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Multiple functions of microsomal triglyceride transfer protein

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Abstract

Microsomal triglyceride transfer protein (MTP) was first identified as a major cellular protein capable of transferring neutral lipids between membrane vesicles. Its role as an essential chaperone for the biosynthesis of apolipoprotein B (apoB) containing triglyceride rich lipoproteins was established after the realization that abetalipoproteinemia patients carry mutations in the *MTTP* gene resulting in the loss of its lipid transfer activity. Now it is known that it also plays a role in the biosynthesis of CD1, glycolipid presenting molecules, as well as in the regulation of cholesterol ester biosynthesis. In this review, we will provide a historical perspective about the identification, purification and characterization of MTP, describe methods used to measure its lipid transfer activity, and discuss tissue expression and function. Finally, we will review the role MTP plays in the assembly of apoB lipoprotein, the regulation of cholesterol ester synthesis, biosynthesis of CD1 proteins and propagation of hepatitis C virus. We will also provide a brief overview about the clinical potentials of MTP inhibition.

Keywords: CD1, MTP, ApoB, Cholesterol, Triglyceride, Lipoproteins

Microsomal triglyceride transfer protein (MTP)

Evidence for an intracellular protein in the lumen of mammalian liver microsomes that transfers neutral lipids, triglycerides and cholesterol esters between phospholipid vesicles was first provided by Wetterau and Zilversmit [1,2]. The protein exhibits significant preference for the transfer of neutral lipids (triglycerides and cholesterol esters) compared to phospholipids. Under non-denaturing polyacrylamide gel electrophoresis conditions, the purified protein migrated as a single band [3]. However, in the presence of 0.1% SDS, two major protein bands were resolved. The P subunit (~58 kDa) was identified as the ubiquitous endoplasmic reticulum (ER) chaperone protein disulfide isomerase (PDI), whereas the larger M subunit (~97 kDa) was unique [3,4]. Therefore, MTP is a heterodimer of two distinct subunits.

The role of PDI in MTP activity

PDI is known to facilitate proper disulfide bond formation during the biosynthesis of nascent proteins. PDI catalyzes disulfide bond formation via its isomerase and shufflase activities; both of these activities are lost when PDI associates with the M subunit. These activities, however, are recovered after disrupting the heterodimer with chaotropic agents, such as guanidine HCl, $NaClO_4$ and KSCN, and non-denaturing detergents, octyl β-glucoside [4-6]. These data indicate that association of PDI with the M subunit involves non-covalent, hydrophobic interactions. This association either physically obstructs active sites present in PDI or instigates a structural change disrupting regions responsible for these activities.

PDI, by itself, lacks lipid transfer activity. Non-covalent association of the M subunit with PDI generates the fully functional lipid transfer complex, MTP. The enzymatic activities associated with PDI are unnecessary when forming an active complex. Missense mutations introduced via site-directed mutagenesis that disrupt PDI's chaperone activities have no effect on heterodimerization with the M subunit and on the formation of a fully functional lipid transfer complex [7]. Disruption of the MTP heterodimer by various agents results in the aggregation of the M subunit and loss of lipid transfer activity [6]. Thus, the role of PDI in the biosynthesis of MTP is more likely related to structural stabilization and solubilization of the complex rather than acting as an active subunit.

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The manner by which the P subunit associates with the M subunit is unknown. Attempts to purify the M subunit and to recombine it with purified PDI have been unsuccessful [8]. Further, endogenous PDI present in the complex could not be exchanged *in vitro* with excess purified PDI [8]. Thus, it has been postulated that the M subunit associates with PDI during translation, although no concrete evidence is available. Once the MTP complex is formed, the two subunits do not dissociate from each other. It remains to be determined whether the complex is degraded *en bloc*, or whether it involves subunit separation followed by selective degradation of the two subunits.

The M subunit

The M subunit belongs to a family of large lipid transfer proteins (LLTP) [9-11]. These proteins share sequence homology and have been predicted to contain similar secondary and tertiary structures. Other members of the family include apolipoprotein B (apoB), lipophorin, and vitellogenin. MTP shares extensive sequence homology with vitellogenin, an ancient protein found in vertebrates involved in the transport of lipids from extra-ovarian tissue to the oocyte. By comparison with the crystal structure of vitellogenin, MTP is predicted to have three major structural domains: N-terminal β-barrel (amino acid residues 22-297), middle α-helical (AA residues 298-603), and C-terminal β-sheet (residues 604-894) [12,13]. The N-terminal β-barrel domain mediates interaction with the N-terminus of apoB; the middle α-helical domain associates with both PDI and apoB; and the C-terminal β-sheet domain contains both the lipid binding and lipid transfer activity of MTP [12,14].

The importance of the M subunit of MTP in apoB-lipoprotein assembly was first realized by the observation that individuals with abetalipoproteinemia lack apoB-lipoproteins in their plasma and have mutations in the *MTTP* gene that result in the loss of lipid transfer activity present in the liver and intestine [15]. Abetalipoproteinemia is a rare autosomal recessive disease [15-17] characterized by a virtual absence of plasma apoB-containing lipoproteins [18]. Due to fat malabsorption and defective lipid transport, intestinal biopsies from abetalipoproteinemia patients grossly demonstrate a whitish coating and histologically visible fat-laden enterocytes. Additionally, these patients have decreased plasma triglyceride and cholesterol levels, altered membrane and lipoprotein lipid compositions, and fat soluble vitamin deficiencies (D, A, K, and E) [19]. The clinical manifestations of abetalipoproteinemia, first described by Bassen and Kornzweig, range from gastrointestinal (steatorrhea, diarrhea, failure to thrive), neurological (absent reflexes, altered sensation and movement, muscle weakness), hematological (acanthocytes, anemia, coagulopathy) to ophthalmological (pigmentary degeneration of the retina and night blindness) symptoms [19].

Lipid transfer activities of MTP

The lipid transfer activities of MTP are measured *in vitro* using donor and acceptor vesicles [1,3]. Donor vesicles consist of unilamellar (one bilayer) phosphatidylcholine with trace amounts of radiolabeled lipids, i.e. triglycerides or cholesterol esters, and cardiolipin. After incubating donor and acceptor vesicles with a source of MTP, DEAE-cellulose (DE52) is added to remove cardiolipin containing vesicles. Acceptor vesicles in the supernatant are then counted and loss of radioactivity is used to calculate % lipid transfer per h.

Wetterau and associates also engineered donor vesicles containing fluorescent cholesteryl ester, cholesteryl 1-pyrenedecanoate, and incubated with acceptor vesicles to quantify MTP activity [6]. Following excitation at 340 nm, pyrene exhibits a complex emission spectra consisting of two peaks. The first emission is observed at 380 nm (monomer emission) and the second is observed at 470 nm (excimer or excited-state dimer emission). Time dependent reduction in excimer/monomer fluorescence ratio was correlated with the lipid transfer activity of MTP. The transformation of the excimer/monomer ratio to lipid transfer activity is not linear and consequently is neither suitable for routine MTP activity measurements nor for comparison of activity in samples of different origins. Subsequently, they reported that the monomer emission of cholesteryl 1-pyrenedecanoate at 380 nm was self-quenched when incorporated at higher concentrations into phosphatidylcholine vesicles [20] and observed that incubation of these vesicles with MTP increased fluorescence due to the binding of cholesteryl 1-pyrenedecanoate to MTP [20]. These studies were further extended to demonstrate that pyrene-labeled phospholipids and triglycerides also bind to MTP [21]. The disadvantages of the use of pyrene include the need to measure emission spectra at two different wavelengths and variability in emission spectra under different conditions and concentrations.

We described a different MTP assay that measures the transfer of nitrobenzoaxadiazol (NBD)-labeled triacylglycerols between vesicles [22]. In this assay, fluorescent lipids are quenched within the phospholipid bilayer of donor vesicles. Incubation of donor vesicles with the acceptor vesicles in the presence of different concentrations of MTP results in an increase in fluorescence with time. Once the fluorescent lipids are transferred to acceptor vesicles the fluorescence is once again quenched. This actual transfer to the acceptor vesicles can be measured after their separation by incorporating cardiolipin and dissolution with isopropanol [23]. Thus,

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the fluorescence increase is due to the exposure of the fluorescent lipids being transferred by MTP between donor and acceptor vesicles, and the saturation of lipid transfer represents the maximum occupancy of MTP with fluorescent lipids. This assay has been further refined to determine the transfer of cholesterol esters and phospholipids [23]. For phospholipid transfer activity, NBD-labeled phosphatidylcholine was a poor substrate as it could not be easily quenched within vesicle membranes. However, NBD-labeled phosphatidylethanolamine incorporated in phosphatidylethanolamine vesicles could be used as donor vesicles to monitor the phospholipid transfer activity of MTP. These assays are simple and amenable to throughput screening.

MTP expression in different tissues

The M subunit expression and MTP activity is predominantly present in differentiated epithelial cells of the small intestine. MTP expression varies both longitudinally throughout the intestine and vertically within the crypt-villus axis [24-26]. Maximal expression occurs starting from the pyloric to intestinal transition in the duodenum, proximal jejunum, decreases towards the distal end and in the ileum, and is nearly absent in the colon [24]. Isolated crypts contain non-differentiated enterocytes and are devoid of both MTP mRNA and protein [26]. A graded increase in mRNA and protein levels occurs as enterocytes mature from the crypts toward the villi.

The liver is another major site of MTP expression in mammals. To date, only hepatocytes have been shown to express MTP. Within the liver, it has been reported that protein expression increases in cells proximal to the central vein and lessens toward the periphery of the lobule opposing the portal triad [27]. The physiological basis for this distribution demands further investigation. The major function of MTP in hepatocytes, as well as the previously discussed enterocytes, is to mobilize dietary and endogenous fat to other tissues through its incorporation into apoB-lipoprotein particles.

Since first described as a liver and intestine specific protein, our appreciation of MTP's tissue distribution has further evolved. Recent studies show that kidney and heart are the 3rd and 4th major organs expressing MTP [28]. Nevertheless, the amounts of MTP mRNA in these tissues are 3-5% of the liver levels. In the kidney, MTP is expressed in the tubular epithelial cells of the cortex but not in glomerular cells [28]. These cells also express apoB and secrete lipoproteins. It has been speculated that cortical cells might synthesize lipoproteins to recycle albumin bound fatty acids and vitamin A from the glomerular filtrate. MTP expressed in cardiac myocytes also functions in the assembly and secretion of apoB-lipoproteins [29-32]. The key purpose for lipoprotein assembly in the heart appears to be to protect this organ against the toxic accumulation of lipids. In animal models of hypoxia and diabetes, MTP activity is reduced and more lipids accumulate in the heart [32-34].

MTP is also expressed in the retina [35]. MTP protein was detected in retinal tissue as well as in a spontaneously arising transformed cell line that was shown to support secretion of apoB lipoproteins. A role similar to that illustrated for the myocardium may also be ascribed to MTP in the retina. Like heart, the retina is susceptible to lipid accumulation with the formation, as well as deposition, of cholesterol crystals. The localized deposition is associated with age-related maculopathy [35]. Further, MTP has also been reported to be expressed in neurons [35]. While speculative, it is likely to play a similar protective role that has been described in heart and retina.

It has long been recognized that MTP is expressed in the yolk sac of certain mammals [36] and is reported to be present in human placenta [37]. Cultured adipocytes, as well as fat tissue extracts from the heart and adrenal glands, also contain MTP [38,39]. Recent studies have shown that immune cells, especially lipid antigen presenting cells, contain both MTP transcripts as well as functional protein capable of transferring lipids [40,41]. Thus, MTP is more widely expressed than anticipated during early studies. MTP expression is high in cells that synthesize lipoproteins, but low in other cells. Low levels of MTP might be sufficient for the biosynthesis of CD1 proteins (see below). Higher amounts of MTP are perhaps needed for the assembly and secretion of apoB-containing lipoproteins. Tissue-specific *cis-* and *trans-* elements responsible for variable MTP expression have yet to be defined.

Recent studies point to the expression of two isoforms of MTP, only in mice, that arise from differential splicing of alternate exon 1 [39,42]. Despite two extra N terminal amino acids in the minor MTP-B isoform, both isoforms have similar lipid transfer properties. The major difference is related to their expression. The expression of the major MTP-A isoform is predominant in the liver, intestine and heart, whereas MTP-B isoform is mainly operative in macrophages and other cells that express low levels of MTP.

Functional consequences of tissue specific ablation of MTP

Two groups have shown that MTP knockout in mice is lethal [43,44]. MTP deficiency leads to embryonic lethality predominantly between E9.5 and E10.5 [43]. Yolk sacs of $MTP^{-/-}$ embryos contained significant amounts of lipid droplets, but lacked VLDL-size lipoproteins in their ER and Golgi compartment. Thus, a reason for

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embryonic lethality might be related to the inability of the yolk sac to synthesize lipoproteins for the delivery of lipids to embryos.

$MTP^{+/-}$ mice on chow diet have half of normal mRNA, protein and activity levels. These mice have similar plasma cholesterol and triglyceride levels as $MTP^{+/+}$ mice, but they have lower levels (reductions of ~28%) of plasma apoB100 [43]. The livers of $MTP^{+/-}$ mice contained higher (~20%) cholesterol and triglyceride levels and showed signs of increased lipid accumulation by Oil Red-O staining. On a high fat diet, $MTP^{+/-}$ mice had ~20% lower levels of plasma cholesterol mainly due to reductions in VLDL/LDL. ApoB production studies showed significant reductions (~20%) in the secretion of newly synthesized apoB100 and apoB48 by isolated primary hepatocytes from $MTP^{+/-}$ mice. Thus, both MTP alleles are required for normal plasma and hepatic lipid levels.

Chang et al. [44] and Raabe et al. [45] have floxed exons 5/6 ($MTTP^{fl(exon5, 6)/fl(exon5, 6)}$) and exon 1 ($MTTP^{fl(exon1)/fl(exon1)}$), respectively, of the MTP gene in mice to facilitate tissue-specific ablation. Chang et al. [44] generated liver specific MTP ablated (L-$MTP^{-/-}$) mice by injecting adenoviruses expressing Cre-recombinase. Hepatic MTP ablation reduced plasma apoB100 and apoB48 mainly due to lower production of apoB-lipoproteins. Moreover, L-$MTP^{-/-}$ were resistant to high-cholesterol diet induced hypercholesterolemia. Thus, hepatic MTP expression has a significant effect on plasma cholesterol levels.

Raabe et al. [45] crossed $MTTP^{fl(exon1)/fl(exon1)}$ mice with mice expressing Cre-recombinase under the control of Mx1 promoter. The Mx1 promoter is activated after the injection of dsRNA leading to the synthesis of Cre-recombinase and deletion of MTP gene. Additionally, they deleted hepatic MTP by injecting adenoviruses expressing Cre-recombinase. Using both these approaches they achieved > 95% hepatic MTP deficiency. L-$MTP^{-/-}$ mice had ~50% less plasma cholesterol and 30-40% less plasma triglyceride compared with floxed mice. They reported > 95% reductions in plasma apoB100 but a modest ~20% reductions in plasma apoB48. L-$MTP^{-/-}$ hepatocytes had several lipid droplets and reduced glycogen levels. These hepatocytes did not show signs of inflammation. Electron microscopic observations showed cytosolic lipid droplets, absence of VLDL-size particles in the ER and Golgi. L-$MTP^{-/-}$ mice were found to be more susceptible to *E. coli* lipopolysaccharide, concavalin A and *P. aeruginosa* exotoxin A induced injury [46].

Khatun et al. crossed $MTTP^{fl(exon1)/fl(exon1)}$ mice with Albumin-Cre mice to obtain liver specific ablation of MTP [47]. MTTP gene deletion in these L-$MTP^{-/-}$ mice reduced triglyceride transfer activity by ~80-85%. Livers of these mice had higher levels of triglyceride, cholesterol and phospholipids. Further, these mice had significantly lower levels of plasma lipids. Lipoprotein synthesis studies revealed that hepatic MTP ablation significantly reduces assembly and secretion of both apoB100 and apoB48 lipoproteins. These three L-$MTP^{-/-}$ studies clearly establish the essential role of hepatic MTP in the production of VLDL and maintenance of plasma cholesterol and hepatic lipids.

Davidson and associates created intestine specific KO mice (I-$MTP^{-/-}$) after crossing $MTTP^{fl(exon1)/fl(exon1)}$ mice with mice that express Cre-recombinase under an inducible Villin promoter activated by tamoxifen [48,49]. Intestinal MTP gene deletion was associated with no weight gain and steatorrhea, gross lipid accumulation, presence of large lipid droplets in the apical portions of the enterocytes and absence of lipoproteins in the Golgi and ER [48]. MTP deficient enterocytes contained about 12-fold and 2-fold increase in triglyceride and free fatty acids, respectively [48]. Plasma lipid analysis revealed significant reductions in plasma triglyceride, cholesterol and free fatty acids due to reductions in both apoB-lipoproteins and HDL. These intestine-specific MTP deficient mice showed almost no absorption of triglycerides and 60-70% reduction in cholesterol absorption. Further, radiolabeling studies with isolated enterocytes revealed that MTP deficiency was associated with significant reductions in apoB48 secretion. These studies establish that MTP activity is essential for the assembly and secretion of apoB48-containing lipoproteins by enterocytes and that intestinal MTP contributes significantly to steady state plasma lipids.

Xie et al. [49] further examined the role of intestinal MTP in the assembly and secretion of apoB48 and apoB100 containing lipoproteins. Enterocytes only synthesized apoB48 due to efficient and complete post-transcriptional editing of the apoB mRNA by Apobec-1 enzyme [50,51]. Ablation of Apobec-1 results in the synthesis of apoB100 by the intestine [50,52]; therefore, they crossed Apobec-$1^{-/-}$ mice with intestine-specific MTP deficient mice to obtain apoB100 expressing intestine specific MTP deficient mice (apoB100-I-$Mttp^{-/-}$) and studied the effect of MTP deficiency on the secretion of apoB100-lipoproteins. They observed that apoB100-I-$Mttp^{-/-}$ mice were more susceptible to death than apoB48-I-$Mttp^{-/-}$ mice when fed high saturated or unsaturated fat but not when fed a high cholesterol diet. Investigators made two novel observations that could explain increased lethality in apoB100-I-$Mttp^{-/-}$ mice. First, they observed that apoB100-I-$Mttp^{-/-}$ mice did not adapt to high fat feeding by increasing the length of the small intestine as apoB48-I-$Mttp^{-/-}$ mice did mainly due to a defect in crypt proliferation. Second, apoB100-I-$Mttp^{-/-}$ enterocytes developed unresolved ER stress

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response. Although not demonstrated, the induction of the ER stress can perhaps be attributed to accumulation of apoB100 in the ER. It is known that unresolved ER stress could lead to cell death [53]. Thus, induction of unresolved ER stress might have contributed to cell death and no increase in villus length.

Iqbal et al. [54] obtain partial MTP gene deletion in the intestine by crossing $MTTP^{fl(exon5, 6)/fl(exon5, 6)}$ mice with Villin-Cre mice. These mice had ~60-70% lower levels of intestinal MTP mRNA and activity compared with floxed mice. [^{3}H]Triolein absorption studies revealed that these mice absorb ~63% less triglyceride in 2 h. These studies showed that partial ablation of MTP gene also has significant effect on acute lipid absorption.

Bjorkegren et al. [55] crossed $MTTP^{fl(exon1)/fl(exon1)}$ mice with α-myosin heavy chain-Cre transgenic mice to generate cardiac myocytes specific ablation of the MTP gene. Heart specific MTP ablation (H-$MTP^{-/-}$) increased triglycerides in cardiac myocytes in fasting mice. They did not report changes in MTP activity in these mice. Further, they showed that MTP inhibitors reduce secretion of apoB-containing triglyceride-rich lipoproteins by these cells. On the other hand, Bartels et al. [56] crossed $MTTP^{fl(exon1)/fl(exon1)}$ mice expressing Cre-recombinase under the control of muscle creatine kinase promoter that is specific for skeletal and cardiac muscle cells. Surprisingly, they did not find any difference in MTP activity because the expression of MTP-A isoform was reduced by > 95% with a concomitant ~3.6-fold increase in the expression of MTP-B isoform. Both these studies, in combination with those involving altering apoB expression in the heart [29-31,55,57], support the notion that lipoprotein assembly by the heart might be to avoid cardiac lipotoxicity associated with influx of free acids during fasting or high fat feeding.

MTP as the precursor to extracellular lipid transport systems

Lipoprotein-based lipid transport systems are also present in the plasma of egg laying animals and the hemolymph of insects. Unlike mammals, the lipid transport vehicles utilized by these organisms are not apoB-based, but instead rely on proteins with similar structure and varying capacities to carry lipids.

Insects synthesize a single multifunctional lipoprotein, lipophorin, within a specialized organ identified as the fat body [58]. Lipophorin typically consists of two apolipoproteins, apolipophorin I and apolipophorin II (~ 240 and 80 kDa, respectively). A third, apolipophorin III (18-20 kDa), may also be present to increase the overall lipid carrying capacity of the lipoprotein [59]. Lipophorin is a phospholipid rich, neutral lipid poor lipoprotein whose density is similar to mammalian HDL (~ 1.15 g/ml). The principal neutral lipid varies between triglyceride and diglyceride depending on the organism, while cholesterol ester is present in very low amounts [60]. Apolipophorin has the capacity to accept lipids via efflux from tissue (i.e. intestine) and can then deliver these to another, distant tissue without undergoing endocytosis. It therefore behaves as a continuous "lipid shuttle" that never leaves the circulation. However, some evidence does suggest that in *Locust migratoria* apolipophorin may be endocytosed similar to certain mammalian lipoproteins through an insect homolog to the mammalian low-density lipoprotein receptor [61].

Egg laying animals utilize a different lipoprotein, vitellogenin. This large apolipoprotein (~ 450 kDa) is synthesized in the liver of vertebrates and the intestines of nematodes. The major function of vitellogenin is to transport lipids to the ovary/oocytes. There it undergoes receptor-mediated endocytosis via a member of the low density-lipoprotein receptor family [62,63]. Vitellogenins, like lipophorins, are phospholipid rich, dense lipoproteins containing ~15% lipid by mass. Vitellogenins described to date contain the bulk of neutral lipids as triacylglycerols and not diacylglycerols.

Apolipophorin, vitellogenin and apoB are functionally related in that each constitutes a vehicle for extracellular lipid transport. Surprisingly, their overall relationship extends to amino acid sequence, conservation of critical cysteine residues identified to be involved in disulfide bond formation, residues required for the development of salt-bridges, as well as an overall maintenance of secondary structure (α-helical and β-sheet). These homologies provide evidence that apolipophorin, vitellogenin, and apoB are distant relatives who have undergone paralogous development [13]. As a group, they comprise the Large Lipid Transfer Protein Gene Family (LLTP) [64]. Microsomal triglyceride transfer protein is also predicted to be a member of the LLTP family based upon sequence homology [13]. Unlike other family members, MTP does not directly participate as a vehicle for transporting lipid to distant tissues. It is restricted to the intracellular compartments of the secretory pathway and is critical for the assembly of apoB and vitellogenin containing lipoproteins. It has been suggested that MTP could be the ancient protein evolved to transfer lipids [9]. The other members may have diverged to carry lipids as a cargo rather than to act as an intracellular shuttle protein for lipid transfer.

Human MTP homologues have been reported throughout a diverse collection of organisms that include mammals [65], birds [66], fish [67], insects [68], and worms [69]. While our appreciation regarding the role of MTP in mammalian apoB lipoprotein assembly and secretion is expansive, its function in organisms that do not express apoB has only recently been studied. As described for human MTP, these more ancient forms

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