Relation of the size and intracellular sorting of apoB to the formation of VLDL 1 and VLDL 2

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Abstract In this study, we tested the hypothesis that two separate pathways, the two-step process and an apolipoprotein B (apoB) size-dependent lipidation process, give rise to different lipoproteins. Expression of apoB-100 and C-terminally truncated forms of apoB-100 in McA-RH7777 cells demonstrated that VLDL particles can be assembled by apoB size-dependent linear lipidation, resulting in particles whose density is inversely related to the size of apoB. This lipidation results in a LDL-VLDL 2 particle containing apoB-100. VLDL 1 is assembled by the two-step process by apoB-48 and larger forms of apoB but not to any significant amount by apoB-41. The major amount of intracellular apoB-80 and apoB-100 banded with a mean density of 1.10 g/ml. Its formation was dependent on the sequence between apoB-72 and apoB-90. This dense particle, which is retained in the cell, possibly by chaperones or association with the microsomal membrane, is a precursor of secreted VLDL 1. The intracellular LDL-VLDL 2 particles formed during size-dependent lipidation appear to be the precursors of intracellular VLDL 1. We propose that the dense apoB-100 intracellular particle is converted to LDL-VLDL 2 by size-dependent lipidation. LDL-VLDL 2 is secreted or converted to VLDL 1 by the uptake of the major amount of triglycerides.—Stillemark-Billton, P., C. Beck, J. Borén, and S-O. Olofsson. **Relation of the size and intracellular sorting of apoB to the formation of VLDL 1 and VLDL 2.** ***J. Lipid Res.*** **2005.** 46: **104–114.**

Supplementary key words chaperones • microsomal triglyceride transfer protein • intracellular retention • apolipoprotein B • very low density lipoprotein

Immunoelectron microscopy (1) and kinetic studies (2, 3) indicate that VLDLs are assembled in two major steps (4, 5). The first step occurs during the translation of apolipoprotein B (apoB) and gives rise to a premature particle (2, 6) we refer to as a primordial lipoprotein. Major amounts of lipid are added in the second step, resulting in bona fide VLDL (2, 7, 8); a second precursor of VLDL, an apoB-free "lipid droplet" in the smooth endoplasmic reticulum (1, 9) whose assembly requires microsomal triglyceride transfer protein (MTP), may also be involved (10). VLDLs are secreted in two forms: large, triglyceride-rich VLDL 1 and smaller, triglyceride-poor VLDL 2. Overproduction of VLDL 1 is linked to conditions such as insulin resistance and type II diabetes (11).

The lengths of C-terminally truncated forms of apoB-100 are inversely related to the amount of lipid in the lipoproteins they assemble; assembly with apoB-100 results in VLDL (12). This size-dependent lipidation of apoB does not fit the two-step model; in particular, it cannot explain why apoB-48 has the ability to assemble VLDL but apoB-40 lacks this ability (8).

In this study, we tested the hypothesis that two separate pathways, the two-step process and an apoB size-dependent lipidation process, give rise to different lipoproteins. Our results indicate that although apoB can assemble VLDL 1 once it reaches the size of apoB-48, the size-dependent process gives rise to LDL-VLDL 2 particles first when apoB-100 is reached. In contrast to apoB-48 (2), the major intracellular form of apoB-100 is much denser than expected from the size/density relation, and it is retained in the secretory pathway. The sequence between apoB-72 and apoB-90 is essential for the formation of this intracellular particle, which is the precursor of the secreted VLDL 1.

MATERIALS AND METHODS

Materials

Immunoprecipitin, Eagle's minimal essential medium (with and without methionine), and Williams' medium E with Glutamax were from Life Technologies (Paisley, Scotland). Nonessential amino acids, glutamine, penicillin, and streptomycin were from ICN Biomedicals (Costa Mesa, CA). Fetal calf serum was from

Abbreviations: apoB, apolipoprotein B; BiP, binding protein; GRP94, glucose regulatory protein 94; LDLR, low density lipoprotein receptor; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase.

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Manuscript received 3 August 2004 and in revised form 28 September 2004 and in re-revised form 20 October 2004.

Published, JLR Papers in Press, November 1, 2004.
DOI 10.1194/jlr.M400296-JLR200

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JRH Biosciences, and rabbit immunoglobulin was from DAKO. Brefeldin A was from Epicentre Technologies (Madison, WI). Collagenase type IV, methionine, sodium pyruvate, disodium carbonate, PMSF, pepstatin A, and leupeptin were from Sigma (St. Louis, MO). Trasylol (aprotinin) was from Bayer Leverkusen (Germany). *N*-Acetyl-Leu-Leu-norleucinal was from Boehringer Mannheim. Amplify, [^{35}S]methionine-cysteine (Pro-mix), Rainbow molecular weight markers, and the ECL Western blot analysis system were from Amersham. Ready Safe was from Beckman (Fullerton, CA). Geneticin was from Duchefa. Antibodies to the chaperones binding protein (BiP), protein disulfide isomerase (PDI), glucose regulatory protein 94 (GRP94), and calreticulin were from Affinity BioReagents (Golden, CO). Primaria cell culture dishes were from Becton Dickinson Labware (Franklin Lakes, NJ). All enzymes for DNA work were from New England Biolabs (Beverly, MA). Dynabeads were from Dynal Biotech (Oslo, Norway). All chemicals for SDS-PAGE and alkaline phosphatase-conjugated goat anti-rabbit and rabbit anti-mouse immunoglobulins were from Bio-Rad (Hercules, CA).

Culture, metabolic labeling, and treatment of McA-RH7777 cells

McA-RH7777 cells were cultured in the presence of oleic acid as described (2). The cultures were fed daily and split twice weekly. They were pulse-labeled with [^{35}S]methionine-cysteine (Pro-mix) and chased in culture medium supplemented with 10 mM methionine as described (2). Treatment with brefeldin A and inhibition of MTP were carried out as described (13). Briefly, McA-RH7777 cells were pretreated with brefeldin A (15 min), pulse-labeled (30 min), and chased (30 min) in the presence of brefeldin A. During the chase, initiated nascent chains were converted to full-length apoB, but brefeldin A blocked further processing to VLDL. In the next step, apoB-100 was allowed to form VLDL during a 180 min chase in the presence of oleic acid but in the absence of brefeldin A. Control cells were compared with cells in which MTP was irreversibly inhibited.

Generation of apoB constructs and transfection of McA-RH7777 cells

The carboxyl-terminal 18% and 28% of apoB-100 were amplified by PCR with the following oligonucleotides: 5′-CGACGCGTCCTAGGTTCTGACGTCCG-3′ and 5′-GGGGTACCCTATCTTCTCAGCTTTGAAGACACTTCT-3′ [B-50+(72–90)] and 5′-CCATCGATCTAGGTTCTGACGTCCGTGT-3′ and 5′-GGGGTACCCTAGAGGATGATAGTAAGTTCTCCTG-3′ [B-41+(72–100)]. PCR fragments were digested with *Mlu*I-*Kpn*I [B-50+(72–90)] or *Cla*I-*Kpn*I [B-41+(72–100)] and ligated into the apoB-100 L-L plasmid (14). The resulting plasmids encode the amino-terminal 50% of apoB-100 fused to the carboxyl-terminal 18% of apoB-100 [B-50+(72–90)] or the amino-terminal 41% of apoB-100 fused to the carboxyl-terminal 28% of apoB-100 [B-41+72–100].

ApoB-41 was obtained by digesting the apoB-100 L-L plasmid with *Cla*I-*Kpn*I and ligating the purified fragment with the following oligonucleotide: 5′-CGATTGATAGTAAGGTAC-3′. The plasmids were transfected into McA-RH7777 cells with Tfx50 (Promega) and maintained in medium containing 600 μg/ml geneticin.

Approximately 3–4 weeks after transfection, colonies were picked and maintained in selective culture medium. Stable clones were selected by PCR of cDNA with the following oligonucleotides: 5′-CGACGCGTCCTAGGTTCTGACGTCCG-3′ and 5′-CCTGTTGTTCCCAGTGGTA-3′ [B-50+(72–90)] and 5′-CCATCGATCTAGGTTCTGACGTCCGTT-3′ and 5′-CCTGTGTGTTCCCAGTGGTA-3′ [B-41+(72–100)]. The RNA was converted to cDNA by reverse transcriptase PCR (Taqman; Applied Biosystems), and the PCR products from both DNA and cDNA were compared on an agarose gel. McA-RH7777 cells were also transfected with carboxyl-terminal-truncated human apoB-53, apoB-72, and apoB-80 (from Zemin Yao, University of Ottawa Heart Institute, Ottawa, Canada) using DOSPER liposomal transfection reagent (Boehringer Mannheim). The cells were maintained in culture medium containing 800 μg/ml geneticin. Approximately 3–4 weeks after transfection, colonies were picked and maintained in selective culture medium. Two weeks before experiments, geneticin was omitted from the culture medium.

Primary cultures of mouse hepatocytes

Mouse hepatocytes were isolated essentially as described for rat hepatocytes (15). The liver was perfused first with Hanks' balanced salt solution containing 0.6 mM EGTA, 20 mM HEPES, and 10 mM sodium hydrogen carbonate without calcium or magnesium, pH 7.4 (7–8 min, 40–50 ml/min) and then with Williams' medium E with Glutamax supplemented with penicillin (50,000 IU/l), streptomycin (50 mg/l), 0.28 mM sodium ascorbate, 0.1 μM sodium selenite, and 400 mg/l collagenase type IV (8–10 min, 40 ml/min). The cells were filtered through a 250 μm nylon filter and a 100 μm cell strainer and washed by centrifugation at 50 *g* three times for 1 min each at 4°C in Williams' E medium with Glutamax containing glucose (3 g/l), insulin (Actrapid; Novo Nordisk; 28.6 U/mg), sodium ascorbate, sodium selenite, and the antibiotics described above. The cells were seeded (70,000 cells/cm^2) in 10 ml of medium in Primaria cell culture dishes. After 4 h, the medium was replaced with fresh medium, and the cells were cultured for 13 h before use. Control experiments with cells from human apoB-100 transgenic mice demonstrated that the secretion of apoB-100 VLDL was stable for at least 24 h after the initiation of the experiment but started to decline after 3 days in culture and had nearly ceased by 4 days. This is in agreement with previous observations (16). To investigate the influence of the LDL receptor (LDLR) on the secretion of pre-VLDL, human apoB-100 transgenic mice were crossed with LDLR-null ($LDLR^{-/-}$) mice (16).

Sucrose gradient ultracentrifugation

Sucrose gradient ultracentrifugation of lipoproteins was performed as described (2). Before centrifugation, the conditioned medium or microsomal extract was supplemented with 0.1 mM leupeptin, 1 mM PMSF, 1 mM pepstatin A, 5 mM *N*-acetyl-Leu-Leu-norleucinal, and 100 Kallikrein inhibitory units (KIU)/ml aprotinin.

The luminal content of the microsomes was extracted by the deoxycholate-carbonate method (13) or the carbonate method (17) as modified (18). Virtually all of the apoB-100 and apoB-48 can be extracted from McA-RH7777 microsomes by the deoxycholate-carbonate method (13). Thus, the amount of primordial particles containing apoB-100 and apoB-48 in the deoxycholate-carbonate extract provides an estimate of the total intracellular pool.

Other methods

ApoB was isolated by immunoprecipitation and SDS-PAGE (2, 19). Immunoblotting was carried out as described (8). Specific immunoglobulins were isolated from rabbit anti-human apoB antiserum by affinity chromatography on LDL immobilized to Sepharose. The specific immunoglobulins were eluted with 3 M sodium thiocyanate from the affinity column and coupled to Dynabeads (M-450 Tosylactivated; Dynal Biotech).

To isolate apoB-containing primordial lipoproteins, we extracted the luminal content from microsomes (13). Density fractions containing the primordial particles were isolated by gradient ultracentrifugation and immunoaffinity purified by incubation with human apoB antibodies coupled to Dynabeads for 2 h at 4°C in PBS, pH 7.4. The precipitate was washed three times with

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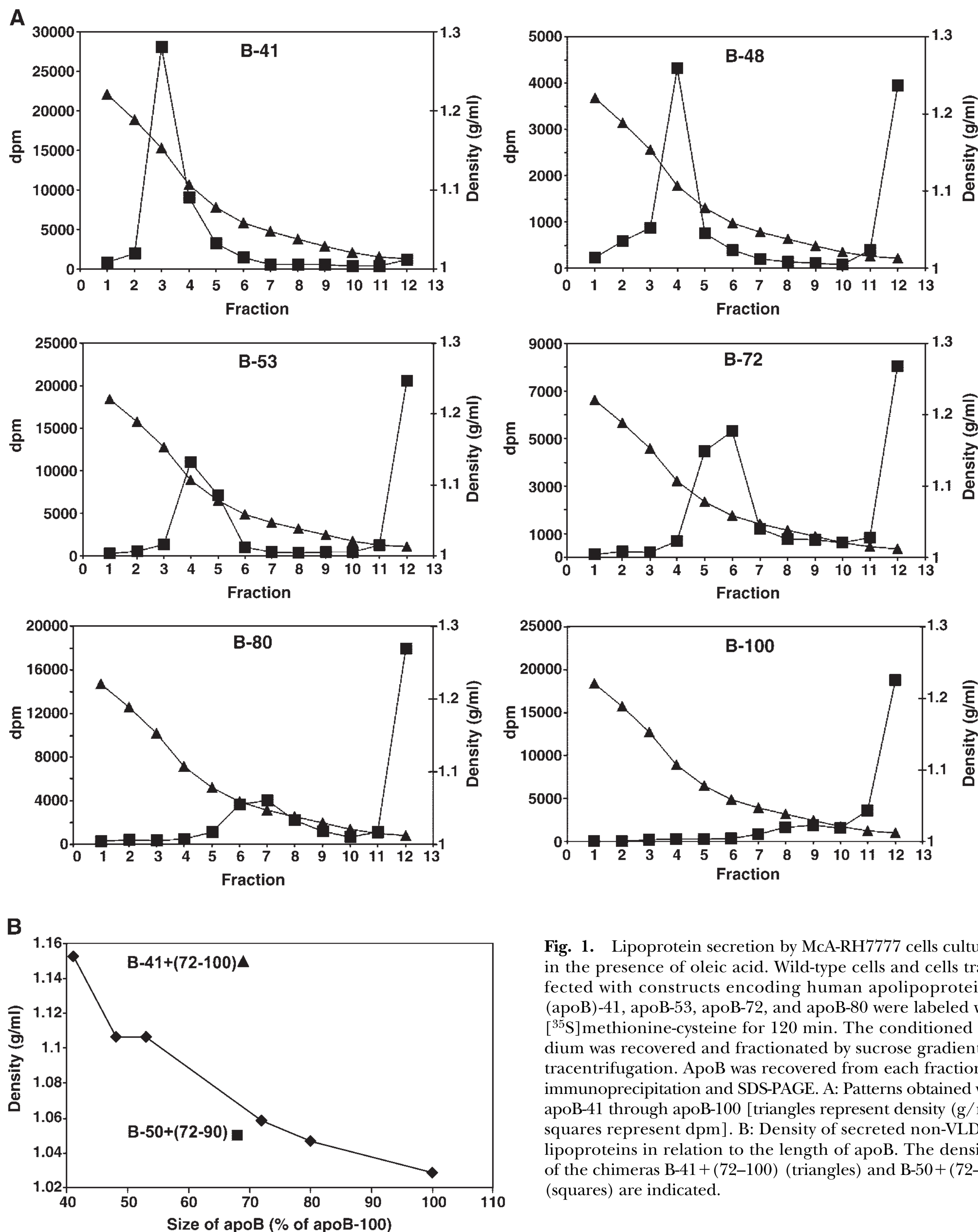

Fig. 1. Lipoprotein secretion by McA-RH7777 cells cultured in the presence of oleic acid. Wild-type cells and cells transfected with constructs encoding human apolipoprotein B (apoB)-41, apoB-53, apoB-72, and apoB-80 were labeled with [^{35}S]methionine-cysteine for 120 min. The conditioned medium was recovered and fractionated by sucrose gradient ultracentrifugation. ApoB was recovered from each fraction by immunoprecipitation and SDS-PAGE. A: Patterns obtained with apoB-41 through apoB-100 [triangles represent density (g/ml); squares represent dpm]. B: Density of secreted non-VLDL 1 lipoproteins in relation to the length of apoB. The densities of the chimeras B-41+(72–100) (triangles) and B-50+(72–90) (squares) are indicated.

PBS and incubated for 5 min at 95°C in sample buffer to release the proteins. The solubilized precipitate was subjected to SDS-PAGE with 10% gels and blotted with antibodies to BiP, calreticulin, GRP94, PDI, and immunoglobulins from nonimmunized rabbit or mouse.

RESULTS

Lipoproteins assembled and secreted from McA-RH7777 cells transfected with different lengths of apoB

Sucrose gradient ultracentrifugation was used to investigate the lipoproteins secreted by McA-RH7777 cells trans-

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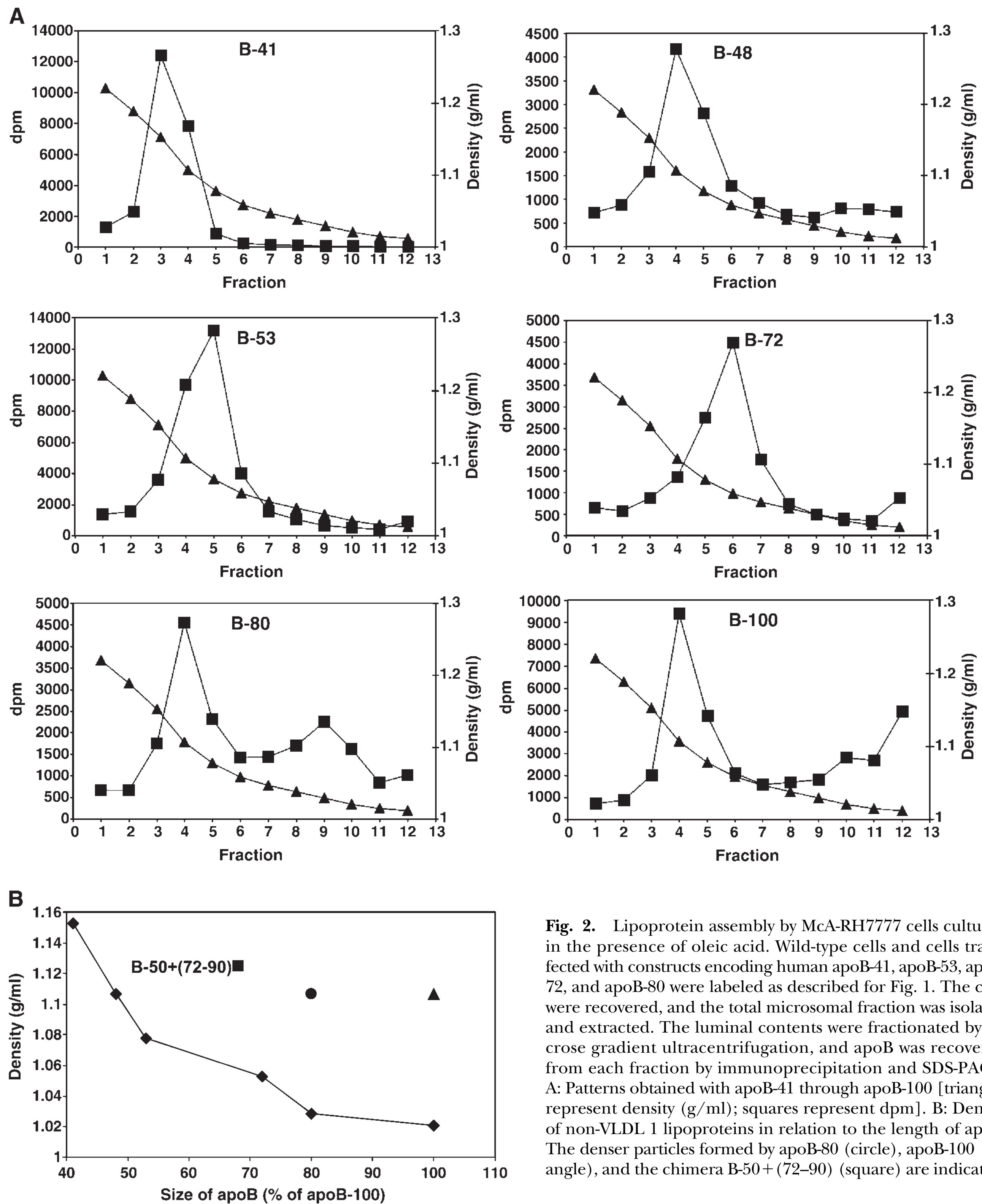

Fig. 2. Lipoprotein assembly by McA-RH7777 cells cultured in the presence of oleic acid. Wild-type cells and cells transfected with constructs encoding human apoB-41, apoB-53, apoB-72, and apoB-80 were labeled as described for Fig. 1. The cells were recovered, and the total microsomal fraction was isolated and extracted. The luminal contents were fractionated by sucrose gradient ultracentrifugation, and apoB was recovered from each fraction by immunoprecipitation and SDS-PAGE. A: Patterns obtained with apoB-41 through apoB-100 [triangles represent density (g/ml); squares represent dpm]. B: Density of non-VLDL 1 lipoproteins in relation to the length of apoB. The denser particles formed by apoB-80 (circle), apoB-100 (triangle), and the chimera B-50+(72–90) (square) are indicated.

fected with apoB-41, apoB-53, apoB-72, and apoB-80 as well as the species secreted by endogenous apoB-100 and apoB-48. Purified human lipoproteins were used to characterize the gradient. The most buoyant VLDL particles (VLDL 1) were confined to the two top fractions (11 and 12). The total VLDL fraction (VLDL 1 and VLDL 2) banded in the four top fractions (9–12). LDL was present in fractions 5–9, and HDL was present in fractions 3–6.

Only a small amount of apoB-41 was secreted as VLDL 1. All other apoB species examined assembled substantial amounts of VLDL 1. Thus, 7.5% of the total secreted pool of apoB-41 was recovered with VLDL 1; the corresponding

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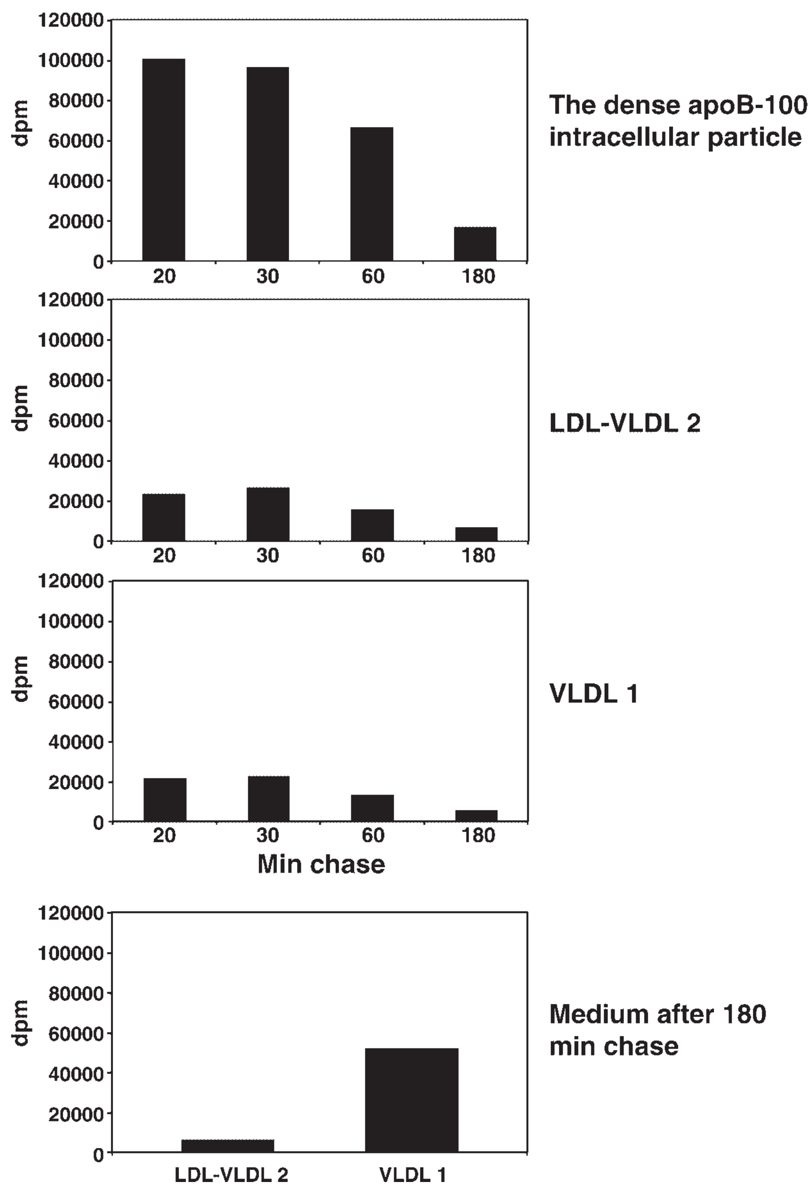

Fig. 3. Pulse-chase analysis of the turnover and secretion of the different apoB-100-containing lipoproteins in McA-RH7777 cells. The cells were cultured in the presence of oleic acid, labeled for 10 min with [^{35}S]methionine-cysteine, and chased for the indicated times. After each chase, the microsomal fraction was isolated and extracted, and the luminal content was fractionated by sucrose gradient ultracentrifugation. ApoB-100 was recovered from each fraction by immunoprecipitation and SDS-PAGE, and the radioactivity of apoB-100 in the dense intracellular particle and in LDL-VLDL 2 and VLDL 1 was determined. To determine the amount of radioactive apoB-100 that was secreted with VLDL 1 and LDL-VLDL 2 into the medium, the cells were chased for 180 min. The conditioned medium was collected and fractionated by sucrose gradient ultracentrifugation, apoB-100 was recovered from each fraction as described above, and the radioactivity was determined. To allow a comparison between apoB-100 in the secretory pathway and the conditioned medium, we corrected for recovery of apoB-100 during the isolation of the microsomal fraction.

figure for apoB-48 was 47%. This indicates that the ability to assemble VLDL 1 increases severalfold between apoB-41 and apoB-48 (**Fig. 1A**). Thus, linear elongation of apoB-41 to apoB-48 allowed the formation of VLDL 1. A chimera consisting of apoB-41 fused to the carboxyl terminus of apoB-100 [B-41+(72–100)] corresponding in length to apoB-69 failed to assemble VLDL 1, demonstrating that the ability to assemble VLDL 1 is not just a matter of the size of apoB.

ApoB-41 through apoB-100 were secreted on a denser non-VLDL 1 particle whose density was inversely proportional to the length of apoB (Fig. 1B). In the case of apoB-100, this particle, which we refer to as LDL-VLDL 2, banded in the LDL and VLDL 2 density regions. These results indicate that particles with a density of LDL-VLDL 2 were formed by size-dependent lipidation of apoB-100. They also indicate that the non-VLDL 1 particles assembled by truncated forms of apoB are analogs of the LDL-VLDL 2

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particles formed by apoB-100. We refer to them as LDL-VLDL 2 analogs.

To determine if the assembly of apoB size-dependent particles was dependent on the linear elongation of apoB or only on the size of the protein, we created a chimera consisting of apoB-50 and the carboxyl terminus of apoB-100 [B-50+(72–90)] and corresponding in length to apoB-68. The density of the assembled particle decreased, as would be expected from a linear increase from apoB-50 to apoB-68 (Fig. 1B). However B-41+(72–100) failed to acquire the expected density of apoB-69 (Fig. 1B), which indicates that the size-dependent lipidation is not just a matter of the size of apoB.

Because these results were based on stable transfections, the cell background (selected clones) might have influenced the results. If so, apoB-48 and apoB-100 lipoproteins would also be influenced. We therefore used endogenous lipoproteins as an "internal" standard. In all of the experiments reported here, the lipoproteins formed by apoB-48 and apoB-100 had the expected density, both in the secretory pathway and after secretion (data not shown).

Analysis of the contents of the secretory pathway revealed that all apoB species assembled apoB size-dependent particles with a density that corresponded to the secreted particle (**Fig. 2A**). The size of apoB was inversely related to the density of the non-VLDL 1 particle, except for apoB-80 and apoB-100, which formed particles of similar density (Fig. 2B). The only VLDL 1 species that showed a significant intracellular pool was apoB-100-containing VLDL (Fig. 2A).

ApoB-80 and apoB-100 were also present in a third form in the secretory pathway (Fig. 2A). This form of apoB banded with a density of 1.10 g/ml, did not follow the relation between the length of apoB and the density of the particle (Fig. 2A, B), and could not be detected in significant amounts in the culture medium (Fig. 1A). This particle, which we refer to as the dense B-80/B-100 intracellular particle, must be taken into account as an intracellular precursor in the assembly of VLDL 1.

To further investigate this, we carried out a pulse-chase experiment in which the cells were pulse-labeled for 10 min. ApoB-100 radioactivity peaked after a 20–30 min chase (data not shown) (2). We therefore chased the cells for 20, 30, 60, and 180 min and followed the turnover of radioactivity in the dense apoB-100 intracellular particle, LDL-VLDL 2, and VLDL 1. Radiolabeled apoB-100 VLDL in the medium reached a plateau at 180 min (data not shown), indicating that the maximal amount of apoB-100 had been secreted. We next analyzed the medium for radioactive apoB-100 in LDL-VLDL 2 and VLDL 1 after a 180 min chase as a measure of the maximal secretion of radiolabeled apoB-100. The results (**Fig. 3**) demonstrate that the decay in the dense apoB-100 intracellular particle

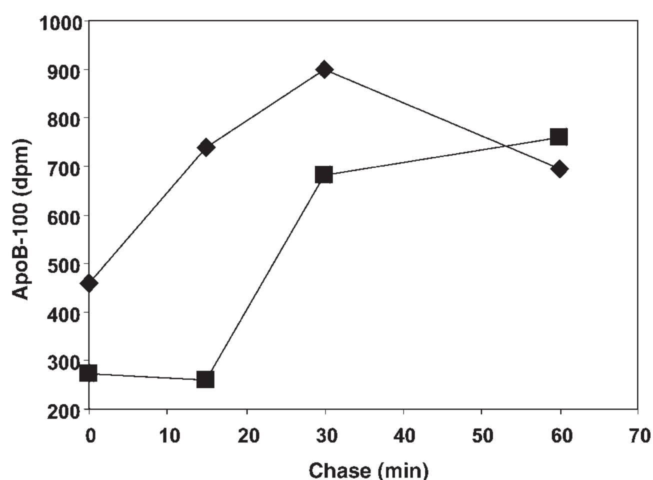

Fig. 4. Pulse-chase analysis of the formation of VLDL 1 (squares) and LDL-VLDL 2 (diamonds) in McA-RH7777 cells. Cells cultured in the presence of oleic acid were pulse-labeled for 10 min with [^{35}S]methionine-cysteine and chased for the indicated times. After each chase, the luminal content of the total microsomal fraction was recovered; apoB in the two fractions was recovered by gradient ultracentrifugation and isolated by immunoprecipitation and SDS-PAGE, and the radioactivity was determined.

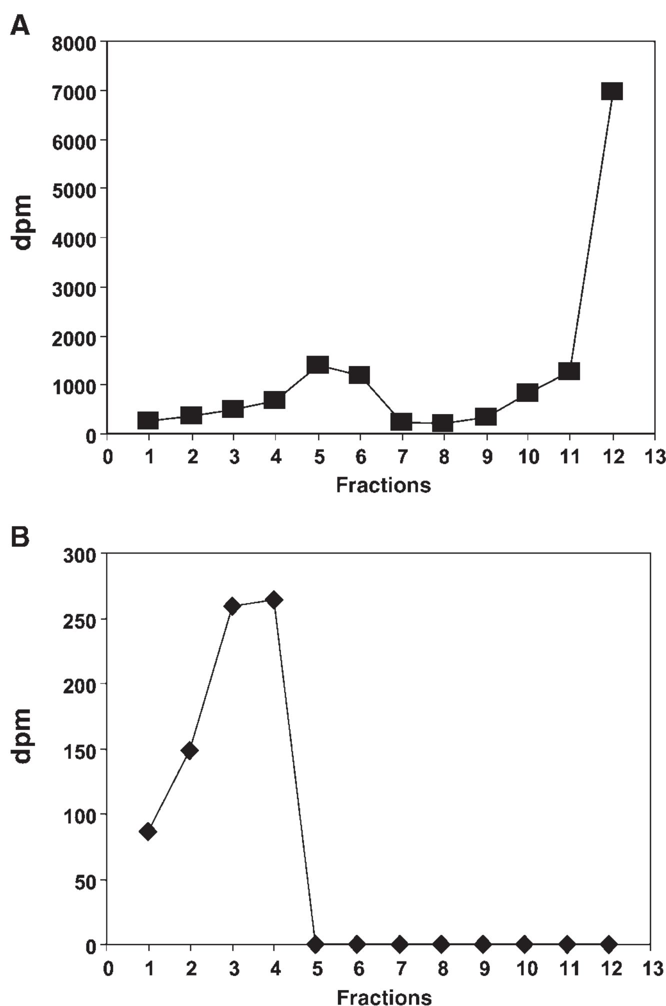

Fig. 5. Secreted (A) and intracellular (B) lipoproteins containing the chimera B-50+(72–90). McA-RH7777 cells stably expressing B-50+(72–90) were labeled as described for Fig. 1. The chimera was isolated from the different lipoprotein species in the culture medium and the secretory pathway (see legends to Figs. 1, 2).

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must be taken into account to explain the secretion of radiolabeled VLDL 1.

The radioactivity in intracellular VLDL 1 and LDL-VLDL 2 was small compared with that in secreted VLDL 1 or in the dense apoB-100 intracellular particle. Moreover, the turnover of intracellular LDL-VLDL 2 and VLDL 1 could not explain the increase in apoB-100 VLDL 1 in the medium. Thus, intracellular VLDL 1 and LDL-VLDL 2 behaved as intermediate pools between the dense apoB-100 intracellular particle and the secreted apoB-100-containing lipoproteins. Not all of the apoB-100 radioactivity in the dense apoB-100 intracellular particle could be recovered in the medium, indicating that a portion of this dense particle is degraded.

The turnover of apoB-100 in intracellular LDL-VLDL 2 and VLDL 1 was very similar. To further investigate the relation between these two apoB-100-containing lipoproteins, we carried out a second pulse-chase experiment (**Fig. 4**). Radioactive apoB-100 was detected in LDL-VLDL 2 by the end of the labeling period, indicating that these particles are assembled relatively close to the translation of apoB-100. LDL-VLDL 2 particles were assembled before VLDL 1, and the turnover kinetics of the two lipoproteins were consistent with a precursor-product relationship. A precursor-product relationship between LDL-VLDL 2 and VLDL 1 is further supported by the observation that LDL-VLDL 2 analogs were the predominant intracellular lipoproteins in cells expressing apoB-48, apoB-53, or apoB-72 (Fig. 2A), which secreted large amounts of VLDL 1 into the medium (Fig. 1A).

Assembly and retention of the dense B-80/B-100 particle

The dense B-80/B-100 intracellular particle (1.10 g/ml) started to appear when apoB reached the size of apoB-80, indicating that the sequence carboxyl terminal of apoB-72 may be important for the appearance of this particle. Consistent with this conclusion, the B-50+(72–90) chimera was secreted as VLDL 1 and as a LDL-VLDL 2 analog (**Fig. 5A**) with the density expected for apoB-68 (Fig. 1B), but the chimera had a higher density than expected in the secretory pathway (Figs. 2B, 5B). Thus, it behaved like the dense B-80/B-100 intracellular particle (Fig. 2B) rather than like apoB-68. The observation that the "internal standards" (i.e., the lipoproteins formed by endogenous apoB-48 and apoB-100) were as expected during these experiments suggested that cell background did not influence the results. Thus, our findings indicate that the sequence carboxyl terminal of apoB-72 increases the density of intracellular apoB-containing particles. They also support the possibility that the dense retained intracellular form of apoB can be converted to VLDL 1 and the LDL-VLDL 2 analog and be secreted.

The dense B-80/B-100 intracellular particle was retained in the cell. Because the binding site for the LDLR lies between apoB-72 and apoB-80, we suspected that the LDLR might be involved in the retention of the dense particle. To assess this possibility, we performed metabolic labeling studies with hepatocytes from human apoB-100 transgenic mice that did or did not express the LDLR

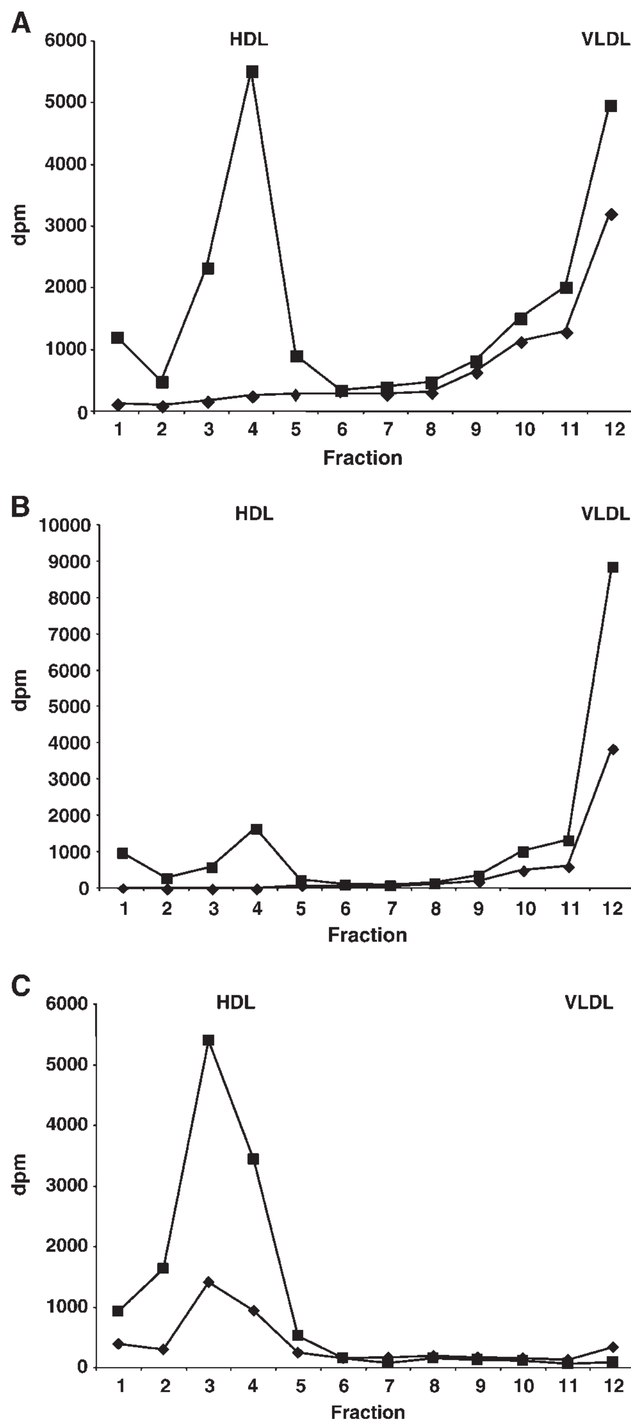

Fig. 6. Secretion of lipoproteins containing apoB-100 and apoB-48 by hepatocytes from LDL receptor-positive (*LDLR*$^{+/+}$) and LDLR receptor-null (*LDLR*$^{-/-}$) mice. Hepatocytes were isolated from human apoB-100 transgenic mice (A) and *LDLR*$^{-/-}$ human apoB-100 transgenic mice (B). C: The intracellular lipoproteins recovered from *LDLR*$^{-/-}$ hepatocytes. The cells were allowed to plate for 4 h, cultured for an additional 13 h, and labeled with [^{35}S]methionine-cysteine for 3 h. A and B: The medium was collected and subjected to gradient ultracentrifugation, apoB-100 and apoB-48 were isolated from each fraction as described in the legend to Fig. 1, and the radioactivity was determined. C: The microsomal fraction was isolated and extracted. The luminal content was fractionated by sucrose gradient ultracentrifugation, apoB-100 and apoB-48 were isolated from the different fractions (see legend to Fig. 1), and the radioactivity was determined. Diamonds, apoB-100; squares, apoB-48.

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(**Fig. 6**). No particle corresponding to the dense apoB-100 intracellular particle was secreted from the $LDLR^{-/-}$ cells (Fig. 6B). However, the dense apoB-100 particle was the dominant apoB-100 lipoprotein in the secretory pathway (Fig. 6C). These results indicate that the LDLR is not involved in the intracellular retention. Because both B-50+(72–90) and B-41+(72–100) were secreted into the medium, the intracellular retention of the dense B-80/B-100 particle does not seem to be dependent on the sequence carboxyl terminal of apoB-72.

Next, we assessed potential mechanisms for the intracellular retention. Dense B-80/B-100 particles and secretable LDL-VLDL 2 analogs with apoB-53 or apoB-72 were isolated from the microsomal luminal content (**Fig. 7**) and analyzed for chaperones. All particles contained calreticulin but not GRP94; however, only the dense B-80/B-100 particle reacted with antibodies to PDI or BiP, as judged from immunoblots (**Fig. 8**). Analysis of lipoproteins extracted from McA-RH7777 microsomes with sodium carbonate showed that a higher proportion of the total pool of the apoB-48-containing LDL-VLDL 2 analog could be extracted with sodium carbonate than could the dense intracellular particle with apoB-100 (48.3 ± 4.5% versus 26.5 ± 3.5%; mean ± SEM; n = 3). Thus, the apoB-48-containing LDL-VLDL 2 analog is less tightly associated with the microsomal membrane than the dense intracellular particle containing apoB-100. Indeed, 73% of the dense intracellular particle containing apoB-100 is relatively tightly associated with the microsomal membrane.

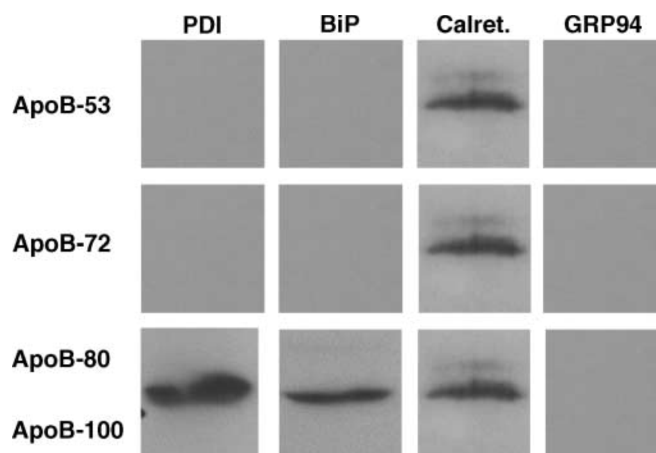

Fig. 8. Coimmunoprecipitation of chaperone proteins with either secretable LDL-VLDL 2 analogs with apoB-53 or apoB-72 or the retained dense particles with apoB-100 or apoB-80 in the microsomal lumen of McA-RH7777 cells. Cells were transfected with human apoB-53, apoB-72, or apoB-80. The total microsomal fraction was recovered, and the luminal content was extracted with sodium carbonate and deoxycholate and fractionated by sucrose gradient ultracentrifugation. The fractions described for Fig. 6 were subjected to SDS-PAGE with 10% gels and blotted with antisera to protein disulfide isomerase (PDI), binding protein (BiP), calreticulin (Calret.), and glucose regulatory protein 94 (GRP94). Nonspecific reactions were detected by blotting with nonimmune antibodies (not shown).

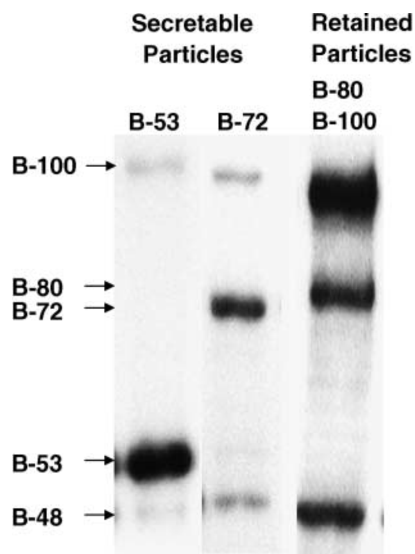

Fig. 7. SDS-PAGE of the isolated secretable LDL-VLDL 2 analogs with apoB-53 or apoB-72 and the retained dense particles (with apoB-100 or apoB-80) in the microsomal lumen of McA-RH7777 cells. McA-RH7777 cells transfected with apoB-53, apoB-72, or apoB-80 were labeled with [^{35}S]methionine-cysteine for 120 min. The total microsomal fraction was recovered, and the luminal content was extracted with sodium carbonate and deoxycholate and fractionated by sucrose gradient ultracentrifugation. Fractions corresponding to the intracellular apoB-53 and apoB-72 lipoproteins as well as the dense lipoproteins containing apoB-80 or apoB-100 were recovered and purified by immunoprecipitation (nondenaturing conditions) with an antibody to apoB. The recovered fractions were analyzed by SDS-PAGE and autoradiography.

Length of apoB and the posttranslational requirement for MTP

The carboxyl-terminal portion of apoB-100 could be related to the posttranslational need for MTP activity during the conversion to VLDL (13). If so, MTP would be required to form VLDL containing apoB-100 and possibly apoB-80 but not VLDL containing apoB-48 or apoB-53. To address this question, we performed pulse-chase studies with brefeldin A and an MTP inhibitor as described (13). Inhibition of MTP after the completion of apoB markedly decreased the accumulation of bona fide VLDL in the culture medium by all forms of apoB investigated (**Fig. 9**). Thus, the length of apoB does not appear to be related to the posttranslational need for MTP.

DISCUSSION

In this study, we investigated the types of particles assembled and secreted by McA-RH7777 cells expressing different lengths of apoB. One of these particles showed an inverse relationship between its density and the size of apoB (i.e., the longer the apoB, the more lipids were added). This relation has been seen before (12) and taken as an indication of a linear assembly of VLDL. In part, this appears to be the case, because the end product, which was reached at an apoB size of apoB-100, was a particle that banded in the LDL-VLDL 2 density region. Con-

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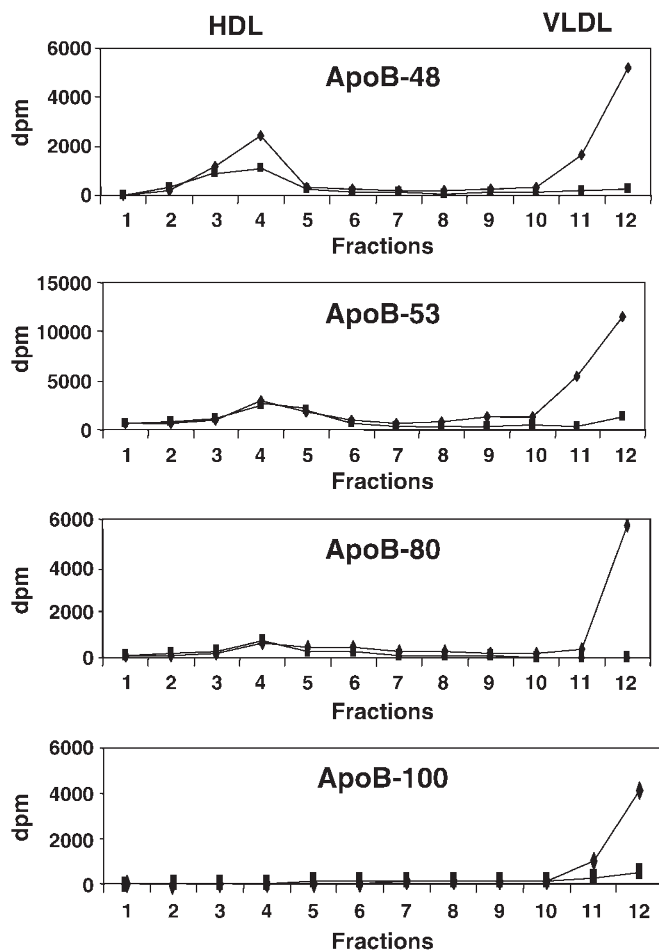

Fig. 9. ApoB-100, apoB-48, and truncated forms of human apoB (apoB-53 and apoB-80) require active microsomal triglyceride transfer protein (MTP) posttranslationally to assemble VLDL. After pretreatment with brefeldin A for 15 min, McA-RH7777 cells were pulse-labeled with [^{35}S]methionine-cysteine for 30 min and chased in the presence of excess unlabeled methionine; both pulse and chase were performed in the presence of brefeldin A. MTP was inhibited by incubation with 10 μM BMS 192951 for 15 min under ultraviolet irradiation. Control cells were treated in the same way, except that no inhibitor was added. After MTP inhibition, the cells were chased for 180 min in the presence of oleic acid without brefeldin A. The culture medium was collected and subjected to gradient ultracentrifugation. ApoB from each fraction of the gradient was recovered by immunoprecipitation and SDS-PAGE, and the radioactivity was counted. Diamonds, control cells; squares, MTP-inhibited cells.

sistent with the notion that the size of apoB has a major effect on the assembly process, B-50+(72–90), a chimera corresponding in length to apo-B68, secreted a lipoprotein with the expected density of an apoB-68. However, B-41+(72–100), a chimera corresponding in length to apoB-69, failed to assemble a particle with the expected density of apoB-69. Thus, length is apparently not the only variable of importance for size-dependent assembly.

VLDL 1 assembly appears only to require a minimal size of apoB. The ability to assemble VLDL 1 increased dramatically when the protein increased in size from apoB-41 to apoB-48. This finding is in agreement with previous results (8) showing that released apoB-100 nascent chains with the size of apoB-40 formed small amounts of VLDL, whereas a dramatic increase occurred when the nascent chain reached the size of apoB-48. ApoB-41 did not acquire this ability when elongated to apoB-69 with the carboxyl terminus of apoB-100, even though this region contains amphipathic β-strands (from the β_2 domain) of the type that exist between apoB-41 and apoB-48 (20). Thus, the portion of the β_1 domain (of amphipathic β-strands) between apoB-41 and apoB-48 seems to be essential for the second step of VLDL 1 assembly. Our results differ somewhat from those reported by McLeod et al. (21), which indicated that apoB-37 avidly forms VLDL (i.e., that the last portion of the β_1 domain is not needed for the quantitative assembly of VLDL). We cannot explain this discrepancy because the same methodology was used in both studies.

The portion of apoB-100 between apoB-51 and apoB-53 has a high requirement for MTP during the assembly process (22). It is unlikely that this increased need for MTP reflects the ability of apoB to go through the second step, because the apoB-51 to apoB-53 region is carboxyl terminal of apoB-48, where this ability seems to be acquired. We also sought to determine if this high requirement for MTP could explain the posttranslational need for MTP that we previously reported (13). The observation that all forms of apoB investigated required MTP posttranslationally to assemble VLDL argues against such a possibility. We conclude that VLDL 1 and VLDL 2 are formed by different pathways.

The observation that two pathways lead to the secretion of apoB-100-containing lipoproteins may explain the heterogeneity of secreted particles containing apoB-100. In addition to VLDL 1 and VLDL 2, LDL-like particles may be secreted directly (23). Like VLDL 2, such particles may also be produced by the apoB size-dependent process.

Why are intestinally derived chylomicrons larger than hepatically derived VLDLs? Our results indicate that once apoB reaches a size that permits it to proceed through the second step of the assembly process, it can acquire the major load of neutral lipids. Thus, the most likely answer to the question is that the intestine provides a larger amount of lipid for the second step than the liver. In support of this possibility, large chylomicrons are assembled in the intestine of apoB-100-only mice (24)

When apoB reached apoB-80, a denser apoB species appeared in the secretory pathway: a dense B-80/B-100 intracellular particle that is the major species of both apoB-80 and apoB-100 in the cell. Its formation seems to involve the sequence on the carboxyl-terminal side of apoB-72. This was confirmed by experiments with B-50+(72–90). This chimera (corresponding in length to apoB-68) accumulated in the culture medium as VLDL 1 and as an LDL-VLDL 2 analog with the expected density of apoB-68. However, the intracellular particle had a density similar to that of the dense B-80/B-100 intracellular particle (i.e., much higher than expected for apoB-68).

The dense apoB-100 intracellular particle was the precursor to secreted VLDL 1, as judged from the results of

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pulse-chase studies. Intracellular LDL-VLDL 2 and VLDL 1, however, behaved as intermediates between the dense apoB-100 intracellular particle and the secreted VLDL. One possibility is that the dense apoB-100 intracellular particle is converted to LDL-VLDL 2 or VLDL 1 before secretion. However, pulse-chase experiments that focused on the turnover of these two VLDL species demonstrated that LDL-VLDL 2 with apoB-100 are assembled before apoB-100 VLDL 1, and the kinetics were consistent with a precursor-product relationship. Such a relationship is supported by the observation that in the case of apoB-48 through apoB-72, the LDL-VLDL 2 analog was the only detectable intracellular source of apoB, whereas VLDL 1 accumulated in the culture medium. This strongly indicates that the LDL-VLDL 2 particles are converted to VLDL 1 shortly before secretion, consistent with previous observations (2). In conclusion, it is possible that the dense apoB-100 intracellular particle is first converted to LDL-VLDL 2, which in turn can acquire more neutral lipid to become VLDL 1.

The dense B-80/B-100 intracellular particle was retained in the cell. The region between apoB-72 and apoB-100 contains the binding site for the LDLR (25, 26), which has been linked to the retention and intracellular degradation of apoB (27–30). Analysis of $LDLR^{+/+}$ and $LDLR^{-/-}$ hepatocytes from transgenic mice expressing human apoB-100 showed no significant amounts of the dense apoB-100 particle in the culture medium, indicating that its retention is not mediated by the LDLR. Because the chimeras B-50+(72–90) and B-41+(72–100) were secreted, the retention is not attributable to the sequence carboxyl terminal of apoB-72.

We also assessed the interactions between chaperones and the secretable LDL-VLDL 2 analog with the dense intracellular particle. LDL-VLDL 2 analogs with apoB-53 or apoB-72 could be separated from endogenous particles containing apoB-100. These secretable LDL-VLDL 2 analogs were not associated with BiP or PDI. In contrast, a fraction enriched in the apoB-80/B-100 dense particle was associated with both BiP and PDI. This may indicate that BiP and PDI, resident in the endoplasmic reticulum (31), are involved in the retention of the lipoproteins in the secretory pathway and that dissociation from these chaperons is important for the secretion of the LDL-VLDL 2 analog. Thus, an interaction between the apoB-80/B-100 dense particle and BiP and PDI may explain the retention of the particle. When evaluating these results, it should be kept in mind that we were unable to remove the LDL-VLDL 2 analog with apoB-48 from the fractions containing the retained particles. However, because the apoB-48 LDL-VLDL 2 analog is avidly secreted, it is likely that it behaves as apoB-53 and apoB-72.

BiP is involved in the folding process and interacts with misfolded proteins. Our results may therefore indicate that apoB-100 and apoB-80 are misfolded on the dense particle. Interestingly, we have shown that apoB-100 starts to leave the cell when it is present on an LDL-sized particle but is retained when present on smaller particles (32). Conversely, apoB-48 is avidly secreted on HDL-sized particles (2).

Although it is well established that BiP retains misfolded proteins in the cell (31), we cannot conclude that PDI is involved in this process. However, PDI is an endoplasmic reticulum protein that may have several roles in VLDL assembly. PDI is a subunit of the functional MTP complex and is also involved in the isomerization of disulfide linkages in protein folding. It is possible that a halt in the lipidation of apoB-100 or a misfolding of the protein may lead to an interaction with PDI that could contribute to retention.

Another potential mechanism for the retention of the dense apoB-100 intracellular particle is an interaction with the microsomal membrane. This particle was much more tightly associated with the microsomal membrane than the LDL-VLDL 2 analog containing apoB-48. Because partially lipidated apoB may interact with the translocon (33), the dense intracellular particle with apoB-100 might represent such a form of apoB-100. Addition of lipids to create a LDL-VLDL 2-sized particle may release apoB-100 from the translocon as well as from BiP and PDI, allowing it either to proceed along the secretory pathway or to be converted to VLDL 1 in the second-step compartment (8). Conversion of the dense retained form of apoB to VLDL 1 and LDL-VLDL 2 is supported by the pulse-chase experiments discussed above and by the behavior of the chimera B-50+(72–90) (see above).

In conclusion, we propose that apoB-100 assembles into a partially lipidated dense precursor particle that is retained in the cell unless it is further converted into LDL-VLDL 2 by apoB size-dependent lipidation. LDL-VLDL 2 in turn can proceed through the secretory pathway to be secreted or converted to VLDL 1 in the second step of the assembly process. JLR

The authors thank Maria Heyden and Elin Björk for excellent technical assistance and Stephen Ordway for editing the manuscript. This work was supported by grants from the Swedish Research Council, the Swedish Heart and Lung Foundation, the Novo Nordic Foundation, and Nestlé.

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