The Evolution of Microsomal Triglyceride Transfer Protein and its Role During the Assembly of ApoB-lipoproteins

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List of Abbreviations

MTP, Microsomal triglyceride transfer protein

dMTP, *Drosophila* MTP

hMTP, Human MTP

PDI, Protein disulfide isomerase

ApoB, apolipoprotein B

MGAT, Acyl-CoA:monoacylglycerol acyltransferase

DGAT, Acyl-CoA:diacylglycerol acyltransferase

LDL, Low-density lipoprotein

LDL-R, Low-density lipoprotein receptor

ER, Endoplasmic reticulum

BSA, Bovine serum albumin

PCTP, Phosphatidylcholine transfer protein

PITP, Phosphatidylinositol transfer protein

FABP, Fatty-acid binding protein

β^N, MTP N-terminal β-barrel domain

α, MTP alpha helical domain

β^C, MTP C-terminal β-sheet1

β^A, MTP C-terminal β-sheet2

TAG, Triacylglycerol

PL, Phospholipid

CE, Cholesterol ester

Abstract

The microsomal triglyceride transfer protein (MTP) transfers lipids (triacylglycerols, cholesteryl esters, and phospholipids) *in vitro* and is required for the secretion of apoB-lipoproteins *in vivo*. Inhibitors of MTP lipid transfer activity significantly increase the degradation of apoB and reduce its secretion. Therefore, transfer of lipids to apoB by MTP is believed to be essential for the assembly of apoB-lipoproteins. In the following studies we explored the structures and activities of evolutionarily distinct MTP homologs in order to better understand how MTP might be assisting apoB-lipoprotein assembly. We describe specific and sensitive *in vitro* assays to measure MTP cholesteryl ester and phospholipid transfer activities using fluorescent lipids. These activities are dependent on MTP concentration and demonstrate saturation kinetics. In contrast to the human MTP that transfers all lipids, we observed that the *Drosophila* MTP is deficient in the transfer of triacylglycerols and cholesteryl esters but does transfer phospholipids. Similarly the zebrafish MTP, like the human MTP, transfers triacylglycerols while the *C. elegans* MTP lacks this activity. Despite differences in their abilities to transfer lipids, all MTP orthologs assisted in the assembly and secretion of human apoB48-lipoproteins. Furthermore, the amounts of apoB secreted with the assistance of different MTP proteins were augmented by increasing triacylglycerol synthesis (the addition of oleic acid or expression of acyl-CoA:monoacylglycerol acyltransferase and acyl-CoA:diacylglycerol acyltransferase proteins) and decreased by treating the cells with triacsin C, an inhibitor of triacylglycerol synthesis. We subsequently showed that the secondary and tertiary structures in the orthologs were highly conserved while their primary amino acid sequences were less conserved. Interestingly the β^C domains as well as helices 4-6 in the

α–helical domains demonstrated greater conservation in vertebrate MTPs than the other structural domains.

Together these data reveal that the phospholipid transfer activity is the most ancient activity associated with MTP and is sufficient to generate an apoB-lipoprotein particle. Triacylglycerol transfer activity was acquired during evolution and is specific to vertebrate MTP proteins. This activity is associated with greater conservation in the β^C domain and helices 4-6 in the α-helical domain. Therefore, we propose that the amino acid content contained in the β^C domain as well as helices 4-6 might be critical for the robust triacylglycerol transfer activity in vertebrates.

Introduction

1. Lipoproteins

Organisms utilize a variety of vehicles to transport water insoluble lipids within aqueous environments. Intracellular proteins shuttle lipid molecules between, and within, sub-cellular compartments. In contrast, bulk lipids are escorted throughout the extracellular milieu as lipid-protein micelles generally referred to as lipoproteins. The typical lipoprotein structure consists of an amphipathic surface, composed of phospholipids, free cholesterol, and proteins that surrounds a hydrophobic core. The polar groups of surface components project from the particle's exterior while their more hydrophobic entities, i.e. the fatty acid tails of lipids and water insoluble protein domains, face inward away from the hydrophilic environment. Within the particle's hydrophobic core resides neutral lipids such as triacylglycerols and cholesteryl esters.

1.1 Lipoprotein transport systems

The utilization of lipoproteins as extracellular lipid transporters can be identified as early as nematodes and are present in all higher organisms (Fig. 1). These include the vitellogenins expressed in egg laying animals, the lipophorins that circulate throughout the hemolymph of insects, and apoB-lipoproteins secreted from the liver and intestine of vertebrates (1-3). Each lipoprotein requires a specialized apoprotein that behaves as a structural support and facilitates the formation of the lipid-protein complex.

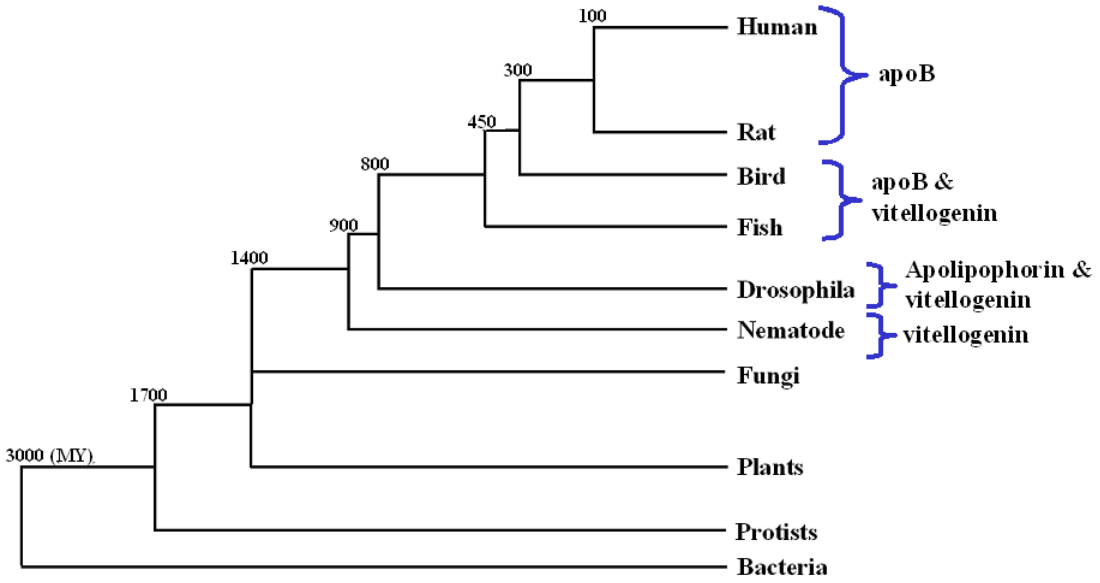


Figure 1. Lipoprotein utilization. The evolution of organisms (from bacteria to mammals) is depicted as a rooted tree. The values indicate when the ancestors of present-day organisms are proposed to have diverged (MY = millions of years ago). The apoproteins utilized by each organism to generate their preferred lipoproteins are shown. Mammals (rats and humans) are solely dependent upon apoB-lipoproteins for the dissemination of dietary and endogenous lipids in contrast to birds and fish that use both apoB and vitellogenin. Insects prefer the reusable lipid shuttle, apolipophorin, while nematodes synthesize a variety of vitellogenins. At present, no lipoproteins have been reported from organisms that predate the emergence of nematodes (fungi, plants, protests, and bacteria).

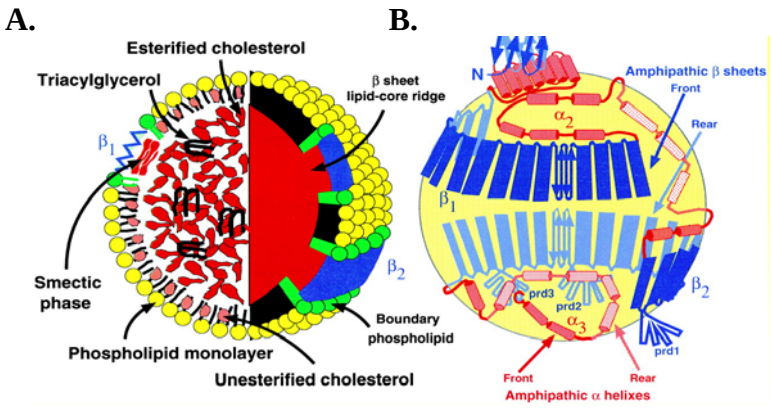
1.2. Apolipophorin and vitellogenin: Insects synthesize the multifunctional lipoprotein, lipophorin, within a specialized organ referred to as the fat body (4). The insect fat body shares functions analogous to the mammalian liver and adipocyte. Lipophorin typically consists of two apolipoproteins, apolipophorin I as well as apolipophorin II (~ 240 and 80 kDa, respectively). A third, apolipophorin III (18-20 kDa) increases the particle's capacity to carry lipids and may also associate with the lipoprotein (5). Lipophorin is a phospholipid rich and neutral lipid poor lipoprotein. The mature particle typically attains a density of 1.15 g/ml (6). Depending on the insect, triacylglycerols represent the major

species of neutral lipids while cholesteryl esters contribute minimally to the overall lipid content of the particle's core (6). Lipophorin is believed to obtain lipids via an efflux mechanism and delivers its cargo to distant tissues without undergoing endocytosis. The protein, therefore, behaves as a continuous "lipid shuttle" never leaving the circulation (5). Recent reports, however, demonstrate that in *Locust migratoria,* a fraction of the lipophorin undergoes receptor-mediated endocytosis (7). Analogous to the process described for the mammalian lipoprotein, apolipoprotein B (apoB), endocytosis of lipophorin requires a homolog to the mammalian low-density lipoprotein receptor.

Vitellogenin is a large apoprotein (~450 kDa) synthesized in the liver and intestine of egg laying animals that is required to supply nutrients (i.e. amino acids and calcium) to the developing oocyte. After its assembly and secretion, vitellogenin enters the cells by receptor-mediated endocytosis through a process that shares similarities with both apolipophorin and apoB (2). Upon endocytosis, the vitellogenin undergoes enzymatic cleavage in unusual lysosomal compartments referred to as yolk platelets (8;9) producing smaller, functional polypeptides. The 136-kDa lipovitellin is generated as a product of this proteolytic cleavage. Vitellogenins, like lipophorins, are phospholipid rich dense lipoproteins containing up to ~12 % lipid (2) and typically demonstrate floatation densities greater than 1.25 g/ml (8). However, unlike the lipophorins, triacylglycerols constitute the majority of neutral lipid associated with the vitellogenin particle.

1.3. ApoB-lipoproteins: Vertebrates, including mammals, birds, and fish utilize apoB-lipoproteins to transport lipids to distant tissues (3). The apoB-lipoprotein consists of two

essential components, a specific apoprotein, apolipoprotein B, in conjunction with associated lipids (Fig. 2). Unlike the vitellogenin and lipophorin particles the apoB-lipoproteins carry bulk quantities of neutral lipids. Consequently, these lipoproteins are more buoyant and float at lesser densities. The majority of apoB particles that are secreted float at a density less than 1.006 g/ml. However, in cell culture systems particle densities can vary between less than 1.006 to 1.21 g/ml (10). The broad range of densities that apoB-lipoproteins can attain demonstrates the flexibility apoB possesses for carrying various quantities of lipid. Recently Wang and colleagues reported a model that describes how specific protein domains in the apoB protein absorb and desorb from the particle depending on the surface pressure it experiences (11). The authors speculate that this unique property of the apoB polypeptide is critical for maintaining the structural integrity of the lipoprotein particle as it undergoes lipolysis in the plasma. The intravascular lipolysis of a lipoprotein involves its transformation from a larger to a smaller diameter particle. In contrast the intracellular assembly of apoB-lipoproteins involves the maturation from a small particle to a larger particle through its association with greater amounts of neutral lipids. Although not speculated on by the authors, the process by which apoB absorbs and desorbs from the particle may be just as important during its assembly.



From Segrest, JP. et al. (2001) *J Lipid Res.* 42(9):1346-67

Figure 2. The structure of an apoB-lipoprotein particle. ApoB-lipoproteins contain the structural protein apoB as well as various species of lipids. The organization of lipid within the lipoprotein particle (**A.**) and the predicted association of the apoB polypeptide with the particle (**B.**) are illustrated. ApoB wraps around the particle's surface providing a scaffold to the lipoprotein. Besides its structural requirement, the polypeptide also contains motifs that function as ligands for intracellular and extracellular proteins. The primary lipid species contained in the apoB-lipoprotein include phospholipids, triacylglycerols and cholesteryl esters. Phospholipids and free cholesterol represent surface lipids while the more hydrophobic triacylgylcerols and cholesteryl esters are packaged within the core. Minor amounts of other lipids (i.e. diacylglycerols) are also present.

2. Apolipoprotein B and lipid: essential components of mature apoB-lipoprotein particles.

2.1 Apolipoprotein B: ApoB is a large, non-exchangeable structural protein that requires the constant association with lipid. In humans two apoB polypeptides, apoB100 and apoB48 (~550 kDa and 210 kDa) respectively, are translated from a single mRNA (12). ApoB100 is primarily synthesized in the liver whereas apoB48, 48% of full-length apoB100, is expressed in epithelial cells of the small intestine. ApoB48 is generated by the tissue- and site-specific deamination of the apoB100 transcript (12). The conversion of cytosine → uracil at nucleotide 6666 introduces a stop codon (UAA) that creates a

truncated yet functional protein capable of forming a buoyant lipid-rich particle. The apoB48 is indispensable during the assembly and secretion of chylomicron particles from intestinal epithelial cells. In contrast, apoB100 resides on the very-low density lipoproteins secreted by the liver.

The amphipathic character of apoB allows it to interact with the hydrophilic environment of the plasma as well as the exceptionally hydrophobic core of the lipoprotein particle. Structurally, the polypeptide contains a pentapartite arrangement of α-helices and β-sheets (Fig. 2 & 3). The $\beta\alpha_1$ domain folds independently as a globular structure (3) while the remainder of the polypeptide is presumed to wrap around the lipoprotein particle. Helices α_2 and α_3 are similar to those present on the exchangeable lipoproteins (i.e. apolipoprotein A (13) and apolipoprotein C (14)) that associate and dissociate between lipoproteins in the plasma. The β-sheet domains (β_1 and β_2) are extremely hydrophobic and require a constant association with lipid. The β-sheets' dependence on lipid is considered to prevent the apoB polypeptide from exchanging between lipoproteins in the plasma.

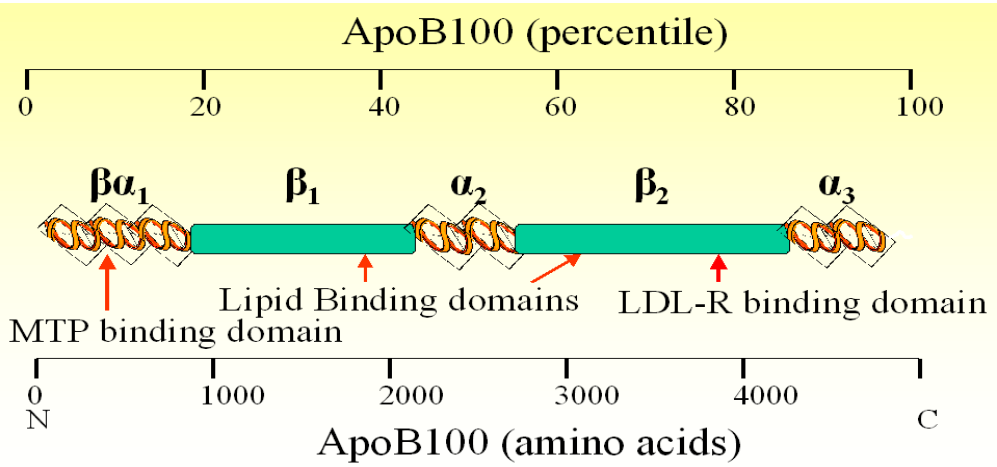


Figure 3. The secondary structure of apolipoprotein B. The apoB polypeptide consists of 4536 amino acids. Its secondary structure is predicted to contain a pentapartite arrangement of α-helices and β-sheets (3). The $\beta\alpha_1$ domain, located at the N-terminus, is globular and interacts with the Microsomal Triglyceride Transfer Protein (MTP). The α-helical domains (α_2 & α_3) are similar to those present in the exchangeable apolipoproteins. ApoB's β-sheets (β_1 & β_2) are extensive, comprising ~50% of the protein, and require the constant association with lipid. The apoB48 contains only the first β-sheet lipid-binding domain and lacks the LDL-receptor (LDL-R) binding region present on apoB100.

Genetic studies and animal models have exhaustively demonstrated the prerequisite for apoB during the assembly of triacylglycerol-rich lipoprotein particles. Humans harboring mutations that generate truncated or non-functional apoB polypeptides characteristically present with moderate to significantly reduced levels of apoB-lipoproteins and plasma lipids, a condition referred to as hypobetalipoproteinemia (15). Numerous mutations in the apoB gene have been linked to this disease (16-20). Similar plasma lipoprotein profiles have been reported in mice containing transgenically truncated apoB as well as those in which the apoB gene has been knocked out (21-23). Although mice harboring homozygous deletions of apoB die in utero, viable heterozygotes exhibit reduced levels of apoB-lipoproteins and plasma lipids (22;23). In contrast the mice expressing a human apoB transgene demonstrated increased amounts of circulating apoB-lipoprotein particles compared to control mice (24). Thus, apoB is an essential component of the lipoprotein particle and altering apoB expression dramatically affects plasma lipoprotein and lipid levels.

2.2 Lipid: The lipid component is also vital to the apoB-lipoprotein particle and influences its assembly and secretion. Full-length apoB cannot be isolated independent of lipids and refolding of the polypeptide following the removal of its associated lipid has not been attained. It is appreciated that intracellular lipid levels modulate the assembly and secretion of apoB (25;26). The post-prandial increase in plasma apoB-lipoproteins is attributed to greater absorption of monoacylglycerols and fatty acids by the enterocyte resulting in a subsequent rise in intracellular lipid pools. Similarly, supplementing oleic acid to growth media augments lipoprotein secretion in cell culture (26). Inhibiting cellular pathways responsible for lipid biosynthesis by gene knockout or knockdown, as well as chemical inhibition, also reduces apoB secretion (27;28). Interestingly, the secretion of apoB-lipoproteins is not only modulated by triacylglycerols and cholesteryl esters, but also by phospholipids. When the gene for phosphatidylethanolamine N-methyltransferase was interrupted in mice, the secretion of apoB-lipoproteins and plasma lipids was similarly reduced (29). Therefore a cell's capacity to synthesize lipids modulates the secretion of apoB-lipoproteins.

3. Microsomal Triglyceride Transfer Protein

Besides apoB and lipid availability other key collaborators necessary for the assembly of, but not associated with, the circulating apoB-lipoprotein have been described. Of these the Microsomal Triacylglyceride Transfer Protein has generated considerable interest as a potential therapeutic target to reduce circulating levels of apoB (30). The disease abetalipoproteinemia is characterized by the nearly complete absence of plasma apoB-lipoproteins in addition to extremely low plasma cholesterol levels and lipid soluble

vitamin deficiencies (31). Unlike hypobetalipoproteinemia, linkage to the apoB gene locus could not be demonstrated in these patients. Using genetic approaches Wetterau and colleagues showed that mutations in the MTP gene segregate among individuals exhibiting the abeta phenotype (32).

Since the seminal work of Wetterau and associates additional studies have confirmed the requirement for MTP during apoB-lipoprotein assembly (33). Reconstitution of MTP activity in heterologous systems rescued apoB secretion, while tissue specific liver knockout models recreated the apoB and lipid deficiencies present in abetalipoproteinemia (10;34;35). Furthermore, several naturally occurring mutations in the MTP gene have since been documented in individuals with abetalipoproteinemia (36-41). While wildtype MTP reconstitutes apoB secretion in cell culture, mutated proteins do not (37;42). More recent work has distinguished that MTP is required during the early stages of assembly and prevents the aberrant folding of apoB as well as its degradation by proteosomes (43;44). Although it is well established that MTP expression is obligatory for the secretion of apoB the mechanism by which this protein supports apoB-lipoprotein assembly remains undefined and speculative.

3.1 MTP is a Heterodimer Composed of M and P Subunits: MTP was first isolated from mammalian liver microsomes as a protein that accelerates the transfer of neutral lipids, triacylglycerols and cholesteryl esters, between phospholipid vesicles (45;46). The protein was subsequently shown to exhibit preference for the transfer of neutral lipids (triacylglycerols and cholesteryl esters) compared to phospholipids (47;48). Under non-denaturing polyacrylamide gel electrophoresis conditions the purified protein

demonstrated a single band. However, in the presence of 0.1% SDS, two major protein bands were resolved, the M and P subunits of ~ 97 and 58 kDa polypeptides, respectively (Fig. 4). The P subunit was identified as the ubiquitous endoplasmic reticulum chaperone protein disulfide isomerase (PDI), whereas the larger polypeptide was primarily expressed in liver hepatocytes and intestinal epithelial cells (49;50).

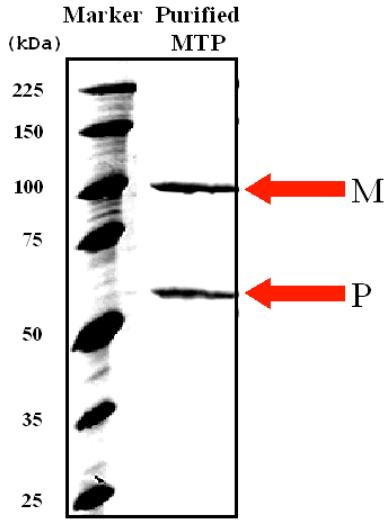


Figure 4. MTP is a heterodimer consisting of M and P subunits. Our lab has purified rat MTP to homogeneity from liver homogenates using FPLC. The protein's purity is revealed by SDS-PAGE analysis followed by staining with Coomassie reagent. The band at ~100 kDa (M) represents the large subunit, while the 55 kDa, small subunit (P), protein disulfide isomerase is also present.

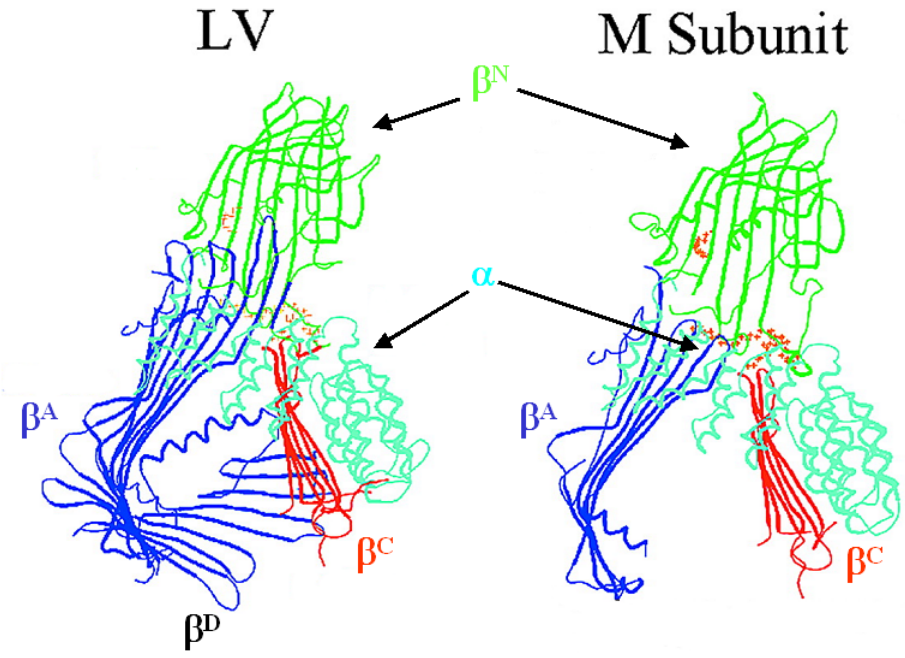
3.2 The P Subunit: PDI facilitates proper folding during the biosynthesis of nascent proteins. In vitro, PDI activity can be measured by its ability to refold denatured proteins via the catalysis of disulfide bond formation utilizing its isomerase and shufflase (rearrangement of pre-existing disulfide bonds) activities. When PDI is in association with the M subunit these chaperone activities are lost. However, both the shufflase and isomerase activities are recovered after disrupting the heterodimer with guanidine HCl

and chaotropic agents ($NaClO_4$ and KSCN) that reduce hydrophobic interactions or by non-denaturing detergents, i.e. octyl β-glucoside (50;51). These data indicate that the association of PDI with the M subunit involves non-covalent hydrophobic interactions.

PDI by itself does not transfer lipids. Non-covalent association of the M subunit with PDI is absolutely required to generate the fully functioning lipid transfer complex, MTP. Not only are the enzymatic activities associated with PDI lost when it is present in the heterodimer, they are also unnecessary to form the active complex. The introduction of missense mutations by site-directed mutagenesis that disrupt PDI's chaperone activities have no effect on its ability to form a heterodimer with the M subunit (52). Moreover, the lipid transfer complex generated using mutant PDI is fully functional. It not only transfers lipids but also supports the secretion of apoB. While PDI activity is restored by the disruption of the MTP heterodimer, aggregation of the M subunit and loss of lipid transfer activity results (51). Thus, the role of PDI in the biosynthesis of MTP is more likely related to maintaining structural stability and solubility of the complex rather than as an active subunit. PDI might also be responsible for retaining the complex in the endoplasmic reticulum via its C-terminally located -KDEL sequence since a retention signal is absent from the M subunit. MTP escaping the endoplasmic reticulum would be recycled from the Golgi via the KDEL receptor's affinity for PDI.

3.3 The M subunit: The *Mttp* gene encoding the large M subunit of MTP is located on human chromosome 4 and translated into an 894 amino acid, 97 kDa protein. As previously discussed, the M subunit requires interaction with PDI to maintain solubility, activity, and localization to the endoplasmic reticulum (53;54). While the ubiquitously

expressed protein disulfide isomerase represents the P subunit, the M subunit is unique and responsible for MTP's lipid transfer activity. The M subunit is primarily expressed in the liver and intestine although other tissues have recently been demonstrated to produce significantly lesser amounts (55-57). As yet, the structure of the M subunit has not been resolved by x-ray diffraction. However, the crystal structure of a homologous protein, lipovitellin, has been reported at 1.8 Å (58-61) (Fig. 5).



Modified from Anderson et al. (1998) Structure 6:895 and Hussain et al. (2003) JLR 44:22

Figure 5. Structures of lipovitellin and the M subunit of MTP. Lipovitellin contains five structural domains, an N-terminal β-barrel (β^N), central helical domain (α), and three β-sheets (β^C, β^A, and β^D). A large cavity is created by the β-sheets located in the protein's C-terminus. Phospholipid molecules were identified within the C-terminal cavity in the lipovitellin structure. Human MTP shares homology with lipovitellin (LV) and was modeled based upon the crystal structure obtained for this protein (62-64). Similar to lipovitellin, the MTP structure contains a β^N, α, and two β-sheets (β^C, β^A) that generate a pocket in its C-terminus. The lipid pocket of human MTP may be involved in its ability to transfer lipids.

The lipovitellin molecule contains five structural domains. The N-terminus consists of an 11-strand β-barrel surrounding a single 14 amino acid α-helix (βN). Formation of the β-barrel is stabilized by a disulfide linkage between two cysteine residues located in strands 8 and 9 as well as the presence of several internal salt bridges (61). Following the β-barrel are 18 α-helices (α domain) that precede three C-terminal β-sheets (β^C, β^A, and β^D) to complete the structure. Together, the α domain and β-sheets enclose a cavity volume measuring ~ 60,000 $Å^3$. The cavity's lateral and inferior boundaries are defined by β-sheets while a portion of its superior border is created by α-helices. Lipovitellin contains 16% (w/w) non-covalently bound lipid with a stoichiometry of ~ 35 lipid molecules / lipovitellin (61). The majority of this lipid occupies the C-terminal cavity, however, a single molecule of phospholipid was also detected in the β-barrel located at lipovitellin's β^N domain (60).

Mann et al. (63) proposed a hypothetical model for the M subunit based upon the homology it shares with lipovitellin. In this model, both of the cysteine residues that participate in an essential disulfide bridge within lipovitellin's β-barrel are conserved. Mutation of either cysteine results in an insoluble protein (63). Buried charged amino acids responsible for critical salt bridge formation in lipovitellin are also conserved in the MTP structure. Missense mutations that disrupt salt-bridge formation adversely effect the solubility and function of MTP (63). Replacing the asparagine at position 531 or glutamic acid at position 570 with alanine decreased the MTP solubility, while introducing a histidine or alanine in place of arginine 540 had no effect on complex solubility, but dramatically reduced MTP lipid transfer activity. These studies effectively demonstrated that the presence of specific amino acids responsible for intramolecular

interactions in lipovitellin are required to maintain the overall three-dimensional character of the M subunit. Furthermore, they are critical for sustaining MTP solubility and function. Using similar methodology, the C-terminus of MTP was modeled (62;64) (Fig. 5). Two β-sheets (β^C and β^A) were predicted to generate a large cavity as described in lipovitellin. The cavity formed in MTP does not appear to be restricted by a third sheet (β^D) inferiorly as observed in the lipovitellin structure. Hussain et al. (62) have proposed that the C-terminal β-sheets represent the lipid transfer domain in MTP.

3.4 MTP lipid transfer activity: MTP was first purified from bovine liver as a protein that transfers triacylglycerols between phospholipid vesicles in vitro (49). Further studies showed that the heterodimeric complex transfers numerous lipids between membrane vesicles and is most likely its principle function in vivo (48). In vitro, its activity is predominantly directed towards the transfer of triacylglycerols, cholesteryl esters, and phospholipids (48). MTP lipid transfer has been kinetically described by a shuttle mechanism (47). Briefly, MTP first binds transiently to a membrane, extracts lipid, dissociates from the membrane, and then delivers this lipid to another membrane. A single binding site is present for triacylglycerols and cholesteryl esters, while both a slow and a fast site exist for the transfer of phospholipids (48). Whether these are independent sites or a single site that binds multiple types of lipid with different affinities remains to be determined.

Other studies have implied a physical mechanism that MTP might utilize to transfer lipids. Two α-helices located at the entrance of the C-terminal cavity of MTP are similar to those found on viral fusion proteins (64). The A (amino acids 725-736) and

B (amino acids 781-786) helices contain nine and five amino acids, respectively. When the helical structure of either A or B is disrupted, MTP associates with PDI and remains soluble, but loses its capacity to transfer lipids. In addition when helix A was independently expressed as a short peptide it disrupted membrane vesicles releasing their contents (64). The authors concluded that the helical structure of A and B are critical to MTP lipid transfer activity and that helix A interacts with phospholipid membranes to disrupt their architecture while helix B transfers lipid to the cavity located in MTP's C-terminus.

4. ApoB-lipoprotein Assembly

The assembly of intracellular lipids with apoB begins in the endoplasmic reticulum. Lipid is added to the growing polypeptide co-translationally (65). Modeling and biochemical studies suggest that amino acids 1-912, or the initial ~20% of apoB's N-terminus can independently associate with lipids (66). In contrast, the β-sheet domains are thought to acquire lipid through an active process of delivery. Accumulating evidence suggests that apoB particle assembly occurs in two-steps (67) (Fig. 5). Initial lipidation produces a dense particle containing a high protein to lipid ratio. This primordial particle contains phospholipids with relatively minimal amounts of core lipids. The primordial particle subsequently undergoes addition with bulk neutral lipids through an unknown process that generates a large, buoyant nascent lipoprotein.

4.1 The role of MTP in apoB-lipoprotein assembly: Three independent functions, apoB-binding, membrane association and lipid transfer, of MTP have been appreciated (62).

Currently all three activities are believed to be required for the assembly and secretion of apoB-lipoproteins (68;69). Upon its translation the apoB can undergo two fates: (1.) the formation of a lipoprotein particle or (2.) degradation (Fig.6). While the amount of intracellular lipid significantly influences which direction the process goes, the presence of MTP is ultimately required for assembly to take place.

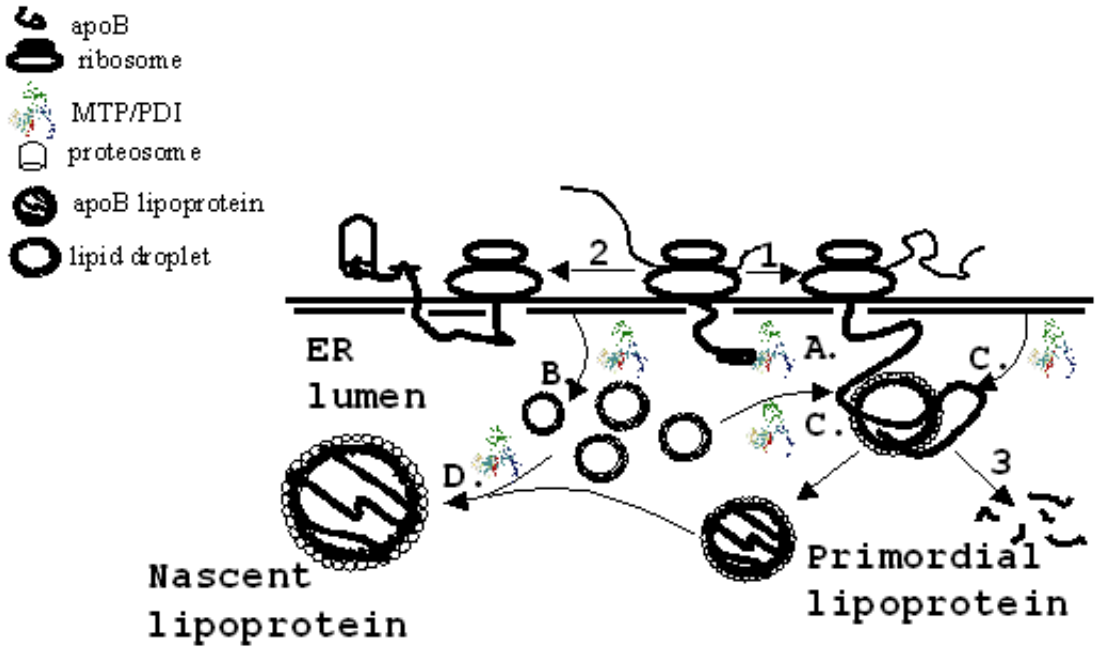


Figure 6. The role of MTP in apoB lipoprotein assembly. The assembly of an apoB-lipoprotein begins with the co-translational translocation of the apoB polypeptide across the membrane of the endoplasmic reticulum (65). In the absence of MTP (**2**), apoB undergoes retrograde translocation and is degraded by proteosomes. When MTP is present (**1**), apoB is not degraded but is instead directed to assemble a lipoprotein particle. MTP is thoroughly required for this to occur. To assemble the particle, MTP might interact with the N-terminus of apoB (**A.**) and transfer lipid from lumenal lipid droplets or the membrane of the endoplasmic reticulum to the polypeptide in a co-translational process (**C.**). This results in a minimally lipidated primordial lipoprotein particle. Evidence also suggests that MTP is involved in the accumulation of lipids in the lumen of the endoplasmic reticulum (**B.**) (70). These lipids may be used during primary or secondary lipid addition to apoB. Whether or not MTP plays a direct role in the second step core expansion of the primordial particle to generate a nascent lipoprotein (**D.**) remains controversial.

Initially apoB enters the lumen of the endoplasmic reticulum by co-translational translocation through the Sec61 complex (71;72). In the absence of a functioning MTP heterodimer, as occurs in abetalipoproteinemia, apoB undergoes retrograde translocation from the lumen into the cytoplasm where the peptide is extensively ubiquitinylated, bound by cytosolic heat shock proteins and degraded by proteosomes (73-75). When the cell's capacity to carry out ubiquitinylation is enhanced, or the expression of Hsp70 and Hsp90 increased, the degradation of apoB is amplified (75).

ApoB undergoes translocation into the lumen of the endoplasmic reticulum in the absence of MTP (72)(71), however expression of the microsomal protein is required for lipoprotein assembly to occur. In the presence of MTP, the tendency for apoB to undergo degradation is significantly reduced and the polypeptide is directed toward its assembly into a lipoprotein particle. Following transient formation of the initial MTP-apoB complex, MTP could transfer lipids to the β domains of apoB creating a minimally lipidated, primordial lipoprotein particle thus preventing its incorrect folding and degradation. Compounds that inhibit the lipid transfer activity of MTP reduce apoB secretion (68;76). Interestingly, lipid transfer inhibitors also decrease membrane and lumenal lipids not associated with apoB indicating that MTP is also involved in modulating the lipid content of the endoplasmic reticulum (77). After its formation, the primordial particle may then undergo endoplasmic reticulum associated degradation (ERAD) (78), be secreted as a dense lipoprotein particle containing minimally associated lipids, or acquire bulk amounts of neutral lipids to generate the nascent lipoprotein. The bulk acquisition of neutral lipids has been coined "core expansion". In this step the

primordial particle undergoes the addition with bulk lipid possibly through a fusion process that uses lumenal lipid droplets. Although evidence suggests the existence of such a step (62;79), the actual mechanism and its functional components remain obscure. The involvement of MTP during second step maturation is also controversial. However, reports demonstrating its requirement to generate lipid droplets within the endoplasmic reticulum (77;80) suggest that MTP might be involved indirectly.

5. MTP Homologs

MTP is required in organisms that assemble and secrete apoB-lipoproteins. This includes all vertebrates (i.e. mammals, birds and fish). Recently, an insect homolog to human MTP, *Drosophila* MTP (81), as well as a nematode protein with limited homology to human MTP were identified (82). How these proteins compare to the mammalian MTPs has yet to be fully appreciated.

***5.1 Insect and nematode MTPs*:** *Drosophila* MTP contains 894 amino acids that share ~22% identity with human MTP. The *Drosophila* MTP was shown to co-immunoprecipitate with human apoB after co-expression in COS cells (81). Furthermore, the insect MTP rescued the secretion of apoB34 as well as apoB41, two truncated apoB polypeptides. Interestingly, in contrast to human MTP the lysates prepared from cells that transiently expressed *Drosophila* MTP did not transfer triacylglycerols in vitro (81).

The *dsc-4* gene of *C. elegans* was isolated as a suppressor of the slow germline development observed in clk-1 mutants (82). The product of *dsc-4* is 892 amino acids,

but shares only minimal identity to human MTP (13%). Reporter gene analysis indicates that *dsc-4* is expressed in the worm intestine (82). It is interesting to note that the phenotype resulting from a *dsc-4* knockdown can be recreated using siRNA toward one of the vitellogenins (*vit*-5) expressed by *C. elegans* (82). More importantly the effect of dsc-4 or *vit*-5 knockdown was not additive. Sellers et al. (42) have reported that vitellogenin secretion from COS cells requires MTP expression. Together, these data suggest that dsc-4 and vit-5 might function in the same pathway and that dsc-4 is the MTP homolog in nematodes. However experimental evidence for this is required.

6. Present Studies

Two fundamental processes influence the amount of apoB-lipoproteins in the plasma. First is their intracellular assembly and secretion followed by the eventual lipolysis of lipoprotein particles and their uptake by target tissues. The removal of apoB-lipoproteins from the plasma via receptor-mediated endocytosis has been extensively studied (83). However, their intracellular assembly is less well understood.

The requirement for MTP during apoB-lipoprotein assembly is indisputable. Yet, how it supports this process remains an area of controversy and speculation. In the following studies we have attempted to further understand how MTP functions to assemble apoB-lipoprotein particles. While knockout mice and small molecule inhibition have been previously used to examine MTP's role in apoB-lipoprotein assembly we focused on studying evolutionarily distinct MTP homologs to understand how MTP might be supporting this process.

The MTP lipid transfer activities were evaluated by first developing in vitro assays that allow the sensitive detection of MTP's cholesteryl ester and phospholipid transfer activities using fluorescent lipids. We subsequently show that *Drosophila* MTP cannot transfer neutral lipids (triacylglycerols and cholesteryl esters) in vitro. However, the insect MTP transfers phospholipids. Furthermore, the *Drosophila* MTP supports the secretion of larger apoB polypeptides and that this secretion responds to treatment with oleic acid. We subsequently identify and compare different MTP proteins expressed in both vertebrate and invertebrate organisms. The vertebrate MTPs transfer triacylglycerols in vitro. The triacylglycerol transfer activity in vertebrate MTPs is associated with increased conservation in specific domains (β^C and helices 4-6 and 15-17 in the α domain). Lastly, we show that although the *Drosophila* MTP does transfer triacylglycerols, enzymes that increase intracellular triacylglycerol synthesis modulate apoB secretion supported by this MTP homolog.

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Transfer of cholesteryl esters and phospholipids as well as net deposition by microsomal triglyceride transfer protein

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Abstract Microsomal triglyceride transfer protein (MTP) activity is classically measured using radioactive lipids. We described a simple fluorescence assay to measure its triacylglycerol (TAG) transfer activity. Here, we describe fluorescence-based methods to measure the transfer of phospholipids (PLs) and cholesteryl esters (CEs) by MTP. Both transfer activities increased with time and MTP amounts and were inhibited to different extents by an MTP antagonist, BMS197636. We also describe a method to measure the net deposition of fluorescent lipids in acceptor vesicles. In this procedure, negatively charged donor vesicles are incubated with MTP and acceptor vesicles, and lipids transferred to acceptors are quantified after the removal of donor vesicles and MTP by the addition of DE52. Lipid deposition in acceptor vesicles was dependent on time and MTP. Using these methods, TAG transfer activity was the most robust activity present in purified MTP; CE and PL transfer activities were 60–71% and 5–13% of the TAG transfer activity, respectively. The method to determine lipid transfer is recommended for routine MTP activity measurements for its simplicity. These methods may help identify specific inhibitors for individual lipid transfer activities, in characterizing different domains involved in transfer, and in the isolation of mutants that bind but cannot transfer lipids.—Rava, P., H. Athar, C. Johnson, and M. M. Hussain. **Transfer of cholesteryl esters and phospholipids as well as net deposition by microsomal triglyceride transfer protein. *J. Lipid Res.* 2005.** 46: **1779–1785.**

Supplementary key words lipoprotein assembly • phosphatidylethanolamine • triacylglycerol • fluorescent lipids • apolipoprotein B • transfer assays • lipid transfer

Microsomal triglyceride transfer protein (MTP) is an essential chaperone for the assembly and secretion of apolipoprotein B (apoB) lipoproteins (for reviews, see 1–5). It is a heterodimeric protein consisting of 97 and 55 kDa polypeptides (5–8). The small subunit is the ubiquitous endoplasmic reticulum resident, protein disulfide isomerase, whereas the large subunit is unique and responsible for the lipid transfer activity present in MTP. There is evidence to suggest that MTP functions in the biosynthesis of apoB lipoproteins through physical association (9–13) with nascent apoB and lipidation of apoB's hydrophobic, lipid binding β-sheets (14). In addition, MTP has been implicated in the import of neutral lipids into the endoplasmic reticulum lumen (15–17), the association with lipid droplets, and the fusion of lipid droplets with nascent apoB primordial lipoproteins (1, 2). In humans, the absence of MTP activity results in abetalipoproteinemia, which is characterized by very low plasma lipid levels and the absence of apoB lipoproteins (18).

The major defining function of MTP is its ability to transfer lipids between small unilamellar vesicles in vitro (7, 19–22). This activity is classically measured using radiolabeled lipids. In this procedure, donor vesicles containing radiolabeled lipids are incubated with acceptor vesicles in the presence of an MTP source (7, 19, 20). After incubation, donor vesicles and MTP are removed by the addition of cationic ion-exchange resins, and the amounts of radiolabeled lipids transferred to acceptor vesicles are quantified by liquid scintillation counting. Recently, we described a simple, rapid, and sensitive assay to measure triacylglycerol (TAG) transfer activity of MTP (23). In this assay, fluorescent TAG was incorporated into donor vesicles and incubated with acceptor vesicles in the presence of an MTP source. During transfer, MTP removes the quenched fluorescent lipids from donor vesicles, exposing the fluorescent moiety. This action is measured as an increase in fluorescence that is subsequently detected using a fluorimeter. Thus, a real-time increase in lipid transfer by MTP can be monitored using this procedure.

In addition to TAG, MTP is known to transfer several other lipids, such as phospholipids (PLs) and cholesteryl esters (CEs) (7, 19–22). Here, we describe simple and sensitive methods to measure the transfer of fluorescently la-

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

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beled PL and CE by MTP. In addition, a method is presented to measure the net deposition of fluorescent lipids by MTP in acceptor vesicles.

MATERIALS AND METHODS

Materials

MTP was purified from bovine liver using the radioisotope assay (7, 19) and rat liver using a kit (Chylos, Inc., Woodbury, NY). Fluorescent (nitrobenzoaxadiazol)-labeled phosphatidylcholines (PCs) and unlabeled PC were purchased from Avanti Polar Lipids (Alabaster, AL). Nitrobenzoaxadiazol-labeled CE, phosphatidylethanolamine (PE), and TAG were from Molecular Probes (Eugene, OR). Thermo Labsystems (Franklin, MA) supplied the black 96-well microtiter plates. Isopropanol and other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Preparation of PL vesicles containing fluorescent PE and CE

Acceptor PC vesicles were prepared as described by Wetterau and associates (7, 19–21). Donor vesicles were also prepared by sonication as described before (7, 19–21, 23). Briefly, unlabeled PE and fluorescent PE were evaporated and sonicated under nitrogen for 45 min at 4°C. CE donor vesicles were prepared similarly using fluorescent CE and unlabeled PC. Vesicles, collected after centrifugation (50,000 rpm, 4°C, 10 min; SW55 Ti), were found to be stable for 1 month. Known amounts of fluorescent lipids were diluted in isopropanol to generate standard curves used to estimate the moles of fluorescent lipids incorporated in the donor vesicles.

Measuring lipid transfer activities

Assays were performed in triplicate on a black 96-well microtiter plate (23). A final reaction mixture (100 μl) contained 3 μl of donor vesicles (1.2 nmol of PC or PE and 100 pmol of fluorescent lipids), 3 μl of acceptor vesicles (7.2 nmol of PC), and an MTP source in 10 mM Tris, pH 7.4, 0.1% BSA, and 150 mM NaCl buffer. The microtiter plate was incubated at 37°C, and at predetermined time points, samples were excited at 485 nm and fluorescence emission was measured at 550 nm using a Victor^3 dual fluorimeter/luminometer (Perkin-Elmer). To determine the percentage of lipid transfer, fluorescence values obtained from control assays containing no MTP source (blanks) were subtracted from sample values and then divided by the total fluorescence present in the vesicles reduced by blanks. Blank values ranged from 10% to 25% of total fluorescence in various preparations. To obtain total fluorescence, 3 μl of donor vesicles was incubated with 97 μl of isopropanol for 5 min.

Measurement of net lipid deposition

To measure net lipid deposition, fluorescent TAG containing negatively charged donor vesicles was prepared (23). To introduce a negative charge, 67.5 nmol of cardiolipin (~7% of total lipids) was added before sonication (7, 19–21). Various amounts of MTP, as well as 3 μl of donor vesicles and 3 μl of acceptor vesicles, were incubated as described above. At predetermined times, fluorescence readings were recorded to quantify the TAG transfer. The reaction mixture was then transferred to microcentrifuge tubes containing 100 μl of DE52 [equilibrated (1:1, v/v) with 15 mM Tris-Cl, pH 7.4, 1 mM EDTA, and 0.02% sodium azide buffer), rotated at 4°C for 5 min, and centrifuged (12,000 rpm, 5 min, 4°C). Supernatants (10 μl) containing only acceptor vesicles were transferred to a microtiter plate, and fluorescence was measured at 5 min intervals after adding 90 μl of isopropanol. Readings obtained at 20 min were used for calculations. The blank values obtained in the absence of MTP were subtracted from the sample values, divided by the total fluorescence reduced by blanks, and multiplied by 100 to determine the percentage of lipids deposited to acceptor vesicles.

Determination of MTP activity in cells and tissues

HepG2 cells grown to confluence in T175 flasks were washed with PBS and then swelled by 2 min incubation at room temperature in hypotonic buffer (1 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, and 1 mM EGTA) (23, 24). The buffer was aspirated, cells were scraped in 750 μl of ice-cold hypotonic buffer containing protease inhibitors and homogenized (20 passages through a 21 gauge needle), the lysates were centrifuged (50,000 rpm, 4°C, 1 h; SW55 Ti rotor), and supernatants were used for lipid transfer assays and protein determination (25). For microsome preparation (7, 19, 23), mouse liver pieces were washed with PBS, homogenized in 50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 250 mM sucrose, and 0.02% sodium azide using a Polytron homogenizer, and centrifuged (10,900 rpm, 30 min, 4°C; Beckman microcentrifuge). Supernatants were adjusted to pH 5.1 with concentrated HCl, mixed in the cold for 30 min, and centrifuged (13,000 rpm, 30 min, 4°C; Beckman microcentrifuge). Pellets were resuspended in 1 mM Tris-Cl, pH 7.6, 1 mM EGTA, and 1 mM $MgCl_2$, vortexed, incubated for 30 min at 4°C, and ultracentrifuged (50,000 rpm, 4°C, 1 h; SW55 Ti rotor), and supernatants were used for lipid transfer assays and protein determination.

RESULTS

PL transfer activity

We previously described a simple, rapid, and sensitive assay to measure TAG transfer activity of MTP (23). Here, we determined whether the same procedure could be used to quantify the PL transfer activity of MTP (**Fig. 1**). Upon the incubation of different amounts of MTP with donor vesicles containing fluorescent PE and acceptor vesicles, fluorescence increased and saturated in a time-dependent manner (Fig. 1A). Each concentration gave a specific curve indicating MTP-dependent increases in fluorescence and was confirmed by plotting the 1 h data against varying amounts of MTP (Fig. 1B). PL transfer was linear between 0.1 and 0.3 μg of MTP and saturated at higher amounts. Next, we studied the inhibition of PL transfer activity by BMS197636. The PL transfer activity was inhibited by ~60% (Fig. 1C). Increased inhibition was not achieved even when the inhibitor concentration was increased to 10 μM (data not shown). Similar results have been described for another MTP antagonist, BMS200150, which is a potent inhibitor of TAG transfer activity but only partially inhibits PL transfer activity (26). The reproducibility of the assay was established by determining the intra-assay and interassay coefficients of variation. The transfer activity in six separate samples using 0.3 μg of MTP was 11.9 ± 1.4%, and variation was found to be 0.12. The average activity in three independent experiments using 0.25 μg of MTP was 9.9 ± 0.96%, and the coefficient of variation was 0.097. These studies indicate that the PL transfer activity of purified MTP could be measured using this method.

CE transfer activity

To study CE transfer, donor PC vesicles containing fluorescent CE were incubated with acceptor vesicles and pu-

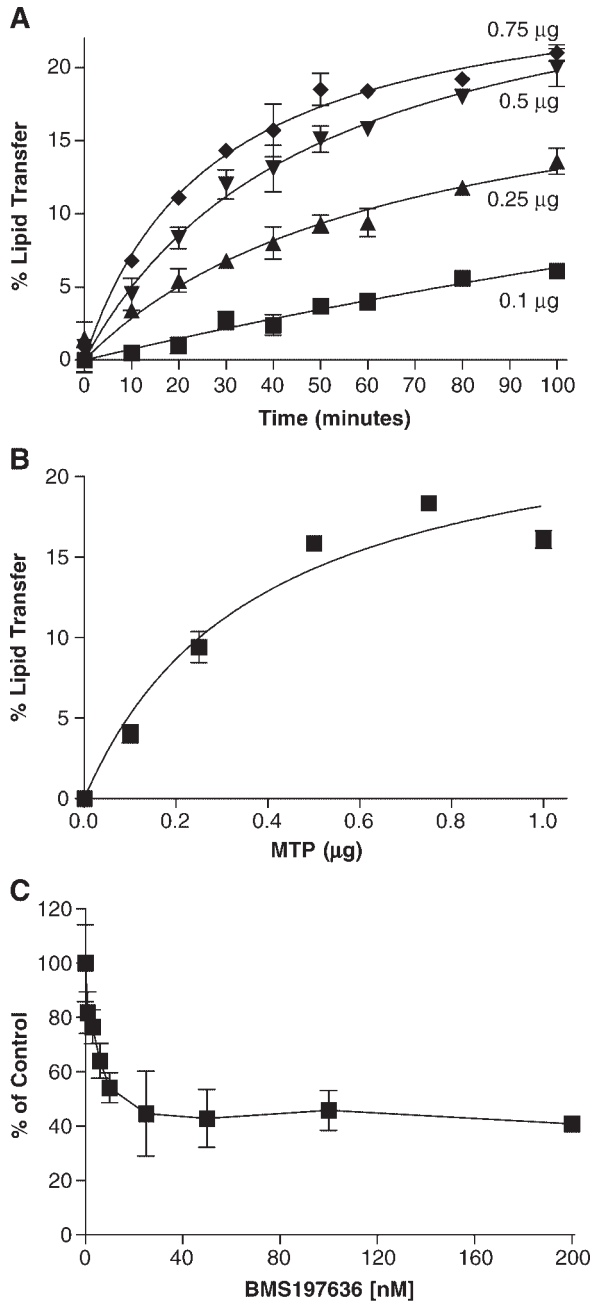

Fig. 1. Phospholipid transfer activity of microsomal triglyceride transfer protein (MTP). A: Different amounts of purified bovine MTP, in triplicate, were incubated with donor vesicles [1.2 nmol of phosphatidylethanolamine (PE) and 100 pmol of fluorescent PE] and with acceptor vesicles [7.2 nmol of phosphatidylcholine (PC)] in 100 µl of 10 mM Tris-HCl buffer containing 0.1% BSA, 150 mM NaCl, and 2 mM EDTA at 37°C. Fluorescence at 550 nm was monitored over time. B: Data from the 60 min time point of A were replotted and subjected to nonlinear regression analysis ($r^2 = 0.9459$) using Prism. C: MTP assay was performed in the presence of the indicated amounts of the MTP inhibitor BMS197636, and fluorescence readings were recorded after 90 min. Line graphs and error bars represent means ± SD. For some points, error bars are not visible because the deviations are smaller than the symbol sizes.

rified MTP (**Fig. 2**). The transfer of CE increased initially and then saturated with time for each of the MTP concentrations used (Fig. 2A). A concentration-dependent, linear increase in CE transfer followed by saturation was also observed using increasing amounts of MTP (Fig. 2B). The intra-assay coefficient of variation using 0.2 µg of MTP was

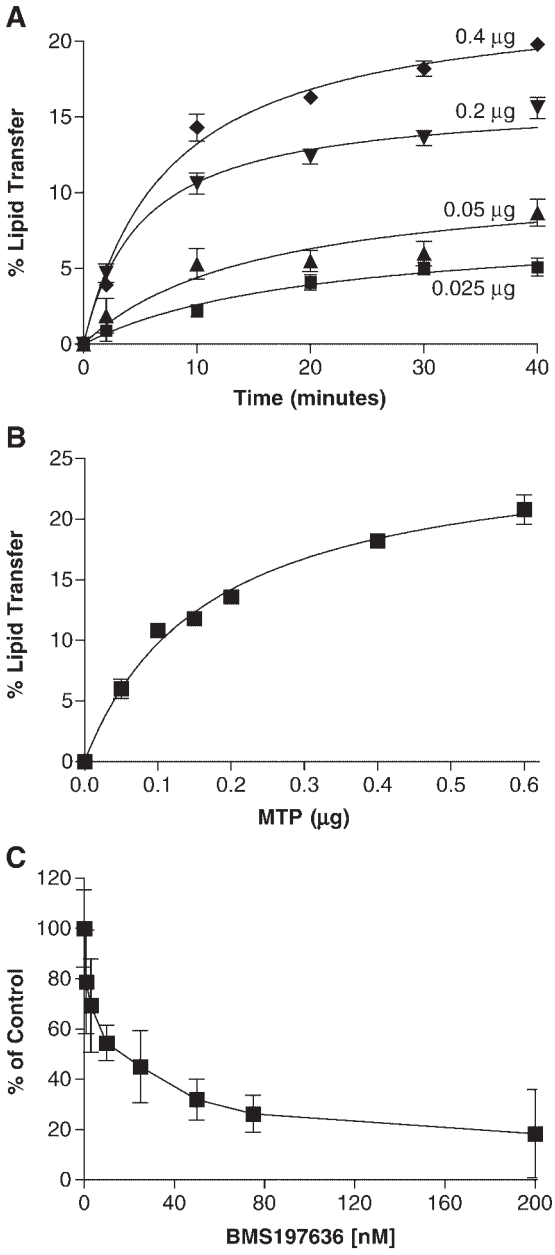

Fig. 2. Cholesteryl ester (CE) transfer activity of MTP. A: Different amounts of purified MTP were incubated with donor (1.2 nmol of PC and 100 pmol of fluorescent CE) and acceptor vesicles as described for Fig. 1. Increases in fluorescence emission at 550 nm were recorded at the indicated time intervals. B: Different amounts of MTP were incubated with donor and acceptor vesicles for 30 min, and the amounts of fluorescent CE being transferred were calculated. A nonlinear regression curve ($r^2 = 0.9842$) was generated using Prism. C: For inhibition, the indicated amounts of the MTP lipid transfer inhibitor BMS197636 were included in the reaction mixture, and fluorescence was measured after 1 h. Line graphs and error bars represent means ± SD (n = 3).

0.09 (n = 6), and the percentage transfer per hour observed in those conditions was 17.4 ± 1.6%. Comparing data from three independent experiments using 0.15 μg of MTP revealed a transfer of 15.0 ± 1.9% (n = 9) and interassay coefficient of variation of 0.127. CE transfer was inhibited by >80% upon increasing concentrations of BMS197636 (Fig. 2C). The IC_{50} values for CE transfer ranged between 15 and 25 nM and were similar to those observed for the inhibition of TAG transfer activity using this inhibitor (23, 26). These studies attest to the suitability of the method for determining CE transfer activity of purified MTP.

Fluorescent lipid transfer measured in cell homogenates and liver microsomes

We then used these assays to study lipid transfer activities in cellular and tissue homogenates. All lipid transfer activities (TAG, CE, and PL) could be measured in HepG2 cell homogenates (**Fig. 3A**). Lipid transfer activities showed time-dependent increases and reached maxima between 20 and 30 min of incubation. The initial rates and maximum amounts of CE transfer were lower than those observed for TAG. PL transfer profiles were similar to those of CE and TAG transfer. The major difference was that PL transfer activity reached a significantly lower maximum transfer.

Next, we measured lipid transfer activity present in mouse liver microsomes (Fig. 3B). All three lipid transfer activities could be measured in microsomal samples using these assays. Again, the major activity observed was TAG transfer followed by CE and PL transfer activities. The initial rates and the maximum amounts of TAG transfer were significantly higher compared with those of CE and PL. These studies indicate that the efficiency of lipid transfer is greatest for TAG followed by those of CE and PL transfer in mouse liver microsomes.

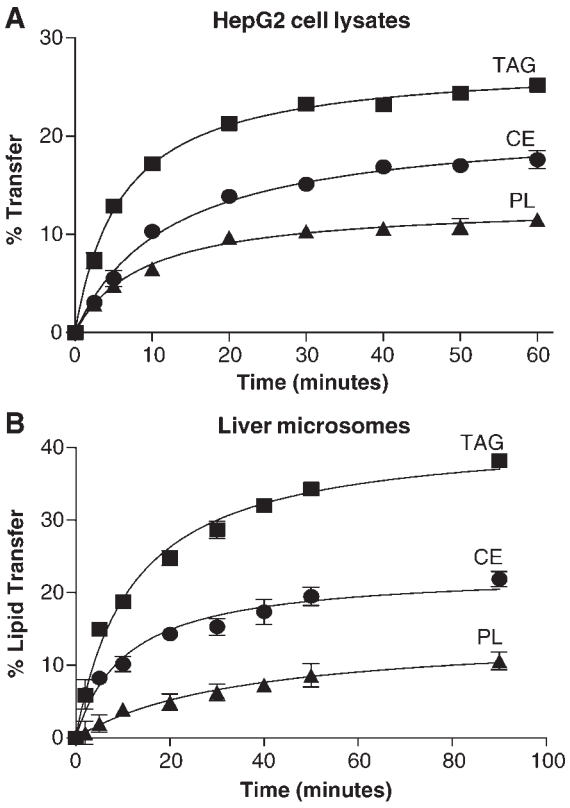

Fig. 3. Lipid transfer activities in HepG2 cells and mouse liver microsomes. A: HepG2 cell lysates were prepared as described in Materials and Methods and used to perform lipid transfer assays in triplicate. Each assay contained 42 μg of protein. Data are expressed as line graphs and error bars representing means ± SD. TAG, triacylglycerol. B: Mouse microsomal contents were prepared as described in Materials and Methods. TAG, CE, or PE lipid transfer activities were measured in triplicate using 21 μg of protein. Mean values are drawn as line graphs and SDs as error bars. Nonlinear regression curve fits were determined using Prism.

Relative lipid transfer activities in MTP

Subsequently, we sought to compare the relationship between TAG, CE, and PL transfer activities measured in purified MTP preparations as well as in cellular and tissue homogenates (**Table 1**). In purified bovine and rat MTP preparations, CE and PL transfer activities were 59–60% and 6–5%, respectively, the TAG transfer activity. These are similar to the relative activities noted by Wetterau et al. (7, 19) in purified bovine MTP using a radioactive assay. In HepG2 cell lysates and liver microsomes, the CE and PL activities were 42–55% and 13–27%, respectively, compared with the TAG transfer activity. Thus, although the relative CE transfer activity in mouse liver microsomes was similar to that of the purified protein, HepG2 cell lysates demonstrated less CE transfer activity compared with purified MTP preparations. This suggests that proteins or other soluble factors in cells or tissues may interfere with this transfer. In contrast, relative PL transfer activities observed in HepG2 cells and liver microsomes were 2- to 4-fold higher than those observed in purified MTP preparations. This is most likely attributable to the presence of other PL transfer proteins, such as PC and phosphatidylinositol

TABLE 1. Specific and relative lipid transfer activities of MTP

MTP Source	Specific Activities (Relative Activities)		
	TAG	CE	PL
Purified bovine	901 ± 36 (100)	533 ± 53 (59)	56 ± 4 (6)
Purified rat	735 ± 45 (100)	438 ± 175 (60)	34 ± 5 (5)
Mouse liver microsomes	9 ± 0.2 (100)	5 ± 1 (55)	1 ± 0.1 (13)
HepG2 cell lysate	4 ± 0.5 (100)	1.5 ± 0.1 (42)	1 ± 0.1 (27)

CE, cholesteryl ester; MTP, microsomal triglyceride transfer protein; PL, phospholipid; TAG, triacylglycerol. Lipid transfer assays were performed using fluorescent lipids as described for Figs. 1–3. The initial rates of lipid transfer (nmol lipid/mg/h) were obtained using low amounts of purified MTP (0.025, 0.04, and 0.1 μg of protein for determining TAG, CE, and PL transfer, respectively) that produced a linear curve for at least 30 min of the assay. Specific activities (% transfer/mg protein/h) were then calculated using time points falling in the linear range for each assay. The absolute rate of lipid transfer (nmol lipid transferred/mg protein/h) was determined by comparing the fluorescence with standard curves. Dividing the specific activity of the lipid transfer in question by the specific activity of TAG transfer and multiplying by 100 provided the relative activities (in parentheses).

transfer proteins (27), in cells and tissues that might transfer fluorescent PE.

Measuring net lipid deposition

To measure the net deposition of lipids in acceptor vesicles, we required a method to separate acceptor vesicles from the donor vesicles and MTP present in the assay. Wetterau et al. (7, 19–21) used cardiolipin and DE52 to achieve this in their radiolabeled assay. First, we determined that the addition of cardiolipin had no effect on the incorporation of TAG in the donor vesicles. The total fluorescence incorporated was 17,821 ± 112 and 17,202 ± 1,162 for donor vesicles with and without cardiolipin, respectively. Second, we confirmed that >99% of the donor vesicles and MTP could be removed from the reaction mixture after incubation with DE52. Third, we determined the effect of the presence of cardiolipin in donor vesicles on the TAG transfer activity of MTP. For this purpose, we performed parallel measurements of TAG transfer with donor vesicles containing, and free of, cardiolipin (**Fig. 4A**). Even though both assays contained the same amounts of acceptor and donor vesicles, as well as MTP, the TAG transfer by MTP from donor vesicles containing cardiolipin was 50% less compared with that obtained with donor vesicles with no cardiolipin and is consistent with published studies (5, 19, 28).

Next, we determined the net lipid deposition to acceptor vesicles. For this, donor as well as acceptor vesicles and MTP were incubated for different times, and fluorescence readings were taken to determine the transfer of TAG. The reaction was then stopped, donor vesicles and MTP were precipitated by the addition of DE52, and the TAG deposited in acceptor vesicles was quantified. The TAG transfer slowly increased with time (Fig. 4B), similar to that observed in the presence of cardiolipin (Fig. 4A). The net deposition of fluorescent TAG to acceptor vesicles increased for 120 min and remained unchanged until 180 min. At saturation, ~50–60% of the TAG was deposited in acceptor vesicles.

We then determined the relative net lipid deposition of various lipids to acceptor vesicles. Donor vesicles containing fluorescent TAG, CE, or PE, as well as cardiolipin, were made (Fig. 4C). Net deposition to acceptor vesicles was measured after the removal of donor vesicles and MTP. The relative activities were 100 ± 4.8%, 71.0 ± 8.5%, and 13.5 ± 5.2% for TAG, CE, and PL, respectively. These relative values are similar to those observed based on lipid transfer measurements (Table 1). Thus, both assays gave similar results concerning relative lipid transfer activities.

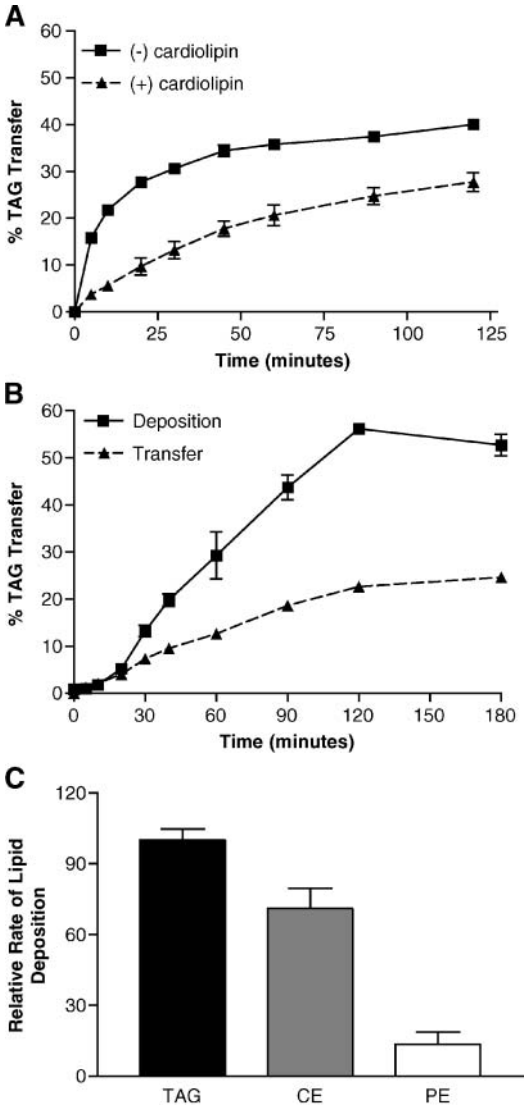

Fig. 4. Measurement of net lipid deposition: A: Effect of cardiolipin on the TAG transfer activity of MTP. Donor vesicles made with and without cardiolipin were used. Each assay in triplicate contained 0.25 μg of purified bovine MTP, 3 μl of donor vesicles (100 pmol of fluorescent TAG, 1.2 nmol of PC with or without 0.081 nmol of cardiolipin), and 3 μl of PC acceptor vesicles, as described for Fig. 1. The microtiter plate was incubated at 37°C, fluorescence was monitored over time, and percentage transfer was determined as described previously. B: Measurement of net deposition of lipids by MTP. Transfer assays were set up in triplicate as described for A containing 0.25 μg of MTP, 3 μl of donor vesicles, and 3 μl of acceptor vesicles in 100 μl assay volume. Percentage lipid transfer was measured as described for Fig. 1. To measure lipid deposition, 100 μl of DE52 anion-exchange resin was added to the reactions at the predetermined times. After centrifugation, 10 μl of supernatant was transferred to a 96-well black microtiter plate. Fluorescence was measured after the addition of 90 μl of isopropanol. C: Relative net lipid deposition by MTP. Net lipid transfer assays were set up in triplicate as described for B. Assays contained 0.25 μg of purified bovine MTP, donor vesicles (100 pmol of different fluorescent lipids, 1.2 nmol of PC, and 0.081 nmol of cardiolipin), and acceptor vesicles. Percentage net lipid deposition was determined at 1 h for TAG as well as CE and at 1.5 h for PE. The specific activity (% transfer/mg protein/h) was then calculated. Dividing the individual specific activities with the specific activity of TAG lipid transfer and multiplying by 100 provided relative net lipid transfer activities. Bar graphs and error bars represent means ± SD.

DISCUSSION

Wetterau et al. (7, 19–21) had published an in vitro method for measuring lipid transfer in microsomal fractions using radiolabeled lipids. Recently, a fluorescent assay was described for MTP that evaluates the transfer of TAG between membrane vesicles (23). This procedure, although rapid and sensitive, also precludes the use, as well

as the disposal, of radioactivity. Here, we describe methods to measure CE and PL transfer by MTP using fluorescent lipids. In addition, we describe a procedure to determine the net deposition of fluorescent lipids to acceptor vesicles.

Although the mechanism associated with MTP lipid transfer has yet to be fully elucidated, it is suggested that MTP binds transiently to lipid membranes, extracts lipid, and ultimately transfers this lipid to another membrane or possibly apoB (21, 22). The assays described here are capable of evaluating the two key events in this process, lipid transfer by MTP and the subsequent deposition of such lipids to acceptor vesicles. The availability of these two methods may be useful for determining whether the two steps occur independent of each other and/or through different domains of MTP.

The first method monitors MTP's capacity to bind and extract lipids from a membrane in the presence of acceptor vesicles. Fluorescence is quenched when lipids are in unilamellar (one PL bilayer) membrane vesicles. Upon association with MTP, the lipid fluorophore is unquenched and detected by the flourimeter. In this process, the real-time lipid transfer is measured and found to be time- as well as concentration-dependent. Furthermore, the process is saturable. The saturation of lipid transfer might indicate that all of the MTP molecules are actively involved in the process.

The second method measures the deposition of fluorescent lipids to acceptor vesicles and is based on the procedure of Wetterau et al. (7, 19–21). In this method, fluorescent lipids deposited in acceptor vesicles are quantified after the removal of MTP and donor vesicles by anion-exchange resin. The amounts of lipids deposited were far greater than those measured during lipid transfer, mostly reflecting the large excess of acceptor vesicles present in the assay. There are two major drawbacks associated with this method. First, it involves an additional step of separating acceptor vesicles from donor vesicles and MTP. Second, the incorporation of negatively charged lipids in the donor vesicles decreases the sensitivity of the assay. Thus, this assay is recommended only when there is a need to measure the process of lipid deposition. Under normal conditions, the measurement of lipid transfer is recommended to measure individual MTP activities. For the routine determination of MTP activity in cell lysates, we recommend measuring TAG transfer.

We observed that PL transfer could not be completely inhibited by MTP antagonists (Fig. 1). Jamil et al. (26) also reported that PL transfer cannot be completely inhibited by MTP antagonists. Three possible reasons were considered. First, we do not think that the incomplete inhibition is attributable to nonfacilitated transfer of PL. All assays have blanks consisting of donor and acceptor vesicles in the absence of MTP. Blank values were not affected by the MTP antagonists, and increases in fluorescence were not observed in blanks. Furthermore, blank values were subtracted during activity calculations, eliminating the contribution, if any, of nonfacilitated transfer. Second, contamination of MTP preparations with PL transfer proteins was considered. Plasma contains PL and CE transfer proteins, whereas cells are known to contain PC and phosphatidylinositol transfer proteins. The purified MTP preparations consisted mainly of two polypeptides corresponding to the known 97 and 55 kDa subunits. None of the known cellular and plasma PL transfer proteins was present in the preparations. Third, BMS197636 might be a specific inhibitor for neutral lipids. Kinetic studies have demonstrated that MTP might contain two PL binding sites and one neutral lipid transfer site (22). The neutral lipid transfer site might also transfer PL. It is possible that the inhibitor might inhibit only one site and not the other, resulting in partial inhibition.

There are some caveats concerning the measurement of PL and CE transfer activities using cell and tissue homogenates. Cells contain other PL transfer activities that contribute to PL transfer activity. The determination of PL transfer activity in cell homogenates is further complicated by the $<60\%$ inhibition of this activity by the available MTP inhibitors. The extent of CE transfer is comparatively lower than that of TAG and is only linear under a small concentration range of MTP. Thus, time course and concentration curves should be measured in the presence and absence of MTP inhibitors to determine the CE transfer activity of MTP in homogenates.

In short, we describe two methods for evaluating lipid transfer activities of MTP using fluorescent lipids. These assays are fast and sensitive and can be used to detect inherent differences in the transfer of various lipids by MTP. The methods measuring the deposition of lipids to acceptor vesicles may be useful in determining the unidirectional transfer of lipids to various acceptors. These methods may be valuable in identifying MTP mutants that are able to bind lipids but cannot transfer them to acceptor vesicles, or those deficient in a specific lipid transfer activity. The availability of methods for measuring different lipid transfer activities of MTP may facilitate the discovery of inhibitors specific for individual lipid transfer activities. The identification of such inhibitors may be valuable in delineating the importance of various lipid transfer activities of MTP in apoB lipoprotein assembly and secretion.

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Phospholipid Transfer Activity of Microsomal Triacylglycerol Transfer Protein Is Sufficient for the Assembly and Secretion of Apolipoprotein B Lipoproteins*

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Human microsomal triacylglycerol transfer protein (hMTP) is essential for apolipoprotein B (apoB)-lipoprotein assembly and secretion and is known to transfer triacylglycerols, cholesterol esters, and phospholipids. To understand the relative importance of each lipid transfer activity, we compared the ability of hMTP and its *Drosophila* ortholog (dMTP) to assemble apoB lipoproteins and to transfer various lipids. apoB48 secretion was induced when co-expressed with either hMTP or dMTP in COS cells, and oleic acid supplementation further augmented secretion without altering particle density. C-terminal epitope-tagged dMTP (dMTP-FLAG) facilitated the secretion of apoB polypeptides in the range of apoB48 to apoB72 but was ~50% as efficient as hMTP-FLAG. Comparison of lipid transfer activities revealed that although phospholipid transfer was similar in both orthologs, dMTP was unable to transfer neutral lipids. We conclude that the phospholipid transfer activity of MTP is sufficient for the assembly and secretion of primordial apoB lipoproteins and may represent its earliest function evolved for the mobilization of lipid in invertebrates. Identification of MTP inhibitors, which selectively affect transfer of a specific lipid class, may have therapeutic potential.

Lipoproteins are lipid-protein complexes that transport lipids, fat-soluble vitamins, and other hydrophobic molecules in the plasma. Apolipoprotein B (apoB)[2] is a structural protein embedded in the phospholipid monolayer on the surface of triglyceride-rich lipoproteins. It has been hypothesized that apoB contains amphipathic α-helical and β-sheet domains (1). Lipidation of the β-sheets is necessary for the assembly of larger apoB polypeptides into lipoprotein emulsion particles. Lipoprotein assembly begins co-translationally and microsomal triglyceride transfer protein (MTP) plays a pivotal role in this process (for reviews, see Refs. 2–6). MTP is absent in abetalipoproteinemia, a disease characterized by the deficit of plasma apoB lipoproteins and low plasma cholesterol levels (7, 8). Reconstitution of MTP in heterologous systems rescues apoB secretion, whereas tissue-specific liver knock-out models recreate the lipoprotein deficiency present in abetalipoproteinemia (9, 10). The signature activity of MTP is its ability to transfer lipids between membranes. It has been demonstrated that MTP lipid transfer activity is necessary for apoB lipoprotein assembly and secretion (for reviews, see Refs. 2–6). More recent evidence suggests that MTP lipid transfer activity is also responsible for lipid accretion within the secretory pathway and that MTP potentially stabilizes lipid vesicles in the endoplasmic reticulum (reviewed in Refs. 2 and 3).

MTP transfers several lipids including triacylglycerols, cholesterol esters, and phospholipids between vesicles *in vitro* (11–14). It has been suggested that MTP transiently interacts with a membrane, extracts lipids, and then delivers them to another lipid acceptor or to nascent apoB lipoproteins (15). Kinetic studies indicate the presence of two lipid-binding sites: one site binds triacylglycerols, cholesterol esters, and phospholipids with a preference for neutral lipids, whereas a second site binds only phospholipids (16). It is not clear whether all MTP lipid transfer activities are required for lipoprotein assembly.

Recently, we identified a human MTP (hMTP) ortholog from the genome of the fruit fly, *Drosophila melanogaster*, that supported the secretion of human apoB34 and apoB41 (17). However, the mechanisms involved in the secretion of apoB lipoproteins by the *Drosophila* MTP (dMTP) have not been elucidated. In this paper, we show that although dMTP is defective in triacylglycerol transfer activity, the phospholipid transfer activity is equivalent to that of hMTP. Thus, phospholipid, but not the triacylglycerol transfer, activity may be necessary for the assembly of primordial lipoproteins.

MATERIALS AND METHODS

Construction of MTP-FLAG Chimeras—hMTP and dMTP expression vectors have been previously described (17). C-terminal FLAG-tagged forms of *Drosophila* and hMTP were produced by PCR, utilizing 3′ antisense primers encoding the FLAG sequence (DYKDDDDK) followed by an in-frame termination codon.

Cell Culture and apoB Secretion—COS-7 cells were grown in Dulbecco's modified Eagle's medium (CellGrow) containing 10% fetal bovine serum (BSA) supplemented with L-glutamine and antibiotics. The cells were initially dislodged from the plate with trypsin and seeded in six-well plates (400,000 cells/well). Transfections were performed using FuGENE 6 transfection reagent (Roche Applied Sciences) according to the manufacturer's instructions. For sequential transfections, apoB expression vectors were initially introduced into COS cells using FuGENE 6 in T175 flasks (7.2×10^6 cells). After 8 h, the cells were detached by trypsin treatment, seeded in six-well plates, and transfected with MTP expression plasmids. At 48 h post-transfection, the media were aspirated and either 1 ml of Dulbecco's modified Eagle's medium or 1 ml of lipid-containing medium (Dulbecco's modified Eagle's medium including 0.4 mM oleic acid complexed with 1.5% BSA and 1 mM glycerol) were added. Following additional 18 h of incubation, the media were collected, protease inhibitors (Sigma) were added, the samples were centrifuged (2,500 rpm, 4 °C, 10 min) to pellet cell debris, and the apoB contents were measured in the supernatants by ELISA (18, 19).

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[2] The abbreviations used are: apoB, apolipoprotein B; BSA, bovine serum albumin; MTP, microsomal triglyceride transfer protein; dMTP, *Drosophila* MTP; hMTP, human MTP; OA, oleic acid; PDI, protein disulfide isomerase; SREBP, sterol regulatory element-binding protein; ELISA, enzyme-linked immunosorbent assay.

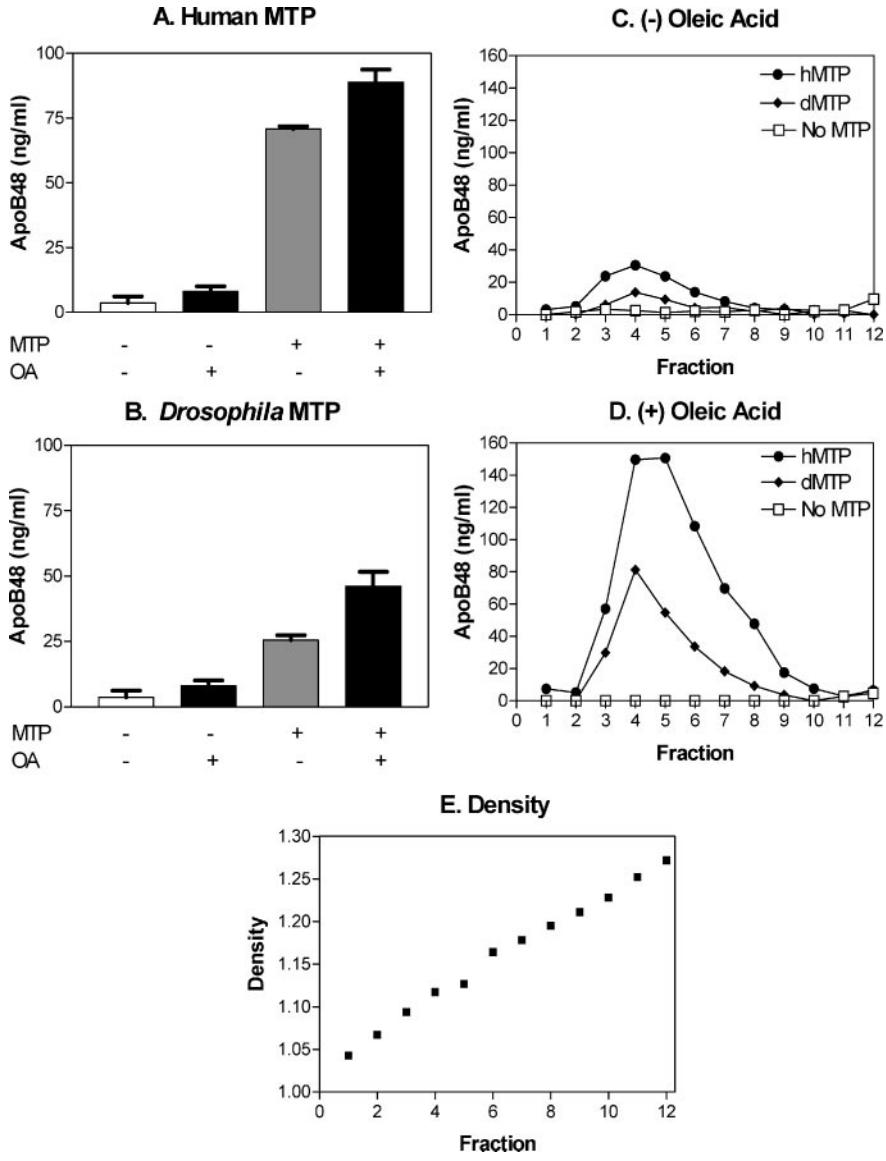

FIGURE 1. **Effect of *Drosophila* MTP and oleic acid on apoB48 secretion.** apoB48 was co-transfected into COS cells with either hMTP (*A*) or dMTP (*B*). After 48 h, the cells received 1 ml of media (−*OA*) or media containing 0.4 mM oleic acid, 1.5% BSA, and 1 mM glycerol (+*OA*). The media were collected 18 h later, and apoB content was determined by ELISA (*A* and *B*). The media were also subjected to density gradient ultracentrifugation (*C–E*) as described under "Materials and Methods," and apoB content in each 1-ml fraction was measured by ELISA. In *A* and *B*, mean apoB concentration is depicted by *bar graphs* ± S.D. ($n = 3$). *C* and *D* show floatation properties of secreted apoB48, and the densities of the fractions are shown in *E*.

The media were also subjected to ultracentrifugation to separate lipoproteins (20). Conditioned media (4 ml) were adjusted to 1.30 g/ml with KBr and overlaid with 2 ml each of 1.24, 1.15, and 1.063 g/ml KBr solutions and 1 ml each of 1.019 and 1.006 g/ml. After ultracentrifugation (SW41 rotor, 40,000 rpm, 17 h, 15 °C), 1-ml fractions were collected, and their apoB content was determined by ELISA. The density in each fraction was measured using a refractometer (Fisher).

Immunofluorescence—COS-7 cells grown on coverslips in 24-well tissue culture plates were transfected with apoB48 and either hMTP- or dMTP-FLAG expression plasmids. Forty-eight hours post-transfection, the cells were fixed and permeabilized in methanol for 15 min at −20 °C. The fixed cells were blocked with phosphate-buffered saline containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3% BSA, and 1% goat serum and incubated with antibodies diluted 1:100 (unless stated otherwise) in the same buffer at room temperature for 1 h. The cells were incubated with mouse anti-FLAG M2 (Sigma) and either rabbit anti-calnexin (Stressgen) or anti-α-mannosidase II (USBiological; 1:25) followed by treatment with Alexa Fluor 488 (green fluorescence)-conjugated goat anti-mouse IgG_1 and Alexa Fluor 594 (red fluorescence)-conjugated goat anti-rabbit IgG_1 (Molecular Probes). The coverslips were mounted in phosphate-buffered saline containing 10% glycerol and 12% triethyldiamine (Sigma) to prevent fluorescent bleaching and visualized using a Bio-Rad Radiance 2000 confocal microscope.

Affinity Purification of MTP-FLAG—COS-7 cells in 150-mm dishes were transiently transfected with either hMTP- or dMTP-FLAG expression plasmids, washed with phosphate-buffered saline, and lysed by incubating in hypotonic buffer (1 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, and protease inhibitor mixture) as previously described (13, 14). The lysates were then centrifuged (SW55 Ti rotor, 50,000 rpm, 1 h, 4 °C), and the supernatants were adjusted to 10 mM Tris, pH 7.4, 150 mM NaCl and mixed by rotation with M2-agarose beads (Sigma) for 3 h at 4 °C. The beads were pelleted by centrifugation (10,000 rpm, 4 °C, 10 s) and washed three times with 10 mM Tris, pH 7.4, 150 mM NaCl, and protease inhibitor. To elute the bound proteins, the beads were rotated with 300 ng/μl of FLAG peptide (DYKDDDDK) in the same buffer for 2 h at 4 °C. The samples were centrifuged (10,000 rpm, 4 °C, 10 s), and the supernatants containing MTP-FLAG were collected.

Lipid Transfer Assays—Radiolabeled lipid transfer assays were performed according to the method described by Wetterau and Zilversmit (11, 12). To prepare unilamellar donor vesicles 1800 nmol of phosphatidylcholine (Avanti Polar Lipids), 3.6 nmol of either [^{14}C]triacylglycerol, [^{14}C]phosphatidylcholine (New England Nuclear), or [^{3}H]cholesterol

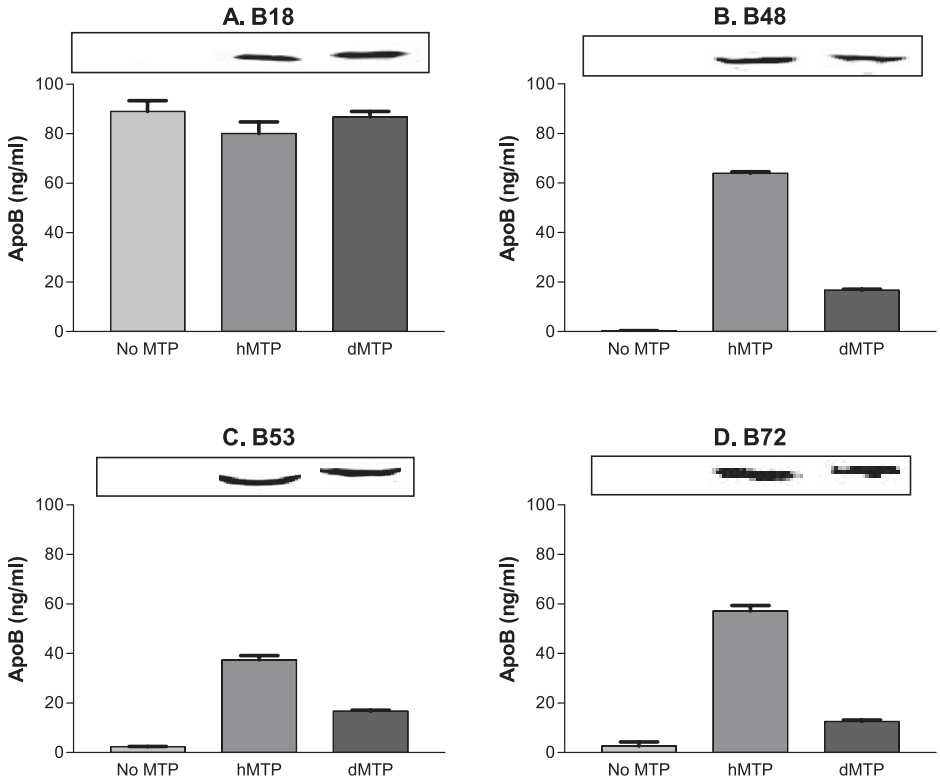

FIGURE 2. ***Drosophila* MTP is less efficient than human MTP at supporting secretion of various apoB polypeptides.** COS-7 cells were first transfected with either apoB18 (*A*), apoB48 (*B*), apoB53 (*C*), or apoB72 (*D*) and then with FuGENE 6 alone (no MTP), hMTP-FLAG (hMTP), or dMTP-FLAG (dMTP) expression plasmids. Following 48 h incubation, growth media was aspirated and replaced with oleate-containing media as described in the legend to Fig. 1. After a further 18-h incubation, the media were collected, and apoB content was determined by ELISA. The mean concentration of apoB is depicted by *bar graphs* ± S.D. ($n = 3$). The cells were then lysed, and equal amounts of protein were applied to SDS-polyacrylamide gels and subjected to Western blotting using M2 anti-FLAG antibody. The protein bands are shown in *insets*. Band intensities were quantified using NIH Image software and used to normalize apoB secretion to cellular MTP content.

ester (Amersham Biosciences), and 135 nmol of cardiolipin (Sigma) were dried under nitrogen, resuspended in 4.5 ml of buffer (15 mM Tris, 40 mM NaCl, 1 mM EDTA, and 0.02% sodium azide, pH 7.4), and sonicated. Acceptor vesicles containing 10,800 nmol of phosphatidylcholine were prepared similarly. The vesicles were then centrifuged (50,000 rpm, 15 °C, 1 h, SW55 Ti rotor), and the top 4-ml fraction was collected for activity determinations. The reaction mixture (final volume, 0.5 ml) contained 100 μl each of donor and acceptor vesicles in 10 mM Tris-HCl, pH 7.4, 0.1% BSA, and 150 mM NaCl. M2 affinity-purified MTP (100 μl) was added to the reaction mixture and incubated for 2 h at 37 °C. The reaction was stopped by the addition of 0.5 ml of DE52 anion exchange resin (1:1, v/v, suspension in 15 mM Tris, 1 mM EDTA, 0.02% sodium azide, pH 7.4.), rotated at 4 °C for 5 min, and centrifuged (12,000 rpm, 4 °C, 10 min). Radiolabeled lipids in supernatants (500 μl) were measured by scintillation counting. Blank assays were performed in the absence of purified proteins. Total radioactivity present in the assays was measured by omitting DE52 from the stop buffer. Lipid transfer (% transfer/2 h) was determined.

Transfer of fluorescently labeled lipids was assayed as described previously (13, 14). Briefly, donor vesicles containing nitrobenzoaxadiazole-labeled triacylglycerols (Chylos, Inc.) and phosphatidylethanolamine (14) were incubated with acceptor vesicles in the presence of equal amounts of total cell protein lysates or purified hMTP- and dMTP-FLAG proteins for the indicated times. Increases in fluorescence during transfer were recorded, and the percentage of transfer was calculated.

MTP Gene Deletion Studies—$MTP^{fl/fl}$Mx1Cre mice (21) were obtained from Jackson Laboratories and bred at SUNY Downstate Medical Center. The mice were injected once with either phosphate-buffered saline or pIpC (250 μg). After 48 h, the livers were collected and used to measure lipid transfer activities of MTP as described before (13, 14).

RESULTS

We previously showed that dMTP promotes human apoB34 and apoB41 secretion (17). Here we explored how dMTP aids in the secretion of apoB. First, we asked whether apoB secretion supported by dMTP is modulated by oleic acid (OA) supplementation. Transfection of COS cells with apoB48 expressing plasmids resulted in barely detectable apoB levels in the media with or without OA supplementation (Fig. 1, *A* and *B*). However, co-transfection with hMTP significantly increased (~20-fold) the secretion of apoB48, and OA supplementation further enhanced its secretion by 25.6 ± 6.9% ($p = 0.003$) (Fig. 1*A*). Similar to hMTP, co-expression of dMTP and apoB48 resulted in increased secretion of apoB48 (~7-fold) that was further augmented (80.8 ± 21.6%, $p = 0.004$) by OA supplementation (Fig. 1*B*). Next, we explored the effect of OA supplementation on the density of secreted lipoproteins. In the absence of OA, apoB secreted from COS cells expressing hMTP or dMTP was present in fractions 3–6 corresponding to a density of 1.1–1.15 g/ml (Fig. 1, *C–E*). Although OA increased the total apoB secreted, it did not change the density of the secreted lipoproteins (Fig. 1, compare *C* and *D*). These studies indicate that apoB secretion driven by dMTP expression is responsive to OA supplementation and produces lipoproteins with properties similar to those secreted in the presence of hMTP.

We observed that dMTP can render apoB48 secretion-competent, but cells expressing dMTP consistently secreted less apoB than those expressing hMTP (Fig. 1). To understand why dMTP was less efficient in promoting apoB secretion, we considered two possibilities. First, the reduction might be due to the lower expression of dMTP compared with hMTP in COS cells. Second, different levels of apoB expression might be due to variations in co-transfection efficiency. To obtain equal expression, the cells were transfected with expression plasmids containing sequences for apoB18 through apoB72 and subsequently transfected with expression vectors containing either hMTP-FLAG or dMTP-FLAG. The relative expression of the different MTPs was determined by Western blotting. apoB18-expressing cells synthesized similar amounts of hMTP- and dMTP-FLAG (Fig. 2*A*, *inset*), and apoB18 secretion was not affected by the co-expression of either MTP (Fig. 2*A*) consistent, with previous studies (22, 23). Next, COS cells were transfected with apoB48 and subsequently transfected with

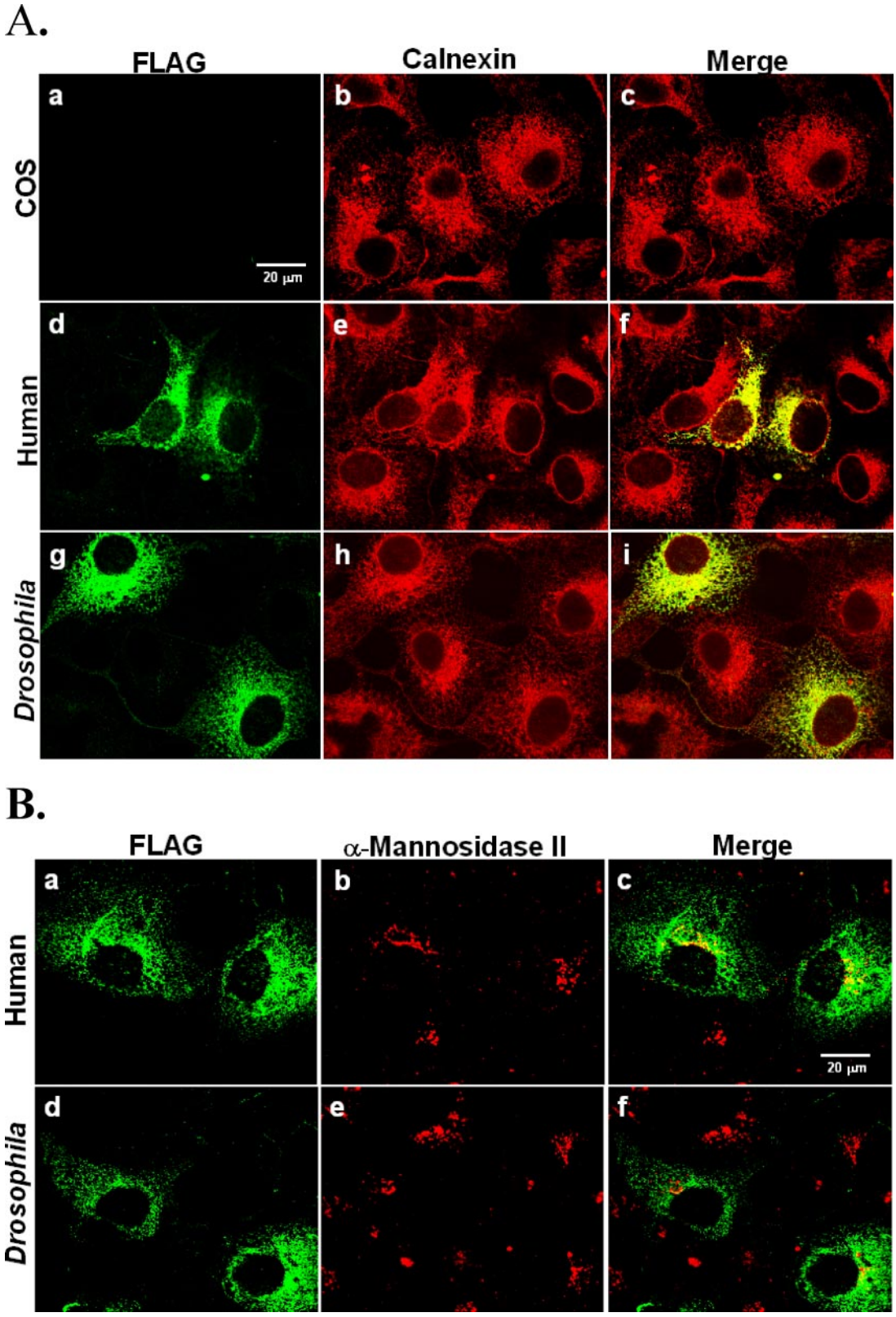

FIGURE 3. **Subcellular localization of human and *Drosophila* MTP-FLAG.** COS cells transfected with apoB48 and either hMTP-FLAG (*d–f*) or dMTP-FLAG (*g–i*) were grown on coverslips in 24-well tissue culture dishes. After 48 h, the cells were fixed and treated as described under "Materials and Methods." *A*, cells were stained with anti-FLAG M2 to label MTP (*panels a*, *d*, and *g*; *green fluorescence*) and anti-calnexin (*panels b*, *e*, and *h*; *red fluorescence*) as a marker for endoplasmic reticulum. Co-localization of MTP with the endoplasmic reticulum was demonstrated in the merged images (*panels c*, *f*, and *i*; *yellow fluorescence*). *B*, cells were incubated with anti-FLAG (*panels a* and *d*; *green*) and anti-α-mannosidase II (*panels b* and *e*; red) to label the Golgi. The merged images (*panels c* and *f*; *yellow*) illustrated co-localization of MTP with the Golgi marker.

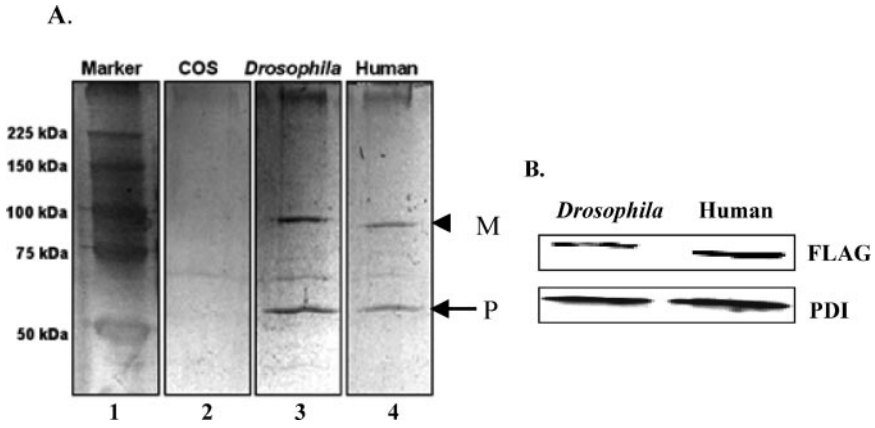

FIGURE 4. **Purification and characterization of hMTP and dMTP.** *A*, MTP-FLAG chimeras were affinity-purified from transiently expressing COS cells. The cell homogenates were applied to M2-agarose beads and washed, and bound proteins were eluted as described under "Materials and Methods." Eluted proteins were applied to polyacrylamide gels, subjected to electrophoresis, and stained. *Lane 1* (Marker), molecular weight markers; *lane 2* (COS), proteins eluted from nontransfected cells; *lane 3* (*Drosophila*), proteins eluted from cells expressing dMTP-FLAG; *lane 4* (Human), proteins eluted from cells expressing hMTP-FLAG. The *arrowhead* and *arrow* point to the M and P subunits of MTP, respectively. Differences in the migration of M subunits are in agreement with previous studies (17). *B*, Western blotting of eluted proteins. Eluted proteins were transferred to nitrocellulose membranes and probed using M2 anti-FLAG or rabbit anti-PDI followed by anti-IgG horseradish peroxidase conjugate.

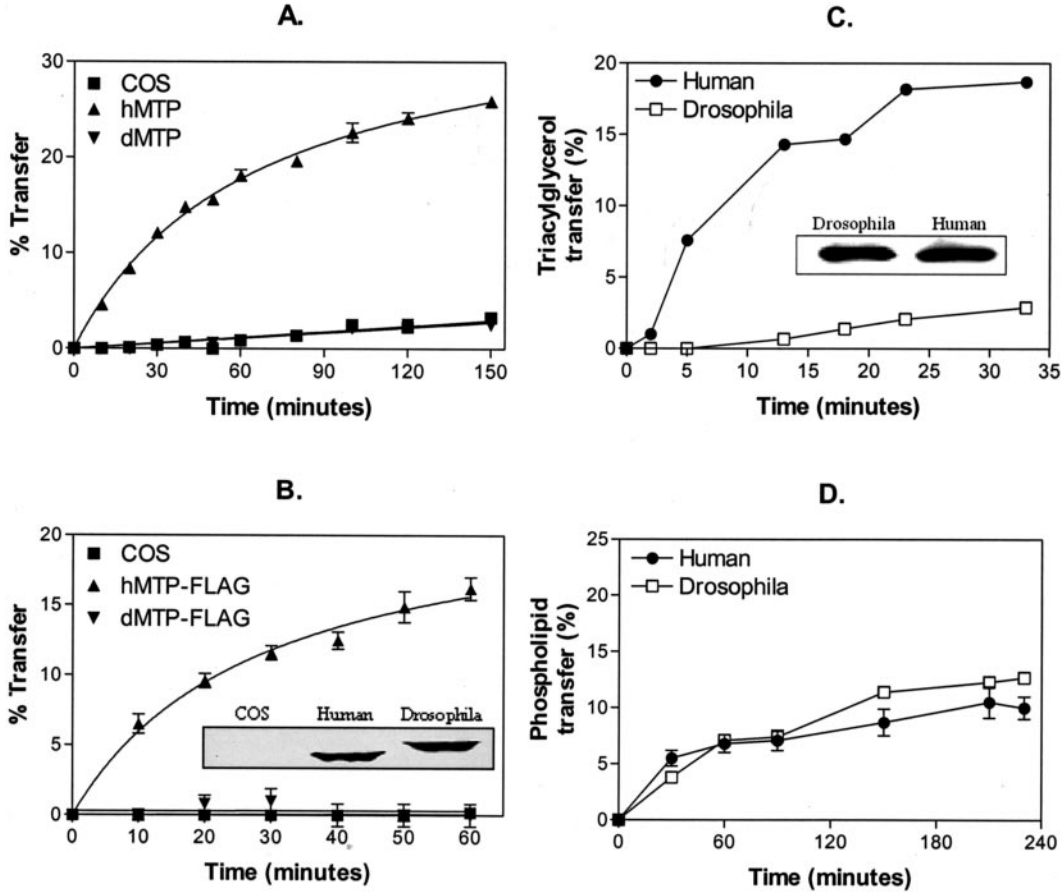

FIGURE 5. **Determination of lipid transfer activities of dMTP and hMTP using fluorescence assays.** COS cells transiently expressing hMTP and dMTP (*A*) or hMTP- and dMTP-FLAG (*B*) were hypotonically lysed, and soluble proteins (30–36 μg) were assayed for triacylglycerol transfer activity using the radiolabeled vesicle transfer assay. In *B* (*inset*), amounts of MTP present in the microsomal contents were visualized by Western blotting. Real time transfer of fluorescently labeled triacylglycerol (*C*), and phosphatidylethanolamine (*D*) was measured using equal amounts of the purified proteins (*C*, *inset*). Fluorescence at 550 nm was monitored over time after excitation at 485 nm.

either hMTP- or dMTP-FLAG. The amount of apoB48 secreted by dMTP-transfected cells was ~33% of the level observed in hMTP-expressing cells (Fig. 2*B*). However, after correcting for MTP protein levels (Fig. 2*B*, *inset*), we calculated that dMTP was ~50% as effective as hMTP in promoting apoB48 secretion. Utilizing similar correction methods it was calculated that dMTP-FLAG was ~50 and ~40% as efficient as the hMTP-FLAG in assisting apoB53 (Fig. 2*C*) and apoB72 (Fig. 2*D*) secretion, respectively. These studies suggest that dMTP can support the secretion of longer apoB polypeptides; however, it is less efficient compared with hMTP.

Consideration was given to the possibility that dMTP is less proficient in supporting apoB secretion because of incorrect subcellular distribution. MTP is present in the endoplasmic reticulum due in part to association with protein disulfide isomerase (PDI) (24, 25) and has also been localized to the Golgi apparatus (26, 27). The subcellular distribu-

tion of the hMTP- and dMTP-FLAG proteins was determined using confocal microscopy (Fig. 3). No staining with anti-FLAG antibodies was seen in nontransfected cells (Fig. 3*A*, *panel a*). However, punctate staining was observed in cells transfected with hMTP- and dMTP-FLAG expression plasmids (Fig. 3*A*, *panels d* and *g*). Calnexin, also detected as punctate cytoplasmic staining (*red*), provides a marker for endoplasmic reticulum localization (Fig. 3*A*, *panels b*, *e*, and *h*). In transfected cells, both hMTP- and dMTP-FLAG staining showed extensive co-localization with calnexin as illustrated in the merged images (Fig. 3*A*, *panels c*, *f*, and *i*, *yellow fluorescence*). In addition to endoplasmic reticulum, hMTP- and dMTP-FLAG (Fig. 3*B*, *panels a* and *d*) co-localized with the Golgi marker protein α-mannosidase II (Fig. 3*B*, *panels b* and *e*). This was confirmed in the merged images (Fig. 3*B*, *c* and *f*, *yellow fluorescence*). These studies indicate similar distribution of dMTP- and hMTP-FLAG chimeras in the endoplasmic reticulum and Golgi apparatus.

Mammalian MTP is a heterodimeric complex of a 97-kDa "M" subunit and a 55-kDa PDI subunit (28, 29). We therefore determined whether dMTP also associates with endogenous PDI. hMTP-FLAG and dMTP-FLAG were affinity-purified using M2 (anti-FLAG monoclonal antibody)-agarose from transiently transfected COS cell lysates (Fig. 4). Polyacrylamide gel electrophoresis followed by silver staining demonstrated two predominant protein bands of ~95 and 55 kDa in dMTP- and hMTP-transfected cells, but not in control cells (Fig. 4*A*, compare control *lane 2* with *lanes 3* and *4*). These were shown to be the M subunit and PDI by Western blotting using specific antisera (Fig. 4*B*), indicating that dMTP interacts with endogenous PDI similarly to hMTP.

We then sought to understand which property of dMTP is critical for its ability to render apoB secretion competent by comparing the specificity of lipid transfer between hMTP and dMTP. Although the lysates from cells that transiently expressed hMTP demonstrated a measurable ability to transfer triacylglycerols (0.71 ± 0.04% triacylglycerol transfer/μg of protein/h), no significant increase was observed using lysates obtained from cells that expressed dMTP (0 ± 0.04% triacylglycerol transfer/μg of protein/h) (Fig. 5*A*). The lack of triacylglycerol transfer activity in dMTP is similar to what we previously reported (17). To demonstrate that dMTP was expressed, we performed similar experiments using lysates from hMTP- or dMTP-FLAG-expressing cells (Fig. 5*B*). Once more, the lysates from cells expressing hMTP-FLAG, but not dMTP-FLAG transferred triacylglycerols. Furthermore, equal amounts of MTP were present in the lysates as assessed by Western blot (Fig. 5*B*, *inset*). Thus, dMTP appeared to lack the ability to transfer triacylglycerols.

Next, we studied the phospholipid transfer activities of human and *Drosophila* MTP. We were unable to measure significant phospholipid transfer in cell lysates obtained from COS cells expressing either of the MTP orthologs (data not shown). We therefore assayed (13, 14) for these activities using purified hMTP and dMTP (Fig. 5, *C* and *D*). The transfer of triacylglycerols by hMTP-FLAG increased with time and reached saturation (Fig. 5*C*). However, increases in triacylglycerol fluorescence in the presence of dMTP were minimal and did not appear to saturate with time. Similar studies with donor vesicles containing fluorescently labeled phosphatidyl-ethanolamine showed that both orthologs were equipotent in time-dependent initial and maximum lipid transfer (Fig. 5*D*). These data indicate that hMTP and dMTP can transfer phospholipids, but dMTP is deficient in triacylglycerol transfer activity.

The lipid transfer activities of purified proteins were also measured employing a classical radiolabeled vesicle transfer assay (11, 12). hMTP-

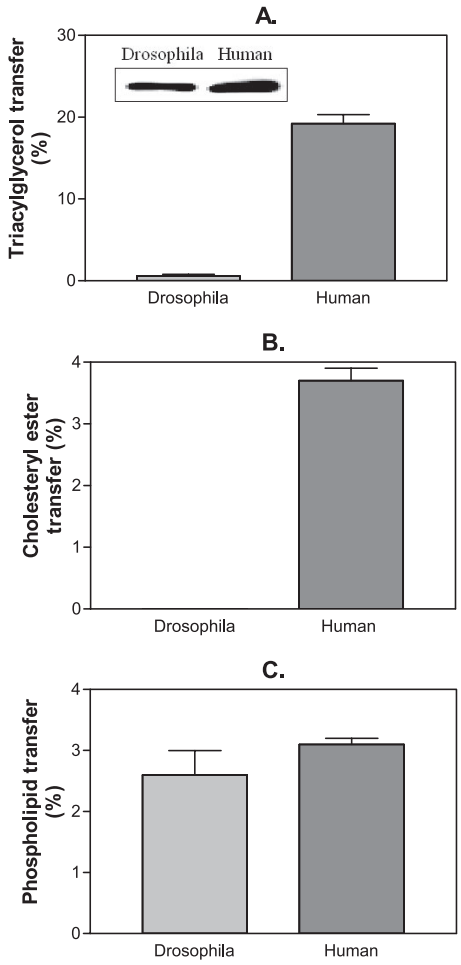

FIGURE 6. **Lipid transfer activity determinations using radiolabel assay.** Similar amounts of purified dMTP-FLAG and hMTP-FLAG were used to measure the transfer of radiolabeled triacylglycerols (*A*), cholesteryl esters (*B*), or phosphatidylcholine (*C*) as described under "Materials and Methods." The *bar graphs* represent the means ± S.D. ($n = 3$).

FLAG showed a robust triacylglycerol transfer activity (19.2 ± 1.1%), whereas only minimal transfer was detected using purified dMTP-FLAG (0.6 ± 0.2%) (Fig. 6*A*), confirming data from Fig. 5. Likewise, hMTP could transfer cholesterol esters (3.7 ± 0.2%); however, this activity was undetectable in dMTP-FLAG (Fig. 6*B*). In contrast to the transfer of triacylglycerols and cholesterol esters, dMTP and hMTP were equally active in transferring phospholipids, (2.6 ± 0.4 and 3.1 ± 0.1%, respectively; Fig. 6*C*). These studies showed that dMTP was deficient in the transfer of neutral lipids, compared with hMTP, but was as efficient as hMTP in phospholipid transfer.

It has been reported that MTP antagonist, BMS 200150, does not affect phospholipid transfer but does inhibit triacylglycerol transfer activity at low concentrations in HepG2 cell lysates (30). We evaluated the effect of BMS 200150 on both phospholipid and triacylglycerol transfer activities using HepG2 cell lysates and purified MTP. Low concentrations of BMS 200150 inhibited triacylglycerol transfer without inhibiting phospholipid transfer activity (Fig. 7*A*) and reduced apoB secretion (Fig. 7*B*) in HepG2 cells. However, low concentrations of the compound inhibited both phospholipid and triacylglycerol transfer activities of the purified MTP (Fig. 7*C*). In addition, we studied the effect of MTP gene deletion on cellular lipid transfer activities. Conditional deletion of MTP gene resulted in a 72% decrease in triacylglycerol activity but had no effect on phospholipid transfer activity in liver homoge-

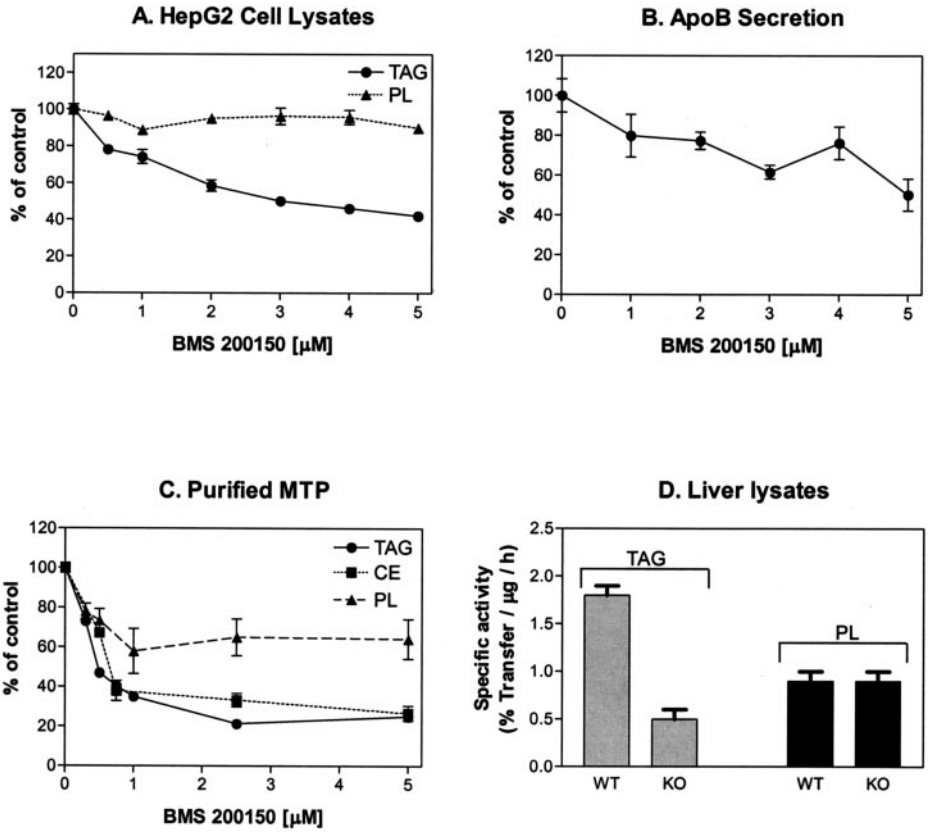

FIGURE 7. **Effect of BMS 200150 on apoB secretion and MTP lipid transfer activities.** Varying concentrations of BMS 200150 (1–5 μM) were added to HepG2 cell lysates (30 μg) or purified rat MTP (0. 05, 0.2, or 0.4 μg of purified protein, for determining triacylglycerol (*TAG*), cholesteryl ester (*CE*), and phospholipid (*PL*) transfer activity, respectively). MTP lipid transfer activities of cell lysates (*A*) and purified MTP (*C*) were measured using the fluorescence-based assay. In *B*, HepG2 cells were treated with inhibitor overnight, and apoB secretion was measured by ELISA. Triplicate assays are represented as the means ± S.D. *D*, $MTP^{fl/fl}$MxCre1 mice were injected with either phosphate-buffered saline or pIpC (250 μg). The liver (100 mg) pieces were homogenized (14). Triacylglycerol and phospholipid transfer specific activities were measured using 20 μg of protein.

nates (Fig. 7*D*). We conclude that incomplete inhibition of phospholipid transfer activity by BMS 200150 in HepG2 cell lysates and no inhibition in *Mttp* gene deleted liver homogenates is due to the presence of other phospholipid transfer activities.

DISCUSSION

The data presented here show that dMTP promotes the secretion of apoB polypeptides but is less efficient than hMTP. Furthermore, dMTP responds to lipid availability, as does its human ortholog. However, although dMTP and hMTP can both transfer phospholipids, dMTP displays virtually no neutral lipid transfer activity. Based on these observations, we propose that the phospholipid transfer activity of MTP may be necessary and sufficient for the assembly and secretion of primordial apoB lipoproteins. The presence of neutral lipid transfer activity may increase the efficiency by which apoB lipoproteins are assembled, or alternatively, might be involved primarily in lipid accretion and second step particle core expansion.

Recently, we showed that dMTP was able to support the secretion of apoB34 and apoB41 (17). These apoB polypeptides do not contain the complete hydrophobic β-sheet domains critical for the assembly of larger lipoproteins. Thus, we had hypothesized that dMTP would not support the secretion of longer apoB polypeptides, which contain more extensive lipid-binding β-sheet domains. Instead, we found that dMTP rescued the secretion of apoB48, which contains the complete β1 domain, as well as apoB72, which contains both the β-1 and a major portion of the β2 domain. These studies suggest that dMTP is capable of lipidating apoB polypeptides with long hydrophobic β-sheets, rendering them secretion-competent.

The nascent apoB polypeptide is believed to interact with the inner leaflet of the endoplasmic reticulum membrane. Its release from the membrane and conversion to a lipoprotein particle is critically dependent on MTP (reviewed in Refs. 2, 6, and 31–34). In the absence of MTP, nascent apoB folds incorrectly and undergoes proteasomal degradation. Although mammalian MTP has been shown to transfer triacylglycerols, cholesteryl esters, and phospholipids between membrane vesicles *in vitro* (11–14), its predominant activity is toward triacylglycerols (12, 14). Several antagonists of the lipid transfer activity of MTP are potent inhibitors of the triacylglycerol transfer activity (14, 30). Similarly, a missense mutation, N780Y, in MTP abolishes triacylglycerol transfer activity and also does not support apoB secretion (35, 36). Hence, it has been assumed that the triacylglycerol transfer activity of MTP is critical for the assembly of nascent apoB into lipoproteins. Here, we have presented evidence that dMTP lacks the ability to transfer triacylglycerols and yet is able to assist in the assembly and secretion of apoB lipoproteins. Thus, phospholipidation of the nascent apoB-polypeptide by MTP may be sufficient to inhibit proteasomal degradation and promote lipoprotein assembly.

It has long been suggested that apoB lipoprotein assembly occurs in two steps. These steps involve the synthesis of primordial lipoprotein particles that contain only small amounts of neutral lipid, followed by the bulk addition of neutral lipid via fusion with triacylglycerol droplets. Based on the present data, we propose that the first step may involve phospholipidation of nascent apoB by MTP followed by the budding of primordial particles from the endoplasmic reticulum membrane. In our previous studies, we observed that the apoB particles secreted in the presence of dMTP and hMTP contained a similar content of triacylglycerols (17). How did these particles acquire triacylglycerols in the absence of a neutral lipid transfer activity? It is known that neutral lipids have low solubility within phospholipid bilayers and tend to diffuse rapidly within the plane of the membrane (37). It is possible that after MTP-dependent priming with phospholipids, the initiating domain of apoB (38) inserts into the membrane and functions to trap neutral lipids

in the form of a small oil droplet between the leaflets of the bilayer. At a critical stage during its translation, apoB is released from the endoplasmic reticulum membrane in the form of a nascent triglyceride core-containing emulsion particle. Subsequently, the particle can be further enriched with neutral lipids by MTP, particularly during second step core expansion.

Comparisons of the lipid transfer activities in hMTP and dMTP revealed that both orthologs were equipotent in transferring phospholipids. Using a classical radiolabel assay, we were unable to measure any neutral lipid transfer activity associated with dMTP. However, employing a more sensitive fluorescent assay, we did detect the transfer of small amounts of triacylglycerols (Fig. 6*D*). This transfer activity, however, did not appear to saturate with time and was ~10-fold lower than that observed in hMTP. Thus, we conclude that the activity detected in the fluorescent assay most likely represents a background, nonspecific transfer and that dMTP lacks the robust neutral lipid transfer activity, which is a hallmark of hMTP.

At first glance, the data obtained with BMS 200150 appear to contradict the conclusion that phospholipid is sufficient for apoB lipoprotein assembly because BMS 200150 inhibits triacylglycerol transfer activity but not the phospholipid transfer activity in HepG2 cell lysates (Fig. 7*A*). However, inhibition of purified MTP by BMS 200150 and MTP gene deletion studies clearly show that the liver expresses other proteins that can transfer phospholipids. In fact, phosphatidyl-choline and phosphatidylinositol transfer proteins have been purified, and their tissue distributions and biochemical characterizations have been reported (for review, see Refs. 39). Thus, with respect to phospholipid transfer, it is difficult to interpret studies using MTP antagonists and whole cell lysates. In contrast, the data presented here show clearly that purified hMTP and dMTP have comparable phospholipid transfer activities. Because dMTP cannot transfer triacylglycerol (Figs. 5 and 6), we suggest that phospholipid transfer activity may be sufficient for the assembly and secretion of apoB lipoproteins.

It was surprising to discover that dMTP lacks neutral lipid transfer activity because insects require exogenous sterols for their growth and viability (40). The major function of mammalian MTP is to assist in the transfer of dietary fat and sterols via chylomicron assembly and secretion. In insects, dietary lipids are transported by lipid transfer particles called lipophorins. Insect fat body, which performs the functions equivalent to vertebrate liver and adipose tissue, synthesizes and secretes high density lipophorins into the hemolymph. These particles acquire lipids from the midgut and fat body via the action of an extracellular lipid transfer particle and become low density lipophorins. These particles then interact with cell surface receptors to deliver lipids to their target tissues (40). Based on the data presented here and previous studies (17, 41), we propose that dMTP may play a crucial role in the intracellular assembly of high density lipophorins by the fat body and that this process may involve the phospholipidation of nascent apolipophorin by MTP.

The phospholipid transfer activity of MTP may also be involved in the secretion of vitellogenin. Sellers *et al.* (41) have recently shown that a mutant hMTP, deficient in triacylglycerol transfer activity, can promote the secretion of *Xenopus* vitellogenin A1. It remains to be determined whether phospholipidation of vitellogenin is required for its secretion. We have recently shown that hMTP transfers phospholipids, but not triacylglycerols, to CD1d, a lipid antigen presenting molecule (42). Therefore, the phospholipid transfer activity of hMTP may also be important for the biogenesis and cell surface expression of CD1d and perhaps other CD1 family members (43).

MTP is a member of the large lipid transfer protein gene family that includes apoB, apolipophorin and vitellogenin (44). It has been suggested that these genes are derived from a common ancestral MTP gene (41, 45). Based on the data presented here, we hypothesize that phospholipid transfer activity might have been evolutionarily the earliest lipid transfer activity acquired by MTP. During evolution, MTP might have acquired neutral lipid transfer activity as an adaptation to accommodate the expanding requirement for triacylglycerol transport in vertebrates.

There are interesting parallels between the evolution of lipid transfer activities and the regulation of lipid biosynthesis. The major pathway that regulates cholesterol and unsaturated fatty acid biosynthesis in mammalian cells is the SREBP pathway, which involves proteolysis of membrane bound transcription factors (46). Seegmiller *et al.* (47) showed that the *Drosophila* genome encodes all the components of the SREBP pathway. However, in contrast to mammals, the *Drosophila* SREBP pathway regulates enzymes involved in the biosynthesis of saturated fatty acids and not sterols. In turn, SREBP function is regulated by phosphatidylethanolamine in *Drosophila* and not sterols (48). It has been suggested that the SREBP pathway evolved to maintain the phospholipid composition of membranes and subsequently acquired sterol regulatory function following gene duplication (47). Thus, both lipid transfer and regulatory mechanisms might have been first developed for phospholipids and later evolved to include other lipids.

Potent MTP antagonists that inhibit both triglyceride and phospholipid transfer activities of MTP and decrease apoB secretion have been identified (49). Most of these compounds have been associated with specific adverse effects, primarily related to hepatic lipid accumulation. Based on the studies presented here, it might be useful to identify compounds that selectively inhibit either of the two lipid transfer activities. Compounds that inhibit only phospholipid transfer and, hence, only the first step in apoB assembly may reduce the number of apoB precursor particles competent to acquire lipids without perturbing MTP-dependent flux of triacylglycerols into the secretory pathway. Such classes of inhibitors may reduce the number of apoB-containing lipoproteins produced by the liver with a minimal disruption in the net export of triacylglycerols. Indeed, antisense oligonucleotide-mediated reduction in apoB expression in mouse liver was recently shown to reduce plasma apoB and cholesterol, without causing liver steatosis (50). Compounds that only inhibit triacylglycerol transfer activity might allow synthesis and secretion of primordial particles with small amounts of neutral lipids and help avoid overt toxicities found associated with MTP antagonists that inhibit both the lipid transfer activities.

In summary, although hMTP can transfer triacylglycerols and cholesterol esters, this activity is absent in dMTP. However, the ability to transfer phospholipids is retained at comparable levels in both the dMTP and hMTP orthologs. Because dMTP can support human apoB secretion, we conclude that phospholipid transfer activity is sufficient to support primordial apoB assembly and to promote lipoprotein assembly in response to lipid availability. The robust neutral lipid transfer activity present in mammalian MTPs might have evolved to increase the efficacy of lipoprotein assembly and to aid in the bulk transport of neutral lipids.

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Acquisition of Triacylglycerol Transfer Activity by Microsomal Triglyceride Transfer Protein During Evolution

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Abbreviations used: ApoB, apolipoprotein B; BSA, bovine serum albumin; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase PCTP, phosphatidylcholine transfer protein; PITP, phosphatidylinositol transfer protein; FABP, fatty-acid binding protein

Abstract

Microsomal Triglyceride Transfer Protein (MTP) is critical for the intracellular assembly and secretion of apoB-lipoproteins. MTP transfers triacylglycerols, cholesteryl esters, and phospholipids between membrane vesicles in vitro. Previously we reported that the *Drosophila* MTP does not transfer neutral lipids but does transfer phospholipids. In this study we evaluate the MTP from different species and compare their protein structures and activities. While the primary structures of the MTP were considerably variable (13-99% amino acid identities when compared to human MTP), the secondary and tertiary structures were highly conserved. The vertebrate proteins shared considerable identity amongst their β^C domain as well as helices 4-6 and 15-17 in the α domain while similar conservation was not observed in the invertebrate MTPs. Activity assays demonstrated that invertebrate MTPs do not transfer triacylglycerols while all vertebrate MTPs contained this activity. From these studies we conclude that MTP triacylglycerol transfer activity was acquired prior to the emergence of vertebrates following the evolution of insects. This corresponds with a shift from relying on phospholipid-rich lipoproteins to the neutral lipid-rich apoB as the predominant carrier of extracellular lipids. Furthermore, the increased conservation in the β^C and helices 4-6 of the α domain may be responsible for the triacylgylcerol transfer activity in vertebrate MTPs.

Introduction

Distinct extracellular lipid transport systems that utilize lipoprotein vehicles evolved more than 900 million years ago. These include the vitellogenins of oviparous animals (1), apolipophorins circulating through the hemolymph of insects (2;3), and the apoB-lipoproteins secreted by vertebrate organisms (4;5). The assembly of apoB-lipoproteins occurs in the endoplasmic reticulum of mammalian liver and intestinal cells. Microsomal triglyceride transfer protein (MTP) is acknowledged to play a critical role during the assembly and secretion of these particles (6-9). In humans, the absence of MTP activity results in abetalipoproteinemia, a disease characterized by the deficiency of plasma apoB and severely reduced lipid levels (10). Besides its role in the assembly of apoB-lipoproteins, recent reports suggest that MTP may be vital to the assembly and secretion of vitellogenins as well as apolipophorin (11;12).

Wetterau and Zilversmit first identified MTP as a heterodimer of M and P subunits that transfers lipids in vitro (13;14). The 97 kDa M subunit is unique and responsible for the protein's lipid transfer activity while the 58 kDa P subunit is the ubiquitous chaperone protein disulfide isomerase (PDI). PDI is inactive in the complex and maintains the solubility of the M subunit (14;15). In vitro assays reveal that mammalian MTP transfers numerous lipids but prefers to use triacylglycerols, cholesteryl esters and phospholipids as substrates when this activity is measured in vitro (16). The MTP lipid transfer activity is essential for the formation of apoB-lipoproteins. Antagonists to this activity increase the intracellular degradation of apoB (17) and reduce the secretion of these particles both in cell culture as well as animal models (18;19).

Recently, we reported that the *Drosophila* MTP does not transfer neutral lipids, but transfers phospholipids in vitro (20;21). We proposed that phospholipid transfer was the most ancient activity associated with MTP, and that the neutral lipid transfer activity was acquired during evolution. In this study we have attempted to define when MTP acquired neutral lipid transfer activity, and whether this acquisition coincides with a change in the protein's structure.

Materials and Methods

Protein alignments and structural analysis: MTP amino acid sequences were acquired by executing an iterative protein-protein BLAST (22) against all non-redundant GenBank CDS translations + RefSeq Proteins + PDB + SwissProt + PIR + PRF protein databases using human MTP (CAA58142) as the query. Full-length proteins that produced significant alignments (E threshold value < 0.0) included: *Bos taurus* (bovine) (CAA55310), *Mus musculus* (mouse) (NP_032668), *Mesocricetus auratus* (hamster) (AAA53143), *Gallus gallus* (chicken) (XP_420662), *Canis familiaris* (dog) (XP_544995), *Sus scrofa* (pig) (NP_999350), *Rattus norvegicus* (rat) (XP_227765), *Pan troglodytes* (chimpanzee) (XP_526779), *Danio rerio* (zebrafish) (NP_998135), *Strongylocentrotus purpuratus* (sea urchin) (XP_788526), *Drosophila melanogaster* (fruit fly) (NP_610075), *Drosophila pseudoobscura* (fruit fly) (EAL33909), *Apis mellifera* (honeybee) (XP_623644), and *Anopholese gambiae* (mosquito) (EAA13951). An incomplete sequence (693 amino acids) for *Tetraodon nigroviridis* (pufferfish) (CAG03740) (E value = 0.0) was also acquired. The *Fugu rubripes* (pufferfish) MTP protein sequence has been reported (20). A partial *Oryzia latipes* (sea squirt) MTP was assembled using protein ESTs (accession numbers: BJ014235, BJ000420, BJ499123, and

BJ735768) accessible from UniGene (23;23). *Xenopus tropicalis* (frog) MTP was acquired from Xenbase (www.xenbase.org). The sequences for *C. elegans* (nematode) (AAR27937), and *C.briggsae* (nematode) MTP (CAE67922) were obtained using an internally conserved region within the M subunit of vertebrate and insect MTPs as the query. Protein alignments were performed using default settings and phylogenetic trees were generated using CLUSTAL W in Biology Workbench (24). Secondary and three dimensional protein structures were resolved using PELE (Biology Workbench) and PHYRE (Protein Homology Analogy Recognition Engine, Imperial College, London), respectively (www.sbg.bio.ic.ac.uk/~phyre). MTP structural domains (β^N, α, β^C and β^A) were determined by aligning each homolog to the corresponding human MTP amino acid sequence (7).

Expression plasmids: Expression vectors containing human MTP and *Drosophila* MTP, as well as apoB48, have bee described previously (20;21). For the expression of zebrafish and *C. elegans* MTPs, full-length cDNA clones were acquired (Open Biosystems and the National institute of Genetics, Mahima, Japan, respectively), amplified by PCR, and subcloned into the mammalian expression vector pCDNA3.1 (Invitrogen). Forward and reverse primers used for PCR amplification included 5'CGGGGTACCGACCCCAAACATGATGCCGG3' and 5'CGGGGTACCCCAGGCCGGCTCAAAGACCTTC3' as well as 5'CGGGGTACCACCAGAGATGTTCTCATCACG3' and 5'CGGGGTACCCAACTACAATCTAAACTGCTCC3' for zebrafish and *C. elegans* MTP, respectively. In order to generate MTP containing C-terminal FLAG epitope tags,

the 3' antisense primers were made to encode the FLAG sequence (DYKDDDDK) followed by an in frame termination codon.

Cell culture and analysis of apoB secretion: COS-7 cells were grown in DMEM (CellGrow), 10% fetal bovine serum supplemented with L-glutamine and antibiotic. Cells were plated in 6 well plates at a density of 400,000 cells per well 24 h prior to DNA transfections. DNA was introduced to cells using Polyfect reagent (Qiagen) according to the manufacturer's instructions. After 48 h, the media was aspirated and 1 ml of lipid-containing media (DMEM, 0.4 mM oleic acid: 1.5% BSA complex, and 1 mM glycerol) was added. Following additional 18 h incubation, the media was collected, protease inhibitor cocktail added (Sigma), and centrifuged (2,500 rpm, 4^{o}C) to pellet cell debris. The presence of apoB was measured using ELISA. Particle density was determined by subjecting conditioned media to a KBr gradient as previously described (21). Briefly, 4 ml of media was brought to 1.30 g/ml with KBr and overlaid with 2 ml each of 1.24, 1.15, and 1.063 g/ml KBr solutions, followed by 1 ml each of 1.019 and 1.006 g/ml. After ultracentrifugation, (SW41 rotor, 40,000 rpm, 17 h, 15^{o}C), 1 ml fractions were collected and apoB content determined. The density of each fraction was measured using a refractometer (Fisher Scientific).

Immunofluorescence: COS cells transfected with MTP-FLAG expression plasmids were grown on coverslips in 24-well tissue culture dishes. Forty-eight hours following transfection, the cells were fixed and permeabilized in methanol for 15 minutes at –20°C. Fixed cells were blocked with phosphate buffered saline containing 1 mM $MgCl_2$, 0.5

mM $CaCl_2$, 3% BSA, and 1% goat serum. Immunofluorescence was performed as previously described (21). Primary and secondary antibodies were diluted accordingly in the same buffer used for blocking: M2 anti-FLAG (Sigma) and anti-calnexin (Stressgen), 1:100; anti-mannosidase II (US Biological), 1:25; anti-PDI (Stressgen), 1:50, Alexa Fluor 488 and Alexa Fluor 594 antibodies (Molecular Probes), 1:100. The cover slips were mounted in phosphate buffered saline containing 10% glycerol and 12% triethyldiamine (Sigma) to prevent fluorescent bleaching and visualized using a Biorad Radiance 2000 confocal microscope.

Affinity purification of MTP-FLAG: The purification of MTP-FLAG chimeras from COS cell lysates that transiently expressed MTP was performed using M2-anti-FLAG agarose (Sigma) as described previously (21). Briefly, cell monolayers were washed with phosphate buffered saline (pH 7.4), incubated for 2 minutes in hypotonic buffer (1 mM Tris-Cl, pH 7.4, 1 mM $MgCl_2$, and 1 mM EGTA), scraped and passaged 20 times through a 25 Gauge needle. Homogenates were then centrifuged (50,000 rpm, SW55 rotor, 4^o C, 1 h) and the supernatant transferred to M2-agarose beads (Sigma). Following incubation (3-5 h at 4^oC), affinity-bound proteins were eluted with 250 ng / μl FLAG peptide (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, and protease inhibitors). M2 agarose beads were pelleted by centrifugation (10,000 rpm, 30 sec, 4^oC) and the supernatant was collected and stored at 4^oC. SDS-PAGE followed by western blotting using M2 anti-FLAG antibody (Sigma) was performed to determine the amounts of MTP-FLAG.

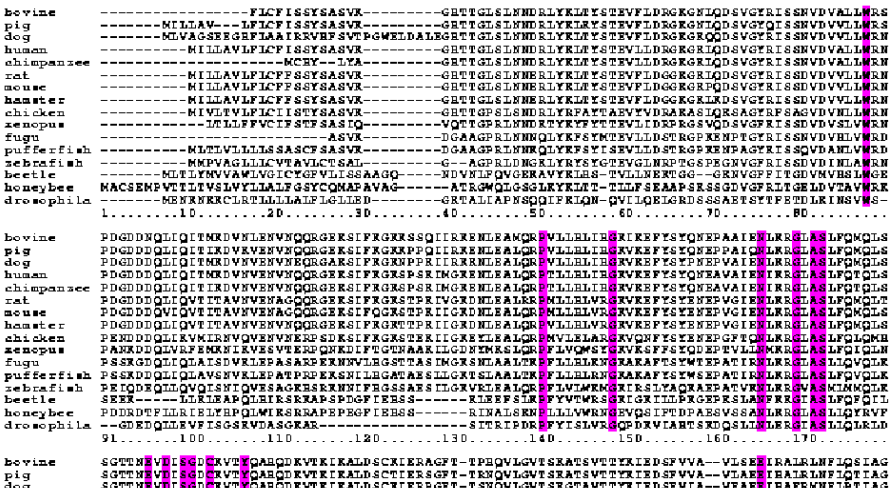
Measuring lipid transfer activity: MTP Lipid transfer activity was assayed using donor vesicles containing phosphatidylcholine and nitrobenzoaxadiazole-labeled triacylglycerols (Chylos Inc.) according to published protocols (25;26). Cell lysates were obtained by hypotonic lysis followed by high-speed centrifugation (SW55 rotor, 50,000 rpm, 4^{o} C, 1 h) to pellet cell debris. To prepare liver homogenates, 100-200 mg of tissues were rinsed with phosphate buffered saline and homogenized in hypotonic buffer containing protease inhibitor cocktail (Sigma) using a Polytron homogenizer (1 minute pulse, setting 5). The homogenates were subsequently first passed through a 20 Gauge needle followed by a 25 Gauge needle (10-15 times each) to lyse the cells. The lysates were centrifuged (SW55 rotor, 50,000 rpm, 1h, 4^{o}C), supernatants were collected, and protein concentrations were determined using Coomassie reagent. Equal amounts of soluble proteins were used to assay triacylglycerol transfer activity by MTP. To study the inhibition of triacylglycerol transfer activity, the antagonist CP-346086 (13) was added to the assay mixture to obtain the appropriate concentration prior to the addition of the MTP source. The final concentration of DMSO did not exceed 0.25%.

Results

Identifying MTP homologs: To identify known or predicted MTP homologs, we searched non-redundant protein databases against the human MTP sequence. Initially, 83 proteins were identified to share homology to human MTP. These included 16 MTP proteins from mammals, birds, fish and insects, as well as the previously reported homologous proteins apoB, apolipophorin, and vitellogenin (27). In addition 3 sequences from *F. rubripes, O. latipes, and X. tropicalis* were obtained as described in Materials

and Methods. The vertebrate and insect MTP sequences were subsequently aligned using CLUSTAL W, and two regions demonstrating significant conservation were recognized (Fig. 1 & 2). The first was located in the N-terminus of MTP (Fig. 1) (amino acids 141-214). This sequence is also present in apoB, vitellogenin, as well as apolipophorin (28), and is therefore not specific to MTP. A second region of conserved residues (Fig. 2) was located in MTP's C-terminus and corresponded to the amino acids 749-823. Unlike the N-terminal sequence, this region was only identified in MTP proteins and was not conserved in apoB, apolipophorin, or vitellogenin. We therefore concluded that the C-terminal sequence was exclusive to the MTP and might be evolutionarily maintained.

A.



B.

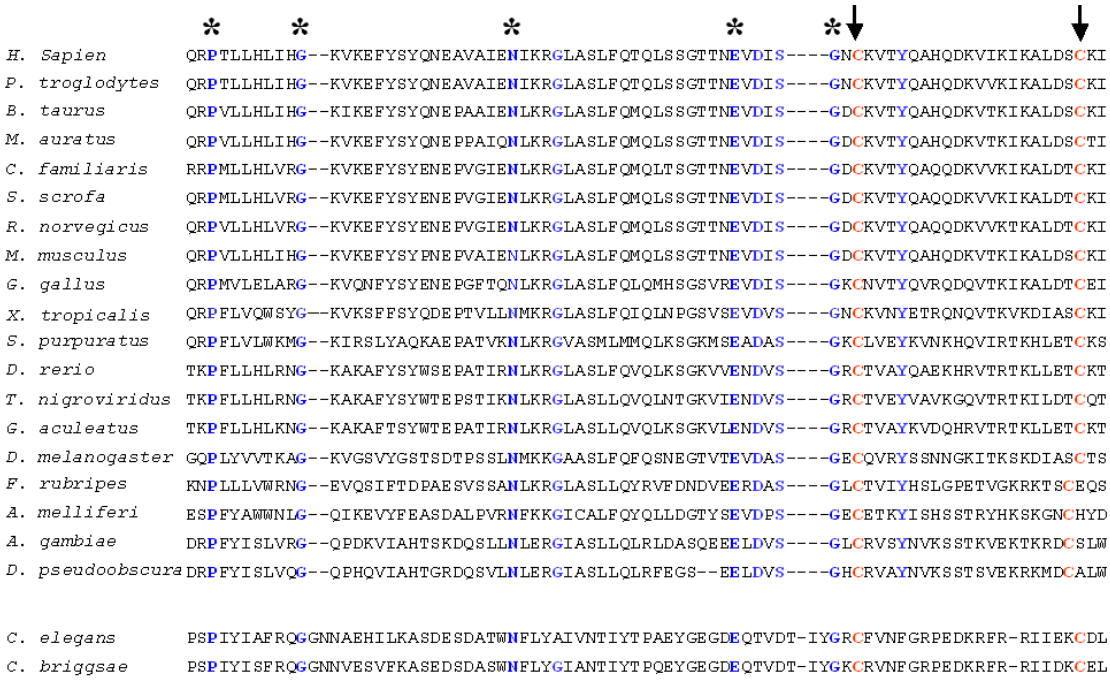
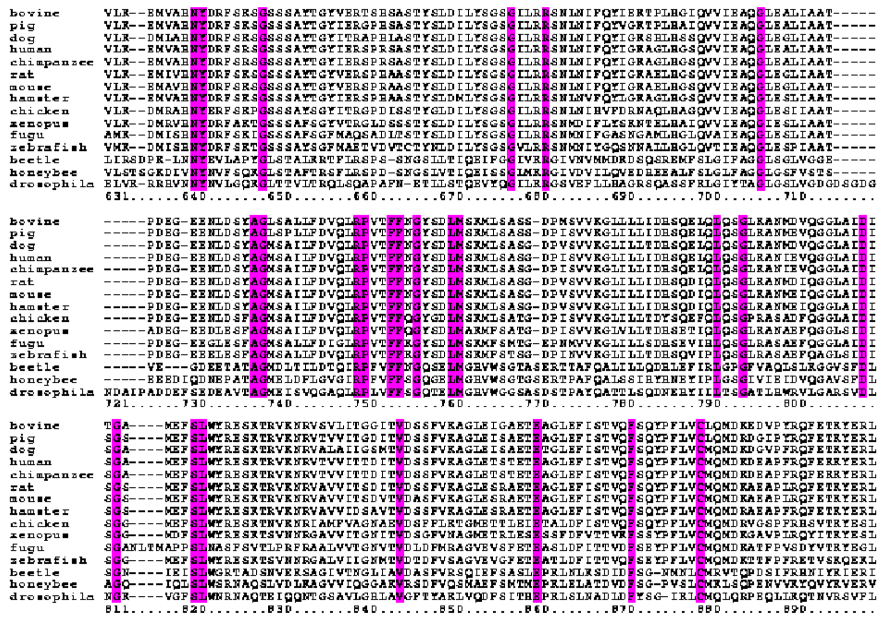


Figure 1. A conserved sequence located in the N-terminus of vertebrate, insect and nematode MTPs. The MTP sequences from vertebrates (*H. sapiens (human), P. troglodytes (chimpanzee), B. taurus (bovine), S. scrofa (pig), C. familiaris (dog), R. norvegicus (rat), M. musculus (mouse), M. auratus (hamster), G. gallus (chicken), D. rerio (zebrafish), F. rubripes (fugu), X. tropicalis (xenopus)* and *T. nigroviridis (pufferfish)*), sea urchin (*S. purpuratus*) and insects (*D. melanogaster (drosophila), T. Castaneum (beetle)* and *A. melliferi (honeybee)*) were aligned using CLUSTAL W (**A.**). Identical amino acids are colored magenta. The amino acid number is depicted as the ruler located beneath each set of sequences. A region consisting of amino acids 141-214 was more conserved than the remainder of the protein domain. The N-terminal 300 amino acids from the MTP of vertebrates, insects, and nematodes were then individually aligned to the human MTP protein sequence. The residues corresponding to the conserved N-terminal sequence for each protein are shown (**B.**). Asterisks indicate totally conserved amino acids. Arrows correspond to the conserved cysteine residues that are also present in vitellogenin, apolipophorin as well as apoB and generate a disulfide linkage in lipovitellin (28).

A.



B.

```
                        * *   *                                *     * **          *                 **
H. sapien          QLRPVTFFNGYSDLMSKMLS-ASGDPISVVKGLILLIDHSQELQLQSGLKANIEVQGGLAIDISG----AMEFSLWYR
P. troglodytes     QLRPVTFFNGYSDLMSKMLS-ASGDPISVVKGLILLIDHSQELQLQSGLKANIEVQGGLAIDISG----AMEFSLWYR
B. taurus          QLRPVTFFNGYSDLMSKMLS-ASSDPMSVVKGLLLLIDHSQELQLQSGLKANMDVQGGLAIDITG----AMEFSLWYR
M. auratus         QLRPVTFFNGYSDLMSKMLS-ASGDPVSVVKGLILLIDHSQDIQLQSGLKANMEIQGGLAIDISG----SMEFSLWYR
C. familiaris      QLRPVTFFNGYSDLMSKMLS-ASGDPVSVVKGLILLTDHSQELQLQSGLKANMDVQGGLAIDISG----AMEFSLWYR
S. scrofa          QLRPVTFFNGYSDLMSKMLS-ASSDPISVVKGLILLIDHSQELQLQSGLKANMEVQGGLAIDISG----SMEFSLWYR
R. norvegicus      QLRPVTFFNGYSDLMSKMLS-ASGDPVSVVKGLILLIDHSQDIQLQSGLKANMDIQGGLAIDISG----SMEFSLWYR
M. musculus        QLRPVTFFNGYSDLMSKMLS-ASGDPVSVVKGLILLIDHSQDIQLQSGLKANMEIQGGLAIDISG----SMEFSLWYR
G. gallus          QLRPVTFFQGYGDLMSKMLS-ATGDPISVVKGLILLTDYSQEFQLQSGPRASADFQGGLAIDISG----GMEFSLWYR
X. tropicalis      QLRPVTFFQGYSDLMAKMFS-ATGDPISVVKGLVLLTDHSETIQLQSGLKANAEFQGGLSIDISG----GMDFSLWYR
D. rerio           QLRPVTFFKGYSDLMSKMFSTSGDPIN-VVKGLILLTDHSQVIPLQSGLRASAEFQAGLSIDISG----GMEFSLWYR
F. rubripes        GLRPVTFFKGYSDLMSKMFSMTGEPNM-VVKGLILLSDHSEVIHLQSGLRASAEFQGGLAIDISGANLTMAPPSLNAS
A. melliferi       GIRPFVFFSGQGELMGHVWSGTGSERTPAFQALSSIHYHNEYIPLGSGIVIEIDVQGAVSFDLAG----QIQLSLWSR
A. gambiae         VMRPLEFFNGKGELMGHVWSGTASEPTPAYQAITLLQDNEERFASHNGVTLALSSTGAISIDLNG----QVTMSLWGR
D. pseudoobscura   QLRPLIFFSGQTELMGHVWGGTASDSTPAYQATTLAQDHEHYIVLASGASLHWRVLGARSVDLNG----KVGFSLWNR
D. melanogaster    QLRPLVFFSGQTELMGHVWGGSASDSTPAYQATTLSQDNEHYIILTSGATLHWRVLGARSVDLNG----KVGFSLWNR

C. elegans         KLPTHHIFKGSTDLLSTVWEADG-RTHKAFEGHVPVRDVRLSVPLLSGLTLDVDSVGAISMRVLA----SAEVSLWNQ
C. briggsae        KLPTNHIFKGSTDLLSTVWDADG-RTHKVFEGHIPVRNTRVSVPLLSGLTVDVDSIGAITLRVLA----SAEVSLWNQ
```

Figure 2. A conserved and specific sequence identified in the C-terminus of vertebrate, insect and nematode MTPs. MTP sequences from vertebrates and insects were aligned using CLUSTAL W as described in Figure 1 (**A.**). The ruler located beneath each set of sequences depicts the numbering of amino acids. Identical residues are colored magenta. A semi-conserved sequence was recognized to contain amino acids

749-823 in the vertebrate and insect MTPs. The terminal 300 amino acids belonging to vertebrate, insect, and nematode MTPs were individually aligned to human MTP and the conserved sequence present among vertebrate and insect MTPs (**A.**) is shown (**B.**). An asterisk indicates identical residues.

It was considered that additional MTP proteins possessing less homology would not meet the required threshold value to be detected when using the full-length human MTP as the query sequence. To improve the sensitivity of our analysis, we then searched for proteins containing the C-terminal conserved sequence identified from the above alignments. In contrast to the 83 proteins recognized by the full-length human MTP, a total of 27 sequences were found to share homology to the C-terminal sequence. Of these 18 were independent MTP proteins, while the others represented redundant sequences. The homologous proteins included each of the MTP obtained during the original search. In addition, two new sequences from the nematode *Caenorhabditis* were identified. The first was previously described as the *dsc-4* gene product in *C. elegans* (29), while the second corresponded to a hypothetical protein from *C. briggsae*. In total, 21 protein sequences (Table 1) were revealed to share homology with human MTP. Each of the proteins was established to represent an MTP homolog based upon sequence length as well as the presence of both the N-terminal and C-terminal conserved sequences (Fig. 1 & 2).

MTP Homolog	Amino acids	Identity (%)	Similarity (%)
H. sapien	894	100	100
P. troglodytes #	881	98	99
S. scrofa	894	89	93
B. taurus	887	87	93
R. norvegicus	896	86	92
M. musculus	894	86	92
M. auratus	895	86	92
C. familiaris #	905	85	92
G. gallus	893	67	81
X. tropicalis $	889	62	n.d.
O. latipes *	554	58	n.d.
F. rubripes	870	56	n.d.
D. rerio	884	54	72
T. nigroviridus # *	693	52	69
S. purpuratus #	1066	25	47
A. melliferi #	894	23	47
D. melanogaster	886	20	45
D. pseudoobscura	889	19	44
A. gambiae *	776	19	47
C. briggsae #	888	15	n.d.
C. elegans	892	13	n.d.

Table 1. MTP homologs. Proteins were identified as homologs to human MTP (criteria included similar number of amino acids as well as containing both the N- and C-terminal conserved regions shown in Fig. 1 and Fig 2). The sequences were subsequently aligned and their identities (%) and similarities (%) compared to human MTP were determined. ($ = sequence that was acquired from Xenbase; # = predicted protein sequence; * = partial amino acid sequence; n.d.= not determined).

Analysis of MTP primary and secondary structures: To further characterize the different MTPs, we performed multiple sequence alignments to determine their overall conservation. The identity of each homolog compared to human MTP was established (Table 1). The vertebrate MTPs exhibited greater than 50% identity and 70% homology, while the identities calculated for the invertebrate MTPs, insects and nematodes, were calculated to be ~20% and 13%, respectively. Human MTP is predicted to contain a well-defined arrangement of secondary structures that includes an N-terminus of

primarily β-sheets, a central region that consists of multiple helices, and C-terminal β-sheets (7;28;30). We next determined whether this secondary structure was conserved in each homolog. The predicted secondary structures were surprisingly similar when comparing the individual proteins (Fig. 3). Both the N- and C-termini consisted of predominantly β-structure, while the ~300 amino acids separating these two regions demonstrated substantial helical content. Thus, while the MTP did not demonstrate considerable conservation in their primary sequences of amino acids, the β-α-β secondary structure was preserved in all homologs.

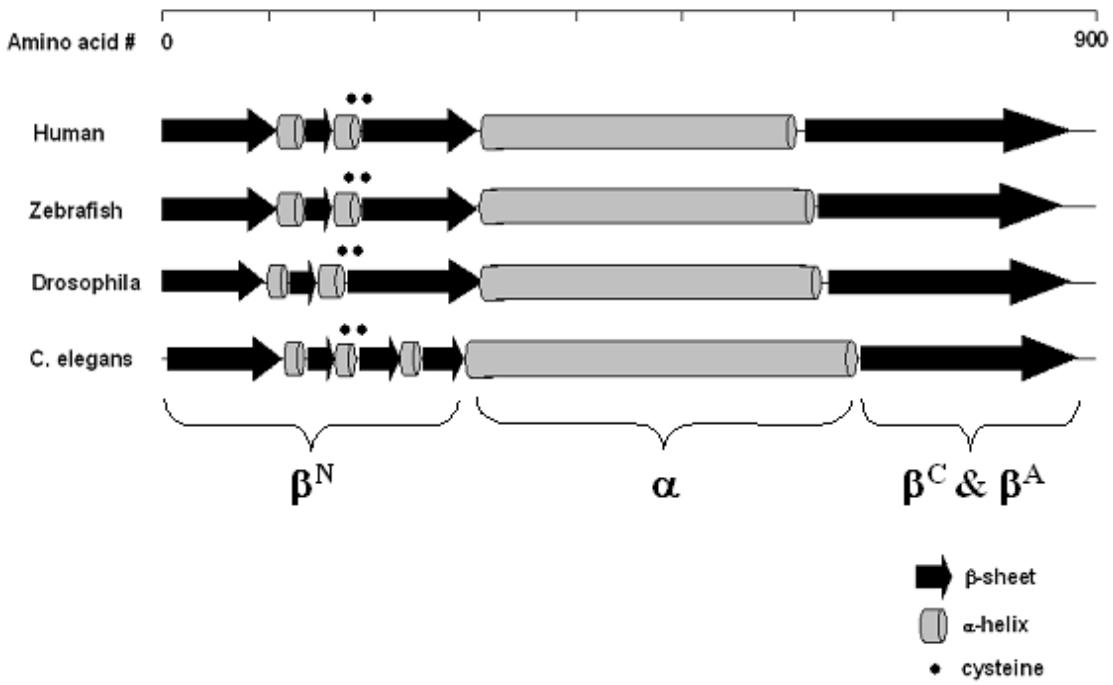


Figure 3. The secondary structure is conserved among the MTP homologs. The secondary structures of each MTP homolog were predicted using the PELE Protein Structure Prediction algorithm (Biology Workbench) and the PHYRE protein structure prediction program (www.sbg.bio.ic.ac.uk/~phyre). The composition of α-helices and β-strands in human, zebrafish, *Drosophila*, and *C. elegans* MTPs are shown. Arrows depict β-strands while cylinders represent α-helices. Each MTP was found to contain a β-α-β structure. The position of the conserved cysteine residues located in the N-terminus of each MTP is also illustrated. Amino acids that generate the 4 structural domains in

human MTP are represented (β^N = N-terminal β-barrel, α = α helical domain, β^C = C-terminal β-sheet1, β^A = C-terminal β-sheet2).

Since the secondary structures were highly conserved between the MTP, we decided to evaluate whether their tertiary structures were also conserved. Protein structures were predicted using PHYRE (Protein Homology / analogY Recognition Engine) (www.sbg.bio.ic.ac.uk/~phyre). The MTP demonstrated four well-defined structural domains (Fig. 4), an N-terminal β-barrel (β^N), helical domain (α), and two C-terminal β-sheets (β^C and β^A). These are consistent with those previously reported for human MTP (7;28;30). These results reveal that the secondary and tertiary structures of the MTP proteins are more conserved than the primary sequences of amino acids.

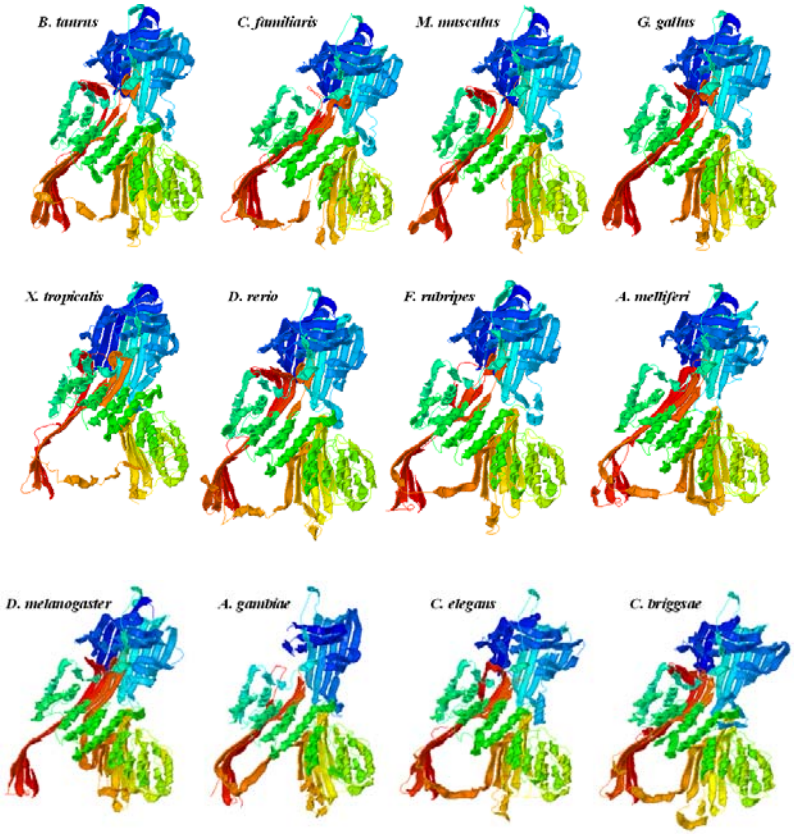


Figure 4. MTP homologs consist of 4 predicted structural domains. The tertiary structure for each MTP homolog was predicted using PHYRE (Protein Homology/analogy Recognition Engine) (www.sbg.bio.ic.ac.uk/~phyre). Four structural domains consistent with those reported for the human MTP were recognized (7;28;30),. These included an N-terminal β-barrel (β^N) (blue), helical domain (α) (green), and two C-terminal β-sheets (β^C and β^A, orange/yellow and red, respectively). The C-terminus of all MTP contained a cavity created by the β^C and β^A domains.

Comparison of structural domains: In our attempts to better understand MTP structure and uncover changes that occurred during its evolution we compared the primary sequence of each domain to the analogous domain in human MTP (i.e. zebrafish β^N domain versus human β^N domain). Human MTP was first separated into its four domains according to the structure reported by Hussain et al. (7). The sequences representing the β^N, α, as well as the β^C and β^A domains in human MTP were then aligned with the full-length proteins from MTP homologs to identify homologous domains. Subsequently, the identities between these domains were deduced (Table 2). We found that the conservation between the individual domains decreased progressing from humans to other mammalian species, fish, insects, and nematodes (moving vertically through the table) and was a similar pattern to that observed for the full-length proteins. Therefore, greater substitution occurred within the individual domains as the evolutionary distance between the MTPs increased. However, when we compared the identity between the domains belonging to a specific homolog and those of human MTP (moving horizontally through the table), we noticed differences between vertebrate and invertebrate MTPs. First, the β^N was less conserved than either the α or β^C and β^A domains in vertebrate MTPs. That is, while the β^N of zebrafish MTP was 40% identical to the β^N in human MTP, the α domain and C-terminal β-sheet domains (β^C and β^A) demonstrated 59% and

65% identity, respectively. A similar pattern was noted for *G. gallus, X. tropicalis, and F. rubripes* MTP. The identity between the β^N and α domains, as well as the β^N and β^C and β^A domains, increased by an average of 14% and 19% in vertebrate MTP, respectively. In contrast, the amino acid identity did not increase amongst the same domains in *Drosophila* MTP and *C. elegans* MTP compared to human MTP. This was characteristic of all invertebrate MTPs studied (data not shown).

Species	Homology (Identity / Similarity (%))			
	overall	β^N	α	β^C & β^A
H. sapiens	100	100/100	100/100	100/100
M. auratus	86	84/90	86/93	90/96
G. gallus	67	60/76	73/86	76/82
X. tropicalis	62	52	66	71
D. rerio	55	40/62	59/77	65/80
F. rubripes	56	49	59	66
D. melanogaster	22	20	24	21
C. elegans	13	14	14	14

Table 2. Comparison of the amino acid conservation determined for the structural domains of MTP. MTP homologs were divided into the four structural domains, N-terminal β-barrel (β^N), α-helical (α), and the two C-terminal β-sheets (β^C and β^A) as described in Materials and Methods and according to Hussain et al.(7). Each domain was subsequently aligned to the analogous sequence in human MTP and the identity was determined.

Our previous analysis suggested that the α and the β^C and β^A domains of vertebrate MTPs were more conserved than the β^N domains. In order to evaluate whether conserved amino acids were restricted to specific subdomains we performed multiple

sequence alignments using the vertebrate homologs. Comparing 13 vertebrate MTP sequences, we found that only 21% of the amino acids were identical in the β^N domain (Fig. 5). In contrast to the β^N domain, 36% of the amino acids were identical in the α domains (Figure 6). Upon further inspection, greater homology was identified in specific helices. We found that the amino acids analogous to those generating helices 4-6 and 15-17 in human MTP were more conserved than the other helices within the α domain. Helices 4-6 and 15-17 contained 54% and 56% identity, respectively while the remainder of the α domain exhibited 27% identity, similar to the value calculated for the β^N domain (21% identity). We then studied the C-terminus (β^C and β^A domains) of vertebrate MTPs (Fig. 7). Although 50% of the residues were identical, the amino acids composing the β^C domain (amino acids 625-721) (57% identity) were more conserved than the β^A domain (amino acids 751-833) (35% identity). These studies show that amino acid substitutions were not acquired and distributed evenly in the vertebrate MTP homologs. The β^C domains, as well as the helices 4-6 and 15-17, are more conserved than the β^N, α, and β^A domains.

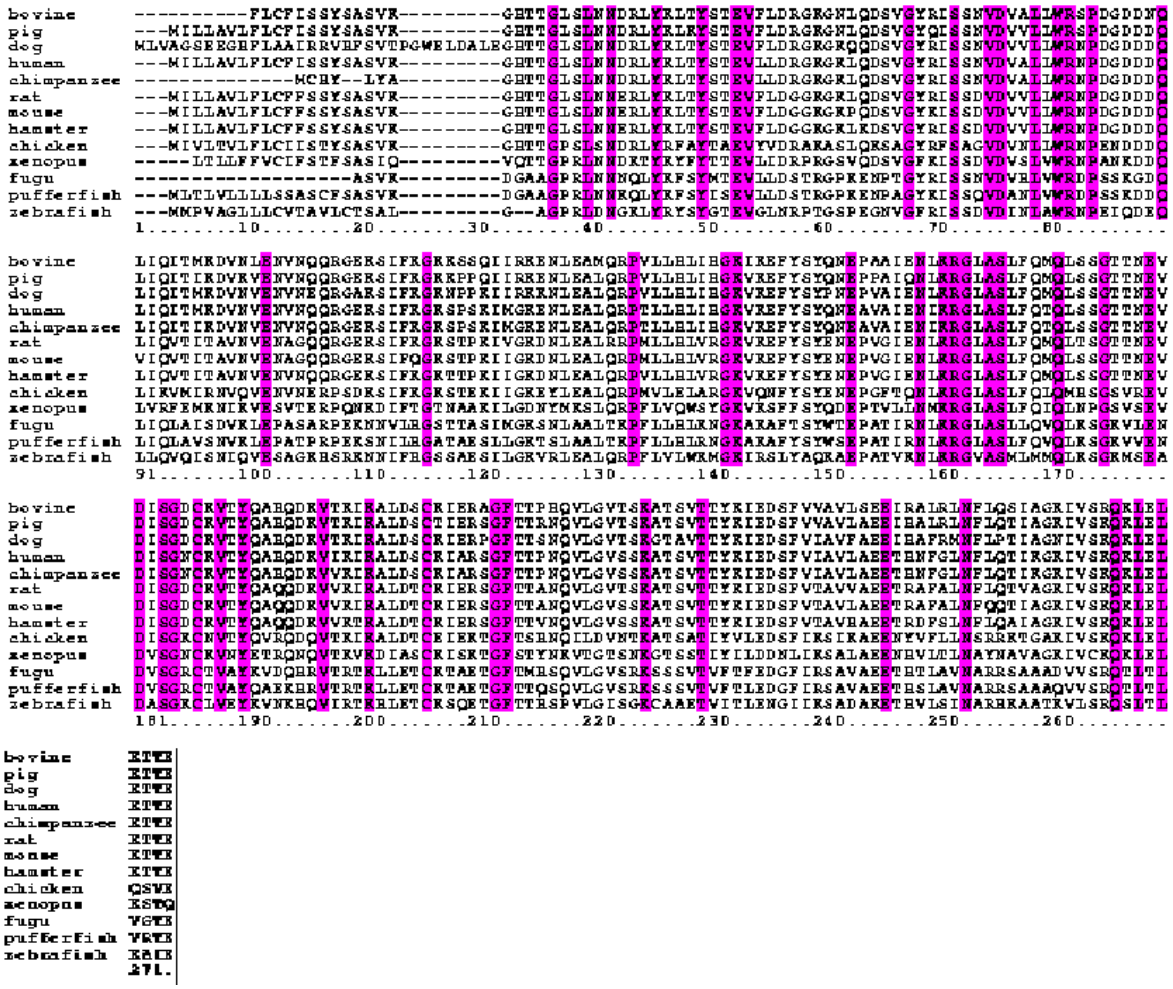


Figure 5. Multiple sequence alignment of the β^N domain of vertebrate MTPs. The amino acids corresponding to the β^N domains of each vertebrate MTP (*H. sapiens (human), P. troglodytes (chimpanzee), C. familiaris (dog), B. taurus (bovine), S. scrofa (pig), M. musculus (mouse), R. norvegicus (rat), M. aureatus (hamster), G. gallus (chicken), X. tropicalis (xenopus), F. rubripes (fugu), D. rerio (zebrafish),* and *T. nigroviridis (pufferfish)*) were identified and aligned as described in Materials and Methods. Identical amino acids are colored magenta. The overall identity was calculated to be 21%. A ruler notes the number of amino acids.

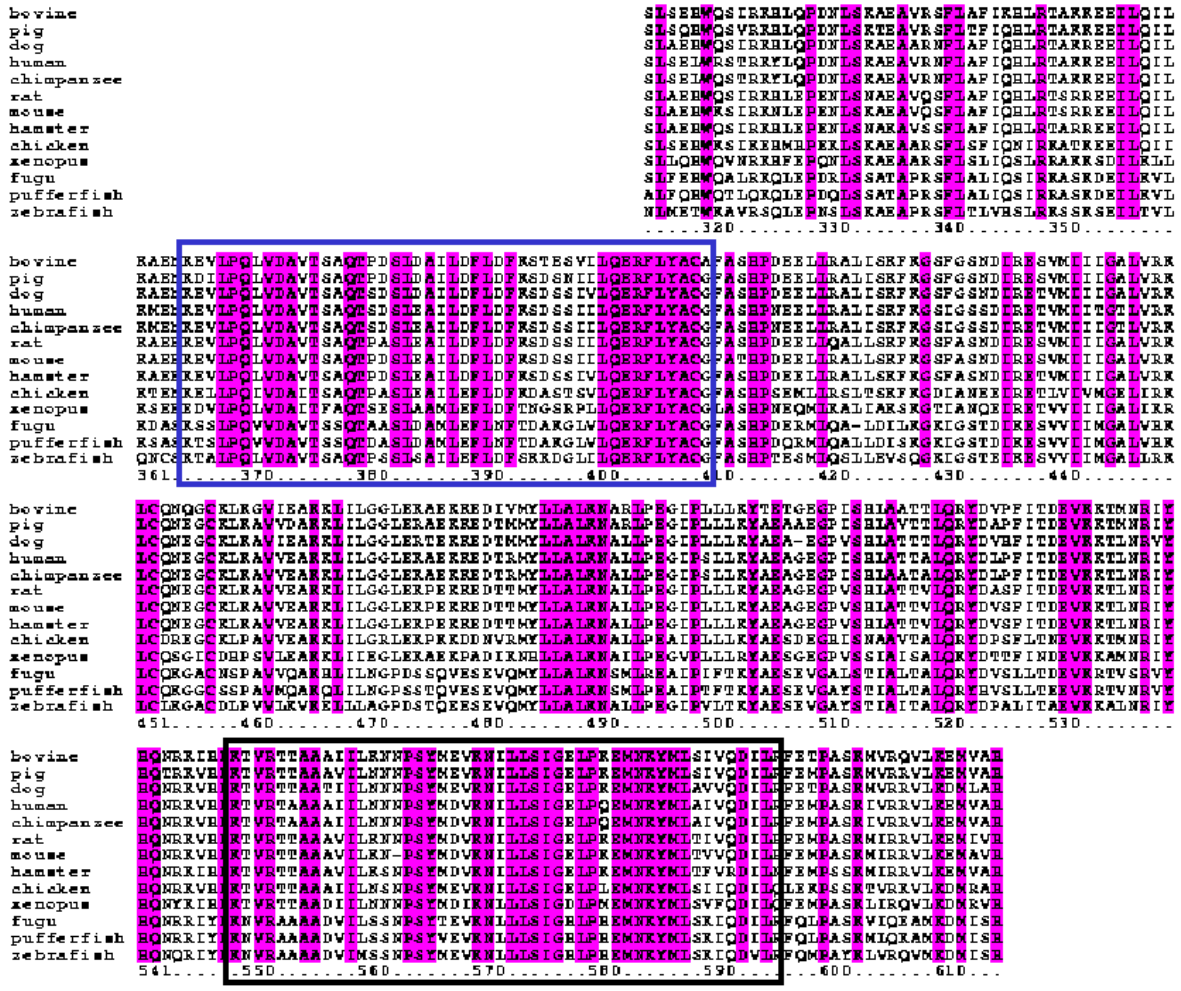


Figure 6. Alignment of the α domains present in the MTP of vertebrates. The α domains of the vertebrate MTPs were identified as described. A multiple sequence alignment was subsequently performed using CLUSTAL W. Identical residues are colored magenta and the overall identity was determined to be 36%. The amino acids corresponding to helices 4-6 (blue box) and 15-17 (black box) were more conserved, 54% and 56% identity, respectively compared to the remainder of the α domain which exhibited only 27% identical residues. The ruler beneath each set of sequences represents the amino acid number.

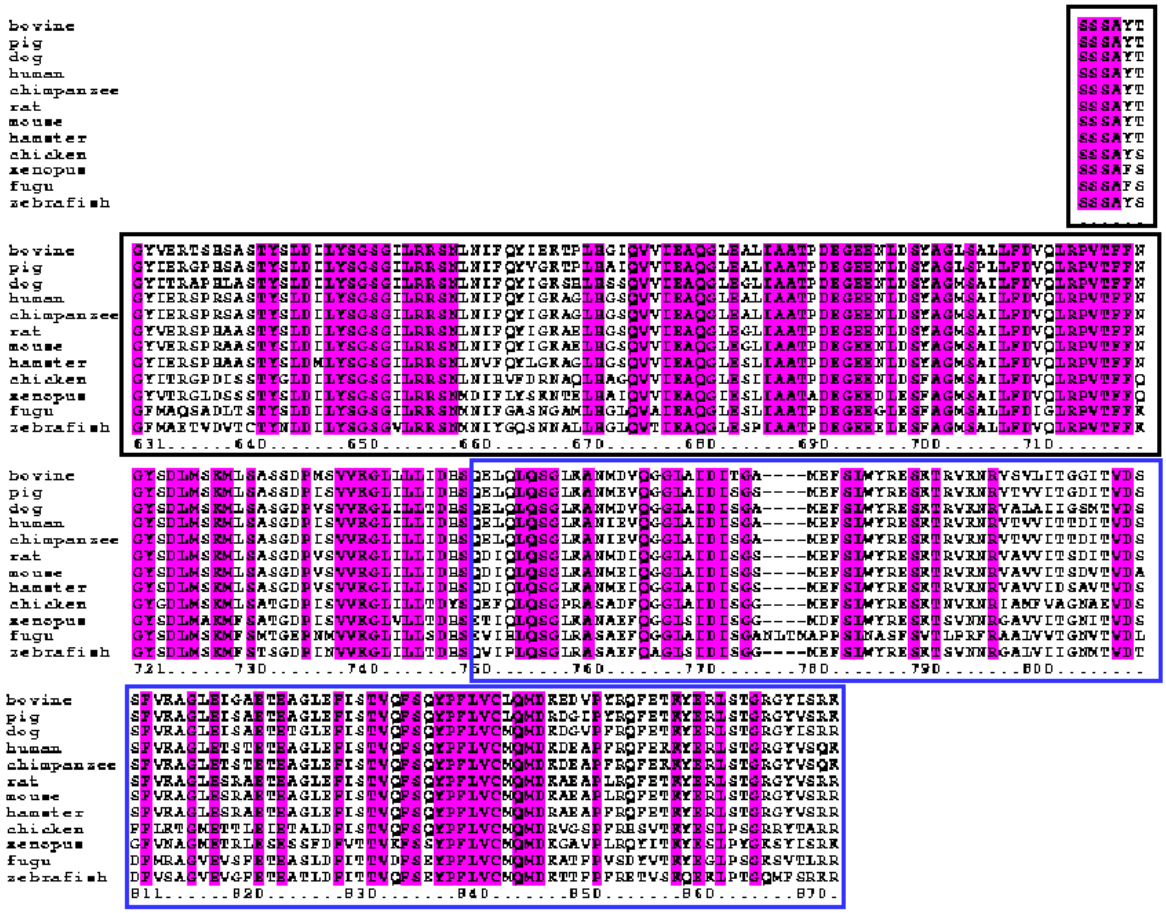
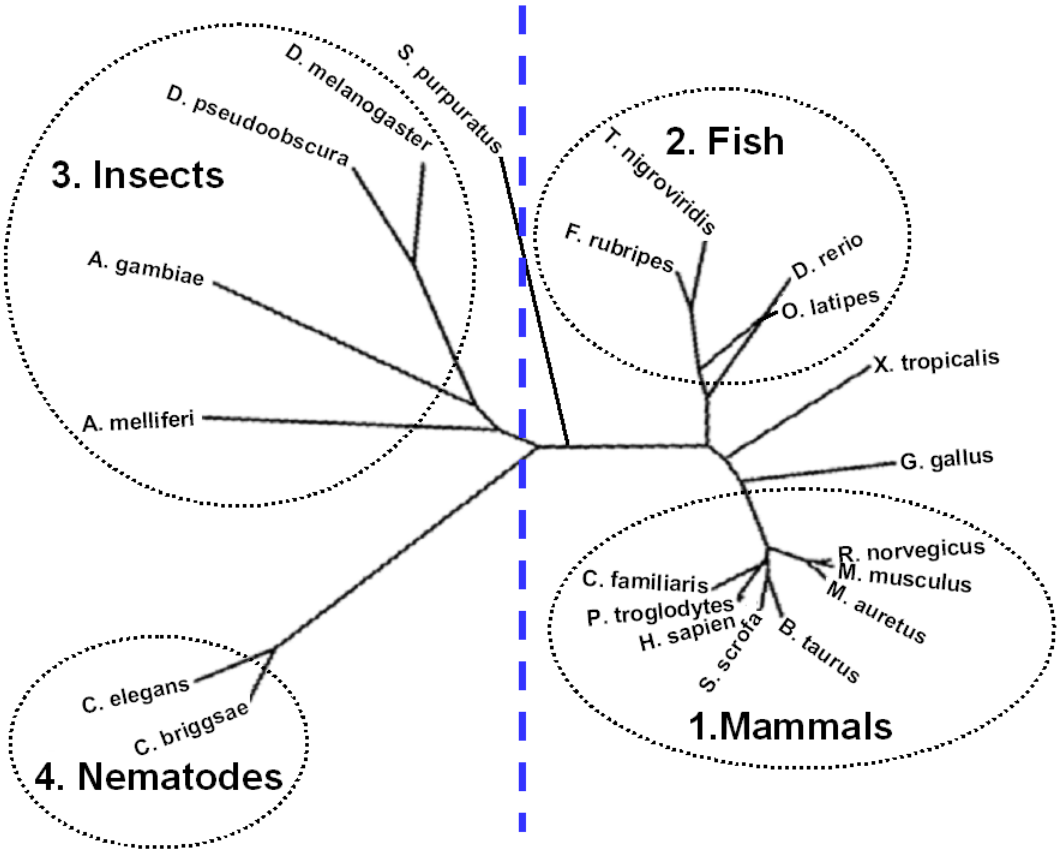


Figure 7. Sequence alignment of the vertebrate MTPs β^C and β^A domains. The C-terminal β^C and β^A domains were identified for each vertebrate MTP, and a multiple sequence alignment was subsequently performed using CLUSTAL W. 50% of the amino acids were conserved (magenta) within the MTP C-terminus compared to only 21% of those in the β^N and 36% of the α domains (Fig. 5 & 6). The sequences responsible for generating β^C (black box) and the β^A (blue box) are shown. β^C was calculated to contain 57% identity while the amino acids in β^A were only 35% identical.

Phylogenetic comparison of MTP homologs: Our studies show that the MTP from nematodes to humans are structurally conserved, but they contain a high degree of dissimilarity when comparing their amino acid sequences. In an attempt to better understand the evolutionary relationship between the MTPs we subsequently generated a phylogenetic tree using the MTP protein sequences (Fig. 8). The MTP segregated into vertebrate and invertebrate clusters that could be further divided into four orthologous

groups. These corresponded to mammals (group 1), fish (group 2), insects (group 3), and nematodes (group 4) (panel A). Each group is predicted to have diverged from a common ancestor at specific times during evolution and to have subsequently co-evolved with a specific lipoprotein (panel B). It has been proposed that MTP is required for the secretion of these lipoproteins (11;12). Although apoB, vitellogenin, and apolipophorin share similarity (27) the proteins are quite distinct from each other in the amount and types of lipids that they transport (1;2;7;31), as well as how they deliver these lipids to tissues. We therefore considered that the MTP belonging to the different groups would have evolved specific activities required for the assembly and secretion of its associated lipoprotein.

A. **Invertebrates** **Vertebrates**



B.

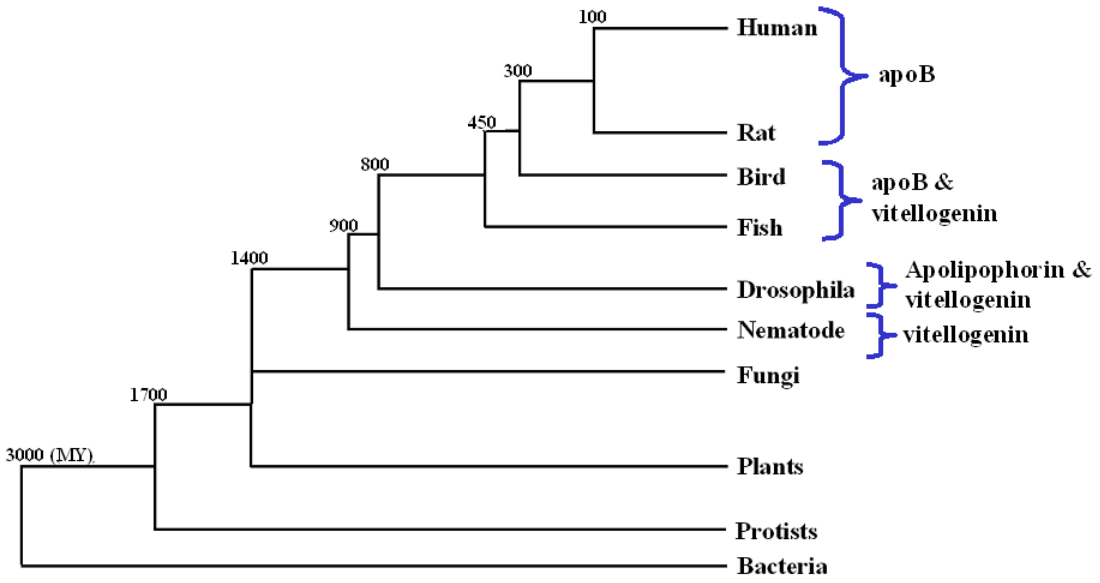
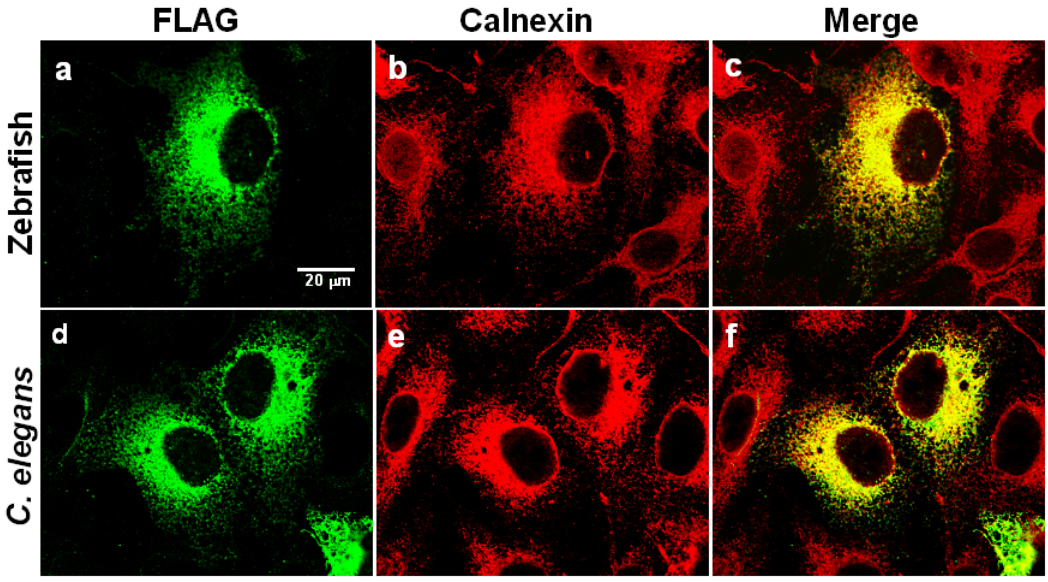


Figure 8. Phylogram of the MTP proteins. Full and partial MTP sequences were used to perform a phylogenetic comparison. MTP protein sequences acquired from BLAST analysis and databank searches (Table 1) were used to generate an unrooted tree (**A.**) using the CLUSTAL W and DRAWTREE programs in Biology Workbench. Sequences belonging to mammals (group 1), fish (group 2), insects (group 3), and nematodes (group 4) are shown. A tree diagram (**B**.) illustrates the evolution of bacteria, yeast, fungi, nematodes, insects, fish, birds, and mammals. The preferred lipoprotein vehicle (apoB, apolipophorin or vitellogenin) utilized as the primary carrier of extracellular triacylglycerols is labeled. The numbers located at breakpoints in the tree indicate the approximate time when a predicted divergence occurred (MY=millions of years ago).

Characterization of zebrafish and C. elegans MTPs: Our alignment data suggests that four orthologous groups that include mammals, fish, insects and nematodes express MTP (Fig. 8). We previously reported that *Drosophila* MTP (group 3), like its human homolog (group 1), was a soluble protein consisting of M and P subunits, and was localized to the endoplasmic reticulum and Golgi apparatus of COS cells (21). Here we investigated, the expression pattern of both zebrafish (Group 2) and *C. elegans* (Group 4) MTPs using indirect immunofluorescence microscopy (Fig. 9). The MTP of *zebrafish* and *C. elegans* were cloned, expressed as FLAG-tagged proteins in COS cells, and

immunofluorescence was performed as described in the Methods. A reticular fluorescent pattern was detected for both zebrafish and *C. elegans* MTPs (green) (Fig. 9A; a, d). Calnexin staining (red) was used to illustrate the location of the endoplasmic reticulum (Fig. 9A; panels b, e). Upon merging the images, overlap (yellow) between the MTP and calnexin signals was observed (Fig. 9A; c, f). The position of the Golgi apparatus was revealed using an antibody to α-mannosidase II (Fig. 9B; panels b, e). Again, the MTP signals (Fig. 9B; a, d) and those depicting the Golgi marker exhibited overlap in the merged images (Fig. 9B; c, f). These results demonstrate that both zebrafish and *C. elegans* MTP are expressed in COS cells and reside within the endoplasmic reticulum and Golgi apparatus.

A.



B.

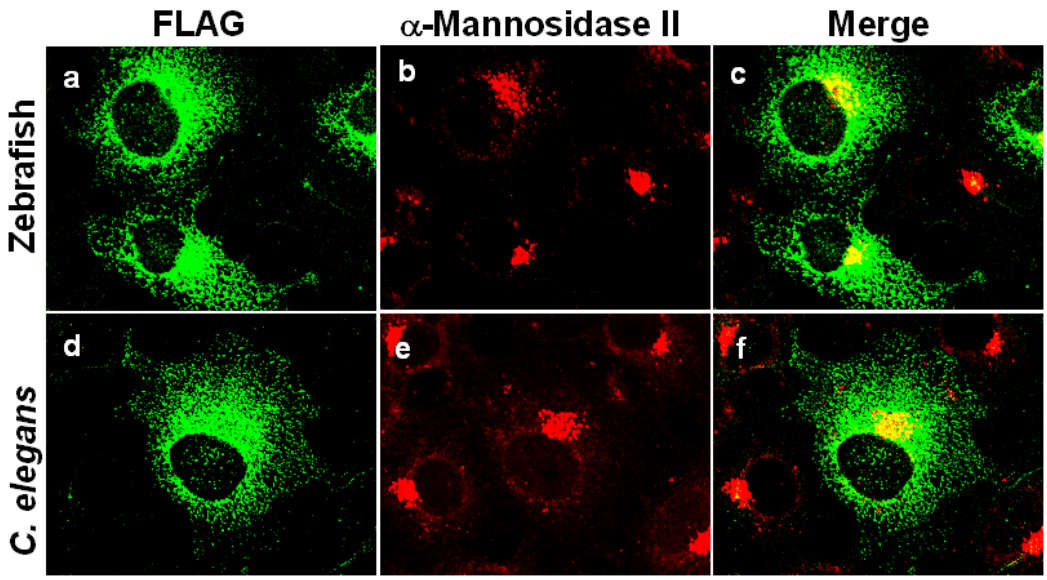
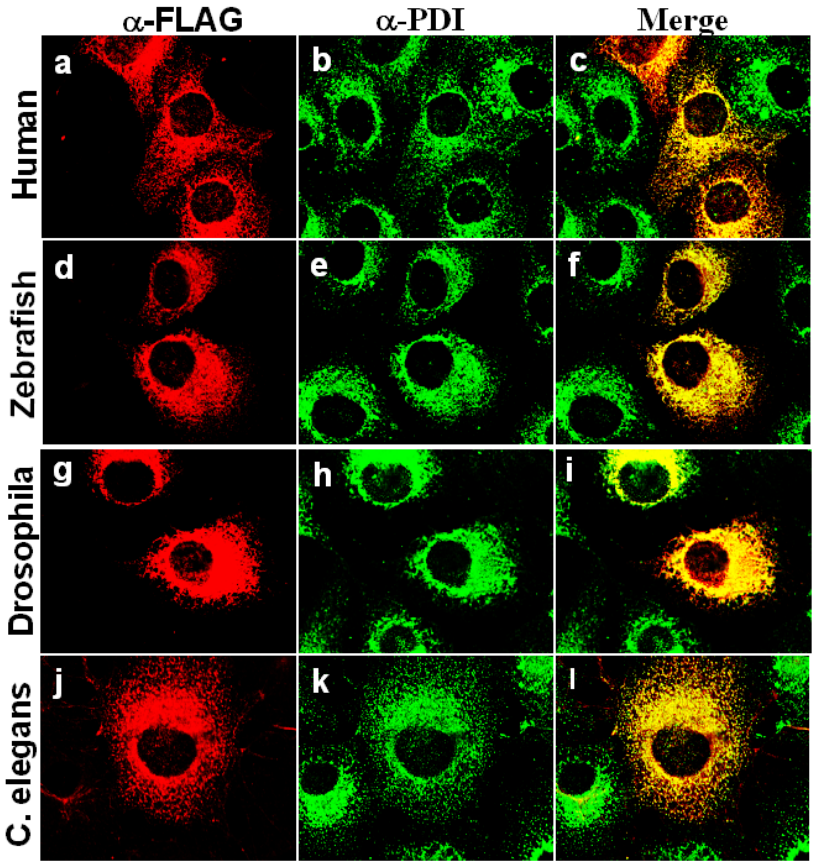


Figure 9. The expression, as well as subcellular localization, of zebrafish and *C. elegans* MTPs. COS cells transiently expressing either zebrafish or *C. elegans* MTP containing C-terminal FLAG epitope tags were grown on coverslips in 24-well dishes. After 48 h, the cells were fixed and treated as described in Materials and Methods. (**A.)** Cells were stained with M2 anti-FLAG (a, d) to label MTP and anti-calnexin (b, e) to illustrate the endoplasmic reticulum. Co-localization of MTP and calnexin is shown in the merged images (c, f) as a yellow color. (**B.**) Cells were incubated with M2 anti-FLAG (a, d) and α-mannosidase II (b, e) to demonstrate the position of the Golgi apparatus. Merged images (c, f) illustrate MTP co-localization with the Golgi.

MTP is known to form a stable and soluble complex with protein disulfide isomerase (PDI) (14;15). We similarly studied the intracellular distribution of zebrafish and *C. elegans* MTPs and endogenous PDI in COS cells (Fig. 10, panel A). For these studies, the human and *Drosophila* MTP were also evaluated. MTP that contained FLAG epitope tags (Fig. 10A; a, d, g, j) as well as PDI (Fig. 10A; b, e, h, k) exhibited punctate staining patterns. Overlap between the signals is indicated as a yellow color in the merged images (Fig. 10A; c, f, i, l). These studies show extensive co-localization of MTP and PDI. It has been shown that MTP requires the association with PDI to maintain

its solubility and activity (15). To determine whether the zebrafish and *C. elegans* MTP were soluble and also associated with PDI COS cells transiently expressing MTP-FLAG chimeras were hypotonically lysed, centrifuged, and the supernatants were incubated with M2 anti-FLAG agarose beads. Affinity bound proteins were competitively eluted by the addition of FLAG peptide and examined using SDS-PAGE followed by western blotting (Figure 10, panel B). M2 anti-FLAG antibody recognized a band migrating at ~100 kDa while the α-PDI antibody revealed a lower molecular weight protein that migrated at ~55 kDa. Therefore, all of the purified homologs consisted of both an M subunit and P subunit, and formed a heterodimeric complex with PDI similar to that of human MTP

A.



B.

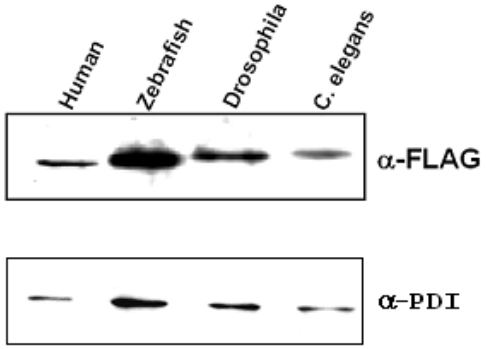


Figure 10. Zebrafish and *C. elegans* MTPs co-localize, and form a complex with protein disulfide isomerase. COS cells transiently expressing either human (a, b, c), zebrafish (d, e, f), *Drosophila* (g, h, i), or *C. elegans* (j, k, l) MTP containing a C-terminal FLAG epitope tag were grown on coverslips in 24 well dishes. After 48 h, cells were fixed and treated as before. (**A.**) M2 anti-FLAG was used to demonstrate MTP (a, d, g, j) while protein disulfide isomerase was stained with α-PDI (b, e, h, k). Merged images (c, f, i, l) reveal the co-localization of MTP and PDI as a yellow color. (**B.**) Homogenates from COS cells transiently expressing human, zebrafish, *Drosophila*, and *C. elegans* MTP were prepared as described in Materials and Methods. Lysates were incubated with M2 (anti-FLAG) agarose beads, and bound proteins eluted by incubating the beads with FLAG peptide. Eluted proteins were analyzed by SDS-PAGE followed by western blotting, and MTP (α-FLAG) as well as protein disulfide isomerase (α-PDI) were demonstrated.

Zebrafish and *C. elegans* MTP support apoB secretion: We next asked whether the different homologs support human apoB secretion. We and others have previously shown that the expression of *Drosophila* MTP can rescue the secretion of human apoB (20;21). However it is not known if the MTP belonging to groups 2 and 4 also support apoB secretion. COS cells were co-transfected with MTP as well as apoB48 expression plasmids (Fig. 11). Consistent with our previous report apoB was not secreted in the absence of MTP while the expression of both human and *Drosophila* MTPs increased secretion appreciably, 120 ± 19 and 25 ± 3 ng/ml apoB, respectively (Fig. 11, panel A). When zebrafish MTP was co-expressed with apoB48, the secretion of apoB (129 ± 80 ng/ml) also increased above background levels and the amounts measured in the media

were similar to those secreted in the presence of human MTP. Although cells expressing the *C. elegans* MTP secreted apoB48 (9 ± 4 ng/ml), the apoB measured in the media was consistently less than the other MTPs studied.

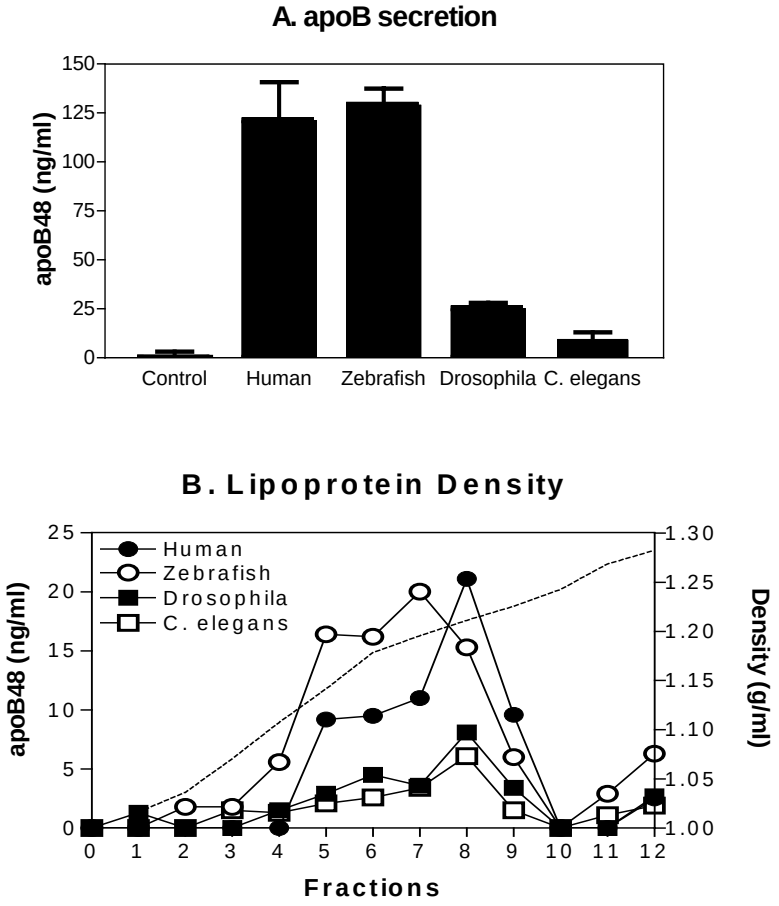


Figure 11. Zebrafish and *C. elegans* MTP support the secretion of human apoB. COS cells transiently expressed MTP (human, zebrafish, *Drosophila*, or *C. elegans*) and apoB48. After 48 h following transfection, media containing oleic acid: BSA complex was added and an additional 24 h incubation was performed with lipid containing media. The amounts of apoB secreted were measured using ELISA (**A.**). Data is illustrated as bar graphs with error bars to represent means and standard deviations. Conditioned media was subjected to density gradient ultracentrifugation and apoB content was determined in 1 ml fractions **(B.)**. The density of each fraction is shown as the dashed line.

The floatation properties of the secreted lipoproteins were subsequently established by subjecting conditioned media to KBr gradient density ultracentrifugation

(panel B). No measurable quantity of apoB was present in fractions 1-3, densities less than 1.1 g/ml, which represent buoyant lipid rich lipoproteins. Although the total secretion of apoB was greater in cells expressing the human or zebrafish MTPs compared to either the *Drosophila* and *C. elegans* MTPs, the density of the secreted particles were similar and ranged between 1.1 – 1.25 g/ml. Very little apoB was measured in fractions 10-12 (density >1.25 g/ml) that distinguish lipid free protein or apoB that might have undergone degradation. These studies show that like the MTP from groups 1 and 3, the homologs representing groups 2 and 4 support the secretion of human apoB as a lipoprotein particle. Furthermore, the MTP do not support the secretion of apoB particles from COS cells with densities less than 1.1 g/ml.

Human and zebrafish MTP transfer triacylglycerols in vitro: We previously showed that while human and *Drosophila* MTP transfer phospholipids, the *Drosophila* MTP is unable to transfer triacylglycerols (21). Therefore, we next asked whether the zebrafish (group 2) and *C. elegans* (group 4) MTPs transfer triacylglycerols (Fig. 12). Lysates containing human MTP demonstrated rapid and significant increases in the amount of triacylglycerols transferred (1.00 ± 0.03 % triacylglycerol transfer/μg protein/h). Likewise, triacylglycerol transfer activity was also detected after the addition of lysates from cells expressing zebrafish MTP to the assay mixture (0.7 ± 0.1 % triacylglycerol transfer/μg protein/h). In contrast, this activity was absent in lysates prepared from cells expressing either the *Drosophila* or *C. elegans* MTPs. We noticed that the triacylglycerol transfer activity was consistently less from lysates containing zebrafish MTP even though greater amounts of zebrafish MTP protein were included in the assays (Fig. 12, panel A,

inset). To further compare the triacylglycerol transfer activity measured in the human and zebrafish MTPs, we performed lipid transfer assays using similar amounts of purified MTP (Fig. 12, panel B and inset). The specific activities of triacylglycerol transfer were again calculated (44 ± 1 and 12 ± 1 % triacylglycerol transfer / h, for human and zebrafish MTP, respectively). The human MTP demonstrated nearly four times greater triacylglycerol transfer activity than the activity measured for the zebrafish MTP. Interestingly, the maximal transfer of lipids achieved by human MTP (10.4 ± 0.4 pmoles) was also greater than that measured for zebrafish MTP (4.6 ± 0.3 pmoles). On average (n = 6), the zebrafish MTP was 27 ± 10 % as efficient as the human MTP in transferring triacylglycerols (Fig. 12, panel C). These data show that zebrafish MTP transfers triacylglycerols in vitro, but is much less efficient than the human MTP at this activity. In contrast, the *Drosophila* and *C. elegans* MTPs are unable to transfer triacylglycerols. Thus, the MTP belonging to groups 1 and 2 transfer neutral lipids while the homologs in groups 3 and 4 are deficient in this activity.

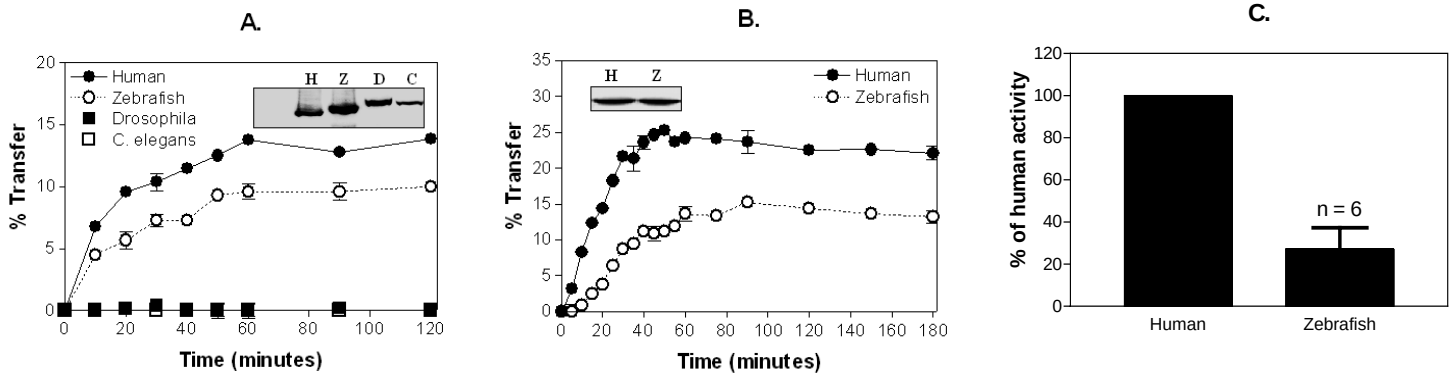


Figure 12. Zebrafish, but not *C. elegans*, MTP transfers triacylglycerols between membranes. COS cells expressing human, zebrafish, *Drosophila*, and *C. elegans* MTP were hypotonically lysed and centrifuged to obtain soluble proteins. A previously described fluorescent assay (25) was then used to measure triacylglycerol transfer activity in the lysates (30 μg protein/assay were used for human, zebrafish, and *Drosophila* MTP, and 50 μg protein/assay for *C. elegans* MTP) (**A.**). Briefly, assays contained 3 μl each of

donor and acceptor vesicles, 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.1% BSA. Changes in fluorescence values were monitored at 550 nm following excitation at 485 nm. The amount of MTP protein added to each assay is shown by western blot (inset, H = human, Z = zebrafish, D = *Drosophila*, C = *C.elegans*). To further evaluate the activities associated with the human and zebrafish MTPs (**B.**), equal amounts of MTP-FLAG protein, as determined by western blotting (inset), were used in the assays. The relative activity of zebrafish and human MTP (n = 6 experiments) was determined after correcting for protein expression (**C.**). Values are represented as means ± standard deviations.

Our present studies, and those previously reported (20;21), clearly illustrate that cells expressing invertebrate MTPs secrete apoB although these homologs do not transfer triacylglycerols to membrane vesicles. We considered that although the MTP of *Drosophila* and *C. elegans* were unable to transfer triacylglycerols to unilamellar phospholipid vesicles they might transfer these lipids to an acceptor particle that contains apoB. To assess this possibility, we replaced the acceptor vesicles in the triacylglycerol transfer assay with apoB-lipoproteins. We then determined whether purified rat MTP could transfer triacylglycerols in the presence of an apoB acceptor (Fig. 13, panel A). Fluorescence did not increase in the absence of low-density lipoproteins (LDL). However, upon the inclusion of low-density lipoproteins, fluorescence increased in a time dependent manner. Furthermore, this increase in fluorescence could be inhibited by the inclusion of the MTP lipid transfer inhibitor, BMS 197636, to the assay mixture. These studies show that MTP can transfer triacylglycerols when LDL is used as an acceptor particle.

We next determined if the MTP-FLAG proteins could also demonstrate this activity (Fig. 13, panel B). Cell lysates containing either human or zebrafish MTP transferred triacylglycerols (4.6 ± 0.2 and 4.5 ± 0.6 % triacylglycerol transfer/μg protein/ h, respectively) to apoB-lipoproteins while the invertebrate MTP lacked this activity.

When triacylglycerol transfer was normalized to the amount of MTP protein used in the assays (Fig. 13, panel C), the zebrafish MTP triacylglycerol transfer activity to apoB (n=3) was ~26% of that measured using human MTP. This value correlates to the relative specific activities calculated for the transfer of triacylglycerols to phospholipid acceptor vesicles by these two homologs (see Fig. 12, panel C).

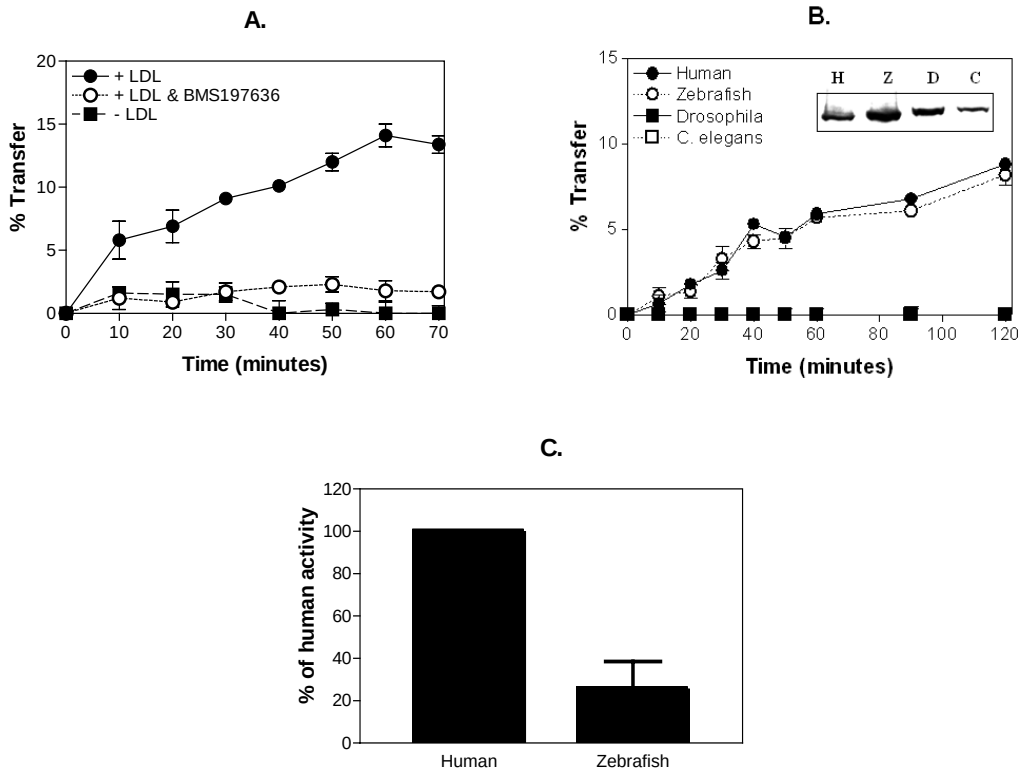


Figure 13. Human and zebrafish MTP transfer triacylglycerols in the presence of apoB-lipoprotein acceptors. Triacylglycerol transfer to an apoB-containing particle (LDL) was measured using an in vitro fluorescent assay as described in the Methods and those previously reported (25). The apoB-lipoproteins were substituted as acceptors instead of phosphatidylcholine vesicles. The changes in fluorescence upon the addition of purified rat MTP (0.5 μg) are shown in the absence and presence of an LDL acceptor (3 μg) and when the MTP lipid transfer inhibitor BMS 197636 (250 nM) is included to the assay mix (**A.**). Cell lysates containing human, zebrafish, *Drosophila*, and *C. elegans* MTP were prepared from COS cells as previously described and used to assay triacylglycerol transfer activity to apoB (**B.**). The transfer activity measured for human and zebrafish MTPs were normalized to the amount of protein present in the assays (**C.**) by western blotting (**B.**, inset). Assays were performed in triplicate and data represented as the mean ± S.D.

Triacylglycerol transfer activity measured from vertebrate liver lysates: Our studies illustrate that the MTP proteins from groups 1 and 2 transfer triacylglycerols while those belonging to groups 3 and 4 do not. The organisms belonging to groups 1 and 2 represent vertebrates. In order to study the evolution of vertebrate MTP, we compared the triacylglycerol transfer activity of bird, amphibian, and mammalian MTPs. Homogenates were prepared from the livers of *X. laevis* (frog), *G. gallus*, *M. musculus*, R. *norvegicus*, and *M. mulatta* (rhesus monkey). Equal amounts of protein were then used to assay for triacylglycerol transfer activity. While all samples contained activity (Fig. 14, panel A) compared to a control (*Xenopus* muscle lysate), homogenates prepared from *Xenopus* liver (1.9 ± 0.3 % transfer/μg protein/h) demonstrated significantly less activity than those from either *G. gallus*, *M. musculus*, *R. norvegicus*, or *M. mulatta* (5.3 ± 0. 1, 4.5 ± 0.1, 4.9 ± 0.2, and 4.7 ± 0.2 % transfer/μg protein/h, respectively) ($p < 0.001$) (Fig. 13, panel A). The triacylgylcerol transfer activities were subsequently measured in the presence of the MTP inhibitor CP-346086 (panel C). The inhibitor reduced activity in a dose dependent manner from each homogenate sample. *Xenopus* MTP was less sensitive to inhibition by CP-346086 than the other homologs. To determine as to whether zebrafish MTP was similarly less sensitive to CP-346086 we performed triacylglycerol transfer assays using lysates from COS cells that expressed either human or zebrafish MTPs (Fig. 13, panel D). Human MTP triacylglycerol transfer activity demonstrated a similar sensitivity to the inhibitor as the other vertebrate homogenates. However, greater concentrations of inhibitor were required to inhibit the transfer activity measured from lysates containing zebrafish MTP. These data show that homogenates prepared from vertebrate livers transfer triacylglycerols and that a potent antagonist to MTP lipid

transfer inhibits this activity. The activity measured from *Xenopus* liver homogenates was significantly less than that measured from other samples. Furthermore, while the MTP triacylglycerol transfer activity of mammal and bird MTPs is efficiently reduced by small molecule inhibition, both the zebrafish and *Xenopus* MTP triacylglycerol transfer activities are less sensitive to inhibition.

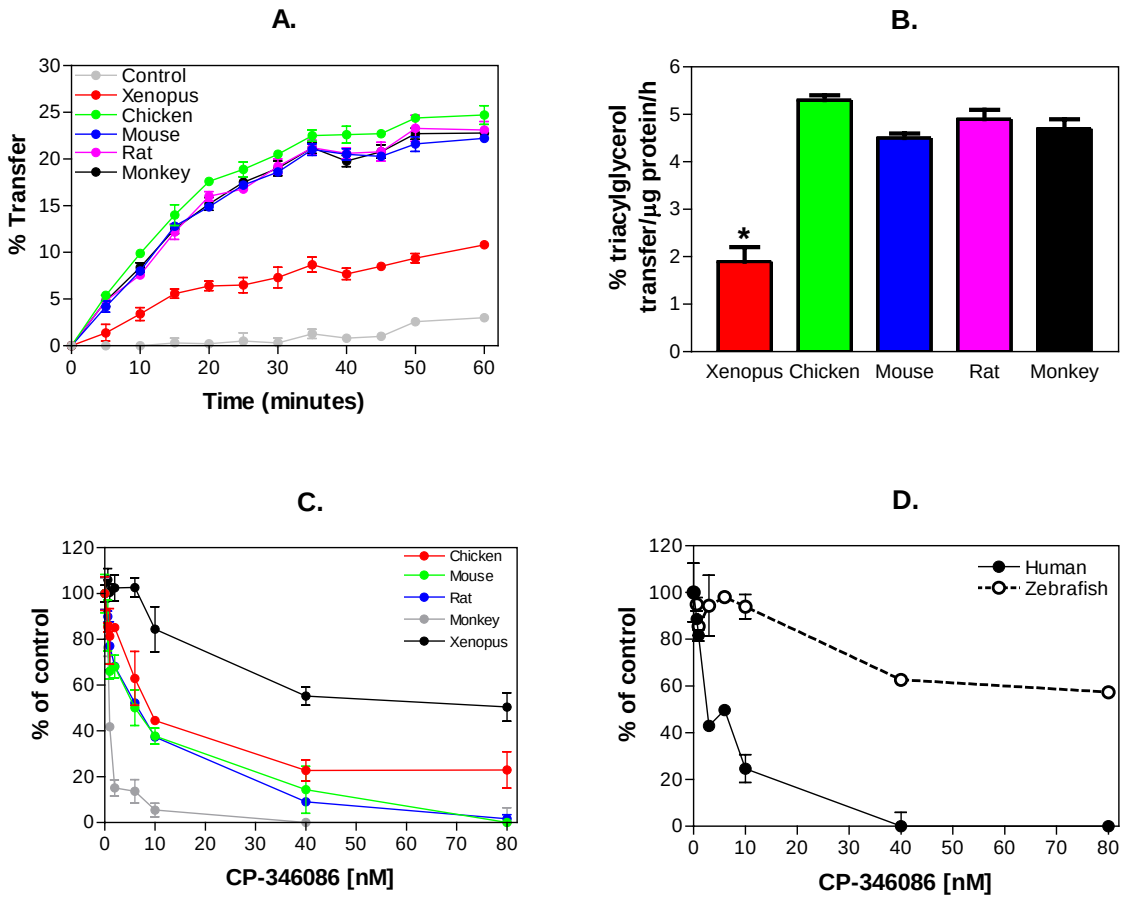


Figure 14. Triacylglycerol transfer activity measured from liver homogenates. Liver homogenates were prepared from 100-300 mg of tissue. Homogenates were incubated in hypotonic buffer (1 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, and 1 mM EGTA), and the cells were subsequently lysed by passage through a 25 Gauge needle. Lysates were centrifuged (50,000 rpm, 1h, 4°C), the supernatant collected, and protein concentrations determined by the Coomassie method (32). To measure triacylglycerol transfer, 10 µg (*G. gallus, M. musculus, R. norvegicus, M. mulatta, X. laevis*) of protein were added to fluorescent assay mixtures as previously described. Incubations were

performed at 37°C and fluorescence at 550 nm was monitored over time after excitation at 485 nm. Panel (**A.**) shows the time dependent increase in triacylglycerols transferred and panel (**B.**) represents the calculated specific activities from each sample (Control = lysates prepared from *Xenopus* muscle). The inhibition of triacylglycerol transfer activity (**C.**) was measured after the addition of CP-346086 to the assay mix at the noted concentrations. The transfer activity was measured for 30 minutes and calculated as previously described. The effect of CP-346086 on human and zebrafish MTPs was studied using lysates (30 μg protein) from COS cells that transiently expressed the proteins (D.). Bar graphs and error bars represent mean ± S.D. (* = $p < 0.05$)

Discussion

We and others previously showed that human MTP, but not *Drosophila* MTP, transfers triacylglycerols in vitro (20;21). In this study we assessed how the MTP protein structure and triacylglycerol transfer activity might be associated. By comparing distinct homologs of the microsomal protein we identified four main orthologous groups that included vertebrate (mammals and fish) as well as invertebrate (insects and nematodes) MTP. Although the primary amino acid sequences were quite variable between the MTP, the proteins contained similar secondary structure (β–α–β). The predicted tertiary structures were also conserved and consisted of four domains (β^N, α, β^C and β^A). The β^C domain as well as helices 4-6 and 15-17 in the α domain, were more conserved than the β^N, α, or β^A domains among vertebrate MTPs. In contrast, none of the structural domains from the invertebrate MTPs showed increased conservation. Furthermore, we show that the triacylglycerol transfer activity is specific to vertebrate MTPs. The proteins from both early vertebrate species (zebrafish and frogs) as well as those that evolved later on (i.e. birds and mammals) transferred triacylglycerols in vitro. The *Drosopohila* and *C. elegans* MTPs, however, lacked this activity. Based on these studies we conclude that MTP acquired its triacylglycerol transfer activity early on during the emergence of

vertebrate organisms and that the β^C domain in concert with helices 4-6 and 15-17 might represent structural elements critical to this activity.

Orthologous proteins accumulate amino acid substitutions as they evolve (33). These changes are incorporated at the same rate and are evenly distributed throughout the entire protein (34) However, domains that are critical for the protein function or to the survival of the organism are less susceptible to acquire inheritable substitutions. Therefore, amino acids that provide an organism with a selective advantage are conserved. We identified MTP orthologs belonging to mammals, birds, frogs, fish, sea urchins, insects, and nematodes. The full-length proteins contained the same β-α-β secondary structure. Furthermore, each protein was predicted to obtain a tertiary structure analogous to that reported for human MTP (7;28;30). This consisted of an N-terminal β-barrel (β^N), central helical region (α), and two C-terminal β-sheet domains (β^C and β^A) that generate a pocket at the C-terminus. Although the proteins were nearly identical by structural analysis their primary sequences of amino acids were considerably different. Therefore, we propose that the MTP structure has been conserved although the MTP amino acid sequences have undergone considerable amounts of substitution as the protein evolved from nematodes to mammals.

Sequence analysis of the structural domains within the MTP homologs revealed that the β^C domain was more conserved than either the β^N or α, or β^A domains in vertebrate but not invertebrate MTPs. MTP's tertiary structure is predicted based upon its homology to the ancient lipid transport protein, lipovitellin (7;28;30). In the MTP structure, both the β^C and β^A domains generate a large cavity in the protein's C-terminus. Lipovitellin possesses a 60 Å cavity that contains phospholipids (35;36). Hussain et al.

has proposed that β^C and β^A form the MTP lipid transfer domain (7). In fact, naturally occurring missense mutations (37) or those created by site-directed mutagenesis (30) within this region of MTP abolish the lipid transfer activity without affecting protein expression or solubility. Here, we show that vertebrate MTP proteins transfer triacylglycerols while invertebrate MTPs are deficient in this activity. Furthermore, the β^C domains in vertebrate MTPs are more conserved than the other protein domains. Based on these data, we propose that the conservation of this domain is critical to the triacylglycerol transfer activity of vertebrate MTP.

In addition to the β^C and β^A domains of vertebrate MTPs, we also show that the helices 4-6 and 15-17 are also conserved. In the lipovitellin model the helices analogous to 4-6 in human MTP are the only structures other than the β^C and β^A domains that contact lipid within the C-terminal cavity (36). Therefore, the conservation of helices 4-6 amongst vertebrate MTP and their association with vertebrate triacylglycerol transfer activity may be similarly attributed. Helices 15-17 are positioned outside the lipid-binding cavity and most likely do not play a role in MTP's lipid transfer activity. However, their location is accessible for protein-protein interactions. Bradbury et al (38) showed using a yeast two-hybrid system that the helices 13-17 in human MTP interact with apoB and function as an apoB-binding domain. Based on our sequence data we believe that the helices 15-17 represent the conserved apoB-binding domain in vertebrate organisms.

Vertebrates rely on apoB-lipoproteins to transport extracellular lipids to distant tissues. MTP is required to assemble and secrete these particles. The absence of MTP (10) or the inhibition of its lipid transfer activity (18) results in reduced levels of plasma

cholesterol and fat-soluble vitamin deficiencies. The evolution of MTP triacylglycerol transfer activity is associated with the utilization of apoB-lipoproteins as the preferred vehicle for vertebrate extra-cellular lipid transport. Prior to the emergence of vertebrates, smaller and less complex organisms existed. At this time the endogenous synthesis of lipid may have been sufficient to support life. However, we suggest that in order to proceed to the next step in evolution greater amounts of lipid than could be synthesized by the organism were required. Therefore with apoB as the efficient lipid carrier and MTP capable to transfer bulk neutral lipids to apoB greater absorption of dietary lipids was possible and provided the prerequisite to vertebrate evolution.

We were unable to identify MTP orthologs from organisms that diverged earlier than *Caenorhabditis*. This suggests that MTP evolved during the emergence of ancestors to the present-day nematodes or that a very early precursor to this protein may share only minimal homology with MTP. We have shown that in contrast to the considerable variability between the MTP protein sequences, its structure is highly conserved. Therefore, an ancestral MTP may contain a secondary or tertiary structure that shares greater similarity to the present-day MTP proteins compared to primary structure. As revealed for lipovitellin, MTP contains an N-terminal β-barrel (β^N) (28). Similar structures are found in other intracellular lipid transfer proteins including phosphatidylcholine transfer protein (PCTP) (39), phosphatidylinositol transfer protein (PITP) (40), and fatty-acid binding protein (FABP) (41). Within the β-barrel of PITP, PCTP and FABP resides a single lipid molecule (39-41). It is noteworthy that a molecule of phospholipid was also present in the β-barrel of lipovitellin (36). Based on the structural homology it shares to PCTP, PITP, FABP, it is also possible that the β-barrel

represents the second phospholipid binding site shown by kinetic analysis to exist in MTP (42). It may also be that MTP evolved from cytoplasmic or as yet unidentified lipid transfer proteins but to transport larger amounts of lipids. To achieve this activity, the α domain and C-terminal cavity (β^C and β^A) might have been acquired through gene duplication, translocations, and/or mutational events. As of yet we have been unsuccessful in finding proteins showing structural homology to either the α or β^C and β^A domains that suggests of such an event.

In conclusion, we have shown that MTP's triacylglycerol transfer activity was acquired prior to the emergence of fish but following the evolution of insects. This acquisition coincides with the progression from relying on the phospholipid-rich lipoproteins (vitellogenin and apolipophorin) to neutral lipid-rich apoB-lipoproteins in order to transport extra-cellular lipids. We also show that the β^C domain and helices 4-6 as well as 15-17 of the α domain are conserved amongst vertebrate MTPs. We suggest that the increased conservation in these regions of the protein is responsible for the robust triacylglycerol transfer activity associated with vertebrate MTPs. Lastly we speculate that the evolution of apoB and MTP provided a critical pathway for evolving organisms (especially vertebrates) to absorb and transport exogenous lipids that could be used as energy or for other cellular processes.

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Triacylglycerol Synthesis Increases apoB48 Secretion Independent of MTP Triacylglycerol Transfer Activity in a Heterologous System

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Abbreviations used: ApoB, apolipoprotein B; BSA, bovine serum albumin; MTP, microsomal triglyceride transfer protein; ER, endoplasmic reticulum

Abstract

The assembly of apoB-lipoproteins requires the expression of the microsomal triglyceride transfer protein (MTP) and is modulated by lipids. The mechanism by which apoB acquires lipids in the endoplasmic reticulum is not known. Currently it is believed that MTP transfers lipids to apoB to prevent proteosome degradation. An insect MTP whose expression rescues apoB secretion has been described (Sellers et al., Journal of Biological Chemistry, 2003). Previously we showed that *Drosophila* MTP does not transfer neutral lipids, however, the homolog does transfer phospholipids in vitro. In this study we determined whether cellular triacylglycerol synthesis modulates apoB secretion assisted by *Drosophila* MTP. The expression of acyl-CoA:monoacylglycerol acyltransferase (MGAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT) increased the amount of apoB secreted from COS cells that also expressed human MTP. Although greater amounts of apoB were secreted the size of the lipoprotein particle was not altered. Similarly, the expression of MGAT and DGAT also increased the apoB secretion that was assisted by *Drosophila* MTP. Both human- and *Drosophila*-dependent apoB secretion were also susceptible to treatment with triacsin C, a potent inhibitor of acyl-CoA synthetase. Based on these results we conclude that apoB secretion is influenced by the cellular synthesis of triacylglycerols, but that this effect is independent of the MTP triacylglycerol transfer activity.

Introduction

The apoB-lipoprotein is a complex assembly of hydrophilic lipids and proteins that surround a hydrophobic core. Packed within the particle's core are the principle component lipids, triacylglycerols and cholesteryl esters (1). The mechanism by which apoB acquires lipids is not well understood and is currently thought to involve two steps (2-4). Initially, a minimal amount of lipid is added to apoB in a co-translational process that generates a dense primordial lipoprotein particle (5). Subsequently, by an unclear mechanism, bulk neutral lipids are loaded onto the primordial particles forming large, lipid-rich buoyant lipoproteins.

The neutral lipids that are utilized during the assembly of an apoB-lipoprotein are synthesized in the endoplasmic reticulum. Triacylglycerols are generated by the integral membrane proteins acyl-CoA:monoacylglycerol acyltransferase (MGAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT) (6-10). MGATs covalently link monoacylglycerol with fatty acyl-CoA to form diacylglycerols (8;9), while DGAT activity catalyzes the synthesis of triacylglycerols by adding activated fatty acids to diacylglycerol substrates (6;10). The disruption of triacylglycerol synthesis adversely affects apoB-lipoprotein secretion. The synthesis and secretion of triacylglycerols are reduced in DGAT1 deficient mice (11). These animals also exhibit decreased post-prandial chylomicron secretion after acute dietary challenge with lipid. In contrast, hepatocytes overexpressing DGAT1 increase apoB secretion from cultured cells (12). In addition to genetic studies, molecular inhibitors also demonstrate the requirement for triacylglycerols during the assembly of apoB-lipoproteins. Pharmacological treatment with triacsin C, an inhibitor of acyl-CoA synthetase, reduces intracellular triacylglycerol

synthesis (13;14). Furthermore, Ginsberg and colleagues showed that the treatment of rat hepatoma cells with triacsin C decreases apoB secretion (14). Collectively these studies demonstrate that the cell's capacity to synthesize triacylglycerols is an important modulator of apoB secretion.

Formation of the primordial lipoprotein particle occurs in the endoplasmic reticulum and requires Microsomal Triglyceride Transfer Protein (MTP) (15). MTP is a heterodimeric complex containing a 97 kDa unique M subunit and the 58 kDa ubiquitously expressed protein disulfide isomerase (16). Wetterau and associates (17) identified MTP by its activity to transfer lipids in vitro. The MTP lipid transfer activity is also most likely its primary activity in vivo as inhibitors of this activity reduce apoB secretion (18;19). While MTP is undoubtedly involved during the first step of apoB-lipoprotein assembly, the mechanism by which it supports this process is not known.

Recently, an insect homolog to human MTP, *Drosophila* MTP, was described that lacked both triacylglycerol and cholesteryl ester transfer activity (20;21). Unexpectedly, expression of the *Drosophila* MTP supported the secretion of apoB. Furthermore, apoB secretion that was assisted by the *Drosophila* MTP was responsive to oleic acid (20) and the lipoprotein particle contained a neutral lipid core containing primarily triacylglycerols (21). In this study we report that expression of MGAT3, as well as DGAT1 and DGAT2, increases apoB48 secretion in a heterologous system. The modulation of apoB secretion by MGAT3, DGAT1, and DGAT2 occurs independent of whether apoB-lipoprotein assembly is supported by the human or *Drosophila* MTPs. In contrast, secretion of apoB17 was not influenced by the expression of either protein. We also show that apoB48 secretion that is assisted by either the human MTP or *Drosophila* MTP is

reduced by treatment with triacsin C. Therefore while the cellular synthesis of triacylglycerols modulates apoB secretion but is independent of the MTP triacylglycerol transfer activity.

Materials and Methods

Reagents, expression vectors, and antibodies: Tissue culture media included Dulbecco's Modified Eagles Medium (DMEM)(Cellgro) containing 10% Fetal Bovine Serum (FBS)(Invitrogen). Triacsin C was purchased from Biomol International. Polyfect transfection reagent was obtained from Qiagen. Expression plasmids for the human, zebrafish and *Drosophila* MTP have been previously described (20). Vectors containing MGAT3 and DGAT1, as well as DGAT2 were generously provided (Dr. D. Cheng). The apoB17 and apoB48 expression plasmids were a kind gift from Dr. ZeminYao.

Measuring apoB secretion from COS cells: COS cells were grown in 12-well tissue culture dishes and transiently transfected with MTP and apoB (apoB17 or apoB48), as well as expression plasmids containing MGAT3, DGAT1, or DGAT2. Following 48h incubation, the media was aspirated and 0.5 or 1.0 ml of lipid-free (DMEM only) or lipid-containing media (DMEM, 0.4 mM oleic acid complexed with 1% BSA, and 1 mM glycerol) was added. When included, triacsin C was supplemented in the media during this step to obtain a final concentration of 0.25 μM. After an additional incubation for 18-24 h, the media was collected and centrifuged (5,000 rpm, 4^{o} C, 5 minutes). The quantity of apoB present in the supernatant was subsequently measured using ELISA. To determine particle density, conditioned media were subjected to a KBr gradient as

previously described (20). Briefly, 4 ml of media were brought to 1.30 g/ml with KBr and overlaid with 2 ml each of 1.24, 1.15, and 1.063 g/ml KBr solutions, followed by 1 ml each of 1.019 and 1.006 g/ml. After ultracentrifugation, (SW41 rotor, 40,000 rpm, 17 h, 15^{o}C), 1 ml fractions were collected and apoB content determined by ELISA. The density of each fraction was measured using a refractometer (Fisher Scientific).

Measuring intracellular lipids: COS cells grown in 6-well plates were transfected as before. Following 48h incubation, the media was aspirated and the cells were pulsed for 18-24 h with 1ml lipid-containing media (DMEM, 5 μCi ^{3}H oleic acid, 0.4 mM oleic acid complexed with 1% BSA, and 1 mM glycerol). Triacsin C (0.25 μM) was added to the media at this time. Following the pulse with radiolabeled oleic acid, the cell monolayers were washed with phosphate buffered saline. To extract lipids, 1 ml of isopropanol was added to the wells and the plates were incubated overnight at 4^{o}C. Subsequently, the solubilized lipids were dried under N_2 (g), and redissolved in 100 μl chloroform:methanol (1:2). Phospholipids, cholesteryl esters, and triacylglycerols were separated by TLC (hexanes:diethyl ether:glacial acetic acid; 80:20:2) and counted using scintillation. The proteins were extracted from the cells by the addition of 0.1 N NaOH, and protein concentrations were measured using Coomassie reagent (22).

Results

The expression of MGAT3, DGAT1 & DGAT2 increase apoB48 secretion: Triacylglycerols are the primary lipid component of the apoB-lipoproteins secreted from hepatoma and intestinal cell lines. To study how proteins involved in triacylgylcerol

synthesis would affect apoB secretion in a heterologous system we expressed MGAT3, DGAT1, and DGAT2 as well as human apoB48 and human MTP in COS cells (Fig. 1). Seventy-two hours following transfection, the amounts of apoB secreted to the media were measured from cells grown in lipid-free (DMEM only) or lipid containing (DMEM, 4 mM oleic acid:BSA complex, and 1 mM glycerol) media (Fig. 1, panel A and panel C, respectively). Consistent with previous studies (20;23;24) apoB48 was not secreted in the absence of MTP, but was present in the media when co-expressed with MTP. Furthermore, upon the expression of the fatty acyl-CoA transferase proteins the amounts of apoB48 secreted were significantly increased, 119% ($p = 0.004$), 123% ($p = 0.004$), and 111% ($p = 0.001$) (Fig. 1, panel A), for MGAT3, DGAT1 and DGAT2, respectively. To explore whether the expression of MGAT3, DGAT1, and DGAT2 were alone sufficient for cells to secrete apoB we expressed these enzymes with apoB48 in the absence of MTP (Fig. 1, panel B). In the absence of MTP, apoB48 was not secreted when the MGAT3, DGAT1 and DGAT2 were expressed. Therefore, although the expression of these proteins increased apoB secretion, MTP was absolutely required. We next sought to determine if the supplementation of oleic acid-containing media to cells that expressed MGAT3, DGAT1 or DGAT2 would produce similar results (Fig. 1, panel C). COS cells were transfected as before and apoB measured. The amount of apoB that was secreted to the media was increased in all samples upon the addition of oleic acid compared to lipid-free media (compare panels A & C). This is consistent with previous studies reporting that the secretion of apoB is increased in the presence of oleic acid (23). Similar to the results obtained with lipid-free media, the cells that expressed the acyltransferase proteins secreted greater amounts of apoB in the media (cells expressing

MTP and apoB48 as well as MGAT3 (321.5 ± 14.5 ng/ml), DGAT1 (308.6 ± 14.5 ng/ml) and DGAT2 (314 ± 3 ng/ml) vs. cells expressing MTP and apoB48 only (228.4 ± 12.1 ng/ml)). These studies illustrate that MGAT3, DGAT1, and DGAT2 expression increases apoB48 secretion from COS cells that assemble and secrete apoB-lipoproteins through the heterologous expression of MTP and apoB48.

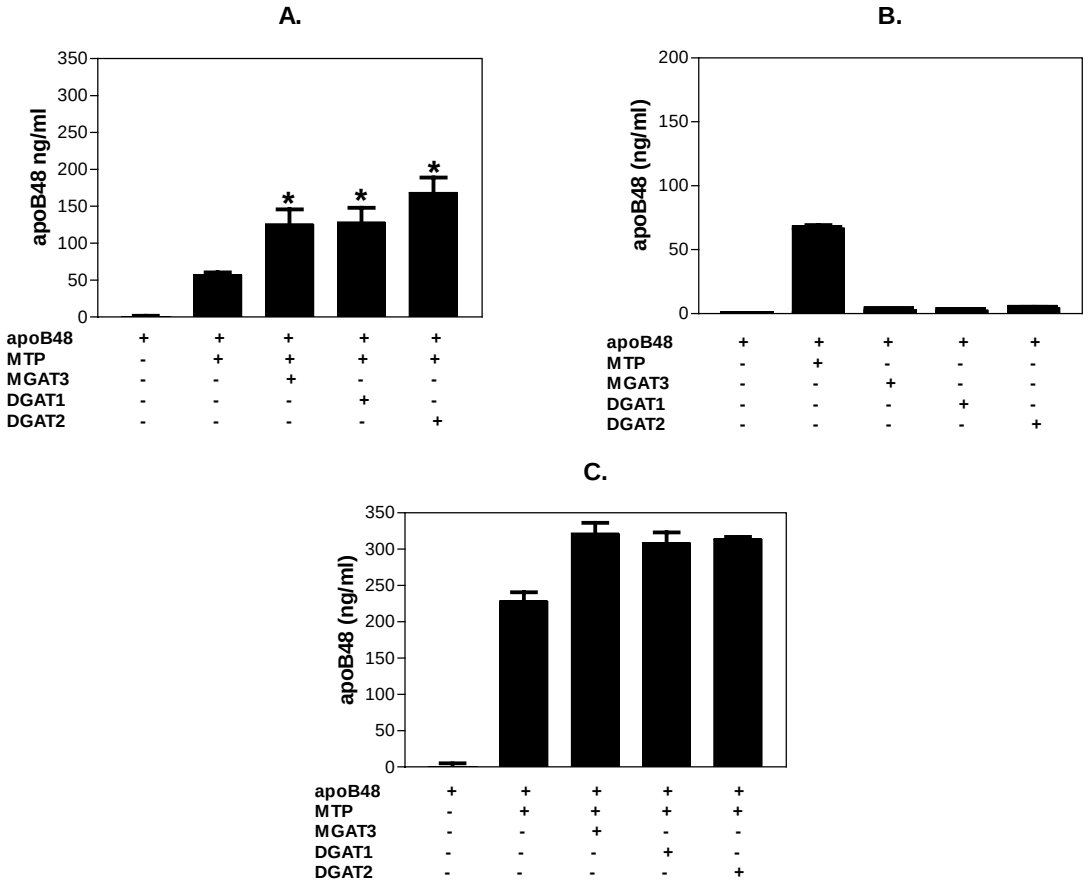


Figure 1. MGAT3, DGAT1 & DGAT2 modulate apoB secretion: COS cells expressing apoB48 and human MTP (**A. & C.**) or apoB48 (**B.**) were co-transfected with MGAT3, DGAT1, or DGAT2. After culturing the cells for 48 h, either lipid-free (**A. & B.**) or lipid-containing media (**C**.) was added and the cells were incubated for an additional 24 h. The amounts of apoB secreted to the media were then measured using

ELISA. Data is represented as bar graphs with means ± standard deviations. Asterisks represent p values < 0.05.

The Effect of MGAT3 and DGAT1 & DGAT2 expression on the secretion of apoB17: The initial 17% of full-length apoB, apoB17, includes the polypeptide's β/α globular domain, but lacks both of its lipid associating β-sheets (1). While both apoB17 and apoB48 are processed in the endoplasmic reticulum and Golgi apparatus, apoB17 does not require the expression of MTP to be secreted (25). To determine if MGAT3, DGAT1, or DGAT2 would affect apoB17 secretion we co-expressed these proteins along with apoB17 and human MTP in COS cells (Fig.2). As shown, apoB17 was secreted independent of MTP (243 ± 5 ng/ml). Furthermore, the amounts of apoB secreted did not change when cells were transfected with either MTP (249 ± 2 ng/ml) or MGAT3 (225 ± 3 ng/ml), DGAT1 (245 ± 3 ng/ml), and DGAT2 (234 ± 1 ng/ml) expression plasmids. This study shows that the secretion of apoB17, unlike that for apoB48, does not respond to the expression of MGAT3, DGAT1, or DGAT2.

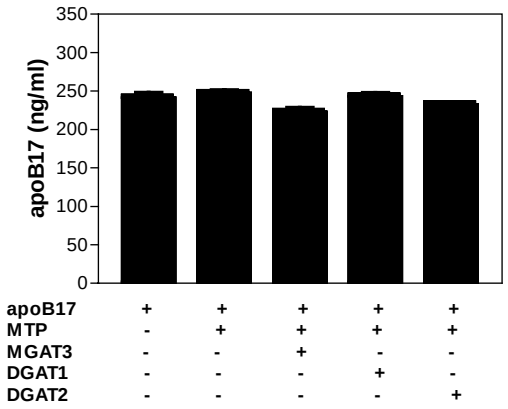


Figure 2. The secretion of apoB17 is not modulated by the expression of MGAT3, DGAT1, or DGAT2: COS cells expressed apoB17 only, apoB17 & human MTP, or

apoB17 & human MTP as well as either MGAT3, DGAT1, or DGAT2. After 48 h following transfection, the media was aspirated and lipid-free media was added to the wells. The cells were then incubated for an additional 24 h. The media were collected and apoB secretion was determined by ELISA. Bar graphs represent the means and error bars are standard deviations.

The expression of MGAT3, DGAT1, or DGAT2 does not affect the floatation properties of secreted apoB: Most apoB-lipoprotein particles secreted from cells in vivo are less than 1.006 g/ml representing very low-density lipoproteins and chylomicrons. When apoB is secreted from heterologous systems, the secreted particles are less buoyant and are present in the high-density fractions (density >1.1 mg/dl) (20;21;26). The differences in the size of the lipoproteins secreted using heterologous systems is suggested to be the result of the cell's capacity to synthesize lipids (26). To determine whether the expression of MGAT3, DGAT1, or DGAT2 alter the densities of the secreted apoB particles, we studied the floatation properties of apoB48 in a KBr gradient (Fig. 3). Consistent with previous experiments the expression of MGAT3, DGAT1, and DGAT2 increased the total amount of apoB secreted. The particles were absent from fractions 1-3 and 10-12, densities 1.03 – 1.08 and 1.28 – 1.33 g/ml, respectively. These represent very-low and low-density lipoproteins as well as proteins that have not associated with lipids. All of the measurable apoB-lipoproteins were present between fractions 4-9 corresponding to a density of 1.1 - 1.26 g/ml. Thus, although expression of MGAT3, DGAT1, and DGAT2 increase the amounts of apoB secreted from COS cells, they do not affect the floatation properties of these particles.

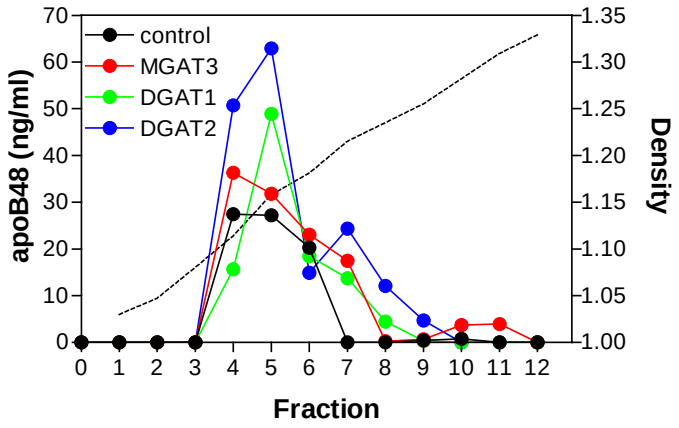


Figure 3. The floatation properties of apoB48 containing lipoprotein particles. COS cells were transfected as before with apoB48, human MTP as well as MGAT3, DGAT1, or DGAT2. After 48 h, the media was aspirated and the cells were grown in lipid containing media for 24 h. The conditioned media was collected and subjected to KBr density gradient ultracentrifugation to measure the floatation properties of the secreted lipoproteins as described in Materials and Methods. 1 ml fractions were collected and apoB content was measured. The dashed line represents the density determined for each fraction.

DGAT2 expression increases apoB48 secretion supported by vertebrate and invertebrate MTPs: Both the expression of zebrafish and *Drosophila* MTP support the secretion of apoB as a lipoprotein particle. We previously reported that zebrafish MTP transfers triacylglycerols but demonstrates only 20% of the activity compared to human MTP using an in vitro lipid transfer assay. Furthermore, the *Drosophila* MTP is completely deficient of neutral lipid transfer activity (20;21). We next studied if the expression of DGAT2 would modulate apoB secretion that is assisted by these MTP. COS cells were made to transiently express human MTP, zebrafish MTP, or *Drosophila* MTP, as well as apoB48 and DGAT2. The amount of apoB secreted was then measured as before (Fig. 4). Cells that were transfected with apoB48 only (control) did not secrete

apoB into the media, however, when apoB was co-expressed with MTP (-DGAT) apoB was secreted. Consistent with previous experiments, the expression of DGAT2 increased the quantity of apoB secreted in the media from cells transfected with human MTP (panel A). COS cells that expressed zebrafish MTP also secrete more apoB in the presence of DGAT2 (panel B). When apoB48 secretion was assisted by the *Drosophila* MTP, the expression of DGAT2 also increased the amounts of secreted apoB (panel C). These studies show that the expression of DGAT2 in COS cells increases the amount of apoB secreted independent of whether secretion is supported by human, zebrafish, or *Drosophila* MTPs.

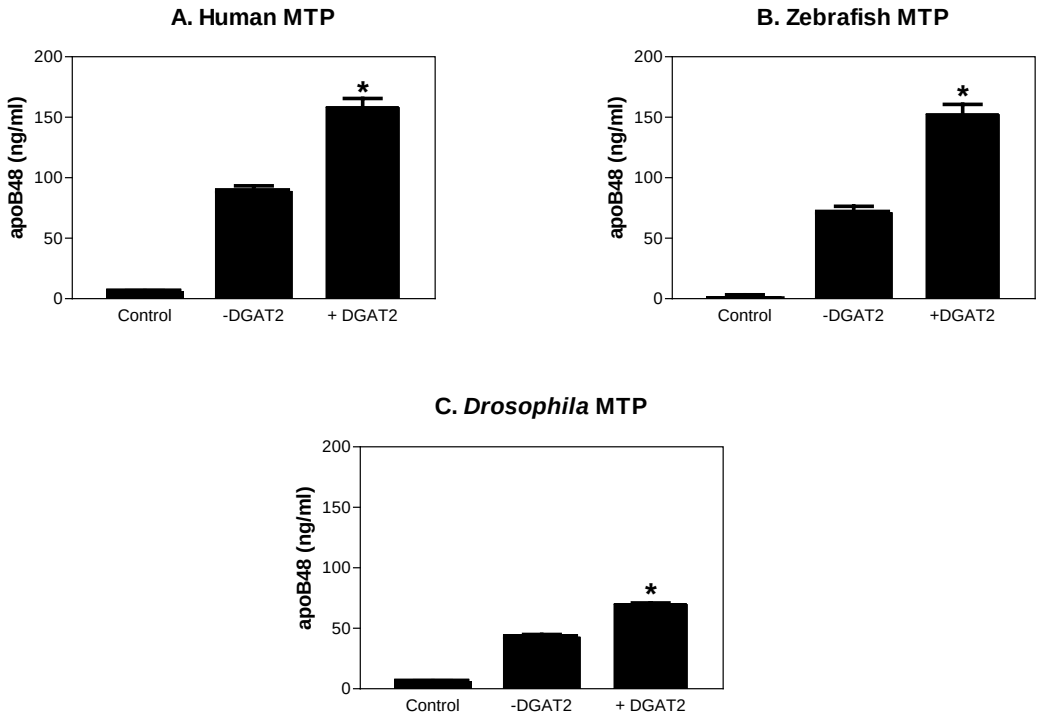
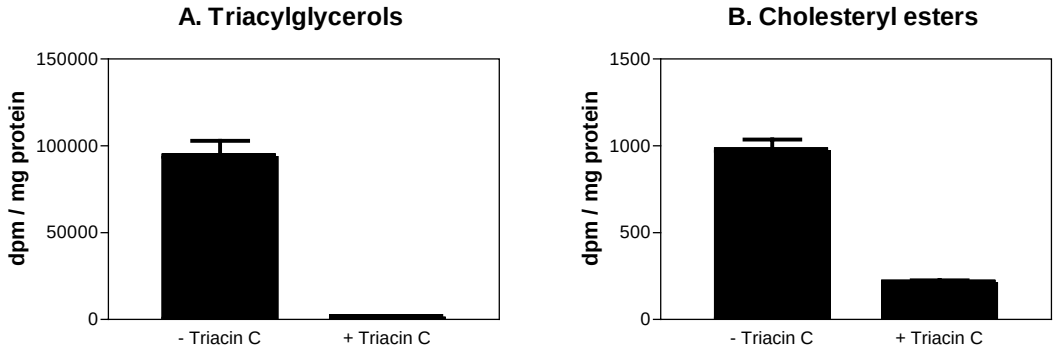


Figure 4. DGAT2 expression increases apoB48 secretion that is supported by human, zebrafish, and *Drosophila* MTPs: COS cells transiently expressed apoB48 and either human (**A.**), zebrafish (**B.**), or *Drosophila* (**C.**) MTPs in the presence (+DGAT2) or absence (-DGAT2) of DGAT2. Control cells expressed apoB48 only. The cells were cultured and apoB secretion was evaluated as previously described using lipid-free

media. The amounts of apoB measured in the media are represented as bar graphs with means ± standard deviations. Asterisks denote p values < 0.05.

Triacsin C decreases apoB secretion: Our results suggest that increasing the synthesis of triacylglycerols augments apoB secretion from COS cells. To determine if decreasing the amount of cellular triacylglyerols has opposing effects on apoB secretion we treated COS cells with triacsin C, a potent antagonist of acyl-CoA synthetase (27). This compound has been reported to decrease both the triacylglycerol and cholesteryl ester synthesis in fibroblasts by 80% and 75%, respectively, at 0.25 μM(27). To determine how triacsin C treatment affects the lipid content of COS cells we pulsed cells with media containing ^{3}H oleic acid in addition to 0.25 μM triacsin C for 24 hours. Lipids were then extracted with isopropanol, separated by thin layer chromatography and the presence of each species (triacylglycerols, cholesteryl esters, and phospholipids) was measured using scintillation (Fig. 5). Consistent with previous reports, triacsin C treatment decreased cellular triacylglycerols (panel A) and cholesteryl esters (panel B). In contrast to its effects on the amounts of neutral lipids, phospholipids were relatively unchanged at this concentration (panel C). These studies confirm that triacsin C reduces the synthesis of both triacylglycerols and cholesteryl esters while not affecting phospholipids in COS cells.



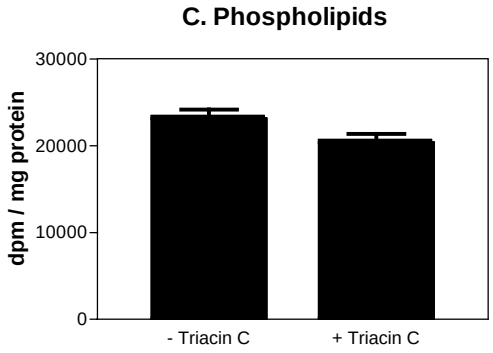


Figure 5. Triacsin C decreases cellular synthesis of neutral lipids: COS cells grown in 6-well dishes were pulsed with DMEM containing 5 μCi ^{3}H oleic acid and 0.4 mM oleic acid complexed to BSA, and 1 mM glycerol, in the presence or absence of 0.25 μM triacsin C, for 24 h. Monolayers were washed, and the lipids were extracted by the addition of isopropanol and dried under N_2 (g). Triacylglycerols (**A.**), cholesteryl esters (**B.**), and phospholipids (**C.**) were separated by TLC and lipids were counted using scintillation. Data is represented by bar graphs showing means ± standard deviations.

We next investigated how triacsin C treatment would affect apoB secretion from COS cells. Cells expressing human MTP, as well as apoB48 were treated with 0.25μM triacsin C for 24 h in the presence or absence of lipid-containing media and apoB secretion was then measured using ELISA (Fig. 6). Triacsin C treatment decreased apoB secretion by 49% and 62% in lipid-free and lipid-containing media, respectively (Fig. 6, panel A). To determine how triacsin C would affect apoB secretion that was assisted by *Drosophila* MTP we performed similar experiments (Fig. 6, panel B). The expression of *Drosophila* MTP was able to rescue the secretion of apoB48 and secretion was modulated by the addition of oleic acid. Treatment with triacsin C decreased the amounts of apoB secreted from cells grown in lipid-free as well as lipid-containing media by ~65%. We next expressed apoB17 alone (Fig. 6, panel C) or with human MTP (Fig. 6,

panel D) in COS cells and subsequently treated the cells with triacsin C. In contrast to its effects on apoB48 secretion, triacsin C treatment did not decrease the secretion of apoB17. These studies show that triacsin C decreases both triacylglycerol and cholesteryl ester synthesis in COS cells. Furthermore, the secretion of apoB48 that is supported by either human or *Drosophila* MTPs is reduced by triacsin C. However, triacsin C has no effect on apoB17 secretion.

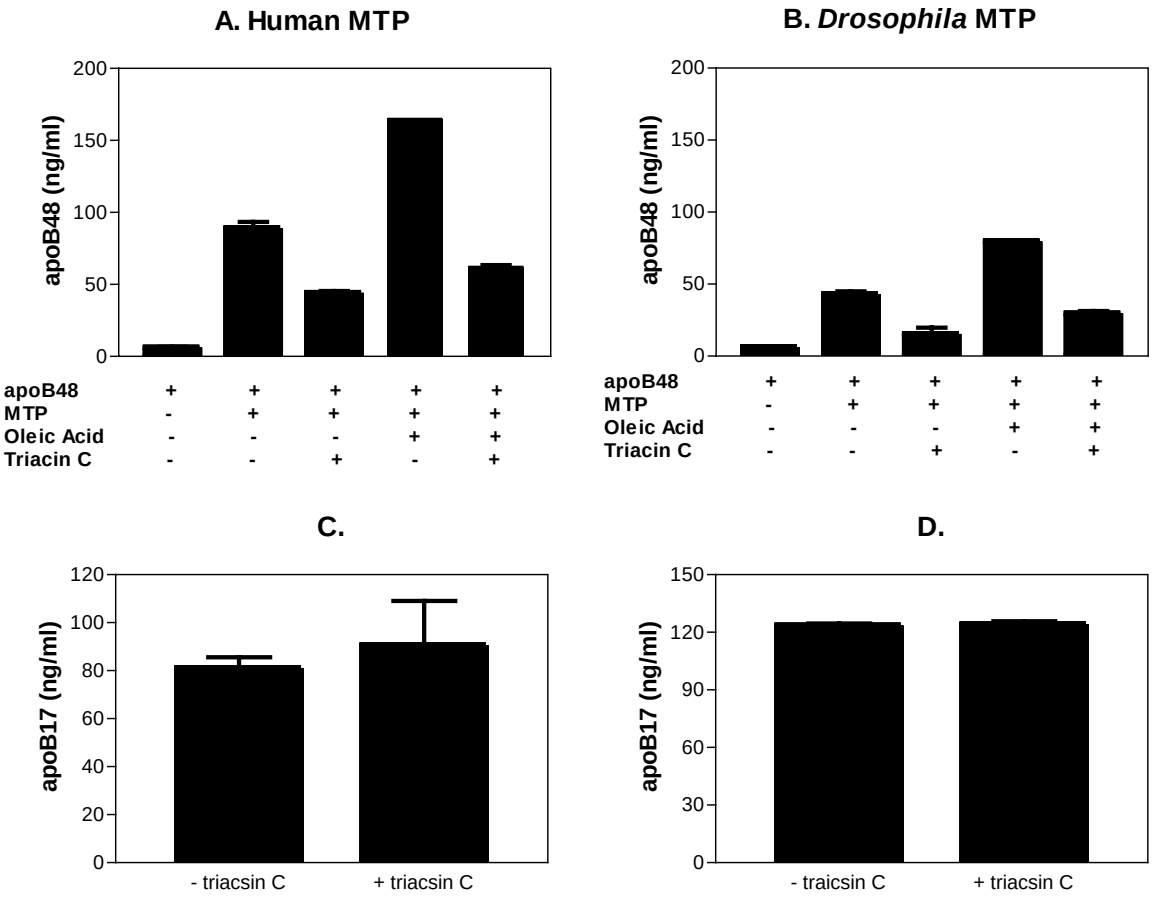


Figure 6. Triacsin C reduces the secretion of apoB48, but not apoB17: COS cells expressing apoB48 alone or apoB48 and either human (**A.**) or *Drosophila* (**B.**) MTPs were treated with 0.25 μM triacsin C for 24 h in the presence of lipid-free or lipid-containing media, and the apoB secretion measured by ELISA. Similarly, apoB17 secretion from cells that expressed apoB17 only (**C.**) or apoB17 and human MTP (**D.**)

was measured in the presence or absence of triacsin C. Bar graphs represent means ± standard deviations.

Discussion

In this study we show that the enzymes catalyzing key steps in triacylglycerol synthesis (MGAT3, DGAT1, and DGAT2) increase the secretion of apoB when apoB-lipoprotein assembly is recreated in a heterologous system. In addition, apoB48 secretion is also increased by DGAT2 when apoB-lipoprotein assembly is assisted by the expression of zebrafish and *Drosophila* MTPs. In contrast, the secretion of apoB48 is decreased from cells treated with triacsin C. The effect of triacsin C on apoB secretion was independent of whether apoB-lipoprotein assembly was assisted by the human or *Drosophila* MTPs. From these data we conclude that triacylglycerol synthesis modulates the assembly and secretion of apoB in COS cells. In addition, although secretion is dependent on the cell's capacity to synthesize triacylglycerols, the effect of increased triacylglycerol synthesis on apoB secretion is independent of whether or not MTP transfers triacylglycerols.

Triacylglycerols are disassembled into their components, glycerol and fatty acids, within the cytosol and then transported to the endoplasmic reticulum where the lipid is re-synthesized (28;29). Acyl-CoA synthetase is required to activate fatty acids during this process (30). The activated fatty acids are then used during the biosynthesis of triacylglycerols by acyl-CoA:monoacylglycerol acyltransferase and acyl-CoA:diacylglycerol acyltransferase. MGAT3, DGAT1, and DGAT2 acylate both monoacyl and diacylglycerols in vitro (8). The expression of DGAT1 increases triacylglycerol content in rat hepatoma cells as well as augmenting the amount of apoB

secreted (12). In this study we show similar increases in apoB secretion using a heterologous system. The amount of apoB measured in the media upon the expression of MGAT3, DGAT1, or DGAT2 in COS cells increased. Furthermore, the modulation of apoB secretion by the expression of MGAT3, DGAT1, or DGAT2 was specific for apoB48. Similar experiments performed using apoB17, showed that the truncated apoB was not responsive to the expression of these proteins. The apoB48 contains hydrophobic β-sheet domains that require the association with lipid. It is likely that the longer apoB is more sensitive to triacylglycerol synthesis than the apoB17 since apoB48 requires neutral lipids to prevent aggregation and degradation.

The acquisition of lipid by apoB to generate a lipoprotein particle is not well understood. Currently, two steps are believed to be required. The first involves the co-translational association of apoB with minimal amounts of lipid to form a primordial particle (2;3;31). This step absolutely requires the expression of MTP. In the absence of this protein, apoB undergoes retrograde translocation to the cytoplasm and is degraded by proteosomes (32;33). MTP transfers lipids in vitro and is presumed to be its predominant activity in vivo (34). When DGAT2 was expressed in COS cells with human MTP and apoB48 we measured significant increases in the amount of apoB secreted to the media. The expression of DGAT2 also resulted in greater secretion of apoB from cells that expressed the *Drosophila* MTP. Therefore, we concluded that increasing cellular triacylglycerol synthesis had a similar effect on apoB-lipoprotein assembly and secretion whether this process was assisted by human or *Drosophila* MTPs. We similarly showed that reducing cellular triacylglycerols decreased the amounts of apoB secreted. Triacsin C is a potent inhibitor of acyl-CoA synthetase, the enzyme required for the addition of

CoA to fatty acids (35). Acyl-CoA synthetase activates fatty acids to be used in lipid biosynthesis. By treating COS cells with this compound we were able to reduce the synthesis of triacylglycerols. The apoB secretion was also reduced by this treatment when its secretion was supported by human MTP. This is consistent with previous studies showing that triacsin C decreases apoB secretion in a rat hepatoma cell line (14). When cells that expressed the *Drosophila* MTP and apoB48 were treated with triacsin C the secretion of apoB was also reduced. Thus, triacsin C reduced apoB secretion independent of whether human or *Drosophila* MTP assisted apoB-lipoprotein assembly.

It is not unexpected that the synthesis of triacylglycerols has a significant effect on apoB secretion that is supported by human MTP. However, DGAT's effect on apoB secretion that was assisted by the *Drosophila* MTP was puzzling. Unlike human MTP, *Drosophila* MTP does not transfer triacylglycerols in vitro (20;21). How then does increasing the synthesis of triacylglycerols augment apoB secretion that is supported by the *Drosophila* MTP? ApoB-lipoproteins contain a neutral lipid core. The acquisition of triacylglycerols by apoB during the formation of a primordial lipoprotein is believed to involve MTP's neutral lipid transfer activity (2;15;31;36). However, the *Drosophila* MTP cannot transfer triacylglycerols. Shelness et al. recently demonstrated that the initial 20% of apoB can associate in vitro with hydrophobic lipids contained within a phospholipid monolayer and suggested that this may signify the initiating event during lipoprotein assembly (37). Furthermore, the N-terminus of apoB (less than apoB34) is secreted independent of MTP with a neutral lipid core containing triacylglycerols (38;39). From our previous studies (20) and the data presented here, we propose a model that

illustrates the difference in apoB-lipoprotein assembly that is supported by human MTP compared with *Drosophila* MTP (Fig. 7).

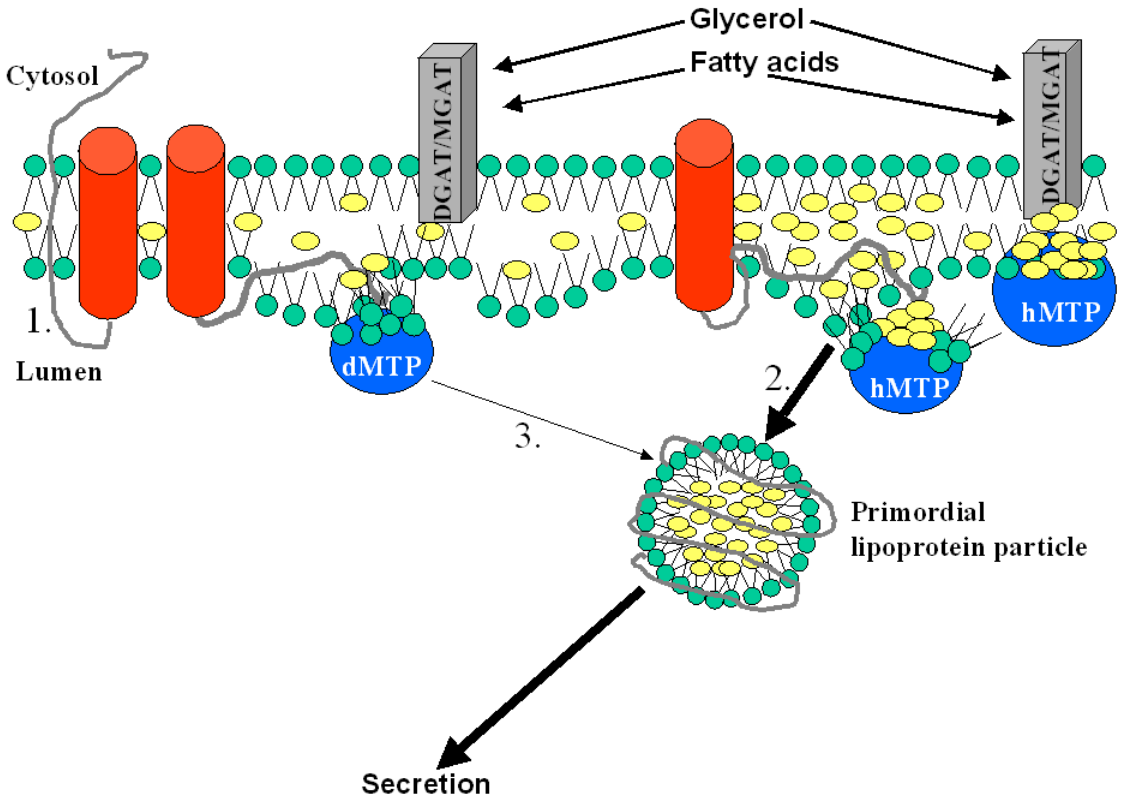


Figure 7. A model showing the difference between apoB-lipoprotein assembly assisted by human and *Drosophila* MTPs. During its translocation into the lumen of the endoplasmic reticulum (ER), the N-terminus of apoB associates with the inner membrane and acquires neutral lipids. In the absence of MTP, apoB undergoes retrograde translocation from the ER (see 1.) and degradation in the cytoplasm. In the presence of MTP, the polypeptide is assembled into an apoB-lipoprotein. Human MTP transfers triacylglycerols and phospholipids in vitro. MTP phospholipid transfer to apoB is required for particle assembly. The human MTP increases triacylglycerol content of the ER membrane and generates membrane blebs (hypothetical protrusions of membrane caused when the local concentration of triacylglycerol is saturated). The apoB polypeptide's lipid binding domains acquire neutral lipid much more efficiently from these areas of concentrated neutral lipid than from regions of membrane where the lipid

content is less. In addition to phospholipids, human MTP transfers triacylglycerols to apoB. This activity increases the efficiency of lipoprotein assembly (see 2.). In contrast, the *Drosophila* MTP only transfers phospholipids to apoB. This is sufficient for apoB secretion but is much less efficient (see 3.). The *Drosophila* MTP cannot modulate the neutral lipid content of the ER membrane or transfer neutral lipids to the polypeptide. Therefore, apoB must rely on its own ability to associate with these lipids in the membrane to form the required core.

Upon its translocation into the endoplasmic reticulum, the N-terminus of apoB associates with the inner membrane. When human MTP is expressed, it creates lipid-rich membrane environments by the transfer of neutral lipids and phospholipids. These hypothetical regions are referred to as membrane "blebs" (protrusions of the membrane that results from saturation of a phospholipid bi-layer with neutral lipids). In support of this, it has been shown that human MTP increases the membrane lipid of the endoplasmic reticulum (40;41). These membrane blebs are saturated with triacylglycerols and provide an optimal location to assemble the lipoprotein. Besides its effect on membrane lipids, human MTP can also perform the direct transfer of triacylglycerols and phospholipids to the apoB further increasing the assembly of secretion-competent lipoproteins. In contrast, *Drosophila* MTP cannot transfer neutral lipids and therefore cannot affect the neutral lipid content of the endoplasmic reticulum. Thus, the neutral lipid is distributed throughout the membrane by diffusion. Although the *Drosophila* MTP transfers phospholipids required for particle assembly, the apoB must rely on its inherent ability to associate with neutral lipid to generate the core. A lipoprotein particle is still produced, however, the efficiency of this process is greatly reduced in the absence of MTP triacylglycerol transfer activity.

In this study we have shown that the expression of MGAT3, DGAT1, and DGAT2 in a heterologous system increases the secretion of apoB. This is independent upon whether lipoprotein assembly is supported by human MTP or *Drosophila* MTP. In contrast, apoB secretion is similarly reduced in cells that express either the human or *Drosophila* MTP when treated with triacsin C. Based on these studies we have described a model defining how the *Drosophila* MTP assists apoB-lipoprotein secretion compared to the human MTP with the fundamental difference being that the human MTP transfers triacylglycerols.

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Discussion

The receptor-mediated uptake of apoB-lipoproteins from the plasma has been thoroughly described (1). However, the intracellular assembly of these particles is less well understood. Apolipoprotein B (apoB) and the Microsomal Triglyceride Transfer Protein (MTP) are essential for the assembly of an apoB-lipoprotein. The apoB is a structural protein (2) that transports bulk lipids and provides a scaffold to the lipoprotein particle. MTP is a microsomal resident whose deficiency results in abetalipoproteinemia, a disease that is characterized by the absence of apoB-lipoproteins in the plasma (3;4). While its absolute necessity during the assembly of an apoB-lipoprotein is generally accepted, how MTP supports this process remains obscure.

Gene knockout (5-7), mutational analysis (8;9), and the use of small molecule inhibitors (10;11) are the methods primarily utilized to study MTP. Over the past 10-15 years, these tools have proved invaluable to understanding apoB-lipoprotein assembly. In the previous studies we describe a different approach to study MTP. We looked at the evolution of MTP structure and function using distinct protein homologs to further assess MTP's role in this process.

Wetterau and associates (12-16), purified MTP from liver microsomes as a heterodimeric protein that transferred many types of lipids in vitro. The classical approach used to study MTP lipid transfer activity assayed the protein-dependent trafficking of radiolabeled lipids between phospholipid vesicles (15). This method is difficult and requires large amounts of MTP. Previously it was shown that fluorescence (nitrobenzoxadiazole)-labeled triacylglycerol could be used to measure the real-time transfer of triacylglycerols by MTP (17). In order to study the other major lipid activities

associated with MTP we developed in vitro assays to measure cholesteryl ester and phospholipid transfer using fluorescent lipids (18). We showed that nitrobenzoxadiazole-labeled cholesteryl esters and nitrobenzoxadiazole-labeled phospholipids can also be used to measure MTP lipid transfer activity. In the absence of an MTP source the fluorescence remained unchanged. However, upon the addition of MTP the fluorescence increased in a time and concentration dependent manner and demonstrated saturation kinetics. MTP dependent fluorescence increases could be obtained with nanogram amounts of protein. Both the cholesteryl ester and phospholipid transfer activities were inhibited by the MTP lipid transfer anatagonist BMS 197636. Thus, these assays provide a sensitive and specific measurement for the transfer of cholesteryl esters and phospholipids by purified MTP in vitro.

Human and bovine MTP transfer many types of lipids, but prefer to transfer triacylglycerols and cholesteryl esters as opposed to phospholipids (12;14). In fact, MTP phospholipid transfer activity is ~5-10% of that measured for neutral lipid transfer (12). We report a similar ratio of activities using purified bovine and rat MTPs as well as partially purified human and mouse MTPs when measuring MTP transfer activity with fluorescent lipids. The phospholipid transfer activity was measured to be only 5-10% of the neutral lipid transfer activity from purified proteins. Therefore, mammalian MTPs contain a robust neutral lipid transfer activity compared to the phospholipid transfer activity. This is independent of whether the direct transfer to phospholipid vesicles (14) or the real-time acquisition of these lipids by MTP in the presence of an acceptor particle was measured (18).

Whether MTP truly prefers to transfer neutral lipids as opposed to phospholipids in vivo remains to be determined. The in vitro assays developed by us (18) and others (15;17) require 35 times the amount of cold phospholipids as compared to fluorescent or radiolabeled lipids (phospholipids, triacylgylcerols, and cholesteryl esters). This ratio is required to maintain the solubility of the neutral lipid in a phospholipid bi-layer as well as to provide an acceptor molecule for MTP to transfer to. Kinetic studies have reported a single binding site for triacylglycerols and cholesteryl esters, while two sites are predicted to exist for the transfer of phospholipids (13). It is not known whether a single site in MTP is responsible for the transfer of these lipids or if three independent sites for triacylglycerols, cholesteryl esters, and phospholipids are present in the molecule. In the first scenario that involves a single site in MTP, the unlabeled phospholipids would equally compete with each of the fluorescent lipids during transfer. Therefore, the reported relative lipid transfer activities showing that triacylglycerols > cholesteryl esters > phospholipids would be an accurate representation. If separate sites in MTP exist for each species of lipid, the transfer of neutral lipids would not compete with the excess phospholipid present in the assays. However, the fluorescent phospholipids would vie with the excess amounts of unlabeled phospholipids for the phospholipid binding site(s). This would significantly underestimate the MTP phospholipid transfer activity. In this scenario the phospholipid transfer activity may in actuality represent the primary lipid transfer activity associated with MTP. At this time we have been unable to determine whether a single site or multiple sites are responsible for the transfer of these lipids in the in vitro assays.

The role of MTP during apoB-lipoprotein assembly is founded primarily on studies that involved its in vitro activities as well as the effect of MTP lipid transfer inhibitors on apoB secretion (19). As discussed, mammalian MTP demonstrates greater neutral lipid transfer activity as opposed to phospholipid transfer activity in vitro. Inhibitors of MTP lipid transfer activity reduce triacylglycerol, cholesteryl ester, and phosopholipid transfer activities in a dose dependent manner at low concentrations (11;17;18;20). Whether in cell culture or animal models treatment with these compounds dramatically reduces apoB secretion and the amount of plasma apoB (11). In contrast the addition of oleic acids to cells increases cellular triacylglycerol concentrations and augments apoB secretion (21). Thus MTP is considered to transfer lipids, primarily triacylglycerols and cholesteryl esters, to apoB during the assembly process. The transfer of neutral lipids is important to generate the hydrophobic core of the apoB-lipoprotein and ensures correct folding of the polypeptide making it secretion-competent.

Recently, an insect homolog of human MTP has been described in the fruitfly, *Drosophila melanogaster* (22). It too exists as a heterodimer of M and P subunits and is localized to the secretory pathway (20). Surprisingly, the *Drosophila* MTP was shown to support the secretion of human apoB34 and apoB41. We show that the expression of *Drosophila* MTP can also rescue the secretion of the longer apoB polypeptides (apoB48 - apoB72). These apoB contain large hydrophobic β-sheet structures predicted to require association with lipid to maintain their integrity and prevent aggregation (2). Although it supported the secretion of human apoB the *Drosophila* MTP is unable to transfer triacylglycerols (20;22). This was demonstrated using a wildtype MTP (20;22) as well as a MTP containing a C-terminal FLAG epitope tag (20) to confirm the protein's

expression. Similarly, cholesteryl ester transfer activity was also found to be deficient in the insect MTP (20). This data demonstrates that the *Drosophila* MTP is deficient in neutral lipid transfer activity.

How then might the *Drosophila* MTP support the secretion of apoB? Unlike neutral lipid transfer activity, the *Drosophila* MTP transferred phospholipids. Since the expression of *Drosophila* MTP supports the secretion of apoB and transfers phospholipids we proposed that the phospholipid transfer activity of MTP is sufficient to assemble a secretion-competent lipoprotein particle. It should be pointed out that the *Drosophila* MTP was able to support significantly less secretion of human apoB (~40% - 50%) compared to the human MTP. This difference may reflect the absence of triacylglycerol transfer activity in the *Drosophila* MTP. While not required for apoB secretion, MTP triacylglycerol transfer activity could affect the efficiency by which these particles are assembled in the endoplasmic reticulum (see later).

While we agree that it is surprising that *Drosophila* MTP supports the secretion of apoB in the absence of a measurable ability to transfer neutral lipids, it is justified according to the hypothetical mechanisms currently available in the literature describing the assembly process (23). In the membrane desorption model, saturation of a phospholipid bi-layer with triacylglycerols is thought to induce membrane “blebs” (portions of the membrane that protrude into the lumen). It has been suggested that apoB or MTP may induce such blebbing by promoting the increased local deposition of triacylglycerols (24). The apoB that associates with this membrane might obtain the required neutral lipids to form its core and then be released to the lumen of the endoplasmic reticulum possibly through the aid of MTP. The first 20% of apoB can be

secreted independent of MTP while longer truncations of the protein absolutely require MTP expression (25). The truncated apoB form lipoproteins and contain both triacylglycerols and cholesteryl esters (26). Recently it was shown that apoB20.1 can spontaneously associate with neutral lipid contained within a monolayer of phospholipids (24). Therefore, the first 20% of apoB is capable to independently associate with lipids in a physiological setting. In our model the N-terminus of apoB initially associates with the inner leaflet of the endoplasmic reticulum according to the desorption theory. MTP might assist in this process by the addition of phospholipids to the apoB as it is translocated into the lumen of the endoplasmic reticulum. MTP might also add phospholipids to apoB as the polypeptide acquires neutral lipids for its core. The acquisition of triacylglycerols may be both dependent and independent of MTP lipid transfer activity. The addition of lipid to apoB by MTP allows apoB to avoid degradation and either self-associate with larger quantities of lipid or acquire bulk lipids through a protein dependent process, possibly involving MTP.

Triacylglycerol synthesis occurs in the membrane of the endoplasmic reticulum and involves acyl-CoA:monoacylglycerol acyltransferase (MGAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT). MGATs catalyze the addition of an activated fatty acid to monoacylglycerols to produce diacylglycerols while DGATs similarly generate triacylglycerols from diacylglycerols (27;28). We expressed these enzymes in COS cells that assembled and secreted apoB-lipoproteins by transfecting them with both apoB and MTP. The expression of MGAT and DGATs increased apoB secretion when the assembly process was supported by human MTP. This is consistent with that previously demonstrated by DGAT1 overexpression in rat hepatoma cells (29).

To our surprise the enzymes also increased apoB secretion that was assisted by expression of the *Drosophila* MTP. This suggested that MTP triacylglycerol transfer activity is not required for the increased apoB secretion measured upon the expression of either MGAT or DGAT. To determine whether decreasing triacylglycerol synthesis would have an opposite effect on apoB secretion we treated cells with triacsin C, a potent inhibitor of acyl-Coa synthetase (30). This compound was reported to reduce the synthesis of triacylglycerols and cholesteryl esters in fibroblast cell lines (31) as well as rat hepatoma cells (32). We showed that triacsin C reduced the cellular triacylglycerols and cholesteryl esters in COS cells. Upon treating COS cells expressing apoB and human MTP with triacsin C the apoB secretion decreased. This is consistent to what others have reported using a hepatoma cell line (32). We then performed similar experiments using cells in which the secretion of apoB-lipoproteins was assisted by the expression of the *Drosophila* MTP. Triacsin C treatment also reduced apoB secretion from these cells. Therefore we concluded that triacylglycerol synthesis modulates the secretion of apoB from a heterologously expressing cell line and that this effect is independent of MTP triacylglycerol transfer activity.

To illustrate how this might be occurring we developed a model demonstrating how the *Drosophila* MTP assists the assembly and secretion of apoB compared to the human MTP. Human MTP transfers triacylglycerols to membrane vesicles as well as apoB (17). It also has been shown to increase the lipid content of the endoplasmic reticulum lumen and membrane (33). We propose that human MTP creates a lipid rich membrane environment that apoB can use during lipoprotein assembly. By its deposition of triacylglycerols, MTP assists in generating membrane blebs (triacylglycerol saturated

regions of membrane) that may increase the efficiency of apoB-lipoprotein assembly according to the desorption model (23). The human MTP transfers phospholipids to apoB required for its assembly into a lipoprotein particle, and also transfers neutral lipids to the polypeptide further enhancing the efficiency of this process. The *Drosophila* MTP transfers phospholipids and can therefore carry out the initial lipidation of apoB. However, the *Drosophila* MTP cannot transfer triacylglycerols and is therefore unable to modulate the membrane deposition of neutral lipids. The apoB must rely on its inherent ability to acquire neutral lipids in order to generate its hydrophobic core. Although evidence that the N-terminus of apoB can acquire lipids independent of MTP (24;26), we believe that it is much less efficient than when this process is assisted by an MTP. Whether the remainder of the protein has the ability to associate with membrane lipids independent of MTP remains to be shown.

While MTP is required for the assembly of apoB-lipoproteins, it is clear that this protein is not only restricted to organisms that utilize apoB to transport extracellular lipids. MTP homologs have been reported in nematodes (34) and multiple insects (22), as well as fish (35), birds, and mammals. We showed that all MTP homologs consist of a similar number of amino acids, however, the conservation of these residues is quite variable. We detected that insect and nematode MTPs contain as little as 20% and 13% identity, respectively. In contrast to the primary structure, the secondary structures for each of the proteins was highly conserved. This consisted of an N-terminal region containing primarily β-strands, a central helical region, and a C-terminus that included significant amounts of β-structure. We used the PHYRE program (http://www.sbg.bio.ic.ac.uk/~phyre) to demonstrate that tertiary structure was also

conserved. Each predicted structure contained a β-barrel located at the N-terminus (β^N), a central region that was composed of multiple helices (α), and a C-terminal cavity enclosed on two sides by β-sheets (β^C and β^A). This suggested that while the protein sequences were only slightly similar in more ancient proteins, MTP's secondary and tertiary structures were highly conserved.

Analysis of the individual structural domains revealed that the α and C-terminal β-sheets (β^C and β^A) were more conserved among vertebrate MTPs compared to their N-terminal β-barrels. Further study showed that the helices 4-6 and 15-17 of the α domain and the entire β^C domain were responsible for the increased calculated identity. The β^C domain generates a lateral border to the C-terminal cavity in MTP (8;36). This cavity contains phospholipids in the crystallized structure of lipovitellin (37). Indeed, mutations in this region have been shown to reduce MTP's ability to transfer lipid (9). Helices 4-6 create the superior border of the lipid cavity and the analogous structures in lipovitellin contact lipid (37). In contrast the helices 15-17 are located outside the cavity. The amino acid residues would not be expected to interact with lipids, but are accessible for protein-protein interactions. We showed that the insect and nematode proteins were deficient of triacylglycerol transfer activity while all of the vertebrate MTPs studied demonstrated robust abilities to transfer this lipid. We therefore concluded that the increased conservation of the β^C domain and helices 4-6 is responsible for the triacylglycerol transfer activity measured in vertebrate organisms. The helices 15-17 are most likely the apoB binding site in MTP as has been speculated by others (38).

Our studies show that MTP acquired its triacylglycerol transfer activity during the same time in evolution as the emergence of vertebrates and the utilization of apoB-

lipoproteins as the primary carrier of extracellular lipids. It is interesting to consider that MTP activity may have played an important role to evolve from invertebrates to vertebrate organisms. Vitellogenin and apolipophorin are the preferred lipoproteins utilized by invertebrate organisms (39;40) while vertebrates utilize apoB-lipoproteins (2). Unlike the phospholipid-rich vitellogenins and apolipophorin, apoB transports enormous amounts of neutral lipids. Since apoB and MTP triacylglycerol transfer activity evolved at a similar time in evolution we propose that the two are interrelated. The apoB-lipoprotein provided the ability to absorb and transport greater amounts of dietary lipids. As a result, vertebrates could grow larger and more complex as they did not have to rely solely on endogenous sources for lipid. MTP in turn co-evolved to transfer triacylglycerols and cholesteryl esters that provided substrates for apoB-lipoprotein assembly.

In conclusion, we suggest that the phospholipid transfer activity of MTP is sufficient for the assembly of an apoB-lipoprotein. However, MTP neutral lipid transfer activity increases the efficiency by which apoB-lipoproteins are assembled. Furthermore, based upon structure and sequence homology, we predict that the β^C domain and helices 4-6 of the α domain are responsible for the triacylglycerol transfer activity associated with vertebrate MTPs. Lastly, we speculate that the evolution of triacylglycerol and cholesteryl ester transfer activity by MTP permitted the efficient assembly of neutral lipid rich lipoproteins. This, in concert with the emergence of apoB-lipoproteins as the preferred extracellular lipid transport vehicle, provided a critical prerequisite for the emergence of vertebrate organisms by allowing greater amounts of energy to be acquired from exogenous sources.

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