The assembly of very low density lipoproteins in hepatocytes requires the microsomal triacylglycerol transfer protein (MTP). This microsomal lumenal protein transfers lipids, particularly triacylglycerols (TG), between membranes in vitro and has been proposed to transfer TG to nascent apolipoprotein (apo) B in vivo. We examined the role of MTP in the assembly of apoB-containing lipoproteins in cultured murine primary hepatocytes using an inhibitor of MTP. The MTP inhibitor reduced TG secretion from hepatocytes by 85% and decreased the amount of apoB100 in the microsomal lumen, as well as that sequestered into the medium, by 70 and 90%, respectively, whereas the secretion of apoB48 was only slightly decreased and the amount of lumenal apoB48 was unaffected. However, apoB48-containing particles formed in the presence of inhibitor were lipid-poor compared with those produced in the absence of inhibitor. We also isolated a pool of apoB-free TG from the microsomal lumen and showed that inhibition of MTP decreased the amount of TG in this pool by ~45%. The pool of TG associated with apoB was similarly reduced. However, inhibition of MTP did not directly block TG transfer from the apoB-independent TG pool to partially lipiddated apoB in the microsomal lumen. We conclude that MTP is required for TG accumulation in the microsomal lumen and as a source of TG for assembly with apoB, but normal levels of MTP are not required for transferring the bulk of TG to apoB during VLDL assembly in murine hepatocytes.

During assembly of very low density lipoproteins (VLDLs) in the liver, triacylglycerol (TG) is concentrated within the hydrophobic core of apolipoprotein (apo) B-containing lipoprotein particles. A microsomal lumenal protein, the microsomal triacylglycerol transfer protein (MTP), has been implicated in the acquisition of TG by nascent apoB for assembly and secretion of VLDLs (reviewed in Refs. 1–4). Individuals with the rare inherited disease abetalipoproteinemia have a defect in the MTP gene and lack detectable MTP protein and MTP lipid transfer activity (5). Despite a normal apoB gene, plasma apoB is barely detectable in these patients. MTP has the ability to transfer TG and other lipids, including cholesteryl esters, diacylglycerols, and phospholipids, between membranes in vitro (6) and has been proposed to transfer TG to nascent apoB-containing lipoproteins in vivo. This idea is consistent with immunoprecipitation studies showing that apoB and MTP interact physically at early stages of VLDL assembly (7–9). MTP is expressed primarily in the liver and intestine as a soluble heterodimer with protein-disulfide isomerase (55 kDa) (10), a ubiquitous protein of the endoplasmic reticulum (ER) lumen that catalyzes disulfide bond formation during protein folding (11). The 97-kDa MTP subunit confers all lipid transfer activity to the heterodimer (12). In in vitro assays, the lipid transfer activity of MTP displays Ping Pong Bi Bi kinetics implying that MTP transfers lipids between membranes via a "shuttle" mechanism (13). The tissue and subcellular location of MTP, and its preference for transferring neutral lipids between membranes in vitro, have suggested that MTP participates in the loading of apoB with TG.

Specific inhibitors of the MTP lipid transfer activity have been developed that lower plasma cholesterol levels by up to 80% in rabbits, hamsters, and rats (14). Complete elimination of the MTP gene in mice is embryonically lethal, probably because apoB-containing lipoproteins are required for transferring lipids from the yolk sac to the developing embryo (15). However, viable mice were generated in which the MTP gene was specifically inactivated in the liver (16, 17). In these mice, compared with wild-type mice, plasma apoB100 levels were reduced by ~90% but apoB48 levels were reduced by only ~20% (16). In support of the hypothesis that MTP provides TG for VLDL assembly, the majority of apoB48 in the plasma of these MTP-deficient mice was present in lipoprotein particles that were only partially lipiddated (16). Moreover, apoB100 secretion from cultured MTP-/- hepatocytes was greatly reduced, whereas apoB48 secretion was only slightly reduced compared with that in MTP+/- hepatocytes (16). Ultrastructural analyses revealed that compared with MTP+/- hepatocytes, MTP-/- hepatocytes contained very few VLDL-sized particles within the ER and Golgi lumina (16). In addition, in mice unable to synthesize apoB in the intestine, chylomircon-sized, lipid particles (without apoB) accumulated in the ER lumen of enterocytes (18) suggesting that ER luminal TG droplets might exist as a source of TG for assembly with apoB. The importance of MTP for apoB secretion is underscored by the demonstration that overexpression of MTP activity in mouse liver increased plasma levels of apoB100 and -B48, as well as TG (19).

MTP has been reported to play several roles in VLDL assem-
bly: (i) as a chaperone that mediates the translocation of newly synthesized apoB across the ER membrane (20–22); (ii) in the co-translational lipidation of apoB during its translocation into the ER lumen (22–24); (iii) in the transfer of the bulk of TG to poorly lipidated apoB in the ER lumen (25, 26); and (iv) in the transfer of TG into the ER lumen for assembly into VLDLs (16, 18). The majority of studies on the role of MTP have been performed in cultured hepatoma cells, either HepG2 or McArdle 7777 cells, that compared with primary hepatocytes, inefficiently secrete VLDLs (21–23, 27–30). Moreover, in hepatoma cells, the activities of several enzymes involved in lipid metabolism are either absent or impaired. The function of MTP has also been investigated in HeLa and COS cells that do not normally secrete lipoproteins but in which apoB secretion was induced by transfection with cDNAs encoding MTP and apoB variants (31–33). Some of the conflicting data on the role of MTP in VLDL assembly might, therefore, be related to the different model systems used. Only a few studies have been performed on the function of MTP in primary hepatocytes (34–36). Although the role of MTP has been investigated in liver-specific MTP knock-out mice (16, 17), detailed studies were not reported on the function of MTP in hepatocytes from these mice. Because many types of genetically modified mice are now frequently used as models of human lipoprotein metabolism it is important to understand the mechanism of lipoprotein assembly in murine hepatocytes.

We have investigated the requirement of MTP for VLDL assembly and secretion in murine primary hepatocytes. Our data show that secretion of apoB100 is markedly more sensitive to inhibition of MTP than is apoB48, and that when MTP is inhibited the apoB48 particles produced are lipid-poor compared with those made in hepatocytes with normal MTP activity. We have also isolated from the microsomal lumen a pool of TG that is not associated with apoB and show that inhibition of MTP reduces the amount of TG within this pool. However, inhibition of MTP does not directly impair the transfer of TG from this pool to lipid-poor apoB-containing particles.

**EXPERIMENTAL PROCEDURES**

**Materials**— Dulbecco’s modified Eagle’s medium (DMEM), Hanks’ solution, collagenase, penicillin, streptomycin, and fetal bovine serum were obtained from Invitrogen. Minimal essential medium (methylene-, free), fatty acid-free bovine serum albumin, oleic acid, phenylmethyl-sulfonyl fluoride, leupeptin, Na-acyetyl-Leu-Leu-norleucine, lactacycin, the protease inhibitor MG132, trypsin, soybean trypsin inhibitor, triclein standard for thin-layer chromatography, digitonin, collagenase type I, insulin, and cycloheximide were purchased from Sigma. Complete protease inhibitor mixture tablets were from Roche Molecular Biochemicals (Laval, PQ, Canada). [35S]Methionine, [3H]oleic acid, protein A-Sepharose CL-4B, Amplify, and rainbow protein molecular weight markers were from Amersham Biosciences (Baie d’Urfe, PQ). Anti-human apolipoprotein B antibodies were purchased from Roche Molecular Biochemicals. BIOCOAT collagen-coated cell culture dishes (60 or 100 mm, 1 cm2). All chemicals used for solubilization, detergent, digitonin-permeabilized lipoprotein, lactate dehydrogenase, from the cells (40). Under the conditions used, >85% of total lactate dehydrogenase activity was released.

**Trypsin Digestion of Proteins in Permeabilized Cells**—For trypsin digestion of proteins exposed to the cytosol, digitonin-permeabilized cells were washed with buffer containing 0.3 m sucrose, 0.1 m KCl, 2.5 mm MgCl2, 1 m sodium-free EDTA, and 10 mM Pipes (pH 6.8) for 5 min on ice. The digitonin-permeabilized cells were then pelleted by centrifugation for 2 min at 14,000 rpm. For immunoprecipitation of secreted apoB, culture medium was centrifuged for 2 min at 3,000 rpm to remove cell debris prior to immunoprecipitation of apoB as described above. For immunoprecipitation of apoB-containing lipoproteins from microsomal lumenal contents under nondenaturing conditions, the lumenal contents were washed with 0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl2, 1 mM sodium-EDTA, and 10 mM Pipes (pH 6.8).

**Immunoprecipitation of Proteins**—Immunoprecipitation of apoB-containing lipoproteins from microsomal lumenal contents under nondenaturing conditions was performed in solubilization buffer containing Tris-HCl (0.63 M, pH 7.4), NaCl (0.75 M), EDTA (25 mM), phenylmethylsulfonyl fluoride (5 mM), and Triton X-100 (5%, v/v). Cellular extracts were centrifuged in a microcentrifuge at 14,000 rpm for 10 min, then the supernatant (1 ml) was incubated overnight at 4 °C with anti-human apoB antibody (7.5 µl). Next, 45 µg of protein A-Sepharose was added and the sample was mixed end-over-end for 2 h at 4 °C. The protein-A–protein complexes were pelleted by centrifugation for 2 min at 14,000 rpm. For immunoprecipitation of secreted apoB, culture medium was centrifuged for 2 min at 3,000 rpm to remove cell debris prior to immunoprecipitation of apoB as described above. For immunoprecipitation of apoB-containing lipoproteins from microsomal lumenal contents under nondenaturing conditions, the lumenal contents were washed with 0.3 M sucrose, 0.1 m KCl, 2.5 mm MgCl2, 1 m sodium-free EDTA, and 10 mM Pipes (pH 6.8). The hepatocytes were washed 3 times in DMEM, then suspended in DMEM containing 10% fetal bovine serum. Cells were plated on collagen-coated dishes (60 or 100 mm, 1 × 106 cells/ml). Cell viability (typically >90%) was estimated by trypan blue exclusion. After hepatocytes had attached to the dish (2 to 3 h), fresh DMEM containing 10% fetal bovine serum was added and the cells were incubated overnight. The next morning, the medium was replaced with fresh serum-free medium containing 0.375 mM oleate and 10% bovine serum albumin.

**Metabolic Labeling of Proteins and Lipids**—For experiments in which proteins were labeled with [35S]methionine, hepatocytes were washed twice with methionine-free minimal essential medium containing 0.3 m sucrose and incubated in medium containing 0.022 mM MTP inhibitor in the same buffer for 2 h to deplete the intracellular methionine pool. Next, the cells were incubated with methionine-free minimal essential medium containing [35S]methionine (100 µCi/ml), 0.375 mM oleate, and 10% bovine serum albumin in the presence or absence of MTP inhibitor. For labeling of TG, hepatocytes were preincubated with DME (no oleate) + MTP inhibitor for 2 h followed by addition of [3H]oleate (5 or 10 µCi/ml).

**Isolation of Microsomal Lumenal Contents**—Cells were collected then homogenized in buffer containing 250 mM sucrose and 300 mM imidazole (pH 7.4) using a hand-operated Dounce homogenizer (37). Cell extracts were centrifuged for 10 min in a microcentrifuge at 14,000 rpm. Lumenal contents were released from microsomes in the supernatant by treatment with 0.1 M sodium carbonate (pH 11) for 25 min at 22 °C, which results in lumenal albumin (5). The samples were centrifuged at 34,000 rpm in a Beckman SW55 rotor for 90 min at 21 °C to separate microsomal membranes (pellet) from lumenal contents (supernatant). Lumenal contents were adjusted to pH 7.4 by addition of 10% acetic acid.

**Immunoprecipitation of ApoB from Cells, Culture Medium, and Lumenal Contents**—For immunoprecipitation of cellular apoB, hepatocytes were washed with phosphate-buffered saline and then homogenized in solubilization buffer containing Tris-HCl (0.63 M, pH 7.4), NaCl (0.75 M), EDTA (25 mM), phenylmethylsulfonyl fluoride (5 mM), and Triton X-100 (5%, v/v). Cellular extracts were centrifuged in a microcentrifuge at 14,000 rpm for 10 min, then the supernatant (1 ml) was incubated overnight at 4 °C with anti-human apoB antibody (7.5 µl). Next, 45 µg of protein A-Sepharose was added and the sample was mixed end-over-end for 2 h at 4 °C. The protein-A–protein A complex was pelleted by centrifugation for 2 min at 14,000 rpm. For immunoprecipitation of secreted apoB, culture medium was centrifuged for 2 min at 3,000 rpm to remove cell debris prior to immunoprecipitation of apoB as described above. For immunoprecipitation of apoB-containing lipoproteins from microsomal lumenal contents under nondenaturing conditions, the lumenal contents were washed with 0.3 M sucrose, 0.1 m KCl, 2.5 mm MgCl2, 1 m sodium-free EDTA, and 10 mM Pipes (pH 6.8). The hepatocytes were washed 3 times in DMEM, then suspended in DMEM containing 10% fetal bovine serum. Cells were plated on collagen-coated dishes (60 or 100 mm, 1 × 106 cells/ml). Cell viability (typically >90%) was estimated by trypan blue exclusion. After hepatocytes had attached to the dish (2 to 3 h), fresh DMEM containing 10% fetal bovine serum was added and the cells were incubated overnight. The next morning, the medium was replaced with fresh serum-free medium containing 0.375 mM oleate and 10% bovine serum albumin.

**Density Gradient Separation of Lipoproteins in Culture Medium and Microsomal Lumenal Contents**—Lipoproteins in the microsomal lumen or culture medium were separated according to density by ultracentrifugation on a sucrose gradient. The sucrose concentration of the samples (5 ml) was adjusted to 12.5% (w/v), the samples were then placed on the top of a step gradient consisting of 2 ml of 49% sucrose and 2 ml of 20% sucrose. The tube was filled with phosphate-buffered saline. All solutions contained a mixture of protase inhibitors. The tubes were ultracentrifuged in a Beckman SW40 rotor at 35,000 rpm for 60 h at 12 °C (39). Eleven or 12 1-ml fractions with densities ranging from 1.006 to 1.21 g/ml were sequentially removed from the top of the tube. The density of each fraction was determined as the weight of 1 ml.
was incubated for 10 min on ice. Proteolysis was terminated by addition of soybean trypsin inhibitor (1 mg/ml), phenylmethylsulfonyl fluoride (1 mM), and leupeptin (0.1 mM) for 10 min on ice. Confirmation that trypsin had degraded proteins exposed on the cytosolic surface of ER membranes was obtained by analysis of the trypsin sensitivity of two microsomal membrane proteins whose active sites are known to be exposed to the cytosol: phosphatidylethanolamine-N-methyltransferase (41) and CDP-choline:diacylglycerol cholinephosphotransferase (41). Analysis of Radiolabeled TG Associated with ApoB in Culture Medium, Lumenal Contents, and Cytosol—Lipids were extracted by the method of Folch et al. (42). Standard TG was added to each sample and solvents were evaporated under a stream of nitrogen. The lipids were separated by thin-layer chromatography in the solvent system hexane:isopropyl ether:acetic acid, 60:40:4 (v/v/v). The band corresponding to authentic TG was visualized by exposure to iodine vapor.

**Isolation and Quantitation of Lumenal TG Not Associated with ApoB**—Hepatocytes were harvested from one dish and homogenized in 1 ml of buffer containing 300 mM imidazole and 250 mM sucrose (pH 7.4) using a hand-held Dounce homogenizer. The homogenate was centrifuged in a microcentrifuge for 10 min at 14,000 rpm. KCl (final concentration of 0.5 M) was added to the supernatant containing microsomes to release any cytosolic TG associated with membranes. The microsomes were isolated by centrifugation of the homogenate for 90 min at 34,000 rpm in a Beckman SW55 rotor at 21 °C and then washed with phosphate-buffered saline. Microsomal lumenal contents were released by sodium carbonate extraction (38) and apoB was immunoprecipitated under nondenaturing conditions, as described above. Immunoblotting confirmed that the supernatant was devoid of apoB. Lipids were extracted from the supernatant and TG was isolated by thin-layer chromatography in the solvent system hexane:isopropyl ether:acetic acid, 60:40:4 (v/v/v). The band corresponding to authentic TG was visualized by exposure to iodine vapor and scraped from the plate.

**Other Methods**—The protein content of samples was determined using the BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as standard.

**RESULTS**

Secretion of Triacylglycerols from Murine Primary Hepatocytes Is Decreased by the MTP Inhibitor, BMS-197636—We investigated the requirement of MTP for assembly of apoB-containing lipoproteins in monolayer cultures of murine primary hepatocytes using the MTP inhibitor BMS-197636. This inhibitor has previously been shown to be a potent (IC50 = 36 nM) inhibitor of the in vitro TG transfer activity of MTP (14). Nanomolar concentrations of BMS-197636 have also been shown to decrease apoB secretion (within 10 min of incubation) from human HepG2 hepatoma cells (14), and TG secretion from McArdle 7777 rat hepatoma cells (25). To establish the effectiveness of this inhibitor in blocking TG secretion from murine hepatocytes, we determined the concentration dependence of TG secretion on the inhibitor. Hepatocytes were incubated for 2 h in the presence of various concentrations of inhibitor (0–10 μM). [3H]Oleate (0.375 mM) was then added for 4 h in medium containing the same concentration of inhibitor. The MTP inhibitor reduced [3H]TG secretion in a dose-dependent manner (Fig. 1). At an inhibitor concentration of 10 μM, TG secretion was decreased by 85%. We therefore used this concentration of inhibitor for all experiments unless otherwise noted. The concentration of inhibitor required to inhibit TG secretion by 50% (~0.5 μM) was approximately an order of magnitude greater than in McArdle hepatoma cells (25). The reason why the inhibitor was less effective in primary murine hepatocytes than in hepatoma cells is not clear but possible explanations include a less efficient uptake of the drug into primary hepatocytes and/or a different metabolism of the inhibitor in primary hepatocytes compared with hepatoma cells. Control experiments were routinely performed to confirm that secretion of radiolabeled TG was inhibited by ~85% in the presence of 10 μM inhibitor.

**The Amount of Newly Synthesized Lumenal ApoB100, but Not ApoB48, Was Greatly Reduced by the MTP Inhibitor**—From previously reported studies, the translocation of apoB100 into the ER lumen appears to be more sensitive to inhibition of MTP activity than apoB48 (16, 34, 35). We, therefore, determined whether or not inhibition of MTP reduced the amount of apoB100 and apoB48 in the microsomal lumen of murine hepatocytes. Synthesis of an apoB100 molecule takes ~10 min, and ~30 min later apoB100 is secreted (data not shown). Hepatocytes were incubated for 2 h without or with MTP inhibitor, after which [35S]methionine was added for 15 or 30 min to label apoB. The cells were harvested, then permeabilized with digitonin under conditions for which ~85% of the cytosolic protein lactate dehydrogenase was released from the cells. Next, trypsin was added to digest proteins exposed to the cytosol. Using this experimental protocol, apoB molecules that had translocated across the ER membrane, and were either located completely within the lumen or were associated with the luminal surface of the ER membrane, would be expected to be protected from trypsin digestion (43). In control experiments we confirmed that trypsin treatment digested the ER membrane proteins exposed to the cytosol. For example, under the same conditions the activities of phosphatidylethanolamine-N-methyltransferase and CDP-choline:diacylglycerol cholinephosphotransferase, whose active sites are on the cytosolic side of the ER membrane (41), were reduced by >90% (data not shown).

ApoB100 and apoB48 were immunoprecipitated from cellular material. Fig. 2 shows that after 15 min of labeling, the amount of lumenal [35S]apoB48 was not significantly altered by the MTP inhibitor, whereas the amount of radiolabeled apoB100 was ~35% less in inhibitor-treated cells than in control cells (Fig. 2). After 30 min of radiolabeling, the amount of [35S]apoB100 that was protected from trypsin digestion was ~85% less in inhibitor-treated cells than in control cells (Fig. 2). In contrast, [35S]apoB48 was equally well protected from trypsin in inhibitor-treated and control cells.

**Inhibition of MTP Impairs ApoB Lipidation**—To investigate whether or not inhibition of MTP prevents addition of lipids to apoB, hepatocytes were incubated ~2 h with the indicated concentrations of MTP inhibitor (MTPI), followed by addition of 5 μCi/ml [3H]oleate (0.375 mM) for 4 h in the presence of the same concentration of inhibitor. [3H]TG was isolated from the culture medium. Data are average ± S.D. of three independent experiments. One error bar is too small to be visible.
worthy that in the previous experiment (Fig. 2) some apoB100 was protected from trypsin and was present in the microsomal lumen of inhibitor-treated hepatocytes. However, Fig. 3 shows that none of this lumenal apoB100 was present in buoyant lipoprotein particles. A possible explanation for this apparent paradox is that a portion of newly synthesized apoB100 is translocated across the ER membrane but remains membrane-associated and is not lipidated to form lipoprotein particles.
In contrast to apoB100, the amount of apoB48 present in the lumen was only slightly affected by the MTP inhibitor (Figs. 2 and 3). However, the density distribution of luminal apoB48 in inhibitor-treated cells was clearly different from that in control cells (Fig. 3). Whereas in control cells apoB48 was present in fractions with a wide range of densities (i.e. in both lipid-rich and lipid-poor lipoproteins), in cells treated with MTP inhibitor most apoB48 was associated with lipid-poor particles (fractions 1–4).

To confirm that inhibition of MTP impaired apoB lipidation, we compared the amount of microsomal luminal TG associated with apoB in hepatocytes incubated ± MTP inhibitor. Lipids of control and inhibitor-treated hepatocytes were labeled with [3H]oleate for 30 min. Microsomes were isolated in the presence of 0.5 M KCl, then luminal contents were released by treatment with sodium carbonate. ApoB was immunoprecipitated from the luminal contents under nondenaturing conditions so that lipids remained associated with apoB (44). We confirmed that immunoprecipitation of apoB was complete by immunoblotting proteins remaining in the supernatant after the immunoprecipitation using an anti-apoB antibody; no apoB was detected in the supernatant (data not shown) indicating that the immunoprecipitation was quantitative. ApoB-associated lipids were extracted from the immunoprecipitate and TG was isolated. In luminal contents of inhibitor-treated cells, 40% less [3H]TG was associated with apoB than in control cells (Fig. 4A, left-hand side).

**Inhibition of MTP Reduces the Amount of Luminal TG Not Associated with ApoB—Ultrastructural analyses have suggested that the lumen of the secretory pathway of enterocytes contains a pool of TG that is not associated with apoB (18). Moreover, Raabe et al. (16) detected VLDL-sized particles within the secretory pathway of livers from MTP−/− mice, but noted that these “droplets” were severely depleted in MTP−/− mice. These studies suggested that MTP might play a role in transferring TG into the ER lumen for VLDL assembly. We, therefore, attempted to isolate TG that was not associated with apoB from the microsomal lumen of hepatocytes. We also asked the question: does inhibition of MTP decrease the amount of TG in this luminal TG pool? Hepatocytes were incubated ± MTP inhibitor for 2 h, then [3H]oleate was added for 30 min to label TG. Next, cells were homogenized and 0.5 M KCl was added to release cytosolic TG adhering to microsomal membranes. Microsomes were isolated and apoB was quantitatively immunoprecipitated from the luminal contents under nondenaturing conditions. Lipids were extracted from the immunoprecipitate and from the supernatant remaining after the immunoprecipitation. The amount of luminal [3H]TG not associated with apoB was ~3-fold greater than that associated with apoB (Fig. 4B). The MTP inhibitor decreased the amount of apoB-free TG in the microsomal lumen by ~45% (Fig. 4A, right-hand side), indicating that MTP participates in the accumulation of this pool of TG. The amount of [3H]TG associated with apoB in the lumen was similarly reduced by ~40% (Fig. 4A). The MTP inhibitor reduced the amount of luminal apoB-free [3H]TG in a dose-dependent manner with maximum reduction being achieved with an inhibitor concentration of 10 μM. The secretion of [3H]TG was similarly inhibited in a dose-dependent fashion (Fig. 1) although the maximum inhibition of [3H]TG secretion was greater than for the decrease in the luminal pool of apoB-free [3H]TG (Fig. 4).

One concern in isolating a luminal TG pool is that some TG from the cytosolic storage pool might have been associated with the isolated microsomes and consequently might have contaminated the luminal TG pool. To avoid this problem, we washed microsomes with 0.5 M KCl to remove any lipid droplets that might have adhered to the cytosolic surface of the membranes. Next, we released the luminal contents and separated the luminal contents from membranes by ultracentrifugation. In a control experiment, cytosol was isolated from hepatocytes that had been incubated for 30 min with [3H]oleate (10 μCi/ml) to label TG, then this cytosolic [3H]TG was mixed with microsomes that had been isolated from unlabeled hepatocytes. We washed the microsomes with 0.5 M KCl to remove adhering cytosolic TG from the membranes and reisolated the microsomes. Luminal contents were released and TG was extracted from the luminal contents. Of the 32,000 dpm/dish of cytosolic [3H]TG added, only 140 dpm of [3H]TG/dish were associated with the luminal contents. In contrast, in a parallel experiment in which hepatocytes were incubated for 30 min with 10...

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**Fig. 4.** Amounts of apoB-associated and apoB-free [3H]TG in the microsomal lumen are reduced by inhibition of MTP. Hepatocytes were incubated with or without MTP inhibitor (10 μM) for 2 h followed by addition of [3H]oleate (10 μCi/ml) ± inhibitor for 30 min. Cells were homogenized and then microsomes were isolated in the presence of 0.5 M KCl to remove adhering cytosolic TG. Microsomal luminal contents were released by sodium carbonate extraction and separated from membranes by centrifugation. ApoB-containing lipoproteins were immunoprecipitated from luminal contents under nondenaturing conditions. Lipids were extracted from the immunoprecipitate and the [3H]TG content was measured (apoB-associated TG). Lipids were also extracted from the supernatant (devoid of apoB) and the apoB-free [3H]TG content was measured. Panel A, the amount of [3H]TG is expressed as a % of that in cells incubated without inhibitor. Data are averages ± S.D. of three experiments each performed in duplicate. Open bars, no inhibitor; filled bars, + inhibitor. Panel B, the amount of microsomal luminal [3H]TG associated with, and independent of, apoB was determined in the absence of MTP inhibitor. Data are from one experiment representative of three similar experiments performed in duplicate.
\[ \text{Inhibition of MTP does not decrease TG transfer to partially lipidated apoB.} \]

Hepatocytes were incubated for 2 h with brefeldin A (BFA, 10 \( \mu \text{M} \)), after which \([\text{3H}]\text{oleate (10 } \mu\text{Ci/ml)}\) was added for 30 min in the presence of BFA. Fifteen min after addition of the radiolabel, MTP inhibitor (MTPI, 10 \( \mu\text{M} \)) was added to half the dishes. At the end of the 30-min labeling period, fresh medium containing 0.375 \( \mu\text{M unlabeled oleate, 10 } \mu\text{M cycloheximide (CHX, to inhibit apoB synthesis), } \pm \text{MTP inhibitor (10 } \mu\text{M) was added and the incubation was continued for an additional 4 h. Panel A, summary of the time course of the pulse-chase experiment. Panel B, culture medium was collected, applied to a sucrose density gradient, and ultracentrifuged for 60 h. Samples of 3 densities were collected: fraction I density, \(-1.02 \text{ g/ml; fraction II density, } 1.02-1.07 \text{ g/ml; fraction III density, } 1.07-1.21 \text{ g/ml.}\) Lipids were extracted from each fraction and TG was isolated. Data are averages \( \pm \) S.D. of three independent experiments. Open bars, no inhibitor; closed bars, \( \pm \) inhibitor.

\[ \text{\( \mu \text{Ci/ml } [\text{3H}]\text{oleate, 3,500 dpm/dish were present in TG in the microsomal lumen. Thus, less than 5\% of the TG in the isolated lumenal TG originated from cytosolic contamination.}} \]

Additional evidence that this lumenal TG pool did not originate from contamination by cytosolic TG was provided by a control experiment in which hepatocytes were pulse-labeled with \([\text{3H}]\text{oleate for 1 h, then incubated for 4 h with } 0.375 \mu\text{M unlabeled oleate.}\) Whereas the inhibitor decreased the lumenal apoB-free \([\text{3H}]\text{TG pool by } \sim45\%, \text{ the amount of cytosolic [3H]TG was increased by } \sim10\% (\text{data not shown}). Therefore, the finding that the labeling pattern of the lumenal TG pool did not reflect that of the cytosolic TG pool provides further evidence that the lumenal TG pool was not the result of contamination by cytosolic TG.

**Inhibition of MTP Does Not Disrupt TG Transfer to Partially Lipidated ApoB**—We next determined the requirement of MTP for TG transfer from the lumenal apoB-independent pool to apoB using the pulse-chase protocol outlined in Fig. 5A. First, hepatocytes were incubated with brefeldin A for 2 h to inhibit reversibly the assembly of VLDLs and cause an accumulation of partially lipidated apoB particles in the microsomal lumen (39). The hepatocytes were then pulse-labeled with \([\text{3H}]\text{oleate for 30 min in the presence of brefeldin A so that radiolabeled TG was transported into the ER lumen but the assembly and secretion of fully lipidated apoB-containing particles remained blocked by brefeldin A (39). We confirmed that under these conditions of brefeldin A treatment, lumenal apoB was present only in lipid-poor particles (data not shown), whereas in the absence of brefeldin A the density distribution of lumenal apoB was as shown in Fig. 3A (left panel). Fifteen minutes after addition of radiolabel (15 min is required before the inhibitor becomes fully active (25)) the MTP inhibitor was added to half the dishes. This experimental protocol results in generation of a pool of lumenal \([\text{3H}]\text{TG not associated with apoB, as well as a lumenal pool of poorly lipidated apoB. After the 30-min radiolabeling period the medium was removed and chase medium, lacking brefeldin A but containing unlabeled oleate and cycloheximide } \pm \text{MTP inhibitor, was added for 4 h. Cycloheximide prevented the entry of any additional newly synthesized apoB into the microsomal lumen (data not shown). Upon removal of brefeldin A, VLDL assembly resumed from the apoB and TG that had accumulated in the lumen. Culture medium was then subjected to density gradient ultracentrifugation to separate secreted lipoproteins into 3 fractions: fraction I density was } \sim1.02 \text{ g/ml; fraction II density was } 1.02-1.07 \text{ g/ml; fraction III density was } 1.07-1.21 \text{ g/ml.} \text{ The amount of } [\text{3H}]\text{TG in the fraction of lowest density (fraction I) was the same in the presence and absence of inhibitor (Fig. 5B). The amount of } [\text{3H}]\text{TG in fraction II was only slightly reduced by inhibition of MTP. The amount of } [\text{3H}]\text{TG associated with lipoproteins in the fraction of the highest density (fraction III) was reduced by 32\% by inhibition of MTP (Fig. 5B). Because the MTP inhibitor was not added until after TG and apoB had independently accumulated in the ER lumen, this experiment demonstrates that inhibition of MTP does not block the transfer of lumenal TG to partially lipidated apoB-containing lipoproteins for VLDL formation.}

**Secretion of ApoB48 Is Less Sensitive to Inhibition of MTP Than Is Secretion of ApoB100**—We next compared the requirement of MTP for the secretion of apoB48 and apoB100. Murine hepatocytes were incubated with MTP inhibitor for 2 h after which \([35\text{S}]\text{methionine was added to inhibitor for 4 h. Fig. 6 shows that 1 } \mu\text{M MTP inhibitor reduced the amount of secreted } [35\text{S}]\text{apoB100 by } \sim70\%, \text{ whereas the amount of } [35\text{S}]\text{apoB48 was decreased by } \sim30\% \text{ and apoB100 secretion was barely detectable. Thus, apoB48 secretion was partially inhibited by a higher dose of MTP inhibitor. However, the secretion of apoB100 was markedly more sensitive to inhibition of MTP than was apoB48.}

To determine whether lipidation of secreted apoB were reduced by inhibition of MTP, hepatocytes were pulse labeled for 4 h with \([35\text{S}]\text{methionine } \pm \text{MTP inhibitor. Lipoproteins of different densities were isolated from the medium by density gradient ultracentrifugation and apoB was immunoprecipitated. ApoB100 was secreted from control hepatocytes primarily as lipid-rich particles (Fig. 7) but was not detected in denser particles. As expected from Figs. 2, 3, and 6, almost no apoB100 was secreted by inhibitor-treated hepatocytes. In contrast, apoB48 secreted by control cells was associated with both lipid-rich and lipid-poor particles, and the density distribution of apoB48 secreted from inhibitor-treated hepatocytes was markedly different from that of control cells (Fig. 7). The amount of apoB48 in lipid-rich particles secreted by inhibitor-treated hepatocytes (fractions 9–12) was very low compared with that of control cells. Moreover, the amount of apoB48 in higher density particles (fractions 1–5) was increased compared with that from control cells (Fig. 7). These data show that apoB100 secretion was almost entirely abolished by the MTP inhibitor, whereas the amount of apoB48 secreted was only slightly affected. However, the apoB48 particles secreted in the presence of the inhibitor were lipid-poor compared with those secreted by control hepatocytes.
Inhibition of ApoB100 Secretion by the MTP Inhibitor Is Independent of the LDL Receptor—Twisk and co-workers (45) have proposed that the net output of apoB from hepatocytes is decreased by two different mechanisms that involve the binding of apoB to the LDL receptor (LDLR). First, because apoB and LDLRs both traverse the secretory pathway, apoB can bind to LDLRs within the secretory route and be targeted for degradation. Second, immediately upon exit from the cell, nascent apoB-containing lipoproteins can be captured by LDLRs on the cell surface, endocytosed, and targeted for lysosomal degradation. Because apoB100 contains the principal LDLR-binding domain, which is absent from apoB48, the secretion of apoB100 is influenced by the presence of LDLRs to a much greater extent than is apoB48 (45).
expect that (i) more apoB100 would be secreted from LDLR would be secreted. If this sequence of events occurred we would almost normal number of apoB48 particles, albeit lipid-poor, from MTP inhibitor-treated cells would be reduced, whereas an domain, apoB48 particles would be much less likely to be re-formed in the presence of MTP inhibitor, these par-

We reasoned that even if a normal number of apoB100 particles had been formed in the presence of MTP inhibitor, these par-

In light of these findings we considered the possibility that the greater sensitivity of apoB100, compared with apoB48, to inhibition of MTP might be because of the presence of LDLRs. We reasoned that even if a normal number of apoB100 particles had been formed in the presence of MTP inhibitor, these particles might be defective (e.g. lipid-poor) and therefore might be unstable in the aqueous environment of the ER lumen. Consequently, these particles might bind to LDLRs, either within the secretory pathway or immediately after secretion, and be degraded. In contrast, because apoB48 lacks the LDLR-binding domain, apoB48 particles would be much less likely to be removed by the LDLR. As a result, the net secretion of apoB100 from MTP inhibitor-treated cells would be reduced, whereas an almost normal number of apoB48 particles, albeit lipid-poor, would be secreted. If this sequence of events occurred we would expect that (i) more apoB100 would be secreted from LDLR−/− cells than from LDLR+/− cells, and (ii) inhibition of MTP would not reduce apoB100 secretion from LDLR−/− hepatocytes if the binding of apoB100 to the LDLR occurred prior to the action of MTP. To test this hypothesis, we incubated hepatocytes from LDLR−/− and LDLR+/− mice for 2 h with MTP inhibitor (MTPI, 10 μM). Next, proteins were labeled for 4 h with 100 μCi/ml [35S]methionine ± MTP inhibitor, medium was collected, and apoB was immunoprecipitated. ApoB was analyzed by polyacrylamide gel electrophoresis and fluorography and the relative amounts of apoB100 (upper panel) and apoB48 (lower panel) were compared by densitometric scanning of the gels. Data are averages ± S.D. from duplicate samples of three independent experiments.

In contrast, another study using hepatocytes from a different line of liver-specific MTP-deficient mice, the secretion of both apoB100 and apoB48 was undetectable (17). The reason for the different results obtained from these two lines of genetically modified mice is not clear. Small amounts of apoB, particularly apoB48 have been detected in the serum of individuals with abetalipoproteinemia (20, 46).

The finding that some newly synthesized apoB100 was translocated across the ER membrane and protected from trypsin digestion in the presence of the inhibitor (Fig. 2), particularly after the 15-min labeling period, might be explained by residual MTP activity. Alternatively, because no buoyant apoB100-containing lipoproteins were detected in the lumen of inhibitor-treated cells (Fig. 3), the protease-protected apoB100 might represent apoB100 that is associated with the luminal surface of the ER membrane because the protease protection assay does not distinguish between apoB that is associated with the luminal leaflet of the membrane and apoB that has been released into the lumen.

Our data using hepatocytes from LDLR−/− and LDLR+/+ mice show that the preferential reduction in secretion of apoB100, compared with apoB48, upon inhibition of MTP cannot be attributed to LDLRs. An alternative explanation might be that apoB100 is preferentially degraded either during, or shortly after, its translocation into the lumen because apoB that is misfolded and/or underlipidated is rapidly degraded by the ubiquitin-proteasome pathway (47, 48). We speculate that because of its large size, apoB100 requires more lipids to attain a stable conformation than does the smaller apoB48. Thus, if lipid availability were reduced by the MTP inhibitor, apoB100 might preferentially be targeted for degradation. We attempted, unsuccessfully, to test this hypothesis by incubating hepatocytes with the proteasome inhibitors lactacystin, N-acetyl-Leu-Leu-norleucinal, or MG132. However, although
these inhibitors have been successfully used in other cell types, the viability of the murine hepatocytes was compromised by equivalent doses of the inhibitors (data not shown).

**MTP Is Required for Accumulation of Lumenal ApoB-free TG as Well as for ApoB Lipidation**—To our knowledge, our study is the first report of isolation of an apoB-free pool of TG from the microsomal lumen of hepatocytes. We consider it unlikely that this pool of TG is the result of contamination by the cytosolic TG pool for the following reasons. (i) Before the lumenal contents were isolated, microsomes were thoroughly washed with 0.5 M KCl, which removes loosely bound proteins and lipid droplets adhering to membranes. (ii) When hepatocytes were incubated with the MTP inhibitor, the amount of cytosolic [³H]TG was unaltered, whereas the amount of apoB-free [³H]TG in the lumenal contents was decreased by 45%. Thus, the pattern of labeling of the lumenal TG did not reflect that of cytosolic TG (3). When cytosolic [³H]TG was mixed with unlabeled microsomes, the amount of [³H]TG in the lumenal apoB-independent pool was only 4% of that found when this TG pool was isolated from radiolabeled cells. Our isolation of the pool of apoB-free TG from the microsomal lumen is consistent with an ultrastructural analysis of mice that were unable to synthesize apoB in the intestine (18). In these mice, chylomicon-sized, lipid-staining particles (that did not contain apoB) accumulated in the ER lumen of enterocytes. In another study (49), lipid-staining particles, the size of VLDLs but lacking immunoreactive apoB, were detected in the lumen of the rough ER of rat hepatocytes. From these studies, a model for VLDL assembly has been proposed in which a TG droplet in the rough ER lumen fuses with a small apoB-containing particle at the junction of rough and smooth ER (49).

A clue to the function of MTP is our observation that the MTP inhibitor decreased by 45% the amount of microsomal lumenal TG not associated with apoB. This finding, combined with our observation that the MTP inhibitor reduced the amount of TG associated with apoB by 40%, supports the electron microscopy studies of Raabe et al. (16) in which disruption of the MTP gene in liver essentially eliminated VLDL-sized particles from the ER and Golgi lumina. Thus, our data suggest that an important function of MTP is to mediate TG accumulation in the ER lumen in a pool not associated with apoB. In light of published data (50–53), it seems likely that this TG is derived primarily from the cytosolic TG pool.

Our experiments do not distinguish between the apoB-independent TG being loosely associated with the luminal surface of the membrane (such a pool would have be released upon sodium carbonate treatment) or floating freely in the lumen. Our laboratory has previously concluded that VLDLs are completely assembled with their full complement of lipid within the rough ER of rat hepatocytes (54). Analysis of the apoB structure suggests that apoB contains a “lipid pocket” that expands as lipids are added (55). Based on evidence that MTP transfers TG between membranes via a shuttle mechanism (13), one possibility is that a sequential addition of TG to apoB occurs on the lumenal surface of the ER membrane. Our observation that apoB48 is distributed among lipoprotein particles with a wide range of densities in the microsomal lumen (Fig. 3) and in the medium (Fig. 7) is also consistent with a sequential addition of TG to apoB. Alternatively, the wide spectrum of densities of apoB48 particles might be the consequence of lipid-poor apoB particles “fusing” with TG droplets of different sizes.

The mechanism by which MTP regulates the supply of TG within the ER lumen for lipoprotein assembly remains to be determined. Mobilization of cytosolic TG via a lipolysis re-esterification cycle is thought to provide the majority (−70%) of TG that is assembled with apoB (50, 51) but the enzymes involved in this lipolysis-esterification cycle have not yet been fully characterized. A microsomal TG hydrolase has been recently implicated in this TG lipolysis re-esterification cycle (52, 53, 56). Interestingly, mice with targeted disruption of the diacylglycerol acyltransferase-1 gene have normal plasma TG levels (57), indicating that a different TG-synthesizing enzyme makes the TG utilized for VLDL secretion. A candidate enzyme is diacylglycerol acyltransferase-2, which is expressed primarily in liver and adipose. The gene encoding this enzyme has recently been identified (58) but its requirement for providing TG for VLDL secretion has not yet been established.

In conclusion, our data demonstrate that MTP is required for TG accumulation within the ER lumen, as well as for apoB lipidation. However, in agreement with studies in hepatoma cells (2), MTP does not appear to transfer the majority of TG to apoB. We, therefore, propose a model (Fig. 9) for the role of MTP in VLDL assembly that incorporates our new findings. When MTP is inhibited, the accumulation of apoB-independent TG in the ER lumen is compromised, resulting in the availability of less TG for assembly with apoB. We speculate that the decreased lipidation of apoB induced by inhibition of MTP is a consequence of the decreased availability of TG in the apoB-independent luminal TG pool.

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