Golgi-associated Maturation of Very Low Density Lipoproteins Involves Conformational Changes in Apolipoprotein B, but Is Not Dependent on Apolipoprotein E

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The major protein component in secreted very low density lipoproteins (VLDL) is apoB, and it is established that these particles can reach sizes approaching 100 nm. We previously employed a cell-free system to investigate the nature of the vesicles in which this large cargo exits the endoplasmic reticulum (ER) (Gusarova, V., Brodsky, J. L., and Fisher, E. A. (2003) J. Biol. Chem. 278, 48051–48058). We found that apoB-containing lipoproteins exit the ER as dense lipid-protein complexes regardless of the final sizes of the particles and that further expansion occurs via post-ER lipidation. Here, we focused on maturation in the Golgi apparatus. In three separate approaches, we found that VLDL maturation (as assessed by changes in buoyant density) was associated with conformational changes in apoB. In addition, as the size of VLDL expanded, apoE concentrated in a subclass of Golgi microsomes or Golgi-derived vesicles that co-migrated with apoB-containing microsomes or vesicles, respectively. A relationship between apoB and apoE was further confirmed in co-localization studies by immunoelectron microscopy. These combined results are consistent with previous suggestions that apoE is required for VLDL maturation. To our surprise, however, we observed robust secretion of mature VLDL when apoE synthesis was inhibited in either rat hepatoma cells or apoE−/− mouse primary hepatocytes. We conclude that VLDL maturation in the Golgi involves apoB conformational changes and that the expansion of the lipoprotein does not require apoE; rather, the increase in VLDL surface area favors apoE binding.

Very low density lipoproteins (VLDL)2 transport endogenously synthesized lipids, particularly cholesterol esters and triglyceride, from the liver to peripheral tissues (for a recent review, see Ref. 1). VLDL assembly is a complex process that consists of at least two steps. In the first step, during its translocation across the endoplasmic reticulum (ER) membrane, nascent apolipoprotein B100 (apoB) associates with lipids provided by microsomal triglyceride transfer protein. This results in the formation of incompletely lipidated “primordial” pre-VLDL particles. In the second step, additional lipid (primarily triglyceride) and other apolipoproteins (e.g. apoE) are added, resulting in the formation of mature, fully lipidated VLDL particles (2).

Although it is established that the first step of VLDL assembly occurs in the ER, the location of the additional step(s) during VLDL maturation is less clear. For example, a number of studies have suggested that the ER is the final site of VLDL maturation (e.g. Refs. 3–6), whereas others have implicated the Golgi complex as a second site of maturation (e.g. Refs. 7–12). To better distinguish between these models, we previously employed a cell-free system in which the budding of ER-derived, apoB-containing vesicles was reconstituted (13). In addition to showing that the transport of apoB from the ER is dependent on Sar1, a required factor for COP II secertory vesicle formation, we obtained preliminary evidence that lipids are added to the pre-VLDL species after ER exit.

Based on our previous evidence, a major goal of this study was to firmly establish the Golgi as the site of VLDL maturation. Another important goal was to study the requirement for apoE in the full lipidation of VLDL. Although apoE has been well characterized as a factor required for the clearance of VLDL from plasma, there are also studies that have implicated apoE in the assembly or secretion of fully lipidated VLDL (14–17). These data are consistent with the observation that a fraction of apoE associates with VLDL within the secretory pathway (18), including secretory vesicles emerging from the Golgi (19).

To address the issues of the site of VLDL maturation and the role of apoE in this process, we have again employed a cell-free system but have supplemented the data with results from immunoelectron microscopy, apoE-deficient primary hepatocytes, and small interfering RNA (siRNA) apoE “knockdown” experiments in rat hepatoma cells. Taken together, our results indicate that the Golgi represents the site of VLDL maturation and that the process of lipid addition to pre-VLDL is associated with apoB conformational changes. Furthermore, we found...
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that neither the assembly nor secretion of mature VLDL requires apoE.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-rat apoE and rabbit anti-rat apoB antisera were provided by Dr. Janet Sparks (University of Rochester School of Medicine). The following antisera and antibodies were purchased from the indicated companies: goat anti-rat apoE antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-rat albumin antiserum (Bethyl Laboratories Inc., Montgomery, TX), mouse anti-mannosidase II antibody (Covance, Princeton, NJ), rabbit anti-calnexin antibody (Stressgen, Victoria, Canada), mouse anti-β-COP antibody (Sigma), and mouse anti-syntaxin 6 antibody (Pharmlingen). Protein A-Sepharose was purchased from Amersham Biosciences. Cell culture media and related supplies were purchased from Invitrogen and Cellgro (Herndon, VA). Protease inhibitor mixture tablets were purchased from Roche Diagnostics. Other reagents were purchased from Sigma unless stated otherwise.

Cell Culture—Until their experimental use, rat hepatoma McA-RH7777 cells (referred to below as McA cells) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 10% horse serum, 2 mM l-glutamine, and 100 μg/ml penicillin, and 100 μg/ml streptomycin in 5% CO2 at 37 °C. The medium was changed every 3 days.

Immunoprecipitation and Western Blotting—Immunoprecipitation under denaturing and nondenaturing conditions and Western blotting were performed as described previously (13).

Preparation of Cytosol and Microsomal Membranes—Rat liver cytosol was prepared as described (13). ER and Golgi membranes (microsomes) were purified by subcellular fractionation as follows. McA cells were grown on four 100-mm tissue culture dishes and preincubated in low serum medium (Dulbecco’s modified Eagle’s medium containing 0.5% (v/v) fetal bovine serum, 0.5% (v/v) horse serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) in 5% CO2 at 37 °C for 16 h. Cells were further incubated for 2 h in the same medium with either bovine serum albumin (BSA; 0.13 mM; hereafter referred to as the “−OA” condition, i.e. without exogenous oleic acid) or, to stimulate triglyceride synthesis, with OA/BSA (0.8 mM OA complexed to 0.13 mM BSA; “+OA” condition). The cells were then metabolically labeled for 3 h by the addition of Express 35S protein labeling mixture (100 μCi/ml per dish; PerkinElmer Life Sciences) in the presence or absence of OA in Cys/Met-free medium. Next, the cells were washed with phosphate-buffered saline and harvested in 2.5 ml of homogenization buffer (10 mM HEPES (pH 7.4), 250 mM sucrose, 0.5 mM dithiothreitol, 1 × EDTA-free protease inhibitor mixture, and 20 units/ml RNase inhibitor). Additional ER and Golgi membranes (microsomes) were homogenized and purified as described (13). The gradient distribution patterns of the subcellular compartment markers were assayed either enzymatically (for mannosidase II, a Golgi marker) or by Western blotting (for calnexin, an ER marker, and syntaxin 6, a trans-Golgi network marker) (20). The fractions containing the appropriate markers were then pooled to provide either ER or Golgi microsomes for experimental use.

Cell-free Generation of ER- or Golgi-derived Vesicles—In vitro ER vesicle budding assays were performed as described (13), and in vitro Golgi budding assays were performed as described (21) with minor modifications. Briefly, Golgi membranes (200 μg) were preincubated with 0.1 mM GTPγS for 5 min at 37 °C. Rat liver cytosol (14 mg of protein), supplemented with 0.5 mM dithiothreitol and an ATP-regenerating system (4.6 IU/ml creatine phosphokinase, 81 mM creatine phosphate, and 28.6 mM ATP), was added, and the total volume was adjusted to 2 ml with HKM buffer (25 mM HEPES-KOH (pH 7.4), 20 mM KCl, and 2.5 mM magnesium acetate). This reaction mixture was incubated for 30 min at 37 °C and was terminated by incubation for 10 min in an ice-water slurry. The remnant (unbudded) Golgi cisternae were separated from the vesicles by differential centrifugation at 17,500 g for 10 min at 4 °C.

Density Gradient Fractionation of Golgi-derived Vesicles—After separation from the membranes (see above), Golgi-derived vesicles were separated from the cytosol by discontinuous sucrose gradient centrifugation at 100,000 g for 90 min at 4 °C. The tube contained a cushion of 200 μl of 2.1 m sucrose overlaid with 200 μl of 20% (w/w) sucrose and 1.8 ml of the cytosol/vesicle mixture (21). All sucrose solutions were prepared in HKM buffer. The cytosol was collected from the loading zone, and the budded vesicles were harvested at the 2.1 m (20%, w/w) sucrose interface.

To further fractionate the vesicles, the material collected from the interface was resuspended in a total volume of 400 μl of 20% sucrose and layered onto a discontinuous sucrose gradient consisting of 300 μl of each of the following sucrose solutions: 50, 45, 40, 35, 30, and 25% (w/w) (21). The tubes were centrifuged at 100,000 g for 18 h at 4 °C. Fractions of 220 μl each were collected from the top of the gradient. Next, apoB and apoE were immunoprecipitated from each fraction with rabbit anti-rat apoB and apoE antisera. To determine the distributions of albumin, the remainder of each fraction was centrifuged at 60,000 g for 1 h at 4 °C in a Beckman TLA100 rotor; pellets were resuspended in sample buffer (125 mM Tris-HCl (pH 6.8), 20% β-mercaptoethanol, and 2.5% bromphenol blue); and Western blot analyses were performed using rabbit anti-rat albumin antisera.

Isolation of Luminal Contents of ER-derived Vesicles and Golgi Microsomes and Sucrose Gradient Separation of ApoB-containing Lipoproteins—Luminal contents were released from microsomes and vesicles by treatment with 0.1 mM sodium carbonate (pH 11) and 0.025% deoxycholate as described (13, 22, 23). The supernatants containing the released lipoproteins were recovered by centrifugation at 60,000 rpm for 60 min at 4 °C in a Beckman TLA100.4 rotor. Lipoproteins were separated according to density by ultracentrifugation on a sucrose gradient (13, 23). In some experiments, the densities of VLDL1 and VLDL2 particle subpopulations were resolved by ultracentrifugation according to Wang et al. (24).

Nondenaturing Immunoprecipitation of ER-derived Vesicles and Golgi Microsomes containing ApoB—ER-derived vesicles and Golgi microsomes (obtained as described above) were immunoprecipitated under nondenaturing conditions (in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2% BSA with rabbit anti-rat apoB antiserum/protein A-Sepharose
beads) to preserve the exposure of apoB domains to the cytosol (13). The supernatant of the immunoprecipitation was reserved; the protein A beads were washed; and the immunoprecipitate was released with NET buffer (150 mM NaCl, 2.5 mM EDTA, and 50 mM Tris-HCl (pH 7.5)) containing final concentrations of 2% SDS and 1% Triton X-100. The released material was re-immunoprecipitated under denaturing conditions in NET buffer containing 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. apoB was also immunoprecipitated under denaturing conditions from the reserved supernatant. The resulting samples were resolved by SDS-PAGE, and the content of radiolabeled apoB was displayed by fluorography.

Protease Susceptibility Studies—To probe the conformation of apoB in either liberated vesicles or microsomes, protease susceptibility assays were performed. Briefly, vesicles or microsomes from radiolabeled McA cells were treated with trypsin at a final concentration of 0.1 mg/ml as described (25). apoB was then immunoprecipitated with rabbit anti-rat apoB antiserum under denaturing conditions. The immunoprecipitates were resolved by SDS-PAGE, and their apoB content was displayed by fluorography.

Immunoelectron Microscopy—For immunoelectron microscopy, a subconfluent population of McA cells was washed with phosphate-buffered saline. The cells were then removed by gentle scraping with the edge of a razor blade and pelleted by centrifugation. The cell pellet was fixed in gelatin and then incubated overnight at 4 °C in a solution containing 20% polyvinylpyrrolidone, 1.6 M sucrose, and 55 mM sodium carbonate buffered in phosphate-buffered saline. The embedded cells were frozen, sectioned, and stained essentially as described (26). Rabbit anti-rat apoB antiserum and goat anti-rat apoE antibody were used at dilutions of 1:200 and 1:100, respectively, and visualized using 10- and 4-nm diameter gold particles conjugated to anti-rabbit and anti-goat antibodies, respectively.

Inhibition of ApoE Expression by siRNA—Lipofectamine PLUS reagent (Invitrogen) was used to transfect McA cells with 10 nM apoE siRNA (catalog no. 16708A, ID 199680, Ambion, Inc.) or 10 nM negative control siRNA-1 (catalog no. 4639) according to the protocol of the manufacturer. After transfection, cells were preincubated in low serum medium in 5% CO2 at 37 °C for 16 h and treated with OA (0.8 mM) for 2 h in the same medium. Cells were then labeled with Express 35S protein labeling mixture for 3 h (100 μCi/ml per dish) in the presence of OA in Cys/Met-free medium. The medium was collected; cells were homogenized; and the Golgi microsomes were isolated by subcellular fractionation as described above. Sucrose density gradient separations of the conditioned medium and the luminal contents of the Golgi microsomes were performed as described (24).

Studies of VLDL Secretion from Mouse Primary Hepatocytes—Male C57BL/6 mice and apoE-deficient mice on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were kept on a 12-h light/12-h dark cycle and were provided with water and a standard mouse chow diet ad libitum.

Hepatocytes were isolated from mouse livers (14–17 weeks old) by a modification of the hepatic portal perfusion described by Klaunig et al. (27). Briefly, the portal vein was cannulated, and the liver was perfused at a rate of 4 ml/min with a calcium- and magnesium-free buffer (10 mM HEPES (pH 7.4), 143 mM NaCl, 7 mM KCl, and 0.2 mM EDTA) and then a collagenase solution (100 units/ml collagenase IV; Sigma). Hepatocytes released from the liver capsule were isolated by two rounds of centrifugation at 50 × g for 3 min. Cells were plated onto 100-mm dishes precoated with collagen (Sigma) at a density of 3 × 106 cells/dish in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum, 0.5% horse serum, 2 mM l-glutamine, 0.2% BSA, 0.6 mM OA/BSA, 0.1 mM insulin (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. After a 2-h attachment period, the medium was changed to Met/Cys-free Dulbecco’s modified Eagle’s medium containing Express 35S protein labeling mixture (200 μCi/ml). To prevent re-uptake of newly secreted VLDL, 10 mg/ml heparin (Sigma) was also added (28). After 3 h of metabolic labeling, the medium was collected; apoB-containing lipoproteins were separated by a KBr/NaBr step density gradient ultracentrifugation (24); and these species were isolated by immunoprecipitation and SDS-PAGE as described above. For quantification of apoB contents in each density fraction, gel bands were excised and assayed by scintillation counting as described previously (29). To ascertain the relationship between the triglyceride and apoB contents of the VLDL1 and VLDL2 density fractions, both Express 35S 35S protein labeling mixture (200 μCi/ml) and 3[H]glycerol (5 μCi/ml) were used for metabolic labeling of primary hepatocytes. In addition to the procedures described above used to assay apoB contents, aliquots of the VLDL fractions were taken to isolate radiolabeled triglycerides by extraction with Dole’s solvent, as we have done before (30). The ratio of the cpm of triglyceride to apoB was then calculated for each VLDL subtraction (VLDL1 and VLDL2).

RESULTS

Stimulation of Triglyceride Synthesis Increases the Content of VLDL-associated ApoB in Golgi Microsomes—In our previous study (13), we employed a cell-free system and inferred that apoB is not fully lipidated in the ER because pre-VLDL density particles are found in ER-derived secretory vesicles. To investigate the mechanism of post-ER VLDL maturation in greater detail, we chose to characterize Golgi microsomes isolated from metabolically labeled McA cells that were treated in the presence or absence of OA, a standard inducer of triglyceride synthesis and VLDL lipidation. Notably, the isolated Golgi microsomes exhibited low contamination with ER membranes (1.7% based on organelle-specific marker assays; see “Experimental Procedures”). The Golgi microsomes were treated with sodium carbonate and deoxycholate (22, 23) to release apoB-containing lipoproteins that were either completely luminal or associated with the inner leaflet of the membrane. The released material was then separated on sucrose density gradients, and the apoB content of each fraction was determined by immunoprecipitation/SDS-PAGE/fluorography. As shown in Fig. 1, the content of apoB in the VLDL fraction (d < 1.005) in Golgi microsomes increased ~5-fold when cells were treated with OA.
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Conformational Changes in ApoB in Golgi Microsomes Are Associated with VLDL Maturation—Although apoB does not have a classical transmembrane segment, we reported previously that it has a domain(s) exposed in ER-derived vesicles (13). To determine whether apoB also exposes a domain in Golgi microsomes, the apoB-containing ER and Golgi vesicles were incubated with trypsin, and the loss of full-length apoB was assessed by immunoprecipitation/SDS-PAGE/fluorography. As shown in Fig. 2A (left panels) and consistent with our previous study (13) ~90% of apoB in ER-derived vesicles was susceptible to protease attack independent of the stimulation of triglyceride synthesis, indicating cytosolic domain exposure.

In contrast to the results for the ER-derived vesicles, a significant fraction (43.8 ± 6.2%, n = 3) of apoB associated with Golgi microsomes was protected from trypsin (Fig. 2A, right panels). This effect was observed even in the absence of OA, which supports the maturation of some VLDL in McA cells under basal metabolic conditions (e.g. Refs. 13 and 28). Notably, the fraction of protected apoB rose to 71 ± 3.6% (n = 3) when maximal VLDL lipidation had been stimulated by OA treatment.

The results suggest that apoB undergoes conformational changes during VLDL maturation in the Golgi. To demonstrate this phenomenon by an independent approach, we performed sequential immunoprecipitation analyses of ER-derived vesicles and Golgi microsomes under nondenaturing and denaturing conditions (see “Experimental Procedures”). As shown in Fig. 2B (left panels) and again in agreement with our previous results (13), after the first (nondenaturing) immunoprecipitation from ER-derived vesicles, apoB was largely depleted from the supernatant independent of the stimulation of triglyceride synthesis by OA. Indeed, densitometric analyses of the fluorograms revealed that only 5.9 ± 1.1% (n = 3; −OA) and 31.1 ± 4.2% (n = 3; +OA) of apoB remained in the supernatant, consistent with significant apoB domain exposure to the cytosol in ER-derived vesicles. In contrast, substantial amounts of apoB were found in the supernatant fractions in Golgi microsomes: 56.3 ± 3.8% (n = 3) under basal VLDL-forming conditions (−OA) and 76 ± 6.9% (n = 3) under conditions that support maximal lipidation (+OA). These data imply that apoB domain exposure in Golgi microsomes is, in general, less than that in the ER-derived vesicles and is inversely related to the proportion of VLDL that is fully lipidated.

Given that apoB conformational changes correlated with the VLDL maturation process in the Golgi, we predicted that the microsomal content of immature apoB-containing lipoproteins (i.e. pre-VLDL or high density lipoprotein density) or mature VLDL would be related to the degree of cytosolic exposure of apoB domains. To test this hypothesis, we isolated Golgi microsomes from McA cells treated with OA and then immunoprecipitated apoB under nondenaturing conditions to separate microsomes with apoB domain exposure or without (remaining in the supernatant). The microsomal contents of apoB-containing lipoproteins from both the supernatant fraction and the original microsome were released with sodium carbonate and deoxycholate (22, 23) and subjected to density gradient separation. ApoB was then immunoprecipitated from each fraction and resolved and identified by SDS-PAGE/fluorography (see “Experimental Procedures”).

As shown in Fig. 3, apoB without domain exposure to the cytosol was enriched in the lipoprotein fractions containing the most lipidated particles (i.e. VLDL) and depleted in those fractions containing poorly lipidated particles (pre-VLDL). Overall,
FIGURE 3. ApoB conformational changes associated with VLDL maturation in the Golgi. Two equal aliquots were taken of Golgi microsomes purified from metabolically labeled McA cells treated with OA. ApoB was immunoprecipitated from one aliquot with anti-rat apoB antiserum and protein A-Sepharose under non-denaturing conditions to deplete microsomes that harbored apoB domains exposed to the cytosol. The depleted supernatant and immunoprecipitated were separated by centrifugation. Microsomes in the supernatant or total microsomes from the other aliquot were then incubated with sodium carbonate and deoxycholate to release their contents, which were subjected to sucrose density gradient centrifugation. ApoB was immunoprecipitated from each fraction; the immunoprecipitates were separated by SDS-PAGE; and the presence of apoB was assessed by fluorography. The density distributions of apoB-containing lipoproteins in the supernatant microsomes and in the total microsomes are shown (A and B, respectively). The apoB bands from repeated experiments were evaluated by densitometry and are plotted as the mean signal intensity ± S.D. (n = 2) for each lane (C). IDL/LDL, intermediate/low density lipoproteins; HDL, high density lipoproteins; Adj., adjusted.

FIGURE 4. Sequential immunoprecipitation analysis of apoE associated with ER-derived vesicles and Golgi microsomes. Metabolically labeled apoB was immunoprecipitated from ER-derived vesicles or from the Golgi microsomes (prepared from McA cells treated with OA) with anti-rat apoB antiserum and protein A-Sepharose under non-denaturing conditions to deplete vesicles or microsomes harboring apoB domains exposed to the cytosol. The depleted supernatants were reserved; the beads were washed; and the immunoprecipitated vesicles or microsomes were released with NET buffer containing 2% SDS and 1% Triton X-100. ApoE in the supernatants (S; lanes 1) and immunoprecipitated vesicles or microsomes (P; lanes 2) was then immunoprecipitated with anti-rat apoE antiserum under denaturing conditions. These immunoprecipitates were separated by SDS-PAGE, and the presence of apoE was assessed by fluorography. The results shown are representative of three independent experiments.

then, by three independent approaches, the same conclusion was reached, viz. that the lipidation of VLDL precursors is associated with apoB conformational changes in the Golgi.

ApoB and ApoE Are Found in Different Subpopulations of ER-derived Vesicles, but Co-migrate in Sucrose Density Gradient Analyses of Golgi Microsomes and Golgi-derived Vesicles — As noted earlier, apoE plays an important role in VLDL metabolism by serving as a ligand for its uptake from plasma. It had long been assumed that apoE secreted by the liver becomes bound to VLDL in the plasma, but it was shown later that a fraction of apoE associates with VLDL within the secretory pathway (18). The site of association was not determined, however.

To address this issue, we performed sequential immunoprecipitation analyses similar to those shown in Fig. 2B. ER and Golgi microsomes were isolated from metabolically labeled McA cells that had been incubated with OA. In vitro ER budding reactions were then performed, and the ER-derived vesicles and Golgi microsomes were separately subjected to immunoprecipitation against anti-apoB antiserum under nondenaturing conditions. The resulting supernatants and the immunoprecipitates were subjected to a second immunoprecipitation with anti-apoE antiserum under denaturing conditions. As shown in Fig. 4, apoE was recovered primarily (>95%) in the suprenatant fraction after nondenaturing apoB immunoprecipitation of the ER-derived vesicles. In contrast, apoB was depleted from this fraction (Fig. 2B), most likely because of its domain exposure in ER-derived vesicles (13). Finding apoB and apoE in different subpopulations of ER-derived vesicles indicates that the association of apoE with apoB-containing lipoproteins occurred post-ER.

In Golgi microsomes, apoE was again recovered primarily (>95%) in the supernatant fraction (Fig. 4), which is enriched for apoB species with low domain exposure, i.e. apoB associated with VLDL density particles (Fig. 3). Although this observation would be consistent with the presence of newly synthesized apoE on nascent Golgi-associated VLDL (31), there is still the possibility that there was a Golgi-associated subfraction of apoE that was separate from VLDL-apoB, as well as a subfraction that co-localized with VLDL-apoB. Thus, we sought to strengthen the evidence for the association in the Golgi of the two apolipoproteins by investigating Golgi-derived vesicles, especially in light of the report that apoE is present on a subset of nascent VLDL particles in Golgi-derived secretory vesicles (19). Thus, Golgi microsomes were prepared from metabolically labeled McA cells treated in the presence or absence of OA, and in vitro Golgi budding reactions were performed (see “Experimental Procedures”). The vesicles were separated from the remnant Golgi membranes by centrifugation and, as expected, were enriched in the coatomer protein β-COP (data not shown). Because the supernatant from the budding reaction contained not only vesicles, but also cytosol, discontinuous sucrose gradient centrifugation was used to isolate the vesicles enriched in β-COP (fractions 5 and 6) (Fig. 5A). Only fraction 5 was taken (fraction 6 represented a sucrose cushion, which was likely impure), and this material was then subjected to discontinuous sucrose gradient fractionation (Fig. 5, B and C) to separate the vesicles. For vesicles that were derived from both the −OA (Fig. 5B) and +OA (Fig. 5C) conditions, the fractional distributions of apoB, apoE, and a non-VLDL-associated secretory protein (albumin) were determined by either immunoprecipitation/
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Effects of the stimulation of lipid synthesis on the distributions of apoB, apoE, and albumin in Golgi-derived vesicles. A, Golgi-derived vesicles from cell-free budding reactions (based on McA cell-derived Golgi microsomes) were separated from the cytosol by centrifugation on a discontinuous sucrose gradient; six fractions (first three, 500 μl each; fourth, 200 μl; fifth, 300 μl; and sixth, 200 μl) were collected from the gradient, and the distribution of β-COP was detected by Western blotting (see “Experimental Procedures”). The top of the gradient is fraction 1. B and C, β-COP-containing vesicles generated from Golgi microsomes purified from metabolically labeled McA cells in the absence and presence of OA treatment, respectively, were subjected to density gradient analysis. For each treatment condition, the vesicles were first separated from the cytosol as described for A, and then fraction 5 was resuspended in a total volume of 400 μl of 20% sucrose, loaded on the top of another discontinuous sucrose gradient, and centrifuged (see “Experimental Procedures”). Ten fractions were collected from the top (fraction 1) of the gradient. The distributions of apoB and apoE were assessed by immunoprecipitation with rabbit anti-rat apoB and rabbit anti-rat apoE antisera, respectively, followed by SDS-PAGE/fluorography. The distribution of albumin was assessed by Western blot analysis. The intensities of the bands were evaluated by densitometry and are plotted on the graphs. The results shown are representative of two independent experiments. Adj, adjusted.

ApatE and apoB residence in McA cells as assessed by thin-section immunoelectron microscopy. ApoB- and apoE-expressing McA cells were grown, harvested, and processed for immunoelectron microscopy as described under “Experimental Procedures.” ApoB residence was established using large (10 nm) gold particle-conjugated antibodies, whereas apoE residence was established using small (4 nm) gold particle-conjugated antibodies. Note the residence of apoE clusters adjacent to apoB (e.g. A–C); however, apoE clusters in the absence of apoB were also observed (e.g. small gold particles) (D). As shown, both apoB and apoE residence was typically observed near membranous structures. Scale bars = 100 nm.

As shown in Fig. 5B, the distributions of apoB and apoE have distinct peaks under basal VLDL assembly conditions (−OA), which largely converged after treatment with OA (Fig. 5C). In marked contrast, the distribution of albumin was distinct from that of either apoB or apoE and was independent of the stimulation of triglyceride synthesis and VLDL lipiddation. These data suggest that, as VLDL matured, there was an increased association of apoE with apoB-containing lipoproteins, consistent not only with apoE residence on some VLDL within Golgi secretory vesicles (19), but also with the finding that more apoE molecules associate with VLDL recovered from liver perfusates when triglyceride synthesis is stimulated (32). Overall, the data in Figs. 4 and 5 suggest a model in which apoE becomes associated with VLDL precursors after their exit from the ER and in which the degree of apoE association correlates positively with the amount of fully lipidated VLDL.

Immunoelectron Microscopic Analysis of McA Cells Indicates Partial Overlap between ApoB and ApoE in Membranous Structures—The foregoing data suggest that fully lipidated VLDL particles in the Golgi should contain both apoE and apoB. Consistent with this suggestion, Hamilton et al. (19) showed that apoE is associated with Golgi and other intracellular membranes and that it co-localizes with VLDL, as assessed by immunoelectron microscopic analysis of hepatic cryothin sections. Moreover, Bergeron and co-workers (33) extended this analysis and suggested partial co-localization between apoE and apoB in a Golgi subdomain. ApoE resided in endosomes, in Golgi “saccular distensions,” and in ~350-nm vesicles, but apoB was restricted to the saccular distensions in sections from rat liver. In addition, these investigators uncovered a distinct localization between albumin and apoE.

To establish the site of apoB and apoE residence in McA cells, we similarly examined the location of these apolipoproteins by immunoelectron microscopy. ApoB and apoE (large and small gold particles, respectively) appeared in adjacent membrane-associated loci (Fig. 6, A–C), but the signal corresponding to apoE also appeared in the absence of apoB (one example is shown in Fig. 6D). These findings are consistent with the rat liver results of Bergeron et al. (33) and with a negative-staining immunoelectron microscopic procedure in which vesicles were left intact. ER-derived vesicles had 2.5 times more signal for (exterior) apoB than Golgi-derived vesicles prepared under basal (i.e. −OA) conditions (data not shown).

ApoE Is Not Required for the Production of Mature VLDL—In addition to its role in VLDL clearance from the plasma, it has been proposed that apoE is required for the assembly or secre-
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Despite these results, a definitive conclusion on the role of apoE in VLDL maturation was complicated by the previous demonstrations that subphysiological levels of apoE are sufficient to support some of its major functions. Specifically, only ~1–3% of wild-type plasma apoE is sufficient to restore impaired hepatic triglyceride secretion, to rescue defective remnant lipoprotein clearance, and to protect from atherosclerosis progression in mouse models (15, 34–36). Therefore, despite the low level of apoE expression in the siRNA-transfected cells, it still may have been sufficient to support VLDL maturation.

To eliminate this possibility, primary hepatocytes from wild-type (C57BL/6) and apoE<sup>−/−</sup> (C57BL/6 background) mice were metabolically labeled in the presence of OA for 3 h, and the density profiles of lipoproteins in the conditioned medium were analyzed as described for the siRNA-transfected McA cells. The medium also contained 10 mg/ml heparin to block apoE-mediated nascent VLDL re-uptake in wild-type hepatocytes (28, 37), which may alter the density distribution of newly secreted VLDL. As shown in Fig. 8, the highly lipidated VLDL<sub>1</sub> was the major VLDL species associated with apoB that was secreted by both wild-type and apoE<sup>−/−</sup> hepatocytes. Most important, the levels of apoB associated with VLDL<sub>1</sub>, as well as the overall density profiles of apoB-containing lipoproteins in the conditioned medium, were essentially identical between wild-type and apoE<sup>−/−</sup> hepatocytes. A final confirmation that apoE was not required for the full lipidation of VLDL was obtained by metabolically labeling the hepatocytes with both [<sup>3</sup>H]glycerol and [<sup>35</sup>S]methionine/cysteine. Four hours later, the conditioned medium was subjected to density gradient centrifugation. Aliquots of the VLDL subfractions (VLDL<sub>1</sub> and VLDL<sub>2</sub>) were used to measure the contents of radiolabeled triglycerides and apoB as we have done before (30).

Expressed as the ratio of the cpm of <sup>3</sup>H-labeled triglycerides to <sup>35</sup>S-labeled apoB, the results from the two types of hepatocytes were essentially indistinguishable for VLDL<sub>1</sub>, VLDL<sub>2</sub>, and total VLDL (VLDL<sub>1</sub> + VLDL<sub>2</sub>): wild-type hepatocytes, 2.0, 1.5, and 2.1; and apoE<sup>−/−</sup> hepatocytes, 2.8, 1.8, and 2.2, respectively.

Taken together, the results obtained with both McA cells and mouse primary hepatocytes do not support a requirement of apoE during VLDL assembly or secretion. As will be discussed below, the apparent increase in the amount of apoE associated with more mature particles may simply reflect more favorable binding conditions on the expanded surface of lipidated pre-VLDL.

**DISCUSSION**

VLDL assembly is a complex process that consists of at least two major lipidation steps. There is comparatively more known about the first step, in which microsomal triglyceride transfer protein-mediated lipid transfer to translocating apoB forms...
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A.

B.

FIGURE 8. ApoE is not required for the secretion of large VLDL from apoE−/− mouse primary hepatocytes. Metabolically labeled primary hepatocytes from wild-type (Wt) or apoE−/− (E−/) mice were incubated with OA and 10 mg/ml heparin (see “Experimental Procedures”). The conditioned medium was subjected to KBr/NaBr step density lipoprotein separation; apoB content of each density fraction was isolated by immunoprecipitation, followed by SDS-PAGE and the protein was visualized by phosphorimaging (A). The apoB content of each fraction was quantified by scintillation counting of the excised gel bands (B). The results shown are representative of three independent experiments. IDL/LDL, intermediate/low density lipoproteins; HDL, high density lipoproteins.

dense primordial apoB-containing lipoproteins in the ER (for a recent review, see Ref. 1). In a previous study, we found that these lipoproteins exit the ER in a Sar1-dependent process, as do general secretory proteins; however, the apoB-containing lipoproteins are contained in a subclass of ER-derived vesicles distinct from those containing other cargo molecules (13). Furthermore, there is a post-ER conversion of these dense lipoproteins to fully lipidated VLDL particles, which are recovered from Golgi microsomes. In this study, we have extended these findings significantly by focusing on the lipidation process that follows the initial formation of the primordial particles. The two major new findings are that the Golgi microsomal VLDL maturation process involves conformational changes in apoB and occurs in the absence of apoE. Notably, the data supporting each of these findings came from more than one experimental approach.

Although there are a number of studies that have favored the hypothesis that VLDL is completely assembled in the ER (e.g. Refs. 3–6 and 38), there are also several reports that support an important role for the Golgi in VLDL maturation. The first such example appears to be from a study in rat hepatocytes suggesting the trans-Golgi as the major site of apoB assembly with triglycerides and phospholipids (7). Other investigators have also concluded that assembly of triglycerides with apoB and other apolipoproteins occurs in the Golgi (8, 11, 39, 40). In fact, it has been estimated that up to 50% of the total VLDL triglycerides and 30–40% of phospholipids are added after pre-VLDL particles exit the ER (40). Although these lipids could have originated in the ER, there is also the possibility that they were produced locally and appended to nascent lipoprotein particles, given the demonstration that the Golgi may provide a second site of lipid synthesis (e.g. Ref. 41) and contains microsomal triglyceride transfer protein (12).

The striking conformational changes we observed in apoB associated with VLDL maturation are supported by several reports in the literature. For example, Bamberger and Lane (39) suggested that apoB interaction with the Golgi membrane is required for VLDL maturation. They found that, in primary chick hepatocytes, apoB resides in the ER for a shorter period of time than in the Golgi. Also, ~50% of apoB in a dense early Golgi fraction was membrane-associated, whereas in a less dense or late Golgi compartment, which presumably contains mature particles, only 20% was bound to membranes. These results agree with our data that the domain exposure of apoB in Golgi microsomes (which inherently establishes membrane association) is inversely related to the density of the apoB-lipoprotein complex. Furthermore, Tran et al. (11) showed that membrane association of apoB is lost in Golgi microsomes coincident with VLDL maturation and that the large fully lipidated particles become exclusively luminal. However, Du et al. (42) reported apoB domain exposure in semipermeabilized HepG2 cells (as assessed by indirect immunofluorescence microscopy) that persisted even when lipid synthesis was stimulated by OA. This result does not, however, contradict our present findings: although the stimulation of lipid synthesis is known to increase lipid loading of pre-VLDL particles, this is not a quantitative process (particularly in HepG2 cells), so there should always be a population of apoB molecules that are incompletely lipidated and would therefore be expected to have domain exposure.

Besides explaining why there would be persistent domain exposure of apoB when lipid synthesis is stimulated, the lack of quantitative conversion of pre-VLDL to mature VLDL is interesting from another perspective. We have found, in the Golgi, apoB associated with particles having VLDL density as well as with particles having low and high density lipoprotein densities (e.g. Figs. 1 and 3). Upon stimulation of lipid synthesis, there was a shift of apoB to more buoyant particles, but incompletely lipidated forms persisted (Fig. 1) that could be secreted (Fig. 7). This differs from other observations by Boren et al. (23) in McA cells: the denser apoB-containing lipoproteins that lost membrane association before they were fully lipidated were retained in the cell and largely degraded. One explanation for this apparent discrepancy is that the previous study used total microsomes, which are predominately of ER origin, whereas we used either Golgi microsomes or Golgi-derived vesicles. As we showed previously (13), apoB that successfully exits the ER does so in vesicles in which the protein is exclusively membrane-associated. Thus, what Boren et al. might have observed is a pool of pre-VLDL that was not incorporated into vesicles and was therefore shunted to a quality control/degradation pathway.

We have also assessed the relationship between apoB and apoE in the secretory pathway, and consistent with other studies (e.g. Refs. 18 and 33), we found evidence that these proteins associated in the Golgi (Figs. 4–6). Most striking was that, under conditions of increased lipid synthesis, the peaks of the density gradient distributions of Golgi-derived vesicles con-
taining apoB and apoE largely coincided, but the albumin peak remained distinct (Fig. 5). Overall, the present and previous results suggest that apoB/apoE-containing Golgi-derived vesicles, which harbor mature VLDL, may represent a distinct subclass of carriers. Specialized vesicles for apoB-containing lipoproteins would be consistent with predictions of the “cargo-selection” hypothesis (e.g. see Refs. 43 and 44) and with the suggestion that the structure of the outer shell of the vesicle coat implies the possibility of vesicle expansion (45), which may be necessary to capture the largest VLDL (>80 nm) in otherwise “standard” sized (40–50 nm) secretory vesicles.

Given these descriptive data on the relationship between apoB and apoE, we were most intrigued by previous suggestions that apoE functions in the secretion of VLDL as the triglyceride content and VLDL size increase. We were initially surprised by our lack of evidence for such a role, but corroborating results were obtained from two independent approaches, viz. the suppression of apoE synthesis by siRNA (Fig. 7) and the study of primary hepatocytes from wild-type and apoE−/− mice (Fig. 8).

One obvious difference between the present and published reports is that the effects of apoE on VLDL triglyceride production were typically studied previously upon apoE overexpression in vivo or in vitro (14–17). In some cases, however, the effects of apoE deficiency were examined; for example, in OATreated hepatocytes from control (wild-type) and apoE−/− mice, the mean sizes of VLDL were 25 and 20 nm, respectively (15). Although there was some increase in diameter (which should reflect increased triglyceride content) with normal apoE expression, the “VLDL” in either group had the diameter range of intermediate-low density lipoprotein particles (46). Thus, it appears that these results are consistent with incompletely lipided apoB-containing particles and not the most lipilated VLDL species (VLDL1; ~80 nm), which is what we monitored for the effects of apoE deficiency.

Another possibility for our not finding evidence for a requirement of apoE in VLDL maturation and secretion is that extracellular apoE is known to recycle through the secretory pathway (40, 47), so it may be possible that this pool has effects on VLDL assembly/secretion and that we would not be able to observe, especially because we added heparin to prevent apoE re-uptake. However, the relative contribution of endogenous and exogenous apoE was tested by two groups (14, 48). The investigators studied apoE−/− mice that received bone marrow transplants from wild-type mice. Sufficient apoE was produced extracellularly so that the plasma cholesterol levels were normalized (because of apoE-mediated uptake of the associated lipoproteins), yet VLDL triglyceride production was unaffected. Thus, it was concluded that the relevant apoE for VLDL assembly/secretion was the intracellular hepatic pool, which is the subject of this study.

Overall, then, the more positive role of apoE in VLDL assembly and secretion in the published reports may not represent a normal function of apoE, but rather effects from apoE overexpression. This would be consistent with the lack of consensus among the previous in vivo and in vitro studies on whether there was increased VLDL particle production, increased triglyceride loading of VLDL, or decreased lipolysis of VLDL after its secretion. Nonetheless, we did find that the peak fractions of Golgi-derived vesicles containing apoE largely coincided with the peak containing apoB when lipid synthesis was stimulated (Fig. 5). Instead of representing a functional relationship between apoE and VLDL assembly, this finding most likely reflects the long-standing observation that apoE preferentially associates with larger lipoproteins, which have a greater surface area and can bind multiple molecules (e.g. Refs. 14 and 18 and references therein).

To conclude, we have presented data that support a model for VLDL maturation as a Golgi-associated process. Furthermore, during this process, apoB undergoes conformational changes, and the expanding lipoproteins recruit more apoE. Surprisingly, this increased association of apoE with apoB in the Golgi appears to be the consequence, not the cause, of VLDL lipidation.

Acknowledgment—We thank Cristina Villagra for technical assistance.

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