The Assembly and Secretion of Apolipoprotein B-48-containing Very Low Density Lipoproteins in McA-RH7777 Cells*

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(Received for publication, November 3, 1999)

We have used an extraction procedure, which released membrane-bound apoB-100, to study the assembly of apoB-48 VLDL (very low density lipoproteins). This procedure released apoB-48, but not integral membrane proteins, from microsomes of McA-RH7777 cells. Upon gradient ultracentrifugation, the extracted apoB-48 migrated in the same position as the dense apoB-48-containing lipoprotein (apoB-48 HDL (high density lipoprotein)) secreted into the medium. Labeling studies with [3H]glycerol demonstrated that the HDL-like particle extracted from the microsomes contains both triglycerides and phosphatidylcholine. The estimated molar ratio between triglyceride and phosphatidylcholine was 0.70 ± 0.09, supporting the possibility that the particle has a neutral lipid core. Pulse-chase experiments indicated that microsomal apoB-48 HDL can either be secreted as apoB-48 HDL or converted to apoB-48 VLDL. These results support the two-step model of VLDL assembly. To determine the size of apoB required to assemble HDL and VLDL, we produced apoB polypeptides of various lengths and followed their ability to assemble VLDL. Small amounts of apoB-40 were associated with VLDL, but most of the nascent chains associated with VLDL ranged from apoB-48 to apoB-100. Thus, efficient VLDL assembly requires apoB chains of at least apoB-48 size. Nascent polypeptides as small as apoB-20 were associated with particles in the HDL density range. Thus, the structural requirements of apoB to form HDL-like first-step particles differ from those to form second-step VLDL. Analysis of proteins in the d < 1.006 g/ml fraction after ultracentrifugation of the luminal content of the cells identified five chaperone proteins: binding protein, protein disulfide isomerase, calcium-binding protein 2, calreticulin, and glucose regulatory protein 94. Thus, intracellular VLDL is associated with a network of chaperones involved in protein folding. Pulse-chase and subcellular fractionation studies showed that apoB-48 VLDL did not accumulate in the rough endoplasmic reticulum. This finding indicates either that the two steps of apoB lipoprotein assembly occur in different compartment or that the assembled VLDL is transferred rapidly out of the rough endoplasmic reticulum.

Immuno-electron microscopy studies have shown that apolipoprotein (apo) B is present in the rough endoplasmic reticulum (ER), but very low density lipoprotein (VLDL)-sized particles are not (1). VLDL particles with immunoreactive apoB first appeared in the smooth termini of the rough ER; the smooth ER contained VLDL-sized particles without immunoreactive apoB (1). Based on these results, a two-step model for the assembly of VLDL was proposed. Dynamic evidence for this model was obtained by pulse-chase studies of apoB-100 and apoB-48 (2, 3). The first step occurs during the translation of apoB and gives rise to a partially lipidated form of apoB (2, 4). In the case of apoB-100, this partially lipidated particle appeared to be loosely associated with the ER membrane (5). In the case of apoB-48, a particle resembling high density lipoprotein (HDL) has been identified. The secretion of this dense, apoB-48-containing, HDL-like lipoprotein varied inversely with that of VLDL (2). Therefore, we hypothesized that this particle is a precursor of apoB-48 VLDL (2). A second VLDL precursor was identified as an apoB-free “lipid droplet” in the smooth ER (1, 6). The assembly of both precursors is dependent on the microsomal triglyceride transfer protein (5, 7, 8).

The mechanism for the second step, fusion of the two precursors (1), is less well understood. We have demonstrated that brefeldin A inhibits the major lipidation of apoB (9). However the exact localization of the brefeldin A-sensitive mechanism in the assembly pathway remains to be elucidated.

Cotranslational or early post-translational degradation of apoB is important in regulating the amount of apoB that passes through the first step (10, 11). This degradation involves ubiquitination and proteasomes (11). Recent results indicate that apoB is completely translocated to the lumen of the ER (12), suggesting that the early post-translational degradation follows the pathway described for misfolded proteins (i.e. the protein is retracted through the translocation channel) (13). However, there is strong evidence that the degradation involves nascent as well as full-length apoB chains (14, 15), suggesting that this proteasomal degradation may also involve other pathways (for reviews, see Refs. 16 and 17).

Studies of VLDL assembly have been hampered by the fact that much of the apoB present in the cell remains associated with the microsomal membrane after carbonate extraction of the luminal proteins and therefore cannot be analyzed. Recently, we developed a procedure that extracts virtually all of the apoB-100 from the microsomal membranes without releasing integral membrane proteins (5). In this study, we used this new extraction procedure in a series of experiments to analyze the assembly of apoB-48 VLDL.

* This work was supported by Grants 7142 and 10435 from the Swedish Medical Research Council and by the Swedish Heart and Lung Foundation, the Swedish Oleo-Margarine Foundation of Nutritional Research, and Novo Nordic Insulin Fond. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: apo-, apolipoprotein; ER, endoplasmic reticulum; VLDL, very low density lipoprotein(s); HDL, high density lipoprotein(s); PAGE, polyacrylamide gel electrophoresis.

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EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimum essential medium, nonessential amino acids, glutamine, penicillin, and streptomycin were obtained from ICN Biomedicals (Costa Mesa, CA). Fetal calf serum was from Biochrom KG (Berlin, Germany) and brefeldin A from Epicenter Technologies (Madison, WI). Methionine, fatty acid-free bovine serum albumin, sodium pyruvate, d saccharate, sodium hydrogene carbonate, phenyl-

methylsulfonyl fluoride, pepstatin A, leupeptin were from Sigma. Rabbit immunoglobulin was from Dako (Glostrup, Denmark), and rabbit anti-trasferrin IgG was from Organon Teknika (West Chester, PA). Trasylol (aprotinin) was from Bayer (Leverkusen, Germany). Immunoprecipitin and Eagle’s minimum essential medium without methionine were from Life Technologies, Inc. N-Acetyl-Leu-Leu-norleucine as well as enzymatic assays for the determination of phospholipids or triglycerides were from Boehringer Mannheim. Amplyphi, F(β)-metho-

mione/cysteine mix, Rainbow protein molecular weight markers, and the ECL Western blotting analysis system were from Amersham Pharmacia Biotech, and ReadySafe was from Beckman (Fullerton, CA). All chemicals used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and alkaline phosphatase-conjugated goat anti-rabbit and rabbit anti-mouse were from Bio-Rad (Hercules, CA). Blue-stabilized sub-

strate for alkaline phosphatase and trypsin (sequencing grade) were from Promega (Madison, WI). Cyagenon bromide-activated Sepha-

rose 4B was from Amersham Pharmacia Biotech, and α-cyano-4-OH cinnamic acid was from Aldrich (Milwaukee, WI). Antibodies to chap-

erones (binding protein, protein disulfide isomerase, glucose regulatory protein 94, and calreticulin) were purchased from Affinity BioReagents (Golden, CO).

Cell Culture—McA-RH7777 cells were cultured as described previ-

ously (2) in Eagle’s minimum essential medium containing 20% fetal calf serum, 1.6 mM glutamine, 8.0 mM NaHCO₃, 1.6 mM sodium pyru-

vate, 140 mg/ml streptomycin, 140 IU/ml penicillin, and 60 mg/ml nonessential amino acids in 5% CO₂ at 37 °C. The cultures were split twice a week and fed daily.

Metabolic Labeling.—The cells were pulse labeled and chased as described (2). Cells and the microsomal fraction were isolated as de-

scribed (18). The luminal content of the vesicles was separated from the vesicle membranes by the sodium carbonate method (19), as modified (18). The luminal content of the microsomes or present in the culture medium was carried out as described (18). SDS-PAGE, autoradiography, and determination of the radioactivity in proteins separated in the gels were performed as de-

scribed elsewhere (21).

Lipid Determination.—Lipids from McA-RH7777 cells were extracted as described by Oegglund and Svennerholm (22) with slight modifications

Phosphatidylcholine and triglycerides were radiolabeled by incubat-

ing the cells for various periods with [3H]glycerol (0.6 μCi/ml of culture medium). Cells were extracted, phosphatidylcholine and triglycerides were separated as described above, and specific radioactivity was de-

termined (dpm/mg). In some experiments, apo-B-containing lipoproteins were isolated by immunofluorometry from the luminal content or the medium. During the extraction of the lipids from these fractions, unlabeled phosphatidylcholine and triglycerides were added as carriers. The lipids were separated as described above, and the spots corresponding to triglycerides and phosphatidylcholine were cut out of the scintillation vials; 1 ml of cyclohexane was added, and the radio-

activity was determined in the presence of ReadySafe scintillation mixture.

Isolation and Characterization of Proteins—Proteins associated with microsomal lipoproteins were isolated and identified as follows. Rat liver microsomes were isolated (23), and the luminal content was ex-

tracted with sodium carbonate (19). The extract (6 ml) was overlaid with 29 ml of phosphate-buffered saline (8 mM disodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, 137 mM sodium chloride, and 2.7 mM potassium chloride, pH 7.4, d = 1.006 g/ml). After centrifugation for 22 h at 40,000 rpm in a Beckman Ti-60 rotor at 4 °C, the gradient was fractionated from the top. The upper one-third of the tube (d < 1.006 g/ml) was collected. Pooled fractions of this supernatant (corresponding to three to five rat livers) were loaded onto a Mono Q column equilibrated with 50 mM Tris-HCl, pH 7.8, with 300 mM sucrose, 1 mM EDTA, 2 mM deoxycholate, 0.5% Triton X-100, 6 μM urea, and 40 mM sodium carbonate. The column was eluted with a linear gradient of sodium chloride (0–250 mM) at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected, and the proteins in each fraction were separated by SDS-PAGE on 10% A, 5% B gels, and stained with Coomassie blue.

Fractions containing the same protein patterns were combined, con-

centrated, and subjected to SDS-PAGE on 3–15% gradient gels. The gels were stained with Coomassie Brilliant Blue, and the bands were cut out, destained with 50 μl of a mixture of 50% ammonium bicarbonate (25 mM) and 50% acetonitrile, dried, and digested for 15 min with 0.1–0.2 mg of trypsin in 20 μl of 50% ammonium bicarbonate (25 mM) with 1 mM aprotinin. Ammonium bicarbonate (100 mM) was added to cover the gels, and incubation was continued for 12 h at 37 °C. Fragments were extracted with 10–50 μl of a mixture of 75% acetonit-

rile and 5% trifluoroacetic acid (in water).

Mass spectra were obtained on a TofSpec-E time-of-flight mass spectrometer (Micromass; Manchester, UK) equipped with a time-lagged focusing unit; TOF2U1 version 3.4 was used for data collection and OPUS version 3.4 for data analysis. α-Cyano-4-OH cinnamic acid (10 mg/ml in water/acetoni trile, 50/50, v/v) was used as matrix without further purification. The α-cyano-4-OH cinnamic acid solution (0.5 ml) was mixed with the gel extract (0.5 ml) on the target and allowed to dry at room temperature. Spectra were collected in reflection mode at an accelerating voltage of 20 kV with a 600-ns delayed extraction and a pulse of approximately 24 kV. Approximately 200 nitrogen laser pulses (3 ns, 337 nm) were carried out on each sample. For external calibra-

tion, a mixture of ACTH and angiotensin II was used (protonated 2465.2 and 1046.5, respectively). The peptide mass fingerprinting software program MS-Fit was run over the Internet. Monoisotopic masses were used for these measurements; mass tolerance was ± 200 ppm.

Biochemical determination of a monoclonal antibody against Rat Retinol—To de-

etermine the effects of deoxycholate/carbonate extraction (5) on integral microsomal proteins, we first generated a monoclonal antibody against riboforin. BALB/c mice were immunized with solubilized microsomal membrane proteins from rat liver (23). Positive hybridomas were identified by enzyme-linked immunoassortent assay with the antigen (solu-

bilized rat liver microsomes) and analyzed by Western blot of solubi-

lized rat liver microsomes. One hybridoma reacted with a 6-kDa

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protein and was recloned to monoclonality. The immunoglobulins were isolated from the hybridoma culture medium and coupled to cyanogen bromide-activated Sepharose 4B as recommended by the manufacturer (Amersham Pharmacia Biotech) and used for immunoadsorption experiments (23). Using this immunoadsorbent, we recovered the 60-kDa protein that reacted with the monoclonal antibody. This protein was cut out of the Coomassie-stained gel, digested with trypsin, and analyzed by mass spectrometry as described above. A data search identified the protein as rat riboforin.

RESULTS

Turnover of Intracellular ApoB-48 and Secretion of ApoB-48 VLDL—In contrast to ordinary sodium carbonate extraction, the deoxycholate/carbonate procedure (5) extracted virtually all of the apoB-48 (93 ± 1%; n = 5) from the microsomes. As judged from immunoblot studies of calnexin (see Ref. 5) and riboforin (Fig. 1A), deoxycholate/carbonate extraction did not release integral membrane proteins.

Upon gradient ultracentrifugation, the major amount of apoB-48 extracted by the deoxycholate/carbonate method migrated in the HDL density range (Fig. 1B). Using a modified gradient, we compared the densities of secreted apoB-48 and deoxycholate/carbonate-extracted apoB-48. The major amount of the secreted apoB-48 was present in the VLDL and the HDL density regions, as described previously (2). ApoB-48 that banded in the HDL density region (apoB-48 HDL) migrated in the same position as the apoB-48 extracted from the microsomes (Fig. 1C); we will refer to this form of apoB-48 as intracellular apoB-48 HDL. Thus, intracellular apoB-48 HDL and secreted apoB-48 HDL have very similar buoyant densities, and each migrated in the gradient in the expected position for a lipoprotein (in comparison with a nonlipidated protein of similar molecular weight) (Fig. 1C, III). The membrane-associated apoB-48, like apoB-100 (5), was extracted from the microsomes as a tentative lipoprotein.

To determine if intracellular apoB (both B-100 and B-48)-containing HDL is associated with lipids and contains a lipid core, we began by estimating the incubation time needed to obtain steady-state labeling of the phosphatidylcholine and triglyceride pools of the cell. The cells were incubated with (3H)glycerol (0.6 µCi/ml of culture medium) for 0, 1, 2, 5, 8, and 22 h. After each incubation, phosphatidylcholine and triglycerides were isolated, and the specific radioactivity (dpm/mg) was determined. Triglycerides reached a plateau after 8 h; phosphatidylcholine plateaued between 1 and 8 h, after which the specific radioactivity decreased (data not shown). We therefore incubated the cells for 8 h with (3H)glycerol (0.6 µCi/ml culture of medium). The specific radioactivities of the total phosphatidylcholine (4,025 dpm/µg of lipid) and triglyceride (2,044 dpm/µg of lipid) pools of the cell were determined. The apoB-containing lipoproteins in the HDL density region of the deoxycholate/carbonate extract of the microsomes were isolated by immunoaffinity chromatography, and the radioactivity in phosphatidylcholine and triglycerides was determined. Assuming that the specific radioactivity of the glycerolipids in this apoB fraction was the same as that of the total cell, we estimated the weight ratio between triglycerides and phosphatidylcholine in intracellular apoB HDL to be 0.85 ± 0.15 (n = 3; molar ratio, 0.70 ± 0.09), indicating less triglyceride than phospholipid. Thus, intracellular apoB HDL has an immature lipid core.

The labeled cells were also chased for 2 h, and apoB HDL in the medium was isolated by gradient ultracentrifugation followed by immunoaffinity chromatography. Analyzed as described above, the weight ratio between triglycerides and phosphatidylcholine was 0.27 ± 0.10 (n = 3; molar ratio 0.22 ± 0.08), indicating that this particle has a lipid core.

Next, we performed pulse-chase experiments to follow the turnover of intracellular apoB-48 HDL and correlated the findings with VLDL assembly and the appearance of apoB-48 HDL in the medium. The cells were pulse labeled with [35S]methionine/cysteine for 10 min and chased for 0–120 min. Radioactive apoB-48 was first seen in the microsomal intracellular apoB-48 HDL (Fig. 2). Not until maximal apoB-48 radioactivity was reached in this fraction did any significant amount of apoB-48 radioactivity appear in the VLDL fraction. In fact, the decrease in the apoB-48 radioactivity which followed this maximum accounted for the increased radioactivity in apoB-48 VLDL and in secreted apoB-48 HDL.

VLDL Assembly and the Size of Nascent ApoB Polyptides—To determine the length of apoB required for VLDL assembly, we performed pulse-chase studies of nascent apoB chains. To obtain a continuous series of apoB polypeptides of different lengths which could be tested in the assembly process, we truncated apoB with cycloheximide, detached the nascent
polypeptides from the ribosomes with puromycin, and chased them through the secretory pathway of the cell into the medium (4). The cells were pulse labeled for 10 min and chased for 0–30 min. After each chase period, the cells were treated with cycloheximide and puromycin and then chased for another 180 min in the presence of cycloheximide and puromycin to allow the nascent chains to form lipoproteins and be secreted into the medium. The medium (containing full-length apoB-100/48 as well as the nascent apoB polypeptides that were released into the secretory pathway and secreted during the 180-min chase) was subjected to gradient ultracentrifugation. ApoB was recovered from each fraction by immunoprecipitation and analyzed by SDS-PAGE.

The major amount of apoB radioactivity was recovered with lipoproteins in the VLDL density range (Fig. 3A). After a 0-min chase, apoB nascent polypeptides corresponding to approximately apoB-40 and longer were associated with the VLDL fraction. However, an only small amount of nascent apoB chains shorter than apoB-48 was incorporated into VLDL. The antiserum used to immunoprecipitate apoB recognized nascent apoB chains in the medium at least as short as apoB-20 (Fig. 3B). Nascent polypeptides of the size of apoB-20 to apoB-40 banded in the HDL density region and were incorporated into apoB-48 HDL (Fig. 5, A and B). These results demonstrate that apoB-20 and longer nascent chains can assemble apoB-48 HDL and that apoB-40 is the approximate minimal length for VLDL assembly. However, efficient VLDL assembly requires apoB chains about the size of apoB-48.

Proteins Associated with Microsomal VLDL—To determine if proteins other than known apolipoproteins were associated with luminal lipoprotein particles (i.e. could be involved in the second step of VLDL assembly), we isolated the luminal content of rat livers and characterized proteins in the d < 1.006 g/ml fraction. Three fractions (I–III in Fig. 4) isolated from this supernatant by ion-exchange chromatography were characterized by major proteins with molecular masses around 70 kDa (I), 60 kDa (II), and 100, 70, and 50 kDa (III). After separation by SDS-PAGE and Coomassie staining, the stained bands (1–5 in Fig. 4) were cut out of the gel and digested with trypsin; the tryptic fragments were identified by the matrix-assisted laser desorption ionization-time of flight technique (Table I). All isolated proteins were identified by data-base search as chaperones: band 1 was identified as binding protein, band 2 as protein disulfide isomerase, band 4 as calcium-binding protein 2, and band 5 as calreticulin. Band 3 did not correspond to any protein in the rat data base, but a search of the mouse data base identified it as glucose regulatory protein 94.

The identities of these proteins were confirmed by immunoblotting of the VLDL fraction with antibodies against binding protein, protein disulfide isomerase, glucose regulatory protein 94, and calreticulin (Fig. 5, lane 1). In these experiments, the supernatant (containing VLDL) was dialyzed and lyophilized before it was subjected to electrophoresis and immunoblotting. The predominant amount of chaperones was recovered in the infranatant after this centrifugation (not shown). This infranatant (depleted of VLDL by the ultracentrifugation) was subsequently recentrifuged under the same conditions at pH 7.4 or 11, and the supernatant was recovered and immunoblotted as described above. No or very small amount of immunoreactive chaperones were detected in this supernatant (Fig. 5, lane 2 (pH 7.4) and lane 3 (pH 11). These results indicate that the recovery of chaperones in the supernatant depends on the presence of VLDL and does not reflect the fact that chaperones are abundant microsomal proteins. The experiments were designed so the two supernatants (Fig. 5, lanes 2 and 3) could be compared directly with the VLDL fraction (Fig. 5, lane 1).
Table I

Identification of proteins isolated from microsomal VLDL

| Identified protein | NCBI access. No. | Band 1 | Band 2 | Band 3 | Band 4 | Band 5 |
|-------------------|-----------------|-------|-------|-------|-------|-------|
|                   |                 | 11/13 (84%) | 13/15 (86%) | 12/13 (92%) | 9/11 (81%) | 9/10 (90%) |
| Tryptic fragments matching theoretical fragments | Peptide mass | Position in identified protein | Peptide mass | Position in identified protein | Peptide mass | Position in identified protein | Peptide mass | Position in identified protein | Peptide mass | Position in identified protein | Peptide mass | Position in identified protein |
| 1 | 1430.8 | 102–113 | 910.5 | 446–453 | 1015.5 | 396–404 | 909.4 | 427–433 | 974.5 | 112–120 |
| 2 | 1460.9 | 354–387 | 1002.6 | 71–76 | 1031.6 | 539–546 | 999.6 | 498–505 | 1019.5 | 144–151 |
| 3 | 1512.8 | 325–336 | 1081.7 | 256–264 | 1081.6 | 62–76 | 1178.5 | 259–268 | 1147.6 | 143–151 |
| 4 | 1566.8 | 61–74 | 1227.6 | 377–386 | 1187.5 | 385–395 | 1282.7 | 244–254 | 1219.7 | 88–98 |
| 5 | 1677.9 | 82–96 | 1355.7 | 377–387 | 1485.8 | 435–446 | 1656.6 | 484–497 | 1452.7 | 25–36 |
| 6 | 1816.1 | 198–214 | 1424.8 | 197–208 | 1752.0 | 415–428 | 1769.7 | 271–286 | 1784.8 | 206–222 |
| 7 | 1888.1 | 165–181 | 1729.9 | 411–425 | 1865.0 | 460–466 | 1852.8 | 330–344 | 2519.1 | 186–207 |
| 8 | 1934.1 | 475–492 | 1780.9 | 83–98 | 2030.1 | 117–135 | 2124.9 | 410–426 | 2760.2 | 225–248 |
| 9 | 1975.0 | 602–617 | 1854.0 | 287–301 | 2250.2 | 117–135 | 2246.9 | 462–483 | 2960.2 | 225–248 |
| 10 | 1999.2 | 493–510 | 1965.1 | 232–248 | 2287.1 | 429–448 | 2191.2 | 512–530 | 364–384 |
| 11 | 2149.0 | 307–324 | 2418.3 | 352–371 | 2452.3 | 244–265 | 2452.7 | 365–384 | 244–265 |
| 12 | 2684.3 | 172–196 | 2542.3 | 244–265 | 2542.3 | 329–348 |
| 13 | 2895.5 | 134–163 | 329–348 |
Assembly and Secretion of ApoB-48-containing VLDL

DISCUSSION

This study shows that deoxycholate/carbonate extraction releases apoB-48 from the microsomal membrane without releasing integral membrane proteins. During sucrose gradient ultracentrifugation, the released intracellular apoB-48 VLDL comigrated with the secreted apoB-48 HDL, and both displayed the characteristics of lipoproteins. The intracellular particle contained both phosphatidylcholine and triglyceride at a molar ratio of 0.7, indicating a lipid core. These analyses were based on the incorporation of [3H]glycerol into the two glycerolipids and the assumption that the specific radioactivities of triglycerides and phosphatidylcholine in the lipoprotein are similar to those observed in the cell. The analyses were carried out under conditions in which very small changes in the specific radioactivities of the two lipids were observed in the cell, indicating a steady-state situation. We believe that these conditions increase the possibility that the specific radioactivities of phosphatidylcholine and triglycerides of the intracellular lipoproteins are similar to those in the cell. Interestingly, the secreted apoB HDL had a significantly lower molar ratio of phosphatidylcholine to triglyceride (0.22) than the intracellular lipoprotein. We suggest two possible explanations for this difference. The first explanation is that intracellular apoB HDL contains both apoB-48 and apoB-100, whereas the secreted apoB-48 HDL contains virtually only apoB-48 (2). It is possible that apoB-48 assembles with less neutral lipid during the cotranslational lipidation. This mechanism is consistent with what we observed during studies of the cotranslational lipidation of apoB in HepG2 cells (4). The second explanation is that the secreted lipoprotein is an underlipidated form that has failed to form VLDL. This mechanism is consistent with our previous observation of an inverse relationship between apoB-48 VLDL and the secreted apoB-48 HDL (2).

In turnover studies, radioactive apoB-48 first appeared in the intracellular apoB-48 HDL and was not observed in VLDL in significant amounts until after the radioactivity had reached a maximum in intracellular apoB-48 HDL. The subsequent decrease in the radioactivity in intracellular apoB-48 HDL accounted for all of the increase in radioactivity in apoB-48 VLDL and in the secreted apoB-48 HDL. These results show that apoB-48 HDL is produced in the first step of the assembly process and is the precursor for VLDL produced during the second step. Moreover, intracellular apoB-48 HDL appears to be the precursor for secreted apoB-48 HDL.

These findings, together with our previous demonstration of an inverse relationship between secretion of apoB-48 HDL and apoB-48 VLDL (2), provide the basis for a model of apoB-48 VLDL and HDL. These results show that apoB-48 HDL is produced in the first step of the assembly process and is the precursor for VLDL produced during the second step. Moreover, intracellular apoB-48 HDL appears to be the precursor for secreted apoB-48 HDL.

Structural Requirements for Assembly of ApoB-48 VLDL—This study also demonstrated that apoB must reach the size of apoB-20 for the HDL-like particle to be assembled and secreted and must reach the size of apoB-40 to undergo the second step of VLDL assembly. In these experiments, we used our previously described method (4) to generate a series of truncated forms of apoB. In contrast to transfection studies, this method uses endogenous apoB and therefore avoids artifacts that can occur from the overexpression of a foreign gene or during the selection of stably transfected cells. The observation that apoB-40 can be assembled into VLDL supports the results of studies with transfected forms of apoB truncated at the carboxyl terminus (24), which indicated that apoB-37 can be assembled into VLDL. Not until apoB has reached the size of apoB-48, however, is there a quantitatively important recruitment of precursor particles to the second step of the assembly process.

ApoB-48 (as well as apoB-40) contains the amino-terminal globular domain and the major amount of the amphipathic β-sheet domain (25). The amino-terminal globular domain is important for the assembly of apoB lipoproteins (26), whereas the amphipathic β-sheet domain seems to be important for the strong and irreversible interaction between apoB and the lipid core. However, the same type of structure is present in the region between apoB-20 and apoB-40 (i.e. on the nascent chains
that are secreted with the HDL-like particle but not with VLDL. Thus, our results define more specifically the structural requirements for apoB to participate in the second step of VLDL assembly. Interestingly, although some nascent apoB chains as short as apoB-40 were assembled into VLDL, the efficiency of VLDL assembly increased rapidly when apoB reached the size of apoB-48. This could indicate that the carboxyl-terminal 17% of apoB-48 (i.e., apoB-40–apoB-48), predicted to be amphipathic α-helices (25), has a central role in the interaction between apoB-48 HDL and the second step. The results obtained in McA-RH7777 cells, which produce bona fide VLDL, differ completely from those obtained with the same method in HepG2 cells, which do not produce significant amounts of VLDL (4). In HepG2 cells, the length of nascent apoB-100 chains is inversely related to the density of the lipoprotein particles they assemble, ranging from HDL to low density lipoprotein (4). Thus, in a cell that does not produce VLDL, the lipidation is proportional to the length of the translated polypeptide. These observations reflect a fundamental difference between the cotranslational assembly process in HepG2 cells (4, 28) and the two-step process for VLDL assembly in McA-RH7777 cells (2).

Chaperone Proteins in VLDL Assembly—Another major observation in this study is that VLDL particles isolated from the secretory pathway were associated with several chaperone proteins. Recently, Linnik and Herscovitz (27) independently demonstrated a similar network of chaperones associated with apoB, independent of lipidation, in HepG2 cells. HepG2 cells may be regarded as a model system for the first (cotranslational) step of VLDL assembly (4, 28). Thus, these important results demonstrate that the first step in the assembly process is guided by chaperones (27). Our observations demonstrate that chaperones are also involved in the second step, when the particles have the density of VLDL. Thus, the whole assembly process seems to be guided by chaperones. A similar network of chaperones has been identified in association with other immature proteins in the secretory pathway. For example, influenza hemagglutinin is associated with binding protein, glucose regulatory protein 94, calreticulin, and calnexin before the homotrimer is formed (29, 30). We did not identify calnexin associated with the intracellular lipoproteins, which is logical because it is an integral membrane protein, and the experiment was designed to investigate lipoprotein particles in the luminal content. One explanation is that these lipoproteins

**Fig. 6.** Accumulation of apoB-48-containing lipoproteins in different subcellular fractions in McA-RH7777 cells. Panel A, subcellular fractionation of McA-RH7777 cells. The cells were homogenized in a Dounce homogenizer, and the microsomal fraction was subjected to ultracentrifugation in a sucrose gradient. The gradient was unloaded from the bottom into 23 fractions, which were analyzed for NADPH cytochrome c reductase and galactosyl transferase. The enzyme activities are shown as arbitrary units (NADPH cytochrome c reductase) and dpm of [3H]galactose transferred/60 min (galactosyl transferase). Western blots of each fraction were probed with anticalnexin (panel B). Pools I, II, III, and IV are indicated. Panel C, distribution of apoB-48 VLDL in the different subcellular fractions during a pulse-chase experiment. McA-RH7777 cells cultured in the absence of oleic acid were labeled with [35S]methionine/cysteine for 30 min and chased, first in the absence of oleic acid for 120 min and then in the presence of oleic acid for 0–60 min. Subcellular fractionation was carried out after each chase period (with oleic acid); and the luminal contents of the four pools (I–IV in panel A) were recovered and subjected to gradient ultracentrifugation. ApoB was recovered from each fraction of the gradient by immunoprecipitation and analyzed by SDS-PAGE and autoradiography.

**Fig. 7.** Two-step model of VLDL assembly. In the first step of VLDL assembly, apoB is cotranslationally translocated to the lumen of the endoplasmic reticulum to form a pre-VLDL particle. The pre-VLDL can acquire the major amount of lipids in the second step, which either occurs immediately before the VLDL particle is transferred out of the rough ER or occurs in a compartment other than that in the first step. A network of chaperones is associated with the VLDL particle that is under assembly. BiP, binding protein; PDI, protein disulfide isomerase; CaBP2, calcium-binding protein 2; GRP 94, glucose regulatory protein 94.
already have lost their contact with calnexin, as the interaction between apoB and calnexin is important for early events in the assembly process (31). Alternatively, the interaction between this chaperone and the lipoprotein may be disrupted by the extraction procedure.

Localization of ApoB-48 during the Assembly Process—The final observation made in this paper is that apoB-48 VLDL accumulated in a compartment separate from the rough ER; no apoB-48 VLDL was detected in the rough ER. One possible explanation for this observation is that the second step of VLDL assembly occurs not in the rough ER but in a smooth membrane compartment (pool IV). Alternatively, apoB-48 VLDL may accumulate, but not be assembled, in pool IV. Three observations argue against the latter possibility. First, no apoB-48 VLDL was detected in any of the rough ER fractions, even though they contained considerable amounts of radioactive apoB-48. Second, the results were the same irrespective of which extraction procedure (carbonate or deoxycholate/carbonate) was used to extract apoB-48 from the microsomes. Third, the appearance of apoB-48 VLDL in pool IV corresponded to the start of the second step during the oleic acid incubation. However, we could not exclude completely the possibility that VLDL is assembled in the rough ER. Thus the very small pool of VLDL in the rough ER may be the result of a rapid transfer of the assembled VLDL out of this compartment. Such a mechanism could be compatible with our failure to detect VLDL in the rough ER. Kinetics studies in rat indicate that the transfer out of the ER is the rate-limiting step in secretion (32). This rate-limiting step could be the conversion of pre-VLDL to VLDL. The observation that VLDL are associated with a network of chaperones, which contains ER retention signals, could suggest that the lipoprotein is retained in a post-rough ER, pre-Golgi smooth membrane compartment until the chaperones have left the particle.

In conclusion, the results presented here support the two-step model for the assembly of apoB-48 VLDL (Fig. 7). The process starts with the translation of the protein, forming a full-length, partially lipidated, pre-VLDL intracellular apoB-48 HDL. The pre-VLDL acquire the major amount of lipids in the second step, forming bona fide VLDL. The second step occurs either in a smooth membrane compartment or immediately before the particle is transferred out of the rough ER. The entire assembly process involves a series of chaperone proteins.

Acknowledgments—We thank Anita Magnusson for technical assistance and Stephen Ordway for excellent editing of the manuscript.

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J. Biol. Chem. 2000, 275:10506-10513.
doi: 10.1074/jbc.275.14.10506

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