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Proteomic and Lipid Characterization of Apolipoprotein B-free Luminal Lipid Droplets from Mouse Liver Microsomes

*IMPLICATIONS FOR VERY LOW DENSITY LIPOPROTEIN ASSEMBLY**

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The assembly of very low density lipoproteins involves the formation of a primordial, poorly lipidated apoB-containing particle in the endoplasmic reticulum, followed by the addition of neutral lipid from luminal lipid droplets (LLD). However, the lipid and protein compositions of LLD have not been determined. We have isolated LLD from mouse liver microsomes and analyzed their lipid and protein compositions. LLD are variably sized particles relatively poor in triacylglycerol (TG) content when compared with the lipid composition of cytosolic lipid droplets (CLD). They are devoid of apoB, adipophilin, and albumin but contain numerous proteins different from those found on CLD, including TG hydrolase (TGH), carboxylesterase 1 (Ces1), microsomal triglyceride transfer protein (MTP), and apoE. Ectopic expression of TGH in McArdle RH7777 hepatoma cells resulted in decreased cellular TG levels, demonstrating a role for TGH in the mobilization of hepatic neutral lipid stores. The isolation and characterization of LLD provide new supporting evidence for the two-step assembly of very low density lipoproteins.

Most of the current models of hepatic very low density lipoprotein (VLDL)[4] assembly describe a "two-step" process (1–5). The first step involves the formation of small, partially lipidated, apolipoprotein B (apoB)-containing VLDL precursor particles in the lumen of the endoplasmic reticulum (ER). The second step encompasses the transfer of the bulk of triacylglycerol (TG) to the precursor particles to form larger, fully lipidated VLDL particles. VLDL assembly is regulated by the availability of lipids, such as TG, which composes over 50% of VLDL lipids (6). Despite intensive research in this area, the source of the lipids transferred during the second step lipidation, the location where this occurs and the mechanism of lipid transfer have not been clearly defined. While the initial formation of the VLDL precursor particles in the ER is well accepted, the addition of bulk lipid (mainly TG) has been suggested to take place both in the ER (1, 7–9) and in the Golgi (8, 10–13). The source of TG for the second step lipidation of apoB is believed to be preformed intracellular lipid droplets (LD). There are at least three types of LD in the apoB-lipoprotein producing tissues (such as liver and intestine): the cytosolic lipid droplets (CLD), the apoB-containing lipid droplets (VLDL and its precursors), and the luminal apoB-free lipid droplets (LLD). The pool of stored TG for the provision of VLDL-TG substrates most likely resides in the lumen of organelles where VLDL is assembled (7, 14, 15). The existence of the LLD has first been observed within the smooth ER in 1976 (1), and this observation was further supported by electron microscopy studies using apoB-deficient mice (9). Microsomal triglyceride transfer protein (MTP), a heterodimeric protein confined to the lumen of the ER and to a lesser amount to the Golgi, has been implicated to play a major role in the formation of the LLD. Generation of LLD was greatly diminished in liver-specific MTP knock-out mice (16) and in hepatocytes treated with MTP inhibitors (17, 18).

The mechanisms by which the lipids from LLD are transferred to the apoB-containing primordial pre-VLDL particle during the second step remain speculative. Some models of VLDL assembly suggest "fusion" between the LLD and the primordial pre-VLDL particle (1, 3). However, the occurrence of any fusion between the partially lipidated apoB particles, and LLD has never been experimentally demonstrated. Another possible mechanism may involve an indirect transfer of lipids from LLD to the primordial pre-VLDL particle through a series of enzymatic events. It has been reported that up to 70% TG in VLDL is derived from hydrolysis and re-esterification of the preformed TG stores (19–21). One of the candidate lipases that could be involved in this process is an ER luminal carboxylesterase Ces3, also termed triacylglycerol hydrolase (TGH) (14, 22). Inhibition of TGH activity leads to decreased mobilization

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[4] The abbreviations used are: VLDL, very low density lipoproteins; apoB, apolipoprotein B; apoE, apolipoprotein E; BSA, bovine serum albumin; CE, cholesteryl ester; Ces1, carboxylesterase 1; CLD, cytosolic lipid droplets; DMEM, Dulbecco's modified Eagle's medium; DTBP, dimethyl 3,3′-dithiobispropionimidate; ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; GC, gas chromatography; HS, horse serum; KLH, keyhole limpet hemocyanin; LLD, luminal lipid droplets; McA, McArdle RH7777; MTP, microsomal triglyceride transfer protein; PDI, protein-disulfide isomerase; TBS, Tris-buffered saline; TG, triacylglycerol; TGH, triacylglycerol hydrolase.

of stored TG and VLDL secretion (23). However, it is obscure which TG storage pool TGH accesses. *In vitro* studies have indicated that apoB-containing particles are not substrates for TGH (24). Because TGH localizes exclusively to the lumen of the ER, we hypothesized that ER-localized LLD are the candidate substrates for TGH. In this study, we isolated LLD from mouse livers and determined their lipid and protein compositions.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and horse serum (HS) were purchased from Invitrogen Canada (Burlington, Ontario, Canada). Bovine serum albumin (BSA), Freund's complete and incomplete adjuvants and Complete™ protease inhibitor mixture tablets were from Roche Applied Sciences Diagnostics (Laval, Quebec, Canada). Anti-FLAG antibodies conjugated to agarose beads were from Sigma-Aldrich. Anti-porcine (22) and anti-human (25) TGH antibodies were generated in our laboratory. Polyclonal anti-apoB antibodies were purchased from Chemicon International (Temecula, CA). Anti-human apoE antibodies were from BioDesign (Saco, ME). Polyclonal antibodies against albumin were a generous gift from Dr. Dennis E. Vance (University of Alberta). Anti-protein-disulfide isomerase (PDI) polyclonal antibodies were from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Horseradish peroxidase-conjugated secondary antibodies, and dimethyl 3,3′-dithiobispropionimidate (DTBP) were from Pierce Biotechnologies. [9,10(n)-^{3}H]Oleic acid (10 Ci/mmol), protein A-Sepharose CL-4B beads, full-range rainbow protein molecular weight markers, HMW native protein standards, and immunoblotting reagents were obtained from Amersham Biosciences-Pharmacia Canada (Oakville, Ontario, Canada). All other reagents were of analytical grade or higher.

Isolation of Microsomal Luminal Contents—Microsomes from female C57BL/6J mouse liver homogenates were prepared essentially as previously described (26). In brief, mice were exsanguinated via cardiac puncture, and livers were removed and washed with ice-cold Tris-buffered saline (TBS). Livers were then homogenized with a motor-driven Potter-Elvehjem homogenizer (Wheaton Science Products, Millville, NJ) at medium speed for 10 strokes in homogenization buffer (250 mM sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA) to yield 20% (w/v) homogenate. Cellular debris were removed by centrifugation at 500 × *g* for 10 min. Crude mitochondrial pellets were obtained by centrifugation of the 500 × *g* supernatant at 15,000 × *g* for 10 min, and microsomes (pellet) and cytosol (supernatant) were separated by centrifugation of the 15,000 × *g* supernatant at 106,000 × *g* for 1 h. Microsomes were then resuspended in 20 mM Tris-HCl, 0.5 M NaCl (pH 7.4) to remove peripherally associated membrane proteins and lipid droplets. Washed microsomes were recovered by centrifugation at 106,000 × *g* for 1 h. Microsomes were suspended in 1 mM Tris-HCl, pH 8.8 for 30 min on ice, followed by centrifugation at 106,000 × *g* for 1 h to obtain luminal contents (supernatant) and microsomal membranes (pellet) (26, 27). Protease inhibitor mixture was included in all of the solutions used in this isolation procedure.

Isolation of Microsomal LLD by Density Gradient Ultracentrifugation—Microsomal luminal contents were adjusted to 50 mM Tris-HCl, 150 mM NaCl (pH 7.4) and immunoprecipitation with anti-apoB polyclonal antibodies in the absence of detergents was performed as previously described (17). 2 ml of post-IP supernatant was combined with an equal volume of glycerol and transferred to a Beckman Ultra-Clear™ centrifuge tube (Palo Alto, CA). Samples were overlaid with 4 ml of buffer containing 250 mM sucrose, 1 mM EDTA, and 20 mM Tris, pH 7.4, and an additional layer of 4 ml of TBS. The solution was centrifuged at 35,000 rpm in a Beckman SW40 rotor for 2 h at 8 °C. Fractions of 2 ml were collected from the bottom of the tube (6 fractions in total).

Immunoblotting—For the analyses of density gradient fractions, the top three fractions were concentrated 15-fold using Millipore Amicon® Ultra-4 Centrifugal Filter units (Mississauga, Ontario, Canada). 40-μl aliquots of each concentrated fraction were analyzed by 4–15% polyacrylamide gels containing SDS.

For particle size analysis by native gradient polyacrylamide gel electrophoresis (PAGE), 25-μl aliquots of each sample were mixed with electrophoresis sample buffer without SDS or β-mercaptoethanol, and electrophoresed in 2–10% polyacrylamide gels in the absence of SDS.

Proteins were transferred to nitrocellulose membranes. Immunoblotting with anti-porcine TGH polyclonal antibodies were performed as previously described (22). Immunoblotting with other antibodies (directed against apoB, apoE, PDI, and albumin) were performed according to protocols provided by the suppliers.

Protein Identification by Mass Spectrometry—Coomassie Blue-stained proteins in polyacrylamide gels were excised and an automated in-gel tryptic digestion was performed on a MassPrep Station (Waters Corp., Milford, MA). Proteins in the gel were destained, reduced (dithiothreitol), alkylated (iodoacetamide), digested with trypsin (Promega Sequencing Grade Modified) and the resulting peptides were extracted from the gel and analyzed via LC/MS/MS (the Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, Canada).

LC/MS/MS was performed on a CapLC HPLC (Waters Corporation, Milford, MA) coupled with a Q-ToF-2 mass spectrometer (Waters Corporation). Tryptic peptides were separated using a linear water/acetonitrile gradient (0.2% formic acid) on a Picofrit reversed-phase capillary column, (5 micron BioBasic C18, 300 Å pore size, 75 micron ID × 10 cm, 15 micron tip from New Objective Inc., Woburn, MA) with an in-line PepMap column (C18, 300 micron ID × 5 mm from LC Packings, Sunnyvale, CA) used as a loading/desalting column.

Protein identification from the resulting MS/MS data were done by searching the NCBI non-redundant data base using Mascot Daemon (Matrix Science, UK). Search parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide.

Generation of Rabbit Anti-mouse TGH Antibodies—Rabbits were immunized with a synthetic peptide corresponding to the C terminus of mouse TGH conjugated to keyhole limpet hemocyanin (KLH) (KLH-C-ESAQRPSHREHVEL). 0.5 mg of this antigen (0.5 ml) was combined with 0.5 ml of Freund's complete

adjuvant, and the emulsion was injected subcutaneously into two rabbits. Two booster intramuscular injections were given at 4-week intervals with 0.5 mg of antigen in Freund's incomplete adjuvant. Rabbits were bled prior to (preimmune) and 8 weeks after the initial immunization; sera were prepared, and titer was determined. The sera were then affinity-purified with the same TGH peptide conjugated to BSA and stored at −80 °C.

Immunoprecipitation—For immunoprecipitation using anti-mouse TGH antibodies, 1 ml of luminal content was adjusted to 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Protease inhibitor mixture was added according to the manufacturer's instructions. The sample was incubated with anti-mouse TGH antibodies described above end-over-end at 4 °C overnight. 20 μl of washed protein A-Sepharose beads were added and incubated with the sample end-over-end for 2 h at 4 °C. The protein-beads complex was pelleted by centrifugation for 30 s at 6,000 × *g*. Immunoprecipitates were washed and solubilized by boiling in SDS sample buffer. Proteins were separated by 10% SDS-PAGE and subjected to immunoblotting using anti-mouse TGH and anti-apoE antibodies.

Cross-linking—Primary rat hepatocytes were prepared by collagenase perfusion of the liver as previously described (23). Primary hepatocytes were electroporated with a plasmid containing the human TGH cDNA with a FLAG epitope tag (24). Electroporation of primary rat hepatocytes was performed based on previously published protocols (28, 29). Briefly, aliquots of 1.5×10^7 cells were combined with 15 μg of plasmid DNA in DMEM containing 10% fetal bovine serum, then incubated on ice for 10–15 min. Electroporations were performed on a BTX ECM630 Electrocell Manipulator. Immediately after electroporation, cells were plated on collagen-coated tissue culture dishes and incubated overnight. Subsequently, cells (electroporated and non-electroporated controls) were incubated with 8 mM DTBP in phosphate-buffered saline for 50 min at room temperature, and then aspirated. Excess DTBP was quenched by incubation in TBS for 5–10 min. Cells were harvested in TBS containing 1% Triton X-100 and Complete™ protease inhibitor mixture. TGH was immunoprecipitated with anti-FLAG antibodies. Immunoprecipitated proteins bound to protein A-Sepharose beads were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, and immunoblotted with indicated antibodies.

Gas Chromatography Analysis of Lipids—The mass of cholesterol, phospholipids, cholesteryl ester (CE), and TG were quantified by gas chromatography. Lipids from cytosol, microsomes, luminal contents, and LLD were extracted in the presence of tridecanoylglycerol (internal standard) (30, 31).

Gel Filtration Chromatography—Luminal apoB-free contents were applied to a Superose 6 size exclusion fast protein liquid chromatography (FPLC) column (Amersham Biosciences, Uppsala, Sweden). For TG analysis, eluted fractions were mixed in-line with the Infinity™ Triglyceride Reagent (Thermo Fisher Scientific, Inc., Waltham, MA) using a post-column T-connector/Solvent Delivery Module (model 110B, Beckman Coulter, Mississauga, Ontario, Canada) and passed through a CH-30 Column Heater (Eppendorf, Mississauga, Ontario, Canada) set at 37 °C. Reaction products were monitored at 500 nm in real-time using a Programmable Detector Module (model 166, Beckman Coulter). For protein analysis, fractions (2 ml) were collected every 4 min from the 22nd to 58th minute. Fraction 1 (eluted from the 22nd to 26th minute) contains VLDL-sized particles and fractions 5–8 (eluted from the 38th to 54th minute) contain HDL-sized particles based on the elution of plasma lipoproteins used as standards. Proteins in collected fractions were precipitated with 2 volumes of ice-cold acetone for 30 min at −20 °C, resuspended in 50 μl of SDS-PAGE sample buffer, and analyzed by SDS-PAGE and immunoblotting.

Cell Culture—McArdle RH7777 (McA) cells were obtained from American Type Culture Collection (Manassas, VA). McA cells stably transfected with human TGH cDNA have been produced and maintained in our laboratory (24). Cells are cultured in DMEM containing 10% fetal bovine serum (v/v) and 10% HS. All incubations were performed at 37 °C, in an atmosphere enriched with 5% CO_2 and in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin.

Metabolic Labeling of Lipids—Wild-type McA cells and McA cells stably expressing human TGH (24) were incubated with serum-free DMEM containing 0.5% BSA and 0.4 mM [^{3}H]oleic acid (125 μCi/μmol) for 4 h. At the end of the incubation, media were aspirated, and cells were washed with DMEM/0.5% BSA, followed by three washes with ice-cold phosphate-buffered saline. Cells were harvested and cytosolic and luminal contents were isolated as described above. Lipids were extracted (32) in the presence of non-labeled lipid carriers, applied to TLC plates and developed to one-third of the height with chloroform-methanol-acetic acid-water (25:15:4:2, by volume) to separate phospholipids, followed by development in heptane-isopropyl ether-acetic acid (60:40:4, by volume) to separate neutral lipids. Lipids were visualized by exposure to iodine; bands corresponding to various lipid classes were scraped off of the plates, and the associated radioactivity was determined by scintillation counting.

Other Methods—7.5 mM oleic acid/10% BSA stock solution was prepared as previously described (33). Protein concentration was determined by the Bio-Rad Protein Assay kit using BSA as a standard.

RESULTS

Isolation of LLD—To isolate mouse liver LLD, female C57BL/6J mice were fasted overnight to accumulate liver TG stores, livers were harvested, and microsomes were prepared. The commonly used procedure to release luminal contents from organelles utilizes alkaline Na_2CO_3. However, this procedure also strips loosely associated peripheral membrane proteins. To preserve the integrity and protein content of LLD we used hypotonic 1 mM Tris-HCl, pH 8.8 to release luminal contents. Compared with 0.2 mM Na_2CO_3, pH 12, 1 mM Tris-HCl released only about one-fourth of the total protein released by Na_2CO_3 (data not shown). However, the majority of luminally localized apoB and TGH were released efficiently by the hypotonic buffer treatment (Fig. 1).

Luminal contents contain apoB-containing particles (VLDL and its precursors) in addition to LLD. ApoB-containing particles were removed by immunoprecipitation with anti-apoB

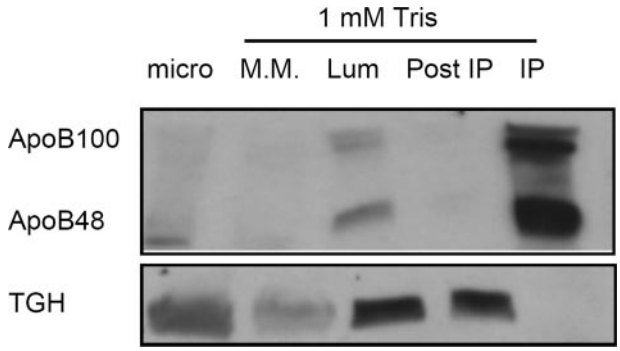

FIGURE 1. **Release of luminal contents from hepatic microsomes.** Salt-washed microsomes (*micro*) were suspended in 1 mM Tris-HCl, pH 8.8 and centrifuged to prepare microsomal soluble luminal contents (*Lum*) and microsomal membranes (*M.M.*). ApoB was immunoprecipitated from luminal contents with anti-apoB antibodies using non-denaturing conditions to yield an immunoprecipitate (*IP*) and a post-IP supernatant (*Post IP*). Equal volumes of each sample were loaded.

antibodies (Fig. 1). The post-apoB immunoprecipitation (*Post IP*) supernatant contains non-apoB-associated luminal proteins including those associated with LLD. The majority of TGH is recovered in this supernatant and is not present in the apoB immunoprecipitation pellet.

Lipid Composition of LLD—Lipid analysis of subcellular fractions isolated from fasted animals revealed that about 93% of cellular TG resided in the CLD and 7% in microsomes. 30% of luminal TG associated with apoB and 70% was found in the post-IP supernatant. The observed partition of TG to various subcellular locations suggested that LLD compose a relatively small pool of intracellular neutral lipid stores.

Floating lipid fractions (LLD) were isolated from the apoB-free post-IP fraction by glycerol-sucrose density centrifugation. About half of lipids present in the post-IP supernatant were recovered in LLD. Lipid content of LLD (fractions 4–6) was analyzed by thin-layer chromatography (Fig. 2*A*) and gas chromatography (Fig. 2*B*). Significant differences were found in the lipid composition of LLD and CLD. While CLD contained about 80% TG and less than 20% glycerophospholipids, LLD were less enriched in TG (ranging from 40% to 60%) and contained a near equal percentage of phospholipids (Fig. 2, *A* and *B*).

Protein Composition of LLD—All biological lipid droplets contain a specific set of associated proteins. To determine the protein composition of LLD, a proteomic approach was employed. Proteins present in combined fractions 4–6 were resolved by SDS-PAGE, and individual protein bands were subjected to analysis by mass spectrometry (Fig. 3*A* and Table 1). The LLD-associated proteins include TGH and a homologous protein of unknown function, carboxylesterase1 (Ces1), VLDL secretion-related proteins (MTP and apoE), and ER-resident proteins (such as heat shock proteins). The identification of MTP and apoE is of particular interest as these proteins are implicated participants in VLDL assembly. MTP has been suggested to play an important role in the transfer of lipid from the cytosol/ER membranes to the lumen of the ER (34). It forms an obligatory heterodimer with PDI, which was also found in the analysis (Table 1). Some mitochondrial and cytosolic proteins were also found in the analysis. They represent possible con-

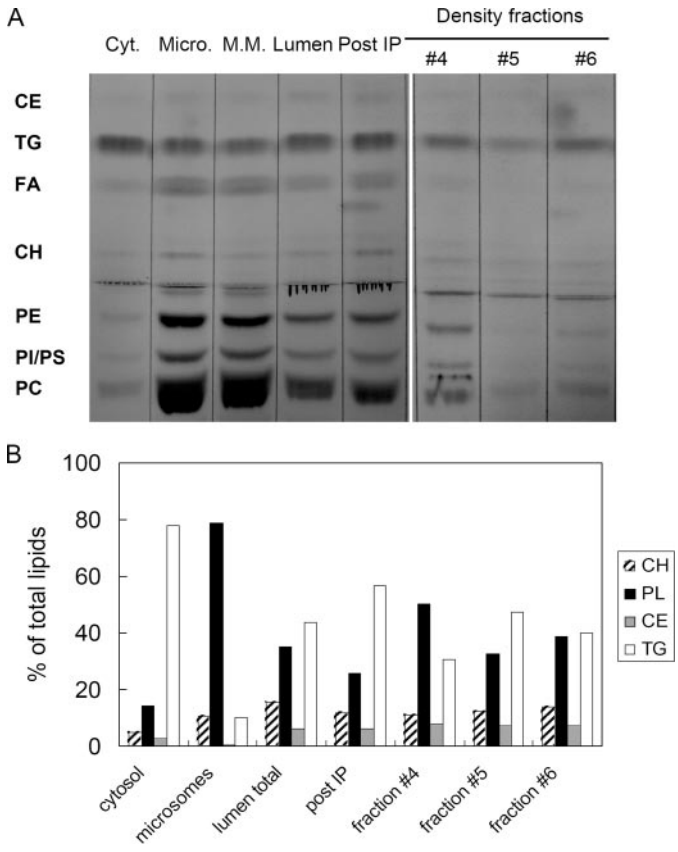

FIGURE 2. **Lipid composition of LLD.** Mouse liver cytosol (*Cyt.*), microsomes (*Micro*), microsomal membranes (*M.M.*), and microsomal luminal contents (*Lumen*) were isolated. Luminal contents were depleted of apoB by immunoprecipitation under non-denaturing conditions, and the post-IP supernatants (*Post IP*) were subjected to density fractionation via centrifugation. Fractions were collected from the bottom of the gradient. Fractions 4–6 represent floating fractions (density range from 1.027 to 1.004). Lipids were extracted and analyzed by TLC (*A*) and GC (*B*). For TLC analysis livers from three mice (about 2.4 g) were homogenized in 12 ml of homogenization buffer. 4 ml of 1 mM Tris-HCl, pH 8.8 were used to resuspend/lyse microsomes and microsomal membranes. 0.5% of the total cytosolic fraction and 2.5% of the microsome-derived fractions were used for analyses. The figure is representative of three independent preparations. *CE*, cholesteryl ester; *TG*, triacylglycerol; *Ch*, unesterified (free) cholesterol; *PL*, phospholipids; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol; *PS*, phosphatidylserine.

taminants that were non-specifically adsorbed to the droplets during preparation. Importantly, transmembrane proteins from either the ER or the Golgi were not found in LLD preparations. The CLD coat protein, adipophilin, was also absent from the LLD both by proteomic and immunoblotting analyses, indicating lack of contamination of LLD with CLD.

The presence of apoE and TGH in the LLD fractions was confirmed by immunoblotting (Fig. 3*B*). The LLD were devoid of albumin (secretory protein), which partitioned to the bottom of the gradient (fractions 1–3). Interestingly, although both TGH and apoE partitioned into LLD, the levels of the two proteins varied in the individual fractions, suggesting a degree of heterogeneity in the LLD protein composition.

ApoE/TGH Association—Apolipoproteins play a role in the inhibition and activation of lipolytic activities (35–37). Therefore, we wished to address whether apoE and TGH are present on separate or on the same LLD and, if both of these proteins can be found on the same lipid droplet, we sought to determine

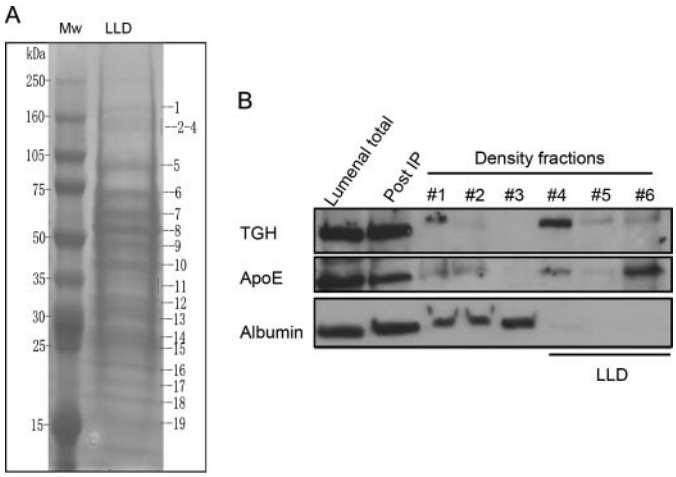

FIGURE 3. **Protein profile of LLD.** *A*, combined LLD fractions were subjected to SDS-PAGE followed by staining with Coomassie Blue. The identities of proteins indicated by numbers on the *right* are listed in Table 1. The protein profile is representative of three independent analyses. *B*, luminal contents and fractions from density centrifugation were analyzed for the presence of TGH, apoE, and albumin by immunoblotting. LLD fractions were concentrated 15-fold before analysis. The result is a representative of several preparations.

TABLE 1

Identification of LLD-associated proteins by mass spectrometry

LLD-associated proteins separated by SDS-PAGE were identified by LC/MS/MS. Raw data obtained from database were evaluated according to significance of ions scores and species where matching peptides were found. Reproducible results from two independent experiments are presented.

Category	Name	GI no.	Band
Carboxylesterases	Triacylglycerol hydrolase (TGH)	16716505	7,8
	Carboxylesterase 1	20070717	8
VLDL secretion-related	Apolipoprotein E	6753102	12
	MTP	15215161	5
ER-resident proteins	BiP	2598562	6
	Heat shock protein 8	76779312	6
	Heat shock 70 protein	1661134	6
	Tumor rejection antigen gp96	6755863	5
	Calreticulin	13097432	9
	Protein-disulfide isomerase	860986	9
	Protein-disulfide isomerase-associated 3	6679687	9
	Protein-disulfide isomerase-associated 4	86198316	6
	Microsomal protease ER-60	1583929	9
	Sorbitol dehydrogenase 1	22128627	11
	Carbonic anhydrase III	10717134	14
Possible contaminants	Albumin	26986064	7,8
	Clathrin heavy chain 1	51259242	1,2
	Complement component 3	23956044	2
	Transferrin	17046471	6
	Catalase	1066114	8
	Alcohol dehydrogenase 1 (class I)	32449839	11
	Regucalcin	6677739	13
	Ribosomal proteins	Multiple	

whether these two proteins directly interact with each other. Immunoprecipitation of TGH under non-denaturing conditions (in the absence of detergent) resulted in a significant recovery of apoE in the immunoprecipitation pellet (Fig. 4*A*), indicating the presence of apoE on the same LLD as TGH. This result was confirmed by reciprocal immunoprecipitations with anti-apoE antibodies (data not shown). In order to explore the possibility of a direct interaction between TGH and apoE, cross-linking was performed in intact cells, followed by TGH immunoprecipitation under denaturing conditions and immunoblot analysis. ApoE co-immunoprecipitated with TGH after cross-linking suggests spatial proximity of the two proteins and possible direct interaction (Fig. 4*B*).

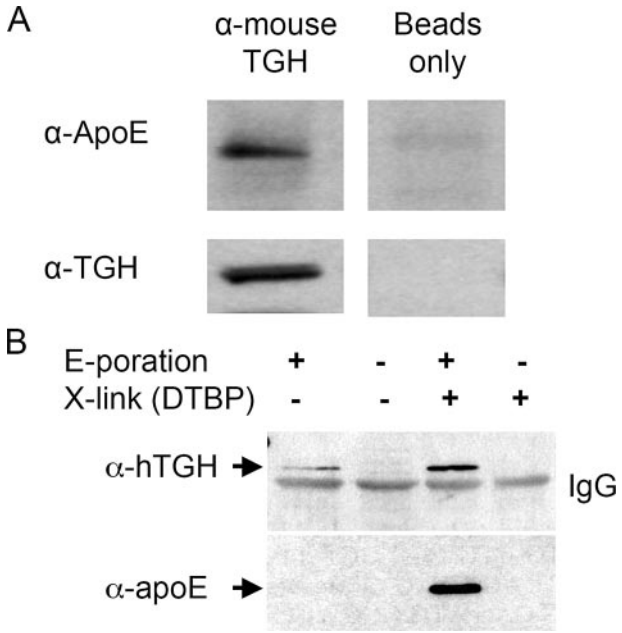

FIGURE 4. **TGH associates with apoE.** *A*, immunoprecipitations with α-mTGH antibodies were performed in the absence of detergents. Immunoprecipitates were analyzed for the presence of TGH and apoE by immunoblotting. The specificity of immunoprecipitations was assessed by parallel experiments using protein A-Sepharose beads without inclusion of the α-mTGH antibodies (beads only). *B*, cross-linking of apoE with TGH. Hepatocytes were electroporated with or without a plasmid encoding TGH-FLAG cDNA, and cross-linked with DTBP as described under "Experimental Procedures." Cells were then homogenized and immunoprecipitation with anti-FLAG antibodies was performed in buffer containing 0.1% SDS and 0.5% Triton X-100. The presence of TGH and apoE were determined by immunoblotting.

LLD Are Heterogeneous in Size—The unique lipid composition of LLD (Fig. 2) suggested that these particles might be relatively dense, resembling high density lipoproteins in size. Gel filtration chromatography of apoB-free luminal contents was performed to determine the size of LLD (Fig. 5*A*). TG eluted in three distinct peaks with sizes corresponding to VLDL, HDL, and smaller-than-HDL. The peak at >54 min corresponds to the elution of free glycerol. The source of luminal glycerol is unclear; however, it might be derived from lipolysis of membrane or luminal lipids.

Eluted fractions from the sizing column were collected and analyzed for the presence of TGH, apoE, and MTP (Fig. 5*B*). TGH was present in most fractions (26–58 min), with the highest enrichment in particles corresponding to small HDL (42–50 min). Interestingly, apoE co-eluted with TGH present in the larger molecular mass fractions (presumably less dense LLD), while MTP co-eluted with TGH present on smaller particles. No significant levels of MTP co-eluted with apoE. It is unlikely that apoE or TGH are present as free proteins in the lipoprotein-sized fractions. The M_r of ApoE and TGH are about 35 and 60 kDa, respectively and the M_r of the obligate MTP/PDI heterodimer is ~150 kDa. Neither TGH nor apoE were detected to any significant amount in fractions 22–25 corresponding to VLDL and none of the fractions contained immunodetectable apoB100 or apoB48.

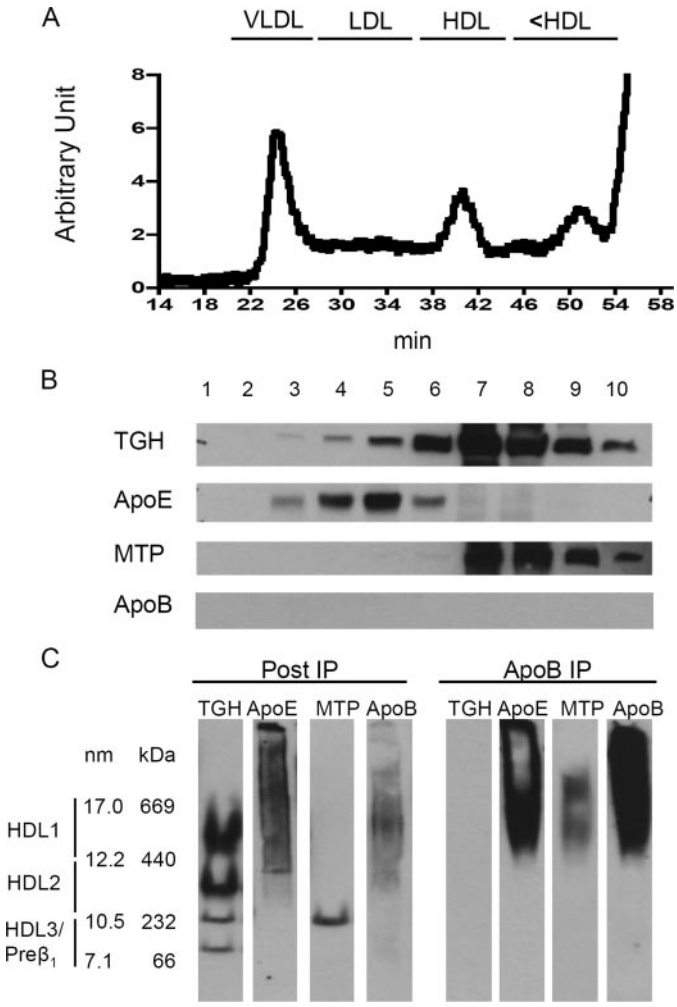

FIGURE 5. **TGH associates with small LLD that may contain apoE or MTP but not apoB.** *A*, FPLC gel filtration profile of microsomal luminal apoB-free contents. Post-apoB immunoprecipitation supernatant was applied to a FPLC gel filtration column, and fractions were assayed for the presence of TG as described under "Experimental Procedures." The elution profile of plasma lipoprotein standards (VLDL, LDL, HDL) is indicated. *B*, fractions collected every 4 min (2-ml fractions), and proteins were precipitated with ice-cold acetone. The presence of TGH, apoE, and MTP were assessed by immunoblotting. Each lane corresponds to eluted fractions. *Lane 1*, 18–21 min; *lane 2*, 22–25 min; *lane 3*, 26–29 min; *lane 4*, 30–33 min; *lane 5*, 34–37 min; *lane 6*, 38–41 min; *lane 7*, 42–45 min; *lane 8*, 46–49 min; *lane 9*, 50–53 min; *lane 10*, 54–57 min. *C*, ApoB was immunoprecipitated from luminal contents and beads (*apoB IP*) and supernatants (*Post IP*) were resolved by 2–10% native PAGE. The presence of TGH, apoE, MTP, and apoB were determined by immunoblotting. Mobility of known lipoprotein standards and the sizes of molecular mass markers are indicated on the *left*.

Similar results were obtained when post-IP contents were analyzed using a native gradient PAGE approach (Fig. 5*C*, *left panel*). TGH migrated as four distinct bands corresponding to relatively small particles ranging in size from HDL1 (17.0 nm) to HDL3 (10.5 nm). ApoE migrated as a smear corresponding to sizes larger than HDL2 (12.2 nm) that partially overlapped with TGH containing particles. MTP migrated as a distinct band with the size of HDL3 (10.5 nm), overlapping with one of the TGH bands, but not with apoE. A small amount of residual apoB was found in the post-IP fraction in this preparation. Immunoprecipitated luminal apoB-containing lipoproteins exhibited slower mobility as expected for larger size particles (Fig. 5*C*, *right panel*). Both apoE and MTP were present in the apoB immunoprecipitation pellet, which is in agreement with published data (38–40), while TGH was absent, supporting our previous results (24).

TGH Decreases Neutral Lipid Stores—TGH has been proposed to play a role in the mobilization of intracellular neutral lipid stores for VLDL assembly (14, 15). However, it is not entirely clear, which lipid pool TGH accesses. The association of TGH with LLD suggests that LLD are the likely TG storage pool utilized by TGH for VLDL assembly. To explore this hypothesis, McA cells stably transfected with human TGH cDNA, as well as wild-type McA cells (lacking TGH expression), were incubated with radiolabeled oleic acid and examined for intracellular and secreted lipid content. A trend toward decreased luminal TG in TGH-expressing cells was observed, but this trend did not reach statistical significance (Fig. 6*A*). However, when levels of cytosolic lipids were examined in parallel, a marked decrease in TG labeling was observed in TGH-expressing cells (Fig. 6*B*), while no significant changes in PC labeling were seen in either luminal or cytosolic content. Correspondingly, a 3-fold increase in media TG labeling was obtained from TGH-transfected cells (Fig. 6*C*), suggesting the existence of TGH-mediated alterations in intracellular neutral lipid stores and the redirection of the lipolytic products to VLDL secretion.

DISCUSSION

To our knowledge, this is the first study reporting isolation and characterization of hepatic LLD that may serve as a reservoir of lipids for VLDL assembly. The isolation was performed using conditions that did not affect LLD integrity and did not strip peripherally associated proteins. CLD contain a phospholipid monolayer and specific coat proteins that play a role in the droplet metabolism (reviewed in Refs. 41, 42). This study suggests that like the CLD, LLD also contain a variety of proteins on their surface. The LLD proteome differs significantly from that of CLD, which is expected given their physical separation in distinct subcellular compartments. Some proteins are common for both LLD and CLD, such as the ER chaperone BiP. It is not clear how BiP, an ER luminal protein, becomes associated with a cytosolic lipid droplet. In the majority of CLD preparations from various cells reported to date, the presence of calnexin (a transmembrane ER protein) was identified, suggesting that CLD exist in a continuum with the ER bilayer (43, 44). The proximity to and/or possible interaction of CLD with the ER, and mitochondria was also suggested by imaging (45). Neither transmembrane proteins nor known CLD coat proteins (adipophilin, TIP-47) were found on LLD. Instead, LLD contained apoE, which may perform a lipid droplet coat function, and MTP, which plays a crucial role in apoB-containing lipoprotein assembly (4, 46). Genetic ablation of apoE in mice results in the impairment of VLDL-TG secretion (47–49) and leads to intracellular accumulation of lipids (50). Conversely, overexpression of apoE in mice promotes VLDL-TG production (51). Interestingly, recent *in vitro* experiments demonstrated that large VLDL can be secreted from apoE-deficient hepatocytes when these cells are supplemented with exogenous fatty acid (52), suggesting that apoE is not absolutely required in this process. Although a definitive role that apoE plays in VLDL assembly

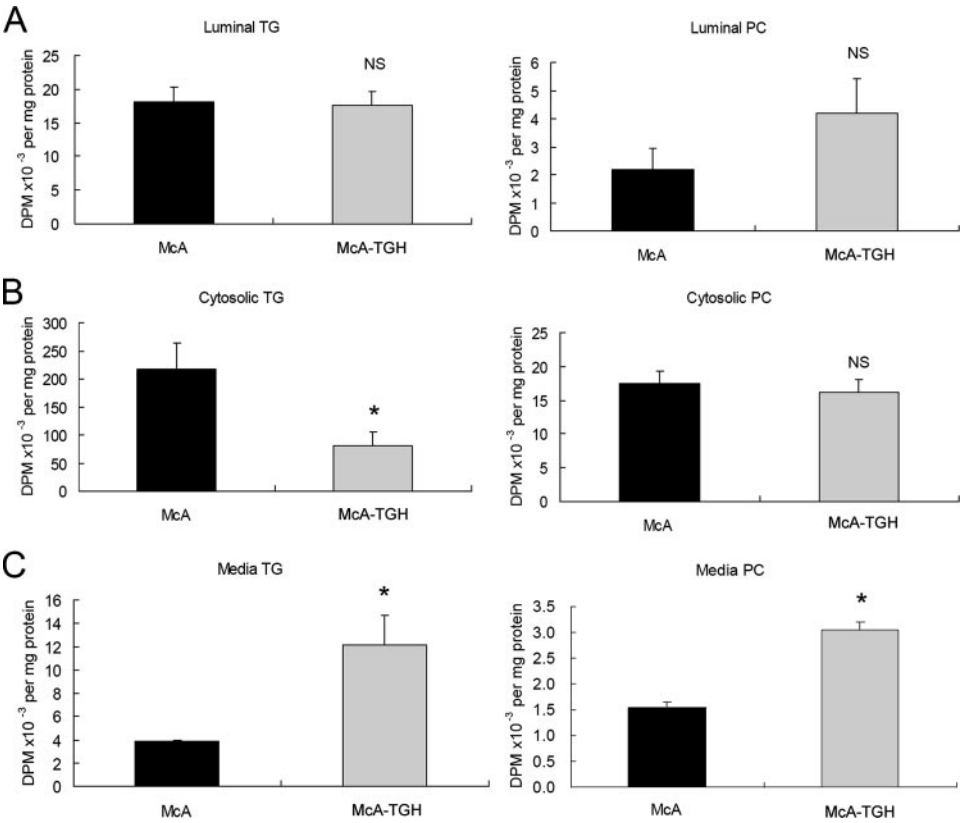

FIGURE 6. **Ectopic expression of TGH decreases neutral lipid stores in McArdle RH7777 cells.** Wild-type McA cells (*McA*) or McA cells stably expressing human TGH (*McA-TGH*) were incubated with [^{3}H]oleic acid for 4 h. Cells were homogenized, LLD (*A*) and CLD (*B*) were isolated, and media (*C*) were collected. Lipids were extracted and separated by thin-layer chromatography. Radioactivities associated with TG, CE, and PC were determined by scintillation counting. *, $p < 0.01$.

and secretion has not been demonstrated, an enticing hypothesis is that apoE may be involved in the mobilization of LLD for lipidation of apoB. Because apoE and TGH may directly interact (Fig. 4), it is possible that apoE modulates TGH function. However, this has not yet been experimentally demonstrated. MTP has been also implicated in the biogenesis of LLD, in addition to its crucial role in the co-translational/co-translocational lipidation of apoB. Genetic ablation of MTP expression (16) or chemical inhibition of MTP activity (17, 18) was shown to decrease LLD levels. The observed co-migration of MTP with LLD supports its proposed role in LLD generation. MTP may also be involved in transporting lipids (phospholipids, TG and CE) from LLD to nascent apoB-containing particles (53–55). Another protein of interest found associated with LLD is carboxylesterase 1 (also known as ES-x or Ces1). ES-x shares a high degree of homology with TGH and therefore may play a role in hepatic lipid metabolism (14, 56).

Compared with CLD that contain 80% TG (Fig. 2*B*), LLD have higher phospholipid/TG ratios. Because phospholipids form a lipid monolayer surrounding the neutral lipid core of LD, the higher phospholipid/TG ratio in LLD suggests smaller particles. Gel filtration chromatography revealed that the apoB-free lipid particles are heterogeneous, ranging from the size of VLDL to HDL. LLD of various sizes might represent particles with different roles or particles at different stages of biogenesis/hydrolysis. Recently, it was reported that a special class of peripheral CLD, much smaller than the major population of large CLD in white adipose tissue, are the site of active lipolysis (57). Similarly, our results suggest that TGH associates primarily with the smaller HDL-size lipid droplets and is absent from the larger VLDL-size particles.

Several groups have reported that the maturation of VLDL (the addition of bulk lipid to primordial apoB lipoproteins) may occur in the Golgi (8, 10–12). One of the key questions that needs to be resolved in the Golgi-localized apoB-lipidation model is the source of non-lipoprotein associated TG in the Golgi. Because TG is synthesized by the ER-localized acyltransferases DGAT1 and DGAT2 (58, 59), LLD generated in this organelle would be required to be sorted into transport vesicles and exported to the Golgi. However, some LLD, at least the subpopulation that bears TGH, could not undergo this transport process because TGH is exclusively localized to the ER and has not been detected in the Golgi (24). Therefore, non-TGH-containing LLD would have to be sorted from TGH-containing LLD into transport vesicles in the ER before being exported to the Golgi. It is likely that LLD in the Golgi would be depleted of other ER proteins identified in our screen but would not be expected to acquire any new proteins because the Golgi does not contain any luminal resident proteins. ApoE and apoB were reported to be present in different subpopulations of ER-derived transport vesicles (52), and thus it is possible that apoE-containing LLD that do not contain TGH are transferred to the Golgi.

Our previous metabolic labeling studies showed that expression of TGH in McA cells significantly increased the turnover of preformed TG (33) and that inhibition of TGH in hepatocytes had the opposite effect (23). In this study, cytosolic neutral lipid levels were depleted markedly in TGH-expressing McA cells, but no significant decrease of luminal neutral lipid levels was observed (Fig. 6). Because luminal TG comprises a relatively minor fraction of total TG storage in hepatocytes, these studies suggested TGH-mediated depletion of cytosolic TG pools in addition to mobilization of luminal TG for VLDL assembly. It is not clear how TGH, a luminal lipase, would access cytosolic TG. One possibility is that TG could egress from CLD that are in continuum with the ER membrane and TGH would mobilize such membrane-associated TG. Up to 3 mol% of TG can be solubilized within phospholipid membranes without disturbing bilayer structures (60–62). Because proteomic analyses of CLD identified the presence of ER resident proteins, such as BiP and calnexin (44, 63) it is also plausible that CLD-ER association represents a hemifusion of the cytosol-facing phospholipid

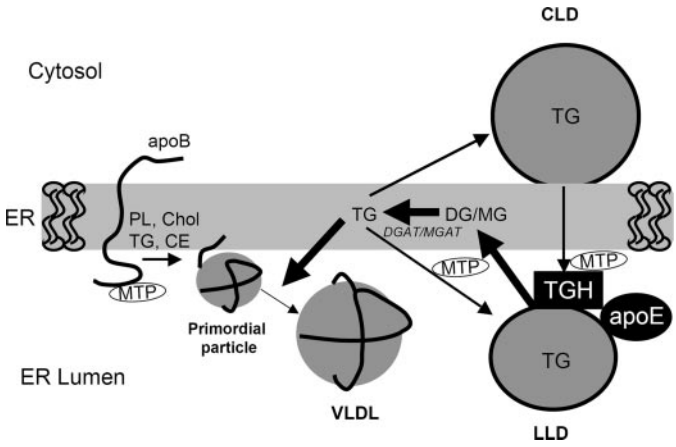

FIGURE 7. **Model for the role of LLD in VLDL assembly.** TGH localizes to the lumen of the ER, associates with LLD, and mobilizes this pool of TG for VLDL assembly. The hydrolysis of TG by TGH may be modulated through interactions with apoE. LLD serve as a direct source for VLDL-TG. TG from the CLD replenishes LLD. The transfer of TG from CLD to LLD may involve MTP and TGH. Excessive TG can be returned to either LLD or to CLD (futile cycle).

monolayer of the ER with a phospholipid monolayer surrounding CLD, providing access of luminal ER proteins (TGH, MTP) to CLD. Such a mechanism would be analogous to that proposed for peroxisome-CLD interactions in yeast (64). It is unlikely that a cytosol-localized lipase would be involved in the provision of substrates for VLDL assembly based on studies by Pease *et al.* (65) who reported that fatty acids released from CLD by overexpression of cytosolic hormone-sensitive lipase were directed for mitochondrial oxidation but not for VLDL assembly. LLD may represent intermediate reservoirs that regulate the transfer of neutral lipids from CLD to VLDL. The proposed model is shown in Fig. 7. In this model, TG for the lipidation of primordial apoB-containing particles is provided through lipolysis/re-esterification of pre-existing ER-associated TG pools. TG that is in excess can be returned to either LLD or to CLD (futile cycle). An unresolved issue regards the possible access of TGH and MTP to cytosol-derived TG. If hemifusion of the ER leaflet with CLD takes place, TG would be expected to become available for either hydrolysis by luminal lipases or for binding to MTP.

In conclusion, we have isolated LLD and demonstrated the presence of proteins linked with VLDL assembly on these droplets, providing further insight in to the molecular mechanism of VLDL assembly. Further research should clarify the exact role of apoE and TGH in the utilization of LLD for apoB lipidation.

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