the membrane-bound pool.

Table 5. Ability of membrane-bound apo B of subcellular fractions to compete with LDL-apo B in e.l.i.s.a.

The ability of apo B in each subcellular fraction to compete with immobilized LDL for a limiting amount of polyclonal antibody was determined by e.l.i.s.a. as described in the Methods and materials section. Closed vesicles were incubated in the absence of added detergent, and open vesicles were incubated in the presence of 0.5% taurocholate (Arion *et al.*, 1976). Competition is expressed as percentage inhibition of binding of antibody to immobilized LDL at a single concentration of fraction protein selected to give limited inhibition (rough microsomes, 0.18 mg; smooth microsomes, 1.38 mg; *trans*-Golgi, 0.032 mg; *cis*-Golgi, 0.206 mg). The actual percentage inhibition is that observed in the experiment. The expected percentage inhibition for closed vesicles is calculated from the apo B of each membrane fraction and that for opened vesicles from the apo B of each total fraction. Thus, if all of the membrane-bound apo B is exposed at the cytosolic surface, the expected inhibition in closed vesicles would be equal to the actual inhibition. The values given are averages of three separate assays on one preparation. Similar results were obtained on different preparations, although the percentage inhibition differed with added protein.

Fraction	Inhibition of binding (%)			
	Closed vesicles		Opened vesicles	
	Actual	Expected	Actual	Expected
Rough microsomes	15.43	22.09	28.53	31.00
Smooth microsomes	66.24	72.57	77.16	81.39
trans-Golgi	17.64	18.52	45.32	42.25
cis-Golgi	39.31	44.75	48.13	54.65

Topography of membrane-bound apo B in subcellular fractions

To probe the topography of the membrane-bound form of apo B, we have determined the ability of the protein in closed vesicles and in vesicles opened by using taurocholate (Arion *et al.*, 1976) to compete with immobilized LDL for polyclonal antibody in the e.l.i.s.a. assay (Table 5). Control experiments demonstrated that the subcellular fractions remained sealed. The vesicle-content proteins were labelled with [^{3}H]leucine, and there was no loss of the labelled protein during the e.l.i.s.a. incubation. The expected inhibition was calculated by assuming that all of the membrane-bound pool of apo B in closed vesicles is expressed at the cytosolic side of the membrane and that both membrane and content apo B are available in opened vesicles. Apo B in closed vesicles was able to compete with that of immobilized LDL by

between 70 and 100%. The membrane-bound form of apo B in both endoplasmic reticulum and Golgi fractions is therefore expressed at the cytosolic side of the subcellular fraction (Table 5).

Investigations of HEP-G2 cells have suggested that mRNA for apo B is constitutive and unchanged even when lipid secretion is increased up to 7-fold (Pullinger *et al.*, 1989). Recently it has been demonstrated that oleate stimulates apo B secretion from HEP-G2 cells and at the same time inhibits intracellular degradation of apo B (Dixon *et al.*, 1991). We have previously suggested that in rat liver apo B is packaged with lipid in the Golgi region and may be transported from the endoplasmic reticulum to the Golgi region in a membrane-associated form (Higgins, 1988). Recent investigations in which the *N*-terminal 15–17% of the mRNA for apo B was translated in cell-free systems have demonstrated that the newly synthesized truncated apo B is translocated across the endoplasmic-reticulum membrane and is glycosylated (Chuck *et al.*, 1990; Pease *et al.*, 1991). A large proportion of the apo B remains membrane-bound; however, these studies have produced conflicting results concerning the topography of bound apo B in relation to the endoplasmic-reticulum membrane (Chuck *et al.*, 1990; Pease *et al.*, 1991). Our observations suggest that the membrane-bound form of apo B is at the cytosolic side of the secretory compartment membranes. This is consistent with studies by Davis *et al.* (1990) which indicate that the apo B in rat liver microsomes is available for digestion by trypsin. An attractive model which takes all of these observations into account is that apo B is synthesized in excess and remains associated with the endoplasmic reticulum/Golgi membrane until packaged with lipid. Excess apo B may be degraded, possibly in a membrane-bound form. At present little is known of the intracellular sites or molecular details of association of apo B with membranes or of the mechanism of packaging of apo B with lipid. Nor is anything known of the potential site or regulation of apo B degradation, although the observations reported here suggest that the site of this degradation might be the Golgi membranes.

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