Suppression of cytosolic triacylglycerol recruitment for very low density lipoprotein assembly by inactivation of microsomal triglyceride transfer protein results in a delayed removal of apoB-48 and apoB-100 from microsomal and Golgi membranes of primary rat hepatocytes

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Abstract **Cellular apoB in primary rat hepatocyte cultures was pulse-labeled with [^{35}S]methionine for 1 h. Cells were then chased with excess unlabeled methionine for periods of up to 16 h in the presence or absence of BMS-200150, an inhibitor of microsomal triglyceride transfer protein (MTP). The secretion of apoB-48-VLDL was more sensitive to MTP inhibition than was apoB-100-VLDL. Inhibition of MTP had no inhibitory effect on the secretion of denser particles (apoB-48 HDL and apoB-100 HDL). BMS-200150 delayed the net removal of newly synthesized apoB-48 and apoB-100 from the microsomal and Golgi membranes, but not from the corresponding lumenal compartments. Only minor proportions of the microsomal lumen apoB-48 and apoB-100 (12–16% and 17–19%, respectively) were present as VLDL irrespective of whether MTP was inactivated or not. The HDL fraction contained most of the lumenal apoB-48 (67–73%) and a somewhat smaller proportion of apoB-100 (44–47%). The remainder of the lumenal apoB was associated with the IDL/LDL fraction. These proportions were unaffected by MTP inactivation. Excess labeled apoB which accumulated in the membranes in the presence of BMS-200150 was degraded. Inhibition of MTP prevented the removal of pre-synthesized triacylglycerol (TAG) from the hepatocytes as apoB-VLDL. Under these conditions intracellular TAG accumulated mainly in the cell cytosol, but also, to a lesser extent, in the microsomal membranes. The results suggest that inactivation of MTP inhibits a pathway of VLDL assembly which does not involve the bulk lumenal compartments of the microsomes. Suppression of this pathway ultimately prevents the net transfer of cytosolic TAG into mature apoB-VLDL.**—Hebbachi, A-M., A-M. Brown, and G. F. Gibbons. **Suppression of cytosolic triacylglycerol recruitment for very low density lipoprotein assembly by inactivation of microsomal triglyceride transfer protein results in a delayed removal of apoB-48 and apoB-100 from microsomal and Golgi membranes of primary rat hepatocytes.** ***J. Lipid Res.*** **1999.** 40: **1758–1768.**

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Recent studies (1–4) have confirmed and extended an earlier proposal that the assembly of hepatic VLDL occurs in a stepwise fashion which involves, at some stage, a bulk transfer of lipid to apoB (5). The later work provided evidence that the first step of VLDL assembly required the correct folding of translocating apoB into a conformation which was compatible with subsequent lipid addition. Misfolding of apoB at this stage resulted in its degradation, probably via the ubiquitin-dependent proteasomal pathway (6, 7). Whether or not the initial "stabilization" phase of apoB in VLDL synthesis requires association with lipids is uncertain. What is clear, however, is that this step is distinct from that which involves the bulk transfer of TAG to apoB resulting in the production of mature particles of VLDL (1–4). The intracellular site(s) at which bulk TAG transfer occurs is obscure and may differ for apoB-48- and apoB-100-containing VLDL particles (2, 3).

Microsomal triglyceride transfer protein (MTP) plays a crucial role in one or more of the events involved in VLDL assembly and evidence has recently been presented that, under certain circumstances, it may become rate-limiting for lipoprotein assembly (8). MTP is located in the lumen of the endoplasmic reticulum (ER) including those elements of the ER which co-sediment with mitochondria, the mitochondria-associated membrane (MAM) (9). MTP consists of a 97-kDa subunit and protein disulfide isomerase (PDI) and, in vitro, MTP is able to transfer TAG and cholesteryl ester between liposomal vesicles. However, neither the site(s) nor the mechanism involved

Abbreviations: MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; apoB, apolipoprotein B; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ER, endoplasmic reticulum; DMSO, dimethyl sulfoxide.

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in the MTP-dependent transfer of TAG to apoB in the secretory pathway of the intact cell are known. Controversy also exists as to whether MTP is required exclusively during the "stabilization" phase of apoB (10–12) or whether there is an additional requirement at the bulk lipid transfer step (4). It seems likely that a precise conformation of the non-lipid-binding N-terminal domain of translocating apoB is a prerequisite for subsequent MTP-dependent lipid addition to upstream lipid binding sites (13–15). A physical interaction between MTP and apoB is probably required at this stage, at least in HepG2 (16). The detailed role, however, of MTP in the mobilization and transfer of endogenous TAG into the secretory pathway remains to be established. In particular, the kinetics of apoB and TAG transport through the lumen and membranes of the various subcellular organelles when MTP is inactivated remains to be described in detail. This is particularly important in view of the apparent difference in the assembly mechanism of apoB-48- and apoB-100-VLDL (1–4, 17, 18). It is possible that this difference may, at least in part, result from a differential requirement for MTP.

Several in vitro models of VLDL assembly are currently in use (19). Of these, the human hepatoma cell line HepG2 has provided valuable information but the cell line does not catalyze the bulk lipid addition step (20–22). The rat hepatoma cell line McA-RH7777 overcomes this difficulty and has been successfully used to study aspects of the role of MTP in VLDL assembly (4, 10, 12). We have used primary cultures of rat hepatocytes to study the intracellular kinetics of TAG and apoB during VLDL assembly for the following reasons. First, unlike McArdle cells, primary cultures efficiently mobilize large quantities of intracellular TAG for the synthesis of physiological quantities of VLDL (19, 23). Second, primary cultures are not dependent upon the presence of extracellular fatty acids for lipid mobilization as are McA-RH7777 cells (4). Finally, primary cultures permit studies of the effects of prior in vivo manipulation on molecular aspects of VLDL assembly (19). For these reasons, we have used this model, in conjunction with the MTP inhibitor BMS-200150 (24) to trace the intracellular movement of apoB-48, apoB-100, and endogenous cytosolic TAG through the secretory apparatus of the cell when MTP is inactivated.

EXPERIMENTAL PROCEDURES

Materials

Waymouth's medium (methionine-free) and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco Ltd., Paisley, Scotland. Methionine was added to the medium as required. [^{35}S]methionine (1175 Ci/mmol) was from New England Nuclear (Boston, MA). Bovine serum albumin (BSA, fatty acid-free), sodium oleate, glycerol, dexamethasone, glutamine, alanine, serine, lactate, pyruvate, methionine, anti-sheep IgG-alkaline phosphatase conjugate and Protein A-Sepharose CL4B were obtained from Sigma (Poole, Dorset, U.K.). The triacylglycerol (TAG) assay kit (GPO-PAP kit) and anti-(human apoB) antiserum were obtained from Boehringer-Mannheim (Lewes, Sussex, U.K.). Anti-rat apoB antiserum was raised in rabbits as described previously (18). Acrylamide-bisacrylamide (40% w/v) was obtained from Bio-Rad Laboratories Ltd. (Hemel Hempstead, U.K.). Male Wistar rats were purchased from Harlan UK Ltd. (Bicester, U.K.). Tissue culture plates and plastic disposables were obtained from Becton Dickinson (Cowley, Oxford, U.K.).

Preparation and culture of rat hepatocytes

Hepatocytes were prepared under sterile conditions by collagenase perfusion of the livers of male Wistar rats maintained under the conditions described previously (25). The isolated cells were suspended in Waymouth's medium MB 752/1 containing methionine (0.33 mm), fetal bovine serum (10% v/v), penicillin (90,000 U/l), streptomycin (90,000 μg/l), glutamine (3.6 mm), alanine (0.36 mm), and serine (0.45 mm). The viability of the hepatocytes was measured by Trypan Blue exclusion and was usually between 92 and 95%. Occasional preparations were obtained in which cell viability was lower than 85%. These were discarded. The cell suspension (0.65×10^6 cells/ml) was plated out onto dishes previously coated with rat-tail collagen (26) until the cells formed a monolayer (4 h). During this period, the cell medium contained oleate (0.75 mm) bound to albumin (0.5%) prepared as described by Van Harken, Dixon, and Heimberg (27). This treatment increased the availability of intracellular stored TAG for subsequent incorporation into VLDL. After cell attachment, the medium was removed, the monolayer was washed twice with PBS, and the cells were cultured for 0.5 h in serum-free, methionine-free medium containing the above antibiotics and amino acids and, in addition, dexamethasone (1.0 μm), pyruvate (1 mm), and lactate (10 mm). This is subsequently referred to as supplemented medium. At the end of this period a trace amount of [^{35}S]methionine (1175 Ci/mmol; 200 μCi) was added to each dish for a further period of 1 h (pulse period). The medium was removed and the monolayer was washed twice with PBS. At this stage, some dishes were removed to measure label incorporation into cellular apoB. To the remaining cells was added supplemented medium containing unlabeled methionine (10 mm). The cells were cultured for further periods of 1, 2, 4, or 16 h in the presence or absence of BMS-200150 (15 μm in 20 μl of dimethyl sulfoxide (DMSO)), after which the medium was removed and the cells were harvested. Control dishes contained 20 μl of DMSO only. In some experiments, the MTP inhibitor was either present or absent during the 1-h pulse-label period. In these cases, the inhibitor was added 0.5 h before the start of the pulse-label, at the stage at which the cells were transferred to the methionine-free medium. After the 1-h pulse, cells were treated as described above, and then chased for 2 h either with or without the MTP inhibitor as during the pulse-label. At 16 h, 15 μm BMS-200150 had no effect on cell viability as measured by Trypan Blue dye exclusion. The viability of control cells (DMSO only) was 96.1 ± 1%; those containing the inhibitor were 96.5 ± 1.1% viable.

Preparation of subcellular fractions from cultured rat hepatocytes

Subcellular fractions, endoplasmic reticulum, Golgi, and cytosol were prepared from cultured rat hepatocytes by a method which was adapted from that described previously (28, 29). Briefly, at the end of each culture period, the medium was removed and used for isolating fractions containing VLDL (IDL + LDL) and HDL (see below). The cell monolayer was washed with ice-cold PBS and the cells were scraped into ice-cold PBS using a rubber scraper. Cell pellets were obtained by low-speed centrifugation (2 min at 1000 *g*). After removal of PBS, all further manipulations were carried out at 0–4°C. The cell pellet was incubated for 15 min with a hypotonic buffer (0.125 g cells/2.0 ml) containing 5 mm Tris/HCl and 1 mm $MgCl_2$ (pH 7.4), followed by addi-

tion of an equal volume of 0.5 m sucrose to the cell suspension to bring the sucrose concentration to 0.25 m. A mixture of protease inhibitors was added to minimize proteolysis (29). After homogenization of the cells using a motor-driven Potter-Elvehjem homogenizer subcellular fractions (Golgi, microsomes, and cytosol) were prepared as described previously (28). About one-third of the total homogenate volume was used for preparing the microsomes and cytosol and the remaining two-thirds were used for preparing Golgi apparatus. The microsomal and Golgi pellets were washed in ice-cold 0.25 m sucrose prior to resuspension in 100 mm sodium carbonate (pH 11.0) at a concentration of at least 1 mg/protein per ml (30). This procedure served to open the vesicles and released the lumenal contents from the membranes. These fractions were separated by ultracentrifugation. In control experiments, the original vesicles were resuspended in 0.25 m sucrose instead of sodium carbonate solution. Ultracentrifugation of the unopened vesicles did not result in the detection of any TAG in the sucrose-containing supernatant. Thus the unopened vesicles were not contaminated significantly with non-vesicular TAG. The purity of the microsomal and Golgi fractions was assessed by determination of the specific activities and recoveries of the marker enzymes for the corresponding organelle (NADPH-cytochrome c reductase for endoplasmic reticulum and UDP-galactose galactosyltransferase for Golgi apparatus) as described previously (29). In the present work, the mean recovery of NADPH-cytochrome c reductase in the microsomal fraction was 39.1 ± 0.6% of the total present in the whole homogenate. The corresponding value for UDP-galactose galactosyltransferase in the Golgi was 40.2 ± 6.1%. From measurements of the specific activities of each marker enzyme in the two subcellular fractions it was calculated that cross-contamination of the Golgi with microsomes was 0.93% and that of the Golgi with microsomes was 14.0%.

Separation of VLDL (IDL + LDL) and HDL from the cell medium and microsomal lumen

At the end of each culture period, the cell medium was centrifuged in a Beckman 50.4 fixed angle rotor for 16 h at 40,000 rpm. The floating VLDL was separated from the denser particles ($d > 1.006$) in the infranate by tube-slicing. The density of the infranate was adjusted to 1.063 g/ml by the addition of KBr and centrifuged under the same conditions for a further 16 h. The $d < 1.063$ floating fraction containing the combined IDL and LDL was obtained by tube slicing. The $d > 1.063$ infranate contained the HDL. The contents of the microsomal lumen were fractionated into corresponding density ranges in an identical manner (29).

Immunoprecipitation, SDS-PAGE, and radioassay of labeled apoB-48 and apoB-100

Labeled apoB was immunoprecipitated from the cells, subcellular fractions, and from the VLDL, LDL and HDL fractions of the medium and microsomal lumen, using a polyclonal anti-rat apoB antibody raised in rabbits and Protein-A Sepharose as described previously (17). The resulting Protein-A Sepharose bead suspension was heated with sample buffer containing 20 μg of rat plasma VLDL apoB. After centrifugation, the supernatant containing the immunoprecipitated labeled apoB-48 and apoB-100 was subject to SDS-PAGE electrophoresis in a 3–20% gradient polyacrylamide gel (17). Bands containing apoB-48 and apoB-100 were visualized with Coomassie Brilliant Blue R and the dried gel was exposed to X-ray film for 48 h. Bands containing labeled B-48 and B-100 were excised from the gel and solubilized with 30% H_2O_2 and NCS tissue solubilizer. After neutralizing the solubilizer with glacial acetic acid, scintillation fluid (Optiphase) was added and radioactivity determined in a Beckman LS-6500 scintillation counter.

Other analytical methods

Cellular protein was determined colorimetrically by the method of Lowry et al. (31). Cellular, subcellular and VLDL TAG mass were determined enzymically after Folch extraction of the total lipid fraction (32) using the GPO-PAP kit from Boehringer-Mannheim (33). VLDL apoB (total mass) was determined using an adaptation of the enzyme-linked immunoabsorbent assay with anti-sheep IgG antibody and anti-human apoB anti-serum (34).

Statistical analysis

All statistical tests (ANOVA:two factor with replication, and paired *t*-test) were performed using the data analysis package in Microsoft Excel for Windows 95, version 7.0. Significance was determined at the 95% confidence interval ($P < 0.05$).

RESULTS

Cellular apoB was pulse-labeled in the absence of the MTP inhibitor followed by chase periods of up to 16 h, in the presence or absence of BMS-200150. Suppression of MTP during the chase period inhibited the secretion of newly synthesized apoB-48 and apoB-100 associated with VLDL (**Fig. 1**). A small proportion of the labeled apoB secreted during the chase appeared in particles of density greater than that of VLDL (Fig. 1). At 2 h of chase, most of this dense-particle label was associated with particles in the HDL-density range (d 1.063–d1.21) (**Table 1**). Inactivation of MTP had little or no effect on the secretion of labeled apoB-48 and apoB-100 associated with these high density particles. The kinetics of secretion of the dense apoB-containing particles differed from those of secretion of apoB associated with VLDL. For instance, whereas secretion of the latter continued for up to 4 h after the pulse-label, most of the labeled apoB HDL was secreted during the first hour of the chase, with little or no further secretion thereafter. ApoB-100 was secreted into the HDL fraction of the medium as well as apoB-48. In the absence of BMS-200150 the ratio of labelled B-48:B-100 in the HDL was broadly similar to that in the VLDL.

Whereas the secretion of newly synthesized apoB ceased after 4 h, hepatocytes continued to secrete unlabeled apoB associated with VLDL for up to 16 h following the pulse. Inactivation of MTP suppressed the secretion of VLDL apoB, measured as total mass, during the whole of this period (**Fig. 2**). The extent of this suppression resembled that of labeled apoB-48-VLDL rather than that of apoB-100-VLDL. BMS-200150 also suppressed the secretion of VLDL triacylglycerol (TAG) with a potency that was somewhat greater than that observed for VLDL apoB, especially after 16 h of chase (Fig. 2). This differential inhibition led to a general decrease in the TAG:apoB ratio in the presence of the MTP inhibitor, indicating the presence of smaller VLDL particles under these conditions (**Table 2**).

Suppression of labeled VLDL apoB output was accompanied by an accumulation of intracellular labeled apoB-48 and apoB-100 in the presence of the MTP inhibitor (**Fig. 3**) at least up to 4 h of chase. At 16 h, however, this difference had been abolished, suggesting that the excess

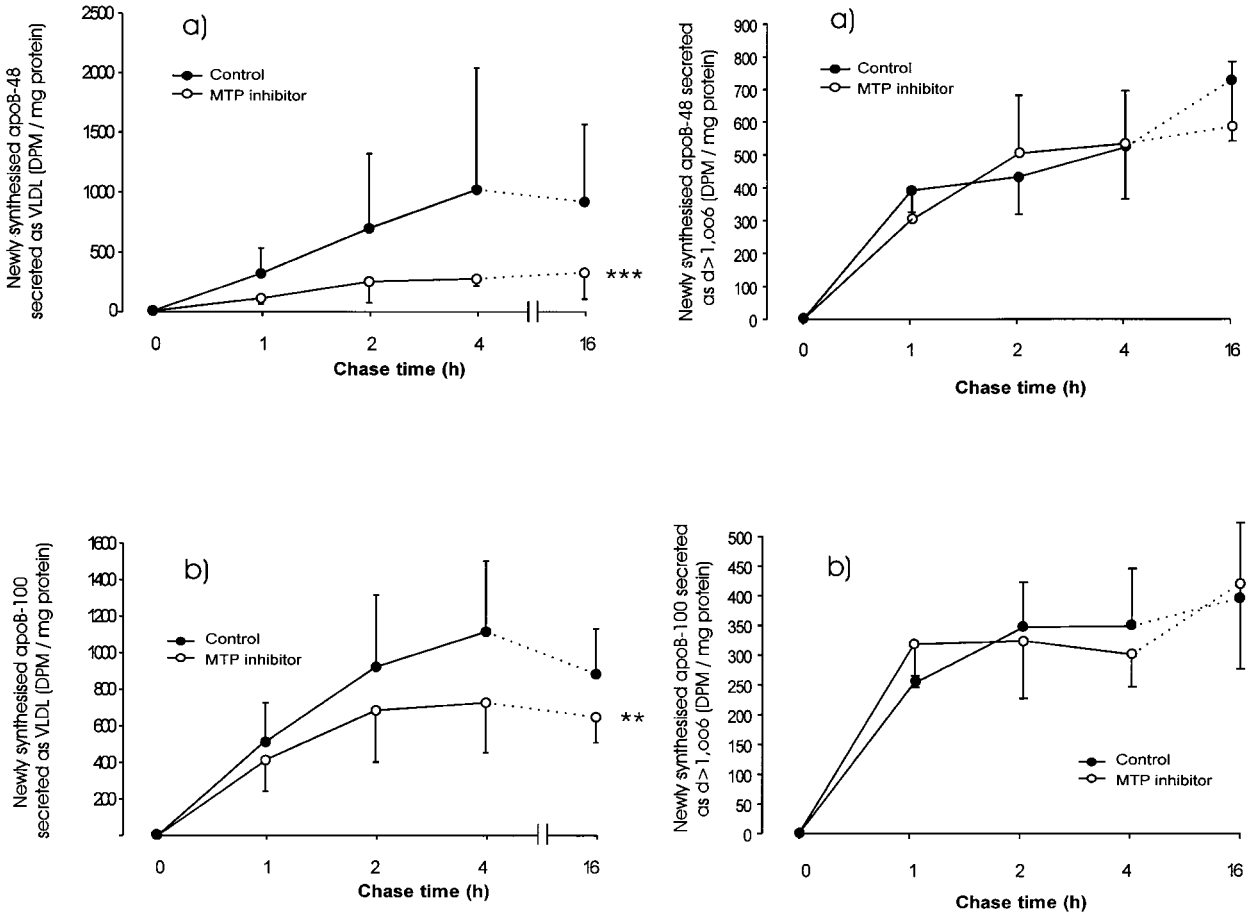

Fig. 1. The effect of MTP inactivation, by BMS-200150, on the secretion of newly synthesized apoB-48 and apoB-100 as d < 1.006 VLDL and d > 1.006 particles. Hepatocytes were pulse-labeled [^{35}S]methionine for 1 h and then chased with 10 mm unlabeled methionine for up to 16 h. The VLDL fraction was separated from the d > 1.006 particles and the amounts of label associated with apoB-48 and apoB-100 in each fraction were determined. The curves marked ** and *** were significantly different from controls ($P < 0.01$ and $P < 0.001$, respectively, by ANOVA, n = 5).

apoB remaining within the cell after 4 h in the presence of BMS-200150 had been degraded. This was confirmed by comparison of the amount of newly synthesized apoB present in the cells at the end of the pulse with the sum of those remaining within the cell and secreted into the medium in the presence and absence of the MTP inhibitor. For apoB-100, in the absence of BMS-200150, the amount remaining after 16 h was 57.9 ± 16.8% of the peak label after the pulse. The corresponding amount in the presence of the MTP inhibitor was 45.1 ± 11.9% ($P = 0.09$). For apoB-48 the corresponding values were 54.4 ± 14.9% and 37.4 ± 8.0% ($P = 0.07$) (n = 5).

TABLE 1. Distribution of secreted apoB between IDL/LDL and HDL

Medium Fraction	BMS-200150	ApoB-100	ApoB-48
		dpm/mg protein	
IDL/LDL	−	69 ± 34	52 ± 7
	+	78 ± 22	69 ± 27
HDL	−	91 ± 4	127 ± 14
	+	123 ± 10	120 ± 19
HDL (% of total)	−	61.6 ± 11.4	70.8 ± 0.9
	+	62.7 ± 4.8	65.6 ± 4.7

Labeled apoB-containing lipoproteins secreted into the cell medium at 2 h after the pulse were isolated by sequential flotation. Labeled apoB-48 and apoB-100 were separated by SDS-PAGE. Each value is the mean ± SEM of three independent hepatocyte preparations.

At the end of the pulse, and at each time-point during the chase period, the microsomal fraction of the cells was obtained and separated into membrane and lumenal compartments by treatment with sodium carbonate. Most of the newly synthesized apoB-48 and apoB-100 at the end of the pulse were associated with the microsomal membrane, with relatively little contained in the lumenal compartment. In the absence of the MTP inhibitor, the kinetics of labeled apoB-100 net disappearance from the membrane during the chase were similar to those for apoB-48. Net removal of the apoB label from the microsomal membrane occurred more slowly when MTP was inactivated (**Fig. 4**). These differences were most apparent between 0 and 4 h of the chase period, after which they were largely eliminated. The effects of the MTP inhibitor in this respect were similar for both apoB-100 and apoB-48. By contrast, there was no significant effect of BMS-200150 on the net

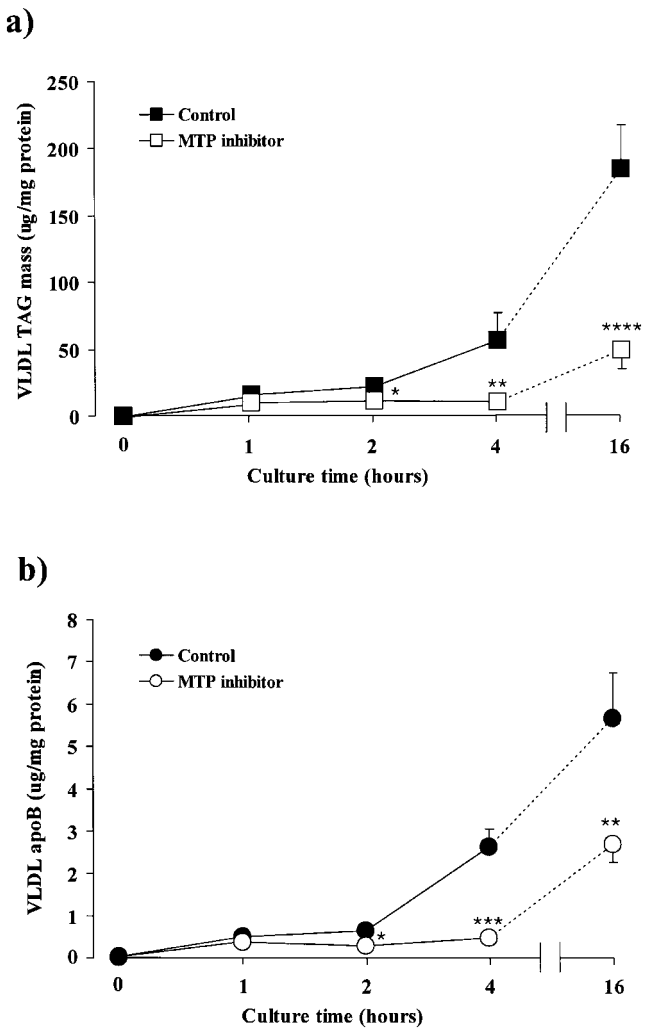

Fig. 2. The effects of MTP inhibition on the secretion of apoB mass and TAG mass in VLDL. Hepatocytes were cultured in the presence of oleate (0.75 mm) for 4 h. The medium was removed, the cells were washed with PBS, and fresh, serum-free, oleate-free Waymouth's medium was added. Cells were cultured under these conditions for the periods of time indicated, either in the absence or presence of the MTP inhibitor (BMS-200150). At each time-point, the medium was removed and the fraction containing VLDL was isolated by ultracentrifugation. The apoB content was determined by ELISA and the TAG content was measured enzymically. Each value represents the mean ± SEM of seven independent hepatocyte preparations. Values marked *, **, and *** are significantly different from the controls ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

disappearance of the smaller quantity of labeled apoB-48 and apoB-100 from the microsomal lumen.

In other experiments, the density distribution of labeled apoB in the microsomal lumen was determined by sequential ultracentrifugation of the lumenal contents (see Experimental Procedures). Because of the relatively small amounts of label in the lumen, more dishes were used in this experiment for the preparation of microsomes. At the end of the pulse the amounts of labeled apoB VLDL as a proportion of the total lumenal apoB were relatively low although this proportion was somewhat greater for apoB-100 (17.5–19.5% of total) compared to that for apoB-48 (11.9–16.4% of the total). The amounts of label associated with the IDL + LDL density range were also relatively small. The HDL fraction accounted for the largest proportion of the lumenal apoB content **Table 3** and **Fig. 5**. How-

TABLE 2. Effect of MTP inactivation on the composition of secreted VLDL

Culture Time	TAG:ApoB Ratio BMS-200150 Absent	TAG:ApoB Ratio BMS-200150 Present
h	*μg/μg*	
1	20 ± 3.5	14 ± 4.3
2	27 ± 6.5	21 ± 5.2
4	19 ± 5.8	18 ± 4.0
16	32 ± 7.0	15 ± 3.8

Secreted VLDL was isolated by ultracentrifugation of the cell medium. ApoB was measured by ELISA and TAG was determined enzymically. Each value is the mean ± SEM of five independent hepatocyte preparations. Over the whole time course the presence of BMS-200150 gave rise to a significant decrease in the TAG:apoB ratio ($P < 0.01$ by ANOVA).

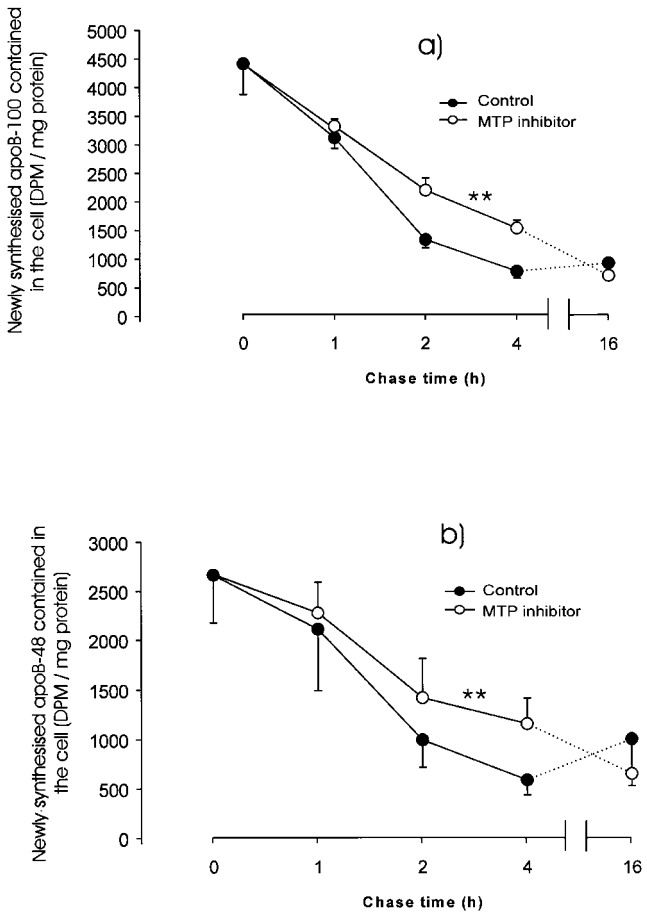

Fig. 3. The effect of MTP inhibition on the loss of total cellular apoB-100 and apoB-48. Hepatocytes were pulse-labeled and chased as in the legend to Fig. 1. The apoB-48 and apoB-100 contents were measured immediately after the pulse (0 time) and then at each subsequent chase time-point. The curves marked ** were significantly different from controls at 2 h and 4 h ($P < 0.01$ by ANOVA, n = 5).

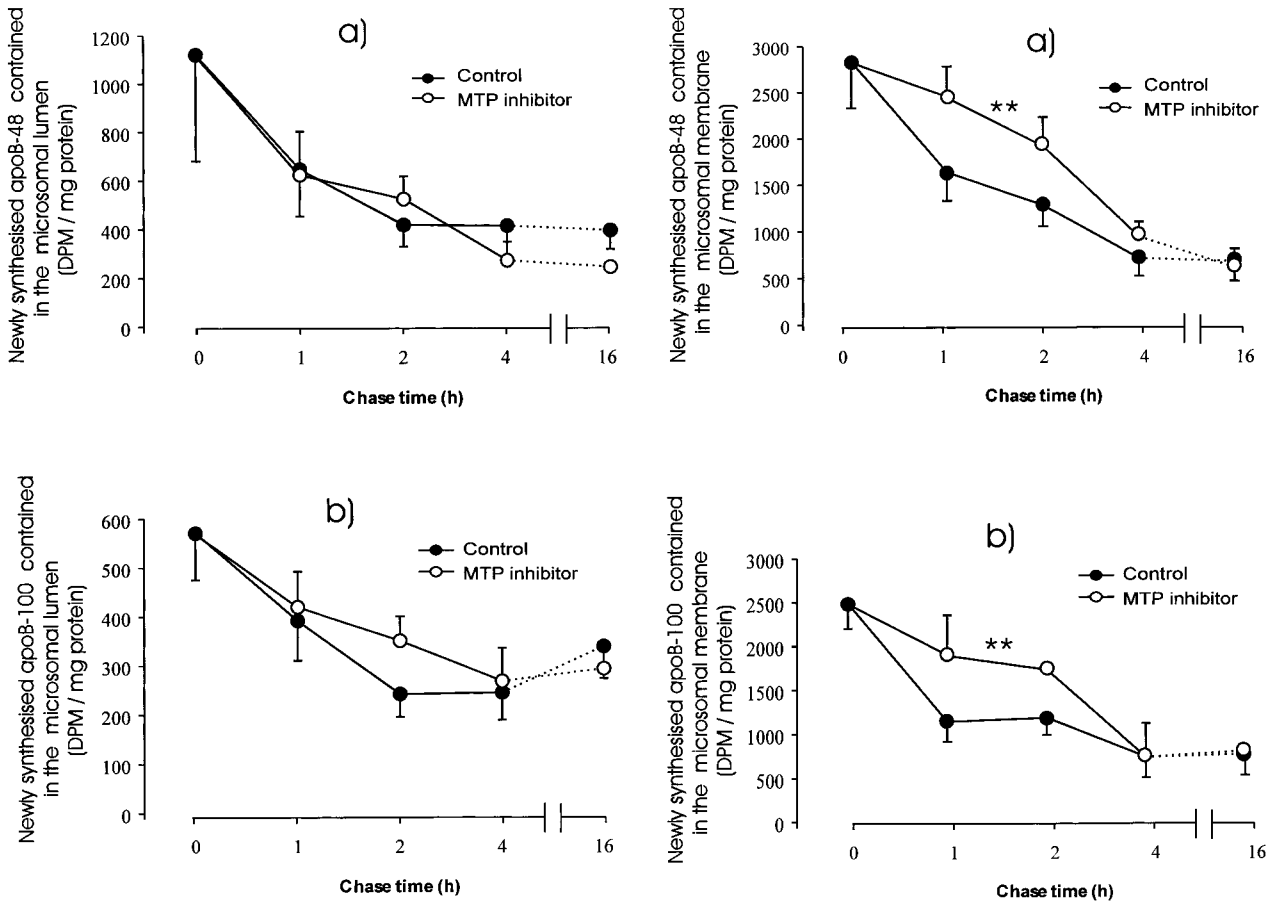

Fig. 4. The effect of MTP inhibition on the loss of apoB-48 and apoB-100 from the microsomal membranes and lumen. Hepatocytes were pulse-labeled and then chased as in the legend to Fig. 1. The cells were then homogenized and the microsomal membranes and lumenal contents were separated after sodium carbonate treatment. The curves marked ** were significantly different from controls at 2 and 4 h ($P < 0.01$ by ANOVA, n = 5).

ever, this proportion was smaller for apoB-100 HDL than for apoB-48 HDL (Fig. 5). There was no change in the relative amounts of label in these density ranges after 2 h of chase. Neither did the presence of BMS-200150 during the chase affect these proportions (Fig. 5). Interestingly, the apoB-100:apoB-48 ratio in the lumenal VLDL was higher than that in the secreted VLDL (1.37 ± 0.08 at 2 h, compared to 0.62 ± 0.09, respectively, $P < 0.01$) and, unlike the secreted VLDL, was unaffected by the presence of BMS-200150.

TABLE 3. Distribution of labeled apoB between HDL and (IDL + LDL) in lumenal lipoproteins

	HDL ApoB	
Time after Pulse	ApoB-100	ApoB-48
h	*% of HDL + (IDL + LDL) apoB*	
0	66.1 ± 1.2	81.9 ± 5.8
2 (−)	62.1 ± 2.9	68.1 ± 8.9
2 (+)	77.8 ± 3.3	72.5 ± 0.1

Hepatocytes were pulse labeled for 1 h in the absence of BMS-200150. They were then cultured for a further 2 h with 10 mm methionine (chase) either in the absence (−) or presence (+) of 15 μm BMS-200150. Values are the mean ± range of two independent hepatocyte preparations.

In further experiments, we studied the consequences of MTP inactivation on the behavior of newly synthesized apoB-48 and apoB-100 in the membrane and lumenal contents fractions of the Golgi. Owing to the large number of dishes required for isolation of a sufficient quantity of Golgi vesicles, it was not possible to carry out a complete time course similar to that for the microsomal fraction. These studies were, therefore, limited to a single 2-h chase period following the 1-h pulse label (**Table 4**). At this point, the effects of MTP on microsomal membrane apoB accumulation were maximal. In this experiment, similar to the microsomes, at the end of the pulse there was again a large excess of labeled apoB associated with the Golgi membrane compared to that in the lumenal contents. In the absence of BMS-200150, there was a significant net disappearance of labeled apoB from the Golgi membrane, particularly of apoB-100, at 2 h after the pulse-label. However, these changes were virtually abolished when MTP was inactivated. These effects of BMS-200150 in the Golgi membrane were not accompanied by any dif-

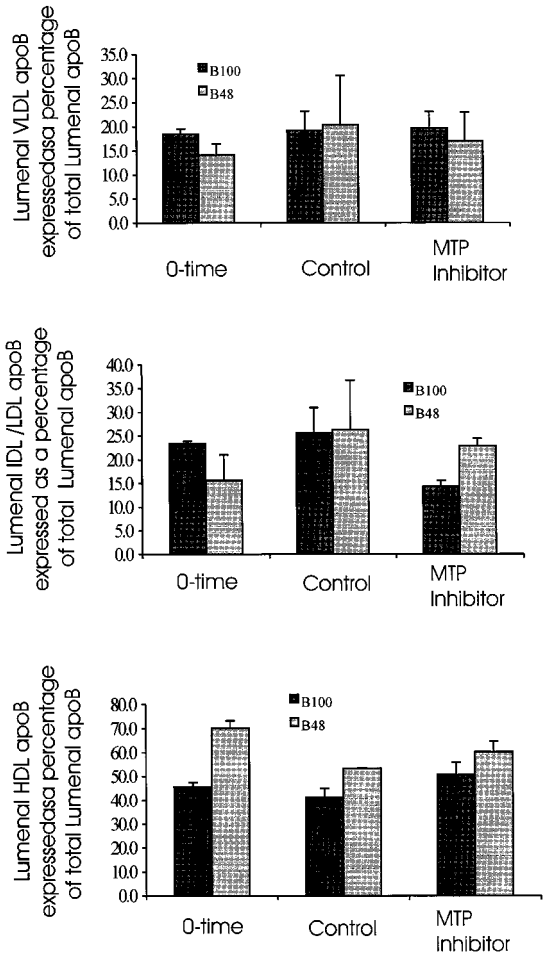

Fig. 5. The effect of MTP inhibition on the proportion of apoB-48 and apoB-100 appearing in the lumenal VLDL, IDL/LDL, and HDL fractions. Cells were pulse-labeled for 1 h with [^{35}S]methionine and then chased for 2 h with 10 mm unlabeled methionine. The cells were then homogenized and the microsomal membranes and lumenal contents were separated after sodium carbonate treatment. The lumenal apoB was then separated into particles with densities of d $<$ 1.006 (VLDL), d 1.006–d1063 (IDL + LDL) and d $>$ 1.063 (HDL) by sequential flotation. The first pair of blocks refers to labeled apoB at the end of the pulse (zero time of chase). The second pair and third pair of blocks represent labeled apoB at the end of the 2 h chase in the absence and presence, respectively, of BMS-200150. The values plotted are the mean ± SEM of three independent hepatocyte preparations.

ferences in the smaller amounts of labeled apoB-100 or apoB-48 associated with the Golgi lumen (Table 4).

The effect of MTP inactivation on the transfer of TAG out of the cell cytosol was determined by measuring the cell cytosolic TAG concentrations at the end of the 4-h culture period in the presence of oleate (0.75 mm). This value was compared with similar measurements made 1, 2, 4, and 16 h after exogenous oleate was removed, during which time BMS-200150 was either present or absent. Changes in the TAG contents of the microsomal membrane and lumen fractions were also made under the same conditions. To present a clear picture of the changes in the distribution of hepatocellular TAG under these conditions, the data have been normalized to give the TAG content of each compartment per g of original hepatocytes. The results of this experiment are shown in **Fig. 6**. At the end of the 4-h culture period in the presence of oleate, most of the cellular TAG was associated with the cytosolic compartment. Subsequent culture in the absence of BMS-200150 resulted in the net removal of TAG from the cytosolic pool. By contrast, when MTP was inactivated, there was no net decline in the concentration of cytosolic TAG. Inhibition of MTP also attenuated the net removal of the smaller quantity of TAG associated with the microsomal membrane but had no significant effect on the quantity of TAG in the microsomal lumen (Fig. 6).

DISCUSSION

Sequential assembly of VLDL: the role of MTP

There is general agreement that VLDL is assembled in at least two distinct and identifiable stages (1–4, 35). The first step involves the priming of newly synthesized apoB by locking it into a secretion-competent conformation. This process may require the association of apoB with a small quantity of neutral lipid and the detailed mechanism may differ subtly for apoB-100 and apoB-48 (1–3). The second step involves the bulk transfer of neutral lipid to the secretion-competent form of apoB to produce mature VLDL. The intermediate particles (Step 1) of VLDL assembly have been identified intracellularly in the rat McA-RH7777 cell line (1, 2) and in intact rat liver (3). At

TABLE 4. Inactivation of MTP delays removal of newly synthesized apoB from Golgi membranes

Golgi Fraction	Chase Time	MTP Inhibitor	ApoB-48	Ratio Inhibited: Control	ApoB-100	Ratio Inhibited: Control
	h		*dpm/mg protein*		*dpm/mg protein*	
Membrane	0		6998 ± 2108		6091 ± 1862	
	2	−	4797 ± 1333		2795 ± 344	
	2	+	6984 ± 1687[a]	1.54 ± 0.30[a]	4985 ± 1565[a]	1.66 ± 0.35[a]
Lumen	0		821 ± 400		758 ± 394	
	2	−	513 ± 212		491 ± 234	
	2	+	605 ± 240	1.17 ± 00.15	501 ± 238	1.03 ± 0.19

Hepatocytes were pulse labeled with [^{35}S]methionine for 1 h and chased for 2 h either in the presence or absence of BMS-200150. The Golgi fractions were isolated and labeled apoB-100 and apoB-48 were isolated from the Golgi membrane and Golgi lumen. Results are the mean ± SEM of four individual hepatocyte preparations.

[a] Significantly different ($P < 0.05$) from values obtained in the absence of the MTP inhibitor.

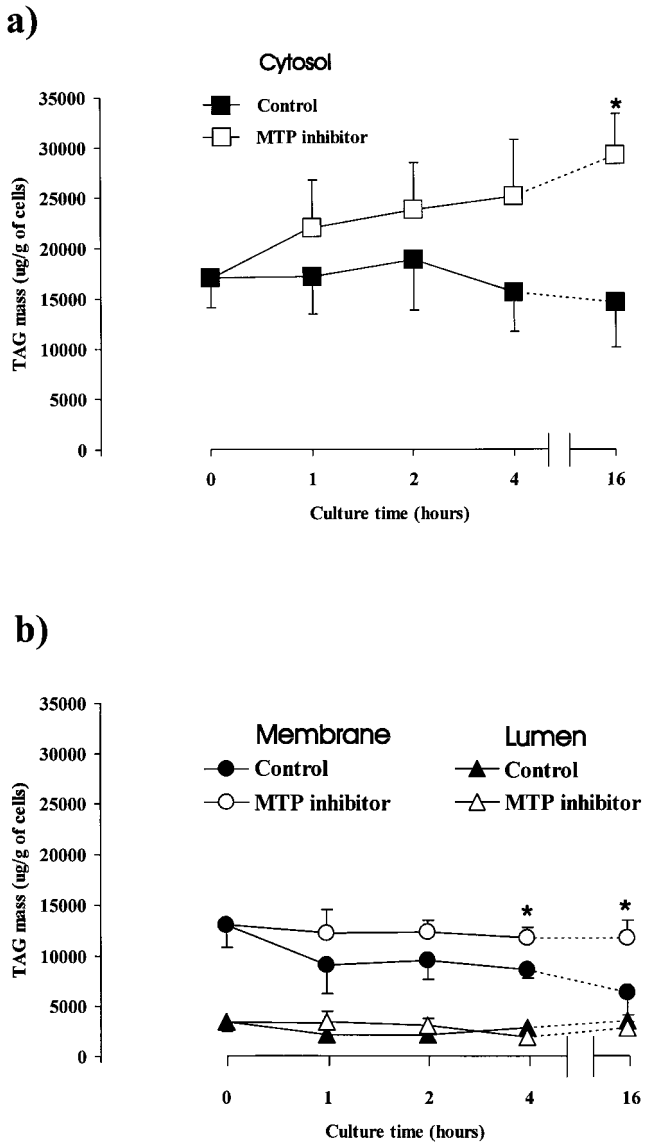

Fig. 6. Effect of MTP inhibition on the removal of TAG of the cell cytosol (a) and TAG associated with endoplasmic reticulum membrane and lumenal content (b). Hepatocytes were cultured as described in the legend to Fig. 3. After removal of oleate-containing medium, cells were washed with PBS. At this stage some dishes were taken and used for preparing the endoplasmic reticulum. The remaining dishes were cultured for further periods of 1, 2, 4, and 16 h in the presence or absence of the MTP inhibitor (BMS-200150, 15 μm). Membrane and lumen of the ER fractions and the cytosol were prepared after each culture period and TAG mass in each compartment was assayed. The total amount of TAG in cytosol (a) was calculated by multiplying the μg of TAG per mg of cell protein by the total cell protein per g of cells. The total amount of TAG in the ER membrane and lumen was calculated using NADPH:cytochrome c reductase as a measure of ER fraction recovery per g of cells. The results are given as means ± SEM of four independent hepatocyte preparations. Values marked * are significantly different from the corresponding controls ($P < 0.05$).

least some of the precursor form of apoB-48-VLDL was present in the lumen of the microsomes as a dense particle (apoB-48-HDL), whereas the precursor of apoB-100-VLDL was loosely associated with the membrane (2, 12). It is possible that, in the intact cell, both forms are loosely membrane-bound but the apoB-48 precursor is more easily detached during preparation of the lumenal contents. The physical properties of these intermediate products of assembly are similar to those of the dense apoB-100-containing particles secreted by HepG2, a liver cell line which appears to lack the capacity to catalyze the bulk transfer of lipids to the dense VLDL precursor forms (20–22). In the present work, using primary hepatocytes, both newly synthesized apoB-100 and apoB-48 were secreted as dense, HDL-like particles (Fig. 1, Table 1), similar to those described above. These particles probably represent or are related to the Step 1 intermediates of VLDL assembly which have escaped the bulk lipid addition stage. Secretion of these dense particles comprised only a relatively small proportion of the total secreted apoB-100 and apoB-48, most of which emerged from the cell as mature VLDL. Most of the newly synthesized apoB-100 and apoB-48 HDL was secreted within the first hour after the pulse with very little secretion thereafter. By contrast, newly synthesized apoB continued to be secreted as mature VLDL for up to 4 h of chase. These results suggest that there are at least two populations, or pools, of labeled apoB present at the end of the pulse: *a*) a pool that is disconnected from the bulk transfer step and is rapidly secreted as poorly lipidated particles, and *b*) a VLDL precursor pool that undergoes continuous association with neutral lipid for at least 4 h after synthesis.

After the pulse-label, inactivation of MTP by BMS-200150 suppressed the secretion of apoB-100 VLDL and apoB-48 VLDL in primary hepatocytes (Fig. 1), as shown previously in McA-RH7777 cells (4, 10–12). However, there were no effects on the secretion of apoB-48 HDL and apoB-100 HDL. The latter observations cannot, however, be unequivocally interpreted as meaning that MTP is not required for the first step of VLDL assembly. Thus, it is possible that a large proportion of dense but secretory competent forms of newly synthesized apoB were already present in the cell at the end of the pulse, and these were rapidly secreted during the first hour of the chase irrespective of the presence or absence of BMS-200150. Alternatively, it is possible that inhibition of the continued translation of apoB by BMS-200150 during the chase, as suggested by Gordon and colleagues (10) in McArdle cell cultures and by Benoist and Grand-Perret (7) in HepG2, affected the final density distribution of the labeled apoB-containing particles. To check this possibility, we determined the effect of MTP inhibition during the cellular labeling of apoB in a 1-h pulse on the subsequent secretion of labeled apoB-HDL and apoB-VLDL during a 2-h chase under the same conditions. This protocol was similar to that used previously to study this aspect in McArdle cells (10). After a 1-h pulse, the amounts of label associated with cellular apoB-48 and apoB-100 in the presence of BMS-200150 were 1716 ± 145 and 1070 ± 246 dpm/mg cell protein, respectively (average of four dishes from two experiments). The corresponding control values (cells containing DMSO only) were 4470 ± 904 and 3114 ± 616 dpm/mg, respectively. This inhibitory effect of BMS-200150

during the translation of apoB is consistent with the suggestion that MTP protects apoB against co-translational degradation (7). During the chase period, the amounts of label associated with the apoB-48 and apoB-100 HDL were lower (730 ± 256 and 345 ± 105 dpm/mg cell protein, respectively) when MTP had been inhibited during the pulse, than when MTP was active during this period (911 ± 281 and 516 ± 131 dpm/mg, respectively). The corresponding amounts secreted with apoB-48 VLDL and apoB-100 VLDL were 454 ± 174 and 167 ± 85 dpm/mg, respectively (MTP inactive) and 1990 ± 507 and 859 ± 296 dpm/mg, respectively (MTP active). Under these conditions, therefore, inhibition of MTP affected apoB translation in such a way as to suppress the secretion of the Step 1 lipid transfer products during the assembly of VLDL in primary cultures of rat hepatocytes.

Inactivation of MTP results in simultaneous delays in the removal of cytosolic TAG and of microsomal membrane apoB and TAG

The complete assembly of VLDL in primary cultures of rat hepatocytes is not dependent upon an extracellular source of fatty acid as is the case in the rat hepatoma cell line McA-RH7777 (4). VLDL secretion in primary cultures of hamster hepatocytes, which secrete apoB exclusively as apoB-100 VLDL, also occurs independently of an extracellular source of fatty acid (23). In primary hepatocytes, the major VLDL TAG precursor pool is located in the cytosol of the cell (36–38). This pool of TAG can be rapidly mobilized and, in some as yet unknown way, is able to access the site(s) of bulk lipid transfer to apoB. The intracellular site(s) involved has not yet been identified. As MTP is a protein of the microsomal lumen, it probably does not have direct access to the cytosolic pool of TAG on the opposite side of the microsomal membrane. Thus, the nature of the linkage between inhibition of MTP and the accumulation of cytosolic TAG (Fig. 6) is obscure. In particular, TAG accumulation in the cytosol cannot, in itself, be interpreted in terms of a role for MTP in the bulk lipid addition step of VLDL assembly. Nevertheless, the results suggest that, in some way, inhibition of MTP ultimately gave rise to a decreased net transfer of cytosolic TAG into mature VLDL and that this disturbance was associated with an increase in the quantity of TAG occurring with the microsomal membranes (Fig. 6) and with a perturbation of the normal kinetics of newly synthesized apoB transfer from microsomal and Golgi membranes into secreted VLDL. In the presence of the MTP inhibitor, the amount of cytosolic TAG actually increased, despite the secretion of some TAG as VLDL. We have previously observed increases in total cellular and secreted TAG during hepatocyte culture in the absence of extracellular fatty acids (39). Some of this material is derived from de novo synthesis. Most, however, is derived from another source, possibly cellular phospholipid.

Nature, origin, and fate of microsomal lumenal apoB

At the end of the 1-h pulse, lumenal apoB made only a relatively small contribution to the total amount of newly synthesized apoB in the microsomal fraction, most of which was associated with the membrane (Fig. 4). A similar distribution of labeled apoB has previously been observed in McA-RH7777 cells (12) and in freshly prepared rat hepatocytes (29). Of the lumenal apoB, only a small proportion of either apoB-100 or apoB-48 was associated with particles in the VLDL density range. The most highly labeled apoB-containing lumenal particles were those associated with HDL (Fig. 5). A similar density distribution of apoB-containing particles has previously been demonstrated in the microsomal lumen of freshly prepared hepatocytes (29) and, in intact rat liver, a large proportion of lumenal apoB was present as particles of density >1.02 g/ml (3, 40).

In the present work there was no significant increase in the proportion of lumenal VLDL apoB during a 2-h chase. Neither was there any change in the proportion of apoB associated with lumenal HDL during this period (Fig. 5). Each type of particle disappeared from the lumen at the same rate. This pattern of change seemed inconsistent with a product/precursor relationship between lumenal apoB-VLDL and apoB-HDL. The origin of the various forms of lumenal apoB in any case remains obscure. In rat McArdle hepatoma cells, Rustaeus et al. (12) have provided evidence that carbonate extraction of microsomes releases small amounts of both apoB-HDL and apoB-VLDL from the membrane into the lumen. Extraction of the denser form of apoB from the membrane could be improved using deoxycholate. This improvement was not so evident for apoB-VLDL. If primary rat hepatocytes behave in a similar manner, then the present work suggests that the apoB-VLDL and apoB-HDL that appear in the microsomal lumen after carbonate extraction were originally associated with the microsomal membrane in the intact cell. If this is the case, it is difficult to explain why MTP inhibition had little effect on the kinetics of carbonate extractable apoB-HDL and VLDL that appear in the lumen (Figs. 4 and 5) whilst there were quite pronounced effects on membrane-bound apoB from which they were presumably derived (Fig. 4). Our present results, however, provide no information about the distribution of newly synthesized apoB between VLDL and HDL in the microsomal membrane, nor how this distribution is affected by BMS-200150. Irrespective of the origin of the small amounts of newly synthesized lumenal apoB, our results support the suggestion that membrane-associated VLDL-apoB is a precursor of VLDL-apoB secreted into the medium (12).

Inhibition of MTP delays the removal of labeled apoB from the Golgi membrane

During the 1-h pulse, Golgi membrane apoB-48 and apoB-100 became heavily labeled as a result of transfer of membrane-bound apoB originating in the RER. When BMS-200150 was present during the chase, the net removal of both isoforms of apoB from the Golgi membrane was delayed, similar to that which occurred in the microsomal membrane (Table 3). This was an unexpected result as there have not, so far, been any reports of MTP expression within the Golgi apparatus. The possibility of

substantial contamination of Golgi membranes with microsomes was ruled out by quantification of marker enzymes. The fate of the apoB in the Golgi membrane is unknown. In contrast to the effects on the Golgi membrane, inhibition of MTP had no effect on the removal of labeled apoB from the lumen of the Golgi apparatus (Table 3). Various reports have provided evidence that implicates the Golgi subcompartment as an active component of the VLDL assembly process (41–43), particularly in the acquisition of phospholipids (40). There is also strong evidence for a Golgi-mediated degradation of apoB (40, 44, 45).

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