Microsomal triglyceride transfer protein in CaCo-2 cells: characterization and regulation

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Abstract As microsomal triglyceride transfer protein (MTP) is required for the assembly and secretion of apoB-containing lipoproteins by intestinal epithelial cells, the characterization and regulation of MTP in human intestinal cells, CaCo-2, was studied. CaCo-2 cells express MTP mRNA of 4.2 kb and a 97 kDa subunit of MTP protein. Similar to the expression of apoB mRNA, MTP mRNA expression was dependent upon cell differentiation and was directly related to the ability of the cells to assemble and secrete apoB-containing lipoproteins. MTP mRNA expression was highest in fully differentiated cells, with small but detectable amounts found in undifferentiated cells. Under conditions of increased apoB secretion by oleic acid, phosphatidylcholine, or lysophosphatidylcholine, MTP mass, MTP activity, and MTP gene expressions were not altered. In cells treated with calcium ionophore or phorbol 12-myristate 13-acetate, no relationship could be established between apoB secretion and MTP mRNA or activity. Similarly, in cells treated with sphingomyelinase, trifluoperazine, verapamil, okadaic acid, vanadate, or monensin, agents that decrease apoB secretion, no corresponding decrease in MTP activity or mass was observed. The results suggest that the various mediators of apoB secretion alter steps in lipoprotein assembly and secretion that are not dependent on MTP activity. CaCo-2 cells have an abundant supply of MTP for the assembly of lipoproteins when apoB secretion is stimulated by dietary lipids.—**Mathur, S. N., E. Born, S. Murthy, and F. J. Field.** Microsomal triglyceride transfer protein in CaCo-2 cells: characterization and regulation. *J. Lipid Res.* 1997. **38:** 61–67.

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The processes involved in the assembly of chylomicrons and their eventual secretion into the lymphatics by small intestinal absorptive cells remain poorly understood. What is well recognized, however, is the important role played by apolipoprotein (apo) B-48 in transporting lipids from the gut (reviewed in refs. 1, 2). ApoB-48, the amino-terminal half of apoB-100, is synthesized in the endoplasmic reticulum of the intestinal cell by a novel post-transcriptional modification of apoB mRNA. ApoB-48 is required for the synthesis, assembly, and secretion of the chylomicron particle. Most of the information to date, however, would indicate that intestinal triacylglycerol-rich lipoprotein production occurs independently of changes in apoB-48 gene expression or biosynthetic rates (3–5). What appears to be important for normal lipoprotein assembly is the association of apoB-48 with neutral lipids during the translocation of apoB into the lumen of the endoplasmic reticulum (2). If neutral lipids are unavailable, most of apoB-48 is degraded prior to its entry into the secretory pathway. During times of lipid flux, in contrast, more apoB-48 is preferentially translocated into the secretory pathway resulting in increased amounts of apoB being secreted.

Microsomal triglyceride transfer protein (MTP) also plays a crucial role in the normal assembly and secretion of triacylglycerol-rich lipoproteins (2, 6–8). MTP is present in the lumen of endoplasmic reticulum of hepatocytes and enterocytes and is believed to catalyze the transfer of neutral lipids to the apoB molecule. In individuals who have a genetic defect in MTP, i.e., abetalipoproteinemia, apoB-containing lipoproteins are not transported by intestinal cells despite normal synthesis of apoB (6–9).

Our laboratory has had a long interest in the regulation of apoB synthesis and secretion by CaCo-2 cells, a cell culture model for small intestinal absorptive cells (10). We have previously characterized the developmental regulation of apoB editing and secretion, investigated the regulation of apoB synthesis and secretion by lipid and nonlipid mediators, and recently we have implicated p-glycoprotein in normal lipoprotein assembly and secretion (4, 11–23). In the present study, because of the importance of both apoB and MTP in intes-

Abbreviations: MTP, microsomal triglyceride transfer protein; apo, apolipoprotein; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid; HDL, high density lipoprotein; PMA, phorbol 12-myristate 13-acetate.

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tinal lipoprotein secretion, we have characterized MTP in CaCo-2 cells and have investigated the regulation of MTP gene expression, mass, and activity under conditions that have been demonstrated to alter the secretion of triacylglycerol-rich, apoB-containing lipoproteins by these cells. The results show that apoB and MTP gene expression parallel each other in CaCo-2 cells during different stages of differentiation. Neither MTP mass nor activity is regulated by mediators that augment or disrupt the secretion of apoB-containing lipoproteins, suggesting that MTP may be constitutively expressed in CaCo-2 cells much like that of apoB.

MATERIALS AND METHODS

Materials

Purified bovine MTP, plasmid pRC/MTP, and goat anti-bovine MTP primary antibody were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ. [α^{32}P]dCTP, [^{14}C]glycerol trioleate and [N-methyl ^{3}H]dipalmitoylphosphatidylcholine were purchased from DuPont NEN, Boston, MA. Rabbit anti-goat IgG, Fc fragment specific, peroxidase conjugate was supplied by Pierce, Rockford, IL. Sodium taurocholate, oleic acid, lysophosphatidylcholine, phosphatidylcholine, eicosapentaenoic acid, sphingomyelinase, trifluoperazine, verapamil, ionophore, phorbol 12-myristate 13-acetate, okadaic acid, vanadate, brefeldin A, monensin, phenylmethylsulfonyl fluoride, leupeptin, cardiolipin, and triolein were obtained from Sigma, St. Louis, MO.

Cell culture

CaCo-2 cells were grown in T-75 flasks as described previously (23). They were subcultured on polycarbonate micropore membranes (24.5 mm diameter) inserted in Transwells (Costar, Cambridge, MA). Cells were used 14 days after plating and medium was changed every 2 days.

RNA isolation and Northern blot analysis

RNA from CaCo-2 cells was isolated using guanidinium thiocyanate–phenol–chloroform method as described by Xie and Rothblum (24). Cells from two filters were pooled to isolate RNA. The RNA was resolved by electrophoresis on 1% agarose gel in 1 × MOPS and 0.66 M formaldehyde. After transfer of the RNA to a positively charged NYTRAN PLUS filter, 0.45 μm, the RNA on the membrane was immobilized by irradiation of the wet membrane at 0.12 J/cm^2.

The plasmid pRC/MTP containing the entire coding sequence of human MTP 97 kDa subunit was grown and digested with HindIII and Xbal to release the insert coding for human MTP. This insert was then isolated and purified by gel electrophoresis. The radiolabeled probe was prepared by labeling 45 ng of the MTP insert with [α^{32}P]dCTP using a random priming DNA labeling kit from Boehringer Mannheim, Indianapolis, IN. Hybridization was performed for 18 h at 68°C in 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 10% SDS and 0.5% blocking reagent (Schleicher & Schuell, Keene, NH). Blots were washed 3 × 20 min at 60°C, with 20 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA and 1% SDS. The blots were exposed to Kodak XAR-5 film at −70°C for up to 5 days. The mRNA on the blots was quantitated using HP ScanJet cx scanner equipped with a transparency adapter and Sigma gel software from Jandel Scientific Software, San Rafael, CA. RNA isolated from proper controls and treatment groups were applied on the same gel. For each blot, RNA loaded on the gel was normalized using the density of 28S and 18S RNA stained with ethidium bromide.

MTP activity assay

MTP activity in CaCo-2 cells was estimated by a modification of the method described by Wetterau et al. (6). Cell homogenization and subsequent procedures were performed at 4°C. CaCo-2 cells were suspended in 0.45 mL of 15 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (15/40 buffer). This buffer was supplemented with 1 mM phenylmethylsulfonyl fluoride and 5 μg leupeptin/ml. The cells were homogenized at 30,000 rpm for 1 min with TISSUE tearor model 985-370, Biospec Products Inc. One part deoxycholate solution (0.56%, pH 7.5) was added to 10 parts homogenate while mixing. After incubation of the sample for 30 min at 4°C, the cell homogenate was centrifuged at 103,000 ***g*** for 1 h. The supernatant was collected and dialyzed for 18 h against 15/40 buffer. This supernatant, designated as CaCo-2 MTP extract, was used to assay MTP activity. The MTP activity contained in this preparation was measured by determining triglyceride transfer from donor to acceptor unilamellar vesicles (25). Donor and acceptor vesicles were prepared by suspending dried lipids in 15/40 buffer and sonication (Sonics and Materials Inc., Danbury, CT) for 30 min at room temperature. The unilamellar vesicles were separated from large vesicles by centrifugation at 105,000 ***g*** for 1 h. The vesicles were used within 4 h after ultracentrifugation. The triglyceride transfer assay volume of 1 ml contained 100–140 μg protein of CaCo-2 MTP extract, 74 μM bovine serum albumin, donor vesicles (100 μM egg phosphatidylcholine, 0.14 μM [^{14}C]glycerol trioleate, 13 μM bovine heart cardiolipin, 0.01% butylated hydroxytoluene (BHT) and acceptor vesicles (400 μM egg phosphatidylcholine, 0.77 μM glycerol trioleate, 0.15 nM [N-methyl ^{3}H]dipalmitoylphosphatidylcholine and 0.01%

butylated hydroxytoluene). Each sample was assayed in duplicate by incubation of this mixture for 1 and 2 h at 37°C. The reaction was terminated by addition of 0.5 ml of DEAE-cellulose (50% w/v in 15/40 buffer). After 5 min of thorough mixing, the donor vesicles bound to the DEAE cellulose were selectively sedimented by centrifugation at 12,000 *g* for 4 min. An aliquot of the supernatant containing acceptor vesicles was taken to determine radioactivity. The ^{14}C and ^{3}H counts were used to calculate the percent recovery of the acceptor vesicles and the percent triglyceride transfer using first order kinetics. Appropriate blanks without MTP extract were run for each experiment to correct for triglyceride transfer in the absence of MTP.

MTP mass

The CaCo-2 cells were harvested from filters by scraping with a plastic spatula in 1.5 ml M199 and centrifugation at 12,000 *g* for 5 min. The cell pellet was solubilized in 50 μL 1 × RIPA buffer [1%, v/v, Triton X-100, 0.1%, w/v sodium dodecyl sulfate, 0.5% w/v, sodium deoxycholate, 20 μM leupeptin, 10 mM sodium phosphate, pH, 7.5, 5 mM EDTA, 5 mM EGTA (ethylene glycol-*bis*(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 100 mM sodium chloride, 1 mM PMSF, 20 mM methionine, and 1 mM cysteine]. After dilution with 50 μL of 10 mM Tris-HCl, pH 7.4, and vortexing for 10 sec, the nuclear material was sedimented by centrifugation at 13,000 *g* for 5 min. The supernatant was mixed with 1 volume of 2.5 × Laemmli sample buffer and proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 5% stacking gel and an 8% running porous gel as described by Doucet, Murphy, and Tuana (26). The proteins were transferred to a PVDF membrane at 100 V for 1 h using a buffer containing 10% methanol, 25 mM Tris, and 192 mM glycine, pH 8.0. The membranes were blocked for 30 min at 37°C with 3% non-fat dry milk in 50 mM Tris-HCl, 80 mM sodium chloride, 0.2% v/v, NP-40, 0.01% Antifoam A (30% solution, Sigma, St. Louis, MO). The blot was then incubated with 10 μL/10 mL goat anti-bovine MTP primary antibody for 1 h at room temperature and washed three times. It was followed by 1 h incubation with 5 μL/10 mL rabbit anti-goat IgG, Fc fragment specific, peroxidase conjugate (Pierce, Rockford, IL.). After removal of excess secondary antibody, the peroxidase bound to the primary antibody and antigen was detected using high sensitivity IBI Enzygraphic Web (Eastman KODAK Co., New Haven, CT) or with ECL Western blotting detection reagent from Amersham (Arlington Heights, IL). The mass of MTP on the blots was quantitated using HP ScanJet cx scanner equipped with a transparency adapter and Sigma gel software from Jandel Scientific Software, San Rafael, CA.

RESULTS

Characterization of MTP in CaCo-2 Cells

MTP mRNA in CaCo-2 cells was readily detected by Northern blotting (**Fig. 1,** left panel). Its size of approximately 4.2 kb was similar to that reported by Shoulders et al. (7) in human liver and intestine. In CaCo-2 cells, apoB gene expression is highly dependent upon cell confluency and differentiation (4, 21, 27–29). To investigate whether MTP expression was likewise dependent upon cell differentiation, steady state levels of apoB and MTP mRNA were estimated in CaCo-2 cells grown for 1, 2, 3, 8 (confluent), or 14 (fully differentiated) days (Fig. 1, right panel). Although detected in relatively very small amounts, mRNA for both proteins was observed on day 1. On day 8, however, when cells were first confluent, the amount of apoB and MTP mRNA significantly increased in parallel. The highest amounts of mRNA were observed after cells were fully differentiated on day 14. Again, similar increases in both apoB and MTP mRNA were observed.

MTP mass in CaCo-2 cells was estimated by immunoblotting using an antibody directed at the 97 kDa subunit of MTP. A distinct band having the same mobility as bovine MTP was observed (**Fig. 2**). The intensity of the band was directly proportional to the amount of protein applied to the gel.

The activity of MTP was estimated by measuring the transfer of triolein from donor to acceptor vesicles in the presence or absence of an MTP extract prepared from CaCo-2 cells. As shown in **Fig. 3,** the transfer of triolein between vesicles was linearly related to the amount of MTP extract, up to 200 μg of protein, and for up to 2 h of incubation.

Regulation of MTP

To determine whether MTP gene expression or activity was regulated by changes in the secretion of apoB-containing lipoproteins, CaCo-2 cells were incubated with a variety of lipid and nonlipid mediators that have been shown to alter apoB secretion in CaCo-2 cells. **Table 1** shows these results. The lipid modulators, 500 μM oleic acid, 1 mM taurocholate + 500 μM phosphatidylcholine, and 1 mM taurocholate + 250 μM lysophosphatidylcholine, despite causing significant increases in apoB secretion, did not alter message levels or activity of MTP. Eicosapentaenoic acid, which weakly promotes lipoprotein production compared to oleic acid and decreases apoB mRNA levels in CaCo-2 cells (15), appeared to decrease MTP mRNA levels as well. The activity of MTP in cells incubated with 250 μM eicosapentaenoic acid, however, was maintained despite a decrease in mRNA levels.

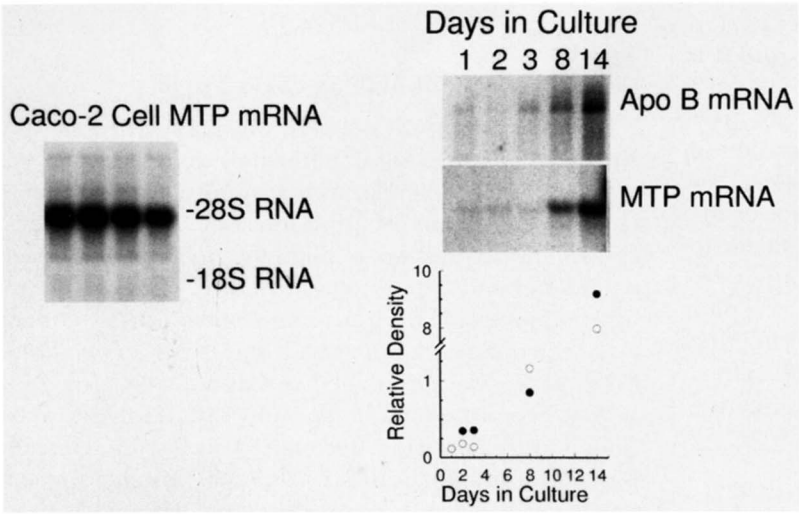

Fig. 1. ApoB and MTP mRNA abundance. Total RNA was isolated and analyzed as described in Methods. The insert from plasmid pRC/MTP containing the entire coding sequence of human MTP 97 kDa subunit was used to probe for MTP mRNA. The apoB mRNA was detected with a cDNA probe 59035 obtained from American Type Culture Collection (Rockville, MD). mRNA shown in the left panel was isolated from control CaCo-2 cells grown on filters for 14 days. To obtain sufficient quantities of RNA for analysis, CaCo-2 cells were grown on a plastic surface for 1, 2, 3, 8, or 14 days after plating (right panel). The relative density values in the figure are an average of 3 RNA preparations for each day; apoB mRNA (●); MTP mRNA (○). A representative blot is shown in the right panel.

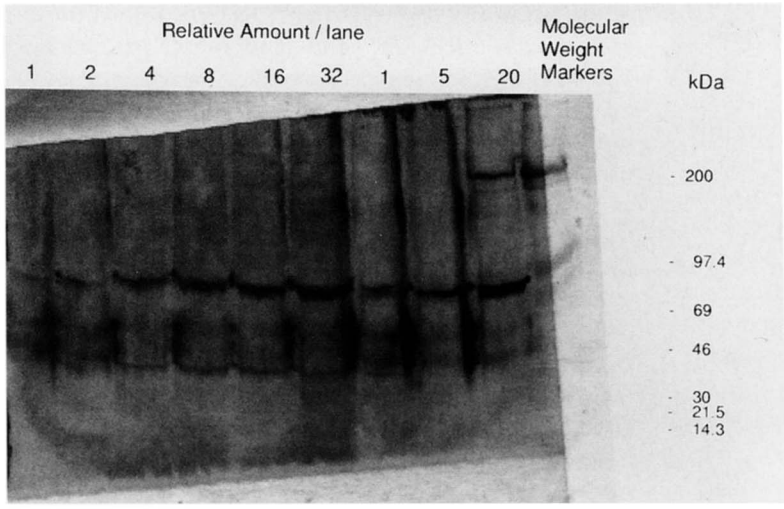

Fig. 2. Western blot for CaCo-2 cells and bovine MTP. CaCo-2 cell extracts, prepared as described in Methods, or purified bovine MTP were resolved by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to PVDF membrane and MTP was detected by using a primary goat anti-bovine MTP antibody and a secondary rabbit anti-goat antibody conjugated with peroxidase. Lanes 1–6: increasing amounts of CaCo-2 cell extract; lanes 7–9: increasing amounts of purified bovine MTP.

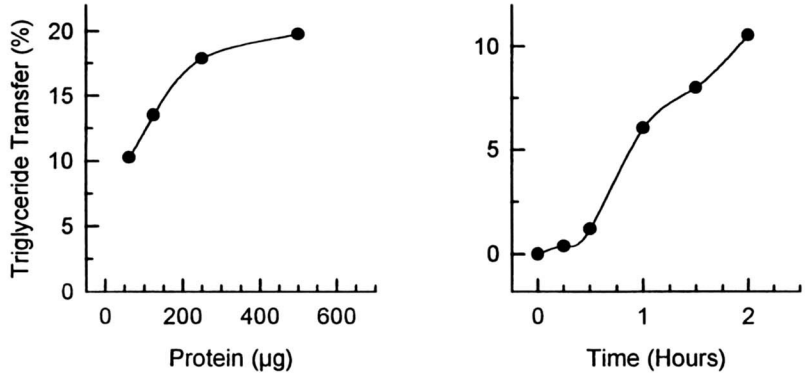

Fig. 3. Triglyceride transfer activity in CaCo-2 cell extracts. CaCo-2 cells were solubilized in buffer containing 0.056% deoxycholate. MTP activity was estimated by determining triglyceride transfer from donor to acceptor unilamellar vesicles as described in Methods. To determine the effect of protein concentration, the assay mixture was incubated for 2 h (left panel). For the time course, 70 µg of protein per assay was used (right panel). Data from one experiment with assays performed in duplicate are shown. Similar results were obtained from 3 separate preparations.

TABLE 1. Effect of lipids and non-lipid mediators on apoB secretion, MTP mRNA, and MTP activity in CaCo-2 cells

	ApoB Secretion[a]		MTP mRNA	MTP Activity
Control			1.0 ± 0.2	100 ± 6
Sodium taurocholate	−	(12, 13, 22)	1.2 ± 0.5	122 ± 2[b]
Sodium taurocholate + lysophosphatidylcholine[c]	+++	(13)	0.9 ± 0.3	126 ± 2
Sodium taurocholate + phosphatidylcholine[c]	++	(12, 22)	1.7 ± 0.7	108 ± 1
Oleic acid	+++	(15, 19)	1.7 ± 0.6	107 ± 3
Eicosapentaenoic acid	+	(15)	0.5 ± 0.0[b]	92 ± 3
Sphingomyelinase	−−−	(11)	0.8 ± 0.3	96 ± 2
Trifluoperazine	−−	(20)	0.8 ± 0.4	111 ± 3
Verapamil	−−−	(20)	0.7 ± 0.2	100 ± 5
Ionophore	−−−	(12)	0.2 ± 0.1[b]	115 ± 4
Phorbol 12-myristate 13-acetate	−−−[d]		0.2 ± 0.1[b]	151 ± 4[b]
Okadaic acid	−−	(14)	1.3 ± 0.2	125 ± 5[b]
Vanadate	−−−[d]		1.2 ± 0.5	136 ± 8[b]
Brefeldin A	−−−[d]		ND	83 ± 1[b]
Monensin	−−[d]		ND	101 ± 3

CaCo-2 cells grown on polycarbonate micropore filters (24.5 mm diameter) for 14 days were incubated with the lipid or non-lipid mediators for 24 h. mRNA or MTP activity was measured as described in Methods. The values for mRNA represent mean ± SEM of 3 preparations. Values for MTP activity are mean ± SEM of 6 filters. The data are derived from several experiments. The values are expressed relative to the controls run for each experiment. For each experiment, the data were analyzed by SIGMASTAT software (Jandel Scientific Software, San Rafael, CA) using one-way ANOVA and Bonferroni *t*-test to compare controls with the treatment groups at $P < 0.05$.

[a]Stimulation (+) or inhibition (−) of apoB secretion. Reference cited for the observation in parentheses.

[b]Significantly different from controls at $P < 0.05$; ND, not determined.

[c]The treatment did not change MTP mRNA or activity compared to cells incubated with taurocholate alone.

[d]Unpublished observations.

Agents that interfere with apoB secretion such as 100 mU/ml sphingomyelinase, 50 μM verapamil, or 50 μM trifluoperazine failed to alter either MTP activity or MTP mRNA levels. In contrast, 1 μM calcium ionophore A 23187, and the phorbol ester, 1 μM phorbol 12-myristate 13-acetate (PMA), which have been shown to inhibit apoB secretion, significantly decreased mRNA levels of MTP with an increase in its activity. A modest increase in MTP activity without any change in MTP mRNA levels was observed when apoB secretion was reduced in cells given 1 μM okadaic acid or 10 μM vanadate. Only in cells given 1 μg/ml brefeldin A was a reduction in apoB secretion accompanied by a decrease in MTP activity. In data not shown, the mass of MTP was not altered significantly by any of the agents tested. Thus, no definite relationship was observed between MTP activity, MTP mass, and modulation of apoB secretion by lipid or non-lipid mediators in CaCo-2 cells.

DISCUSSION

ApoB gene expression, apoB mRNA editing, and apoB mass secretion by CaCo-2 cells are all highly dependent upon cellular differentiation (4, 21, 27–29). Non-confluent undifferentiated cells have little capacity to assemble and secrete apoB-containing lipoproteins (29). In contrast, soon after confluency, when cells have morphological and biochemical properties of mature enterocytes, apoB expression is fully developed and cells readily respond to lipid influx by secreting triacylglcyerol-rich apoB-containing lipoproteins (15, 17, 30, 31). If MTP were required for apoB lipoprotein assembly and secretion by CaCo-2 cells, one would have expected that MTP expression would occur simultaneously with the expression of apoB. That is precisely what was observed in different stages of cellular differentiation. The results suggest that in CaCo-2 cells, these two genes are turned on together. The importance of this parallel expression for apoB and MTP is supported by studies demonstrating that transfection of both genes into cells that normally do not secrete lipoproteins is essential for lipoprotein secretion to occur (32–34). The present results also provide evidence that, similar to what is observed in normal human enterocytes (6, 7), CaCo-2 cells contain significant amounts of the 97 kDa subunit of MTP and a MTP mRNA that is of similar size, 4.2 kb.

Neither MTP activity nor MTP mass changed in response to lipid mediators that are known to stimulate apoB secretion. Moreover, the nonlipid mediators that moderately stimulated MTP activity, in fact, reduced apoB secretion in CaCo-2 cells. Except for eicosapentaenoic acid, calcium ionophore, and phorbol ester, which for unexplained reasons decreased mRNA levels, mRNA levels of MTP were unaltered in cells incubated with the other mediators of apoB secretion. In the two-

step model for apoB-48 lipoprotein assembly, MTP catalyzes the first lipidation step of apoB-48 to form a dense "HDL-like" particle (2, 35). The second step which converts these particles into large triacylglycerol-rich lipoproteins is independent of MTP activity (2, 35). It is apparent from the results of the present study that the various mediators of apoB secretion alter steps in lipoprotein secretion that are not dependent on MTP activity and thus will not affect the first lipidation step in chylomicron assembly. The reported half-life for the 97 kDa subunit of MTP in HepG2 cells is 4.4 days (36). This rather long half-life makes it unlikely that potential inhibitors of MTP protein synthesis or MTP gene transcription will have an immediate impact on MTP activity, and thus, disruption of lipoprotein secretion. This likely explains our observations with PMA and calcium ionophore, both of which caused a marked decrease in MTP mRNA levels without reducing the activity or the amount of protein present. Similarly, in Hep-G2 cells, insulin was shown to cause a decrease in MTP mRNA levels and decrease apoB secretion without altering MTP activity (36). In data not shown, experiments were performed over 48 h in cells incubated in medium containing oleic acid and phosphatidylcholine and still, MTP protein levels were not changed. In another recent report by Brett et al. (37), a discordance between apoB-containing lipoprotein secretion and MTP expression was also observed. In this report, VLDL secretion was markedly decreased in livers of diabetic or suckling rats without an observed decrease in MTP mass or activity.

It has been proposed that inhibition of MTP could provide a mechanism for lowering plasma cholesterol and triacylglycerol levels in humans by interfering with lipid transport by the gut and/or liver (2, 8). Our present results and those of others (36) would suggest that prolonged inhibition of MTP synthesis or gene transcription would be required to alter the amount of MTP protein, and therefore, activity levels. It has been demonstrated, however, that the promoter of the MTP gene can be regulated (38). In transfected Hep-G2 cells, cholesterol was shown to increase promoter activity, whereas insulin decreased the activity. In a recent dietary study performed in hamsters, high fat feeding for 31 days was shown to increase message levels of MTP in liver and MTP mRNA and mass in the intestine (5). Moreover, intestinal MTP mRNA was elevated by 24 h after instituting the high fat diet, suggesting a more rapid regulation of MTP expression in the intestine.

The results suggest that the MTP is present in excess in enterocytes for the normal synthesis and secretion of apoB-containing lipoproteins. Thus enterocytes containing normal MTP expression have sufficient amounts of the protein to meet the general demands for transport of lipids from their site of synthesis to the site of lipoprotein assembly. Only under pathological conditions, such as abetalipoproteinemia, where enterocytes have abnormal expression of the MTP gene and its protein product, does the transport of lipids to the site of lipoprotein synthesis by MTP become a limiting step for the synthesis and transport of triacylglycerol-rich apoB lipoproteins (6–9). Thus, an abundant supply of MTP and apoB required for normal lipoprotein assembly in enterocytes provide a mechanism for the absorption and transport of large loads of dietary lipids.

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