Identification of Two Classes of Lipid Molecule Binding Sites on the Microsomal Triglyceride Transfer Protein†

Amy Atzel‡,§,|| and John R. Wetterau*,⊥

Division of Lipoprotein Research, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575, and Department of Metabolic Diseases, Bristol-Myers Squibb, P.O. Box 4000, Princeton, New Jersey 08543-4000

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ABSTRACT: The gene for the microsomal triglyceride transfer protein (MTP) is defective in subjects with the genetic disease abetalipoproteinemia, indicating that MTP is essential for the assembly of apolipoprotein B containing lipoproteins. *In vitro*, MTP is a lipid molecule binding protein that catalyzes lipid transport between membranes by a shuttle mechanism. In this study, the lipid binding properties of MTP were examined. MTP was incubated with donor phosphatidylchloine vesicles of varying neutral lipid composition. MTP was subsequently reisolated by ultracentrifugation, and MTP-bound lipid was quantitated. When the triolein content of the vesicles was increased up to 4 mol %, neutral lipid binding to MTP increased proportionately, while phosphatidylcholine binding appeared to remain constant around two molecules per MTP. Using phosphatidylcholine emulsions containing 60 mol % triolein as the donor particles resulted in only a slight increase in triolein binding to MTP. The highest triolein:MTP ratio observed was (0.20−0.25):1. Differences in the neutral and phospholipid binding properties of MTP were observed by measuring the transport of lipid from MTP to acceptor vesicles. Transport of triolein was rapid and complete, while phosphatidylcholine transport was biphasic, containing rapid and slow phases. These results indicated that MTP contains more than one class of lipid molecule binding site. Measurements of fluorescent lipid transport from donor vesicles to MTP supported this hypothesis. The transport of pyrene-labeled triglyceride from donor particles to MTP was rapid, while phosphatidylcholine transfer had fast and slow phases. From these data, we propose that MTP contains at least two distinct classes of lipid molecule binding sites that differ in function. The fast site or sites are responsible for lipid transport.

The microsomal triglyceride transfer protein (MTP)[1] is a soluble heterodimeric enzyme (Wetterau et al., 1990, 1991a) that catalyzes the intermembrane transport of triglyceride, cholesteryl ester, and phosphatidylcholine (Wetterau & Zilversmit, 1984, 1985). MTP is present in mammalian liver and intestine, and is located subcellularly within the microsomes of these tissues (Wetterau & Zilversmit, 1986). The primary sequences of both the small (apparent molecular mass of 58 kDa) and large (apparent molecular mass of 88 kDa) MTP subunits have been deduced from their cDNA sequences [Edman et al. (1985) and Sharp et al. (1993), respectively]. The 58 kDa subunit of MTP is protein disulfide isomerase (PDI) (Wetterau et al., 1990), a ubiquitous multifunctional resident endoplasmic reticulum protein (Freedman, 1989). The recent finding that mutations in the gene for the large subunit of MTP are a cause of abetalipoproteinemia (Sharp et al., 1993; Shoulders et al., 1993), a genetic disease characterized by the absence of circulating apolipoprotein B (apoB) containing lipoproteins (Kane & Havel, 1989; Gregg & Wetterau, 1994), indicates that MTP is of fundamental importance in plasma lipoprotein biogenesis.

Previous kinetic analyses revealed that MTP transports lipid between membranes by a shuttle mechanism (ping-pong Bi-Bi kinetics) (Atzel & Wetterau, 1993), indicating that MTP binds and transports lipid molecules between membranes. To begin to elucidate the relationship between MTP structure and function, we have further investigated MTP−lipid molecule interactions. The goal of this study was to determine the number and nature of the lipid molecule binding sites on MTP, and elucidate how they contribute to lipid transport. The results indicated that there are two distinct classes of lipid molecule binding sites present on MTP and that the sites differentially bind and transport phospholipid and neutral lipid.

MATERIALS AND METHODS

Materials. Radiolabeled lipids, [2-*palmitoyl*-9,10-^{3}H(N)]-dipalmitoylphosphatidylcholine ([^{3}H]phosphatidylcholine), [*dioleoyl*-1-^{14}C]phosphatidylcholine, [*carboxyl*-^{14}C]triolein ([^{14}C]triolein), and [*oleate*-1-^{14}C]cholesteryl oleate ([^{14}C]-

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* To whom correspondence should be addressed.

‡ University of Cincinnati College of Medicine.

§ Current address: Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210.

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[1] Abbreviations: MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; CETP, cholesteryl ester transfer protein; apoB, apolipoprotein B; Py-PC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; Py-CE, cholesteryl 1-pyrenedecanoate; Py-TG, 1,2-dioleoyl-3-(pyren-1-yl)decanoyl-*rac*-glycerol; Py-butyrate, pyrene butyrate; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine.

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cholesteryl oleate), were purchased from New England Nuclear (Hoffman Estates, IL). Cholesteryl 1-pyrenedecanoate (Py-CE), 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (Py-PC), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) were purchased from Molecular Probes (Eugene, OR). Egg phosphatidylcholine, dioleoylphosphatidylcholine, triolein, 1,2-dioleoyl-3-(pyren-1-yl)decanoyl-*rac*-glycerol (Py-TG), cholesteryl oleate, cardiolipin, and pyrene butyrate (Py-butyrate) were purchased from Sigma (St. Louis, MO). All lipids were stored in chloroform under N_2 gas at −20 °C.

Protein Isolation. Bovine liver MTP was purified as previously described (Wetterau et al., 1991b), dialyzed into assay buffer (40 mM NaCl, 1 mM ethylenediaminetetraacetate, 0.02% NaN_3, and 15 mM Tris, pH 7.4), and stored at 4 °C. PDI was isolated by a modification of the method of Hillson et al. (1984) as described by Wetterau et al. (1990).

Vesicle Preparation. Both radiolabeled and fluorescent lipid vesicles were prepared as follows. Lipids (0.1−5 μmol total lipid) were mixed in organic solvent. The solvent was evaporated under a stream of N_2 gas, up to 1 mL of assay buffer was added to the dried lipid, and the mixture was sonicated in a bath sonicator (Laboratory Supplies Inc., Hicksville, NY) under a N_2 atmosphere until visual clarity was obtained. The lipid specific activity was measured by counting an aliquot of the final vesicle preparation. Vesicles were used in experiments within 4 h of preparation.

Binding Assay. MTP (50−200 μg) in assay buffer was incubated with radiolabeled vesicles for 4 h at 23 °C. In preliminary experiments, similar results were found using shorter incubation times (30 min), indicating that the binding was not time-dependent within the time frame of the assay. After incubation, the mixture (<0.8 mL) was layered on top of ~5 mL of 31% sucrose (density = 1.13 g/mL) and spun at 159000*g* for 48 h. At this density, MTP sediments toward the bottom of the tube while vesicles float. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis and silver stain analysis of centrifuge tube fractions showed a gradient of MTP with greater than three-fourths of the MTP judged to be in the bottom third of the tube and none in the top third. The centrifugation conditions were chosen to maximize MTP recovery and minimize the contamination of vesicles in the bottom of the tube.

In a typical binding experiment, the centrifuge tubes were fractionated into 2 mL aliquots and analyzed. Protein was quantitated using the Pierce BCA assay (Rockford, IL) with a bovine serum albumin standard, and lipid was quantitated by scintillation counting. Approximately 50% of the initial MTP was recovered in the bottom fraction of the tube after 48 h of centrifugation. This bottom fraction was used to calculate the lipid binding stoichiometry and to measure lipid transfer from MTP to acceptor membranes (see below). Shorter centrifugation times resulted in a lower MTP yield in the bottom fraction; however, the binding stoichiometries were similar. To demonstrate that lipid binding was specific for the MTP complex, control binding assays using an equivalent amount of pure PDI were performed. Radioactivity in the bottom fractions of tubes containing PDI was the same as background levels found when vesicles were centrifuged alone.

Gel Permeation Chromatography. In studies used to validate the binding assay, centrifuge tube fractions recovered following the binding assay were characterized by fractionation on a 1 × 60 cm Bio-Gel Al5-M gel permeation column (Bio-Rad, Richmond, CA). The column was equilibrated in assay buffer, and then either the top or the bottom fraction from the ultracentrifugation tube was applied to the column and eluted. Radioactivity found in the bottom fraction eluted in the same position as MTP, while radioactivity in the top fraction eluted in the void volume as vesicles (Figure 1).

Radiolabeled Lipid Transfer Assay. Lipid transfer assays were performed as previously described (Atzel & Wetterau, 1993). Donor vesicles contained egg phosphatidylcholine, 5 mol % cardiolipin, and various [^{14}C]triolein concentrations as indicated. Acceptor vesicles contained egg phosphatidylcholine. In some triolein transfer assays, acceptor vesicles contained trace amounts of [^{3}H]phosphatidylcholine which was used to monitor the recovery of acceptor vesicles. Recovery was typically greater than 95%. Assays contained 50 nmol of donor and 200 nmol of acceptor phosphatidylcholine in 0.5 mL of assay buffer. Following the transfer reaction, the donor vesicles were selectively precipitated with DEAE-cellulose, [^{3}H]phosphatidylcholine or [^{14}C]triolein in an aliquot of the supernatant was quantitated by scintillation counting, and the mass of lipid transferred from donor to acceptor vesicles was calculated.

Lipid Transfer from MTP to Acceptor Membranes. The rate of transfer of radiolabeled lipid from MTP to vesicles was measured by incubating 10−30 μg of MTP containing bound lipid (isolated as described above) with 100 nmol of unlabeled phosphatidylcholine acceptor vesicles for varying lengths of time at 23 °C. The transfer reactions were terminated by precipitation of MTP with DEAE-cellulose in a manner similar to that used to precipitate donor vesicles in the lipid transfer assay (Atzel & Wetterau, 1993). Radioactivity in the supernatant, representing lipid transferred from MTP to acceptor vesicles, was quantitated by scintillation counting. Radioactivity contributed by nonprecipitated MTP (<1% total radioactivity) was measured in control assays where acceptor vesicles were omitted. This background value was subtracted from that obtained in the presence of acceptor vesicles to calculate net lipid transfer. Initial lipid bound to MTP (B_i) was defined as cpm added to each dissociation reaction. Lipid bound to MTP at various time points (B_t) was calculated as B_i − (cpm transferred to acceptor vesicles). Data were plotted as percent bound to MTP versus time or as a logarithmic transformation of the fraction bound versus time [$\ln(B_t/B_i)$ versus time].

Fluorescence Assays. Donor particles composed of 100% Py-PC, 90:10 phosphatidylcholine:Py-TG, or 90:10 phosphatidylcholine:Py-CE were prepared by sonication as described above. At these concentrations in the donor particles, the pyrene-labeled lipids are poorly fluorescent at 380 nm due to self-quenching. An aliquot of prepared vesicles was added to a cuvette containing assay buffer and placed in a Perkin Elmer LS 50-B luminescence spectrometer (Norwalk, CT) at ambient temperature (23 °C). At zero time, an aliquot of either MTP (10−80 μg) or buffer was added to make a final volume of 2 mL, and the time-dependent change in fluorescence intensity was measured. Excitation and emission wavelengths were 342 and 380 nm, respectively.

Quenching constants (K_Q) for the fluorescent lipid molecules bound to MTP were deterrmined. MTP containing bound lipid molecules was isolated by gel permeation

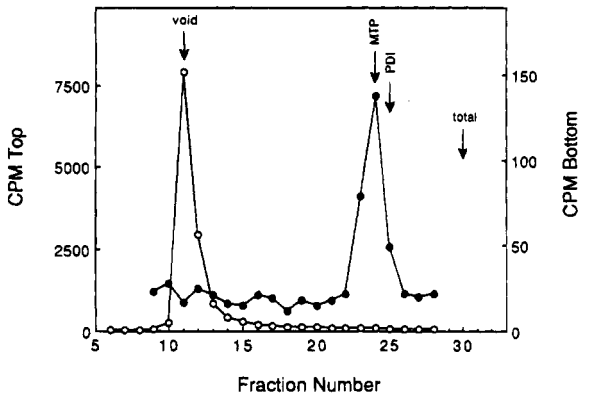

FIGURE 1: Gel permeation chromatography of ultracentrifugation fractions from the lipid binding assay. To assess the binding assay procedure, the top fraction (open circles) and the MTP-containing bottom fraction (solid circles), following the ultracentrifugation step in a lipid binding assay (described under Materials and Methods), were run on a Bio-Gel A15-M column, and the eluted [^{14}C]triolein radioactivity was quantitated. Of the radioactivity applied to the column, 85% was recovered in the eluted fractions. The elution positions of MTP and PDI alone are indicated by arrows. All the radioactivity in the top fraction eluted in the void volume, corresponding to the elution position of vesicles, while the radioactivity in the bottom fraction coincided with the elution position of MTP.

chromatography as described above. Two molar stock solutions of acrylamide in assay buffer or KI in assay buffer supplemented with 0.1 mM sodium thiosulfate were prepared. Aliquots of the quenchers were added to a cuvette containing MTP, and the fluorescence intensity was measured. K_Q was calculated according to the equation: $F_0/F = 1 + K_Q[Q]$, where F_0 is unquenched fluorescence, F is quenched fluorescence (corrected for dilution resulting from the addition of quencher), and [Q] is the final concentration of quenching reagent (Lakowicz, 1983).

RESULTS

Lipid Binding Assay. To characterize the lipid binding properties of MTP, an assay was developed to measure lipid molecules bound to MTP following incubation of MTP with vesicles. MTP, PDI, or assay buffer alone was incubated with radiolabeled lipid vesicles, layered over 31% sucrose, centrifuged, and fractionated. The amount of radiolabeled lipid found in the bottom fraction of tubes containing MTP was 5−10 times that found in control tubes containing either PDI or assay buffer alone (data not shown). When the bottom fraction of tubes containing MTP was subjected to gel permeation chromatography, all the radioactivity eluted at the same position as MTP (Figure 1). These results illustrate several points. First, that the centrifugation procedure efficiently separates protein from vesicles; second, that radioactivity sedimenting with MTP is not associated with vesicles in that the elution position of MTP is not shifted (i.e., MTP is not bound to vesicles); and third, that the binding of lipid by MTP requires the large subunit of MTP since PDI alone does not bind lipid.

Lipid Binding Stoichiometry. MTP was incubated with egg phosphatidylcholine vesicles containing a trace of [^{3}H]-dipalmitoylphosphatidylcholine and varying amounts of [^{14}C]-triolein. The mixture was centrifuged as described above, and the MTP-containing bottom fraction was assayed for protein and radioactivity. When the results from all the experiments were combined, the observed phosphatidylcholine binding stoichiometry was 1.9 ± 1.0 (average ± SD, $n = 9$), phospholipd to MTP (mol/mol). A greater than 10-fold increase in the amount of total lipid in the incubation mixture did not significantly increase the binding of either phosphatidylcholine or triolein to MTP when the ratio of triolein to phosphatidylcholine in the donor vesicle was constant (data not shown). In addition, the [^{3}H]phosphatidylcholine binding remained relatively constant when the triolein composition of the donor vesicles was varied (Figure 2). Subtle changes in phosphatidylcholine binding to MTP in response to changes in the assay conditions would be difficult to detect due to the variability of the binding assay.

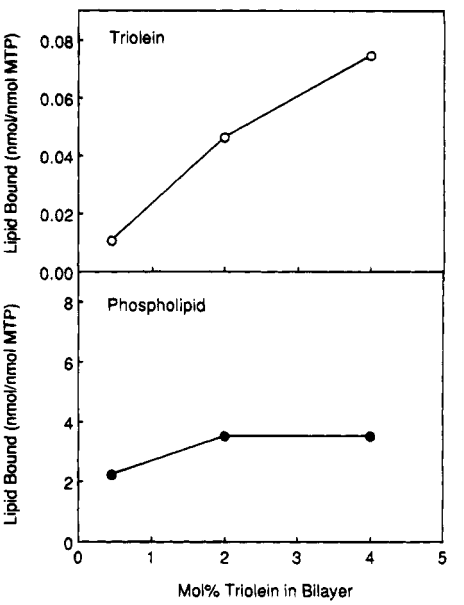

FIGURE 2: Effect of the triolein concentration in the bilayer on triolein and phospholipid binding to MTP. Lipid binding to 100 μg of MTP was measured following incubation with donor vesicles (1.8 μmol of phosphatidylcholine) containing varying concentrations of triolein as indicated. Triolein binding (top panel) increased with donor vesicle triolein concentration, while phospholipid binding (bottom panel) remained relatively constant.

To calculate lipid binding in these experiments, it was assumed that the radiolabeled dipalmitoylphosphatidylcholine is representative of the egg phosphatidylcholine. To validate this assumption, two independent binding experiments were performed with dioleoylphosphatidylcholine vesicles containing a trace of radiolabeled dioleoylphosphatidylcholine. A 3.0:1 binding ratio, phospholipid:MTP, was observed. The greater than 1:1 phospholipid stoichiometry indicates that there is more than one lipid molecule binding site on MTP.

When vesicles with varying compositions of [^{14}C]triolein (0.5−4 mol %) were used in binding assays, triolein binding to MTP was proportional to its mole concentration in the donor membrane (Figure 2). The low triolein binding values (approximately 0.1:1, triolein to MTP at 4 mol % triolein in the donor vesicle) suggested that the neutral lipid binding site on MTP was not fully occupied. Attempts to saturate the neutral lipid site were of limited success. Varying the incubation time did not change the neutral lipid stoichiometry. By using a 60 mol % triolein emulsion as the donor particle, a maximum triolein occupancy of (0.20−0.25):1 was observed. The similar levels of triolein binding could be explained if one assumes that MTP does not penetrate deeply into the membrane but, rather, it interacts primarily with the membrane surface which is saturated with triolein at around

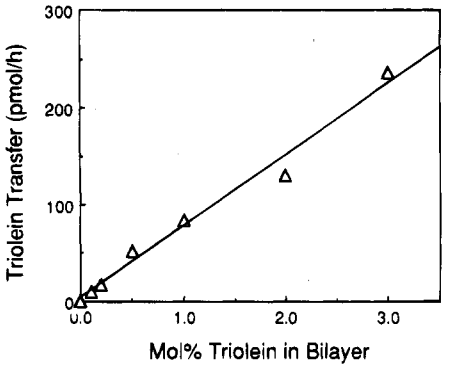

FIGURE 3: Effect of the triolein concentration in the bilayer on triolein transfer. The triolein transfer assays were performed as described under Materials and Methods. The assays were for 20 min at 23 °C and contained 200 ng/mL MTP. Donor vesicles contained increasing concentrations of triolein as indicated. Acceptor vesicles were 100% phosphatidylcholine. Triolein transfer increased with increasing triolein concentration in the donor vesicles.

Table 1: Effect of Triolein Concentration in the Bilayer on Phospholipid Transfer[a]

mol % triolein in bilayer	lipid transfer (nmol/h)	
	phosphatidylcholine	triolein
0	4.9	0
0.5	3.5	0.11
3.6	4.2	0.43

[a] The phosphatidylcholine and triolein transfer assays were performed as described under Materials and Methods. The assays were for 1 h at 23 °C and contained 200 ng/mL MTP. Donor vesicles contained increasing concentrations of triolein as indicated. Acceptor vesicles were 100% phosphatidylcholine. The data presented are the average of duplicate measurements which agreed within 6% of the average.

4 mol % (Hamilton et al., 1983). At triolein concentrations greater than 4 mol %, the triolein forms an oil phase in the bilayer, or an emulsion is formed. Thus, the surfaces with which MTP interacts would have similar triolein concentrations whether the donor particle was a vesicle or an emulsion.

When the triolein concentration in the donor vesicles was increased up to 4 mol %, triolein binding to MTP was increased proportionally. To determine how the neutral lipid concentrations in the donor membranes affect MTP-mediated lipid transport, donor vesicles were prepared with different concentrations of triolein in the donor membranes, and the rate of neutral lipid transport was measured. By increasing the concentration of triolein in the membrane, the triolein transfer rate was increased (Figure 3). Thus, neutral lipid transport is highly sensitive to the neutral lipid composition of the membrane. In contrast, when the triolein concentration in the donor vesicles was increased, phosphatidylcholine transfer was relatively constant (Table 1). Similar results were obtained with cholesteryl ester. Cholesteryl ester binding to MTP and transport were both increased when the cholesteryl ester contents of the donor membranes were increased (data not shown). The binding and transport rates of cholesteryl oleate were comparable to those of triolein.

Identification of Two Classes of Binding Sites. MTP containing bound radiolabeled lipid was incubated with unlabeled phospholipid acceptor vesicles at 23 °C, and the rate of radiolabeled lipid transfer from MTP to the vesicles was measured. As seen in Figure 4A, almost all (greater than 80%) of the triolein or cholesteryl oleate was transferred

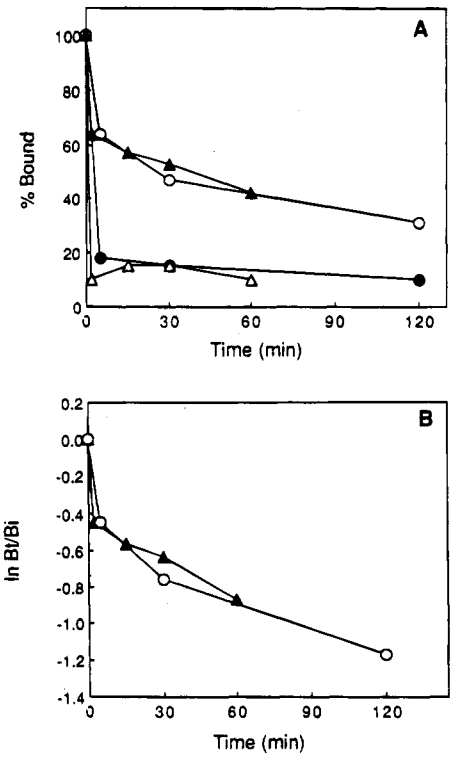

FIGURE 4: Neutral lipid and phospholipid transfer from MTP to acceptor vesicles. MTP containing bound lipid was prepared by incubating 200 μg of MTP with donor vesicles (1 μmol of phosphatidylcholine) containing 4 mol % [^{14}C]triolein or [^{14}C]-cholesteryl oleate. The MTP was reisolated by ultracentrifugation. Lipid transfer experiments were then carried out with the MTP containing bound lipids as described under Materials and Methods. (A) Representative time courses are shown for [^{14}C]triolein (open triangles) and [^{3}H]phosphatidylcholine (solid triangles) transfer from MTP to acceptor vesicles. In a second experiment, [^{14}C]cholesteryl oleate (solid circles) and [^{3}H]phosphatidylcholine (open circles) transfer from MTP to acceptor vesicles was measured. (B) Logarithmic transformation of data presented in panel A for the transfer of phosphatidylcholine from MTP which contained bound phosphatidylcholine and triolein (solid triangles) or phosphatidylcholine and cholesteryl oleate (open circles), to acceptor vesicles.

from MTP to vesicles rapidly, whereas phospholipid transfer from MTP to acceptor vesicles was biphasic. A fraction of the total bound phospholipid was transferred at a rate comparable to that of neutral lipid, while the remainder was transferred slowly. Figure 4B is a logarithmic transformation of the phospholipid data in Figure 4A. The curved line illustrates the complex nature of phospholipid transfer.

Fluorescent Lipid Binding. Pyrene-conjugated lipid was used to further characterize phospholipid and neutral lipid binding to MTP. When MTP was added to donor particles containing self-quenched pyrene-labeled cholesteryl ester, triglyceride, or phosphatidylcholine, the fluorescence emission at 380 nm increased, indicating that MTP extracted the lipid molecules from the membranes. Figure 5 shows typical time courses for neutral lipid (Py-CE, Py-TG) and phospholipid (Py-PC) binding to MTP. All neutral lipid binding was rapid (within minutes) while a portion of the phospholipid binding was rapid and a portion slow (within hours). No significant fluorescence increase was observed when PDI was added to vesicles, which again demonstrates the specificity of the binding assay. The transfer of a second fluorescent phospholipid species, NDB-PE, from donor vesicles to MTP also had rapid and slow phases (data not shown).

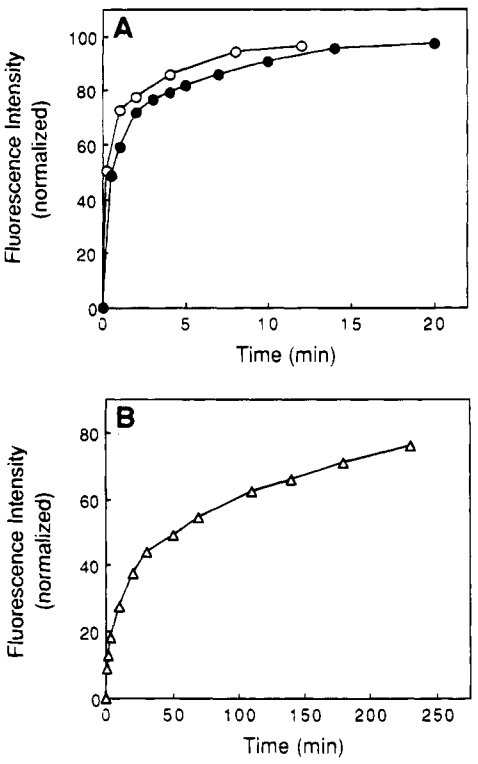

FIGURE 5: Neutral lipid and phospholipid transport from donor vesicles to MTP. (A) Shown are representative fluorescence lipid transfer assays using donor particles prepared as described under Materials and Methods. MTP (80 μg) was added to donor particles (30 nmol of total lipid) at zero time, and the increase in fluorescence intensity for Py-TG (solid circles) or Py-CE (open circles) was measured. Fluorescence in the absence of MTP was subtracted, and the data were normalized to the maximum fluorescent intensity change observed. (B) Time course for Py-PC transfer. MTP (12 μg) was added to 2 nmol of Py-PC vesicles, and the increase in fluorescence intensity was measured. Fluorescence in the absence of MTP was subtracted, and the data were then normalized to the maximum fluorescent intensity change. The maximum fluorescent intensity increases for Py-TG, Py-CE, and PyPC were 147, 85, and 900 arbitrary units, respectively.

Table 2: Fluorescence Quenching Constants for Pyrene-Labeled Phosphatidylcholine, Cholesteryl Ester, and Triglyceride[a]

	K_Q (M^{-1})[b]	
	iodide	acrylamide
Py-PC	1.98 ± 0.62 (12)[c]	1.71 ± 0.93 (17)
Py-CE	2.39 ± 0.45 (8)	1.35 ± 0.35 (10)
Py-TG	2.35 ± 0.18 (6)	0.92 ± 0.14 (6)
Py-butyrate	57.3 ± 4.4 (8)	298 ± 24 (13)

[a] Iodide and acrylamide quenching constants for pyrene-labeled phosphatidylcholine, cholesteryl ester, and triglyceride were determined as described under Materials and Methods. For comparison, the quenching constants for pyrene butyrate, a soluble pyrene conjugate, were determined. [b] Values are expressed as mean ± standard deviation. [c] Number of measurements.

The local environment of the pyrene-labeled lipid bound to MTP was probed by fluorescence quenching with acrylamide and iodide. Aliquots of either quencher were added to MTP containing bound Py-PC, Py-CE, or Py-TG. The fluorescence of all three probes was poorly quenched by either quencher when compared to the quenching of a soluble pyrene compound, Py-butyrate. K_Q values for iodide (a negatively charged quencher) and acrylamide (a neutral quencher) are shown in Table 2 for Py-butyrate and the pyrene-lipids. Py-butyrate quenching exhibited K_Q values approximately 25−175 times greater than K_Q values for the pyrene-lipids. This indicates that the fatty acid chains of all three lipid species are buried in the MTP molecule and thus shielded from the aqueous environment containing the quenchers.

DISCUSSION

Using lipid binding and lipid transport assays, two distinct classes of lipid binding sites on MTP have been identified. Phospholipid bound to MTP at a ratio of approximately 2:1, phospholipid:MTP, while triolein binding to MTP at its highest observed level was (0.2−0.25):1, triolein:MTP. Neutral lipid and phospholipid were transported from MTP to vesicles at different rates. Triolein, cholesteryl oleate, and a portion of the phospholipid were rapidly transported from MTP to acceptor vesicles. The remainder of the phospholipid was transported slowly with an approximate turnover time of 4 h. Fluorescent lipid transfer from donor particles to MTP also showed different rates of lipid transport. Triolein and cholesteryl oleate were transported to MTP rapidly (within minutes), while a portion of the phospholipid was transported fast and a portion, slow.

We propose the following model for MTP that is consistent with the transport and binding properties of MTP. In this model, MTP contains two different classes of lipid molecule binding sites. One class is responsible for catalyzing lipid transfer and can be occupied by triglyceride, cholesteryl ester, or phospholipid. On the basis of the lipid transport specificity of MTP (Wetterau & Zilversmit, 1985), this site or sites appears to selectively bind and transport triglyceride and cholesteryl ester compared to phospholipid. Neutral lipid transfer is disproportionately fast when compared to its membrane concentration. In the absence of data to demonstrate two or more distinct fast sites (for example, one site for neutral lipid and one for phospholipid), we propose the simplest model in which there is one fast site that binds and transports triglyceride, cholesteryl ester, and phospholipid. Given the current methodology and the relatively high levels of phospholipid (compared to neutral lipid) binding to MTP, it is not possible to determine if phospholipid and neutral lipid compete with each other for binding to the same site or sites on MTP.

A second class of lipid binding sites on MTP appears to be specific for phospholipid. This class transports lipid slowly and is distinct from the site which rapidly transports lipid molecules between membranes. Currently one can only speculate about the precise role of this site or its relationship to the fast site. Although the role of this stable binding site is unclear, there is precedent for similar sites in the literature. For example, phosholipase A_2 contains an activator phospholipid binding site which is necessary for enzyme activity (Adamich et al., 1979). The differential binding of phospholipid and neutral lipid to MTP not only indicates the presence of more than one class of lipid binding site but also suggests they have distinct functions.

Figure 2 illustrates that neutral lipid binding to MTP is dependent on its concentration in the donor membrane. We were unable to demonstrate an effect of donor vesicle neutral lipid content on phospholipid binding. Although triolein binding and cholesteryl oleate binding increase with increasing neutral lipid content in membranes, their binding to MTP

did not approach that of phosphatidylcholine. This may in part be explained by the limited solubility of neutral lipid in membranes. Above 4 mol % neutral lipid in a membrane, an oil phase occurs resulting in the formation of a hydrophobic lipid core that is not exposed to the surface (Hamilton et al., 1983). Under these conditions, phospholipid availability at the membrane surface is 25 times that of neutral lipid, regardless of particle composition. This could explain the overall higher level of phospholipid binding to MTP and why competitive binding between phospholipid and neutral lipid could not be detected. The similar levels of triolein binding to MTP using bilayer vesicles or emulsions (which contain a neutral lipid core) as the donor particle suggest that MTP interacts with membrane surfaces. MTP may have minimal direct interaction with the neutral lipid core of an emulsion.

MTP is one of only a few known proteins that bind neutral lipids. Proteins such as the cholesteryl ester transfer protein (CETP), triglyceride lipases, and cholesteryl esterases have no obvious sequence homologies to MTP, yet these proteins share neutral lipid binding capabilities. CETP is functionally similar to MTP in that it catalyzes the transport of neutral and phospholipid between membranes. It binds cholesteryl ester at a 1:1 ratio and phospholipid at about a 10:1 mole ratio (lipid:protein) (Swenson et al., 1988). CETP also has a higher affinity for membranes than MTP (Pattnaik & Zilversmit, 1979; Atzel & Wetterau, 1993) and, on the basis of its amino acid composition, an overall more hydrophobic character than MTP (Drayna et al., 1987; Sharp et al., 1993) which perhaps explains these lipid binding differences. Of the neutral lipid binding proteins, the structure of fungal lipase is the best understood. Crystallographic studies have shown that the triglyceride binding site is buried within the enzyme (Brzozowski et al., 1991). MTP also appears to have buried lipid binding sites. Fluorescence quenching of pyrene-labeled lipid molecules bound to MTP indicated that the fatty acyl chains were shielded from the aqueous solute environment. As summarized in Table 2, Py-PC, Py-TG, and Py-CE fluorescence were poorly quenched by either iodide or acrylamide (small K_Q) while a soluble pyrene analog, Py-butyrate, was readily quenched (large K_Q). The shielding of extremely hydrophobic lipids from the surrounding aqueous solute would be a thermodynamically stable means for MTP to solubilize and transport hydrophobic ligands.

The precise role of MTP in lipoprotein assembly is not known. Defects in MTP have been shown to cause abetalipoproteinemia (Sharp et al., 1993; Shoulders et al., 1993). The absence of lipoprotein-like structures in electron micrographs of liver or intestinal biopsies from abetalipoproteinemic subjects (Avigan et al., 1984; Greenwood, 1976) suggests that MTP is involved in the early stages of very low density lipoprotein or chylomicron assembly. In the presence of MTP, apoB secretion is regulated posttranscriptionally [reviewed in Borén et al. (1993), Dixon and Ginsberg (1993), and Sniderman and Cianflone (1993)]. When intracellular triglyceride is available, apoB is efficiently assembled into lipoprotein particles and secreted. If triglyceride is limited, apoB is degraded intracellularly. MTP-mediated transport of lipid to apoB may enhance the stability of apoB and allow it to form a secretion-competent particle. MTP would be ideally suited to communicate the neutral lipid content of a lipoprotein-secreting cell to apoB in that MTP-mediated transfer of neutral lipid is directly proportional to the neutral lipid concentration in the membrane (see Figure 3). In addition, Lin et al. (1994) have shown that hamster MTP mRNA and protein levels are increased by increased fat content in the diet. Hence, fat may increase MTP-mediated triglyceride transport by two independent mechanisms: one in which MTP levels are increased, and a second in which the MTP transport is increased due to increased triglyceride concentrations in membranes.

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