Adenovirus-Mediated Overexpression of Microsomal Triglyceride Transfer Protein (MTP): Mechanistic Studies on the Role of MTP in Apolipoprotein B-100 Biogenesis†

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ABSTRACT: The intracellular concentration of the microsomal triglyceride transfer protein large subunit (*l*MTP), the abetalipoproteinemia gene product, is tightly controlled. To date, attempts at overexpressing *l*MTP in vivo or in vitro have been unsuccessful. We successfully overexpressed *l*MTP in HepG2 cells using an adenoviral vector containing an *l*MTP cDNA, AdMTP. AdMTP-transduced HepG2 cells overexpressed MTP activity. They secreted increased amounts of apoB-100 lipoproteins with LDL and HDL density into the medium. *l*MTP overexpression alone minimally changed the density profile of apoB-containing lipoproteins, but addition of oleic acid shifted the profile toward lower densities. Oleic acid had a greater stimulatory effect on apoB-100 secretion in control HepG2 cells than in AdMTP-transduced cells, because (i) adenoviral transduction per se suppressed protein synthesis, affecting apoB-100 and albumin equally, and (ii) adenoviral transduction partially attenuated the increase in triglyceride synthesis in response to oleic acid supplementation. AdMTP treatment greatly diminished the intracellular degradation of apoB-100, but in comparison with recombinant virus containing luciferase cDNA (AdLuc), it caused no change in its biosynthetic rate. It greatly reduced, but did not eliminate, its proteasomal degradation. Our study constitutes the initial demonstration that adenovirus-mediated transfer of *l*MTP markedly stimulates MTP expression which in turn stimulates apoB-100 production. The mechanism involves a downregulation of ubiquitin-proteasome-mediated degradation without any change in synthetic rate.

Apolipoprotein (apo) B-100 is an obligate protein component of very low-density lipoproteins (VLDL) and their metabolic products, intermediate-density (IDL), and low-density lipoproteins (LDL). It is a ligand for the LDL receptor, and its plasma concentration, like that of LDL, is a major determinant of atherosclerosis susceptibility (reviewed in refs *1* and *2*). The plasma concentration of apoB-100-containing lipoproteins is determined by a balance between its production by the liver and its catabolism. The transcription of the apoB gene is constitutive and the intrahepatic apoB mRNA level is generally not affected by various metabolic manipulations (reviewed in refs *3*−*5*). The major site of control of production of apoB by the liver appears to be at the translational and posttranslational levels by varying the extent of intracellular degradation of newly synthesized apoB. In the human hepatoma cell line, HepG2, about 30−60% of newly synthesized apoB-100 is degraded intracellularly and only 40−70% is secreted (*3*−*5*). A number of factors impact on the production and secretion of apoB-100 by liver cells. One important factor is microsomal triglyceride transfer protein (MTP), an endoplasmic reticulum (ER) protein that appears to be required for the secretion of newly synthesized apoB-100. In nonapoB producing cells, transfection of apoB expression vectors does not induce apoB secretion by these cells unless MTP is produced at the same time (*6*, *7*). The apoB produced in cells that do not express MTP appears to be completely degraded intracellularly and never gets secreted.

Microsomal triglyceride transfer protein (MTP) is a heterodimeric protein composed of a 97 kDa subunit (designated *l*MTP for the large subunit of MTP in this study) and a 58 kDa subunit, which has been identified as protein disulfide isomerase (PDI) (*8*) (reviewed in refs *9* and *10*). PDI is a ubiquitous multifunctional ER protein (reviewed in refs *11* and *12*). It catalyzes the insertion of disulfides into folding proteins and corrects errors in disulfide bonding by rearranging them should they occur. The large subunit of MTP, *l*MTP, is expressed mainly in the liver and small intestine (*13*−*15*), the major organs that synthesize and secrete apoB. Mutations in the *l*MTP gene were found to be the basis of abetalipoproteinemia, an autosomal recessively inherited syndrome characterized by the almost complete absence of apoB-containing lipoproteins in the circulation (*13*, *16*−*18*).

Both apoB and MTP expression are crucial to the production of VLDL by the liver. Transgenic mice that overexpress apoB produce increased amounts of apoB-containing lipoproteins (*19*, *20*), whereas patients with heterozygous hypobetalipoproteinemia who have mutations in the apoB gene (*21*) as well as mice with targeted disruption of one apoB allele (*22*) exhibit markedly reduced apoB-containing lipoproteins in the circulation. These observations

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underscore the importance of a normal level of apoB expression in determining apoB production by the liver and suggest that, under certain circumstances, apoB availability is rate limiting. On the other hand, mice heterozygous for *l*MTP gene inactivation have been found to have an attenuated plasma VLDL/IDL/LDL response to high-fat high-cholesterol diet feeding (*23*). Furthermore, specific MTP inhibitors have been shown to downregulate apoB secretion from HepG2 cells in a dose-dependent manner (*24*−*27*). These studies suggest that MTP may be rate limiting under other circumstances. However, whereas transgenic mice overexpressing apoB have been reported (*19*, *20*), transgenic mice that overexpress an *l*MTP-transgene have not been presented. Furthermore, successful overexpression experiments using transient or stable transfection of *l*MTP in cultured hepatocytes or hepatoma cell lines have not been reported. Indeed, Jamil et al. (*27*) commented on their failure to increase MTP expression by this approach, which suggests that *l*MTP gene transcription may be tightly regulated and any attempt to upregulate its expression may be doomed to failure. It is conceivable that the cell guards against *l*MTP overexpression to prevent the latter from heterodimerizing with all the available PDI and removing it from a pool required for other important cellular functions. Therefore, the question remains open whether apoB or MTP expression is rate limiting in the liver in the biogenesis of apoB-containing lipoproteins under normal circumstances. It is also possible that under basal conditions the amount of *l*MTP (and MTP) expressed optimally protects against the early phase of apoB degradation (*10*, *24*, *28*) and its overexpression would not further enhance apoB secretion. To address these issues, we have studied the effects of induced overexpression of *l*MTP in HepG2 cells by the powerful technique of adenovirus-mediated gene transfer (*29*, *30*). Our observations provide new insight into the role of MTP in the biogenesis of apoB-containing lipoproteins.

EXPERIMENTAL PROCEDURE

Reagents. Nitrocellulose membrane was from Schleicher & Schuell. *N*-Ethylmaleimide (NEM), rabbit polyclonal antibody against bovine ubiquitin antiserum, L-α-phosphatidyl choline, oleic acid, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN), cardiolipin and triolein were from Sigma. Gamma-Bind G Sepharose, [1(3)-^{3}H]glycerol and glycerol tri[1-^{14}C]oleate were from Amersham. Lactacystin was obtained from CalBiochem. Mouse monoclonal antibody against ubiquitin and goat polyclonal antibodies against apoB and apoA-I were from Chemicon. Rabbit polyclonal antibody against albumin was from Research Diagnostic, Inc. [^{35}S]-Methionine, methionine, RPMI 1640, and methionine-free RPMI 1640 were from ICN. Monoclonal antibody against apoB (1D1) was kindly provided by Dr. R. W. Milne (Ottawa Heart Institute). Polyclonal antibodies against *l*MTP were kindly provided by Dr. David Gordon (Bristol-Myers Squibb Pharmaceutical Research Institute). Mouse monoclonal antibody against PDI was from StressGen Biotechnologies Corp.

Cell Culture. HepG2 cells were from American Type Culture Collection and were maintained at 37 °C in an atmosphere with 5% CO_2 and in RPMI 1640 medium (GibcoBRL) containing 10% fetal calf serum (FCS, HyClone), penicillin (100 unit/mL), and streptomycin (100 μg/mL) (GibcoBRL).

Generation of Recombinant Adenoviral Vectors. Human *l*MTP cDNA was cloned into the *Bgl*II site of the pAvCvSv shuttle plasmid vector, which contains a cytomegalovirus promoter, translation enhancer sequence, and SV40 polyadenylation signal. The pAvCvSv shuttle plasmid vector containing the full-length human *l*MTP cDNA was cotransfected with pJM17 into human embryonic kidney 293 cells using LipofectAMINE (GibcoBRL). Two weeks after transfection, infectious recombinant adenoviral vector plaques were picked, propagated, and screened for *l*MTP sequences by polymerase chain reaction. Large scale production of the recombinant adenovirus was performed as described previously (*31*). The purified virus was dialyzed overnight at 4 °C against a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol, aliquoted and stored at −80 °C.

Transduction of HepG2 Cells by Recombinant Adenovirus. HepG2 cells were cultured in either 24-well plates (Falcon) or 162 cm^2 flasks (Costar) in RPMI 1640 medium containing 10% FCS, penicillin (100 unit/ml) and streptomycin (100 μg/mL) until 70% confluence. The cells were then infected with the recombinant virus containing *l*MTP cDNA (Ad-MTP), luciferase cDNA (AdLuc), or an equal amount of dialysis buffer (mock) in 0.2 mL/well or 6 mL/flask of RPMI 1640 medium containing 2% FCS for 1 h. After that, 1 mL/well or 20 mL/flask of RPMI 1640 containing 10% FCS was added for further incubation as indicated.

MTP Activity Assay. Two days postinfection, HepG2 cells were harvested and the microsomes were prepared as described earlier (*32*) with the exception that a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 mM leupeptin was used as homogenizing buffer. The microsome pellets were suspended in 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM EDTA, 1 mM PMSF, and 1 mM leupeptin. The protein concentration in the microsome preparation was measured with Bio-Rad DC protein assay reagent. Nine volumes of microsome preparation was mixed with 1 vol of 0.54% deoxycholate (pH 7.4), and the mixture was incubated at 4 °C for 30 min. MTP activity assay was performed by mixing 100 μg of microsome protein with donor and acceptor vesicles in 15 mM Tris-HCl (pH 7.4) containing 40 mM NaCl, 1 mM EDTA, and 0.02% NaN_3 as described previously (*13*).

Pulse-Chase Analysis and Immunoprecipitation. Two day postinfection, HepG2 cells were preincubated for 20 min in methionine-free medium followed by a 30-min pulse labeling with L-[^{35}S]methionine (100 μCi/mL). Chase was initiated by replacing the labeling medium with RPMI 1640 medium containing 10% FCS and cold methionine (15 g/L). In experiments where we studied the effect of oleic acid, oleic acid−albumin complex (360 μM oleic acid) was present in the medium during preincubation and pulse-chase periods; medium for the control cells contained an equal concentration of fatty-acid-free albumin (3.2 mg/mL). At the times indicated, the culture medium was taken out and cell debris removed by low-speed centrifugation. It was then mixed with an equal volume of a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 1 mM PMSF, 0.1 mM ALLN, 5 mM NEM, and complete protease inhibitors (Boehringer Mannheim) for immunoprecipitation as described below. The cells were washed with cold PBS, and lysed for 2.5 h at 4 °C in 2% sodium cholate

in Hepes-buffered saline (50 mM Hepes, 200 mM NaCl) containing 1 mM PMSF, 0.1 mM ALLN, 5 mM NEM and complete protease inhibitors (Boehringer Mannheim). Cell lysates were then centrifuged at 10000*g* for 10 min. The cleared supernatant was used for immmunoprecipitation. Immunoprecipitation was done as described previously (*33*). Aliquots of cleared medium or cell lysate were incubated with the antibody indicated at 4 °C on a gyrating platform for 1 h. Gamma-Bind G Sepharose beads were then added and incubation was continued for 5 h. The beads were washed three times and immunoprecipitates were released by boiling for 5 min in SDS−PAGE buffer in the presence of 5% 2-mercaptoethanol and analyzed by SDS−PAGE on 4 to 15% gradient gel or 6% gel (for apoB) or on 4 to 20% gradient gel (for albumin and apoA-I). Gels were dried and autoradiographic image was captured by a phosphor storage system (Cyclone, Packard) and analyzed by OptiQuant Image Analysis Software. Data were expressed as DLU (digital light units).

Immunoblot Analysis. Western blot analysis was performed on cell lysates as described previously (*32*). In brief, cells were washed with cold PBS and lysed in a buffer containing 0.5% sodium deoxycholate, 0.5% Triton X-100, 5 mM EDTA, 150 mM NaCl, 62.5 mM sucrose, 1 mM PMSF, and 50 mM Tris (pH 7.4). Insoluble material in the cell lysate was removed by centrifugation at 10000*g* for 10 min. Aliquots of cleared cell lysate were mixed with SDS−PAGE buffer containing 5% 2-mercaptoethanol and heated to boiling for 5 min. The proteins were separated by SDS−PAGE and transferred overnight onto nitrocellulose membranes, probed with polyclonal anti-MTP antibody followed by appropriate peroxidase-conjugated secondary anti-IgG; detection was by enhanced chemiluminescence (ECL kit, Amersham). Immunoblot analysis of apoB in the cell lysate and culture medium was performed in the immunoprecipitated samples. ApoB was probed with peroxidase-conjugated monoclonal anti-apoB (1D1). The relative intensity of the immunoblot bands was quantified by AlphaImagerTM 2000 Documentation & Analysis system (Alpha Innotech Corporation).

Sucrose Gradient Ultracentrifugation of Lipoproteins. The lipoproteins present in the medium were separated by sucrose gradient ultracentrifugation as described by Borén et al. (*34*). Each gradient was formed by layering the following from the bottom of the tube: 2 mL of 47% sucrose, 2 mL of 25% sucrose, 5 mL of sample in 12.5% sucrose, and 3 mL of PBS. The gradients were centrifuged in a Beckman SW40 rotor at 35 000 rpm at 4 °C for 65 h. Gradients were unloaded from the top of the tube into 12 fractions. Lipoproteins in each fraction were precipitated by trichloroacetic acid and washed with acetone, and apolipoproteins were recovered by elution into SDS−PAGE buffer as described (*35*, *36*), and resolved by 6% SDS−PAGE. ApoB was visualized by immunoblot analysis using a monoclonal antibody (1D1) against human apoB as described above.

Triglyceride Synthesis. [^{3}H]Glycerol was used as a tracer to measure triglyceride synthesis. Two days posttransduction, the culture medium was replaced by the medium containing [^{3}H]glycerol (2 μCi/mL) in the presence of either oleic acid−albumin complex (360 μM oleic acid and 3.2 mg albumin/mL) or albumin alone for a 30 min incubation. After incubation, the cells were washed with cold PBS, and the cellular lipids were extracted with chloroform/methanol (1:2, vol/vol) (*37*). The lipids were separated by thin-layer chromatography on a Whatman silica gel 60 plate developed in petroleum ether/diethyl ether/acetic acid (80:20:1, vol/vol/vol). The triglyceride spots were identified by iodine vapor and counted in a beta counter.

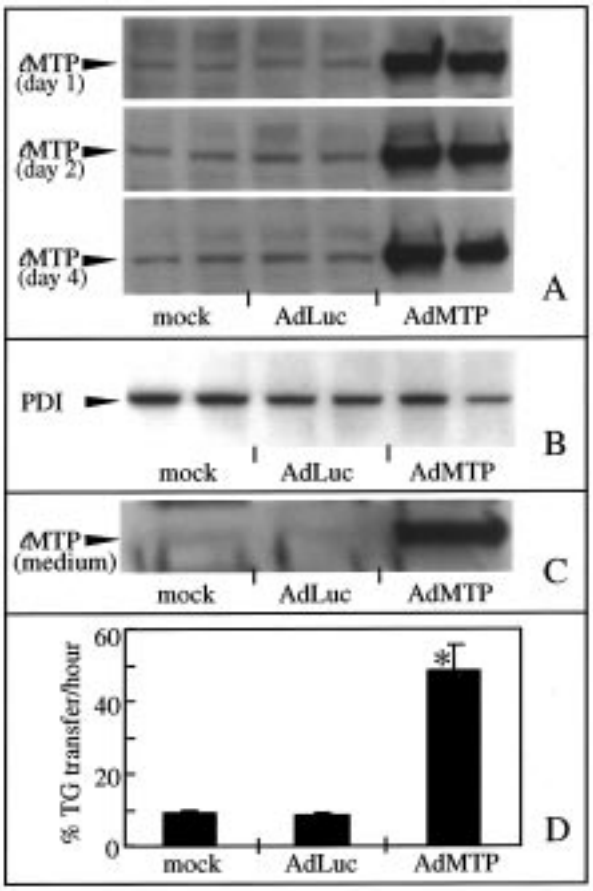

FIGURE 1: AdMTP induces MTP expression. (A) HepG2 cells were cultured in 24-well plates until about 70% confluence and then infected with AdMTP, AdLuc, or buffer (mock). One, 2 or 4 days posttransduction, the cells were lysed and *l*MTP was determined by immunoblotting as described in the Materials and Methods. (B) Two days posttransduction, PDI in the cell lysate was determined by immunoblotting. (C) Two days post-transduction, the culture medium was collected and *l*MTP was determined by immunoblotting. (D) Two days post-transduction, the microsome was prepared and MTP activity was determined as described in the Materials and Methods. Data are expressed as mean ± SEM ($n = 3$) and statistical significance was determined by two-tailed *t*-test. (*) $P < 0.01$ vs mock or AdLuc.

RESULTS

Adenovirus-Mediated Transfer of MTP Large Subunit (lMTP) cDNA Leads to Accumulation of MTP in HepG2 Cells. We produced AdMTP, an E1a-deleted adenoviral vector containing the human *l*MTP cDNA driven by a cytomegalovirus promoter, by a method described in the Materials and Methods. Another vector, AdLuc, had a similar structure except that a luciferase cDNA instead of *l*MTP cDNA was inserted into the vector. We transduced HepG2 cells with AdMTP or AdLuc; as an additional control, we also mock-transduced the cells with a equal volume of dialysis buffer. The relative amount of intracellular *l*MTP in the treated cells was determined by Western blotting on day 1, 2, or 4 following transduction. As shown in Figure 1A, there was marked stimulation of intracellular *l*MTP accumulation

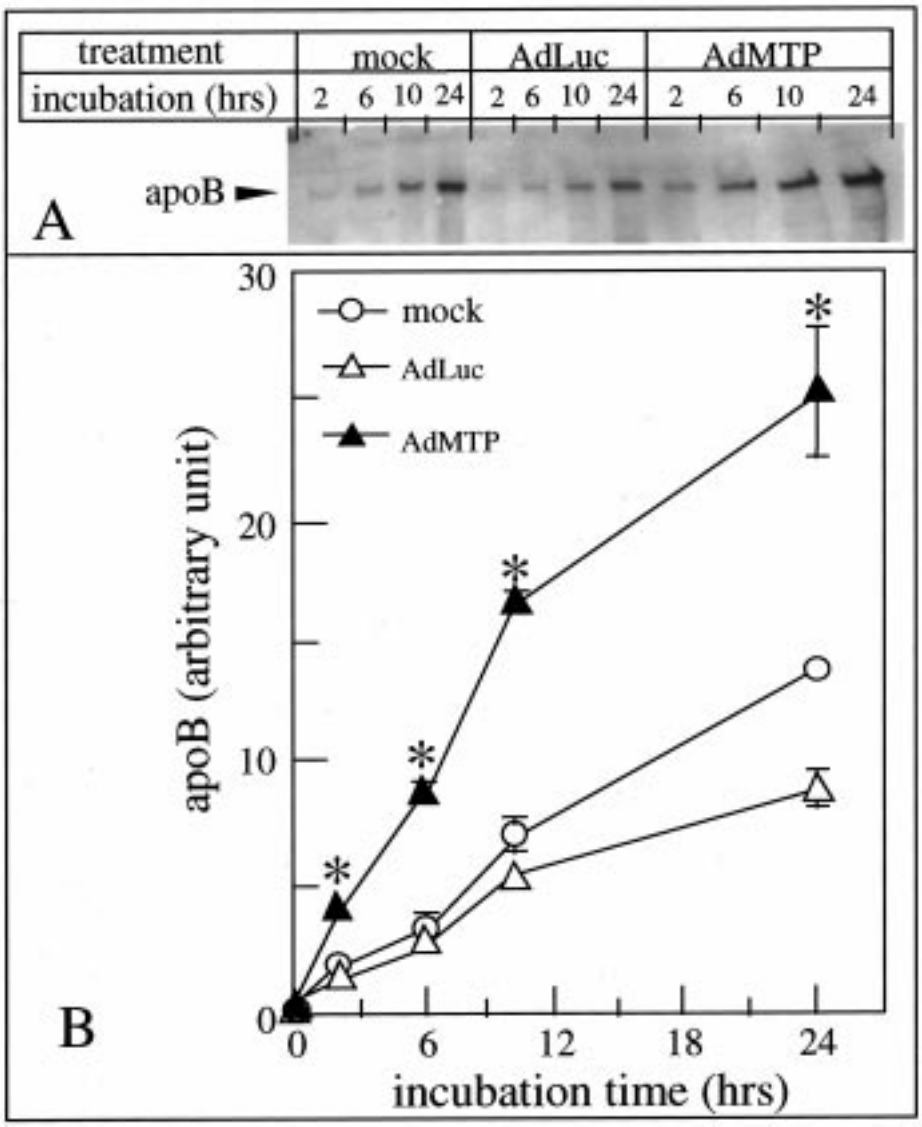

FIGURE 2: AdMTP stimulates apoB secretion. HepG2 cells were cultured in 162 cm^2 flasks until about 70% confluence, and then infected with AdMTP, AdLuc, or buffer (mock). One day posttransduction, the culture medium was changed and aliquots of the culture medium (0.5 mL) were collected at various times of a further 24 h incubation. ApoB in the medium was then analyzed by immunoblotting as described in the Materials and Methods. One representative of three apoB immunoblots is shown in the upper panel (A). ApoB quantification by densitometry is shown in the lower panel (B). Data are expressed as mean ± SEM ($n = 3$) and statistical significance was determined by two-tailed *t*-test. (*) $P < 0.01$ or less vs the corresponding value for mock or AdLuc.

at all three time points (~9-fold increase). In contrast, AdLuc was without effect on the expression of *l*MTP at any of the time points. Therefore, AdMTP was highly effective in inducing *l*MTP production in HepG2 cells. We further examined whether the upregulation of *l*MTP would have any effect on the expression of PDI, the small subunit of MTP. By Western blot analysis at day 2 (Figure 1B), there was a minimal reduction in PDI expression in AdMTP- and AdLuc-treated cells compared with mock-treated cells.

*l*MTP normally forms a heterodimer with PDI which contains an ER-retention signal. *l*MTP, which does not have an ER-retention sequence, is thought to be retained in the ER solely as a result of its binding to PDI. We asked whether *l*MTP overexpression might saturate the capacity of PDI to retain the protein in the ER and the excess *l*MTP might be secreted into the culture medium. We analyzed for the presence of *l*MTP in the culture medium by Western blotting. Indeed, by this technique, a significant amount of the protein was detected in the medium of AdMTP-transduced cells. In contrast, *l*MTP was not detectable in the medium of AdLuc-, or mock-transduced cells (Figure 1C).

To express lipid-transfer activity, *l*MTP must be complexed with PDI (*38*). We examined whether the induced overexpression of *l*MTP in HepG2 cells was associated with a change in MTP activity, which would imply that the *l*MTP formed a functional complex with PDI. We isolated microsomal fraction from mock-, AdLuc-, and AdMTP-treated cells and assayed for MTP activity (*13*). We found that AdLuc transduction did not alter MTP activity but AdMTP treatment stimulated MTP activity of HepG2 cells by approximately 5-fold (Figure 1D). This degree of stimulation is lower than the 9-fold increase in *l*MTP accumulation. It is unclear whether the increase in MTP activity is limited by the availablity of PDI that heterodimerizes with *l*MTP to form the functional MTP protein.

AdMTP-Induced MTP Overexpression Stimulates apoB-100 Secretion by HepG2 Cells. To determine whether AdMTP transduction alters apoB-100 production and secre-

tion by HepG2 cells, we determined the relative amounts of apoB-100 secreted into the medium in 24 h of culture by SDS−PAGE followed by Western blotting, starting at day 1 following transduction. Mock- and AdLuc-transduced cells were used as controls. It is clear from Figure 2 that adenovirus transduction per se inhibits apoB-100 secretion by HepG2 cells. At 24 h the amount of apoB-100 detected in the culture medium of AdLuc-treated cells was reduced to 65% of the mock-transduced cells. In contrast, AdMTP transduction markedly stimulated apoB-100 secretion. At 24 h, apoB-100 in the medium from AdMTP-transduced cells was 84% higher compared with mock-transduced cells; in comparison with the AdLuc-transduced cells, the apoB-100 concentration in the medium of AdMTP-transduced cells was 180% higher, indicating that there was marked enhancement in the amount of apoB secreted into the medium by HepG2 cells that were overexpressing *l*MTP.

Density Profile of apoB-100-Containing Lipoproteins Induced by AdMTP Treatment of HepG2 Cells. We compared the density profile of the apoB-100 lipoprotein particles produced by HepG2 cells treated with buffer (mock), AdLuc, or AdMTP (Figure 3). The lipoproteins secreted into the medium between 24 and 48 h of treatment were fractionated by sucrose gradient ultracentrifugation. Individual fractions were analyzed by SDS−PAGE and Western blotting. As shown in Figure 3B, the apoB-100-containing lipoproteins secreted from HepG2 cells had a density that overlaps LDL and HDL densities. AdLuc transduction reduced apoB-100 lipoproteins in all fractions, but especially those in the LDL range. AdMTP treatment produced only a minimal change in the density distribution of the apoB-100 containing lipoproteins. Compared to mock controls, the major increase happened toward the lighter end of the density range, affecting the LDL and large HDL-like particles, resulting in a relative reduction in the smallest HDL-sized particles. Compared with AdLuc controls, AdMTP treatment caused a marked increase in apoB-100 in the lighter (LDL-sized and very large HDL-sized) fractions because the apoB-100 bands in these fractions were much lighter in the AdLuc cells. Therefore, the density profile of the lipoproteins produced by HepG2 cells in response to the induced MTP overexpression was changed minimally by the MTP overexpression with a slight preference for the larger particles compared with the AdLuc and mock controls.

Overexpression of lMTP Enhances apoB-100 Production by HepG2 Cells. To understand the underlying mechanism by which *l*MTP overexpression stimulates apoB secretion by HepG2 cells, we performed pulse-chase experiments to examine the fate of newly synthesized apoB. Two days posttransduction, HepG2 cells were labeled with [^{35}S]-methionine for 30 min and chased with excess unlabeled methionine for 1 h. The amount of [^{35}S]methionine-labeled apoB-100, apoA-I, and albumin in the total cell lysate and culture medium was quantified by immunoprecipitation followed by SDS−PAGE and autoradiography as described in the Materials and Methods. As shown in Figure 4A, AdLuc transduction led to an ∼50% reduction of [^{35}S]apoB-100 in the cell lysate and an ∼70% reduction of [^{35}S]apoB-100 secreted into the culture medium, compared with the mock-transduced controls. This effect of AdLuc indicates that infection of HepG2 cells by these adenoviral vectors inhibits apoB synthesis and secretion. In AdMTP-transduced

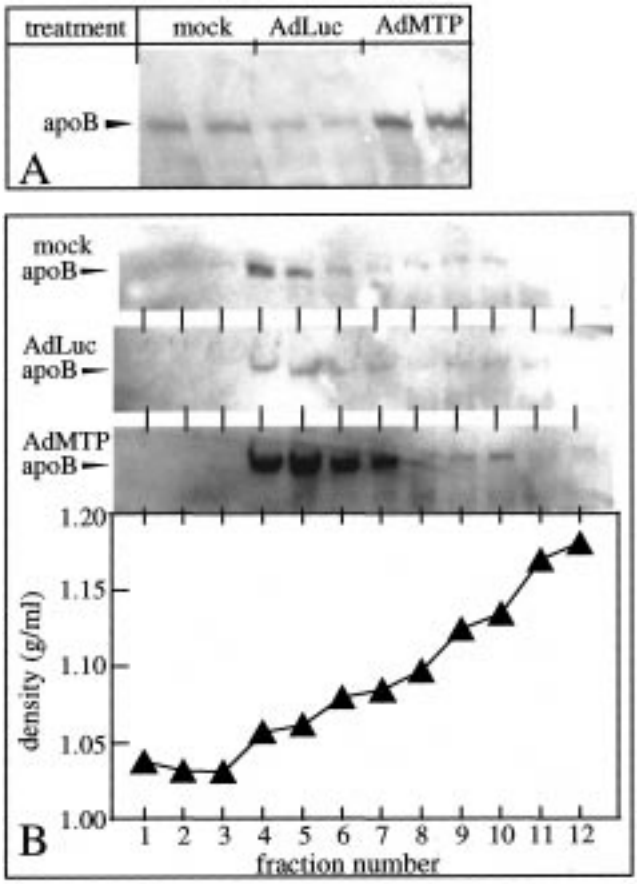

FIGURE 3: Sucrose gradient ultracentrifugation analysis of AdMTP-stimulated apoB-containing lipoproteins. HepG2 cells were cultured in 162 cm^2 flasks until about 70% confluence, and then infected with AdMTP, AdLuc, or buffer (mock). Each group consisted of two flasks. One day posttransduction, the culture medium was changed for another 24 h incubation. After the 24 h incubation, the culture medium was harvested. An aliquot of the culture medium was used for apoB immunoblotting (A). The culture medium from each treatment condition was pooled for lipoprotein separation by sucrose gradient ultracentrifugation as described in the Materials and Methods. ApoB in the fractions of the gradient was analyzed by immunoblotting (B).

cells, the intracellular [^{35}S]apoB-100 was restored to mock-control levels. The amount of [^{35}S]apoB-100 secreted into the medium was increased about ∼6-fold compared with AdLuc-transduced cells, being more than twice that in control cells treated with buffer alone. Therefore, MTP overexpression increases the production of apoB-100, possibly by protecting the intracellular apoB-100 against degradation and facilitating its secretion into the medium.

To determine whether the AdMTP effects are specific for apoB-100, we examined two other secretory proteins, apoA-I and albumin, in the same experiment. It is apparent that adenoviral vector transduction reduced the amount of both [^{35}S]apoA-I and [^{35}S]albumin inside the cell as well as in the medium (Figure 4, panels B and C). The results are similar whether AdLuc or AdMTP was used for transduction, indicating that AdMTP treatment neither protected the intracellular [^{35}S]apoA-I or [^{35}S]albumin, nor did it facilitate the secretion of these proteins into the culture medium. The results were much more evident when we examined the ratio of [^{35}S]apoB-100/[^{35}S]albumin in the medium (Table 1); the AdMTP-treated cells displayed an ∼4-fold increase over mock-treated cells, and an ∼10-fold increase over AdLuc-

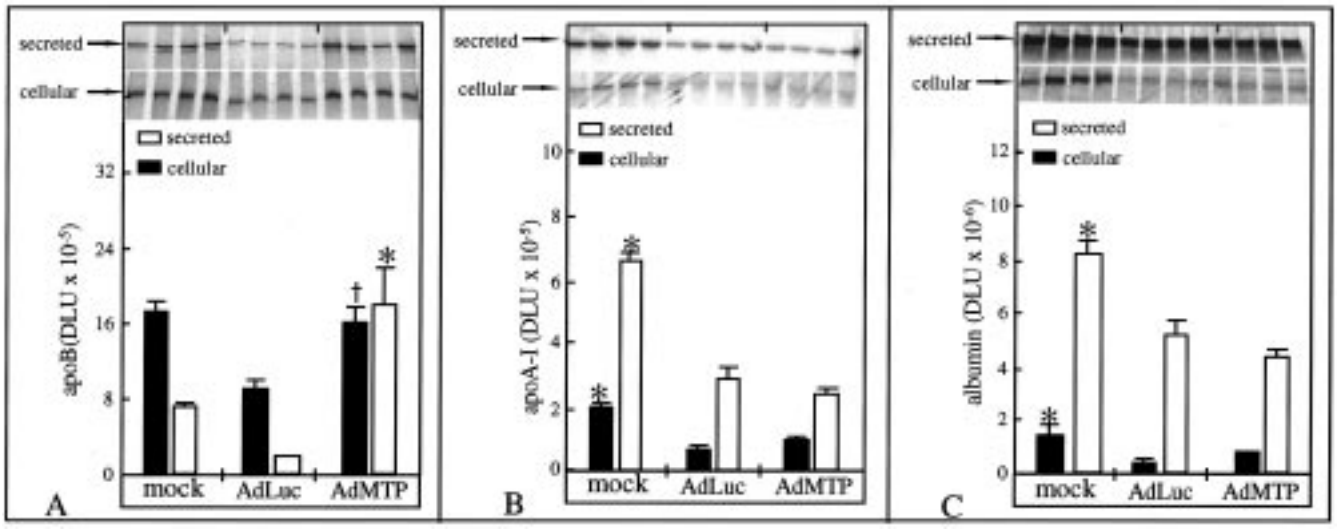

FIGURE 4: AdMTP effects on apoB, apoA-I and albumin secretion. HepG2 cells were cultured in 24-well plates until about 70% confluence, and then infected with AdMTP, AdLuc, or buffer (mock). Two days post-transduction, the cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for 1 h. After the chase, apoB, apoA-I, and albumin in the culture medium and cell lysate were immunoprecipitated and quantified by SDS−PAGE as described in Materials and Methods. Data are expressed as mean ± SEM ($n = 4$) and statistical significance was determined by two-tailed *t*-test. (A) Cellular and secreted apoB. (*) $P < 0.05$ vs mock, $P < 0.001$ vs AdLuc; (†) $P < 0.005$ vs AdLuc. (B) Cellular and secreted apoA-I. (*) $P < 0.001$ or less vs the corresponding value for AdLuc or AdMTP. (C) Cellular and secreted albumin. (*) $P < 0.01$ or less vs the corresponding value for AdLuc or AdMTP.

Table 1: AdMTP Effects on ApoB and ApoA-I Secretion: Amount of ApoB-100 and ApoA-I Secreted Relative to the Amount of Albumin Secreted[a]

treatment	apoB/albumin ratio	apoA-I/albumin ratio
mock	0.095 ± 0.014	0.081 ± 0.005[b]
AdLuc	0.040 ± 0.002	0.059 ± 0.006
AdMTP	0.413 ± 0.089[c]	0.056 ± 0.003

[a] Data are derived from the experiment shown in Figure 4 and expressed as mean ± SEM ($n = 4$) and statistical significance was determined by two-tailed *t*-test. [b] $P < 0.05$ vs AdLuc, $P < 0.01$ vs AdMTP (for apoA-I/albumin ratio). [c] $P < 0.02$ vs mock, $P < 0.01$ vs AdLuc (for apoB/albumin ratio).

treated cells. In contrast, a minor reduction was observed in the [^{35}S]apoA-I/[^{35}S]albumin ratio in both AdLuc- and AdMTP-treated cells, which suggests that the MTP effect does not extend to another apolipoprotein, apoA-I (Table 1).

AdMTP Protects Newly Synthesized apoB from Intracelluar Degradation. We wished to examine the detailed kinetics of newly synthesized apoB-100 accumulation in HepG2 cells and its secretion into the medium following AdMTP transduction. Transduced HepG2 cells were pulse-labeled with [^{35}S]methionine for 30 min and chased for 0, 30, 60, and 120 min. The intracellular and secreted [^{35}S]-apoB were quantified by immunoprecipitation and SDS−PAGE at the different time points. In mock-transduced HepG2 cells, in agreement with previous reports on apoB-100 biosynthesis (*33*, *39*), as [^{35}S]apoB-100 was secreted into the medium (Figure 5, panel A-1), intracellular [^{35}S]-apoB-100 level went down (Figure 5, panel A-2). However, the total [^{35}S]apoB-100 obtained by adding the intracellular and secreted labeled protein did not stay constant; it decreased substantially with time, such that, by 120 min, only about 40% of the initial [^{35}S]apoB-100 remained (Figure 5C, top), indicating that about 60% of the newly synthesized apoB was degraded and never got secreted.

In AdLuc-transduced cells, there was marked inhibition of secretion of [^{35}S]apoB-100 into the medium (Figure 5, panel A-1) during a time that intracellular [^{35}S]apoB-100 was decreasing (Figure 5, panel A-2). At the end of 120 min, only about 20% of the total (intracellular + secreted) [^{35}S]-apoB-100 remained (Figure 5C, top). Therefore, adenoviral vector transduction of HepG2 cells stimulates intracellular degradation of apoB-100. In contrast, AdMTP transduction stimulated [^{35}S]apoB-100 secretion into the medium (Figure 5, panel A-1). When we summed up the cellular and secreted [^{35}S]apoB-100, there is clearly protection of newly synthesized apoB-100 in AdMTP-infected cells such that, at 120 min, ~55% of the [^{35}S]apoB-100 was accountable compared with the 0 min value. Compared with the AdLuc-transduced cells, this constitutes a better than 2-fold protection (Figure 5C, top).

To examine whether the AdMTP effects are specific for apoB-100, we studied the effects of AdMTP, AdLuc, and mock transduction on the fate of newly synthesized albumin in the same experiment. As evident from Figure 5, panels B-1, B-2, and C, both types of adenoviral vector, AdMTP and AdLuc, produced marked inhibition of [^{35}S]albumin secretion (Figure 5, panel B-1). They were associated with a similar extent of degradation of the intracellular [^{35}S]-albumin of approximately 20% of mock controls at 120 min (Figure 5C, bottom). In conclusion, the effects of MTP overexpression on apoB-100 secretion (stimulation) and intracellular degradation (inhibition) are specific for apoB-100 and not extended to another secretory protein, albumin.

AdMTP Transduction of HepG2 Cells Influences Degradation but Not Synthesis of apoB-100 Compared with AdLuc Transduction. From the last set of experiments, we conclude that AdMTP protects against the intracellular degradation of apoB-100. However, the design of the experiments in Figure 5 does not allow us to conclude whether the biosynthetic rate of apoB-100 is affected by MTP overexpression. To address this issue, we examined the incorporation of [^{35}S]methionine into apoB-100 and albumin during a short pulse of 10 min and a more prolonged labeling period

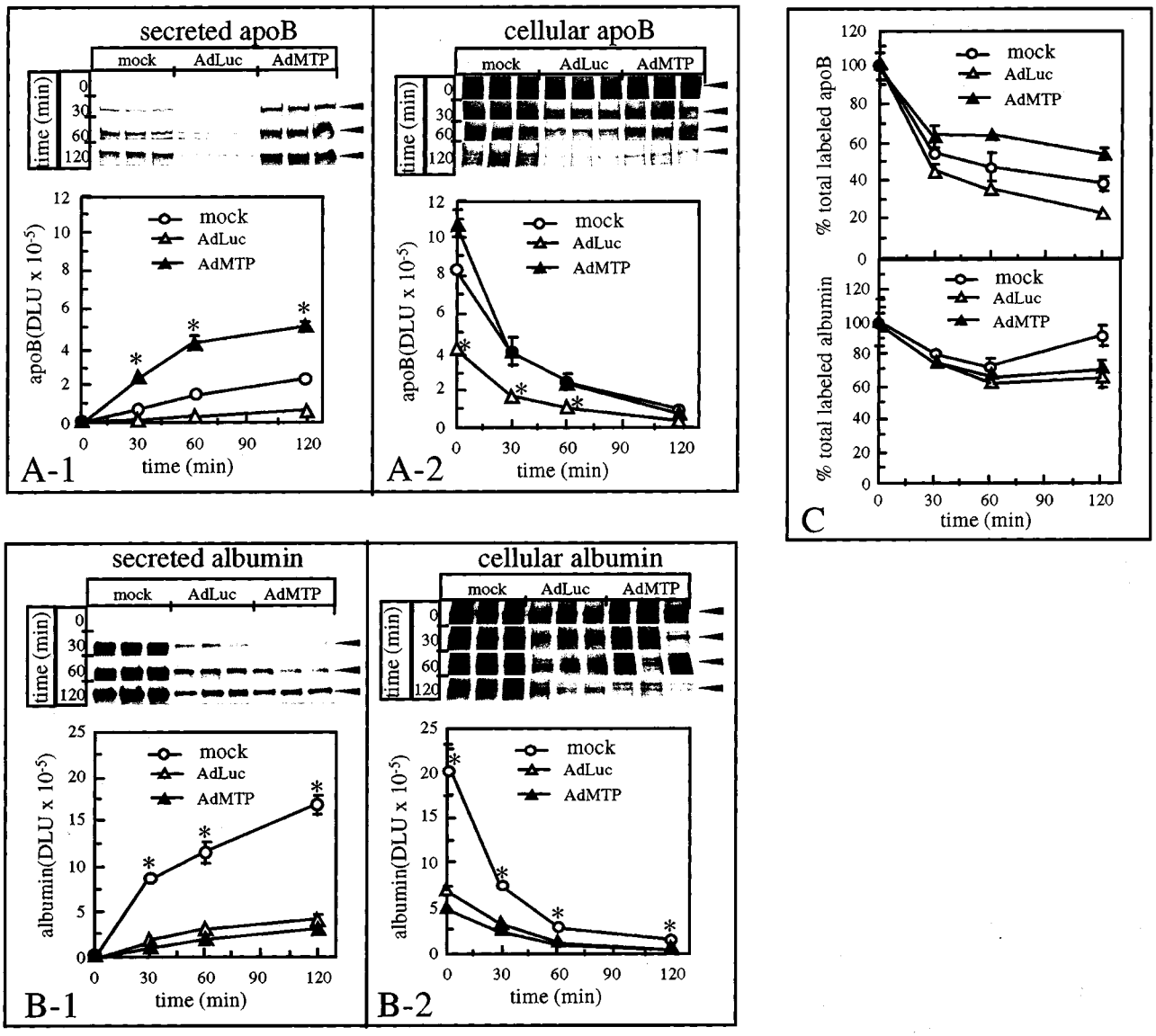

FIGURE 5: Effect of AdMTP on intracellular and secreted apoB and albumin. HepG2 cells were cultured in 24-well plates until about 70% confluence and then infected with AdMTP, AdLuc, or buffer (mock). Two days posttransduction, the cells were pulse labeled with [^{35}S]-methionine for 30 min and chased for 0, 30, 60, 120 min. After the chase, apoB and albumin in the culture medium and in the cell lysate were immunoprecipitated and quantified by SDS−PAGE as described in the Materials and Methods. Data are expressed as mean ± SEM ($n = 3$) and statistical significance was determined by two-tailed t-test. (A-1) Secreted apoB. (*) $P < 0.01$ or less vs the corresponding value for mock or AdLuc. (A-2) Cellular apoB. (*) $P < 0.05$ or less vs the corresponding value for mock or AdMTP. (B-1) Secreted albumin. (*) $P < 0.002$ or less vs the corresponding value for AdLuc or AdMTP. (B-2) Cellular albumin. (*) $P < 0.02$ or less vs the corresponding value for AdLuc or AdMTP. (C) Total labeled apoB (upper panel) and albumin (lower panel) remaining during the chase.

of 30 min. In the short pulse, the effect of degradation would be greatly minimized and we would be examining biosynthesis preferentially. As shown in Figure 6, adenoviral infection with either AdMTP or AdLuc produced an inhibition of albumin and apoB-100 synthesis at the 10 min time point. There was no difference whether AdMTP or AdLuc was used as they were equally potent in reducing [^{35}S]-methionine incorporation into albumin or apoB-100 compared with buffer (mock) controls. At 30 min of labeling, [^{35}S]methionine incorporation into albumin continued to be linear (Figure 6A), indicating that, during this time, there is no significant degradation of this protein. However, for apoB-100, the [^{35}S]methionine incorporation curves for the two types of adenovirus-infected cells diverged markedly (Figure 6B). The curve for AdMTP stayed linear, indicating that intracellular degradation of the newly synthesized protein was not a major factor during this time, whereas the curve for the AdLuc-infected cells took a downward turn, indicating that the newly synthesized apoB-100 was being degraded rapidly. We note that the mock-transduced HepG2 cells also displayed a biphasic curve, indicating that, in the absence of any adenovirus transduction, there was also significant degradation of [^{35}S]methionine-labeled apoB-100 in the cell such that there was actually no net gain in radioactivity incorporated into apoB-100 at 30 min of labeling compared with the 10 min time point. These experiments indicate that the major mechanism for the protective effect of MTP overexpression on apoB-100 in HepG2 cells is not by stimulating its synthesis, but by inhibiting its degradation inside the cell.

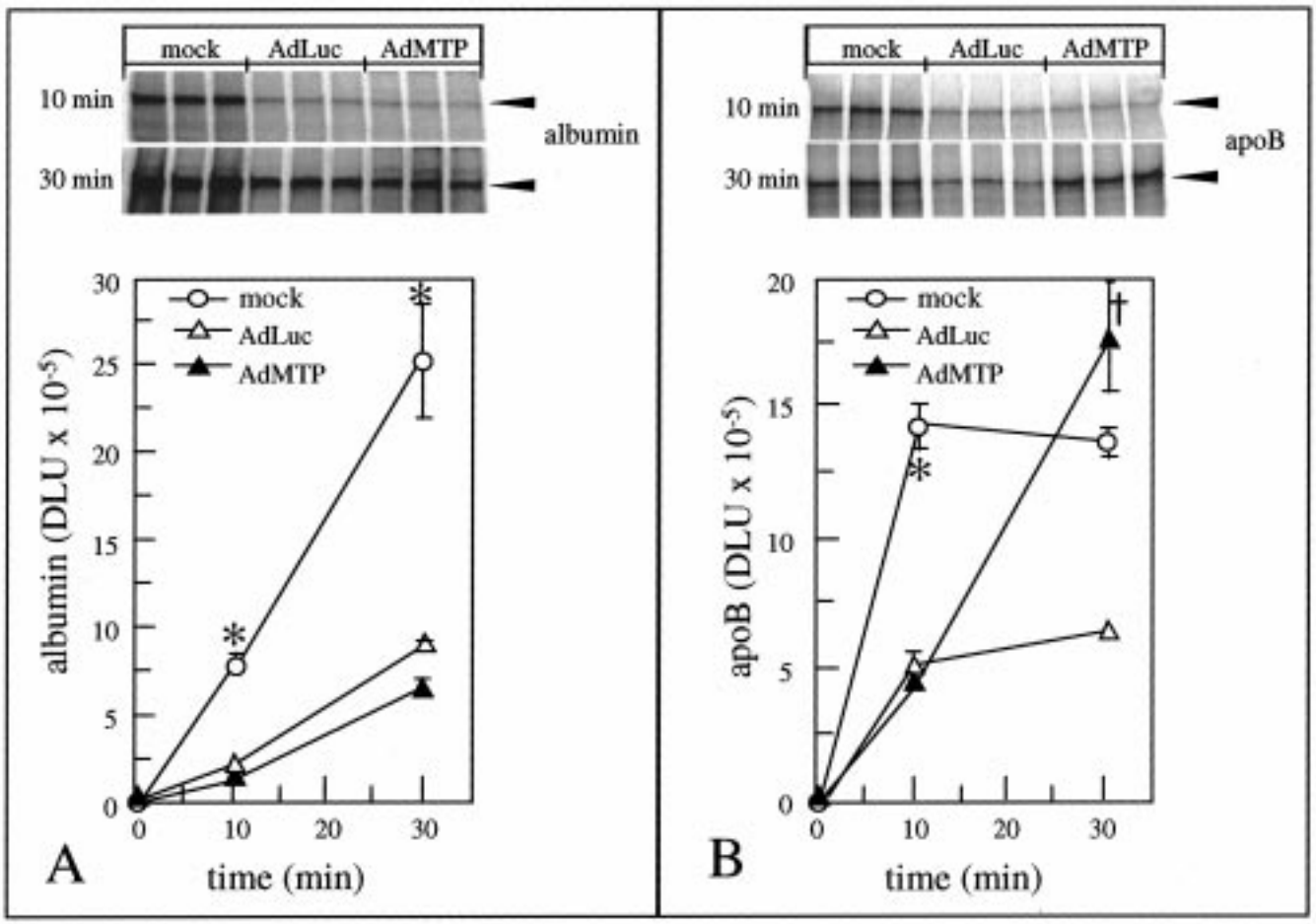

FIGURE 6: Effect of AdMTP on initial apoB and albumin synthesis. HepG2 cells were cultured in 24-well plates until about 70% confluence and then infected with AdMTP, AdLuc, or buffer (mock). Two days posttransduction, the cells were pulse labeled with [^{35}S]methionine for 10 or 30 min. After the labeling, the cells were lysed, and apoB and albumin in the culture medium and in the cell lysate were immunoprecipitated and quantified by SDS−PAGE as described in the Materials and Methods. Data are expressed as mean ± SEM (n = 3) and statistical significance was determined by two-tailed t-test. (A) Cellular albumin. (*) $P < 0.02$ or less vs the corresponding value for AdLuc or AdMTP. (B) Cellular apoB. (*) $P < 0.001$ or less vs the corresponding value for AdLuc or AdMTP. (†) $P < 0.01$ vs the corresponding value for AdLuc.

Effect of Oleic Acid Supplementation on AdMTP-Stimulated apoB-100-Containing Lipoproteins. Oleic acid supplementation stimulates triglyceride synthesis in HepG2 cells and has been reported to stimulate apoB-100 secretion by these cells (*40*). We studied the interaction of oleic acid supplementation and *l*MTP overexpression in HepG2 cells. AdMTP transduction stimulated the accumulation of apoB-100 in the medium of HepG2 cells compared with AdLuc-transduced cells. However, the presence or absence of oleic acid in the culture medium produced only a minor difference in these vector-transduced cells (Figure 7A, also see next section for quantitation). We next analyzed the density distribution of the apoB-100-containing lipoproteins in the medium by sucrose gradient ultracentrifugation (Figure 7B). Oleic acid addition had little or no effect on the density distribution of the apoB-100-containing lipoproteins in AdLuc-treated cells which secreted only a small amount of the lipoproteins. In AdMTP-transduced cells, the presence of oleic acid resulted in a definite shift in the distribution of the apoB-100 in the medium toward lighter particles that are VLDL/LDL-sized with a concomitant decrease in the small HDL-sized particles (Figure 7B).

Effect of Oleic Acid on apoB-100 and Albumin Distribution in the Cell and in the Medium. To examine the effect of oleic acid supplementation on apoB biogenesis, we pretreated HepG2 cells with oleic acid for 20 min. The cells were then labeled with [^{35}S]methionine for 30 min and chased with excess unlabeled methionine for 1 h. The amount of ^{35}S-labeled apoB in the cell and medium was measured, comparing buffer (mock)-, AdLuc-, or AdMTP-treated cells in the presence or absence of oleic acid (Figure 8A). The presence of oleic acid stimulated ^{35}S-labeled apoB-100 accumulation markedly both inside the cell and in the medium in the mock control, in which the amount of [^{35}S]-apoB-100 secreted went up from 43 to 56% of the total (secreted + cellular) [^{35}S]apoB-100. The amount of radiolabeled apoB-100 present either inside the cell or in the culture medium was much lower following AdLuc treatment in the absence of oleic acid. In the presence of oleic acid, there was significant stimulation of [^{35}S]apoB-100 accumulation in both compartments, but the final level is still much lower than that in either the mock or AdMTP-treated cells. In AdMTP-transduced cells, the addition of oleic acid caused a minor but significant ($p < 0.02$) increase in the amount of [^{35}S]apoB-100 secreted into the culture medium and no change in intracellular [^{35}S]apoB-100. In parallel experiments, the absence or presence of oleic acid had no effect on the intracellular or secreted ^{35}S-labeled albumin under

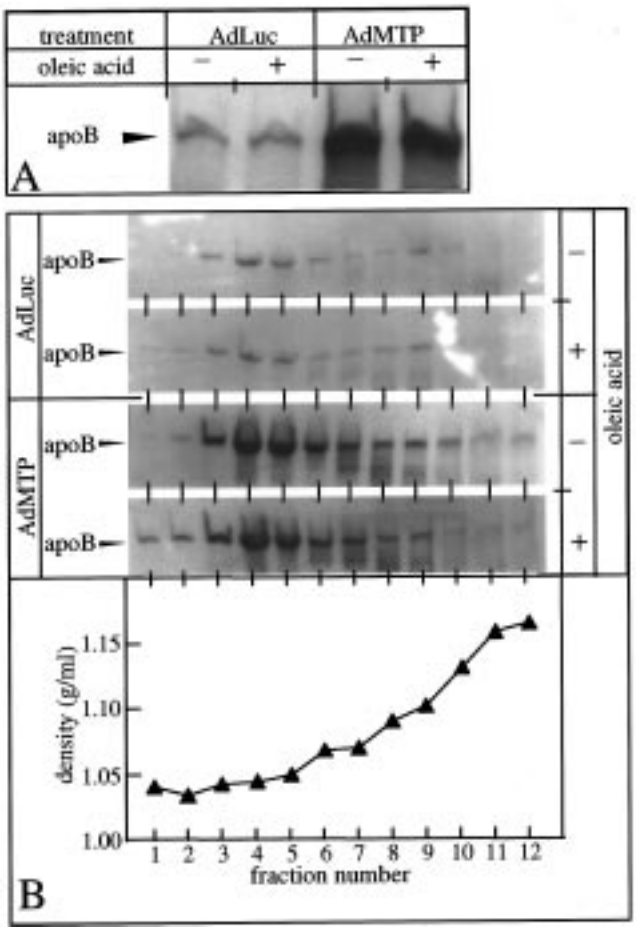

FIGURE 7: Effect of oleic acid supplementation on AdMTP-stimulated apoB-containing lipoproteins. HepG2 cells were cultured in 162 cm^2 flasks until about 70% confluence, and then infected with AdMTP or AdLuc. One day posttransduction, culture medium was replaced by medium containing either oleic acid-albumin or albumin alone for another 24 h incubation. After the 24 h incubation, the culture medium was harvested. Aliquots of the culture medium were used for apoB immunoblotting (A) and for lipoprotein separation by sucrose gradient ultracentrifugation (B) as described in the Materials and Methods. ApoB in the fractions of the gradient were analyzed by immunoblotting.

Table 2: Effect of Oleic Acid on ApoB Secretion: Amount of ApoB Relative to Albumin Secreted in the Absence and Presence of Oleic Acid[a]

	apoB/albumin ratio	
treatment	−oleic acid	+oleic acid
mock	0.2368 ± 0.0087	0.6784 ± 0.0526[b]
AdLuc	0.2212 ± 0.0072	0.5269 ± 0.03266[c]
AdMTP	1.1311 ± 0.0982[d]	1.4550 ± 0.1460[e]

[a] Data are derived from the experiment shown in Figure 8 and expressed as mean ± SEM ($n = 4$) and statistical significance was determined by two-tailed *t*-test. [b] $P < 0.001$ vs mock in the absence of oleic acid. [c] $P < 0.001$ vs AdLuc in the absence of oleic acid. [d] $P < 0.001$ vs mock or AdLuc in the absence of oleic acid. [e] $P < 0.005$ vs mock in the presence of oleic acid, $P < 0.001$ vs AdLuc in the presence of oleic acid.

all three (mock, AdLuc, or AdMTP) treatment conditions (Figure 8B). We calculated the secreted ^{35}S-labeled apoB-100/albumin ratio under the various experimental conditions (Table 2). As observed previously (*40*, *41*), the presence of oleic acid stimulated this ratio 2.8-fold in mock-treated cells ($p < 0.001$), 2.3-fold in AdLuc-treated cells ($p < 0.001$) and 1.28-fold in AdMTP-treated cells. The slight increase in the AdMTP-treated cells was not significant statistically ($p = 0.11$).

Table 3: Effect of Oleic Acid on Triglyceride Synthesis[a]

	[^{3}H]glycerol incorporated into triglyceride (cpm)	
treatment	−oleic acid	+oleic acid
mock	372 ± 103	3043 ± 179[b]
AdLuc	243 ± 54	1178 ± 188[c]
AdMTP	303 ± 35	1753 ± 363[d]

[a] Two days posttransduction, the culture medium was replaced by the medium containing [^{3}H]glycerol (2 μCi/mL) in the presence of either oleic acid-albumin or albumin alone for a 30 min incubation. After the incubation, the cellular lipids were extracted and analyzed by thin-layer chromatography. Data are expressed as mean ± SEM ($n = 3$) and statistical significance was determined by two-tailed *t*-test. [b] $P < 0.001$ vs mock in the absence of oleic acid. [c] $P < 0.01$ vs AdLuc in the absence of oleic acid, $P < 0.02$ vs mock in the presence of oleic acid. [d] $P < 0.02$ vs AdMTP in the absence of oleic acid, $P < 0.05$ vs mock in the presence of oleic acid.

We note that oleic acid had a much larger absolute effect on apoB-100 secretion in mock than AdMTP-treated cells (see Figure 8A), although AdMTP was a potent stimulator of apoB-100 production. There are probably two reasons for this paradox. One reason is that adenoviral infection per se inhibits protein secretion in HepG2 cells (see Figure 8B). Another possibility is that adenoviral infection might interfere with the stimulatory effect of oleic acid on triglyceride synthesis. To address this issue, we measured the effect of oleic acid supplementation on triglyceride synthesis in HepG2 cells under the three treatment conditions (Table 3). We found that oleic acid stimulated triglyceride synthesis 8.2-fold in control (mock) HepG2 cells. Adenoviral vector transduction using AdLuc reduced this stimulation by about 50%. Similarly, the stimulatory effect on triglyceride synthesis of oleic acid supplementation in AdMTP-transduced cells was limited to 5.7-fold, about 43% less than that in mock controls. Thus, the relative inhibition of the triglyceride biosynthetic response in adenoviral vector-treated cells may also be a factor in partially limiting the amount of apoB-100 secreted in response to oleic acid treatment.

Role of the Ubiquitin-Proteasome Pathway in AdMTP-Mediated Protection of Cellular apoB-100 in HepG2 Cells. We showed that *l*MTP overexpression following AdMTP transduction leads to a downregulation of the intracellular degradation of apoB-100 without any change in its biosynthetic rate (Figure 6). Since a major mechanism for the intracellular degradation of apoB-100 is via the ubiquitin−proteasome pathway (*32*, *33*, *42*−*45*), we examined the effect of AdMTP transduction on the polyubiquitination of intracellular apoB-100, a step that commits the protein to proteasomal degradation. As shown in Figure 9 (left panel), ubiquitinated apoB-100 is heterogeneous in size and is prominent in control (mock) HepG2 cells, with a size distribution similar to that reported previously (*32*, *33*). Following AdLuc treatment, there is little change in this pattern, with maximum distribution of the ubiquitinated apoB-100 in the 250 to >550 kDa range. Following AdMTP treatment, the amount of ubiquitinated apoB-100 was greatly reduced. The difference in distribution of ubiquitinated apoB-100 between AdMTP and AdLuc-transduced cells is quite reproducible in multiple experiments. It suggests that *l*MTP

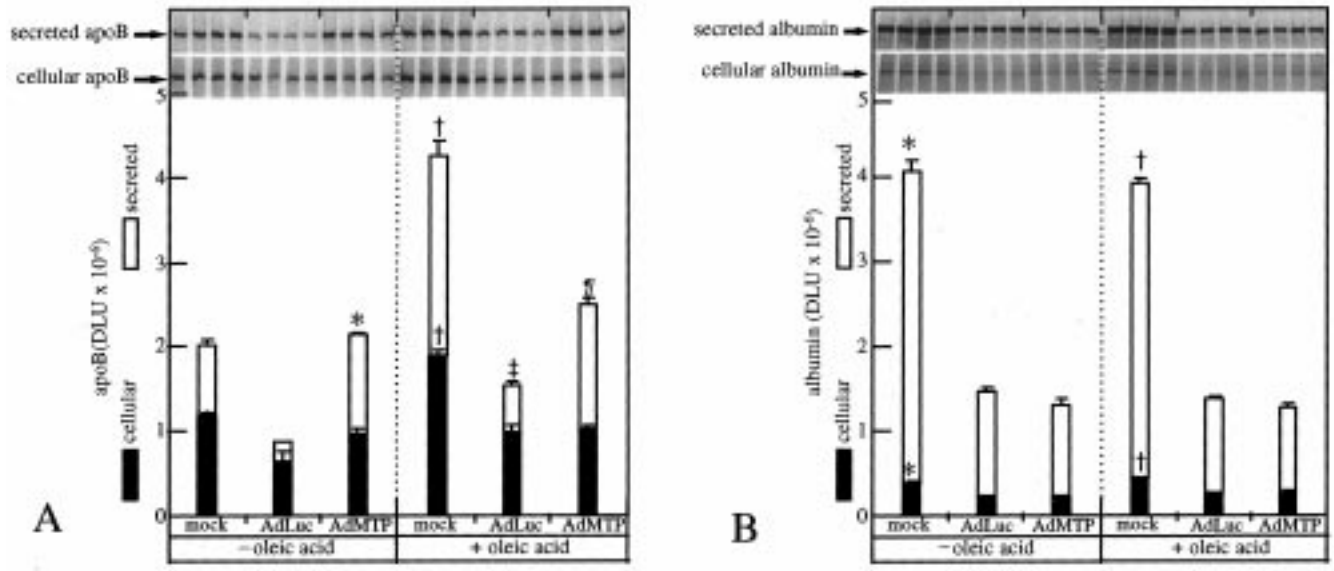

FIGURE 8: Effect of oleic acid on apoB and albumin secretion. HepG2 cells were cultured in 24-well plates until about 70% confluence and then infected with AdMTP, AdLuc, or buffer (mock). Two days posttransduction, the cells were preincubated with methionine-free medium for 20 min, pulse labeled with [^{35}S]methionine for 30 min and chased for 1 h. During the preincubation and pulse-chase periods, the culture medium contained either oleic acid-albumin or albumin alone. After the chase, apoB and albumin in the culture medium and in the cell lysate were immunoprecipitated and quantified by SDS−PAGE as described in the Materials and Methods. Data are expressed as mean ± SEM ($n = 4$) and statistical significance was determined by two-tailed *t*-test. (A) Cellular and secreted apoB. (*) $P < 0.005$ vs mock in the absence of oleic acid, $P < 0.001$ vs AdLuc in the absence of oleic acid; (†) $P < 0.001$ vs the corresponding value for mock in the absence of oleic acid. (‡) $P < 0.001$ vs AdLuc in the absence of oleic acid; (¶) $P < 0.02$ vs AdMTP in the absence of oleic acid. (B) Cellular and secreted albumin. (*) $P < 0.001$ vs the corresponding value for AdLuc or AdMTP in the absence of oleic acid; (†) $P < 0.001$ vs the corresponding value for AdLuc or AdMTP in the presence of oleic acid.

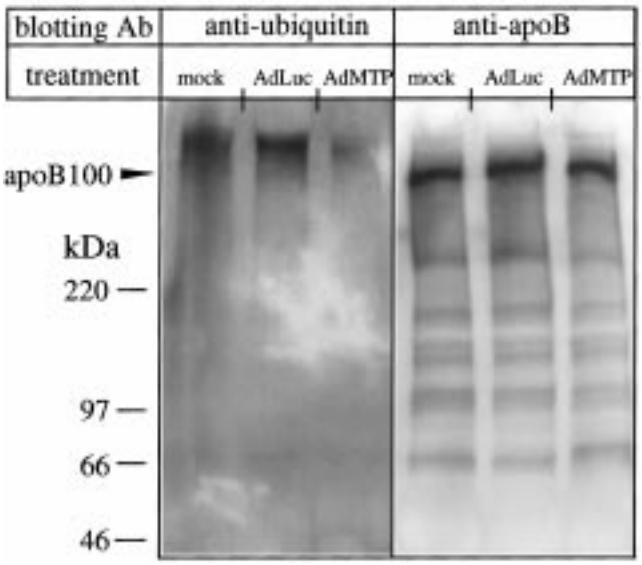

FIGURE 9: Effect of AdMTP on apoB polyubiquitination. Two days posttransduction, HepG2 cells were lysed and the apoB in the cell lysate was immunoprecipitated by polyclonal antibody against apoB and separated by SDS−PAGE and immunoblotted with anti-ubiquitin antibody (left panel). The membrane was stripped and immunoblotted with monoclonal antibody against apoB (1D1) (right panel).

overexpression rescues intracellular apoB-100 from proteasomal degradation. We note, however, that this protective effect of AdMTP was incomplete, because a significant amount of the intracellular apoB-100 was still ubiquitinated and tagged for proteasomal degradation.

To study the contribution of proteasome-mediated degradation on apoB-100 economy in the various experimental conditions, we examined the effect of a proteasome-specific inhibitor, lactacystin, on apoB-100 accumulation in mock-, AdLuc-, and AdMTP-treated HepG2 cells (Figure 10A). In mock-treated cells, lactacystin stimulated the yield of apoB-100 in the intracellular compartment before and after the 60 min chase. However, it was without effect on the amount secreted into the medium following the chase, indicating that the intracellular degradation of apoB-100 was inhibited, but its secretion was not facilitated. Exactly analogous findings were obtained with AdLuc-transduced cells. In AdMTP-transduced cells, the situation was different in that, in addition to the increase in intracellular apoB-100 recovered both before and after the chase, the amount recovered in the medium (secreted) was also stimulated 2.7-fold. To examine if these effects of lactacystin are specific for apoB-100, we performed parallel experiments on another apolipoprotein, apoA-I. We found that lactacystin was without effect on apoA-I dynamics (Figure 10B). Therefore, the protective effect of lactacystin was specific for apoB-100 and the protection was enhanced in the presence of *l*MTP overexpression induced by AdMTP transduction. The results of these experiments corroborate the finding of residual amounts of ubiquitinated apoB-100 in HepG2 cells following AdMTP transduction (Figure 9); they indicate that the protective effect of MTP overexpression is incomplete and additional protection can be afforded by the addition of lactacystin.

DISCUSSION

Microsomal triglyceride transfer protein (MTP) plays a major role in regulating the production of apoB-containing lipoproteins. It has been demonstrated that there may be an MTP-dependent "window" in the VLDL assembly process that occurs after the completion of apoB-100 translation but before the major amount of lipids is added to the VLDL particle (*46*). Much of the mechanistic studies on the

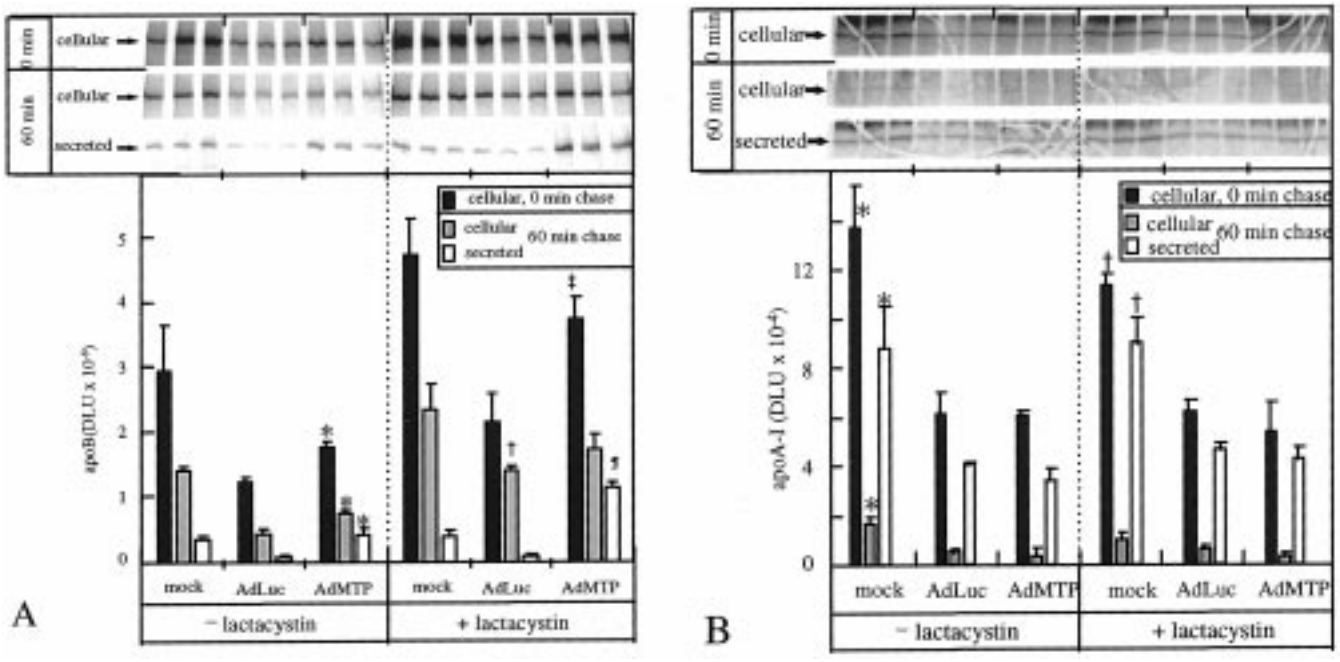

FIGURE 10: Effect of lactacystin on apoB and apoA-I secretion. HepG2 cells were cultured in 24-well plates until about 70% confluence and then infected with AdMTP, AdLuc, or buffer (mock). Two days posttransduction, the cells were preincubated with methionine-free medium for 20 min, pulse labeled with [^{35}S]methionine for 30 min and chased for 1 h either in the presence or absence of lactacystin. After the chase, apoB and apoA-I in the culture medium and in the cell lysate were immunoprecipitated and quantified by SDS−PAGE as described in the Materials and Methods. Data are expressed as mean ± SEM ($n = 3$) and statistical significance was determined by two-tailed t test. (A) Cellular and secreted apoB. (*) $P < 0.02$ vs the corresponding value for AdLuc in the absence of lactacystin; (†) $P < 0.005$ vs AdLuc in the absence of lactacystin; (‡) $P < 0.001$ vs AdMTP in the absence of lactacystin; (¶) $P < 0.005$ vs mock and AdLuc in the presence of lactacystin, $P < 0.005$ vs AdMTP in the absence of lactacystin. (B) Cellular and secreted apoA-I. (*) $P < 0.05$ or less vs the corresponding value for AdLuc or AdMTP in the absence of lactacystin; (†) $P < 0.02$ or less vs the corresponding value for AdLuc or AdMTP in the presence of lactacystin.

regulation and action of MTP have been performed in hepatoma cell lines (*25*−*27*, *46*−*48*). Use of MTP inhibitors in these systems has demonstrated that there is strong dose-dependent inhibition of apoB-100 production by these compounds (*25*−*27*). However, the expression of *l*MTP appears to be tightly regulated, and attempts to increase its expression in hepatoma cells via transient transfection have been unsuccessful (*27*). We have produced several transgenic mouse lines containing an *l*MTP gene driven by a liver-specific promoter [phosphoenolpyrevate carboxykinase (*49*)]. None of the lines expressed detectable levels of the transgene mRNA (Chang, B.H.-J., and Chan, L. unpublished material). Thus, it appears that *l*MTP expression cannot be stimulated acutely, and little is known concerning the effect of upregulation of MTP on apoB synthesis and secretion. To understand how an enhancement of MTP activity affects apoB production, it would be best to acutely stimulate MTP activity by a maneuver that is effective and specific for MTP. We have found that adenovirus-mediated gene transfer of *l*MTP into cultured HepG2 cells is a useful model to study the effect of acute upregulation of *l*MTP on apoB-100 biogenesis. Other experimental manipulations have been shown to upregulate MTP expression, e.g., long-term fat feeding stimulates *l*MTP mRNA and protein in the small intestine (but not the liver) in the hamster (*14*). However, such maneuvers are indirect and likely work via other pleotrophic effects that in turn affect apoB biogenesis.

MTP is a heterodimeric protein consisting of two subunits. In bovine liver, there is an approximately 5-fold excess of the small subunit, PDI, compared to the large subunit, *l*MTP (*8*). We found that *l*MTP gene transfer in HepG2 cells stimulated MTP activity approximately 5-fold (Figure 1D), though the increase in *l*MTP protein in these experiments was ~9-fold (Figure 1A). Perhaps the availability of PDI limits the amount of functional MTP that can be produced in these cells. The intracellular concentration of PDI was unchanged by the AdMTP treatment (Figure 1B). Interesting, substantial amounts of *l*MTP was detected in the culture medium in AdMTP-transduced cells (Figure 1C), indicating that the excess free *l*MTP that was uncomplexed with PDI was secreted. This is consistent with the fact that there is no ER-retention signal in *l*MTP, and the protein is normally kept in the lumen of the ER via its binding to PDI, an intrinsic ER protein with an ER retention sequence, KDEL (*50*). Under the conditions of our experiment, therefore, the degree of stimulation of MTP appears to be limited by the availability of PDI which complexes with *l*MTP and retains it within the ER.

MTP overexpression was found to stimulate the production of apoB-100 by HepG2 cells. Therefore, apoB-100 mRNA availability and apoB-100 production are not rate limiting in these cells. Furthermore, it is clear that the amount of *l*MTP and MTP expressed under basal conditions does not optimally protect against apoB degradation. Our study represents the first successful attempt in inducing *l*MTP overexpression in a liver cell line. Not only can we conclude that MTP is rate limiting in the biogenesis of apoB-100-containing lipoproteins, we can further infer that under basal conditions a substantial proportion of the apoB-100 synthesized is destroyed by the proteasome pathway and increasing basal MTP production enhances apoB production by reducing this nonproductive pathway.

The density profile of the *l*MTP overexpression-induced apoB-100-containing lipoproteins is changed only minimally in HepG2 cells following transduction using AdLuc or AdMTP (Figure 3). This observation is consistent with the role of MTP in facilitating an early phase in apoB-100 lipidation and stabilization without a significant effect on the addition of the major amounts of lipid on the lipoprotein particle which occurs at a later phase; a similar conclusion on MTP action was reached by Rustaeus et al. (*46*) using an MTP inhibitor in another liver cell line McA-RH7777. We note that van Greevenbroek et al. (*51*) proposed that MTP may also be involved in later steps in bulk lipidation of apoB-containing (predominantly apoB-100) lipoproteins in Caco-2 cells, because the addition of an MTP inhibitor BMS-200150 inhibited the secretion of chylomicron/VLDL particles with little effect on IDL/LDL density lipoproteins. The different conclusions reached by us and Rustaeus et al. (*46*), on one hand, and van Greevenbrock et al. (*51*), on the other, may be related to the fact that we and Rustaeus et al. used hepatoma cells whereas van Greenenbrock et al. used a colon carcinoma cell line as the experimental model. Furthermore, the experimental conditions are sufficiently different in these studies that the results probably should not be compared directly. Finally, we note that HepG2 cells is not the best model to study the bulk lipidation of apoB-100, because this hepatoma cell line does not secrete significant amounts of VLDL and appears to be defective in this step (*52*).

In contrast to the effect of *l*MTP overexpression alone, which had only minor effects on the relative density of the apoB-100-containing lipoproteins produced by HepG2 cells, the addition of oleic acid to the culture medium significantly shifted the lipoprotein profile toward lighter densities such that more of the particles were in the VLDL/LDL range in cells overexpressing *l*MTP (Figure 7). A similar effect of oleic acid on lipoprotein profile in untransduced HepG2 cells has been noted in the past (*41*). The fact that MTP upregulation per se has little effect on the density profile whereas oleic acid supplementation shifts the profile of the apoB-100-containing lipoproteins toward lighter densities supports the thesis that MTP facilitates the initial phase of apoB lipidation on the nascent apoB polypeptide but has no effect on the bulk lipidation during lipoprotein assembly. In contrast to the lack of effect of MTP, the increased triglyceride synthesis and availability in response to the addition of oleic acid to the culture medium was shown to stimulate the addition of the major amount of lipids to the lipoprotein particle. Again, as alluded to above, there may be limitations in using HepG2 cells as a model to study the bulk lipidation of apoB-100-containing lipoproteins.

Benoit and Grand-Perret (*43*) showed that inhibition of MTP stimulates the intracellular degradation of apoB-100 and proteasome inhibitors prevented the effect of an MTP inhibitor on apoB synthesis. We and others have shown recently that there is substantial cotranslational degradation of apoB-100 in the absence of MTP inhibition (*32*, *45*). Here we showed that acute upregulation of MTP reduced the intracellular degradation of apoB-100. It did this at least in part by downregulating the polyubiquitination of apoB-100, further reducing the proteasomal degradation of apoB. However, the proteasome pathway was not entirely circumvented because there is still significant ubiquitination of apoB-100 in these cells (Figure 9). Furthermore, the addition of a specific proteasome inhibitor, lactacystin, conferred additional protection to the newly synthesized apoB, indicating that a 5-fold increase in MTP activity fails to completely prevent the newly synthesized apoB from degradation via the ubiquitin-proteasome pathway (Figure 10A). The inability of MTP to totally prevent apoB degradation may be related to the fact that MTP only protects against apoB degradation in a very narrow window in lipoprotein assembly, which, as discussed above, occurs after the completion of apoB-100 translation but before the major amount of lipids is added to the lipoprotein particle. Our observation supports this essential but limited role of MTP in apoB lipoprotein biogenesis.

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