Quantification of apolipoprotein B-48 and B-100 in rat liver endoplasmic reticulum and Golgi fractions

Ian J. CARTWRIGHT and Joan A. HIGGINS*

Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

We have developed a method for measurement of apolipoprotein (apo) B-48 and apo B-100 in blood and subcellular fractions of rat liver based on SDS/PAGE followed by quantitative immunoblotting using ^{125}I -Protein A. Standard curves were prepared in each assay using apo B prepared from total rat lipoproteins by extraction with tetramethylurea. Subcellular fractions (rough and smooth endoplasmic reticulum and Golgi fractions) were prepared from rat liver and separated into membrane and cisternal-content fractions. For quantification, membrane fractions were solubilized in Triton X-100, and the apo B was immunoprecipitated before separation by SDS/PAGE and immunoblotting. Content fractions were concentrated by ultrafiltration and separated by SDS/PAGE without immunoprecipitation. Quantification of apo B in subcellular fractions and detection of apo B by immunoblotting yielded consistent results. In all fractions apo B-48 was the major form, accounting for approximately three-quarters of the total apo B. By using marker enzymes as internal standards, it was calculated that all of the apo B was recovered in the endoplasmic reticulum and Golgi fractions, with approximately 80 % of each form of apo B in the endoplasmic reticulum. More than 90 % of the apo B of the rough- and smooth-endoplasmic-reticulum fractions was membrane-bound, whereas approx. 33 and 15 % of the apo B of the *cis*-enriched Golgi fractions and *trans*-enriched Golgi fractions respectively were membrane-bound.

INTRODUCTION

Apolipoprotein B (apo B) is the major non-exchangeable apoprotein essential for secretion of very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) [1–4]. Apo B occurs in two forms differing in molecular mass: apo B-48, which is synthesized largely in the intestine in humans, and apo B-100, which is synthesized largely in liver. In the rat, both forms are synthesized and secreted by the liver. Apo B-48 and apo B-100 are products of the same gene. Apo B-48 is a truncated form of apo B-100 and produced through the post-transcriptional modification of the mRNA for apo B-100 by a single base change, producing a stop codon at position 2153 [5–7]. This is an important modification, because the truncated apo B-48 lacks the binding site for the apo B/E (LDL) receptor [3,4]. In addition, secretion of apo B-48 and apo B-100 by rat liver appear to be independently regulated by nutritional and developmental factors [2–8].

We have previously investigated the intracellular events in the synthesis, assembly and secretion of VLDL by rat liver [9–11]. Briefly, these studies have suggested that a triacylglycerol-rich precursor with a low phospholipid, cholesterol and apo B content is formed in the cisternae of the endoplasmic reticulum. These particles move to the Golgi region, where they are assembled with apo B, phospholipid and cholesterol to form completed VLDL for secretion. Several investigations, including our own, suggest that apo B does not behave like a secreted protein such as albumin [12–16]. At least a part of the newly formed apo B is associated with the endoplasmic reticulum rather than released into the cisternal space. There have been no previous investigations to determine the intracellular distribution and the pool sizes of apo B, although such information is necessary for investigations of the mechanisms involved in assembly of apo B with lipid and its regulation. In the present study we have developed a quantitative method for assay of apo B-48 and apo B-100 in subcellular fractions.

METHODS

Animals

For all studies male Sprague–Dawley rats (150–300 g body wt.) were used. These were housed in the University Animal House and fed standard rat chow. For most experiments the animals were used between 09.00 and 10.00 h.

Materials

Sheep-anti-(human apo B) serum was purchased from Boehringer. By using radial immunodiffusion or immunoblotting after SDS/PAGE, the antiserum was found to cross-react with rat apo B [11]. Donkey anti-(sheep IgG) serum and non-immune sheep serum were purchased from Scottish Antibodies, Law Hospital, Carlisle, Lanarkshire, Scotland, U.K. Biotinylated anti-(sheep IgG) serum and avidin–alkaline phosphatase were purchased as a kit from Vector Labs, Peterborough, U.K. ('ABC reagents'). Other chemicals were purchased from Sigma. ^{125}I -Protein A was purchased from Amersham.

Preparation of subcellular fractions

Total microsomes, rough microsomes, smooth microsomes, Golgi *trans*-enriched and Golgi *cis*-enriched fractions were prepared from rat liver as described previously [9,10,17], with the modification that a cocktail of proteinase inhibitors was added to the homogenate and subcellular fractions to minimize proteolysis [pepstatin (0.5 mg), leupeptin (5.0 mg), chymostatin (5.0 mg), antipain (5.0 mg) and aprotinin (5.0 mg) dissolved in 10 ml of water and used at a final dilution of 1:100; phenylmethanesulphonyl fluoride (PMSF) in ethanol was added to give a final concentration of 6 μM]. The vesicular fractions were separated into membrane- and cisternal-content fractions by treatment with Na_2CO_3 as described previously [10]. The enrichment and recoveries of marker enzymes were determined as described previously, using glucose-6-phosphatase for endoplasmic reti-

Abbreviations used: apo, apolipoprotein; VLDL and LDL, very-low- and low-density lipoproteins; PMSF, phenylmethanesulphonyl fluoride; TMU, tetramethylurea; TTBS, Tris-buffered saline containing 0.5 % Tween.

* To whom correspondence should be sent.

culum, galactosyltransferase for Golgi and 5' nucleotidase for plasma membrane.

Immunoprecipitation of apo B from subcellular fractions

Aliquots of total liver homogenate, subcellular fractions and membrane fractions, each equivalent to 0.5 g of starting liver, were diluted to 0.5 ml with Tris buffer (10 mM-Tris/150 mM-NaCl, pH 7.4) and mixed with an equal volume of solubilization buffer (10 mM-sodium phosphate buffer/140 mM-NaCl, pH 7.4, containing 0.5% sodium deoxycholate and 0.5% Triton X-100). Samples were then mixed with 0.2 ml of primary antibody [sheep anti-(human apo B) serum diluted 1:50 with Tris buffer] and left for 1–2 h at 4 °C. This was followed by the addition of 0.4 ml of secondary antibody [donkey anti-(sheep IgG) serum diluted 1:5 in Tris buffer] and the samples left overnight at 4 °C. The immunoprecipitate was isolated by centrifugation at 3500 g for 10 min. In some experiments both the immunoprecipitate pellet and the supernatant were retained for analysis. The immunoprecipitation technique was not satisfactory when applied to the cisternal-content fractions. Instead, aliquots of these equivalent to 3 g of starting liver were concentrated to 1.0 ml with Centricon 30 microconcentrator tubes (Amicon) and used directly without immunoprecipitation. In some experiments to compare the membrane and content fractions using the same procedures the membrane fractions were dissolved in sample buffer directly without immunoprecipitation. Although there was less apo B in the samples applied in this case, the same results were obtained in terms of apo B per mg of membrane protein.

Preparation of apo B standards

Rat blood was taken by exsanguination. To minimize proteolysis PMSF (20 μ M) and EDTA (100 μ M) were added. The red cells were pelleted by centrifugation at 4000 rev./min (r_{av} , 12 cm) for 10 min and NaN_3 (1.5 μ M) added to the plasma. The relative density of the serum was adjusted to 1.21 with solid KBr, and total lipoproteins were floated by centrifugation for 24 h at 30000 g. Solid NaCl was added to the total lipoprotein solution to a final concentration of 0.195 M, and the apo B was precipitated by addition of an equal volume of tetramethylurea (TMU), followed by mixing for 1 min. The apo B was pelleted by centrifugation at 3500 rev./min for 5 min, washed in 2 ml of distilled water, dissolved in sample buffer (see below) and stored at –20 °C for up to 2 months without detectable degradation.

Preparation of samples for electrophoresis

Immunoprecipitates of apo B and their supernatants from total liver homogenates, vesicular fractions and membrane subfractions and the concentrated cisternal content fractions were delipidated with ethanol/diethyl ether (3:2, v/v) followed by two changes of diethyl ether. The precipitates were dissolved in sample buffer [25 mM-Tris (pH 6.5)/5 mM-EDTA/10 mM-dithiothreitol/10% (w/v) SDS], boiled for 1 min, and iodoacetic acid was added to a final concentration of 50 mM. Apo B standards were prepared in the same way, except that the delipidation step was omitted. All samples were then assayed for protein content by the method of Lowry *et al.* [18], with BSA as standard.

Electrophoresis and electrotransfer of apo B

Aliquots of apo B standard (1–40 μ g of protein), immunoprecipitates and supernatants from subcellular fractions (100 μ g

of protein) in sample buffer were separated on gradient gels (3–20%, w/v) of polyacrylamide using a constant voltage (70 V) for 20 h. In some experiments gels were Coomassie Blue- or silver-stained. In others the proteins were transferred from the gels on to nitrocellulose membranes by using a Bio-Rad Transblot Cell with a heat exchanger at 500 mA for 60 min. For transfer the gel was trimmed and washed in the blotting buffer [Tris/glycine, pH 7.4, containing 20% (v/v) 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 the blotting buffer. The efficiency of the electrotransfer of apo B was checked by staining the gels with Coomassie Blue after transfer.

Immunostaining of apo B

After electrotransfer the nitrocellulose was blocked by washing in Tris-buffered saline containing 0.5% Tween 20 (TTBS) and 2.5% (w/v) BSA for at least 2 h, followed by the primary antibody diluted 1:1000 in the same buffer for at least 1 h. The membranes were washed with three changes of TTBS, the last wash also containing 2.5% BSA (approx. 30 min each), followed by the secondary antibody complexed to biotin diluted 1:1000 in TTBS containing 0.1% crystalline BSA (at least 1 h). The membranes were washed with three changes of the buffer as described above, followed by the Vector ABC avidin-alkaline phosphatase reagent prepared at a dilution of 1:1000 (30 min). The membranes were washed again with three changes of buffer, followed by a final wash in Veronal buffer (150 mM, pH 9.6) for 15 min. The enzyme was detected by colour development in Nitro Blue Tetrazolium (0.1 mg/ml), 5-bromo-4-chloroindol-3-yl phosphate (0.05 mg/ml) and MgCl_2 (50 mM) in Veronal buffer.

Quantitative immunoblotting of apo B

Apo B was quantified in subcellular fractions by an immunoassay which employed ^{125}I -Protein A, essentially as described by Quinn *et al.* [20]. A standard curve was prepared by using a range of concentrations of the apo B standard (1–40 μ g of protein) separated on 3–20%-polyacrylamide gels. The amount of protein applied to the gel was determined in aliquots of the apo B preparation in sample buffer by using the Lowry method [18] with BSA in sample buffer as a standard. The amount of apo B-48 and B-100 in each sample was determined by separating known amounts of the apo B standard by SDS/PAGE. The apo B-48 and apo B-100 bands were sliced from the gel and dissolved in 1.0 ml of 30% (v/v) H_2O_2 . The samples were dried, dissolved in sample buffer, and protein was determined by the method of Lowry *et al.* [18] by using a standard curve prepared by running a range of concentrations of BSA on SDS/3–20%-PAGE gels and treating them in exactly the same way as the apo B samples. In total rat plasma lipoproteins, 57% of the total apo B was apo B-48 and 42% was apo B-100. Silver staining of gels confirmed that no other protein bands, except apo B-48 and apo B-100, were present when up to 50 μ g of the standard was separated by SDS/PAGE.

After separation of a range of concentrations of apo B standards by SDS/PAGE, apo B-48 and apo B-100 were electrotransferred on to nitrocellulose membranes. The membranes were stained with Amido Black (0.1%, w/v, in methanol/acetic acid/water, 9:2:9, by vol.) and destained in methanol/acetic acid/water (95:2:3, by vol.). After blocking overnight in TTBS containing 2.5% BSA, the nitrocellulose membranes were incubated with primary antibody (1:1000) for 2 h, washed with three changes of TTBS (the last containing 2.5% BSA) (30 min each), incubated with secondary antibody [donkey anti-(sheep IgG) serum diluted 1:500] for 2 h, and washed with three changes of TTBS (30 min each). The nitrocellulose membranes were then incubated for 24 h in 20–50 ml

of an immunoassay buffer [9% NaCl/10 mM-Tris/HCl (pH 7.4)/0.2% SDS/0.5% Triton X-100/0.5% BSA/0.01% NaN₃] containing 1 μ Ci of ¹²⁵I-Protein A. The membranes were washed five times (30 min each) with the immunoassay buffer and air-dried. The Amido Black-stained apo B-48 and apo B-100 bands were cut from the membrane and counted in a γ -radiation counter.

For the quantification of apo B in subcellular fractions, immunoprecipitates and supernatants of subcellular fractions and concentrated cisternal-content fractions were separated by SDS/PAGE followed by electrotransfer on to nitrocellulose and quantitative immunoblotting. For each assay of unknown samples a series of apo B standards were applied to each gel and a new standard curve prepared. This allowed for decay of the specific radioactivity of ¹²⁵I-Protein A and for any experimental variation in the procedures used.

RESULTS AND DISCUSSION

Preparation of a standard curve for assay of apo B-48 and apo B-100

Apo B prepared by TMU extraction of rat lipoproteins contained only two detectable bands apo B-48 and apo B-100 (Fig. 1). Apo B-48 contributed 57% to the total apo B and apo B-100 42%. This is a similar distribution to that reported by others for apo B of plasma lipoproteins [21]. Apo B-48 was

Fig. 1. Apo B-48 and apo B-100 of rat lipoproteins

Apo B was purified from total rat lipoproteins by TMU extraction, and a range of concentrations were applied to 3–20% gels and separated by SDS/PAGE as described in the Methods section. (a) Illustrates a silver-stained gel, and (b) shows proteins after electrotransfer on to nitrocellulose and immunoblotting as described in the Methods section. The amounts of protein applied were 0.5 μ g (lane 1), 1.0 μ g (lane 2), 2.5 μ g (lane 3), 5.0 μ g (lane 4) and 10 μ g (lane 5). Prestained markers were run in lane 6.

Fig. 2. Quantitative immunoblotting of apo B

A range of concentrations of apo B were separated by SDS/PAGE, electrotransferred on to nitrocellulose membrane and quantified using ¹²⁵I-protein A as described in the Methods section. In (a) the radioactivity bound is plotted against the concentration of apo B-100 and in (b) against concentration of apo B-48.

calculated as 57% of the protein applied to the gel and apo B-100 as 43%. The standard curves showed a linear relationship with protein up to 10 μ g, the highest concentration investigated (Fig. 2). In some preparations the apo B-100 band was resolved into two bands with close mobilities. This has been reported by others for rat apo B, and the bands have been designated apo B-95 and apo B-100 or PI and PII [21,22]. We have taken both of these bands as apo B-100 in the present study.

A number of investigations have quantified apo B in serum samples [22–25]. These studies have produced inconsistent results because of the absence of an absolute protein standard for calibration. Our determinations of apo B in subcellular fractions therefore do not yield absolute values because they are based on the Lowry method [18] and use BSA as a standard, which may overestimate apo B by approx. 7–13% [23–24]. However, they provide relative values for apo B in different subcellular fractions measured under the same conditions.

The anti-(apo B) antibody used in these studies is polyclonal. Thus it might be anticipated that the radioactivity (c.p.m.) bound/mol to apo B-48 would differ from that bound to apo B-100 in relation to the number of epitopes. This was found to be the case: the radioactivity bound/ μ g of apo B-100 was approx. 30% greater than that bound per μ g of apo B-48. (Fig. 2). This suggests that the polyclonal antibody recognizes epitopes throughout the length of the apo B molecule, with a greater concentration of epitopes in the part of the molecule not included in apo B-48.

Determination of apo B in subcellular fractions

The methods used for preparation of *trans*-enriched Golgi membranes, *cis*-enriched Golgi membranes and endoplasmic-reticulum fractions have been used previously in this laboratory.

Table 1. Distribution of protein, phospholipid and marker enzymes in subcellular fractions of rat liver

Subcellular fractions were prepared and marker enzymes assayed as described in the Methods section. Specific activities are expressed in terms of mg of subcellular fraction-protein. Enrichment is the activity of the fraction divided by that of the original homogenate. Yield is the amount of enzyme activity recovered in the total subcellular fraction as a percentage of that in the homogenate. The values given are the means from between 4 and 15 assays using different rat livers $\pm$ s.d. A plasma-membrane preparation prepared as described by Hubbard *et al.* [41] showed a 40-fold enrichment of 5'-nucleotidase and a recovery of 17%.

(a) Protein and phospholipid

Fraction	Protein (mg/g of liver)	Phospholipid (μ mol/g of liver)
Homogenate	205.87 $\pm$ 43.14	70.52 $\pm$ 7.41
Total microsomes	28.54 $\pm$ 2.04	9.94 $\pm$ 0.52
Smooth microsomes	8.87 $\pm$ 2.13	5.14 $\pm$ 1.06
Rough microsomes	9.89 $\pm$ 0.12	6.34 $\pm$ 0.98
<i>trans</i> -Golgi	0.34 $\pm$ 0.027	0.25 $\pm$ 0.018
<i>cis</i> -Golgi	2.10 $\pm$ 0.143	1.56 $\pm$ 0.019

(b) Glucose-6-phosphatase

Fraction	Specific activity (nmol/min per mg of protein)	Enrichment	Yield (%)
Homogenate	47.04 $\pm$ 3.44		
Total microsomes	144.26 $\pm$ 2.28	3.06	42.86
Smooth microsomes	181.30 $\pm$ 16.98	3.85	16.61
Rough microsomes	232.97 $\pm$ 22.18	4.97	23.79
<i>trans</i> -Golgi	10.81 $\pm$ 2.12	0.23	0.04
<i>cis</i> -Golgi	41.60 $\pm$ 6.66	0.88	0.91

(c) UDP-galactose galactosyltransferase

Fraction	Specific activity (pmol/min per mg of protein)	Enrichment	Yield (%)
Homogenate	12.53 $\pm$ 1.16		
Total microsomes	17.60 $\pm$ 2.10	1.40	19.47
Smooth microsomes	86.06 $\pm$ 11.9	6.86	29.591
Rough microsomes	30.80 $\pm$ 3.49	2.46	11.91
<i>trans</i> -Golgi	572.00 $\pm$ 48.50	45.67	7.48
<i>cis</i> -Golgi	169.60 $\pm$ 15.50	13.54	13.82

(d) 5'-Nucleotidase

Fraction	Specific activity (nmol/min per mg of protein)	Enrichment	Yield (%)
Homogenate	67.88 $\pm$ 3.02		
Total microsomes	64.74 $\pm$ 4.26	0.95	13.27
Smooth microsomes	76.70 $\pm$ 3.33	1.12	4.88
Rough microsomes	38.00 $\pm$ 2.89	0.55	2.70
<i>trans</i> -Golgi	39.58 $\pm$ 4.68	0.58	0.28
<i>cis</i> -Golgi	18.31 $\pm$ 1.84	0.27	0.27

The specific activities of marker enzymes and enrichment of fractions are similar to those reported previously by ourselves and by other workers (Table 1). The endoplasmic-reticulum fractions (microsomes) contain galactosyltransferase, the marker for *trans*-Golgi membranes with a small enrichment compared with the total homogenate. The *trans*-enriched Golgi fraction has

Fig. 3. Apo B in immunoprecipitates of subcellular fractions of rat liver

Subcellular fractions were prepared, solubilized and the apo B immunoprecipitated as described in the Methods section. A 100 μ g portion of the protein of the immunoprecipitate and the supernatant from the immunoprecipitate were applied to gels. Lanes 1, 5 and 9, apo B standard, 10 μ g; lanes 2-4, supernatants from immunoprecipitates of total endoplasmic reticulum (lane 2), *trans*-enriched Golgi (lane 3) and *cis*-enriched Golgi (lane 4); lanes 6-8, immunoprecipitates from total endoplasmic reticulum (lane 6), *trans*-enriched Golgi (lane 7) and *cis*-enriched Golgi (lane 8).

Table 2. Distribution of 125 I-Protein A binding to immunoprecipitates and supernatants from subcellular fractions

Subcellular fractions were prepared, solubilized and immunoprecipitated as described in the Methods section. The immunoprecipitate and the supernatants were separated by SDS/PAGE, electrotransferred to nitrocellulose and the amount of 125 I-Protein A bound was determined as described in the Methods section. There was no visible apo B band in the supernatant fractions; however, using the standards as a guide, the area of the nitrocellulose in the region of apo B was cut out and counted for radioactivity, together with the apo B bands of the immunoprecipitated fractions. The results given are typical for several experiments; however, as the specific radioactivity of protein A varies, the d.p.m. values differ between experiments.

Fraction	125 I-Protein A bound to apo B (d.p.m.)			
	Immunoprecipitates		Supernatants	
	Apo B-100	Apo B-48	Apo B-100	Apo B-48
Total microsomes	36 578	41 125	177	102
<i>trans</i> -Golgi	27 548	30 544	132	174
<i>cis</i> -Golgi	34 301	37 802	147	177

very low to undetectable contamination with endoplasmic-reticulum membranes using glucose-6-phosphatase as a marker. The *cis*-enriched fraction is contaminated with glucose-6-phosphatase to the same level as the homogenate and is only enriched with the galactosyltransferase marker by 13-fold. However, as the *cis* fraction is always produced during our fractionation procedures, we have used it in subsequent assays. The identification of the *trans*-enriched Golgi fraction and the *cis*-enriched Golgi fractions was made originally by Erhreich *et al.* [26] on the basis of morphological appearance and is supported by the sequential appearance of secreted protein through these fractions [27] and the presence of terminal glycosyltransferases [28,29]. This identification has been recently confirmed by Futterman *et al.* [30] by immunoblotting with antibodies against mannosidase II, as a *cis*-element marker, and sialyltransferase, as a *trans*-element marker.

After immunoprecipitation of apo B from solubilized subcellular fractions there was no apo B of either form detectable in the supernatant fraction (Fig. 3). Immunoprecipitation of apo B is therefore complete. In the preparations from subcellular fractions, faint immunoreactive bands appeared between apo B-48 and apo B-100. These may be due to degradation of apo B-100, either during the isolation of subcellular fractions or as a consequence of the intracellular hydrolysis of apo B in the liver

Table 3. Apo B-48 and apo B-100 content of microsome and Golgi fractions

Subcellular fractions were prepared and the apo B content determined by quantitative immunoblotting as described in the Methods section. The results are the average of four separate determinations on different rat livers $\pm$ S.E.M. The values given are expressed as μ g of apo B per mg of subcellular-fraction protein. There were $146.13 \pm 6.22 \mu$ g of apo B-100 and $367.00 \pm 25.17 \mu$ g of apo B-48/g wet weight of the original liver used.

Fraction	Content (μ g/mg of protein)	
	Apo B-100	Apo B-48
Total microsomes	1.64 ± 0.08	4.88 ± 0.29
<i>trans</i> -Golgi	6.66 ± 0.71	17.14 ± 1.60
<i>cis</i> -Golgi	5.08 ± 0.42	12.10 ± 1.27

Table 4. Distribution of apo B in subcellular organelles of rat liver

The total amount of apo B in each subcellular fraction was calculated using the data of Table 2. Glucose-6-phosphatase is used as an internal standard for endoplasmic reticulum and galactosyltransferase for the *trans*-enriched Golgi fraction. The amount of apo B in the total liver was calculated directly. Recovery is the sum of apo B in endoplasmic reticulum and Golgi compared with that in the total homogenate (%).

Fraction	Content (μ g/mg of protein)	
	Apo B-100	Apo B-48
Total liver	146.13 ± 6.22	367.00 ± 25.71
Total endoplasmic reticulum (glucose-6-phosphatase)	103.32	308.42
Total Golgi (galactosyltransferase)	30.27	77.91
Recovery...	133.59 (91.4 %)	386.33 (105.3 %)

cell. As all preparations contained a cocktail of proteinase inhibitors, we favour the latter explanation. Davis and co-workers [13,16,31] have noted similar bands in subcellular fractions of rat liver and suggested that they were due to apo B degradation during intracellular processing. When 125 I-Protein A was used to determine the amount of apo B in the supernatant and immunoprecipitate fractions, 98 % of the radioactivity was found in the apo B-48 and apo B-100 bands of the immunoprecipitate (Table 2), confirming the conclusion that immunoprecipitation results in complete removal of apo B from the solubilized subcellular fractions. Only a few per cent of the radioactivity in the immunoprecipitate were found in the faint bands below apo B-100.

The total microsome fraction contained 1.6μ g of apo B-100 and 4.9μ g of apo B-48 per mg of membrane protein. The Golgi fractions contained approx. 4 times more apo B per mg of protein than the microsomes (Table 3). The proportion of apo B as apo B-48 was 75 % in the endoplasmic reticulum, 73 % in the *trans*-enriched Golgi membranes and 71 % in the *cis*-enriched Golgi membranes (Table 3). A similar percentage distribution of the two forms of apo B has been reported for liver microsomes [21]. By using glucose-6-phosphatase as an internal standard it can be calculated that, in 1 g of liver, the total endoplasmic reticulum contains 103 μ g of apo B-100 and 308 μ g of apo B-48,

whereas the galactosyltransferase-containing Golgi elements contain 30 μ g of apo B-100 and 78 μ g of apo B-48 (Table 4). In this calculation the apo B content of endoplasmic reticulum has been corrected for contamination with *trans*-Golgi membranes using galactosyltransferase as internal marker. No correction is necessary for the *trans*-Golgi elements, as these contain insignificant amounts of glucose-6-phosphatase. Essentially all of the apo B in the homogenate is found in the two fractions, with 72 % of the apo B-100 and 80 % of the apo B-48 of the total homogenate in the endoplasmic reticulum and 20 % of the apo B-100 and 21 % of the apo B-48 in the *trans*-Golgi membranes. There is therefore no significant pool of apo B outside the secretory compartment.

Distribution of apo B-48 and apo B-100 in membranes and content fractions of subcellular fractions

The apo B content of smooth microsomes expressed per mg of protein was approximately twice that of the rough microsomes. A similar ratio was found by Borchardt & Davis [13], although they did not quantify apo B directly. This is an unexpected result, as the rough microsomes are the site of synthesis of apo B and might be expected to contain a larger fraction of the protein. However, the smooth-microsomal fraction is more heterogeneous than the rough and undoubtedly contains Golgi elements and elements transitional between rough and smooth endoplasmic reticulum and between endoplasmic reticulum and Golgi. Alexander *et al.* [32] used a cytochemical method to detect apo B in intact liver and showed apo B associated with the endoplasmic-reticulum membrane and also associated with particles in the transitional region of the rough- and smooth-membrane cisternae. The apo B content of smooth microsomes may thus be made up of several pools.

When rough microsomes, smooth microsomes, and *cis* and *trans*-enriched Golgi preparations were subfractionated, striking differences were found in the distribution of apo B between the membranes and the cisternal-content fractions (Fig. 4 and Table 5). In the rough endoplasmic reticulum, only 6 % of the recovered apo B-48 was in the cisternal content, and there was no detectable apo B-100 in the cisternal content; 95 and 99 % of the total microsomal apo B-100 and apo B-48 respectively were recovered in the membrane and content fractions. There was slightly more apo B in the smooth-microsome contents, with 4 % of the apo B-100 and 8 % of the apo B-48 in the cisternal contents. Recoveries in this case were 95 and 94 % for apo B-100 and apo B-48 respectively; 26 % of the apo B-48 and 33 % of the apo B-100 were recovered in the *cis*-enriched Golgi membrane fraction, whereas in the *trans*-enriched Golgi, only 12 % of the apo B-48 and 19 % of the apo B-100 were recovered in the membranes. These observations indicate that the apo B in the endoplasmic reticulum is predominantly membrane-bound and that, even in the *trans*-Golgi fraction, some apo B remains membrane-bound. The distribution of apo B in subcellular fractions and in membrane and content fractions detected by immunoblotting (Fig. 4) was consistent with the quantification of the fractions.

A number of investigators have concluded that apo B is associated with the endoplasmic-reticulum membrane. Bamberger & Lane [14] labelled apo B with [3 H]leucine in oestrogen-induced chick hepatocytes and found that 50 % of the radiolabelled apo B remained associated with endoplasmic-reticulum membranes. Davis *et al.* [16] found that 56 % of the apo B-48 and 70 % of the apo B-100 were lost on trypsin treatment of microsomal fractions, indicating that at least this proportion of apo B is partly exposed on the outside of the membrane vesicle. There was no loss of apo B from the Golgi preparations on treatment with trypsin. The major difference between our results and those of other investigators is that we

Fig. 4. Apo B in subcellular fractions of rat liver

Subcellular fractions were prepared, the proteins separated by SDS/PAGE, electrotransferred on to nitrocellulose and immunostained for apo B as described in the Methods section. Total subcellular fractions are illustrated in (a), membrane fractions in (b) and content fractions in (c). Lanes 1, 5 and 9 are *trans*-enriched Golgi fractions, lanes 2, 6 and 10 are *cis*-enriched Golgi fractions, lanes 3, 7 and 11 are smooth endoplasmic reticulum and lanes 4, 8 and 12 are rough endoplasmic reticulum. The Golgi-content fractions were concentrated 5-fold and the endoplasmic-reticulum fractions were concentrated 10-fold in order to obtain immunoblots of apo B in these fractions (see the Methods section).

Table 5. Distribution of apo B in membrane and cisternal content subfractions of endoplasmic reticulum and Golgi preparations

Subcellular fractions were prepared and the apo B content was determined by quantitative immunoblotting as described in the Methods section. The values given are expressed as μg of apo B/mg of subcellular-fraction protein. The results are the averages of three separate determinations $\pm$ S.E.M. Abbreviations: n.d., not detectable.

Fraction	Content ($\mu\text{g}/\text{mg}$ of protein)	
	Apo B-100	Apo B-48
Smooth endoplasmic reticulum		
Total fraction	1.23 ± 0.09	3.64 ± 0.21
Membrane fraction	1.12 ± 0.09	3.31 ± 0.14
Content fraction	0.05 ± 0.01	0.30 ± 0.04
Rough endoplasmic reticulum		
Total fraction	0.58 ± 0.02	1.96 ± 0.13
Membrane fraction	0.55 ± 0.02	1.74 ± 0.19
Content fraction	n.d.	0.11 ± 0.03
<i>trans</i> -Enriched Golgi		
Total fraction	6.48 ± 0.16	17.46 ± 0.25
Membrane fraction	1.15 ± 0.01	2.04 ± 0.09
Content fraction	4.91 ± 0.20	14.65 ± 0.19
<i>cis</i> -Enriched Golgi		
Total fraction	5.33 ± 0.22	12.27 ± 0.40
Membrane fraction	1.62 ± 0.12	2.93 ± 0.34
Content fraction	3.35 ± 0.12	8.07 ± 0.28

find almost all of the apo B is membrane-bound in the endoplasmic reticulum. However, ours is the only study in which apo B has been directly quantified.

Contribution of apo B taken up by endocytosis

Endocytic vesicles, which may contain LDL particles, have been reported as contaminants of the Golgi preparations [33,34]. To check the possible contribution of LDL apoB to our preparations, we injected ^{125}I -apo B-labelled LDL into the portal vein of rats and, after 15 min, the peak time for labelling of endocytic vesicles, we isolated Golgi and endoplasmic-reticulum membranes. The maximum recovery of the ^{125}I in the different fractions as a percentage of that in the homogenate was around 3% in the smooth endoplasmic reticulum and *cis*-Golgi fractions, and this represented maximum of 3-fold enrichment compared with the homogenate (Table 6). The amount of apo B in the subcellular fractions amounted to between 2 and 10 ng/g of liver

Table 6. Contribution of internalized LDL to apo B in subcellular fractions

^{125}I -labelled LDL (74000 d.p.m., equivalent to 563 ng of apo B) in 0.1 ml of 0.14 M-NaCl were injected intraportally into anaesthetized rats. Subcellular fractions were prepared as described in the Methods section, and the amount of apo B determined by γ -radiation counting. Results are expressed as d.p.m. of apo B in subcellular fraction prepared from 1 g of liver in the first column. In the second column the recovery of d.p.m. in each subcellular fraction is given as a percentage of the total in the homogenate. In the third column the enrichment of the ^{125}I in each fraction relative to the homogenate is shown. More than 90% of the LDL radioactivity was recovered in the particulate fractions.

Fraction	Radioactivity (d.p.m./g of liver)	Recovery (%)	Enrichment (fold)
Homogenate	6100	100	
Rough endoplasmic reticulum	66	1.08	0.3
Smooth endoplasmic reticulum	214	3.51	0.8
<i>trans</i> -Golgi	84	1.38	2.8
<i>cis</i> -Golgi	196	3.21	3.2

(Table 6). This compares with 7 μg of apo B recovered in the Golgi fraction from 1 g of liver and 182 μg in the microsomes from 1 g of liver. It is unlikely, therefore, that contamination of Golgi and endoplasmic-reticulum fractions by endosomes can account for a significant amount of the apo B content. Other investigators have also found less than 1% of injected LDL were recovered in Golgi fractions prepared using the same methods [35–37].

While this work was in progress, Hamilton *et al.* [38] published a report that claimed that Golgi preparations prepared by methods based on that of Erhreich *et al.* [26] were grossly contaminated with endocytic vesicles containing chylomicron and VLDL remnants. They found that Golgi membranes prepared after intravenous injection of ^{125}I -LDL were enriched 50–70-fold compared with the homogenate, whereas multi-vesicular bodies which contain endosomes had an enrichment of 100-fold. In our experiments the *trans*-Golgi enriched fraction contained approx. 1% of the injected LDL and this was enriched approx. 3-fold compared with the homogenate. The explanation for the differences between our results and those of Hamilton *et al.* [38] may be due to the fact that these investigators intubated rats with ethanol before isolation of Golgi fractions, as described in Erhreich *et al.*'s [26] original procedure. This treatment has not been used generally because it is known to perturb lipoprotein metabolism [39].

In earlier studies we examined the association of lipids with apo B by incorporation of labelled palmitic acid into triacylglycerol and phospholipid, followed by cell fractionation, and immunoprecipitation of apo B from the subcellular-content fractions. These results indicated that approx. 8, 11, 26 and 57% of the labelled lipids of the rough microsomes, smooth microsomes, *cis*-enriched Golgi and *trans*-enriched Golgi respectively were immunoprecipitated with an antibody to apo B. The present results indicate that there is also an increase in the proportion of apo B in the cisternal contents from rough endoplasmic reticulum (~3%) to the smooth endoplasmic reticulum (~5%) to the *cis*-enriched Golgi (~70%) to the *trans*-enriched Golgi (~85%).

We have previously suggested that the Golgi membranes are the main site of assembly of the apo B with the VLDL lipids [11]. The observations reported here are consistent with this suggestion and indicate that apo B remains associated with the endoplasmic

reticulum membrane rather than moving into the cisternal space. The mRNA for apo B appears to be constitutive and does not change when VLDL secretion by HEP-G2 cells increases 7-fold [40]. Thus regulation of VLDL assembly and secretion does not appear to be at the level of gene expression. An attractive hypothesis is that apo B is produced in excess, and the protein which is not packaged with VLDL for secretion is degraded within the endoplasmic-reticulum/Golgi compartment. As most of the apo B of trans-enriched Golgi is cisternal, the most likely candidate for the site of degradation or recycling of apo B is the endoplasmic reticulum or *cis*-Golgi compartment.

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