methods

A simple, rapid, and sensitive fluorescence assay for microsomal triglyceride transfer protein

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Abstract Microsomal triglyceride transfer protein (MTP) is critical for the assembly and secretion of apolipoprotein B (apoB) lipoproteins. Its activity is classically measured by incubating purified MTP or cellular homogenates with donor vesicles containing radiolabeled lipids, precipitating the donor vesicles, and measuring the radioactivity transferred to acceptor vesicles. Here, we describe a simple, rapid, and sensitive fluorescence assay for MTP. In this assay, purified MTP or cellular homogenates are incubated with small unilamellar donor vesicles containing quenched fluorescent lipids (triacylglycerols, cholesteryl esters, and phospholipids) and different types of acceptor vesicles made up of phosphatidylcholine or phosphatidylcholine and triacylglycerols. Increases in fluorescence attributable to MTP-mediated lipid transfer are measured after 30 min. MTP's lipid transfer activity could be assayed using apoB lipoproteins but not with high density lipoproteins as acceptors. The assay was used to measure MTP activity in cell and tissue homogenates. Furthermore, the assay was useful in studying the inhibition of the cellular as well as purified MTP by its antagonists. This new method is amenable to automation and can be easily adopted for large-scale, high-throughput screening.—Athar, H., J. Iqbal, X-C. Jiang, and M. M. Hussain. **A simple, rapid, and sensitive fluorescence assay for microsomal triglyceride transfer protein. *J. Lipid Res.* 2004.** 45: **764–772.**

Supplementary key words lipoprotein assembly • cholesteryl esters • phospholipids • triacylglycerol • apolipoprotein B

Microsomal triglyceride transfer protein (MTP) is a dedicated chaperone that is required for the assembly of apolipoprotein B (apoB) lipoproteins [for reviews, see refs. (1–6)]. It is believed that MTP transfers lipids to nascent apoB in the endoplasmic reticulum and renders it secretion-competent by forming primordial lipoprotein particles [for reviews, see refs. (1–9)]. The importance of MTP's lipid transfer activity in apoB secretion has been established by three independent approaches. First, mutations in MTP have been correlated with the absence of apoB lipoproteins in abetalipoproteinemia (10, 11). Second, antagonists that inhibit MTP's lipid transfer activity in vitro have been shown to decrease apoB secretion in vivo (12–14). Third, the coexpression of MTP with apoB has been demonstrated to facilitate apoB secretion in cells that do not express apoB and MTP (15, 16). In addition to its lipid transfer activity, MTP is known to physically interact with apoB [for reviews, see refs. (1, 2)]. Recently, MTP was implicated in the import of triacylglycerols (TAGs) from cytosol to the lumen of the endoplasmic reticulum (17–19). Thus, it appears that MTP is a multifunctional protein (2) that plays a crucial role in the transport of TAG within intracellular organelles and in its secretion out of the cells.

MTP was identified and purified by Wetterau and Zilversmit (20, 21) based on a radioisotope assay. In this assay, radiolabeled TAGs are incorporated into donor vesicles consisting of phosphatidylcholine (PC) and cardiolipin. These vesicles are incubated with acceptor vesicles in the presence of MTP. After 1–3 h of incubation, the cardiolipin-containing donor vesicles are allowed to bind to DE52 and removed by centrifugation. Radioactivity remaining in the supernatant is quantified by scintillation counting. This procedure is labor-intensive and time-consuming. Negatively charged lipids, such as cardiolipin, are known to inhibit the lipid transfer activity of MTP (22). Because of the multiple steps involved in this procedure, it is difficult to automate. Thus, it might be advantageous to have a simple, one-step procedure to measure MTP activity.

Several pharmaceutical companies have identified antagonists that inhibit MTP activity (12–14, 23). A general approach taken by these companies was to identify compounds that decrease apoB secretion by HepG2 cells and then to determine their ability to inhibit MTP activity [for reviews, see refs. (3, 23)]. The primary screening involv-

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Abbreviations: apoB, apolipoprotein B; CE, cholesteryl ester; PC, phosphatidylcholine; PL, phospholipid; MTP, microsomal triglyceride transfer protein; TAG, triacylglycerol.

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ing the inhibition of apoB secretion might have been attributable to the difficulties involved in evaluating large numbers of compounds for MTP inhibition using a multistep radioactive assay (20, 21). This two-step screening has resulted in the identification of compounds that inhibit MTP activity and decrease apoB secretion. Surprisingly, different compounds identified by various companies are structurally very similar (23). Unfortunately, these compounds cause significant accumulation of TAG in cells. Could this be the result of the selection of compounds that are potent inhibitors of apoB secretion? Is it possible to identify compounds that inhibit MTP activity but that have a partial effect on lipoprotein secretion? For this purpose, it might be desirable to develop methods that can screen compounds primarily for their ability to inhibit the lipid transfer activity of MTP. Here, we describe a simple, rapid, and sensitive method to assay MTP activity that is amenable to automation and high-throughput screening.

MATERIALS AND METHODS

Materials

MTP was purified from bovine liver using the radioactivity assay (20, 21) and has been used previously (24–29). PC and TAG were from Avanti Lipids (Alabaster, AL). Fluorescence (nitrobenzoxadiazole)-labeled TAG was from Molecular Probes (Eugene, OR). Vesicles containing fluorescence-labeled cholesteryl ester (CE) and phospholipid (PL) were from Roar Biomedical, Inc. (New York, NY) and Cardiovascular Target, Inc. (New York, NY), respectively. Isopropanol and other chemicals were from Sigma Chemical Co. (St. Louis, MO). Acceptor vesicles were prepared as described by Wetterau and associates (20, 21, 30–32). Donor vesicles were also prepared according to their procedure except that cardiolipin was omitted and radiolabeled TAGs were replaced with fluorescence-labeled TAGs. Known amounts of fluorescent lipids were diluted in isopropanol to generate a standard curve, and amounts of labeled lipids in vesicles were determined after their disruption with isopropanol. The amounts of triolein in vesicles were quantified by a colorimetric assay (Infinity™ Triglyceride Reagent Kit; Sigma). The MTP inhibitor BMS200150 (diphenyl-propyl-piperidinyl-dihydroisoindole) has been described (12) and was a kind gift from Dr. Haris Jamil of Bristol-Myers Squibb (Princeton, NJ).

Transfer assay

The assay was done in Microfluor® 2 Black "U" Bottom Microtiter® plates (Thermo Labsystems, Franklin, MA). To the wells, we added 3 μl of donor (450 nmol of PC and 14 nmol of TAG per milliliter), 3 μl of acceptor (2,400 nmol PC/ml) vesicles, 10 μl of 10 mM Tris-HCl buffer, pH 7.4, 2 mM EDTA, 150 mM NaCl, distilled water to make the final assay volume of 100 μl, and purified MTP (0.1–1.5 μg) in triplicate. In some experiments, NaCl and BSA were added to obtain final concentrations of 150 mM and 1 mg/ml, respectively. Plates were incubated at 37°C or at room temperature for different time periods and read with a fluorescence plate reader (7620 Microplate Fluorimeter; Cambridge Technology, Watertown, MA) using 460 nm excitation and 530 nm emission wavelengths. To determine blank values, MTP was omitted from the wells. Total fluorescence in donor vesicles was determined by adding 97 μl of isopropanol to 3 μl of donor vesicles. To study MTP inhibition, different concentrations of BMS200150 were added to the reaction mixture before the addition of MTP. MTP activity (percentage transfer) was calculated by the following equation: percentage transfer = (arbitrary fluorescence units in assay wells − blank values)/(total fluorescence units − blank values) × 100. The specific activity is expressed as percentage transfer per micrograms per hour.

Transfection of Cos-7 cells

Cos-7 cells were grown (37°C, 5% CO_2, humidified chamber) in DMEM (Cellgro Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Life Technologies, Rockville, MD). Cells ($\sim 1 \times 10^6$) were plated in 75 cm^2 flasks 24 h before transfection. At the time of transfection, cells were ~50–60% confluent. The MTP expression vector (15) pRc-hMTP (7 μg; expresses human MTP under the control of the cytomegalovirus promoter) was introduced into Cos-7 cells complexed with 21 μl of FuGENE-6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Cells were maintained at 37°C and 5% CO_2 in 7 ml of medium for ~72 h. Cos-7 cells were also treated with FuGENE-6 alone (mock transfection) and used as controls.

Determination of MTP activity in cell homogenates

MTP activity in cellular homogenates was determined as described by Jamil et al. (12). Confluent cell monolayers were washed with ice-cold sterile PBS, pH 7.4, scraped in 5 ml of PBS, transferred to 15 ml conical tubes, and pelleted down by centrifugation (2,500 rpm, 10 min, room temperature). At this point, cell pellets can be stored at −70°C. For homogenization, 750 μl of homogenization buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, and 5 mM EDTA) and 7.5 μl of protease inhibitor cocktail (catalog number P 2714; Sigma) were added to the cell pellets. Cells were then suspended by repeated aspirations through a needle (29G, 1 1/2 inches) attached to a 3 ml syringe and homogenized on ice in a ball-bearing homogenizer (clearance ≈ 0.253 inches, 10 passages). Cell homogenates were stored on ice, and protein concentrations were determined by the Bradford method (33) using Coomassie Plus Reagent (Pierce, Rockford, IL) and BSA as standards. Cell homogenates were diluted with homogenization buffer to a protein concentration of ⩽1.5 mg/ml. MTP was released from microsomes by deoxycholate treatment (12). For this purpose, cell homogenates were adjusted to 0.054% deoxycholate by the addition of one-tenth volume of 0.54% sodium deoxycholate, pH 7.4, and left on ice for 30 min with occasional mixing. Cell membranes were subsequently removed by centrifugation in a SW55 Ti rotor at 50,000 rpm for 1 h at 10°C. The supernatants were dialyzed in 12–14 kDa cutoff dialysis bags against 15 mM Tris-HCl, pH 7.4, 40 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, with the first change after 1 h and the second change after 2 h followed by overnight dialysis. Cell homogenates were removed from the dialysis bag and used for protein determination and MTP assay. For inhibition studies, HepG2 cells were incubated with different concentrations of the MTP inhibitor BMS200150 for 24 h, and cell homogenates obtained from the cells were used for MTP assay.

To develop a more rapid procedure to assay cellular MTP, we evaluated the procedure described by Chang, Limanek, and Chang (34) for cell disruption. In this procedure, cells are first exposed to a hypotonic buffer and then scraped off the plates. Exposure to hypotonic buffers results in swelling of the cells, and scraping breaks these cells. Cell monolayers were washed twice with ice-cold PBS and once with 5 ml of 1 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 1 mM $MgCl_2$ at 4°C. Cells were then incubated for 2 min at room temperature in 5 ml of ice-cold 1 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 1 mM $MgCl_2$. The buffer was aspi-

rated, and 0.5 ml of the same buffer was added to cells. Cells were scraped and collected in ice-cold tubes, vortexed, and centrifuged (SW55 Ti rotor, 50,000 rpm, 10°C, 1 h), and supernatants were used for MTP assay and protein determination.

Determination of MTP activity in rat liver microsomes

Rat liver microsomes were prepared as described by Wetterau and Zilversmit (20, 21) with slight modifications. Briefly, 2 g of rat liver was cut into small pieces and washed twice with ice-cold PBS. Pieces were then homogenized in 2 ml of 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM sucrose, and 0.02% sodium azide using a Polytron homogenizer and centrifuged (Beckman microcentrifuge, 10,900 rpm, 30 min, 4°C). Supernatants were retained and adjusted to pH 5.1 with concentrated HCl, stirred in the cold for 30 min, and centrifuged (Beckman microcentrifuge, 13,000 rpm, 30 min, 4°C). Pellets were suspended in 2 ml of 1 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 1 mM $MgCl_2$, vortexed, and ultracentrifuged (SW55 Ti rotor, 50,000 rpm, 10°C, 1 h), and supernatants were used for MTP assay and protein determination.

Recommended assay conditions

Four different conditions (blank, total, positive control, and test) are recommended for each assay. In all assays, the reaction is started by the final addition of the MTP source. Pipette 3 μl each of acceptor and donor vesicles onto fluorescence microtiter (black) plates. Add 10 μl of 10 mM Tris, pH 7.4, containing 2 mM EDTA and 10 μl of 1% BSA stock in 1.5 M NaCl. In blanks, add the needed amount of control buffer (which contains the MTP source in positive control and test samples) and make up the volume with water to 100 μl. In positive controls, add a known amount of the MTP source and make up the volume with water to 100 μl. In tests, add unknown samples and make up the final assay volume. For totals, add 3 μl of donor vesicles and 97 μl of isopropanol only. Incubate at 37°C for 30 min. Measure fluorescence units using excitation and emission wavelengths of 460–470 and 530–550 nm, respectively. In case of low transfer activity, the incubation time can be increased. In fact, the same titer plate can be used several times to measure increases in fluorescence with time. However, the fluorophore is unstable in isopropanol over long periods of time. Thus, for periods longer than

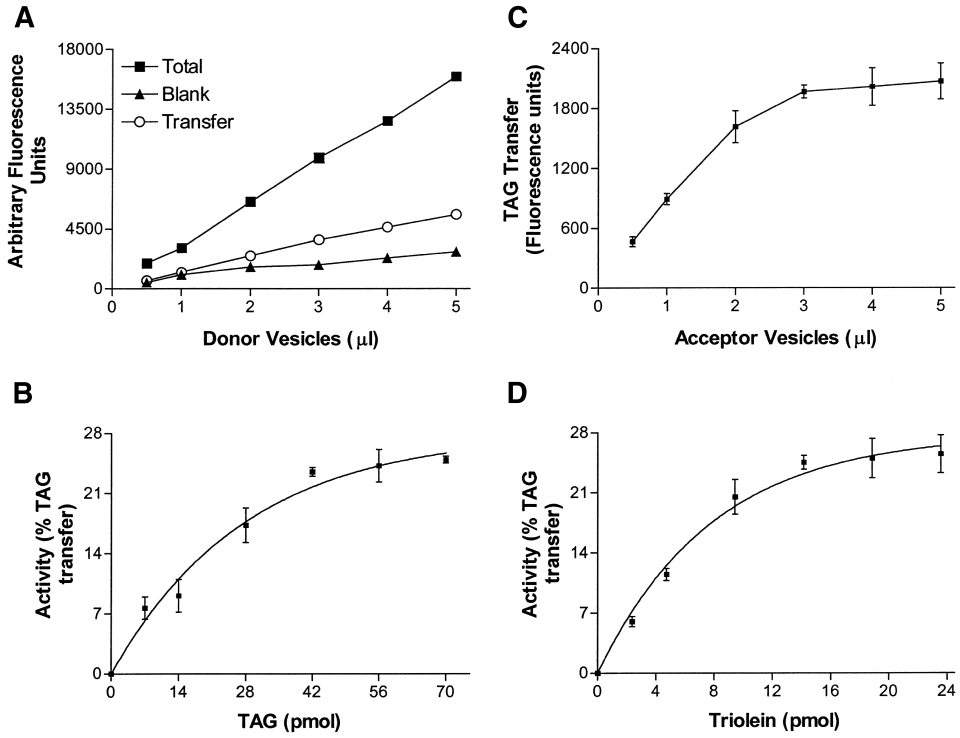

Fig. 1. Effect of different amounts of donor and acceptor vesicles on the transfer of triacylglycerol (TAG) by microsomal triglyceride transfer protein (MTP). A: Total. Different indicated volumes of donor vesicles were disrupted by the addition of 100 μl of isopropanol, and the fluorescence units were measured immediately. Note that this value represents the total amounts of fluorophore present in the vesicles and is not a simple sum of "blank" and "transfer" values. Blank. Different volumes of donor vesicles and 3 μl of acceptor vesicles were incubated in assay buffer (100 μl) as described in Materials and Methods, and fluorescence units were measured after 30 min of incubation at 37°C. Transfer. Incubations were the same as those described for blank except that these samples also contained 0.5 μg of purified MTP. This represents the amount of lipids being transported by MTP at a given time. During transfer, MTP-bound fluorescent lipids are most likely exposed to the aqueous environment and are detected by the fluorimeter. B: The data from A were used to calculate the percentage transfer of TAG as described in Materials and Methods. The TAG concentration in donor vesicles was 14 pmol/μl. C: Donor vesicles (3 μl) were incubated (30 min, 37°C) with different volumes of acceptor vesicles along with 0.5 μg of purified MTP. The fluorescence units were obtained by subtracting blank fluorescence units from units observed in assay tubes. D: The data from C were used to calculate percentage transfer as described in Materials and Methods. For this purpose, blank and total fluorescence units were determined in triplicate simultaneously, as described in Materials and Methods. Line graphs and error bars represent means ± SD (n = 3).

30 min, use total values from readings determined at 30 min or at earlier times. The assay ingredients, including vesicles and positive controls, are available from Chylos, Inc. (Woodbury, NY).

RESULTS

Optimization of assay conditions

To standardize a fluorescence assay for MTP, TAGs were incorporated into small unilamellar PC vesicles (donor vesicles). It was anticipated that the encapsulation would result in the quenching of the fluorophore. Indeed, disruption of increasing amounts of donor vesicles with isopropanol resulted in enhanced measurable fluorescence (**Fig. 1A**, total). This represents the total amounts of fluorophore present in the vesicles. Before disruption, this fluorescence is not detectable because it is quenched in vesicles. It was also envisioned that donor vesicles would be stable and would not release the fluorophore in the absence of MTP. To determine the stability, donor vesicles were mixed with acceptor vesicles and the fluorescence in the absence of MTP was measured after 30 min (Fig. 1A, blank). The blank fluorescence values ranged between

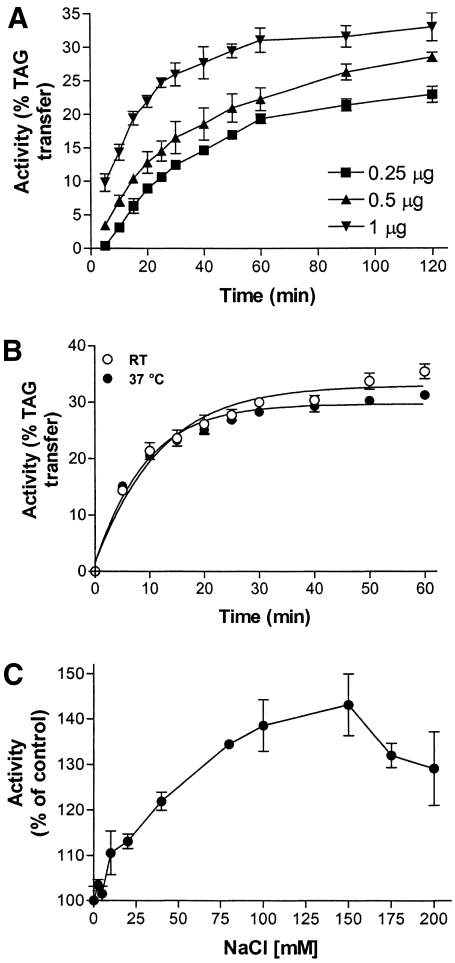

Fig. 2. Effect of time, temperature, and NaCl on TAG transfer activity of MTP. A: Time course. Different indicated amounts of MTP were incubated with donor and acceptor vesicles as described in Materials and Methods. Appropriate totals and blanks were included as described in Materials and Methods. Fluorescence readings were measured at the indicated times in triplicate using a microplate reader. The percentage transfer of TAG is plotted against time. B: Temperature. The experiment was performed on two different microtiter plates. Purified MTP (0.25 µg/well) was incubated with donor and acceptor vesicles in triplicate. One plate was incubated at 37°C, and the other was left at room temperature (RT). Fluorescence readings were taken at different time points at room temperature (22°C). C: Effect of NaCl. Donor vesicles (3 µl), acceptor vesicles (3 µl), and MTP (1 µg) were incubated in triplicate for 30 min in 1 mM Tris-HCl, pH 7.4, and 2 mM EDTA in the presence of various indicated concentrations of NaCl. Line graphs and error bars represent means ± SD.

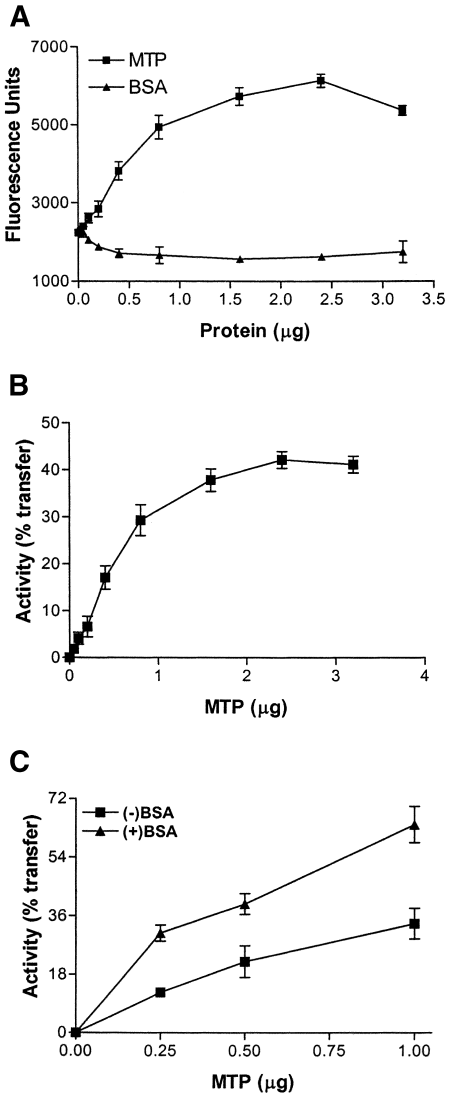

Fig. 3. Specificity of TAG transfer activity: A: Donor [3 µl; 450 nmol of phosphatidylcholine (PC) and 14 nmol of TAG per milliliter] and acceptor (3 µl; 2,400 nmol PC/ml) vesicles were incubated with different indicated amounts of MTP or BSA in triplicate for 30 min. Fluorescence units were obtained after subtracting blanks from the assay tubes. B: Data from A for MTP were used to determine percentage transfer activity. For BSA, these values were negative and were not plotted. C: The assay was performed in the absence and presence of BSA (0.1%) using various indicated amounts of purified MTP. Line graphs and error bars represent means ± SD.

13% and 19% [15.7 ± 2.7% (average ± SD; n = 3)] of the totals. The blank values probably represent the small leakage of the fluorophore. It was then hypothesized that the extraction of TAG from donor vesicles by MTP for transfer would manifest as increased detectable fluorescence. Incubation of constant amounts of MTP and acceptor vesicles with increasing amounts of the donor vesicles resulted in increased detection of fluorescence, indicating the transfer of TAG by MTP (Fig. 1A, transfer). The "transfer" represents the amounts of TAG being transferred by MTP between vesicles. During transfer, the MTP-bound fluorophore is most likely exposed to the aqueous environment and is now detected by the fluorimeter. The fluorescence units were 40–47% higher than the blank values. Next, the data were used to calculate the percentage transfer of TAG (Fig. 1B). The percentage transfer activity increased up to 2 μl of the donor vesicles (28 pmol of TAG) and appeared to saturate thereafter.

Next, we studied the effect of different concentrations of acceptor vesicles. In these experiments, constant amounts of MTP and donor vesicles were incubated with different volumes of acceptor vesicles (Fig. 1C). The amounts of TAG transferred increased with increasing amounts of the acceptor vesicles and saturated at 2 μl. The increases in the transfer at lower concentrations of acceptor vesicles indicated that these vesicles were limiting in the assay conditions. However, at 2 μl and above, the assay became independent of the acceptor vesicle concentrations. Note that there was no decrease in detectable fluorescence with increasing concentrations of acceptor vesicles. This indicates that MTP transfers lipids between vesicles and does not cause the unidirectional deposition of lipids into the acceptor vesicles. Under these conditions, MTP was in the process of transferring ~20% of the total TAG present in the donor vesicles (Fig. 1D). In subsequent studies, 3 μl of the acceptor vesicles was used.

To determine the intra-assay coefficient of variation (CV), we performed the transfer assay in 10 tubes using 0.5 μg of MTP, 3 μl of donor, and 3 μl of acceptor vesicles. The percentage transfer observed was 19.9 ± 1.8 (mean ± SD; n = 10), and the intra-assay CV was 0.09. Similarly, we also evaluated the interassay variations. Comparison of seven different independent determinations performed in triplicate using 1 μg of MTP revealed an interassay CV of 0.19. The percentage transfer observed in these experiments was 34.5 ± 3.0.

Experiments were then performed to determine the effect of time, temperature, and NaCl concentrations required for MTP activity (**Fig. 2**). At all of the different concentrations of MTP used, TAG transfer activity increased with time up to 30 min (Fig. 2A). After that time, the transfer activity began to saturate. Next, we studied the effect of temperature on transfer activity (Fig. 2B). The lipid transfer activity of MTP was the same at room temperature (22°C) and at 37°C, indicating no significant effect of temperature on activity. This probably indicates that MTP is optimally active at 22°C. We also determined the effect of NaCl on MTP activity (Fig. 2C). The addition of increasing concentrations of NaCl up to 150 mM resulted in increased MTP activity. Higher concentrations of NaCl appear to inhibit transfer activity. We conclude that 30 min incubations and 150 mM NaCl are optimum to determine MTP activity.

Next, we studied the specificity of the assay using different concentrations of purified MTP and BSA on TAG transfer (**Fig. 3**). The addition of increasing amounts of MTP resulted in enhanced detectable fluorescence (Fig. 3A). In contrast, the presence of BSA decreased the amounts of detectable fluorescence and may represent either quenching of the released fluorescence by BSA or stabilization of the donor vesicles by BSA, preventing the basal fluorophore leakage. In Fig. 3B, the data were converted to measure the percentage of TAG undergoing transfer between vesicles. Under the experimental conditions, the amounts of TAG being transferred reached saturation at 2 μg of MTP. At saturation, ~40% of the total TAG was in the process of transfer and probably represented the maximum binding capacity of MTP.

Because BSA decreased the fluorescence units in blank samples, we reasoned that it may have a positive effect on MTP assay. Furthermore, the presence of BSA may decrease the loss of MTP and vesicles by adsorption to tube

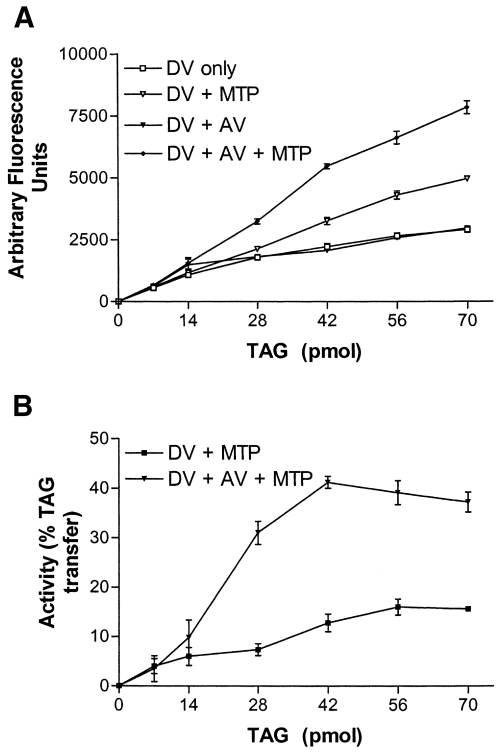

Fig. 4. Role of acceptor vesicles in lipid transfer by MTP. A: Different amounts of donor vesicles (450 nmol of PC and 14 nmol of TAG per milliliter) were incubated without acceptor vesicles and MTP (DV only), with 0.5 μg of purified MTP (DV + MTP), with 3 μl of acceptor vesicles (DV + AV), or with 3 μl of acceptor vesicles (2,400 nmol PC/ml) and 0.5 μg of purified MTP (DV + AV + MTP) in assay buffer (100 μl). Fluorescence units were measured after 30 min of incubation at 37°C. B: The data from A were used to calculate percentage transfer as described in Materials and Methods. Line graphs and error bars represent means ± SD.

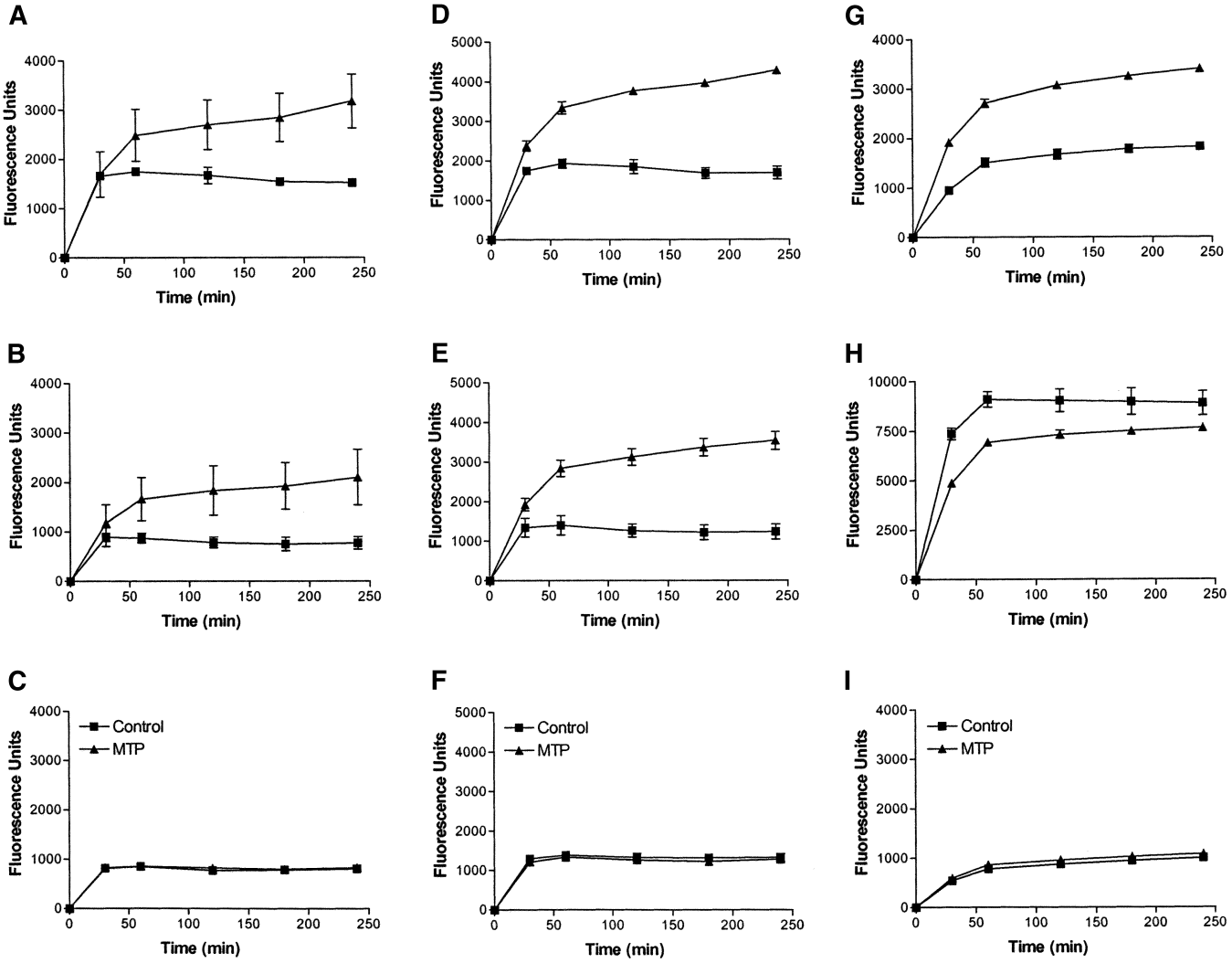

Fig. 5. Transfer of various lipids in the presence of different acceptors. Three different types of donor vesicles (3 μl) containing TAG (A–C), cholesteryl ester (D–F), or phospholipids (G–I) were used. The acceptor vesicles (3 μl; 2,400 nmol PC/ml) were small unilamellar vesicles (PC/TAG vesicles; A, D, and G), apolipoprotein B (apoB) lipoproteins (VLDL and LDL; B, E, and H), and HDLs (10 mg protein/ml; C, F, and I). Different donor and acceptor vesicles were incubated without (control) or with 1 μg of purified MTP (MTP) in triplicate for the indicated times, and fluorescence was measured as described in Materials and Methods. Line graphs and error bars represent means ± SD.

surfaces. To test this hypothesis, we added BSA (1 mg/ml) in the assay. As shown in Fig. 3C, the activity measured in the presence of BSA was almost twice that observed in the absence of BSA. This was mainly attributable to decreased blank values in the presence of BSA. The blank values in the presence and absence of BSA were 12.5 ± 0.7% and 18.5 ± 0.5% (n = 3) of the totals. Thus, the inclusion of BSA improves the sensitivity of the assay, most likely by preventing the leakage of the fluorophore from the donor vesicles.

Subsequently, we compared the specific activity of MTP determined by radiolabel and fluorescence assays. The specific activity (percentage transfer per microgram per hour) in various preparations using the radiolabel assay was 12.5 ± 2.4 (n = 27). The specific activity by the fluorescence method in the absence of BSA was 92.6 ± 19.7 (n = 20), whereas the specific activity determined in the presence of BSA was 204.4 ± 33.1 (n = 7). Thus, specific activities measured by the fluorescence assay were higher than those observed using the radiolabel assay. The higher specific activities may be attributable to the higher sensitivity of the assay. Another reason for the difference may be different parameters used in these two as-

TABLE 1. Determination of MTP activity in cells using the deoxycholate method

Cells	Protein per Assay	Fluorescence	Specific Activity
	μg	*% change*	*% transfer/μg/h*
Cos-7 cells	70.9	0.9 ± 0.6	0.026 ± 0.017
Cos-7 cells + MTP[a]	51.0	10.2 ± 1.0	0.401 ± 0.04
HepG2 cells	47.2	23.2 ± 0.6	0.982 ± 0.024
Caco-2 cells	48.6	19.5 ± 1.4	0.801 ± 0.057

MTP, microsomal triglyceride transfer protein.
[a] Cos-7 cells transiently transfected with MTP expression vectors.

says. In the radiolabel assay, the amount of TAG transferred to acceptor vesicles is measured. In contrast, the fluorescence assay measures the amount of TAG being transferred by the MTP.

Role of acceptor vesicles

The MTP assay consists of three components: donor vesicles, acceptor vesicles, and MTP. Obviously, donor vesicles and MTP are required. We reasoned that MTP could transfer lipids between donor vesicles and that the acceptor vesicles may not be needed for activity measurements (**Fig. 4**). Incubation of increasing amounts of donor vesicles with (DV + AV) or without (DV only) acceptor vesicles gave similar fluorescent readings, indicating little transfer of TAG in the absence of MTP (Fig. 4A). These data are in agreement with the blanks in Fig. 1A. Incubation of increasing amounts of donor vesicles with MTP (DV + MTP) resulted in some increase in fluorescence, indicating some transfer of lipids. In contrast, a significant increase in fluorescence was observed when acceptor vesicles were included in the reaction mixture (DV + AV + MTP). The data were then used to calculate percentage transfer of TAG (Fig. 4B). In the absence of acceptor vesicles (DV + MTP), the TAG transfer ranged between 4% and 16%. In the presence of acceptor vesicles (DV + AV + MTP), however, MTP was engaged in transferring almost 40% of TAG present in donor vesicles. These data demonstrate that the presence of acceptor vesicles greatly facilitates the transfer process.

Transfer of different lipids to various acceptors by MTP

We studied the effect of different types of acceptor vesicles on the transfer of various lipids by MTP. First, we used PC or PC/TAG vesicles as acceptors. The percentage transfers observed in triplicate with these acceptors were 33 ± 2.6 and 30.9 ± 1.3, respectively, indicating that the absence of TAG in the acceptor vesicles does not have any significant effect on the transfer activity.

MTP is known to transfer other lipids besides TAG (20). Thus, experiments were performed to evaluate the suitability of this assay to study the transfer of CEs and PLs in addition to TAG (**Fig. 5**). In this experiment, we used small unilamellar vesicles (PC/TAG vesicles), apoB lipoproteins (which contained both VLDL and LDL), and HDL as acceptors and studied the transfer over a period of 4 h. This experiment was performed before the optimization of conditions described in Fig. 2. In these early experiments, we had to incubate for a longer period of time to measure the transfer activity. MTP transferred TAG when donor vesicles were incubated with small unilamellar PC/TAG vesicles (Fig. 5A) and apoB lipoproteins (Fig. 5B) but not when incubated with HDL (Fig. 5C). The transfer resulted in a 109–172% increase in fluorescence units after 4 h of incubation (Fig. 5A, B). Similarly, MTP transferred CE in the presence of PC/TAG vesicles (Fig. 5D) and apoB lipoproteins (Fig. 5E) but not in the presence of HDL (Fig. 5F). MTP was able to transfer PLs when donor vesicles were incubated with PC/TAG vesicles; the increase in fluorescence was 186% at 4 h (Fig. 5G). However, studies of the transfer of PL by MTP in the presence of apoB lipoproteins as acceptors were difficult to interpret (Fig. 5H). This was attributable to a significant increase in the background fluorescence when donor vesicles were incubated with apoB lipoproteins in the absence of MTP (compare controls in Fig. 5G, H; also note the different *y* values in these panels). Again, MTP did not transfer PL when HDL was used as an acceptor (Fig. 5I). These

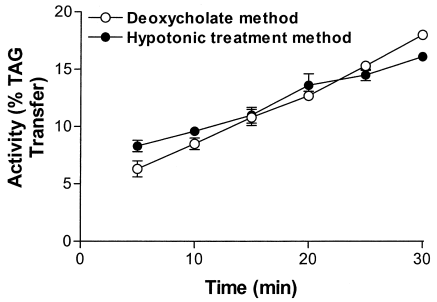

Fig. 6. Comparison of two methods to measure MTP activity in cell homogenates. MTP activity was measured in HepG2 cell homogenates prepared by either the deoxycholate or the hypotonic buffer treatment method as described in Materials and Methods. For transfer assays, 40–50 μg of cellular proteins was used in triplicate, and the activity was measured for up to 1 h. Line graphs and error bars represent means ± SD.

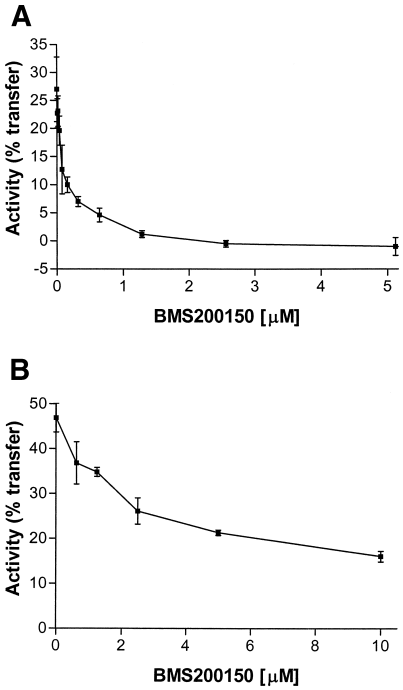

Fig. 7. Inhibition of MTP activity by BMS200150. A: Purified MTP (1 μg) was incubated with donor and acceptor vesicles for 30 min in the presence of the indicated concentrations of the MTP inhibitor BMS200150. B: HepG2 cells were incubated in triplicate with the indicated concentrations of BMS200150 for 24 h. Cells were washed, and lysates were prepared using the deoxycholate method described in Materials and Methods. For transfer assays, 40–50 μg of cellular proteins was used in triplicate. Line graphs and error bars represent means ± SD.

studies show that MTP actively transfers both neutral lipids, TAG and CE, when small unilamellar vesicles and apoB lipoproteins are used as acceptors.

Measurement of MTP activity in cells

The next question was to determine whether this assay could be used to measure MTP activity in cells (**Table 1**). We released microsomal MTP by deoxycholate treatment described by Jamil et al. (12). Cos-7 cells were transfected or not with MTP expression vector to determine the specificity of the MTP assay (Table 1). As expected, no MTP was detectable in mock-transfected Cos-7 cells. However, transfection with MTP expression vectors resulted in increased MTP activity in Cos-7 cells, in agreement with other studies (15, 29). Similarly, MTP activity could be measured in cellular homogenates of HepG2 and differentiated Caco-2 cells. These studies indicate that the new method can be used to determine cellular MTP activity.

The deoxycholate method is cumbersome, involves several steps, and requires at least 2 days. To simplify this procedure, we evaluated the method of Chang, Limanek, and Chang (34), which involves the disruption of cells by hypotonic buffers and requires far less time to prepare cell extracts for assay (**Fig. 6**). Both methods gave similar MTP activity. Thus, hypotonic treatment is a better procedure to measure cellular MTP activity because less time and fewer manipulations are required.

Next, attempts were made to determine the MTP activity in liver. Rat liver microsomes were subjected to hypotonic buffer treatment, and released contents were used for MTP activity measurements. The specific activity (percentage transfer per microgram per hour) of MTP in liver microsomal contents was 0.498 ± 0.09 (mean $\pm$ SD, $n = 9$).

Inhibition of MTP activity

Experiments were then performed to measure the effect of MTP antagonists on its activity (**Fig. 7**). For this purpose, we used an MTP inhibitor, BMS200150, described by Jamil et al. (12). As shown in Fig. 7A, increasing concentrations of BMS200150 resulted in a dose-dependent inhibition of the purified MTP activity. The IC_{50} was 0.08 μM and was in the range reported by others (12). Next, HepG2 cells were incubated with different concentrations of the inhibitor for 24 h, and homogenates were prepared using the deoxycholate method (12) and assayed for MTP activity. As shown in Fig. 7B, increasing concentrations of BMS200150 resulted in decreased cellular MTP activity. The IC_{50} value of $\sim$1.3 μM is in agreement with published studies (12). The differences in the IC_{50} values obtained for the purified MTP and cell lysates may be attributable to the presence of other proteins in cell lysates that might interact with the inhibitor and decrease its efficacy. These studies indicate that the assay is useful in measuring MTP activity and its inhibition by antagonists.

DISCUSSION

The method described here to measure MTP activity is simple and rapid. It is based on the determination of increases in fluorescence attributable to the binding of fluorophore with MTP that occurs during the transfer of lipids between donor and acceptor vesicles. The method shows a linear relationship with all three components of the assay mixture and time (Figs. 1–4). This procedure reproduces the lipid transfer properties of the MTP as determined by radioactive assay. It faithfully measures cellular activity in cells known to express MTP and does not measure it in cells that do not express MTP (Table 1). Furthermore, it displays similar inhibitory properties of antagonists that were identified using the radioisotope assay (Fig. 7).

MTP shows significantly higher activity in the presence of acceptor vesicles (Fig. 4). The low lipid binding activity of MTP in the absence of acceptor vesicles provided us with a unique opportunity to understand the role of different acceptor vesicles in the lipid transfer process. These studies showed that MTP efficiently transfers lipids in the presence of unilamellar vesicles and apoB lipoproteins (Fig. 5). Under the conditions of this assay, we observed very low to no lipid transfer activity when HDL was used as an acceptor. It is not clear why HDL did not act as an acceptor. It should be noted that HDL has been used as an acceptor with LDL to demonstrate lipid transfer by MTP (21). We have not tried to study the transfer of lipids from LDL to HDL in our studies.

In summary, we have described a simple and rapid fluorescence assay for the measurement of MTP activity. The advantages of the new method include ease, rapidity, sensitivity, avoidance of the use of negatively charged lipids, versatility in studying different lipid transfer activities by purified and cellular MTP, ability to measure inhibitory activities of antagonists, and forestalling the use of radioactivity. The fluorescence assay described here can be easily automated and used for large-scale, high-throughput screening. This approach may identify compounds that partially inhibit MTP activity and possibly minimize the unwanted side effects related to TAG accumulation in cells. It is becoming clear that MTP is a multifunctional protein that may have functions other than being a dedicated lipoprotein assembly chaperone. Some of the compounds identified via screening based on the fluorescence assay may be useful in the identification of other functions of MTP unrelated to lipoprotein assembly and secretion.

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REFERENCES

1. Hussain, M. M., J. Shi, and P. Dreizen. 2003. Microsomal triglyceride transfer protein and its role in apolipoprotein B-lipoprotein assembly. *J. Lipid Res.* **44:** 22–32.
2. Hussain, M. M., J. Iqbal, K. Anwar, P. Rava, and K. Dai. 2003. Microsomal triglyceride transfer protein: a multifunctional protein. *Front. Biosci.* **8:** S500–S506.
3. Bakillah, A., and A. El Abbouyi. 2003. The role of microsomal triglyceride transfer protein in lipoprotein assembly: an update. *Front. Biosci.* **8:** D294–D305.

4. Shelness, G. S., and J. A. Sellers. 2001. Very-low-density lipoprotein assembly and secretion. *Curr. Opin. Lipidol.* **12:** 151–157.
5. Gordon, D. A., and H. Jamil. 2000. Progress towards understanding the role of microsomal triglyceride transfer protein in apolipoprotein-B lipoprotein assembly. *Biochim. Biophys. Acta.* **1486:** 72–83.
6. Wetterau, J. R., M. C. M. Lin, and H. Jamil. 1997. Microsomal triglyceride transfer protein. *Biochim. Biophys. Acta.* **1345:** 136–150.
7. Hussain, M. M. 2000. A proposed model for the assembly of chylomicrons. *Atherosclerosis.* **148:** 1–15.
8. Davis, R. A. 1999. Cell and molecular biology of the assembly and secretion of apolipoprotein B-containing lipoproteins by the liver. *Biochim. Biophys. Acta.* **1440:** 1–31.
9. Fisher, E. A., and H. N. Ginsberg. 2002. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **277:** 17377–17380.
10. Wetterau, J. R., L. P. Aggerbeck, M-E. Bouma, C. Eisenberg, A. Munck, M. Hermier, J. Schmitz, G. Gay, D. J. Rader, and R. E. Gregg. 1992. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. *Science.* **258:** 999–1001.
11. Shoulders, C. C., D. J. Brett, J. D. Bayliss, T. M. E. Narcisi, A. Jarmuz, T. T. Grantham, P. R. D. Leoni, S. Bhattacharya, R. J. Pease, P. M. Cullen, S. Levi, P. G. H. Byfield, P. Purkiss, and J. Scott. 1993. Abetalipoproteinemia is caused by defects of the gene encoding the 97 kDa subunit of a microsomal triglyceride transfer protein. *Hum. Mol. Genet.* **2:** 2109–2116.
12. Jamil, H., D. A. Gordon, D. C. Eustice, C. M. Brooks, J. K. Dickson, Jr., Y. Chen, B. Ricci, C-H. Chu, T. W. Harrity, C. P. Ciosek, Jr., S. A. Biller, R. E. Gregg, J. R. Wetterau. 1996. An inhibitor of the microsomal triglyceride transfer protein inhibits apoB secretion from HepG2 cells. *Proc. Natl. Acad. Sci. USA.* **93:** 11991–11995.
13. Haghpassand, M., D. Wilder, and J. B. Moberly. 1996. Inhibition of apolipoprotein B and triglyceride secretion in human hepatoma cells (HepG2). *J. Lipid Res.* **37:** 1468–1480.
14. Benoist, F., and T. Grand-Perret. 1997. Co-translational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein—synchronized translation studies on HepG2 cells treated with an inhibitor of microsomal triglyceride transfer protein. *J. Biol. Chem.* **272:** 20435–20442.
15. Gordon, D. A., H. Jamil, D. Sharp, D. Mullaney, Z. Yao, R. E. Gregg, and J. Wetterau. 1994. Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc. Natl. Acad. Sci. USA.* **91:** 7628–7632.
16. Leiper, J. M., J. D. Bayliss, R. J. Pease, D. J. Brett, J. Scott, and C. C. Shoulders. 1994. Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product, mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. *J. Biol. Chem.* **269:** 21951–21954.
17. Raabe, M., M. M. Véniant, M. A. Sullivan, C. H. Zlot, J. Björkegren, L. B. Nielsen, J. S. Wong, R. L. Hamilton, and S. G. Young. 1999. Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. *J. Clin. Invest.* **103:** 1287–1298.
18. Wang, Y. W., K. Tran, and Z. M. Yao. 1999. The activity of microsomal triglyceride transfer protein is essential for accumulation of triglyceride within microsomes in McA-RH7777 cells—a unified model for the assembly of very low density lipoproteins. *J. Biol. Chem.* **274:** 27793–27800.
19. Kulinski, A., S. Rustaeus, and J. E. Vance. 2002. Microsomal triacylglycerol transfer protein is required for lumenal accretion of triacylglycerol not associated with Apo B, as well as for Apo B lipidation. *J. Biol. Chem.* **277:** 31516–31525.
20. Wetterau, J. R., and D. B. Zilversmit. 1984. A triglyceride and cholesteryl ester transfer protein associated with liver microsomes. *J. Biol. Chem.* **259:** 10863–10866.
21. Wetterau, J. R., and D. B. Zilversmit. 1985. Purification and characterization of microsomal triglyceride and cholesteryl ester transfer protein from bovine liver microsomes. *Chem. Phys. Lipids.* **38:** 205–222.
22. Jamil, H., J. K. Dickson, Jr., C-H. Chu, M. W. Lago, J. K. Rinehart, S. A. Biller, R. E. Gregg, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. *J. Biol. Chem.* **270:** 6549–6554.
23. Chang, G., R. B. Ruggeri, and H. J. Harwood, Jr. 2002. Microsomal triglyceride transfer protein (MTP) inhibitors: discovery of clinically active inhibitors using high-throughput screening and parallel synthesis paradigms. *Curr. Opin. Drug Discov. Dev.* **5:** 562–570.
24. Hussain, M. M., A. Bakillah, and H. Jamil. 1997. Apolipoprotein B binding to microsomal triglyceride transfer protein decreases with increases in length and lipidation: implications in lipoprotein biosynthesis. *Biochemistry.* **36:** 13060–13067.
25. Hussain, M. M., A. Bakillah, N. Nayak, and G. S. Shelness. 1998. Amino acids 430–570 in apolipoprotein B are critical for its binding to microsomal triglyceride transfer protein. *J. Biol. Chem.* **273:** 25612–25615.
26. Bakillah, A., H. Jamil, and M. M. Hussain. 1998. Lysine and arginine residues in the N-terminal 18% of apolipoprotein B are critical for its binding to microsomal triglyceride transfer protein. *Biochemistry.* **37:** 3727–3734.
27. Bakillah, A., N. Nayak, U. Saxena, R. M. Medford, and M. M. Hussain. 2000. Decreased secretion of apoB follows inhibition of apoB-MTP binding by a novel antagonist. *Biochemistry.* **39:** 4892–4899.
28. Bakillah, A., and M. M. Hussain. 2001. Binding of microsomal triglyceride transfer protein to lipids results in increased affinity for apolipoprotein B: Evidence for stable microsomal MTP-lipid complexes. *J. Biol. Chem.* **276:** 31466–31473.
29. Tietge, U. J. F., A. Bakillah, C. Maugeais, K. Tsukamoto, M. M. Hussain, and D. J. Rader. 1999. Hepatic overexpression of microsomal triglyceride transfer protein (MTP) results in increased in vivo secretion of VLDL triglycerides and apolipoprotein B. *J. Lipid Res.* **40:** 2134–2139.
30. Wetterau, J. R., L. P. Aggerbeck, P. M. Laplaud, and L. R. McLean. 1991. Structural properties of the microsomal triglyceride-transfer protein complex. *Biochemistry.* **30:** 4406–4412.
31. Atzel, A., and J. R. Wetterau. 1993. Mechanism of microsomal triglyceride transfer protein catalyzed lipid transport. *Biochemistry.* **32:** 10444–10450.
32. Atzel, A., and J. R. Wetterau. 1994. Identification of two classes of lipid molecule binding sites on the microsomal triglyceride transfer protein. *Biochemistry.* **33:** 15382–15388.
33. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
34. Chang, T. Y., J. S. Limanek, and C. C. Chang. 1981. A simple and efficient procedure for the rapid homogenization of cultured animal cells grown in monolayer. *Anal. Biochem.* **116:** 298–302.