Intracellular events in the assembly of very-low-density-lipoprotein lipids with apolipoprotein B in isolated rabbit hepatocytes

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Isolated rabbit hepatocytes incorporated [³⁵S]methionine into cellular and secreted apolipoprotein B (apo-B), and [³H]glycerol into cellular and secreted triacylglycerol and phospholipids. Newly synthesized apo-B was incorporated into rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), *cis*-Golgi and *trans*-Golgi membranes and was preferentially transferred into the lumen of the RER with specific radioactivities ten times those in the membrane. Radiolabelled apo-B did not equilibrate with pre-existing unlabelled apo-B, and pools of different specific radioactivities were established in different subcellular fractions. Only a small fraction of the newly synthesized apo-B was transferred to the Golgi lumen. In pulse-chase experiments, most of the newly synthesized apo-B in the RER membrane and the RER lumen was degraded.

[³H]Glycerol was incorporated into triacylglycerol and phospholipids in the lumen of the RER, SER, *cis*-Golgi and *trans*-Golgi. However, in contrast with apo-B, all of the radiolabelled lipids in the lumen of the RER, SER and *cis*-Golgi were transferred to the *trans*-Golgi lumen or secreted. Analysis of the lipid composition of the luminal content fractions suggests that, although very-low-density-lipoprotein (VLDL) lipids are present in the endoplasmic reticulum lumen, a large fraction of these is not associated with apo-B. Collectively these observations suggest that assembly of apo-B into complete VLDL is not co-translational, that most lipids become associated with apo-B late in the endoplasmic reticulum compartment and that the lipids are further modified in the Golgi lumen.

INTRODUCTION

Endogenous triacylglycerols secreted by the liver are transported in the circulation in the form of very-low-density lipoproteins (VLDLs). Recent studies of isolated rat hepatocytes and Hep-G2 cells have indicated that VLDL secretion is driven by triacylglycerol availability and that apolipoprotein B100 (apo-B), the major and essential apolipoprotein of VLDL, is synthesized in excess over that required for lipid transport (reviewed in refs. [1–3]). The excess apo-B is degraded intracellularly [4–12]. Studies from our laboratory on isolated rat hepatocytes have shown that newly synthesized apo-B is either incorporated into the membrane of the rough endoplasmic reticulum (RER), transported to the *trans*-Golgi membrane and subsequently degraded, or is translocated across the RER membrane into the lumen where it is found in at least two pools: a heavy pool (*d* 1.21), most of which is degraded *in situ*, and a lighter pool, which moves from the RER lumen through the secretory compartments to the *trans*-Golgi, is packaged with lipid and secreted as VLDL [12]. In hepatocytes from orotic acid-fed rats in which VLDL secretion is inhibited, apo-B is translocated to the heavy pool in the RER lumen and is transferred to the Golgi lumen but the light pool is not formed and apo-B is not secreted. Studies by Boren and co-workers [13] have also found three forms of apo-B in Hep-G2 cells, a membrane-bound form and apo-B in heavy and light particles in the ER lumen. These results suggest that there are at least two steps in the intracellular transit of apo-B which are involved in regulating VLDL secretion: (i) the transfer of apo-B across the RER membrane and (ii) the packaging of apo-B with VLDL lipids. At each of these steps apo-B may be diverted from the normal secretory pathway and degraded. Several models for VLDL assembly have been suggested. The main differences between these lies in the intracellular sites and sequence of addition of VLDL lipids to apo-B, whether this is co-

translational or post-translational and whether assembly takes place in a single or multiple steps. Models for VLDL assembly that take into account recent findings include the following: (i) VLDLs are formed completely in the RER lumen by co-translational association between newly synthesized apo-B and lipids; (ii) apo-B is released into the RER lumen in precursor particles which acquire other lipids sequentially during transit through the RER lumen; and (iii) the apo-B-containing precursor particles are formed in the RER lumen and fuse within the secretory compartment with triacylglycerol-rich particles formed separately. In order to distinguish between these models we have now investigated the intracellular transit of VLDL lipids and its kinetic relationship to apo-B.

Most previous studies of VLDL assembly have used rat hepatocytes, either freshly isolated or in primary culture, or the human hepatoma cell line Hep-G2. However, there are significant differences between rat and human liver. Notably rat liver secretes apo-B48 and apo-B100, whereas human liver secretes only apo-B100. In addition, although Hep-G2 cells are of human origin and secrete only apo-B100, these cells secrete particles of low-density lipoprotein (LDL)/high-density lipoprotein (HDL) rather than normal VLDL particles [14]. Rabbit liver secretes VLDL containing apo-B100 and more closely resembles human lipoprotein metabolism than rats [15,16]. We have therefore performed our investigations on freshly isolated rabbit hepatocytes.

MATERIALS AND METHODS

Materials

Anti-TGN38 (*trans*-Golgi network marker) polyclonal and monoclonal antibodies were a gift from Dr. Banting, University of Bristol [17]. These included polyclonal AW 29 raised in rabbit

Abbreviations used: VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo-B, apolipoprotein B; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; HPTLC, high-performance TLC; MTP, microsomal triacylglycerol-transfer protein.

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and IgG1, a monoclonal antibody that recognizes the extreme N-terminus of TGN38; rabbit anti-p58 (*cis*-Golgi network marker) was a gift from Dr. Saraste, University of Bergen [18,19]. TLC plates (60F254) and nanosil high-performance TLC (HPTLC) plates were obtained from Merck and Camlab respectively. Centricon 30 microconcentrator tubes were purchased from Amicon. PHM-L liposorb was purchased from Calbiochem. [³⁵S]Methionine (cell-labelling grade), ¹²⁵I-Protein A and [³H]glycerol were purchased from Amersham. Other chemicals and materials were as described previously [12].

Animals

All experiments were performed on adult dwarf-lop rabbits (1–2 kg body weight). These were housed in the University Field Labs and fed on standard chow pellets.

Preparation of isolated rabbit hepatocytes

Rabbit hepatocytes were prepared and characterized as described for rat hepatocytes [12] except that the liver was initially flushed out with Krebs' bicarbonate saline containing 1 % (w/v) sodium citrate to prevent blood clotting. The cannulated liver was then rapidly excised and perfusion continued on a hollow metal surgical table heated to 37 °C by circulating water.

Incubation of hepatocytes

Freshly isolated hepatocytes were incubated and their viability was checked before and after incubations as previously described [12]. The ability of the cells to incorporate [³⁵S]methionine into cellular and secreted proteins was similar under all experimental conditions. Hepatocytes were resuspended (50 %, w/v) in modified methionine-free Eagle's minimum essential medium which had been gassed with 95 % O₂/5 % CO₂. Aliquots of volume 2 ml were incubated in 50 ml siliconized flasks, which were gassed at intervals before and during the incubation period. [³⁵S]Methionine (approx. 100 µCi) or [³H]glycerol (approx. 100 µCi) was added and the flasks were incubated for 5 min; 2 ml of 2 mM oleic acid (bound to 1 % fatty acid-free albumin) was then added. The incubation was continued for up to 180 min. The cells were chilled to 4 °C and isolated by centrifugation at 1000 *g* for 5 min. The media were centrifuged at 17500 *g* for 30 min at 4 °C to remove any cell debris. In most experiments, subcellular organelles were prepared from the cell pellets, other-wise cells and supernatants (media) were immediately processed for analysis of apo-B or lipids.

Preparation of subcellular fractions from hepatocytes

Hepatocyte pellets were subjected to osmotic shock by resuspension in 2.5 ml of ice-cold 10 mM Tris/HCl/1 mM MgCl₂, pH 7.4. Then 2.5 ml of 0.5 M sucrose was added and the cells were homogenized using a top-drive Potter-Elvehjem homogenizer, as described previously [12]. Homogenization was checked by inspection of the cell suspension using the light microscope. The membrane and luminal content fractions of RER, smooth endoplasmic reticulum (SER), *cis*-Golgi and *trans*-Golgi were prepared as described previously [12,20–25]. Protease inhibitors were added to the homogenate and to resuspended fractions to minimize apo-B degradation. In some experiments, the luminal contents were further subfractionated on discontinuous KBr gradients consisting of layers of *d* = 1.21, 1.063, 1.02 and 1.006 [12]. In other experiments apo-B was immunoprecipitated from the luminal content fractions as described previously [26]. Briefly, content fractions were neutralized and concentrated using Centricon tubes or an Amicon PM10 filter.

Aliquots were immunoprecipitated using sheep anti-apo-B as primary antibody and donkey anti-sheep IgG as secondary antibody, and immunoprecipitates were centrifuged through a 0.5 M sucrose cushion exactly as described previously [26]. The concentrations of each antibody that completely immunoprecipitated apo-B, detected by immunoblotting, were determined in preliminary experiments [23,26]. Less than 5 % of the radiolabelled apo-B was immunoprecipitated in control experiments in which sheep serum replaced anti-apo-B.

Characterization of hepatocyte subcellular fractions

The purity and degree of cross-contamination of subcellular fractions was determined by marker enzymes as described previously [12,24] and by immunoblotting. Aliquots of subcellular membrane fractions were dissolved in SDS/PAGE sample buffer (see below) and run on 8 % polyacrylamide gels for 7 h at 70 V. The proteins were blotted on to nitrocellulose and incubated with antibodies against p58 for the *cis*-Golgi network and against TGN38 for the *trans*-Golgi network at a dilution of 1:1000. The primary antibodies were detected as described previously [12,23–25] using anti-rabbit, anti-rat or anti-mouse IgG labelled with alkaline phosphatase diluted 1:1000 as secondary antibodies.

Separation and quantification of apo-B100 by SDS/PAGE

Samples were prepared for SDS/PAGE as follows. Cell pellets were washed twice by resuspension to 20 % (w/v) in ice-cold buffer (10 mM Tris/HCl/150 mM NaCl, pH 7.4). Secreted lipoproteins were adsorbed from media by mixing with 10 mg of PHM-L liposorb for 24 h at 4 °C, and then centrifuged at 17500 *g* for 30 min at 4 °C. The concentration of PHM-L liposorb required for complete adsorption was ascertained in preliminary experiments in which the apo-B in the pellet and the supernatant were determined as described below. Total luminal contents and subfractions were concentrated to 40 µl in Centricon 30 tubes. Aliquots of whole hepatocytes, secreted lipoproteins (on liposorb pellets), subcellular membranes, concentrated luminal contents and content subfractions were dissolved in sample buffer (30 mM Tris/HCl, 3 mM EDTA, 3 % SDS, 15 % glycerol, pH 6.5). 2-Mercaptoethanol was then added (final concentration 15 %), and the mixture heated at 60 °C for 20 min. Two sets of samples were separated on each half of 3–20 % gradient gels. Proteins in samples in one-half of the gel were transferred to nitrocellulose membranes, and the mass of apo-B100 was determined by quantitative immunoblotting as described previously [23]; values are expressed as µg/mg of hepatocyte or subcellular fraction protein. The other half of each gel was stained using Coomassie Blue; the apo-B100 bands were excised and digested with 3 ml of H₂O₂ at 100 °C as described previously [23]. The digests were mixed with 10 ml of scintillation fluid and counted for radioactivity. The specific radioactivity of apo-B was calculated and expressed as d.p.m. of [³⁵S]methionine incorporated/µg of apo-B100.

Extraction of lipids

Immediately before lipid extraction, hepatocytes and subcellular fractions were homogenized in distilled water at 4 °C. Aliquots (2 ml) of hepatocytes and subcellular membrane suspensions, media or total luminal contents and subfractions were mixed with 20 vol. of chloroform/methanol (2:1, v/v). After 10 min, the mixtures were centrifuged at 1000 *g* for 10 min and the supernatants removed and shaken vigorously with 4 ml of 50 mM CaCl₂ for 2 min and then re-centrifuged at 2000 *g* for 5 min. The

lower organic phases, containing the lipids, were evaporated to dryness and stored at -20°C until analysed.

Determination of [^3H]glycerol incorporation into lipids

The [^3H]glycerol-labelled lipid extracts were redissolved in 100 μl of chloroform/methanol (1:1, v/v) and separated by one-dimensional TLC, using the solvent system heptane/diethyl ether/acetic acid (60:40:2, by vol.). Lipid-containing bands were visualized by iodine vapour. The iodine was allowed to escape and the phospholipid- and triacylglycerol-containing bands were scraped from the plates and mixed with 3 ml of distilled water to form a slurry; 10 ml of scintillation fluid was added and the samples were counted for radioactivity in a β -scintillation counter.

Quantification of lipids by HPTLC densitometry

Lipid extracts were redissolved in chloroform/methanol (1:1, v/v) and aliquots (1–10 μl) applied in 18 lanes to nanosil 20 cm $\times$ 10 cm HPTLC plates using a Desaga AS30 autospotter. Lipids were separated using the system, heptane/diethyl ether/acetic acid (60:40:2, by vol.). The plates were air-dried and dipped in cupric acetate (3%, w/v) dissolved in phosphoric acid (8%, w/v) for 30 s, air-dried again and heated to 140°C on a hotplate for 30 min. They were then cooled and the amount of each lipid band in each lane was determined using a Camlab Desager CD60 densitometer. The first six lanes on each plate were used for standard curves for phospholipid (egg lecithin), triacylglycerol (triolein), cholesterol oleate and cholesterol mixed in approximately the same proportions as that found in microsomal membranes. Lipid-containing samples were applied to the plate in triplicate in three volumes (1, 5 and 10 μl) to ensure that each lipid was assayed on the straight part of the standard curve.

Pulse-chase studies

To follow the transit of apo-B and lipids through the secretory compartments, hepatocytes (1 g wet weight) were incubated for 30 min with [^{35}S]methionine or [^3H]glycerol for 30 min as above in the absence of oleate. Hepatocytes were isolated by centrifugation at 1000 g for 5 min, resuspended (50%, w/v) in minimum essential medium containing 45 mM unlabelled methionine or glycerol and incubated for different times up to 180 min with oleic acid (2 mM). The appearance of ^{35}S and ^3H in apo-B and lipids respectively was determined at each time point as above. Because the radiolabelled tracer added, but not the amount of

unlabelled precursor, varied by about 20% between individual experiments, the results of single experiments are presented. However, the patterns of the results presented were reproducible when each experiment was repeated several times.

RESULTS

Characterization of subcellular fractions

The specific activities of marker enzymes in RER, SER, *cis*-Golgi and *trans*-Golgi fractions prepared from rabbit hepatocytes were the same as those of subcellular fractions isolated from whole rabbit liver (Table 1) [24,25]. This was also found in previous studies in which rat liver and isolated rat hepatocytes were compared [12,23]. The degree of cross-contamination between subcellular fractions based on assay of marker enzymes has been discussed in detail elsewhere [12,23–25]. However, in this study we have also probed our subcellular fractions by immunoblotting with antibodies against p58 for the *cis*-Golgi network and against TGN38 for the *trans*-Golgi network. The RER, SER and *cis*-Golgi fractions contained the *cis*-Golgi marker, p58, which was

Figure 1 Identification of *trans*-Golgi marker, TGN38, and *cis*-Golgi marker, p58, in subcellular fractions from rat and rabbit liver

Subcellular fractions were prepared as described in the Materials and methods section. The proteins were separated by SDS/PAGE, electroblotted on to nitrocellulose and the *trans*-Golgi network marker, TGN38 (c, d), and the *cis*-Golgi network marker, p58 (a, b), detected by immunoblotting as described in the Materials and methods section. Gc, *cis*-Golgi-enriched fraction; Gt, *trans*-Golgi-enriched fraction. (a, c) Fractions from rabbit liver; (b, d) fractions from rat liver.

Table 1 Specific activity of marker enzymes in subcellular fractions

Subcellular fractions were prepared and the specific activities of UDP-galactose galactosyltransferase, as a Golgi marker, and NADPH-cytochrome *c* reductase, as an endoplasmic reticulum marker, were determined [12,23–25]. The specific activities and recoveries are presented as means $\pm$ S.D. of four separate assays. The maximum cross-contamination of endoplasmic reticulum fractions with Golgi and Golgi fractions with endoplasmic reticulum was determined as described previously [12,25] assuming that all of the UDP-galactose galactosyltransferase is in the Golgi and all of the NADPH-cytochrome *c* reductase is in the endoplasmic reticulum.

Fraction	NADPH-cytochrome <i>c</i> reductase (nmol/min per mg)	Recovery (%)	UDP-galactose galactosyltransferase (pmol/min per mg)	Recovery (%)	Maximum cross-contamination (%)
Homogenate	2.78 $\pm$ 0.05	—	20 $\pm$ 3	—	—
RER	11.60 $\pm$ 2.17	12.1	48 $\pm$ 14	2.1	7
SER	9.31 $\pm$ 0.08	15.3	152 $\pm$ 26	26.7	24
<i>cis</i> -Golgi	3.20 $\pm$ 1.50	1.5	257 $\pm$ 52	14.5	30
<i>trans</i> -Golgi	0.52 $\pm$ 0.18	0.03	762 $\pm$ 56	9.2	5

Figure 2 Incorporation of [³⁵S]methionine into apo-B of hepatocytes incubated with and without oleate

Isolated hepatocytes were incubated with [³⁵S]methionine with and without oleate, and (a) the amounts of and (b) radioactivity in apo-B in the cells and media determined as described in the Materials and methods section. ▲, Cells minus oleate; △, medium minus oleate; ■, cells plus oleate; □, medium plus oleate.

Table 2 Apo-B content of subcellular fractions

Subcellular fractions were prepared from isolated hepatocytes and the apo-B content determined as described in the Materials and methods section. The protein recovered in each fraction per g of hepatocytes is shown in the right-hand column and the apo-B content expressed in terms of this in the left-hand columns. The results are means ± S.D. of eight separate preparations measured in duplicate.

Fraction	Apo-B content (μg/mg of protein)		Protein recovered (mg/g of hepatocytes)
	Membrane	Luminal content	
RER	1.40 ± 0.04	0.86 ± 0.08	1.56 ± 0.31
SER	1.11 ± 0.04	1.41 ± 0.08	2.93 ± 0.49
<i>trans</i> -Golgi	3.12 ± 0.17	7.46 ± 0.43	0.2 ± 0.02
<i>cis</i> -Golgi	1.67 ± 0.05	1.60 ± 0.09	0.85 ± 0.22

Figure 3 Incorporation of [³⁵S]methionine into apo-B in subcellular fractions from rabbit hepatocytes

Isolated hepatocytes were incubated with radiolabelled methionine as described in the Materials and methods section. At timed intervals the cells were isolated by centrifugation and subcellular fractions prepared. The specific radioactivity of apo-B (d.p.m./μg of apo-B) in luminal content (a) and membrane (b) fractions was determined as described in the Materials and methods section. △, RER; ▲, SER; □, *cis*-Golgi; ■, *trans*-Golgi.

absent from the *trans*-Golgi fraction (Figures 1a and 1b). The polyclonal antibody against TGN38, AW 29, showed two bands one of which is probably a partly glycosylated precursor (G. Banting, personal communication). These were present in all fractions, consistent with synthesis in the RER, but at the highest concentration in the *trans*-Golgi fraction, particularly in the rat fractions (Figures 1c and 1d). The monoclonal antibody against TGN38, IgG1, showed only one band in the rat *trans*-Golgi fraction but did not cross-react with the rabbit fractions (results not shown). The immunological data indicate that the *trans*-Golgi fraction does not contain the *cis*-Golgi network but does contain the marker for the *trans*-Golgi network in relatively high concentration.

Most of the data presented in this paper are expressed in terms of mg of isolated fraction protein, which was determined in each experiment. In interpreting some of the results we have also expressed the data in terms of the total amount of each fraction in 1 g of hepatocytes which was calculated using recoveries of marker enzymes [12,23,24]. We have calculated that the total endoplasmic reticulum (NADPH-cytochrome *c* reductase-containing elements) contributes 47 mg of protein/g of hepatocytes and the total *trans*-Golgi (UDP-galactose galactosyltransferase-containing elements) contributes 3.5 mg of protein/g of hepatocytes [24]. The relative proportions of endoplasmic reticulum and Golgi in liver is consistent with results from sterological analysis,

Figure 4 Transfer of pulse-radiolabelled apo-B between compartments of the secretory pathway during chase experiments

Isolated hepatocytes were incubated for 30 min with radiolabelled methionine. The cells were isolated and re-incubated with a large excess of unlabelled methionine. At timed intervals from 0 to 180 min the cells were isolated by centrifugation, membrane and luminal content fractions of subcellular fractions were prepared and the radioactivity in apo-B (b, d) and its specific radioactivity (a, c) were determined as described in the Materials and methods section. Results are expressed as the specific radioactivities (d.p.m./ μ g of apo-B) and d.p.m. in apo-B in the membrane and luminal content fraction per mg of protein of the unopened subcellular fraction. $\blacktriangle$, RER; $\triangle$, SER; $\blacksquare$, *trans*-Golgi; $\square$, *cis*-Golgi.

which have also shown that RER accounts for 55% of the total endoplasmic reticulum membrane [27].

Incorporation of radiolabelled precursors into cell and secreted apo-B

$[^{35}\text{S}]$ Methionine was incorporated into cellular and secreted apo-B of isolated rabbit hepatocytes (Figure 2b). As found previously for rat hepatocytes, secretion of apo-B or lipids was dependent on addition of oleate to the incubation medium (Figures 2a and 2b). Although the hepatocytes secreted apo-B, this had a lower specific radioactivity than that of the cellular apo-B, indicating that the newly synthesized apo-B was preferentially retained in the cells and that intracellular pools of apo-B do not equilibrate. Similar findings have also been reported for rat hepatocytes [12].

Incorporation of $[^{35}\text{S}]$ methionine into apo-B of subcellular fractions

The concentrations of apo-B in isolated subcellular fractions were similar to those described previously for fractions from rabbit liver (Table 2). The highest concentration of apo-B was found in the *trans*-Golgi and 70% of this was in the luminal contents. The lowest concentration was in the RER with 38% in the luminal contents.

Time course

$[^{35}\text{S}]$ Methionine was incorporated into apo-B of RER, SER, *cis*-Golgi and *trans*-Golgi membrane and luminal contents. The specific radioactivity of apo-B in RER membranes rose most

Figure 5 Incorporation of [^3H]glycerol into triacylglycerol and phospholipids in subcellular fractions from rabbit hepatocytes

Isolated hepatocytes were incubated with radiolabelled glycerol as described in the Materials and methods section. At timed intervals the cells were isolated by centrifugation and subcellular fractions prepared. The lipids were extracted, separated by TLC and the d.p.m. in triacylglycerol and phospholipid determined as described in the Materials and methods section. Results are expressed as d.p.m. in triacylglycerol (a, c) or phospholipid (b, d) in the membrane (c, d) or luminal content (a, b) fractions per mg of protein of the unopened subcellular fraction. $\triangle$, RER; $\blacktriangle$, SER; $\square$, *cis*-Golgi; $\blacksquare$, *trans*-Golgi.

rapidly followed by that of the SER, *cis*-Golgi and *trans*-Golgi membranes (Figure 3). The specific radioactivity of apo-B in the RER luminal contents also rose rapidly; that of the SER lumen reached a level about 60% that of the RER, and that in the Golgi lumen remained low. The specific radioactivities of endoplasmic reticulum luminal apo-B were approximately ten times higher than those of apo-B in the membranes, indicating that a selective transfer of newly synthesized apo-B into the endoplasmic reticulum lumen occurs. The pool of radiolabelled apo-B moved only slowly to the Golgi lumen. However, the Golgi lumen contained the highest concentration of apo-B (Table 2), indicating that degradation of newly synthesized apo-B was far more rapid than translocation and secretion of the bulk of apo-B.

Chase experiments

In pulse-chase experiments the d.p.m. and the specific radioactivities of apo-B in the RER membrane and lumen fell rapidly, those in the SER and *cis*-Golgi membrane also fell, and those in the *trans*-Golgi membrane rose (Figure 4). The loss of labelled apo-B/mg of RER was 133000 d.p.m. in 60 min and the gain by the Golgi membrane was 59000 d.p.m. The loss from the RER lumen was 383000 d.p.m. and the gain by the Golgi lumen was 10000 d.p.m. During the chase period less than 1000 d.p.m. were in secreted apo-B. These observations are consistent with our

previous findings in rat hepatocytes that the greater part of newly synthesized intracellular apo-B is degraded in both the membrane-bound pool and the luminal pools. As we previously found in studies of rat hepatocytes [12], the concentration of radiolabelled apo-B in luminal fractions from RER was highest in the dense fractions with 8, 12, 38 and 42% in fractions of $d = 1.006$, $d = 1.02$, $d = 1.063$ and $d = 1.21$ respectively, whereas in the *trans*-Golgi luminal fractions the concentration of radiolabelled apo-B was highest in the lightest fractions with 68, 21, 8.7 and 3% in fractions of $d = 1.006$, $d = 1.02$, $d = 1.063$ and $d = 1.21$ respectively. The loss of radiolabelled apo-B is therefore predominantly from RER membrane and denser particles in the RER lumen.

Incorporation of [^3H]glycerol into lipids of subcellular fractions

Time course

[^3H]Glycerol was incorporated rapidly into hepatocyte fractions reaching a plateau after 20–30 min incubation (Figure 5). Radiolabelled triacylglycerol was found in both membrane and content fractions. As the enzymes involved in synthesis of glycerolipids are located in the endoplasmic reticulum, it would be expected that newly synthesized triacylglycerol destined for transfer to either the cytosol or the lumen for secretion would be transiently present in this membrane. However, the greatest amount of

Figure 6 Transfer of pulse-radiolabelled VLDL components between compartments of the secretory pathway during chase experiments

Isolated hepatocytes were incubated for 30 min with [^3H]glycerol or [^{35}S]methionine. The cells were isolated and re-incubated with a large excess of unlabelled glycerol or methionine. At 0 and 120 min the cells were isolated by centrifugation, membrane and luminal content fractions of subcellular fractions were prepared and the radioactivity in triacylglycerol (a, b), phospholipid (c, d) and apo-B (e, f) were determined as described in the Materials and methods section. Results are expressed as d.p.m. in triacylglycerol, phospholipid or apo-B in the membrane (a, c, e) or luminal content (b, d, f) fraction per mg of protein of the unopened subcellular fraction protein.

radiolabelled triacylglycerol occurred in the *trans*-Golgi membranes. The triacylglycerol contents of the RER, SER, *cis*-Golgi and *trans*-Golgi membranes were 0.053 ± 0.01 , 0.048 ± 0.005 , 0.153 ± 0.024 and $0.230 \pm 0.056 \mu\text{g}/\mu\text{g}$ of phospholipid respectively measured in three separate preparations. The d.p.m. in triacylglycerol therefore reflects the amount of this lipid in the membrane and may represent that which is stable in the bilayer structure.

To determine whether newly synthesized luminal triacylglycerol and phospholipids are associated with apo-B, the luminal content fractions were immunoprecipitated with antibodies against apo-B as described previously [23,26]: 28, 40, 63 and 73 % of the radiolabelled triacylglycerol and 30, 36, 60 and 63 % of the radiolabelled phospholipid were co-immunoprecipitated with apo-B from RER, SER, *cis*-Golgi and *trans*-Golgi luminal contents respectively. These observations are similar to those reported previously for rat liver fractions [26]

and rat hepatocytes (A.-M. Hebbachi, I. J. Cartwright and J. A. Higgins, unpublished work).

Chase experiments

To investigate the relationship between intracellular pools of radiolabelled lipids, hepatocytes were pulsed with [^3H]glycerol for 30 min and chased with a large excess of unlabelled glycerol for 120 min. Radioactivity in the triacylglycerol and phospholipid of RER, SER and *cis*-Golgi lumen and membrane fractions fell during the chase period and those in the *trans*-Golgi rose (Figures 6a–6d). Using marker enzymes it can be calculated that, in subcellular fractions from 1 g of hepatocytes, total loss of d.p.m. from luminal triacylglycerol was 94025 d.p.m. (70777 d.p.m. from the RER and 23248 d.p.m. from the SER) and that gained was 212217 d.p.m. (137217 d.p.m. by the Golgi and 75000 d.p.m. secreted). The total loss of triacylglycerol radioactivity from

Figure 7 Transfer of pulse-radiolabelled triacylglycerols (a) and phospholipids (b) between luminal content subfractions during chase experiments

Hepatocytes were incubated with [^3H]glycerol as described in the legend to Figure 6. The luminal content fractions at each time point were further fractionated on discontinuous gradients ($d = 1.006, 1.02, 1.063$ and 1.21) as described in the Materials and methods section. The lipids were extracted from the subfractions and analysed as described in the Materials and methods section. The radioactivity plotted is expressed as d.p.m. in each fraction per mg of protein of each unopened subcellular fraction in triacylglycerol and in phospholipid. $\triangle$, $d = 1.006$; $\blacktriangle$, $d = 1.02$; $\square$, $d = 1.063$; $\blacksquare$, $d = 1.21$.

Figure 8 Lipid composition of luminal content fractions

Luminal fractions were prepared and the lipids were extracted and analysed by HPTLC as described in the Materials and methods section. In parallel experiments the amount of apo-B was determined as described in the Materials and methods section. The results plotted are expressed as μg of lipid or apo-B in luminal content fractions isolated from 1 mg of protein of the unopened subcellular fraction and are means of determinations on three preparations. The standard deviations were less than 20%. $\blacksquare$, Apo-B; $\square$, cholesterol; $\blacksquare$, cholesterol ester; $\blacksquare$, phospholipid; $\blacksquare$, triacylglycerol.

the endoplasmic reticulum membranes was 938807 d.p.m. (318457 d.p.m. from the RER and 620352 d.p.m. from the SER) and that gained by the Golgi membrane was 276377 d.p.m.

Similar ratios in loss and gain of phospholipid radioactivity were also observed. These observations are consistent with a transfer of all of the newly synthesized endoplasmic reticulum luminal content triacylglycerol and phospholipid through the secretory pathway via the *trans*-Golgi lumen. As the amount of radiolabelled lipids moving into the *trans*-Golgi lumen was twice as high as that leaving the endoplasmic reticulum lumen, there must also be a transfer of newly synthesized lipids from the membranes into the secreted VLDL. In studies on rat hepatocytes we have shown that newly synthesized radiolabelled triacylglycerol is also transferred from the endoplasmic reticulum membrane to the cytosolic lipid droplets and there is no evidence of degradation of lipids. This is in contrast with the fate of apo-B, most of which is degraded without secretion (Figures 6e and 6f).

The luminal content fractions were separated on discontinuous gradients into fractions of $d = 1.006$, $d = 1.021$, $d = 1.063$ and $d = 1.21$. At the beginning of the chase period, the radiolabel in both triacylglycerol and phospholipids in the RER and SER fractions was highest in the most dense fraction with the lowest activity in the lightest fraction (Figure 7). During the chase period the radioactivity in each of the subfractions of the RER, SER and *cis*-Golgi luminal contents fell, whereas that in the lightest fraction of the *trans*-Golgi contents rose (Figure 7). Newly synthesized lipids including those in the dense fractions of the RER are therefore transferred to the VLDL ($d = 1.006$) in the *trans*-Golgi lumen.

Lipid composition of content subfractions

The *trans*-Golgi luminal contents contained about four times

Figure 9 Ratio of d.p.m. in lipid/d.p.m. in apo-B in luminal contents before and after chase experiments

Ratios of radiolabel in luminal triacylglycerol (a) or phospholipid (b) (^3H) to that in luminal apo-B (^{35}S) at the beginning and after 120 min of the chase are plotted. The data are taken from Figure 7. ▨, 0 min chase; ■, 120 min chase.

more lipid per mg of fraction protein than the other fractions. However, all content fractions contained lipids in similar ratios to apo-B (5–7 μg of phospholipid, 3–5 μg of cholesterol ester and 0.8–1.4 μg of cholesterol per μg of apo-B) except for triacylglycerol which had the highest concentration in the RER (40 $\mu\text{g}/\mu\text{g}$ of apo-B) and the lowest in the *trans*-Golgi (20 $\mu\text{g}/\mu\text{g}$ of apo-B) (Figure 8).

Are complete VLDL particles formed in the RER?

If complete VLDL particles are formed co-translationally in the lumen of the RER, it would be expected that the relative amount of radioactivity in all components in each compartment of the secretory pathway would remain constant during the chase experiments. In the radiolabelling experiments, the ratio of d.p.m. in triacylglycerol and phospholipid to that in apo-B in the luminal contents increased sharply between the SER and the *cis*-Golgi (Figure 9). During the chase period the ratio of d.p.m. in triacylglycerol and phospholipid to that in apo-B fell in the *cis*-Golgi and rose in the *trans*-Golgi. These results are due in part to the fact that a large fraction of the newly synthesized apo-B is retained and degraded in the RER lumen; however, they also indicate that lipoprotein particles are further modified by addition of more lipid between the SER and the *cis*-Golgi and that further remodelling occurs between the *cis*-Golgi and the *trans*-Golgi.

DISCUSSION

We have described in detail the characterization of our subcellular fractions from rat and rabbit liver based on marker enzymes and electron microscopy in previous publications [12,23,24]. Briefly, these results indicate that, in rabbit liver fractions, maximal cross-contamination between RER fractions and *trans*-Golgi is less than 10%; the SER contains less than 15% *trans*-Golgi and the *cis*-Golgi contains up to 24% endoplasmic reticulum. We have also shown that our rabbit Golgi preparations are not significantly contaminated with endosomes characterized by uptake of ^{125}I -LDL [24]. In the present study we have used additional markers of the *cis*-Golgi and *trans*-Golgi networks to confirm the identity of our fractions. In interpreting results from subcellular fractions, however, it must be emphasized that the secretory compartment represents a functional continuum in which the endoplasmic reticulum is linked to the *cis*-Golgi through the *cis*-Golgi network and the *trans*-Golgi is linked to the plasma membrane through the *trans*-Golgi network. The different compartments cannot be considered as separate, and use of all four fractions provides information about the whole secretory pathway with overlap between the central regions.

One model suggests that complete VLDLs are assembled in the RER lumen [28]. A number of pieces of evidence presented in this paper are inconsistent with this model. Most of the newly synthesized apo-B is in the dense fraction of the RER luminal contents and is degraded. In parallel experiments, all of the newly synthesized lipids, including those in the dense fraction, are transferred through the secretory pathway. Newly synthesized lipids are also transferred from the membranes into the secretory compartment, although the membrane-bound apo-B is degraded. In chase experiments there is a large increase in the ratio of radiolabelled newly synthesized phospholipid and triacylglycerol relative to radiolabelled apo-B at a point between the SER and the *cis*-Golgi with further modulation of lipid relative to apo-B between the *cis*-Golgi and the *trans*-Golgi lumen. These findings suggest that the apo-B-containing particles acquire a large fraction of lipid after leaving the RER. This is consistent with the immunocytochemical observations that particles of VLDL size in the SER lumen lack apo-B [29] and the fact that only about 28% of newly synthesized radiolabelled lipids in the RER lumen immunoprecipitate with apo-B whereas more than 70% of radiolabelled lipids are associated with apo-B in the Golgi luminal contents. Hamilton [30] has demonstrated that the RER lumen contains triacylglycerol-rich particles of VLDL density and denser apo-B-containing particles. In early studies on rat liver we also found small triacylglycerol-rich particles in the endoplasmic reticulum [22]. Newly synthesized lipids in these particles did not immunoprecipitate with apo-B [26]. Studies of the assembly of truncated apo-B into VLDL by Hep-G2 cells have also led to a two-step model for VLDL assembly in which smaller apo-B-containing precursor particles, the size of which is determined by the length of the apo-B molecules, are produced co-translationally [31]. Later in the secretory compartment these particles fuse with a triacylglycerol-rich particle and this step may be deficient in Hep-G2 cells, which only secrete apo-B in lipoproteins of LDL density, and which lack significant amounts of SER. Our finding that about a third of the newly synthesized lipids in the RER co-immunoprecipitate with apo-B does indicate that assembly of some lipid with apo-B occurs within the RER lumen. However, more lipid is added later in the secretory compartment.

Rusinol et al. [28] have isolated apo-B by immunoaffinity chromatography from the luminal contents of the endoplasmic reticulum and the Golgi and determined the composition of the

associated lipids. Their findings indicate that, apart from a higher concentration of triacylglycerol in the particles in the endoplasmic reticulum lumen compared with those secreted, the lipids associated with apo-B were of a similar composition. On the basis of these observations and the density distribution of the particles, they concluded that VLDL particles are completely formed early in the secretory pathway probably co-translationally. The lipid composition of the luminal contents in our experiments was similar to that found by Rusinol et al. However, we found that about 70 % of the newly synthesized triacylglycerol and phospholipid in the RER lumen did not immunoprecipitate with apo-B. As Rusinol et al. did not report what proportion of the lipids in the luminal content fractions was co-isolated with apo-B, it is not possible to compare their results directly with ours. However, it is clear from work on Hep-G2 cells and isolated rat hepatocytes and from the present findings that a significant fraction of newly synthesized apo-B is incorporated into a dense fraction which is degraded *in situ* [12,13]. In hepatocytes from rats fed on orotic acid, apo-B is only present in this dense fraction [12]. It is not therefore necessary for apo-B to be completely assembled into VLDL for translocation into the RER lumen and transfer to the Golgi lumen.

Experiments using monoclonal antibodies and proteolytic enzymes as topographic probes have shown that part of the membrane-bound apo-B in the RER is exposed at the luminal surface of the membrane [25,32]. An attractive model for translocation of apo-B has been suggested in which newly synthesized triacylglycerol reaches a critical concentration and separates from the membrane bilayer taking apo-B and phospholipids from the luminal surface into the cisternal space of the RER [2]. Alternatively, cholesterol esters or phosphatidylcholine have been implicated in the translocation of apo-B to the RER lumen [33–42]. In the present studies, although some lipids were present in all apo-B-containing fractions, we did not detect any relationship between specific lipids and luminal apo-B. This may be because the lipids bound to apo-B are only a small fraction of the total and/or that a large fraction of the newly synthesized apo-B that is degraded is associated with the wrong or insufficient lipids and is not 'secretion-competent'. Further investigations using specific radiolabels for different lipid classes and sub-fractionation of the luminal contents are necessary to elucidate the role of individual lipids in apo-B translocation.

Two related models have been suggested to account for the acquisition of lipids by apo-B-containing precursor particles. Lipids can be added sequentially or triacylglycerol-rich particles may fuse with the precursor particles. There is some evidence for both of these models. As indicated above, it has been possible to isolate triacylglycerol-rich particles from the lumen of the RER. However, there is also evidence for the involvement of the microsomal triacylglycerol-transfer protein (MTP) in VLDL assembly. MTP is located in the lumen of microsomes and mediates transfer of triacylglycerol, cholesterol esters and phospholipids between membranes and lipoproteins [43–45]. This protein has been shown to be defective in individuals suffering from abetalipoproteinaemia, a genetic disease in which apo-B secretion is blocked [44]. The results in the present study favour the model in which a large fraction of triacylglycerol and phospholipid becomes associated with apo-B at a point between the SER and the *cis*-Golgi. However, the formation of triacylglycerol-rich particles, the formation of apo-B-containing precursor particles and the fusion might all involve the MTP. There also appears to be further acquisition of lipids or remodelling of the VLDL between the *cis*- and *trans*-Golgi compartments, where synthesis of some VLDL phospholipid takes place [20].

The model of VLDL assembly that we believe best fits the available data is that apo-B-rich particles form in the RER lumen. Some of these are not secretion-competent, possibly because the associated lipid composition is incorrect or the apo-B-rich particles are bound by chaperone proteins. These are degraded *in situ*. Triacylglycerol-rich particles form in the endoplasmic reticulum lumen. The secretion-competent apo-B-containing-particles fuse with triacylglycerol-rich particles mainly in the endoplasmic reticulum lumen. Further modification of the lipid composition, including addition of phospholipids, takes place later in the secretory pathway.

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