THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 51, Issue of December 20, pp. 33047–33053, 1996
Printed in U.S.A.
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Inhibition of the Microsomal Triglyceride Transfer Protein Blocks the First Step of Apolipoprotein B Lipoprotein Assembly but Not the Addition of Bulk Core Lipids in the Second Step*

(Received for publication, June 19, 1996, and in revised form, October 2, 1996)

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The microsomal triglyceride transfer protein (MTP) is required for assembly and secretion of the lipoproteins containing apolipoprotein B (apoB): very low density lipoproteins and chylomicrons. Evidence indicates that the subclasses of these lipoproteins that contain apoB-48 are assembled in a distinct two-step process; first a relatively lipid-poor primordial lipoprotein precursor is produced, and then bulk neutral lipids are added to form the core of these spherical particles. To determine if either step is mediated by MTP, a series of clonal cell lines stably expressing apoB-53 and MTP was established in non-lipoprotein-producing HeLa cells. MTP activity in these cells was ~30%, and apoB secretion was 7–33% of that in HepG2 cells on a molar basis. Despite having robust levels of triglyceride and phospholipid synthesis, these cell lines, as exemplified by HLMB53-59, secreted >90% of the apoB-53 on relatively lipid-poor particles in the density range of 1.063–1.21 g/ml. These results suggested that coexpression of MTP and apoB only reconstituted the first but not the second step in lipoprotein assembly. To extend this observation, additional studies were carried out in McArdle RH-7777 rat hepatoma cells, in which the second step of apoB-48 lipoprotein assembly is well defined. Treatment of these cells with the MTP photoaffinity inhibitor BMS-192951 before pulse labeling with [^{35}S]methionine/cysteine led to an 85% block of both apoB-48 and apoB-100 but not apoAI secretion, demonstrating inhibition of the first step of lipoprotein assembly. After a 30-min [^{35}S]methioneine/cysteine pulse labeling and 120 min of chase, all of the nascent apoB-48 was observed to have a density of high density lipoproteins (1.063–1.21 g/ml), indicating that only the first step of lipoprotein assembly had occurred. The addition of oleic acid to the cell culture media activated the second step as evidenced by the conversion of the apoB-48 high density lipoproteins to very low density lipoproteins (d < 1.006 g/ml) during an extended chase period. Inactivation of MTP after completion of the first step, but before stimulation of the second step by the addition of oleic acid, did not block this conversion. Thus, inhibition of MTP did not hinder the addition of bulk core lipid to the primordial lipoprotein precursor particles, indicating that MTP is not required for the second step of apoB-48 lipoprotein assembly.

Lipoproteins containing apolipoprotein B (apoB)[1] are the main carriers of triglyceride and cholesteryl esters in the circulatory system (1). Very low density lipoprotein (VLDL) is a triglyceride-rich particle produced by the liver. In humans, VLDL contains one copy of apoB-100 per particle. In other species, such as rats, mice, rabbits, and dogs, the liver also produces VLDL containing one copy of a smaller form of apoB generated by post-transcriptional deamination of cytidine residue 6666 of the mRNA, converting it to a uridine, changing codon 2153 from a glutamine to a translation stop signal (2). The resulting translation product contains the amino-terminal 48% of full-length apoB-100 and is termed apoB-48. Post-transcriptional editing of the apoB mRNA at the same nucleotide residue also occurs in the intestinal enterocytes of all vertebrates studied to date. ApoB-48 produced in the intestines is packaged into chylomicron particles that are more highly enriched in triglycerides and larger in diameter than VLDL.

Early ultrastructural studies (3) of rat liver demonstrated immunoreactive apoB on VLDL size particles at the junction between the rough and smooth endoplasmic reticulum, while apoB in the proximal regions of the rough endoplasmic reticulum was associated with smaller particles. Furthermore, lipid droplets lacking immunoreactive apoB were identified in the smooth endoplasmic reticulum. These observations were interpreted to indicate that small apoB particles that were formed in the rough endoplasmic reticulum were fusing with preformed lipid droplets from the smooth endoplasmic reticulum at the border between these subcellular structures. These observations formed the basis for a model in which VLDL is assembled in two steps (for review, see Ref. 4). The first step includes all of the events leading up to production of small, dense, apoB-containing primordial lipoprotein precursor particles. The second step is the addition of the bulk lipid by fusion of the small primordial lipoprotein precursors to preformed lipid droplets to form mature VLDL.

A recent study in McArdle RH-7777 rat hepatoma cells (5) confirmed this model by demonstrating that nascent apoB-48 polypeptide chains are cotranslationally complexed with lipids

* This work was supported in part by the Bristol-Myers Squibb Pharmaceutical Research Institute, Swedish Medical Research Council Grant 7142, the Swedish Heart and Lung Foundation, the Nordisk Insulin Fund, the Swedish Oleo-Margarine Fund for Nutritional Research, and the King Gustav V Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by the Howard Hughes Medical Institute via a Howard Hughes postdocotoral fellowship for physicians.

[1] The abbreviations used are: apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PBS, phosphate buffered saline; MEM, minimal essential medium; Me_2SO, dimethyl sulfoxide.

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to form a primordial lipoprotein particle with the density of high density lipoproteins (HDL, d = 1.063–1.21 g/ml). In a distinct second step, the major load of lipid is added to the primordial lipoprotein particle to form a particle of VLDL ($d <$ 1.006 g/ml) density. These two steps could be temporally dissociated by manipulating the fatty acid content of the cell culture medium (5). In the absence of fatty acid, the cells slowly secreted lipid-poor apoB-48 HDL density particles, suggesting that only the first step of assembly had occurred. Upon supplementation of the medium with oleic acid, large quantities of lipid were added to the apoB-48 HDL that remained within the cell, resulting in secretion of VLDL density particles. In contrast, apoB-100 is rapidly assembled into VLDL density particles regardless of lipid supplementation of the medium, precluding the use of apoB-100 to study the second step in this model system. Thus, the second step of apoB-48 VLDL assembly was stimulated by a high level of lipid synthesis in response to fatty acid feeding. In the same study (5), when protein synthesis was blocked with cycloheximide, conversion of apoB-48 HDL to VLDL was partially inhibited regardless of lipid supplementation. This finding suggests that a protein factor may be required to add bulk core lipid to nascent apoB-48 lipoproteins to convert them to VLDL.

Besides apoB and lipid synthesis, the only other known factor required for lipoprotein assembly is the microsomal triglyceride transfer protein (MTP) (for review, see Ref. 6). MTP is a soluble heterodimeric protein composed of a unique 97-kDa subunit and protein disulfide isomerase, a ubiquitous multifunctional protein (7). The active MTP complex resides in the lumen of the endoplasmic reticulum in cells that produce apoB lipoproteins (8). The absence of MTP has been identified as the proximal cause of the autosomal recessive disease abetalipoproteinemia (9, 10). The principal characteristic of this disease is the nearly complete inability to secrete apoB-containing lipoproteins from the hepatocytes and intestinal enterocytes, leading to exceedingly low levels of cholesterol in the plasma (11). In cell culture studies, it has been shown that expression of apoB alone in non-lipoprotein-producing cell lines does not lead to secretion of apoB lipoproteins. Conversely, coexpression of apoB and MTP leads to secretion of apoB lipoproteins (12–14). Furthermore, inactivation of MTP in liver-derived cells with a photoaffinity inhibitor abolishes apoB secretion (15).

Taken together, these studies clearly indicate that MTP is necessary to mediate the assembly and secretion of apoB-containing lipoprotein particles. What is not clear is which step(s) in the lipoprotein assembly pathway is mediated by MTP. Since MTP catalyzes the transfer of lipid between phospholipid surfaces, it is possible that MTP mediates both the first and second step of apoB-48 lipoprotein assembly. In this report, we describe studies in a non-lipoprotein-producing cell line stably expressing MTP and apoB and in the McArdle RH-7777 rat hepatoma cell line, designed to address this question.

EXPERIMENTAL PROCEDURES

Materials

All tissue culture media, sera, and reagents and enzymes and buffers for DNA manipulation were obtained from Life Technologies, Inc. All other chemicals and immunological reagents were from Sigma unless otherwise indicated. McArdle RH-7777 rat hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum, 10 μM nonessential amino acids, MEM vitamins, and 1.0 mM sodium pyruvate. The HeLa-derived HLM-40 cell line that stably expresses the large subunit of human MTP has been described (12). Goat anti-human apoB antiserum was purchased from Biodesign International (Kennebunk, ME). Rabbit anti-rat apoB antiserum has been described (5). Express [^{35}S]methionine/cysteine labeling mixture was obtained from DuPont NEN. Amplify gel-enhancing agent was purchased from Amersham Corp. Protein A-Sepharose CL-4B was obtained from Pharmacia Biotech Inc.

Methods

Plasmid Constructs and Transfections—Plasmids expressing human apoB-53 (pB53) were kindly supplied by Dr. Zemin Yao (Ottawa Heart Institute, Ottawa, Ontario) (12). An expression plasmid containing the hygromycin resistance gene (pRC/hygro) was constructed as follows. The hygromycin expression cassette was excised from commercial vector pREP7 (Invitrogen, San Diego, CA) by digestion with restriction enzymes *Sal*I and *Sma*I and subcloned into pBluescript II KS (Stratagene, San Diego, CA) using standard molecular biological techniques (16). After removal from pBluescript II KS with *Xba*I and *Xho*I, the ends of the 1.86-kilobase pair hygromycin fragment were filled in using DNA polymerase I Klenow fragment and ligated into plasmid pRC/CMV (Invitrogen) previously digested with *Nae*I (New England Biolabs, Beverly, MA) to remove the neomycin resistance gene.

Plasmid pB53 was cotransfected at a molar ratio of 4:1 with pRC/hygro into HLM-40 cells with the Lipofectin Reagent (Life Technologies, Inc.) as described (12). Two days after transfection, the cells were subcultured at a ratio of 1:4 and fed selection medium composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, MEM nonessential amino acids, 1.0 mg/ml active concentration of Geneticin, and 360 units/ml hygromycin (Calbiochem). After 3 weeks of selection, the cells were fed the same medium containing 1.0 mg/ml of Geneticin and 240 units/ml of hygromycin (maintenance medium). Individual hygromycin-resistant colonies were isolated and expanded as clonal cell lines for analysis of apoB expression.

From previous studies (12), it was expected that expression of apoB in the HLM-40 cell line would lead to secretion of this protein into the medium. Thus, conditioned medium from each hygromycin-resistant clonal cell line was assayed for apoB as follows. Cells were grown to confluency in 48-well tissue culture plates. Maintenance medium was removed, and the cells were fed 0.5 ml of lipid-rich medium (RPMI 1640, 3% bovine serum albumin, 0.8 mM oleic acid, 1.0 mM glycerol) for 24 h. Aliquots of media (100 μl) were assayed for apoB by an enzyme-linked immunosorbent assay (17). Several clonal cell lines secreting apoB-53 were identified, one of which (HLMB53-59) was chosen for further analysis. MTP-mediated triglyceride transfer activity was measured in cell extracts as described (12). Buoyant density fractionation by sequential ultracentrifugation and subsequent immunoblot analysis of the lipoproteins secreted from HLMB53 cell lines were carried out as described (12).

In Situ Inactivation of MTP with a Photoaffinity Inhibitor—McArdle RH-7777 rat hepatoma cells were cultured in MEM supplemented with 20% fetal bovine serum, nonessential amino acids, sodium pyruvate, and MEM vitamins (growth medium). All supplements were added according to the recommendations of the manufacturer. McArdle RH-7777 cells grown to 90% of confluency in 10.0-cm dishes were used in all experiments described below.

To establish the parameters for *in situ* inactivation of MTP in McArdle RH-7777 cells, the following experiment was carried out. Cultures were brought to 10.0 μM BMS-192951 in growth medium containing 0.5% Me_2SO. All subsequent manipulations were carried out in the dark until the cells were harvested. After incubation for 1 h under standard cell culture conditions, the cultures were exposed to ultraviolet light at 365 nm for 0–15 min (UVP, San Gabriel, CA) at 4 °C. One culture dish remained in the incubator as the zero time control. The cultures were rinsed 3 times with phosphate-buffered saline (PBS), and extracts were prepared as described (12). To ensure removal of all noncovalently bound BMS-192951, the extracts were exhaustively dialyzed against a buffer containing 15 mM Tris-HCl, pH 7.4, 40 mM NaCl, and 0.02% sodium azide for 16 h at 4 °C. MTP-mediated triglyceride transfer activity was measured in the dialyzed extracts by the standard assay (9).

To determine the effect of *in situ* inactivation of MTP on apoB and apoAI secretion, McArdle RH-7777 cells were treated with 10.0 μM BMS-192951 as above. After exposure to ultraviolet light, the cells were rinsed 3 times with PBS and fed prelabeling medium (methionine- and cysteine-free growth medium containing 15% dialyzed fetal bovine serum) for 1 h. The prelabeling medium was removed, and the cells were rinsed once with PBS and pulse-labeled for 30 min with labeling medium (prelabeling medium plus 200 μCi/ml [^{35}S]methionine/cysteine). The labeling medium was removed, and growth medium was added for 3 h. The media were harvested; adjusted to 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM benzamidine, 5.0 mM EDTA, and 50 μg/ml aprotinin; and centrifuged at 1500 $\times g$ for 5 min at 4 °C to remove loose cells.

The amount of apoB and apoAI in each sample was determined by immunoprecipitation of 0.5-ml aliquots of medium diluted 1:1 with NET buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.005 M EDTA, 0.5% Triton X-100, and 0.1% sodium dodecyl sulfate) as described previously (17). Rabbit anti-rat apoB and rabbit anti-hamster apoAI antisera were utilized at a final dilution of 1:500. Immunoprecipitates were analyzed on 4% SDS-polyacrylamide gels for apoB and 12% SDS-polyacrylamide gels for apoAI. Radioactivity in the apoB and apoAI bands was directly quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell Fractionation—To determine if inhibition of MTP causes a shift in the buoyant density of lipoproteins in the lumen of the endoplasmic reticulum or those secreted into the medium, cultures of McArdle RH-7777 cells were treated as indicated in Fig. 4. Upon completion of the experimental protocol, media were removed, treated with protease inhibitors, centrifuged as described above, and stored at 4 °C for further analysis. Endoplasmic reticulum lumenal extracts were isolated by a modification of the previously published technique (5). The cells were rinsed with and scraped into 5.0 ml of 0.25 M sucrose solution (made in 3.0 mM imidazole buffer, pH 7.4) and pelleted by centrifugation at 3,000 × *g* for 5 min at 4 °C. All subsequent steps were carried out in buffers containing 1.0 mM EGTA, 2.0 μg/ml calpain I inhibitor, 1.0 μM pepstatin A, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 1.0 mM phenylmethylsulfonyl fluoride. After resuspension in 1.0 ml of 0.05 M sucrose solution (made in 3.0 M imidazole buffer, pH 7.4), the cells were transferred to a 1.5-ml microcentrifuge tube and were pelleted by centrifugation for 15 s at 3,000 × *g*. The cell pellets were gently resuspended in 0.5 ml of 0.05 M sucrose solution, transferred to a 2.0-ml Dounce homogenizer, and homogenized by 15 strokes with the tight fitting pestle. After the addition of 52 μl of 47% sucrose solution (made in 3.0 mM imidazole buffer, pH 7.4), the cells were further homogenized by an additional five strokes with the tight-fitting pestle. The homogenates were quantitatively transferred to a 1.5-ml microcentrifuge tube at a final volume of 800 μl and were centrifuged for 10 min at 3,000 × *g* at 4 °C. Pellets were resuspended in 200 μl of 0.25 M sucrose solution and recentrifuged under the same conditions. The supernatants were pooled and recentrifuged once to remove all cellular debris and nonmicrosomal membrane components. Final supernatants (1.0 ml) were added to 0.5 ml of 0.25 M sucrose, 2.5 ml PBS, 0.5 ml 47% sucrose, and 0.5 ml 1.0 M sodium carbonate and incubated for 25 min at room temperature with gentle mixing every 5 min. Samples were brought to 5 mg/ml bovine serum albumin and were centrifuged at 200,000 × *g* for 30 min at 4 °C in a Beckman model TL-100 ultracentrifuge in the TLA 100.3 rotor. The supernatants were removed and neutralized to pH 7.4 with glacial acetic acid, and EGTA and protease inhibitors were added to achieve the final concentrations listed above.

Sucrose Gradient Ultracentrifugation and Immunoprecipitation—Aliquots (5.0 ml) of cell culture media and lumenal extracts were loaded onto sucrose gradients, centrifuged, and fractionated as described (5). Fractions were brought to 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.005 M EDTA, 0.5% Triton X-100 and a final volume of 1.5 ml, and 1.5 μl of nonimmune rabbit serum was added. After incubation for 3 h at room temperature, 75 μl of 5% protein A-Sepharose (w/v) was added for 30 min. The protein A-Sepharose was pelleted by centrifugation at 10,000 × *g* for 2 min, and the supernatants were transferred to a fresh tube containing 10 μl of rabbit anti-rat apoB antisera. All subsequent immunoprecipitation steps were carried out as described (17).

Analytical Methods—Protein concentrations were measured by the Bio-Rad DC protein assay as described by the manufacturer. Data shown are representative of at least two independent experiments.

RESULTS

Analysis of Lipoprotein Production in HeLa-derived Cell Lines—HLM-40 cells are a HeLa-derived cell line stably expressing MTP at approximately ⅓ the level of the human hepatoblastoma HepG2 cell line (12). After cotransfection with plasmids encoding the gene for hygromycin resistance and apoB-53, approximately 100 hygromycin-resistant colonies were chosen and expanded for analysis. Several colonies secreted relatively high levels of apoB-53 as measured by an enzyme-linked immunosorbent assay. One clone, HLMB53-59, was selected for further analysis.

Fig. 1*A* shows the amount of apoB secreted from HLMB53-59 compared with HepG2 cells over 24 h. On a mass basis, the HLMB53-59 cells secreted approximately 18% of the amount of apoB secreted by the HepG2 cells. Taking into account the difference in molecular weight between apoB-53 and apoB-100, the HLMB53-59 cells secreted approximately ⅓ the amount of apoB secreted by the HepG2 cells on a molar basis. ApoB secretion in the other HLMB53 clones was 14–54% of the level of HepG2 cells. MTP activity in all of the analyzed HLMB53 cell lines (data not shown) was the same as that of HLMB53-59 (Fig. 1*B*).

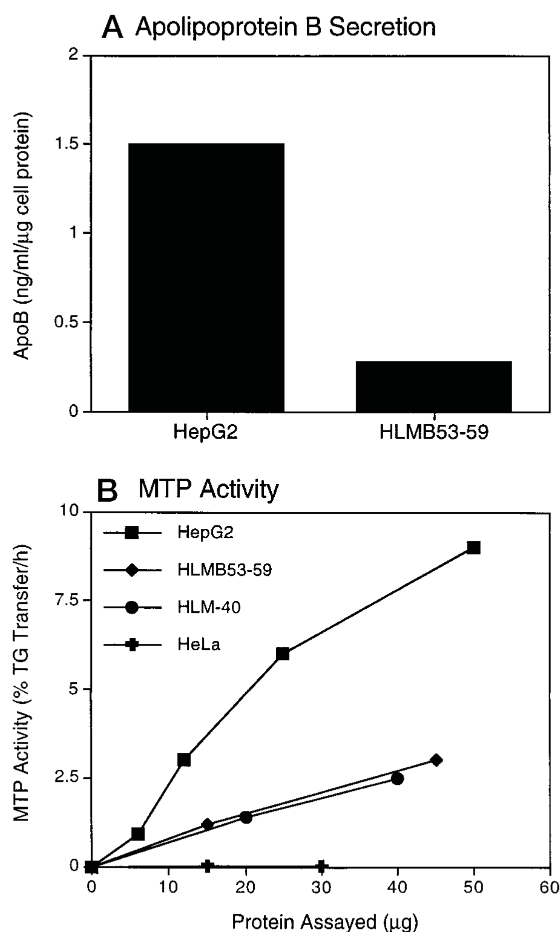

FIG. 1. **Quantitation of ApoB secretion and MTP activity in HeLa-derived cell lines.** *A*, HepG2 and HLMB53-59 cells were grown in parallel triplicate cultures and fed medium supplemented with 0.8 mM oleic acid and 1.0 mM glycerol for 24 h. ApoB concentration was measured in triplicate by enzyme-linked immunosorbent assay. Total cellular protein was measured in whole-cell extracts in duplicate. Data are expressed as ng of apoB secreted per 24 h per ml of culture medium, normalized to total cellular protein. *B*, MTP-mediated triglyceride transfer was measured with an *in vitro* assay based on the percentage of transfer of radiolabeled triglyceride from donor small unilamellar vesicles to recipient small unilamellar vesicles. Data are presented as MTP activity at various dilutions of cell extract. The data shown are representative of at least two independent assays with HepG2 as the reference.

To characterize the lipoprotein particles secreted by these cell lines, the cultures were fed medium containing 0.8 mM oleic acid and 1.0 mM glycerol for 24 h. The conditioned media were collected, and the buoyant density of the apoB-containing lipoproteins was analyzed by sequential ultracentrifugation. Fig. 2 shows that virtually all of the apoB-containing lipoproteins secreted from cell line HLMB53-59 were of HDL density (d = 1.063–1.21 g/ml). In fact, HeLa cells coexpressing apoB-53 and MTP always secrete >90% of the apoB on HDL density particles whether apoB-53 expression is stable (data not shown) or transient (12).

This observation, while consistent with previous reports of expression of truncated apoB polypeptides in hepatoma cells

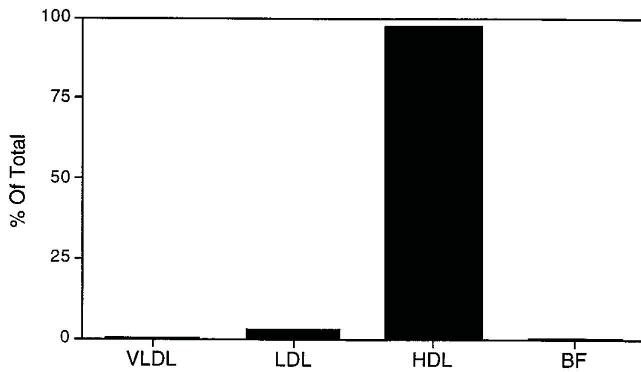

FIG. 2. **Buoyant density analysis of lipoproteins secreted from HLMB53-59 and HLMB100–49 cells.** Confluent cultures of HLMB53-59 cells were fed medium containing 0.8 mM oleic acid and 1.0 mM glycerol for 24 h. Media were harvested and fractionated by sequential ultracentrifugation into VLDL ($d < 1.006$ g/ml), LDL ($d = 1.006$–1.063 g/ml), HDL ($d = 1.063$–1.21 g/ml), and bottom fraction (*BF*, $d >$ 1.21 g/ml) density fractions. ApoB was quantitated by Western immunoblot using a colorimetric reagent to visualize the bands (9). Band intensity was directly measured with a Molecular Dynamics Personal Densitometer Si (Sunnyvale, CA). The data shown are representative of four independent experiments.

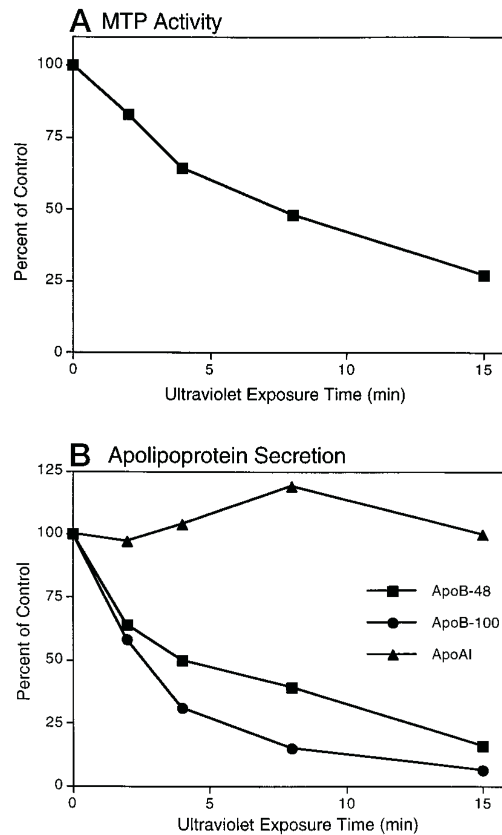

FIG. 3. **Effect of MTP inactivation on ApoB and ApoAI secretion from McArdle RH-7777 rat hepatoma cells.** Duplicate parallel cultures of McArdle RH-7777 cells were treated with BMS-192951 as described under "Experimental Procedures." One set of cultures was harvested, and MTP-mediated triglyceride transfer activity was measured in cell extracts (*A*). The second set of cultures was pulsed for 30 min with [^{35}S]methionine/cysteine. After a 3-h chase, the secretion of apoB-48 (■), apoB-100 (●), and apoAI (▲) was monitored by immunoprecipitation. The data (*B*) shown are representative of two independent experiments.

(18, 19), does not reflect what occurs *in vivo*. Naturally occurring truncated forms of apoB, such as apoB-48, are secreted as lipid-rich VLDL or chylomicron particles from the liver and intestines, respectively. The observation that the HLMB53-59 cell line secreted a similarly truncated apoB polypeptide (apoB-53) on relatively lipid-poor particles of HDL density rather than VLDL density suggested that MTP expression alone reconstitutes the first step of lipoprotein assembly and is insufficient to add bulk neutral lipid to the core of these particles in the second step of lipoprotein assembly.

Analysis of Lipoprotein Production in McArdle RH-7777 Cells—To extend the observation that MTP mediates the first step of lipoprotein assembly and not the addition of bulk core lipid to nascent lipoproteins in the second step, a series of experiments were carried out in a liver-derived cell line. Previous studies have shown that apoB-48 VLDL assembly in McArdle RH-7777 hepatoma cells occurs in a distinct two-step process (5). In the absence of exogenously supplied fatty acid, only the first step occurs resulting in the accumulation of nascent dense lipoprotein particles in the lumen of the endoplasmic reticulum that are slowly and inefficiently secreted from the cell. When the culture media are supplemented with fatty acids, large quantities of neutral lipid are added to the core of the nascent particles, resulting in a distinct shift to lower densities and enhanced efficiency of secretion.

Fig. 3*A* shows the results of experiments to determine the conditions required to achieve *in situ* inactivation of MTP. McArdle RH-7777 cells were treated with BMS-192951 at a concentration of 10.0 μM in 0.5% Me_2SO. Increasing the time of exposure to ultraviolet light led to decreasing levels of MTP activity; approximately 50% of the MTP was inactivated after 8 min of exposure, and greater than 75% was inactivated by 15 min. Preliminary experiments showed that exposure of cells to ultraviolet light at 365 nm for 15 min or less resulted in little or no cell damage as evidenced by no change in the secretion of control proteins or incorporation of [^{3}H]thymidine into cellular DNA.[2]

Two sets of experiments were carried out to determine if inhibition of MTP *in situ* blocks either or both steps of apoB VLDL assembly. In order to study the first step only, MTP was inactivated prior to pulse labeling under conditions that do not activate the second step (5). Parallel cultures of McArdle RH-7777 cells were pretreated with 10 μM BMS-192951 for 1 h in growth medium not supplemented with oleic acid. After exposure to ultraviolet light at 365 nm for 0–15 min, they were pulsed with [^{35}S]methionine/cysteine for 30 min followed by a 3-h chase in medium not supplemented with oleic acid. The amounts of apoB-100 and apoB-48 secreted into the medium were measured by immunoprecipitation. Secretion of both apoB-100 and apoB-48 decreased (Fig. 3*B*) with decreasing MTP activity (Fig. 3*A*). At 15 min, when ~80% of the MTP activity was blocked, apoB-48 secretion was decreased by 85% and apoB-100 secretion was decreased by 95%. Thus, apoB secretion from these cells correlates positively with the level of MTP activity.

Aliquots of cell culture media were also measured for concentrations of apoAI by immunoprecipitation with a rabbit anti-hamster apoAI antiserum. Treatment of McArdle RH-7777 cells with 10.0 μM BMS-192951 and a 15-min exposure to ultraviolet light did not decrease apoAI secretion. Since it is known that MTP does not play a role in secretion of apoAI lipoproteins, treatment of McArdle RH-7777 cells with the photoaffinity inhibitor and ultraviolet light does not inhibit general protein secretion. Thus, the inhibition of apoB secretion was due to inactivation of MTP, as indicated by measurements of cellular MTP activity. Furthermore, since the conditions of this experiment promote only the first step of apoB-48 VLDL production, the decrease of apoB-48 secretion with decreasing

[2] H. Jamil and D. Gordon, manuscript in preparation.

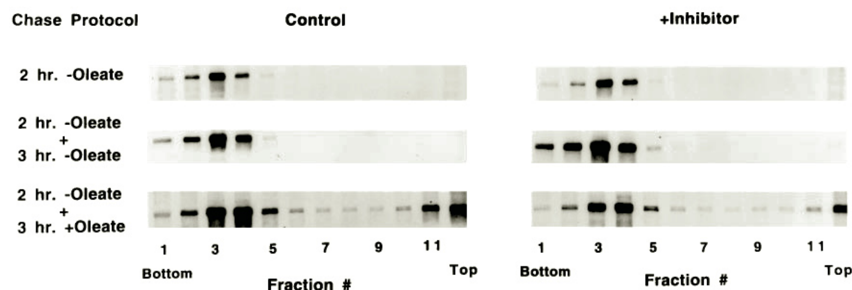

FIG. 4. **Effect of MTP inactivation on the buoyant density of ApoB-48 lipoprotein particles secreted from McArdle RH-7777 rat hepatoma cells.** Parallel cultures of McArdle RH-7777 cells were pulsed with [^{35}S]methionine/cystine for 30 min. At the commencement of the chase, one set of cultures was brought to 0.5% Me_2SO only (control), while the other set was brought to 0.5% Me_2SO and 10.0 μM BMS-192951. One hour later, the cells were exposed to ultraviolet light for 15 min at 4 °C, and the chase was continued for an additional 1 h. After the first 2 h of chase, media from one set (one control and one with inhibitor) of plates were harvested. Of the four remaining plates, two (one control and one with inhibitor) were fed standard growth medium containing 3% bovine serum albumin, and two were fed standard growth medium supplemented with 3% bovine serum albumin and 0.36 mM oleic acid. All cultures were incubated for an additional 3 h under standard cell culture conditions. The media were harvested, and the buoyant density of the apoB lipoproteins was analyzed by sucrose gradient ultracentrifugation as described under "Experimental Procedures." Fractions 1–5 represent the HDL density range (d = 1.23–1.063 g/ml) of the gradient. Fractions 6 and 7 represent the LDL density range (d = 1.063–1.006 g/ml), and fractions 8–12 represent the VLDL density range (d < 1.006) of the gradient.

levels of MTP activity indicates a role for MTP in the first step of VLDL assembly.

To determine if an MTP inhibitor could block the addition of bulk core lipid to nascent apoB-48 lipoprotein particles, McArdle RH-7777 cells were pulsed with [^{35}S]methionine/cysteine for 30 min followed by a 2-h chase in growth medium without the addition of oleic acid. One hour into the chase, MTP was covalently inactivated with 10.0 μM BMS-192951 by exposure to ultraviolet light for 15 min. Control cultures were treated identically, except the inhibitor was omitted. At the end of the first 2 h of chase, the media were removed and replaced with fresh growth medium with or without 0.36 mM oleic acid, and the chase was continued for 3 h. Fig. 4 shows that control cells secreted most of the labeled apoB-48 as HDL density particles when chased with medium not supplemented with oleic acid. The addition of 0.36 mM oleic acid caused a shift of particles from the HDL density fractions to the VLDL density fractions. Inactivation of MTP prior to the addition of oleic acid did not block this conversion. Analysis of the contents of the lumen of the endoplasmic reticulum gave identical results (data not shown), indicating that the density of the secreted particles accurately reflected the state of lipoprotein assembly within the cell.

DISCUSSION

Previous studies have clearly demonstrated that MTP is required for the efficient assembly and secretion of apoB-containing lipoproteins (for review, see Ref. 6). While these studies have been useful in defining the overall requirement for lipid transfer activity in the lipoprotein assembly pathway, they have not determined the exact steps mediated by MTP. Besides translation of the apoB mRNA and translocation of this secretory protein into the lumen of the endoplasmic reticulum, assembly of apoB-48 containing VLDL or chylomicrons requires at least two additional processes. First, the nascent apoB polypeptide must be properly folded and complexed with enough lipid to stabilize the amphipathic, lipid-binding α-helices and β-sheet structures found throughout the protein downstream of the amino-terminal 50–60 kDa (20). This step yields a relatively lipid-poor primordial lipoprotein precursor particle of HDL density. Second, bulk quantities of neutral lipid must be loaded into the core of the particle, increasing its diameter and decreasing its density to that of mature VLDL or chylomicrons (for review, see Ref. 4). Inferences drawn from early indirect studies have led us to propose (6, 21) that MTP mediates the first step of lipoprotein assembly. For instance, an ultrastructural analysis of liver from a homozygous abetalipoproteinemia patient showed no evidence of small, dense lipoprotein particles in the lumen of the endoplasmic reticulum (22). While studies such as these suggest a role for MTP in the first step of apoB-48 lipoprotein assembly, they do not address whether MTP plays a role in the second step. Given its lipid transfer capabilities, MTP may also mediate the loading of bulk neutral lipids to the core of nascent apoB-48 lipoproteins.

In this report, we describe a series of studies designed to address which step(s) in apoB-48 lipoprotein assembly are mediated by MTP. First, MTP and apoB-53 were expressed in HeLa cells, a non-lipoprotein-producing, nonhepatic, nonintestinal cell line that has a robust level of lipid synthesis (~½ the level of HepG2 cells for triglyceride and phosphatidylcholine and 2 times HepG2 levels for cholesteryl ester)[3] but does not express MTP or apoB. Thus, by coexpressing apoB and MTP and analyzing the cell culture medium for the presence of apoB and the physical nature of the apoB lipoproteins, it can be inferred whether either or both steps in lipoprotein assembly involve MTP. The results showed that coexpression of MTP and a close relative to apoB-48, apoB-53, led to the secretion of a particle with the density of HDL when the cells were fed oleic acid and glycerol. Feeding cholesterol in addition to oleic acid did not lead to the formation of less dense particles.

These results can be explained as follows. First, expression of apoB-53 alone in HeLa and other non-lipoprotein-producing cell lines does not lead to lipoprotein secretion (12, 23). Furthermore, it is generally accepted (4) that the product of the first step of apoB-48 lipoprotein assembly is a relatively lipid-poor particle of HDL or similar density that can be secreted from the cell, albeit inefficiently relative to the mature VLDL (4). Thus, the observation that coexpression of apoB-53 and MTP yields secretion of an HDL density particle and not a VLDL density particle suggests that MTP expression along with apoB in an appropriate cell environment is sufficient to reconstitute only the first step in lipoprotein assembly, consistent with the hypothesis that MTP mediates the first step in apoB-48 lipoprotein assembly but not the second step. However, several alternate explanations remain valid. For instance, MTP may not mediate the second step of lipoprotein assembly by itself; some other tissue-specific or pathway-specific factor(s) may be required. This may also be the case with HepG2 cells, which contain active MTP and yet fail to assemble full-size VLDL particles from endogenous apoB-100 (24) or truncated forms of apoB, even those in the size range of

[3] D. A. Gordon and H. Jamil, unpublished observation.

apoB-48 (18, 25). In addition, the level of lipid synthesis in these HeLa-derived cells, while robust, may not be high enough to support apoB-53 VLDL assembly. This is unlikely, because sister cell lines expressing very low levels of apoB also secrete only HDL density particles (data not shown) and, as mentioned above, the HepG2 cells do not secrete VLDL sized particles even despite their high capacity to synthesize all classes of lipids. Third, while structurally similar, apoB-53 and apoB-48 may differ enough to make production of VLDL from apoB-53 impossible.

To distinguish between these possibilities and further extend our observations concerning the role of MTP in the first step of lipoprotein assembly, we turned to the McArdle RH-7777 rat hepatoma cell line. The hepatocytes of certain rodent species, including the rat, produce both apoB-48 and apoB-100 VLDL. While human liver produces only apoB-100 VLDL, the enterocytes of the intestines of all vertebrate species studied produce triglyceride-rich apoB-48 chylomicrons. Thus, this type of cell line is a good model for the intestinal production of triglyceride-rich lipoproteins containing apoB-48 (4). Furthermore, of the commonly studied hepatocyte-derived cell lines, McArdle RH-7777 cells have the most highly developed capability to carry out the second step of apoB-48 lipoprotein assembly (5). In the absence of exogenously supplied fatty acid, McArdle RH-7777 cells produce only the product of the first step of lipoprotein assembly; a relatively lipid-poor apoB-48-containing HDL density particle. This particle is, however, competent to be converted to VLDL upon activation of the second step by the addition of oleic acid to the culture medium.

Fig. 3*B* shows that inactivation of MTP before the pulse-chase protocol, under conditions that only promote the first step of apoB-48 VLDL assembly, leads to inhibition of secretion of both apoB-48 and apoB-100. These data strongly support the conclusion from the HeLa cell experiments that MTP plays a critical role in the first step of lipoprotein assembly. That apoB-100 secretion as well as apoB-48 secretion was blocked suggests that MTP mediates a common process required for assembly of both of these nascent polypeptides into secretion-competent lipoproteins. Since we know that under the conditions of this experiment only the first step of apoB-48 assembly is active, by extension, we can surmise that MTP mediates a similar step in apoB-100 lipoprotein assembly.

To directly address whether or not MTP plays a role in the second step of apoB-48 lipoprotein assembly, MTP was inactivated after the formation of the HDL density particle but before activation of the second step. MTP inhibition clearly did not interfere with the conversion of apoB-48 HDL to VLDL (Fig. 4). Thus, MTP activity is not required for the addition of bulk core lipid to apoB-48 in the second step of apoB-48 lipoprotein production.

This result suggests that the reason why apoB-53 was secreted as an HDL density particle from the HLMB53-59 cells was that another factor(s) besides MTP is required to fully reconstitute assembly of neutral lipid-rich apoB-48 lipoproteins. Evidence for an additional factor comes from two sources. First, Borén, *et al.* (5) showed that pretreatment of McArdle RH-7777 cells with the protein synthesis inhibitor cycloheximide prior to oleate feeding partially blocked the conversion of apoB-48 with HDL density to VLDL density. Further evidence comes from studies of patients with the autosomal recessive disorder, chylomicron retention disease. This disease has been characterized as a defect in the assembly and secretion of chylomicron particles from the intestine. As a result, apoB-48 lipoproteins are retained within the enterocytes, causing a build-up of fat in these cells (11). Clearly, there must be a protein factor, encoded by the chylomicron retention disease gene, that is deficient in these patients, causing a selective defect in the production of apoB-48 particles in the intestine, while maintaining the ability to produce apoB-100 VLDL in the liver. Indeed, biochemical studies of intestinal biopsies from patients afflicted with chylomicron retention disease have shown normal levels of MTP protein and activity, confirming that MTP is not this factor (9). That apoB-48 VLDL assembly appears to require an additional factor, whereas assembly of apoB-100 VLDL does not, suggests that the distal steps in these pathways are fundamentally different. This is consistent with previous studies (25, 26) showing that unlike apoB-48, the assembly of apoB-100 VLDL is closely linked, temporally and spatially, to translocation of nascent apoB polypeptides into the endoplasmic reticulum in a continuous process that may represent only the first step of apoB lipoprotein assembly.

While the identity of the putative factor required for the second step of apoB-48 lipoprotein assembly remains unknown, recent studies have indicated a possible function for this factor. In a recent report (27), it was shown that brefeldin A selectively inhibits the second step in the assembly of apoB-48 VLDL in McArdle RH-7777 cells at a concentration that still allows other secretory proteins as well as the HDL-like primordial particles to be secreted. This suggests that a GTP-binding protein could be involved in the loading of bulk lipid. This additional protein factor most likely plays some role in recruiting, transporting, or joining large quantities of neutral lipid to the nascent apoB-48 lipoprotein within the secretory pathway of the cell.

The studies described herein show, in two different model systems and utilizing two different experimental strategies, that MTP is essential for production of the primordial, HDL density, apoB-48 lipoprotein precursor particles. In addition, MTP most likely mediates a similar process in apoB-100 lipoprotein assembly. In contrast, MTP is clearly not required for the addition of bulk neutral lipid to the core of these apoB-48 HDL density particles. These data provide strong evidence to support the hypothesis that MTP mediates the first step of apoB-48 lipoprotein assembly but not the the second step. In addition, these observations add support for the existence of an additional protein factor required for apoB-48 lipoprotein assembly.

Acknowledgments—We thank Leo Ward for excellent assistance with preparation of the figures. We also thank Drs. Scott Biller, John Dickson, and Ying Chen of the Division of Chemistry, Bristol-Myers Squibb Co. for providing BMS-192951.

REFERENCES

1. Havel, R. J., Goldstein, J. L., and Brown, M. S. (1980) in *Metabolic Control and Disease*, 8th Ed. (Bondy, P. K., and Rosenber, L. E., eds) pp. 393–494, W. B. Saunders, Philadelphia
2. Powell, L. M., Wallis, S. C., Pease, R. J., Edwards, Y. H., Knott, T. J., and Scott, J. (1987) *Cell* **50,** 831–840
3. Alexander, C. A., Hamilton, R. L., and Havel, R. J. (1976) *J. Cell Biol.* **69,** 241–263
4. Innerarity, T. L., Borén, J., Yamanaka, S., and Olofsson, S.-O. (1996) *J. Biol. Chem.* **271,** 2353–2356
5. Borén, J., Rustaeus, S., and Olofsson, S.-O. (1994) *J. Biol. Chem.* **269,** 25879–25888
6. Gordon, D. A., Wetterau, J. R., and Gregg, R. E. (1995) *Trends Cell Biol.* **5,** 317–321
7. Wetterau, J. R., Combs, K. A., Spinner, S. N., and Joiner, B. J. (1990) *J. Biol. Chem.* **265,** 9800–9807
8. Wetterau, J. R., and Zilversmitt, D. B. (1985) *Chem. Phys. Lipids* **38,** 205–222
9. Wetterau, J. R., Aggerbeck, L. P., Bouma, M.-E., Eisenberg, C., Munck, A., Hermier, M., Schmitz, J., Gay, G., Rader, D. J., and Gregg, R. E. (1992) *Science* **258,** 999-1001
10. Shoulders, C. C., Brett, D. J., Bayliss, J. D., Narcisi, T. M. E., Jarmuz, A., Grantham, T. T., Leoni, P. R. D., Bhattacharya, S., Pease, R. J., Cullen, P. M., Levi, S., Byfield, P. G. H., Purkiss, P., and Scott, J. (1993) *Hum. Mol. Genet.* **2,** 2109–2116
11. Kane, J. P., and Havel, R. J. (1989) in *The Metabolic Basis of Inherited Disease*, 6th Ed. (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 1139–1164, McGraw-Hill Inc., New York
12. Gordon, D. A., Jamil, H., Sharp, D., Mullaney, D., Yao, Z., Gregg, R. E., and Wetterau, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7628–7632

13. Leiper, J. M., Bayliss, J. D., Pease, R. J., Brett, D. J., Scott, J., and Shoulders, C. C. (1994) *J. Biol. Chem.* **269,** 21951–21954
14. Patel, S. B., and Grundy, S. M. (1994) *Circulation* **90,** I-186
15. Jamil, H., Gordon, D. A., Eustice, D. C., Brooks, C. M., Dickson, J. K., Jr., Chen, Y., Ricci, B., Chu, C.-H., Harrity, T. W., Ciosek, C. P., Jr., Biller, S. A., Gregg, R. E., and Wetterau, J. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11991–11995
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Lin, M. C. M., Gordon, D., and Wetterau, J. R. (1995) *J. Lipid Res.* **36,** 1073–1081
18. Spring, D. J., Chen-Liu, L. W., Chatterton, J. E., Elovson, J., and Schumaker, V. N. (1992) *J. Biol. Chem.* **267,** 14839–14845
19. Yao, Z., Blackhart, B. D., Linton, M. F., Taylor, S. M., Young, S. G., and McCarty, B. J. (1991) *J. Biol. Chem.* **266,** 3300–3308
20. Segrest, J. P., Jones, M. K., Mishra, U. K., Anatharamaiah, C. M., and Garber, D. W. (1994) *Arterioscler. Thromb.* **14,** 1674–1685
21. Gordon, D. A., Yao, Z., Jamil, H., Yan, M., Wang, S., McLeod, R., and Wetterau, J. R. (1995) *Circulation* **92,** I-362
22. Avigan, M. I., Ishak, K. G., Gregg, R. E., and Hoofnagle, J. H. (1984) *Hepatology* **4,** 1223–1226
23. Thrift, R. N., Drisko, J., Dueland, S., Trawick, J. D., and Davis, R. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 9161–9165
24. Thrift, R. N., Forte, T. M., Cahoon, B. E., and Shore, V. G. (1986) *J. Lipid Res.* **27,** 236–250
25. Borén, J., Graham, L., Wettesten, M., Scott, J., White, A., and Olofsson, S.-O. (1992) *J. Biol. Chem.* **267,** 9858–9867
26. Rusiñol, A., Verkade, H., and Vance, J. E. (1993) *J. Biol. Chem.* **268,** 3555–3562
27. Rustaeus, S., Lindberg, K., Borén, J., and Olofsson, S.-O. (1995) *J. Biol. Chem.* **270,** 28879–28886