

The Low Density Lipoprotein Receptor Prevents Secretion of Dense ApoB100-containing Lipoproteins from the Liver*

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The assembly and secretion of very low density lipoproteins (VLDL) require microsomal triglyceride transfer protein (MTP). Recent evidence also suggests a role for the low density lipoprotein (LDL) receptor in this process. However, the relative importance of MTP in the two steps of VLDL assembly and the specific role of the LDL receptor still remain unclear. To further investigate the role of MTP and the LDL receptor in VLDL assembly, we bred mice harboring “floxed” *Mttp* alleles (*Mttp*^{lox/lox}) and a *Cre* transgene on a low-density lipoprotein receptor-deficient background to generate mice with double deficiency in the liver (*Ldlr*^{-/-} *Mttp*^{ΔΔ}). In contrast to the plasma of *Ldlr*^{+/+} *Mttp*^{ΔΔ} mice, the plasma of *Ldlr*^{-/-} *Mttp*^{ΔΔ} mice contained apoB100. Accordingly, *Ldlr*^{-/-} *Mttp*^{ΔΔ} but not *Ldlr*^{+/+} *Mttp*^{ΔΔ} hepatocytes secreted apoB100-containing lipoprotein particles. The secreted lipoproteins were of LDL and HDL sizes but no VLDL-sized lipoproteins could be detected. These findings indicate that hepatic LDL receptors function as “gatekeepers” targeting dense apoB100-containing lipoproteins for degradation. In addition, these results suggest that very low levels of MTP are insufficient to mediate the second step but sufficient for the first step of VLDL assembly.

The hepatic assembly of very low density lipoproteins (VLDL)¹ is usually described in two well defined steps (1). The first step consists of the formation of a small pre-VLDL particle and takes place in the rough endoplasmic reticulum (ER). In the rough ER, the apoB protein is translated across the membrane and becomes enriched with lipids. If the apoB protein is not properly folded or if the enrichment of lipids is insufficient, the apoB-containing precursor particle will be degraded instead of proceeding to the second step. Microsomal triglyceride transfer protein (MTP) is believed to be crucial for the first step of VLDL assembly because it transfers lipids and also binds to apoB (2–4). Through these actions, MTP is believed to avoid improper folding and premature degradation of apoB (5, 6). A vital role of MTP in the formation and secretion of apoB is further supported by the fact that patients with abetalipoproteinemia, a dominant disorder caused by mutations in the MTP gene (*Mttp*), have low levels of apoB in plasma (7). Although most evidence points toward the necessity of MTP in the first step of VLDL assembly, some studies indicate that the translation of apoB and the formation of the pre-VLDL particle can occur in the absence of normal levels of MTP (1, 8, 9).

In contrast, there is less controversy over the necessity of functional MTP to fulfill the second step of VLDL assembly (1). In this step, an apoB-free lipid particle is synthesized in the smooth ER (MTP-dependent) and fuses with the apoB-containing precursor particle synthesized in the rough ER to form a nascent VLDL particle (MTP-independent) (6, 9–12). The nascent VLDL will be further enriched with lipids in the Golgi apparatus to form a buoyant VLDL particle that will eventually be secreted.

The low-density lipoprotein (LDL) receptor is a key receptor for the endocytosis of circulating VLDL and LDL. The dominantly inherited disorder familial hypercholesterolemia is primarily caused by a dysfunctional LDL receptor, which leads to the accumulation of cholesterol-rich LDL in plasma and premature atherosclerosis (13). The inhibition of intracellular cholesterol synthesis with 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (e.g. with a statins) leads to a compensatory up-regulation of primarily hepatic LDL receptors that increases the uptake of cholesterol-rich LDL particles, lowering plasma cholesterol levels. It has also been suggested that the LDL receptor is involved in the hepatic VLDL assembly by interfering with secretion of VLDL from the liver (13–16).

Most studies of the role of MTP in VLDL assembly have used MTP inhibitors in cultures of immortalized or primary hepatocytes (1, 17) or MTP-negative cells lines (18–20). *In vivo* studies of MTP deficiency have been hampered because inactivation of *Mttp* in mice is embryonically lethal (21). Recently, the *loxP* system (22) was used to create conditional targeting of *Mttp* specifically in the liver (9, 23). These mice were unable to form buoyant VLDL in the liver, which is consistent with the idea that MTP is necessary for the second step of VLDL assembly. However, metabolic labeling studies performed in one of these studies (9) showed that primary hepatocytes from these mice secreted HDL-sized lipoproteins containing apoB48 but not apoB100. We hypothesized that the absence of secretion of small apoB100-containing lipoproteins from the livers of liver-specific *Mttp* knockout mice is due to the presence of hepatic LDL receptors. To test this hypothesis, we conducted plasma lipid, lipoprotein, and metabolic-labeling analyses of liver-specific *Mttp* knockout mice (9) bred onto an *Ldlr*-deficient (*Ldlr*^{-/-}) background (24).

EXPERIMENTAL PROCEDURES

Mouse Models—Mx1-*Cre* transgenic mice (22) containing a conditional *Mttp* allele in which exon 1 of *Mttp* is flanked by *loxP* sites (9) (*Mttp*^{lox/lox} Mx1-*Cre*) were bred with *Ldlr*^{-/-} mice (24) to generate *Ldlr*^{-/-} *Mttp*^{lox/lox} Mx1-*Cre* mice and *Ldlr*^{+/+} *Mttp*^{lox/lox} Mx1-*Cre* mice.

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¹ The abbreviations used are: VLDL, very low density lipoproteins; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; LDL, low density lipoprotein; pI-pC, polyinosinic-polycytidylic ribonucleic acid; PBS, phosphate-buffered saline.

At 21–28 days of age, male *Ldlr*^{-/-} *Mtpp*^{lox/lox} *Mx1-Cre* and *Ldlr*^{+/+} *Mtpp*^{lox/lox} *Mx1-Cre* mice were injected with 500 μ l of polyinosinic-polycytidylic ribonucleic acid (pI-pC) (1 μ g/ μ l, Sigma) every other day for 8 days to delete *Mtpp* (*Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} and *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ}). Littermate *Ldlr*^{-/-} *Mtpp*^{lox/lox} mice and *Ldlr*^{+/+} *Mtpp*^{lox/lox} mice lacking the *Cre* transgene were also injected with pI-pC. The mice had a mixed genetic background (~25% 129/SvJae and ~75% C57BL/6). They were housed in a pathogen-free barrier facility with a 12-h light/12-h dark cycle and were fed rodent chow containing 4.0% fat (Lactamin AB, Kimstad, Sweden). Genotypes were determined by PCR with genomic DNA from tail biopsies.

Lipid Measurements—The mice were fasted overnight before blood sampling. Total plasma cholesterol and triglyceride concentrations were determined with colorimetric assays (INFINITY-triglyceride/cholesterol kits, Sigma) 2 weeks after the final pI-pC injection. The distribution of lipids within the plasma lipoproteins was determined by fractionating mouse plasma (100 μ l pooled from five mice) on a Superose 6 10/30 column (25). Liver pieces (~200 μ g) were homogenized with a polytron (Techtum Lab AB, Umeå, Sweden) in chloroform:methanol (2:1, v/v). Before extraction of triglycerides, a known amount of triphenyladecanoic acid (Sigma) was added to the mixture as an internal standard (26). Triglycerides were separated from other lipids by thin-layer chromatography, transesterified with methanolic HCl (Aldrich), and quantified by gas chromatography as described (26). The lipid transfer activity of MTP in primary hepatocytes was measured by using a commercial kit (Calbiochem Inc., San Diego, CA).

Preparation of ER and Golgi Sub-cellular Fractions—Hepatic ER and Golgi fractions were isolated as described (27–29). In brief, ER fractions were isolated from liver homogenates in buffer A (Tris-HCl, pH 7.4, 25 mM KCl with the addition of protease inhibitors, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 mg/ml Pefabloc SC Plus, all from Roche Applied Science) by two consecutive ultracentrifugations in SW40 Ti rotors at 14,000 rpm for 60 min at 4 °C. Golgi fractions were isolated from homogenate in buffer A isolated from the same livers. The density of the homogenates was first increased with sucrose to a final concentration of 1.07 M and overlaid with 9 + 9 ml of 0.9 M and 0.2 M sucrose in buffer A. The gradients were ultracentrifuged in SW28 Ti rotors at 16,500 rpm for 2 h. The Golgi fractions were extracted from the interface between 0.9 and 0.2 M sucrose, mixed 1:1 with 20% glycerol, and pelleted by an additional spin in SW40 Ti rotors at 28,000 rpm for 90 min. The total protein contents of all samples were determined by the method of Lowry (40).

Western Blot of ApoB—Mouse plasma samples (2 μ l), hepatic ER, and Golgi samples (150 μ g of total protein/well) were fractionated by 4% SDS-PAGE. The apoB proteins were detected with a commercial rabbit antiserum against mouse apoB (Biosite AB, Taby, Sweden). The binding of the primary antibodies was assessed with horseradish peroxidase-labeled donkey anti-rabbit antibodies and ECL Western blotting detection reagents (Amersham Biosciences). The intensity of the apoB48 and apoB100 bands was assessed by densitometry with a Fuji LAS-1000 CCD camera and Image Gauge version 3.45 software (Fuji Film, Sweden).

Real-time PCR—*Mtpp* mRNA levels in the livers of pI-pC-treated *Ldlr*^{-/-} *Mtpp*^{lox/lox} *Mx1-Cre*, *Ldlr*^{+/+} *Mtpp*^{lox/lox} *Mx1-Cre*, and *Mtpp*^{lox/lox} mice were assessed by real-time, quantitative reverse transcriptase-PCR, as described (30).

Primary Hepatocytes—Primary mouse hepatocytes were prepared and cultured essentially as described (31). In brief, mice were anesthetized, the inferior vena cava was cannulated through the right atrium with an arterial catheter (Becton-Dickinson Infusion Therapy AB, Sweden), and the portal vein was cut open for drainage. The livers were perfused with liver perfusion medium (Invitrogen) for 3 min and then with liver digest medium (Invitrogen) for 10 min. The livers were harvested, and the hepatocytes were washed twice with 40 ml of hepatocyte washing medium (Invitrogen). The number of hepatocytes was calculated by trypan blue exclusion, and a fixed number of cells was plated on six-well tissue culture plates (BD Biosciences) in Dulbecco's modified Eagle's medium lacking L-methionine and L-cysteine (Invitrogen) and containing 2.0 mM L-glutamine, 2.5 mM sodium pyruvate, 1 \times minimum Eagle's medium nonessential amino acids, and 6.5% fetal bovine serum (all from Invitrogen), and the cells were allowed to attach for 60 min.

Metabolic Labeling of Primary Hepatocytes—The attached hepatocytes were washed twice with phosphate-buffered saline (PBS), and 1 ml of fresh medium containing 50 μ l of [³⁵S]methionine/cysteine (Promix, 530 Mbq/ml, Amersham Biosciences) was added. The cells were incubated with the labeling medium for 3 h for the straight labeling experiments and for 15 min and 2 h for the pulse-chase experiments.

The labeling media was harvested, and the cells were washed twice with PBS. For the pulse-chase experiments, the cells were then chased with fresh medium lacking [³⁵S]methionine/cysteine for 15 and 30 min or for 1, 2, and 3 h. In all experiments, protease inhibitors were added immediately after the medium had been harvested. In the pulse-chase experiments, the cells (washed twice with PBS) were incubated with PBS containing 100 international units/ml Heparin (LeoPharma AB, Sweden) at 4 °C for 30 min to release cell surface-bound lipoproteins. The PBS containing heparin was harvested and supplemented with protease inhibitors. After two final PBS washes, the hepatocytes were sonicated in 1 ml of PBS containing protease inhibitors for 2–5 s using a 550W sonicator.

Isolation of Lipoproteins from Labeled Primary Hepatocytes—To obtain HDL-sized and less dense lipoproteins, 3 ml of hepatocyte medium and PBS containing heparin or cell lysate was mixed with 0.2 ml of human plasma (used as carrier), the density was increased to 1.21 g/ml with NaBr, and the mixture was ultracentrifuged in an SW40 Ti rotor at 40,000 rpm for >80 h at 10 °C (32). The top 0.5 ml of the tube containing HDL and less dense lipoproteins was collected and immediately delipidated in methanol:diethylether (1:1, v/v) (33). The delipidated proteins were resolved in 200 μ l of loading buffer containing SDS, and 15 μ l was fractionated on a 4–15% SDS-PAGE gradient. The gels were fixed in isopropanol:H₂O:acetic acid (5:13:2), incubated with Amplify (Amersham Biosciences), dried (on a Bio-Rad Model 583 gel dryer), and exposed to film (Hyperfilm, Amersham Biosciences) at -80 °C for 4–10 days. The intensity of the ³⁵S incorporated into apoB48 and apoB100 was assessed by densitometry as described above.

Density Fractionation of ApoB Secreted from Primary Hepatocytes—To separately isolate VLDL, LDL, and HDL subfractions, a density gradient consisting of 3.0 ml each of 1.065, 1.020, and 1.006 g/ml NaCl solutions was layered onto 4 ml of a mixture of 2.5 ml of hepatocyte medium, 0.5 ml of human plasma, and 1 ml of 1.42 g/ml NaBr (final density 1.10 g/ml) (33). This density gradient was ultracentrifuged in an SW40 Ti rotor at 40,000 rpm for at least 14 h at 10 °C to float Svedberg flotation >20 at the top of the gradient and LDL (Svedberg flotation 12–20) at 29 mm from the top. After isolation of the VLDL and LDL subfractions, the density of the remaining 4 ml of the bottom fraction was increased to 1.21 g/ml with NaBr and transferred to another ultracentrifuge tube for an additional run to isolate the HDL subfraction, as described above. All apolipoproteins within the density fractions were immediately delipidated and subjected to SDS-PAGE as described above.

RESULTS

Plasma and Liver Lipids—pI-pC-treated littermate mice (*Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} versus *Ldlr*^{+/+} *Mtpp*^{lox/lox}, and *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} versus *Ldlr*^{-/-} *Mtpp*^{lox/lox}) were fed a chow diet (4.0% fat) until week 7, when all mice were used for experiments (2 weeks after the final pI-pC injection). The liver-specific combination of *Mtpp* reduced the plasma concentrations of cholesterol and triglycerides (Table I). The reduction in plasma triglycerides was similar in *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} and *Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} mice, but the plasma cholesterol reduction was greater in the *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} mice (100 versus 65 mg/dl). Fast-performance liquid chromatography of plasma cholesterol revealed an LDL-cholesterol peak in *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} mice but not in *Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} mice (Fig. 1). In addition, the HDL-cholesterol peak was about 2-fold higher in *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} mice than in *Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} mice (Fig. 1). The liver weight and hepatic triglyceride content were higher in *Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} and *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} mice than in *Mtpp*^{lox/lox} controls (Table I). Of note, livers lacking the LDL receptor weighed less and had a lower triglyceride content than livers with intact LDL receptors independent of *Mtpp*.

Plasma ApoB, Liver *Mtpp* mRNA Levels, and MTP Activity—The plasma apoB48 levels were similar in *Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} and *Ldlr*^{+/+} *Mtpp*^{lox/lox} mice, whereas apoB100 was almost undetectable in the plasma of *Ldlr*^{+/+} *Mtpp* ^{Δ/Δ} mice, as judged by Western blot analysis (Fig. 2). Surprisingly, the plasma of *Ldlr*^{-/-} *Mtpp* ^{Δ/Δ} mice contained apoB100 (Fig. 2). Assessment of the apoB100 and the apoB48 bands by densitometry showed that the average plasma apoB100/B48 ratio was 0.68 ± 0.15 ($n = 8$) in *Ldlr*^{+/+} *Mtpp*^{lox/lox} mice and 1.75 ± 0.53 ($n = 8$) in

TABLE I
Basic characteristics of the study mice

Values are mean \pm S.D. TG, triglyceride; chol, cholesterol.

	<i>Ldlr</i> ^{+/+} <i>Mtpp</i> ^{flox/flox} (n = 10)	<i>Ldlr</i> ^{+/+} <i>Mtpp</i> ^{$\Delta\Delta$} (n = 10)	<i>Ldlr</i> ^{-/-} <i>Mtpp</i> ^{flox/flox} (n = 10)	<i>Ldlr</i> ^{-/-} <i>Mtpp</i> ^{$\Delta\Delta$} (n = 10)
Body weight (g)	26 \pm 2	24 \pm 2	23 \pm 2	22 \pm 2
Liver weight (g)	1.21 \pm 0.12	1.43 \pm 0.15 ^a	1.13 \pm 0.08	1.25 \pm 0.24
Liver TG (mg/g wt tissue)	216 \pm 82	648 \pm 216 ^b	161 \pm 62	318 \pm 84 ^b
Plasma TG (mg/dl)	99 \pm 26 ^c	43 \pm 15 ^{b,d}	136 \pm 24 ^e	42 \pm 24 ^{b,f}
Plasma chol (mg/dl)	119 \pm 23 ^c	54 \pm 22 ^{b,d}	174 \pm 36 ^e	73 \pm 25 ^{b,f}

^a $p < 0.05$ compared to *Ldlr*^{+/+} *Mtpp*^{flox/flox}.

^b $p < 0.005$ compared to *Ldlr*^{-/-} *Mtpp*^{flox/flox}.

^c $n = 13$.

^d $n = 19$.

^e $n = 16$.

^f $n = 14$.

FIG. 1. Distribution of cholesterol (A) and triglycerides (B) in the plasma lipoproteins of the study mice. After a 4-h fast, pooled plasma (100 μ l) from five mice of each genotype was fractionated on a fast-performance liquid chromatography column. \blacktriangle , *Ldlr*^{+/+} *Mtpp* ^{$\Delta\Delta$} ; \blacktriangle , *Ldlr*^{+/+} *Mtpp*^{flox/flox}; \bullet , *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} ; \circ , *Ldlr*^{-/-} *Mtpp*^{flox/flox}.

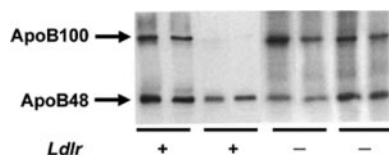
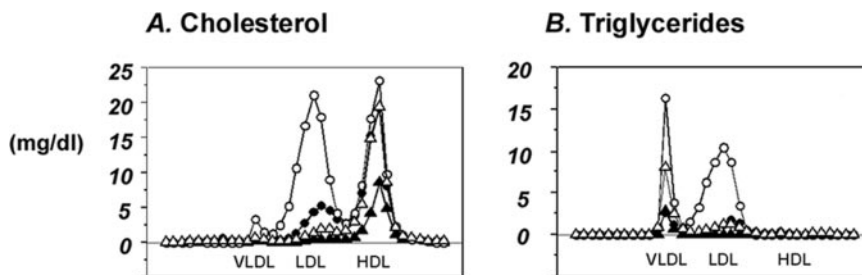


FIG. 2. Detection of ApoB in plasma of the study mice. Western blot analysis of apoB in plasma drawn from eight representative mice after a 4-h fast. ApoB was detected with a rabbit anti-mouse polyclonal antibody.

Ldlr^{-/-} *Mtpp*^{flox/flox} mice. Only in two of the eight *Ldlr*^{+/+} *Mtpp* ^{$\Delta\Delta$} mice examined was an apoB100 band detectable. In both of these cases, the intensity of the apoB100 band was less than 5% of the intensity of the apoB48 band. In contrast and very much to our surprise, the apoB100/B48 ratio in plasma of *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} mice was 1.40 ± 0.39 ($n = 7$). To ensure that the presence of apoB100 in the plasma of *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} mice was not a result of less efficient pl-pC-mediated *Mtpp* recombination related to the absence of *Ldlr*, the level of hepatic *Mtpp* mRNA was measured by real-time PCR. *Mtpp* mRNA expression in the liver of both *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} and *Ldlr*^{+/+} *Mtpp* ^{$\Delta\Delta$} mice was reduced by no more than 95% compared with control *Mtpp*^{flox/flox} mice (data not shown). For the pulse-chase experiments we also measured MTP activity. The MTP activity in *Mtpp* ^{$\Delta\Delta$} hepatocytes was less than 3% of the wild-type and did not differ between *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} and *Ldlr*^{+/+} *Mtpp* ^{$\Delta\Delta$} hepatocytes (62 ± 40 versus 47 ± 39 pmol/100 μ g/hr, $p = 0.55$ ($n = 4 + 4$)).

ApoB100 Secretion from Primary Hepatocytes—Intestinal cells secrete only trace amounts of apoB100-containing lipoproteins (34). The presence of apoB100-containing lipoproteins in the plasma of *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} mice, therefore, indicated that the livers of these mice still were capable of secreting apoB100-containing lipoproteins. However, to rule out the possibility that the presence of these apoB100-containing lipoproteins was caused by their accumulation in the absence of the LDL receptor rather than by reemerging secretion, we analyzed apoB100 secretion from labeled primary hepatocytes. Primary hepatocytes were incubated in the presence of [³⁵S]methionine/cysteine, and the incorporation of this label into apoB was meas-

ured in the medium. The secretion of apoB48 seemed to be unaffected in both *Ldlr*^{+/+} *Mtpp* ^{$\Delta\Delta$} and *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} hepatocytes ($104 \pm 7\%$ versus $93 \pm 4\%$, $n = 3$). ApoB100 in the medium of *Ldlr*^{+/+} *Mtpp* ^{$\Delta\Delta$} hepatocytes was undetectable by densitometry (Fig. 3). In contrast, the amount of apoB100 in the medium of *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} hepatocytes was only reduced by $\sim 30\%$, compared with the medium of *Ldlr*^{-/-} *Mtpp*^{flox/flox} hepatocytes ($69 \pm 23\%$, $n = 4$) (Fig. 3).

Density of ApoB100-containing Lipoproteins Secreted by Primary Hepatocytes—To determine the density of the secreted apoB100-containing lipoproteins, the medium of primary hepatocytes was subjected to density gradient ultracentrifugation to examine the secretion of apoB in the VLDL, LDL, and HDL fractions (33). We suspected that the apoB100-containing lipoproteins secreted by primary hepatocytes from *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} mice would, like the apoB48-containing lipoproteins secreted by *Mtpp* ^{$\Delta\Delta$} primary hepatocytes (9), be of HDL size. Surprisingly, however, an equally intense apoB100 band appeared in the LDL fraction (Fig. 4). The secretion of LDL-sized lipoproteins by hepatocytes from *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} mice is consistent with the LDL cholesterol and triglyceride peaks shown by fast-performance liquid chromatography of their plasma (Fig. 1). In contrast, *Ldlr*^{-/-} *Mtpp* ^{$\Delta\Delta$} hepatocytes did not secrete any VLDL (Fig. 4).

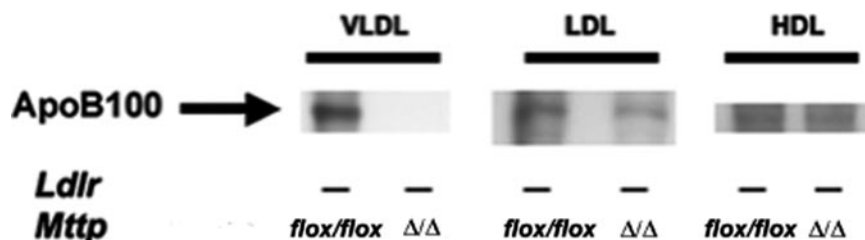


FIG. 4. Density distribution of apoB100-containing lipoproteins secreted from MTP and LDL receptor-deficient primary hepatocytes. Primary hepatocytes from littermate $Ldlr^{-/-}$ $Mttp^{lox/lox}$ and $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ mice were incubated in the presence of [35 S]methionine/cysteine. The media were then subjected to density gradient ultracentrifugations (33). VLDL, $d < 1.006$ g/dl lipoproteins; LDL, $d = 1.006$ – 1.063 g/dl lipoproteins; HDL, $d > 1.063$ g/dl lipoproteins.

Apo100 Synthesis, Turnover, and Sub-cellular Location—To investigate plausible mechanisms underlying the LDL receptor-mediated prevention of dense apoB100-containing lipoprotein secretion from $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ hepatocytes, we performed two pulse-chase experiments: a short 15-min pulse followed by 15- and 30-min chase periods (to estimate the rate of apoB100 *de novo* synthesis), and a 2-h pulse followed by 1, 2, and 3 h chase periods (to estimate apoB100 turnover). As indicated by the intracellular levels of labeled apoB100 after a 15-min pulse and after the subsequent chase periods (Fig. 5A), the biosynthesis of apoB100 appeared similar in $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ and $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ hepatocytes. In contrast, the intracellular turnover of apoB100 seemed more rapid in $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ than in $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ hepatocytes (Fig. 5B, black versus white sections of bars at each time point). In contrast to $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ hepatocytes, labeled apoB100 was detected in the medium of $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ hepatocytes (Fig. 5B, gray section of the bars). However, the overall recovery of a labeled apoB100 was similar from $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ and $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ hepatocytes (Fig. 5B, black versus the entire white bar or white and gray bar at each time point). There were no differences in apoB48 biosynthesis or turnover between $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ and $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ hepatocytes, nor could we detect a difference in heparin-releasable apoB100 from the cell surface (data not shown). Taken together, these results suggest that (i) the LDL receptor does not affect the *de novo* biosynthesis of apoB100, and (ii) the LDL receptor-mediated degradation of apoB100 mainly occurs intracellularly. To investigate the sub-cellular location of the LDL receptor-mediated degradation, we separated hepatic ER and Golgi fractions and measured the relative content of apoB100 in these sub-cellular fractions. As indicated previously (35), the LDL receptor-mediated degradation of apoB100 seemed to occur mainly in the ER, as indicated by the levels of apoB100 in the ER fractions from $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ and $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ hepatocytes ($100 \pm 63\%$ ($Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$) versus $330 \pm 123\%$ ($Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$), $n = 4 + 4$, $p < 0.04$). In contrast, there was a tendency to the inverse relation in the Golgi fractions ($100 \pm 40\%$ ($Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$) versus $77 \pm 54\%$ ($Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$), $n = 3 + 3$, $p = 0.59$).

DISCUSSION

This study shows that the secretion of dense apoB100-containing lipoproteins from the liver is prevented by the LDL receptors. In addition, this study suggests that hepatic apoB100 synthesis can occur despite very low levels of MTP. Thus, normal levels of MTP do not seem to be essential for the first step of VLDL assembly. In contrast, and consistent with the notion that MTP is essential for the transformation of the pre-VLDL particle into a buoyant VLDL particle, the secretion of buoyant VLDL particles was abolished. The fact that dense apoB100-containing lipoproteins that escape co-translational degradation were targeted by mainly intracellular hepatic LDL receptors assigns this receptor a novel “gatekeeping” function that may be an important additional mechanism for the cho-

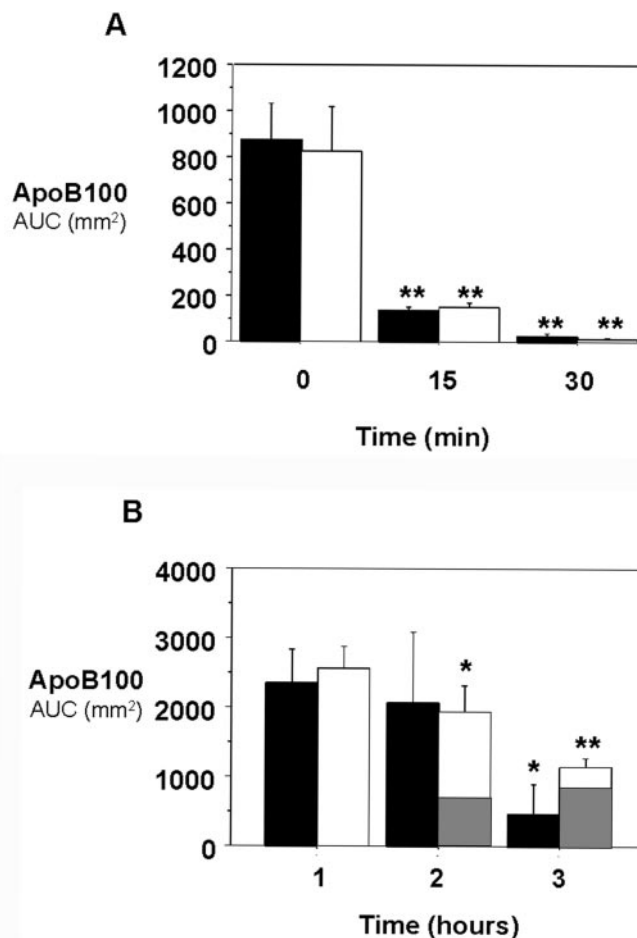


FIG. 5. The effects of the LDL receptor on the synthesis (A) and turnover (B) of apoB100 in MTP-deficient primary hepatocytes. Primary hepatocytes from $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$ and $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ mice were pulsed with [35 S]methionine/cysteine for 15 min and then chased for 15 and 30 min (A) or pulsed with [35 S]methionine/cysteine for 2 h and then chased for 1, 2, and 3 h (B). At each time point, HDL and less dense lipoproteins were isolated from the cell lysate and media by ultracentrifugation (40,000 rpm for >80 h at 10°C), as described in Fig. 3. The intensities of labeled apoB100 were determined by densitometry. Bar graphs show the mean intensity of apoB100 after 0, 15, and 30 min (A) and 1, 2, and 3 h (B) chase periods. Values are mean \pm S.E., $n = 4$, 3, and 3 (A); 3, 4, and 3 (B) ($Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$); 4, 4, and 4 (A); 4, 4, and 3 (B) ($Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$) at each time point. **, $p < 0.005$ compared with the 0-min values (A); *, $p < 0.05$ and **, $p < 0.005$ comparing black bars and white sections of the bars with 1-h values (B). ■, $Ldlr^{+/+}$ $Mttp^{\Delta\Delta}$; □, $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$; grey sections of bars, $Ldlr^{-/-}$ $Mttp^{\Delta\Delta}$ (extracellular).

lesterol-lowering and antiatherosclerotic effects of statins.

The *Lox P* system (22) allows the study of genes with important functions in both adult life and embryonic development. However, this conditional targeting technology has its limitations compared with conventional targeting, as the *Cre*-medi-

ated recombination of the targeted gene is not complete. Using this system, residual activity of the targeted gene will remain. In our case, this is consistent with residual activity of MTP in the *Ldlr*^{+/+} *Mttp*^{ΔΔ} and the *Ldlr*^{-/-} *Mttp*^{ΔΔ} mice. We found that, on average, 69% of apoB100 secretion remained from *Ldlr*^{-/-} *Mttp*^{ΔΔ} hepatocytes despite more than 95% *Mttp* recombination and less than 3% residual MTP activity. In contrast, the same level of residual MTP activity was insufficient to maintain any VLDL synthesis causing fat to accumulate within hepatocytes. In addition, 3% residual MTP activity was not consistent with any apoB100 secretion when the LDL receptor was present (*Ldlr*^{+/+} *Mttp*^{ΔΔ}).

The fact that the secretion of HDL-sized apoB48-containing lipoproteins by *Mttp*^{ΔΔ} livers occurred despite the presence of the LDL receptor is not surprising, because apoB48 does not bind to this receptor and, hence, escapes LDL receptor-mediated degradation. Previously, liver-specific *Mttp*-deficient hepatocytes were shown to secrete apoB48 (9), and the reduction of apoB48 secretion from primary hepatocytes in the presence of an MTP inhibitor was only marginally reduced (17). Although the smaller size of apoB48 protein relative to apoB100 may decrease its vulnerability to protease-mediated degradation, these studies evoke the possibility of a separate hepatic synthetic pathway for the assembly of apoB48-containing lipoproteins (36). Our results do not support this possibility. Rather, they support the traditional view of a common pathway for the assembly of both species of apoB, for the first step of which normal levels of MTP do not seem to be essential.

Interestingly, Kulinski *et al.* (17) recently reported only a minor reappearance of apoB100 secretion from LDL receptor-deficient hepatocytes upon incubation with an MTP inhibitor. The authors concluded that the preferential reduction of apoB100 secretion, compared with apoB48, upon inhibition of MTP cannot be attributed to the presence of LDL receptors. The reason why Kulinski *et al.* (17) only detected a marginal reappearance of apoB100 secretion from LDL receptor-deficient hepatocytes, whereas we, in this study, show that apoB100 secretion remains with an average 69% is most likely because of the fact that they used an MTP inhibitor as compared with our model system, which used a conditional targeting of MTP. It is possible, and even likely, that the presence of MTP together with an inhibitor affects the first step of VLDL assembly differently than the absence of MTP alone. For instance, MTP and the inhibitor could form a complex that hinders the proper folding and lipidation of apoB100. Alternatively, an MTP inhibitor could interact with other proteins that are essential for the proper folding or lipidation of apoB100. Another explanation could be that residual MTP activity in our model was sufficient to support the first step of VLDL assembly. It would be of great interest to determine the secretion of apoB100 from hepatocytes that completely lack both *Mttp* and *Ldlr*.

An inevitable question is why individuals with the rare dominant disorder abetalipoproteinemia, which is caused by mutations in *Mttp* (7), have low levels of apoB100 in plasma. If the first step in VLDL assembly does not require MTP, wouldn't these patients have circulating LDL- and HDL-sized apoB100-containing lipoproteins in their plasma? The obvious answer is that these patients have intact hepatic LDL receptors that would, as in the liver-specific *Mttp* knockout mice, target those particles for degradation before they can be secreted. In theory, a combined abetalipoproteinemia and familial hypercholesterolemia genotype should go with the presence of dense apoB100-containing lipoproteins in plasma. However, to our knowledge, such a patient has not been reported. A similar argument can

be used to understand the absence of apoB secretion from cell lines that lack MTP (18–20).

The fact that the secretion of apoB100 reemerged in the absence of the LDL receptor indicates that hepatic LDL receptors are crucial for regulating the degradation of newly synthesized apoB100. Twisk *et al.* (15) recently showed that LDL receptor-deficient hepatocytes secrete 3.5-fold more apoB than wild-type hepatocytes and that the prevention of VLDL secretion by the LDL receptor occurred both intracellularly and after VLDL secretion. In our model, the LDL receptor completely blocked the secretion of dense apoB100 containing lipoprotein from the liver. In addition, our pulse-chase experiments, including the incubation of primary hepatocytes with heparin and the sub-cellular subfractionation studies, indicate that the LDL receptor targets these particles intracellularly, mainly in the ER. Assuming that there is no biological incentive for the liver to secrete lipid-poor particles, this gatekeeping function by the LDL receptor makes sense. In the presence of normal levels of hepatic MTP (as in the study by Twisk *et al.*), when buoyant VLDL particles also are made, the LDL receptor is likely to prioritize lipid-poor particles for degradation, because these particles have higher affinity for the LDL receptor than VLDL-sized lipoproteins.

A gatekeeping function of the LDL receptor could also help explain the anti-atherosclerotic effects of statins that are unrelated to their ability to lower plasma cholesterol levels. These drugs inhibit 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, a tightly regulated step in the cholesterol biosynthetic pathway (37). Cells respond to the shortage of cholesterol by up-regulating the transcription of cholesterol-regulated genes, including the LDL receptor (38). The current study indicates that the LDL receptor prevents the secretion of dense apoB100-containing lipoproteins from the liver. Because cholesterol carried in small dense particles is believed to be more atherogenic than cholesterol carried in VLDL particles (39), the gatekeeping function of the LDL receptor would prevent hepatic secretion of cholesterol in potentially more atherogenic particles and promote cholesterol secretion in less atherogenic VLDL.

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