THE IMPACT OF MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN ON INTRACELLULAR HEPATIC NEUTRAL LIPID DISTRIBUTION AND TRAFFICKING

BY

PHILIP S. MACARTHUR

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Approved By:

Gregory S. Shelness, Ph.D., Advisor

Paul A. Dawson, Ph.D., Chair

Griffith D. Parks, Ph.D.

John S. Parks, Ph.D.

Ryan E. Temel, Ph.D.
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DEDICATION

This dissertation is dedicated to Jackson and Paris
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<td>ApoB</td>
<td>apolipoprotein B</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CE</td>
<td>cholesteryl ester</td>
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<td>CHO-K1</td>
<td>Chinese hamster ovary cells</td>
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<td>COS</td>
<td>African Green monkey kidney cells</td>
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<td>DG</td>
<td>diglyceride</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<td>dMTP</td>
<td>Drosophila microsomal triglyceride transfer protein</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>hMTP</td>
<td>human microsomal triglyceride transfer protein</td>
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<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LLTP</td>
<td>large lipid transfer protein</td>
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<td>LV</td>
<td>lipovitellin</td>
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<tr>
<td>MBP</td>
<td>maltose binding protein</td>
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<tr>
<td>McA-RH7777</td>
<td>McArdle RH7777 rat hepatoma cells</td>
</tr>
<tr>
<td>mMTP</td>
<td>mouse microsomal triglyceride transfer protein</td>
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<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
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NaCl.................................................................................sodium chloride
PBS.................................................................................phosphate buffered saline
PDI.................................................................................protein disulfide isomerase
PC......................................................................................phosphatidylcholine
PL.......................................................................................phospholipid
PMSF.............................................................................phenylmethylsulfonylfluoride
SDS.....................................................................................sodium dodecyl sulfate
SDS-PAGE..............................................................sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG......................................................................................triglyceride
VLDL..............................................................................very low-density lipoprotein
VTG......................................................................................vitellogenin
Microsomal triglyceride transfer protein (MTP) is a multifunctional protein necessary for conversion of apolipoprotein B into precursor lipoproteins and bulk triglyceride (TG) movement into the endoplasmic reticulum (ER) for precursor lipoprotein expansion. Invertebrate forms of MTP (e.g. Drosophila; dMTP) are capable of phospholipid (PL) transfer, whereas vertebrate forms (e.g. human; hMTP) engage in PL and TG transfer. We hypothesized that PL transfer is the primordial activity of MTP necessary for precursor lipoprotein particle formation, whereas TG transfer is a vertebrate adaptation necessary for trafficking of TG into the ER for 2nd-step assembly. Hence, we assessed hMTP’s ability to promote TG trafficking into the ER of transiently transfected or stable, Dox-inducible, non-hepatic cell lines. In cell lines that expressed hMTP, a 2.5 - 5.0-fold increase in microsomal TG content was observed. The lipid-transfer activity of MTP was necessary for this function as in the presence of a potent MTP inhibitor, BMS-212122, microsomal TG content was similar to mock-transfected cells. To determine if the TG-transfer activity of MTP facilitated TG translocation we compared the TG
content of microsomes from hMTP and dMTP expressing cells. Compared to mock-transfected cells, hMTP increased microsomal TG content by ~2.5-fold while cells transfected with dMTP demonstrated no increase in microsomal TG content. These data indicate that MTP promotes TG trafficking into the ER and that the TG-transfer activity of MTP is essential for this function.

To explore whether the PL and TG-transfer activities of MTP play distinct roles in resistance to hepatic steatosis, we engineered mice with liver-specific expression of hMTP or dMTP. TG-transfer activity in hMTP transgenic mouse liver was increased 2-fold while dMTP mice failed to display an increase compared to wild type. When challenged with a high-fat diet, hMTP mice displayed a mild reduction in liver TG content. Unexpectedly, expression of dMTP caused a 2-fold increase in liver TG, relative to wild type. Plasma lipids and hepatic TG secretion rates were similar among all three genotypes. These results demonstrate that modest hMTP overexpression protects the liver from high-fat, diet-induced fatty liver without increasing plasma lipids, a property that may require MTP’s intrinsic TG-transfer activity.
CHAPTER 1

Introduction
Lipoprotein metabolism and clearance

The mouse liver secretes approximately 25 to 50 mg of TG per day, which is primarily delivered to muscle, heart, and adipose tissue (1). In addition to apolipoprotein B (apoB), these particles contain other apolipoproteins (apo), such as apoCII, apoCIII, and apoE, which function in the metabolism and removal of these lipoproteins from the bloodstream (2-4). These particles are metabolized in the plasma compartment by lipases, releasing free fatty acids and glycerol, which are taken up by peripheral tissues, including muscle and adipose (5). The metabolism of VLDL and chylomicrons within the circulation leads to the generation of successively smaller and higher density particles resulting in a heterogeneous population of apoB-containing lipoproteins ranging from VLDL to intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and chylomicron remnants, respectively (4). TG within apoB-containing lipoproteins is hydrolyzed primarily by lipoprotein lipase (LPL), an enzyme located on the capillary endothelium that requires apoCII for activation (6). Individuals with genetic defects of apoCII display high levels of plasma TG that are comparable to individuals with LPL deficiencies (7-10). Another VLDL apolipoprotein that regulates LPL activity, in this case negatively, is apoCIII (11). The potency of apoCIII as an LPL inhibitor is observed upon its overexpression in transgenic mice, which results in a ~ 10-fold increase in fasting TG concentrations (12). Hepatic lipase (HL) is another lipolytic enzyme responsible for the lipolysis and remodeling of VLDL and its metabolic products. In addition to its lipolytic properties, HL, by virtue of its localization within the sinusoids of the liver, also plays a critical role in the hepatic uptake of VLDL remnants, a process that may be independent of its catalytic activities (13). LPL, as well as HL, hydrolyze the TG from the interior of the lipoprotein particle, releasing free fatty acids for energy production and/or storage (14). As the TG is selectively removed from
the lipoprotein interior, the core of the particle decreases and CE is added by the action of cholesteryl ester transfer protein (CETP). CETP acts to transfer lipid molecules between different lipoprotein species, primarily LDL and HDL, and consequently makes LDL particles CE rich (15). It is the CE-rich LDL that plays an important role in the development of atherosclerotic disease.

LDL particles normally reside within the circulation for 2-3 days (6), and two-thirds of LDL particles are cleared by the low-density lipoprotein receptor (LDLR) (57). The LDLR binds apoB at a region of the protein termed site B, which corresponds to amino acids 3345-3381 (particularly residues 3359-3369), a region highly enriched in positively charged amino acids (16, 17). This site displays similarity to the LDLR binding region in apoE. Hence, the LDLR recognizes and clears particles containing both apoB and apoE. LDLR binding to apoE creates another mechanism for LDL clearance, and also facilitates uptake of chylomicron remnants, which do not contain the LDLR binding site. The receptor mediated binding and uptake of apoB-containing lipoproteins through the LDLR is a complex process, in that a recently described cause of hypercholesterolemia is due to the autosomal recessive hypercholesterolemia (ARH) protein, a putative LDLR adaptor protein, implying that LDLR function depends on other intracellular cofactors (18). ApoB-containing lipoproteins can also interact with the LDL receptor related protein (LRP), the main receptor for chylomicron remnant uptake, as well as other receptors (14, 19).

**Significance and relevance to cardiovascular disease and hepatic steatosis**

Hepatic free fatty acids originating from adipocyte lipolysis, dietary lipids, and de novo synthesis can be utilized for energy via β-oxidation, esterified to form triglyceride (TG), stored
as cytosolic lipid droplets, or packaged as TG into very low-density lipoprotein (VLDL) particles for secretion (Figure 1). An alteration affecting any of these pathways has implications for the etiology of hepatic and plasma lipid and lipoprotein metabolism and the development of metabolic syndrome and its associated comorbidities. For example, reduced TG efflux via the VLDL pathway could lead to cytosolic TG accumulation and eventually, steatosis (20). Conversely, enhanced hepatic production of apoB-containing lipoproteins, which can lead to elevated plasma concentrations, is associated with increased body mass index (BMI), metabolic syndrome, and a 3.2 fold increase in 5-year risk of cardiovascular disease (CVD) (21, 22). CVD, including myocardial infarction, stroke, and peripheral artery disease account for >50% of the annual mortality rate in the United States of America (23). While CVD is positively associated with levels of apoB-containing lipoproteins, levels of high-density lipoproteins (HDL) are inversely correlated with the risk of CVD (24, 25). Normal apoB plasma levels range from 60-120 mg/dL (6), with levels higher than this, leading to elevated TG, cholesterol, or both (2) and being an important risk factor for the development of cardiac events.

A number of genetic disorders can impact apoB and plasma cholesterol levels. Familial hypercholesterolemia (FH) (26) is a disorder caused by defects in the LDLR, which can severely impair the clearance of apoB-containing lipoproteins. Conversely, the disorder termed familial defective apoB100, caused by an arginine to glutamine substitution at amino acid 3,500, also impairs apoB clearance by altering the conformation of apoB near the LDLR binding site (6,16,17).

Other defects in apoB can lead to mild to severe reductions in plasma apoB and lipid concentrations. The disorder hypobetalipoproteinemia (HBL) (27) is characterized by lipid
values below the 5th percentile and can be caused by numerous genetic lesions within the apoB gene. Over forty known frameshift or nonsense mutations produce truncated forms of the apoB protein, ranging in size from apoB2 (2 percent of the amino-terminus) to apoB89 (89 percent of the amino-terminus) (27). ApoB truncations shorter than 27.6 percent of the amino-terminus are usually absent from the plasma due to either decreased production or increased catabolism (27, 28). Patients heterozygous for mutations in apoB would be predicted to have LDL-cholesterol levels 50% lower than normal; however, it appears that some HBL alleles exert dominant-negative effects, as apoB and LDL-cholesterol levels in some HBL heterozygotes are as low as 25% of the normal concentrations (27), suggesting complex interactions in the production and metabolism of apoB-containing lipoproteins. Recently, several missense mutations in apoB have also been implicated in HBL (29, 30).

Another genetic disorder that leads to lower plasma apoB and cholesterol levels is abetalipoproteinemia (ABL), a recessive disorder resulting in fat malabsorption and the virtual absence of apoB-containing lipoproteins. While the phenotype of ABL is similar to severe homozygous HBL, the disorder is unlinked to the gene for apoB. Utilizing a candidate gene approach, the underling cause of ABL was attributed to genetic lesions in the gene for the microsomal triglyceride transfer protein (MTP) (31). MTP is localized to chromosome 4q22-24, and a variety of mutations within the gene have been shown to disrupt MTP function and impair the secretion of apoB-containing lipoproteins (32, 33). Patients with ABL or severe homozygous HBL exhibit a variety of clinical features such as acanthocytosis, fat-soluble vitamin malabsorption, as well as neurologic and ophthalmologic problems (31, 34), although if applied early in life, many of these symptoms can be mitigated by fat-soluble vitamin supplementation and diets low in long chain fatty acids (31).
The inability to properly lipidate and secrete apoB100 from the liver has been associated with forms of non-alcoholic fatty liver disease (NAFLD) in both human and mouse studies (35, 36). Patients with some forms of familial HBL have been shown to have ~5 fold higher hepatic lipid values when compared to non-disease controls (36). Similarly, ABL patients have been shown to have lipid droplet accumulation within the liver and intestine, due to the inability to export lipid on apoB-containing particles (31). Recent studies of patients with non-alcoholic steatohepatitis (NASH) revealed that the synthesis of apoB100 was lower in these patients (37). Some studies have also demonstrated that genetic polymorphisms that reduce MTP expression, also increase susceptibility to NAFLD. Taken together, these data show that mild to severe impairments in apoB-containing lipoprotein production are associated with a wide variety of clinical disorders as a result of either increased or decreased production of apoB-containing lipoproteins.

**Large lipid transfer protein gene family**

Both vertebrates and invertebrates possess systems for the mobilization and transport of lipids. The major players in this process belong to a family of genes coined the large lipid transfer proteins (LLTP). This family includes the amphipathic lipid binding proteins vitellogenin (Vtg), insect apolipophorin II/I (apoLpII/I), apoB, and MTP. Each member has a high molecular weight (~97–500 kDa) and binds to and transports lipids and other apolar molecules (38, 39). Sequence analyses conducted by Mann et al. and Babin et al. determined that each protein shares an amino-terminal β-sheet and an extended α-helix domain, which contains a highly conserved hydrophobic cluster (38, 39). These sequence data, as well as the wide distribution of Vtg in both oviparous vertebrates and invertebrate species, including C. elegans, led to a
consensus that Vtg was the evolutionary progenitor of the LLTP gene family (38). However in a study published in 2003, it was revealed unexpectedly that functional orthologs of MTP exist in invertebrate species (40) and that the secretion of Vtg is dependent on MTP expression (41), resembling the requirement of MTP for the assembly of apoB into lipoprotein particles (42, 43). Subsequent studies revealed that the assembly of insect apoLp II/I into high-density lipophorin (HDLp) particles was also MTP-dependent (44). Together, these findings demonstrated that so-called primitive lipoproteins such as lipovitellin (the processed form of Vtg) and high-density lipophorin (HDLp) (formed by apoLpII/I) did not acquire lipids by autonomous recruitment (45, 46), as previously believed (45), but rather required MTP as an assembly cofactor. In a 2005 review, Shelness and Ledford proposed that MTP may be the most ancient LLTP family member and that it functions in the biosynthesis of all of its evolutionary descendants, including Vtg, apoLpII/I, and apoB (47) (Figure 2). However, they further proposed that the properties of MTP required to achieve these assembly pathways have diverged leading to the creation of lipoprotein classes harboring very different amounts and types of lipids and playing distinct roles in lipid transport and homeostasis.

ApoB and hepatic and intestinal lipid transport

TG is the most calorically dense biological fuel source and can be stored in a relatively inert and benign form within cellular lipid droplets (48). However, due to the hydrophobic properties of TG and other neutral lipids such as cholesterol ester (CE), they must be transported through the bloodstream in the form of lipoprotein particles, in which the neutral lipids are sequestered in the core of the particle, surrounded by a surface monolayer of phospholipid (PL), free cholesterol, and apolipoproteins (2). ApoB100 is an amphipathic, 4,536
amino acid, secretory glycoprotein that functions as the major component of TG-rich VLDL and is essential for the assembly and secretion of VLDL from the liver into the plasma compartment (43, 49). ApoB48, the amino-terminal 48% of apoB100, is the major structural protein on chylomicrons, which are assembled in the intestinal enterocyte and are essential for the transport of dietary lipids across the intestinal epithelium and into the circulation, via the mesenteric lymph (50). The basis for the formation of apoB48 is post-transcriptional modification of the apoB100 messenger RNA (mRNA) by a site-specific cytidine deamination reaction that converts a glutamine codon (CAA), at a position corresponding to amino acid 2,152, to a stop codon (UAA) (6).

The formation of hepatic VLDL particles depends on TG availability. The Intracellular storage pool of TG serves as a primary source of TG making up VLDL. Approximately 70% of VLDL-TG comes from this pool while 30% is derived from de novo TG synthesis (51). Mobilization of cytosolic lipid droplets for VLDL assembly is believed to require a cycle of TG lipolysis and re-esterification (52). Triacylglycerol hydrolase (TGH) is one enzyme that has been proposed to play a role in TG mobilization. Dolinsky et al. (53) propose a mechanism for TGH in which the enzyme hydrolyzes cytosolic lipid-droplet TG that is ER associated. The resulting diglyceride (DG) products are then re-esterified by either DGAT1 or DGAT2 at the ER membrane and then shuttled into the VLDL assembly pathway.

Mature apoB-containing lipoproteins contain a single molecule of apoB48 or apoB100 (54, 55) and are assembled into TG-rich particles having diameters of 30-80 and 75-1200 nanometers (nm), for VLDL and chylomicrons, respectively (56). Therefore, the amount of lipid associated with the apoB-containing lipoproteins varies considerably. However, the function of apoB-containing lipoproteins remains the same:
to package and transport lipid molecules such as TG to extra-hepatic and intestinal cells for energy production or storage (3, 48). Additionally, as each chylomicron and VLDL contains a single molecule of apoB, which remains irreversibly associated with the lipoprotein during its intravascular metabolism, the plasma concentration of apoB reflects apoB lipoprotein particle number and is a strong positive risk factor for atherosclerosis progression as well as other risk factors associated with the metabolic syndrome (15, 16, 17, 57).

**Two-step model of VLDL formation**

The formation of VLDL is considered to occur by a two-step process (Figure 3), as described originally by Alexander et al. (58). The formation of VLDL begins with apoB being simultaneously translated and translocated into the lumen of the ER where an interaction with MTP aids the formation and stabilization of a precursor lipoprotein (43). Unlipidated and under-lipidated apoB is vulnerable to rapid pre-secretory degradation and considerable effort has gone into elucidating the pathways responsible for these co- and post-translational forms of gene regulation (43). The ubiquitin/proteasomal pathway of protein degradation is responsible for a large percentage of pre-secretory apoB turnover, although other mechanisms have also been proposed (59). Leiper et al. determined the necessity of MTP in first-step assembly by demonstrating that a truncated form of apoB was not secreted from COS cells unless apoB was co-transfected with MTP (42, 60). After completion of apoB translation and the first step in lipoprotein formation, it is believed that a relatively small, dense (HDL–LDL) precursor lipoprotein particle is formed and released from the ribosome (61). These studies suggested that apoB containing particles formed within the rough ER must be further lipidated by the
addition of TG (primarily) and some PL (mainly phosphatidylcholine) in a post rough ER compartment before secretion from the hepatocyte (61). Additionally, studies comparing apoB protein size, and lipid composition and density from HepG2 cells showed that apoB protein size was proportionally related to the diameter of the lipoprotein core (62). In these experiments, apoB particles containing apoB80 (amino-terminal 80%) had a radius of ~ 20 nm diameter, significantly lower than mature apoB containing particles secreted from hepatocytes. These studies suggest that apoB co-translationally associates with lipid to form a nascent precursor particle of 20-25 nm in diameter, which is then further lipidated in a second step of bulk lipid addition to form the mature lipoprotein particle. The formation of the first-step primordial particle is an MTP-dependent process (63), in that inhibition of MTP blocks conversion of apoB from an unlipidated form to an HDL sized particle (64).

The second step of apoB containing lipoprotein assembly is likely a highly structured process regulating the expansion of the precursor lipoprotein into a mature VLDL particle. This process may involve multiple chaperones that facilitate the addition of bulk neutral lipid (65), comprised mainly of TG. The intracellular site of second-step assembly is controversial with groups proposing that particle maturation occurs either in the ER (66) or the distal Golgi (67). An essential prerequisite for second-step particle maturation is the creation of secretion-coupled lipid; that is, lipid that has been targeted for VLDL assembly via its translocation from the cytosol into the ER lumen. Although most of the enzymes responsible for TG formation have been identified and characterized (68), the mechanisms by which lipids are mobilized or secreted via transport into the ER are not well understood. However, convincing data has emerged to suggest that MTP plays a critical role in this process, as genetic or pharmacological ablation of MTP activity reduces the neutral lipid content of the microsomal
fraction (69-71).

MTP is necessary for the transport of lipids into the lumen of the smooth ER and inhibition of MTP has been shown to perturb the addition of bulk lipids to the nascent precursor particle. In murine primary hepatocytes, isolation of an ER lumen pool of TG not associated with apoB was found to have a TG content 3-fold higher than the TG associated with apoB (71). MTP inhibition reduced the amount of TG in this pool, indicating the importance of MTP in the movement of bulk lipid into the lumen of the secretory pathway (71). MTP inhibition, however, did not affect the transfer of TG from this pool to lipid poor apoB, suggesting that bulk lipid acquisition by apoB occurs autonomously or is mediated by other factors (71). MTP-/- mouse hepatocytes showed very few VLDL sized particles within the ER or Golgi (71), supporting the requirement of MTP for formation of large lipid droplets in the secretory pathway. Studies done in mice lacking apoB expression in the intestine showed a large amount of apoB-free, large lipid emulsions within the ER that appeared to get stuck in the secretory pathway, and never made it to the Golgi or out of the cell (72). These results demonstrated that large lipid droplets do form in the ER in the absence of apoB, but that apoB is needed to facilitate their movement out of the ER into distal compartments of the secretory pathway (72). Other factors beside MTP are involved in the second step of particle assembly. For instance, generation of the TG-rich particles in the smooth ER is dependent upon ADP-ribosylation factor 1 (ARF-1), a GTP binding protein (48), and the second step of assembly has been shown to be inhibited by liver-fatty acid binding protein (L-FABP) (65). Therefore, the second step of particle assembly also requires many enzymes and cofactors necessary for the formation of the apoB-free, TG-rich emulsions, and also for fusion of the nascent precursor particle with the TG-rich emulsions. In conclusion, MTP may play multiple roles in the
biogenesis of apoB-containing lipoproteins including: 1) chaperone activity involved in apoB folding, 2) co-translational lipidation of the apoB protein, and 3) TG transfer into the ER lumen for apoB-containing lipoprotein assembly (Figure 4) (43, 63, 71).

**Microsomal triglyceride transfer protein**

MTP was first identified based on its capacity to transfer neutral lipids, including TG and CE, between donor and acceptor membrane vesicles, in vitro. MTP is a heterodimer consisting of 58- and 97-kDa subunits. The 58-kDa subunit is the ubiquitous, multifunctional ER localized enzyme, protein disulfide isomerize (PDI), which provides stability to the heterodimer and localizes MTP to the endoplasmic reticulum (ER) by virtue of its KDEL retention signal (73, 74). The unique 97-kDa subunit is responsible for MTP’s lipid transfer functions (55). Dissociation of the MTP subunits causes aggregation of the 97-kDa subunit of MTP and abolishes its lipid transfer activity (73). Because MTP is abundant in the liver and the intestine, a possible essential role in apoB-containing lipoprotein formation was suggested. This prediction was validated by the discovery that the absence of apoB-containing lipoproteins in the human genetic disorder ABL is caused by mutations in the gene for MTP (75). The resulting severe lipid and fat soluble vitamin malabsorption phenotype associated with genetic ablation of MTP is due to the absence of chylomicron formation in the intestine and the absence of apoB-containing lipoproteins in the plasma (76-78). The exact mechanism by which MTP facilitates the initial lipidation of apoB is not understood in detail, although the ability of MTP to transfer lipids to apoB during its translation and translocation into the ER is critically important. Additionally, studies have demonstrated a direct, physical interaction between MTP and apoB (79) at specific sites within the amino-terminal domains of each protein, raising the possibility
that MTP may act as a chaperone to facilitate the proper folding of apoB (74, 80) and implying that MTP plays a chaperone like role early in the translation of apoB and the beginning stages of VLDL assembly.

It has been shown that MTP can bind to and transfer a variety of lipids between donor and acceptor vesicles in vitro (55). It is clear that MTP has a distinct preference for neutral lipids over polar lipids, with the preference being in the order of TG>CE>PL, and an ~ 80% higher preference for TG transfer relative to polar lipids such as DAG and PL (55). The transfer of PL is low, regardless of the head group or charge on the PL molecule (55). Although there is no crystal structure for MTP, because of its homology with VTG, the structure of MTP was superimposed on the known crystal structure of LV (33). The model based on these analyses suggested that, similar to the lipid-binding cavity in the crystal structure of LV, MTP also contains a small lipid-binding cavity, which may be important for in its lipid transfer activity. The lipid-binding cavity of MTP is proposed to be composed of two beta sheet regions guarded by two alpha helixes that aid in lipid acquisition and transfer. Indeed, site-directed mutagenesis of the helixes guarding the lipid-binding cavity of MTP impaired the ability of MTP to transfer lipids between donor and acceptor vesicles (33). This led to the following model for lipid transfer by MTP: 1) Helix A of MTP acts as a fusigenic peptide creating distortions in the donor membrane, possibly exposing the acyl chains of TGs and PLs, which would then interact with Helix B and the hydrophobic lipid binding cavity (33). The lipid would, therefore, be removed from the donor membrane and transferred into the lipid-binding cavity; however, the mechanism by which MTP would then transfer lipid out of the cavity to apoB was not speculated upon. However, the lipid transfer activity of MTP has led to the hypothesis that
MTP shuttles lipids from a particular site, possibly the ER membrane, to apoB during the process of lipoprotein assembly (33).

MTP is also necessary for the trafficking of TG from the cytosol, across the ER membrane into the lumen of the ER. Evidence for this function of MTP come from in vivo studies of MTP liver specific knockout mice conducted by Raabe, et al. which suggest, based on electron microscopy, that the absence of MTP leads to the accumulation of neutral lipid droplets in the cytosol accompanied by disappearance of neutral lipids within the lumen of the ER and Golgi (69). In contrast, in MTP replete but apoB deficient mouse intestine, the presence of lipid droplets in the ER and Golgi compartments was observed (72). Additionally, Wang et al. established a >50% reduction in $[^3]$H-TG and TG mass within the microsomal lumen of McA-RH7777 cells treated with the MTP inhibitor BMS-197636 (70) indicating that MTP plays a role in this process. Similar results were also observed by Kulinski, et al (71). When combined, these data indicate that MTP is necessary for both precursor lipoprotein formation (first-step assembly) (42) and bulk neutral lipid movement into the ER for precursor particle expansion (second-step assembly) (69). However, it is not known whether MTP is the only tissue specific factor required for this lipid translocation pathway. In 2002, Higashi et al. devised a cell free lipid translocation assay and noted a time dependent ~ 0.5 fold decrease in the transfer of radiolabeled TG into MTP containing acceptor microsomes in the presence of the calcium channel blocker Verapamil (81). After determining that Verapamil did not affect the transfer activity of MTP they concluded that additional factors contribute to neutral lipid translocation across the ER membrane (81). These and other data (82) may be indirect evidence that MTP is only one component of a more complex lipid translocation system.
Evolution of lipoprotein assembly pathways and dissection of MTP Function

Physiological similarities and differences in the formation, maturation, and function of apoLpII/I in invertebrates (e.g. Drosophila melanogaster) and apoB-containing lipoproteins in vertebrates have given clues about the adaptations that MTP has undergone through its evolutionary history. The formation of apoLpII/I-containing particles in insects is critical to transport stored lipids from fat body cells to flight muscle (45). In a process similar to apoB-containing lipoprotein formation in vertebrates, apoLpII/I is translated and translocated into the lumen of the ER where it is converted into a high-density lipoprotein precursor by MTP (44). This HDLp precursor particle is then secreted from the fat body cell into the hemolymph, where it functions as an acceptor particle for DG, which is transported from the cytosol across the plasma membrane by the extracellular lipid transfer particle (LTP) (45). As the HDLp accepts DG it expands to a low-density lipophorin (LDLp) particle, which then shuttles lipid to flight muscle. In contrast, apoB-containing lipoprotein precursors in vertebrate species are expanded to mature particles within the secretory pathway using TG that originated in the cytosol. The movement of TG into the ER for second step expansion, a process unique to vertebrates, requires MTP. Presumably, however, the properties of MTP in insects and vertebrates differ to reflect the acquisition of this additional lipid translocation function. In 2003 Sellers et al. demonstrated that CG9342, a gene whose product was ~20% identical to human MTP and was subsequently named Drosophila MTP (dMTP) supported the formation and secretion of apoB34 (amino-terminal 34%) in a non-lipoprotein producing cell system (40). However, dMTP displayed no TG transfer ability, in vitro, and was also resistant to the MTP inhibitor BMS-197636 (40) indicating functional and structural differences between vertebrate and invertebrate MTP. Functional differences between vertebrate and invertebrate MTP were
further explored by Rava et al. in 2006 who reported that dMTP possessed no detectable TG transfer activity, yet had PL transfer activity that was equivalent to the hMTP ortholog (Figure 5) (83). The observations above lead directly to the hypothesis to be tested in this dissertation: First step conversion of apoB and other LLTP family members into lipoprotein precursor proteins is a primordial function of MTP and requires its PL transfer activity, and possibly other chaperone-like functions (38, 69, 70, 84). However, the intracellular second-step expansion of precursor lipoproteins, a process unique to vertebrate liver and intestine, requires translocation of neutral lipids into the ER/Golgi, a process executed by MTP’s intrinsic neutral lipid transfer activity.

**Concluding remarks**

Trafficking of hepatic TG from their default localization in cytosolic lipid droplets into the ER for assembly into apoB-containing lipoproteins is central to the export of hepatic TG for storage in adipocytes, protecting the liver from hepatic lipid accumulation (steatosis), and also underlies the formation of atherogenic lipoproteins. The overall scope of this project was to define the mechanisms underlying TG translocation into the hepatocyte ER, a process that is essential and likely rate limiting for lipoprotein-mediated TG efflux. A major implication of the hypothesis is that vertebrate MTP possesses separable functions in lipoprotein assembly and that each activity could be independently targeted for therapeutic purposes. MTP possesses at least two distinct functions: priming of apoB for nascent lipoprotein formation and apoB-independent neutral lipid translocation (Figure 5). The conclusion that MTP forms at least part of the neutral lipid translocation system responsible for hepatic VLDL assembly is based on altered lipid trafficking observed upon pharmacologic or genetic inactivation of MTP in
hepatocytes and hepatoma cells (69-71). In our studies we examined the independent lipid trafficking potential of MTP using cell-based approaches. These studies established that MTP promotes TG trafficking into the ER and that the TG-transfer activity of MTP is essential for this function. Additionally, transgenic mice were generated that express either *Drosophila* (PL transfer only) or human (PL plus TG transfer) MTP in hepatocytes and we explored whether the PL and TG-transfer activities of MTP play distinct roles in resistance to hepatic steatosis. These studies have provided important insights into the intracellular lipid trafficking routes that have evolved to support whole body lipid homeostasis. Our findings could have important therapeutic implications by establishing that the PL and TG transfer activities of MTP play distinct and separable roles in vertebrate cellular lipid transport and that selective pharmacologic inhibition of PL transfer activity might reduce conversion of apoB into precursor lipoproteins without disrupting TG trafficking into the ER. As apoB particle number best predicts atherogenesis (40, 44, 47, 83) the secretion of fewer molecules of apoB on larger lipoprotein particles could protect the liver from toxic lipid accumulation while simultaneously reducing atherogenesis. This concept has been validated through studies in both mice and humans, which demonstrated that antisense oligonucleotide-mediated reduction in apoB expression reduces plasma levels of apoB, cholesterol, and TG with no adverse effects on liver lipid accumulation (84, 85).
Figure 1. Schematic representation of exogenous and endogenous lipid transport. Dietary lipids are absorbed into enterocytes and packaged into large TG-rich, apoB48-containing chylomicrons. These apoB48-containing particles are released into the circulation and the lipids are hydrolyzed by the action of LPL. The liver removes remnant chylomicrons and the lipid is stored, utilized in cellular processes, or used for lipoprotein assembly. *De novo* synthesis is another major source of TG and cholesterol. In the fasting state, TG-rich, apoB100-containing VLDL particles are released from the liver. They are converted to LDL by the action of LPL and then taken up in both hepatic and extra-hepatic cells in a regulated manner by various receptors (R). Reproduced from Packard CJ, Gaw A, and Shepherd J. *Cholesterol-lowering Agents and Their Use*. (May 2003) In: eLS. John Wiley & Sons Ltd, Chichester.
Figure 2. Evolution of the LLTPs. Vertebrates and invertebrates possess systems for the mobilization and transport of lipids. The major players in this process belong to a family of genes called the LLTPs. This family includes the lipid binding proteins Vtg, insect apoLpII/I, apoB, and MTP. Sequence analyses determined that each protein shares an amino-terminal β-sheet and an extended α-helix domain, which contains a highly conserved hydrophobic cluster. Shelness and Ledford hypothesized that MTP, as the most ancient LLTP protein, functions in the biosynthesis of all of its evolutionary descendants, including Vtg, apoLpII/I, and apoB. Reproduced with permission from Wolters Kluwer Health (Lic # 3124200486426). *Curr Opin Lipidol* 2005 June;16(3):325-32.
Figure 3. Two-step model of hepatic VLDL formation and secretion. The apoB protein is translated within the rough ER, while TG-rich apoB-free lipid droplets are formed within the smooth ER. At the convergence of the rough and smooth ER, the apoB-containing precursor particle fuses with the larger apoB-free, TG-rich lipid droplet, followed by transport to the Golgi apparatus. After glycosylation and complete maturation, the VLDL particles are secreted into the Space of Disse via vesicular trafficking and plasma membrane fusion. Reproduced from Alexander CA, Hamilton RL, Havel RJ. Subcellular localization of apoprotein of plasma lipoproteins in rat liver. J Cell Biol 1976 May; 69(2): 241-63.
Vertebrates have evolved a distinct lipid trafficking pathway involving the formation of an intracellular lipid acceptor particle containing apoB (precursor lipoprotein) and an independent lipid translocation system, which transports lipid from the cytosol into the ER lumen. In first step assembly, MTP acts on apoB cotranslationally possibly by the addition of PL to form precursor particles (pre-VLDL). In a separate function of MTP, cytosolic lipids are mobilized and brought into the ER where they form lipid droplets (secretion-coupled lipids). These lipid droplets fuse with the precursor lipoprotein to form mature chylomicrons or VLDL particles during the second step of particle assembly. The question is whether the distinct TG transfer activity of MTP is responsible for the mobilization of cytosolic lipids and the formation of apoB-free lipid droplets in the ER. This research was originally published in *Biochemical Society Transactions*. John McLauchlin, Hepatitis C virus: viral proteins on the move, *Biochem Soc Trans*, 2009, *Volume 37, pages 986-990* © the Biochemical Society.
Figure 5. hMTP and dMTP can transfer phospholipids, but dMTP is deficient in triacylglycerol transfer activity. COS cells transiently expressing hMTP and dMTP (A) or hMTP- and dMTP-FLAG (B) were assayed for TG-transfer activity using the radiolabeled vesicle transfer assay under conditions of equal protein expression. Real time transfer of fluorescently labeled triacylglycerol (C), and phosphatidylethanolamine (D) was measured using equal amounts of the purified proteins (C, inset). Reproduced from Rava P, Ojakian GK, Shelness GS, Hussain MM. Phospholipid transfer activity of microsomal triacylglycerol transfer protein is sufficient for the assembly and secretion of apolipoprotein B lipoproteins. J Biol Chem 2006 April 21;281(16):11019-27.
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Chapter II
The triglyceride transfer activity of microsomal triglyceride transfer protein promotes neutral lipid accumulation within the microsomal fraction of non-hepatic cells

Philip S. MacArthur, Li Hou, and Gregory S. Shelness
Department of Pathology Wake Forest University School of Medicine Winston-Salem, NC 27157
ABSTRACT

Microsomal triglyceride transfer protein (MTP) is a multifunctional protein necessary for conversion of apolipoprotein B (apoB) into precursor lipoproteins and bulk triglyceride (TG) movement into the endoplasmic reticulum (ER) for precursor lipoprotein expansion. Invertebrate forms of MTP (e.g. *Drosophila*; dMTP) are capable of phospholipid (PL) transfer, whereas vertebrate forms (e.g. human; hMTP) engage in PL and TG transfer. Hence, we hypothesized that PL transfer may be the primordial activity of MTP necessary for precursor lipoprotein particle formation, whereas TG transfer may be a vertebrate adaptation necessary for trafficking of TG into the ER for 2nd-step assembly. We explored this hypothesis by assessing the ability of MTP to promote TG trafficking into the ER in CHO and COS cells transiently transfected with hMTP and in a stable, Dox-inducible, hMTP CHO cell line. In all cell lines tested an ~ 2.5 - 5.0-fold increase in microsomal TG content was observed upon expression of hMTP. The lipid transfer activity of MTP was necessary for this cellular function as in the presence of BMS-212122, a potent MTP inhibitor, microsomal TG content was similar to that observed in mock-transfected cells. To determine whether the TG transfer activity of MTP is important for this lipid trafficking function, we compared the TG content of microsomes from transiently transfected hMTP and dMTP cells. As observed previously, compared to mock-transfected cells, hMTP was able to increase microsomal TG content by ~2.5-fold. In contrast, cells transfected with dMTP demonstrated no increase in microsomal TG content, despite similar levels of protein expression. Taken together, these data indicate that MTP can promote TG trafficking into the ER and that the TG transfer activity of MTP is essential for this function.
INTRODUCTION

A necessary component in hepatic and intestinal lipid homeostasis is the microsomal triglyceride transfer protein (MTP), a 97-kDa protein complexed with the ER localized enzyme, protein disulfide isomerase (PDI) (1). MTP is an essential cofactor for the formation of hepatic very low-density lipoproteins (VLDL) and intestinal chylomicrons (2), being necessary for both precursor lipoprotein formation (first-step assembly) (3) and bulk neutral lipid movement into the ER for precursor particle expansion (second-step assembly) (4). The absence or dysfunction of MTP causes the human genetic disorder, abetalipoproteinemia, which is a phenocopy of homozygous familial hypobetalipoproteinemia, which is caused by the genetic disruption of apoB. Either loss of apoB or MTP abolishes the formation of hepatic VLDL and intestinal chylomicrons causing severe lipid and fat-soluble vitamin malabsorption and gross perturbations in whole body lipid homeostasis.

A critical process needed to maintain the equilibrium of neutral lipids within the hepatocyte is the formation and secretion of VLDL. Widely considered a two-step process (5), the formation of VLDL begins with apoB undergoing cotranslational translocation into the lumen of the ER where an interaction with MTP leads to the formation and stabilization of a precursor lipoprotein (6). Leiper et al. determined the necessity of MTP in first-step assembly by demonstrating that a truncated form of apoB was not secreted from COS cells unless apoB was co-transfected with MTP (3, 7). The second step of the two-step model is the expansion of the precursor lipoprotein into a mature particle as a result of its fusion with luminal, apoB-free, neutral lipid droplets composed primarily of TG. MTP is also necessary in this phase of particle formation by facilitating the trafficking of TG from the cytosol, across the ER membrane into the lumen of the ER. Evidence for this function of MTP comes from in vivo studies of MTP
liver-specific knockout mice conducted by Raabe, et al. which suggest, based on electron microscopy, that the absence of MTP leads to the accumulation of neutral lipid droplets in the cytosol accompanied by the disappearance of neutral lipids within the lumen of the ER and Golgi (4). Additionally, Wang et al. established a >50% reduction in [\(^3\)H]-TG and TG mass within the microsomal lumen of McA-RH7777 cells treated with the MTP inhibitor BMS-197636 (8) indicating that MTP plays a role in the TG translocation process (9). These previous studies demonstrated that genetic or pharmacological disruption of MTP reduces TG content in the ER limiting the TG pool necessary for particle expansion. However, these studies were performed using tissues and cells of hepatic origin that contain all endogenous factors necessary for TG translocation into the ER. While these studies demonstrated that MTP is necessary for TG movement across the ER membrane, it could not be established whether MTP functions as an autonomous neutral lipid translocase or whether MTP is only one essential component of a series of factors required to promote lipid flux into the ER.

To begin to address this question, we utilized non-hepatic cell lines lacking MTP, apoB, and presumably other possible factors and processes necessary for VLDL formation. Human MTP (hMTP), either transiently transfected or under the control of an inducible promoter, was introduced into Chinese hamster ovary (CHO-K1) or African green monkey (COS) cells and the relationship between MTP expression and the trafficking of TG into the microsomal fraction was assessed. To test the hypothesis that the TG transfer activity is required for TG trafficking into the ER, we compared the abilities of hMTP and Drosophila MTP (dMTP) (TG transfer incompetent isoform) to promote TG trafficking in transiently transfected COS-1 cells. Data from these studies support the hypothesis that MTP expression on its own may be necessary and sufficient for bulk neutral lipid movement into the ER for precursor particle expansion (4)
and that the TG transfer activity, unique to vertebrate forms of MTP, is essential for this cellular function.
EXPERIMENTAL PROCEDURES

Cell Culture

COS cells were grown in DMEM (Gibco) containing high glucose (4.5 g/L), 10% FBS, penicillin (100 u/mL), and streptomycin (100 µg/mL). CHO-K1 cells were cultured in Ham’s/DMEM (50:50) containing 10% FBS, antibiotics, and vitamins. CHO-K1/F-hMTP inducible cells were maintained in the same medium but with the addition of 400 µg/mL G418 and hygromycin to maintain selective pressure. All cell lines were maintained at 37°C in an atmosphere containing 5% CO₂.

Transfection of cells

COS and CHO-K1 cells were transfected by either the FuGene 6 or FuGene HD (Roche Molecular Biochemicals, Indianapolis, IN) method using a 2:1 (volume:mass) ratio of FuGene to DNA. Cells were transfected following the manufacturer’s instructions at 50-60% confluence (except where indicated).

Subcellular fractionation and carbonate extraction

CHO-K1, CHO-K1/F-hMTP inducible, and COS cell lines were incubated with 0.8 mM oleate complexed to fatty acid-free bovine serum albumin (BSA) and radiolabeled with [³H]-oleate (10 µCi/mL) for 4 or 24 hours, as indicated. Cells were washed twice with sterile 1 X balanced salt solution (BSS) then gently scraped from the dish in 3 mL of BSS into a 15 mL conical centrifuge tube. Cells were centrifuged at 1,000 rpm for 10 minutes to pellet cells, then the supernatant was aspirated and the cells were resuspended in 1 mL BSS and transferred to a 1.5 ml microcentrifuge tube. The cells were microcentrifuged at full speed for ~ 10 seconds.
The supernatant was aspirated and the cell pellet was overlaid with 0.5 mL cold hypotonic buffer (10 mM Hepes, pH 7.4, 1 mM PMSF, 10 µg/ml leupeptin and pepstatin). The tube was inverted once and cells centrifuged ~ 5 seconds at full speed. The supernatant was removed and then cells were resuspended in an additional 0.5 mL hypotonic buffer. After incubating on ice for 15 minutes the cells were Dounce homogenized (tight “B” pestle). After 20 strokes, 2 M sucrose was added to a final concentration of 250 mM followed by homogenization with 1–3 additional strokes. The homogenate was transferred to a microcentrifuge tube and centrifuged 10 minutes at 1000 x g in 4°C to pellet nuclei and cell debris. The post-nuclear supernatant was transferred to a 11 x 34 mm polycarbonate tube (Beckman) placed in a TLA 100.2 or 120.2 rotor (Beckman) and centrifuged at 240,000 x g (70,000 rpm) for 20 minutes at 4°C in a Beckman tabletop ultracentrifuge (TL-100). The supernatant was removed and the microsomal fraction was washed twice with membrane buffer (10 mM HEPES, pH 7.4, 250 mM sucrose, 10 mM NaCl, 10 mM KCl, 2.5 mM CaCl₂, 2.5 mM MgCl₂, 1 mM PMSF, 10 µg/mL leupeptin and pepstatin) before being resuspended by Dounce homogenization in 0.5 mL membrane buffer (0.3 mL if continuing to carbonate extraction, as described below).

Carbonate extraction. The resuspended microsomes were transferred to a polycarbonate tube to which 33.3 µL of 1 M sodium carbonate, pH 11.5 was added. The microsomes were incubated on ice 1 hour then centrifuged at 240,000 x g for 20 minutes at 4°C. To conduct lipid extraction and analysis, the lumenal contents (supernatant) were transferred into a 12 mL glass tube. Additionally, the microsomal membranes were washed twice with membrane buffer, resuspended in 0.5 mL of membrane buffer and also transferred into a 12 mL glass tube for lipid extraction and analysis.
**Lipid extraction and thin layer chromatography (TLC)**

Lipids were extracted from total microsomes or the lumen and membrane fractions by the method of Bligh and Dyer (10). Samples were adjusted to 0.5 mL with phosphate-buffered saline (PBS) before the addition of 1.88 mL chloroform: methanol (1:2), 10 µL of 20 mg/mL cold triolein and egg yolk lecithin (used as internal standards), and 0.626 mL of chloroform followed by vortexing. Samples were acidified by addition of 0.626 mL of 0.05 % sulfuric acid and vortexed again. After centrifugation at 1,500 rpm for 10 minutes at room temperature, the aqueous phase was removed by aspiration. The organic phase was dried at 60°C under a stream of nitrogen, redissolved in 50 µL of chloroform, and applied to a polyester-backed silica gel TLC plate (PE SIL G, Whatman). Plates were first developed with chloroform: methanol: acetic acid: water (65:45:12:6) by running the solvent front ~ ⅓ up the length of the plate. The plate was air dried and then developed further with heptane: ethyl ether: acetic acid (90:30:1). After air-drying, lipid standards were visualized by incubation in iodine vapor and areas containing PL and TG were cut from the plate and the radioactivity was quantified by liquid scintillation counting.

**MTP inhibitor BMS-212122**

The MTP inhibitor, BMS-212122, was acquired from Bristol-Myers Squibb via a standing Material Transfer Agreement. The inhibitor was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at -80° C. The inhibitor was added to cells at the indicated concentrations such that the final DMSO concentration in all dishes was equivalent and did not exceed 0.05%.
Detection of MTP by immunoblotting

Samples containing indicated amount of microsomal protein or total cell protein, as determined by a BCA assay (Pierce) were resolved by 8% SDS-PAGE. After electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in 5% non-fat milk powder dissolved in a solution of 10 mM phosphate, pH 7.4, 150 mM NaCl and 0.1% Tween-20 (PBS-T) overnight at 4°C while shaking. The membrane was then incubated 2 hours at room temperature in mouse anti-FLAG M2 (Sigma) at a concentration of 2.5 µg/mL in PBS-T containing 2.5% non-fat milk powder. The membrane was washed in PBS-T for 1 hour then incubated for 1.5 hours in anti-mouse IgG conjugated to horseradish peroxidase (Sigma) at a dilution of 1:4000 in PBS-T containing 2.5% non-fat milk powder. This was followed by a 1-hour wash in PBS-T before the immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Blue Ultra Autorad film (Genemate).

Metabolic labeling and immunoprecipitation

Transfected cells were metabolically radiolabeled with 100 µCi/mL [35S]Met/Cys (EasyTag Express Protein Labeling Mix; Perkin Elmer Life Sciences) in Met- and Cys-deficient DMEM for the times indicated. ApoB from media and cell lysates was immunoprecipitated with goat anti-human apoB (Academy Biomedical, Houston, TX), and fractionated by SDS-PAGE. Dried gels were exposed to BioMax MS film in combination with a BioMax TransScreen-LE intensifying screen (Kodak) at −80°C.
**Determination of total cellular lipids**

When the CHO-K1/F-hMTP inducible cells reached 80% confluent they were washed twice with 1x BSS. Cells were then incubated in media only or media containing 1 µg/mL Dox. After 48 hours, cells were washed three times with BSS and lipids were extracted from the cells using hexane:isopropanol (3:2). Extracted lipids were dried under nitrogen then resuspended in chloroform before conducting TLC to separate PL, TG, and CE, as described above. Bands were viewed with iodine vapors, cut from plate and radioactivity was determined by scintillation counting.

**Statistical analysis**

Statistical analysis was performed using unpaired Student’s *t*-tests using GraphPad Prism (GraphPad Software, San Diego, CA). Unless otherwise noted, n=3 for each group or condition. Results are expressed as the mean ± SE. A *p*-value of less than 0.05 was regarded as statistically significant.
RESULTS

Microsomal TG content increases when hMTP is transiently transfected into non-hepatic cells

As a preliminary test of whether MTP functions as an autonomous lipid translocase and, therefore, affects the distribution of neutral lipid within cells, COS-7 or CHO-K1 cells were transiently transfected with hMTP or mock-transfected. The cells were allowed to accumulate hMTP for 24 hours post-transfection before the cells were incubated for an additional 24 hours with BSA-oleate and [³H]-oleate to simultaneously stimulate TG production and radiolabel the cellular lipid pool. To assess subcellular distribution of radiolabeled TG, lipids were extracted from the microsomal fraction by the method of Bligh-Dyer (10), separated by TLC utilizing sequential polar and neutral solvent systems and visualized by exposure to iodine vapor. The bands corresponding to PL and TG were cut from the TLC plate and radioactivity quantified by scintillation counting. At this point we compared the MTP-dependent increase in lipid within the microsomal membrane or lumen by comparison to mock transfected cells. Compared to mock-transfected cells, an ~ 2.5- and 5.0-fold increase in microsomal TG content was observed in COS-7 and CHO-K1 cells expressing hMTP, respectively (Figure 1A). To further validate this observation we generated CHO-K1 cells stably transfected with hMTP. These cells displayed an ~ 3.0-fold increase in microsomal TG compared to wild type CHO-K1 cells (Figure 1B) when expressed as a ratio of [³H]-TG to [³H]-PL. This normalization technique is based on the assumption that MTP’s capacity to engage in TG trafficking should be much higher than PL.
Hence, PL levels will remain relatively static or increase to a much lower extent than TG. Use of this ratio also has the advantage that it is a highly controlled value in that the measurements come from the same load of the TLC plate. These data indicate that the expression of hMTP in non-hepatic cell lines can induce a significant increase in the TG:protein and the TG:PL ratio of isolated microsomes.

**The effect of the MTP inhibitor BMS-212122 on TG translocation**

To confirm that it is MTP’s lipid transfer activity that drives TG redistribution and not other endogenous factors that may be induced by MTP over-expression or other non lipid transfer-related functions of MTP (1), the specific and potent MTP inhibitor BMS-212122 (11) was employed (Figure 2). Previous studies demonstrated that BMS-212122 reduced apoB100 secretion from HepG2 cells by 50% at a concentration 0.03 nM (11). In the studies performed here, COS cells transfected with hMTP displayed a 2.5-fold increase in microsomal [\(^3\)H]-TG (Figure 3A), which confirms our previous results. When cells transfected with hMTP were treated with 5 µM BMS-212122, [\(^3\)H]-TG content of the microsomal fraction was similar to that in the mock-transfected control cells. Importantly, the protein expression of transiently transfected hMTP in the cells treated with or without inhibitor was equivalent (Figure 3B). These data indicate that it is the lipid transfer activity of MTP that is essential for TG translocation and not other putative activities that have been postulated for MTP (1).
Generation of a CHO-K1 cell line with doxycycline (Dox)-inducible expression of FLAG-epitope tagged hMTP (F-hMTP)

To further explore the ability of hMTP to increase the TG content of the microsomal fraction, we established a system in which the expression of MTP in a clonal cell line could be acutely regulated. In addition, to better monitor hMTP expression we employed an amino-terminal FLAG-tagged form of hMTP (F-hMTP). The functional competence of F-hMTP was characterized by monitoring its ability to convert apoB41 (amino-terminal 41%) into a secrutable lipoprotein. Therefore, COS cells were transiently transfected with apoB41 with and without MTP cotransfection (3). Twenty-four hours after transfection, cells were radiolabeled with $[^{35}\text{S}]$-Met/Cys (100 µCi/mL) for 4 hour and apoB41 in the cell lysates (C) and the media (M) was analyzed by immunoprecipitation, followed by SDS-PAGE and fluorography. As observed, in Figure 4, alkaline phosphatase (AP) transfected control cells synthesized apoB41 but were unable to secrete it into the medium (Figure 4, Lanes 1 and 2). However, when apoB41 was cotransfected with native hMTP, abundant apoB41 synthesis and secretion was observed (Figure 4, Lanes 3 and 4). Likewise, cells cotransfected with F-hMTP supported the synthesis and secretion of apoB41 (Figure 4, lanes 5-8), to a similar extent as native hMTP. Hence, the addition of the FLAG epitope to the N-terminus of mature MTP did not interfere with its ability to promote apoB secretion.

To control the expression of F-hMTP, the non-hepatic cell line, CHO-K1, was cotransfected with F-hMTP cloned into the expression vector pTRE2hyg along with the plasmid pTet-On (Clontech). The pTet-On plasmid expresses a Dox-dependent transactivator protein that controls the transcription of the F-hMTP gene, resulting in a regulated and tunable protein expression. Selection of monoclonal cell lines expressing F-hMTP was achieved by
incubating the cells in 800 µg/mL each of the antibiotics G418 and hygromycin. The clonal cell lines were then screened for F-hMTP expression by incubating the cells with 1 µg/ml Dox for 24 hours and monitoring MTP expression by immunoblot analysis. Results demonstrated that 11 cell lines displayed some basal expression (in the absence of Dox) and an increase in MTP expression when incubated 24 hours in the presence of Dox (data not shown). In addition, we established that over a 48-hour time course, near maximal accumulation of F-hMTP occurred at 24 hours with very little to no additional accumulation seen at 48 hours (Figure 5). The clonal cell line in figure 5 was employed in subsequent experiments as it consistently displayed robust expression of MTP with the addition of Dox in combination with a low basal level of MTP expression in the uninduced state.

**Effect of F-hMTP expression on subcellular neutral lipid distribution in Dox-inducible CHO-K1 cells**

Inducible F-hMTP cells identified above were used to assess the relationship between hMTP expression and the TG content of the microsomal fraction. Cells were plated at ~ 50% confluency in 100-mm dishes and cultured overnight. Cells were then incubated for twenty-four hours with [³H]-oleate (10 µCi/mL) and 0.8 mM oleate complexed to BSA in the presence and absence of 1 µg/mL Dox. Under these conditions, F-hMTP synthesis and TG accumulation were simultaneously induced. To assess the subcellular distribution of radiolabeled lipid, cells were Dounce homogenized and the post-nuclear supernatants were then subjected to centrifugation to obtain the microsomal fraction. Lipids were extracted and then separated by thin layer chromatography (TLC). The bands corresponding to PL and TG were cut from the TLC plate and radioactivity quantified by scintillation counting. When normalized to total cell
protein there was an approximately 2-fold increase of $[^{3}\text{H}]-\text{TG}$ within the microsomal fraction of Dox-treated cells (+Dox) relative to the control (-Dox) cells (Figure 6A). To further confirm this result, we also expressed the result as a ratio of $[^{3}\text{H}]-\text{TG}$ to $[^{3}\text{H}]-\text{PL}$ (Figure 6B), which revealed an ~ 50% increase. These increases in microsomal TG content appear to be a direct result of increased MTP expression as a result of Dox treatment (Figure 6C). Furthermore, the $[^{3}\text{H}]-\text{TG}$ content of the cytosol was measured to determine whether hMTP produced a corresponding decrease in cytosolic TG; however, perhaps because of the much larger pool of cytosolic TG, the increase in microsomal TG was not accompanied by a detectable decrease in the cytosolic fraction (data not shown). Nonetheless, these experiments further confirm that in a non-hepatic cell system, the relative abundance of TG within the microsomal fraction increases as a function of hMTP expression.

**Examination of TG accumulation in the ER membrane and lumen after carbonate extraction**

The experiments thus far have focused on assaying radiolabeled TG abundance in total microsomal fractions. However, to further explore hMTP’s capacity to traffic TG in non-hepatic cells, isolated microsomal pellets from wild type, uninduced (-Dox) and induced (+Dox) F-hMTP/CHO-K1 cells were subjected to alkaline extraction using sodium carbonate, pH 11.5 (12), to obtain separate lumenal and membrane fractions. Lipids were then extracted and analyzed as described previously. In the membrane fraction, uninduced F-hMTP/CHO-K1 cells displayed a 5-fold increase in $[^{3}\text{H}]-\text{TG}$ compared to wild type CHO-K1 control cells (Figure 7). This increase may be attributed to basal F-hMTP expression (Figure 5). However, the cells that were treated with Dox displayed an approximate 10-fold increase in $[^{3}\text{H}]-\text{TG}$ compared to
the wild type CHO-K1 control cells and a 2-fold increase compared to the uninduced cells (Figure 7). Likewise, there was a 2.5-fold increase of radiolabeled TG in the microsomal lumen fraction of cells induced with Dox (Figure 7), relative to both the wild type CHO-K1 control cells and the uninduced cells. To further validate these results we confirmed that Dox on its own exerted no effect on CE, PL, or TG biosynthesis and accumulation (Figure 8). Taken together these data indicate that the expression of MTP causes a reproducible increase in the TG content of the microsomal fraction, which perhaps reflects its autonomous role in the trafficking of lipids into the ER for the creation of secretion-coupled lipid for VLDL assembly.

Examination of TG distribution in dMTP transfected COS cells

Although previous work in our laboratory demonstrated that dMTP, and presumably other invertebrate forms of MTP, have the capacity to convert truncated forms of apoB (e.g., apoB41) into precursor lipoprotein particles (13), we hypothesized that dMTP would fail to induce a redistribution of TG into the ER due to its lack of neutral lipid transfer activity (13). The relationship between MTP lipid transfer specificity and the intracellular trafficking of TG was assessed by transient transfection of native hMTP or dMTP into COS cells. The expression of hMTP in COS cells increased the $[^{3}$H]-TG content of microsomes by ~ 2-fold, relative to mock transfected control cells (Figure 9). However, as predicted, cells transfected with dMTP failed to produce a similar increase (Figure 9). To ensure that the differential ability to promote microsomal lipid accretion was not due to differential levels of expression, we created epitope tagged forms of the two MTP orthologs. Unlike hMTP, an amino-terminal FLAG tag on dMTP affected its ability to support the synthesis and secretion of apoB (Figure 10, lanes 5-8). However, when the carboxy-terminal FLAG tag was added to dMTP and hMTP they displayed
equivalent capacities to promote the secretion of apoB34, which were in turn similar to the activities seen for the native proteins (Figure 11). Use of the epitope tagged forms of MTP allowed us to monitor and, if necessary, normalize the relative levels of dMTP and hMTP expression.

Differences in lipid distribution achieved by dMTP versus hMTP were then assessed in a side-by-side comparison of cell lines transfected with dMTP-F or hMTP-F. COS-7 cells were transiently transfected using FuGENE HD with dMTP-F, hMTP-F or human serum albumin modified with the ER retention signal KDEL (HSA-KDEL). We chose HSA-KDEL as the control protein as it will accumulate within the ER similar to dMTP-F and hMTP-F. The cells were allowed to accumulate protein for 48 hours post-transfection before the cells were incubated for an additional 4 hours with BSA-oleate and [3H]-oleate to simultaneously stimulate TG production and radiolabel the cellular lipid pool. To assess the subcellular distribution of radiolabeled TG, lipids were extracted from the microsomal fraction by the method of Bligh-Dyer (10), separated by TLC utilizing sequential neutral and polar solvent systems and visualized by exposure to iodine vapor. The bands corresponding to PL and TG were cut from the TLC plate and radioactivity quantified by scintillation counting. The expression of hMTP in COS cells increased the [3H]-TG content of microsomes by ~2-fold, relative to the HSA-KDEL transfected control cells (Figure 12). However, as predicted, cells transfected with dMTP failed to produce a similar increase (Figure 12). Under conditions of equivalent protein expression determined by relative mass measurements using immunoblot analysis (Figure 12), data from this experiment corroborates our earlier finding that hMTP, and not dMTP, facilitates the trafficking of neutral lipid into the ER creating bulk-neutral lipid for second-step particle expansion. And these data substantiate that the TG transfer activity of hMTP is critical for this cellular process.
DISCUSSION

Previous studies have demonstrated that all orthologs of MTP, ranging from invertebrate (i.e. Drosophila melanogaster) to vertebrate (i.e. human), have the ability to transfer PL between donor and acceptor membrane vesicles, in vitro. Further, all orthologs of MTP are competent to convert all LLTP family members into precursor lipoproteins. However, unique to vertebrate (e.g. human) MTP is the ability to engage in neutral lipid transfer (13). We hypothesized that this function of MTP, which likely evolved during chordate evolution (14), may be a key adaptive function responsible for the trafficking of neutral lipids, predominantly TG, into the ER lumen for second-step apoB particle expansion.

The goal of the current studies was to establish a cell-based system in which the effects of MTP on the trafficking of TG from the cytosol into the ER lumen could be assessed independently of processes and other possible factors that modulate VLDL assembly in native lipoprotein producing cells. For this purpose we chose the non-hepatic, non-lipoprotein producing African green monkey (COS) or Chinese hamster ovary (CHO-K1) cell lines, neither of which express endogenous apoB or MTP (15). We hypothesized that if MTP expression in non-hepatic cells promoted TG translocation into the secretory pathway, the lack of apoB would result in the accumulation of measurable TG within the lumen of the ER. As predicted, the expression of human MTP in both non-hepatic cell models led to a reproducible increase in total microsomal TG content (Figure 1).

Although it might be assumed that increased microsomal lipid content achieved by expression of human MTP is due to its lipid transfer activity, numerous other studies and hypotheses have highlighted functions of MTP that may be unrelated to its capacity to engage in monomolecular lipid transfer between membrane vesicles, in vitro (1, 16). In addition, there is
the possibility that the overexpression of MTP in these cells could induce other genes involved in lipid biosynthesis or trafficking that either directly or indirectly impact microsomal TG content. To address these concerns we used a potent MTP inhibitor, BMS-212122 (Figure 2) (11), to assess how the selective inhibition of MTP’s lipid transfer activity may impact the changes in cellular TG distribution observed in Figure 1. These data revealed that while cells transfected with hMTP displayed a 2.5-fold increase in microsomal \[^3H\]-TG, treatment of this cell population with 5 µM BMS-212122 reduced microsomal \[^3H\]-TG content to levels seen in mock-transfected control cells (Figure 3A). Although it is known that BMS-212122 inhibits the TG transfer activity of MTP, it is not known to what extent the inhibition also impacts PL transfer. Hence, while these studies confirm that the lipid transfer activity of MTP is essential to promote microsomal accumulation of TG, the relative contribution of the TG and PL transfer activity cannot be dissected using this approach.

Although the studies above using transiently transfected cells provided initial evidence for the capacity of hMTP to promote TG accretion within the microsomal fraction, we chose to explore this phenomenon more closely using a clonal cell system where MTP expression could be acutely regulated. For this purpose we generated a CHO cell line that expressed an amino-terminal FLAG tagged form of hMTP (F-hMTP) under the control of a Dox-inducible promoter. Again, as predicted from the results displayed in Figures 1-3, we observed an approximately 2-fold increase of \[^3H\]-TG within the microsomal fraction of Dox-treated cells (+Dox) relative to the control (-Dox) cells (Figure 6). Hence, using several independent systems, we consistently observed an increase in the TG content of microsomes upon expression of MTP in non-hepatic cells, demonstrating for the first time a possible autonomous role of MTP in achieving net lipid
movement into the ER in the absence of both apoB as well as other proteins and pathways that may be unique to native lipoprotein producing cells.

Although our initial goal was to explore the possible MTP-dependent increase in TG content of the entire microsomal fraction, we also sought to dissect the relative contribution of the microsomal membrane and lumenal compartments in lipid sequestration. As expected, both the membrane and lumenal fractions participated in TG acquisition in response to MTP expression (Figure 7). While the presence of TG in the luminal fraction can be rationalized as representing the formation of lipid droplets, which would subsequently participate in second-step assembly, the basis for the membrane component is less clear. In both lipoprotein and non-lipoprotein producing cells, cytosolic lipid droplets undergo lipolysis by triglyceride hydrolases (TGH) (17). DAG released from lipid droplets as well as DAG formed by de novo synthesis, via the glycerol-3-phosphate pathway, are acted on by DGAT1 or DGAT2, both of whose active sites are believed to be located on the cytosolic face of the ER membrane (18). Although TG is likely formed in the cytosol, it rapidly partitions into the ER membrane which, like most bilayers systems, is able to solubilize ~ 2-3 mole % of neutral lipid. However, when this solubility limit is exceeded, phase transitions occur which result in the formation of cytosolic lipid droplets (19). In lipoprotein producing cells, or upon exogenous expression of MTP, an alternative trafficking route may ensue, which requires the TG transfer activity of MTP. The presence of MTP in the ER lumen may remove TG from the ER membrane and deposit it in the ER lumen, thereby enabling a system for cytosol to ER luminal TG flux. The 10-fold increase in membrane associated TG we observed may be an intermediate in the trafficking of TG across the membrane into the ER lumen. Alternatively, it is possible that membrane associated TG represents a separate pool of TG, perhaps dedicated to the
formation of first-step lipoprotein precursor particles. In either case, the concept that MTP may provide a trafficking pathway for cytosolic TG to reach the ER membrane and lumen might also suggest that MTP expression would reduce cytosolic lipid content. To test this, the $[^3]H$-TG content of the cytosol was measured in the absence and presence of Dox; however, perhaps because of the much larger pool of cytosolic TG, relative to microsomal, the increase in microsomal TG was not accompanied by a detectable decrease in the cytosolic fraction. Hence, no precursor-product relationship could be established between cytosolic and lumenal TG, although future studies could more specifically be designed to address this question.

Another element of the hypothesis that was tested here suggests that the TG transfer activity, which is associated with only vertebrate forms of MTP, is necessary to promote increased microsomal TG content. Although selective inhibitors of MTP’s PL and TG transfer activities have not been reported, we took advantage of the different lipid transfer activities of human and Drosophila MTP to assess whether their expression would have different consequences with respect to cellular TG distribution (20-22). As demonstrated in Figure 12, compared to mock-transfected cells, hMTP increased microsomal TG content by ~2.5-fold while cells transfected with dMTP demonstrated no significant increase in microsomal radiolabeled TG. These data indicate that MTP’s capacity to promote TG trafficking into the ER is indeed dependent upon its TG transfer activity. These data also support the hypothesis proposed earlier, that first step conversion of apoB and other LLTP family members may be a primordial function of MTP and requires its PL transfer activity and possibly also chaperone-like functions (22-25). However, the intracellular second-step expansion of precursor lipoproteins, a process unique to vertebrate liver and intestine, requires translocation of neutral lipids into the ER/Golgi, a process executed by MTP’s neutral lipid transfer activity. Hence, the
side-by-side comparisons of neutral lipid trafficking achieved by these two distant MTP orthologs has provided direct evidence of an important evolutionary adaptation in vertebrate species, which underlies their capacity to transport neutral lipid via the protein secretory pathway rather than across the plasma membrane, as occurs in invertebrate fat body cells (26).

Although the experiments above established that hMTP is capable of reconstituting lipid translocation into the microsomal fraction of non-hepatic cells, questions still remain regarding these experimental outcomes. First, these experiments did not directly establish whether such trafficking is quantitatively similar to that achieved by endogenous MTP in hepatic cells. Another issue is whether factors other than MTP also participate in TG translocation. This possibility was highlighted by Higashi et al. who conducted both cell-based and cell-free lipid translocation assays and noted a time dependent ~ 0.5-fold decrease in the transfer of radiolabeled lipids into hepatic microsomes in the presence of the calcium-channel blocker Verapamil (27). As Verapamil was shown to have no effect on cell free MTP-mediated lipid transfer itself, these experiments were interpreted to mean that, in addition to MTP, a Verapamil-sensitive factor was required to achieve efficient flux of TG into the microsomal compartment. The possibility of other factors beside MTP being necessary for TG translocation was also addressed when Herskovitz, et al. determined that C127 cells, a non-hepatic, breast carcinoma line, could effectively secrete apoB41 (28). However, in another study, Sellers and Shelness demonstrated, based on several criteria, that C127 cells possess MTP levels corresponding to 10-20% of those observed in the hepatoma cell line, HepG2. Hence, while it is possible that other factors may modulate TG trafficking into the ER, it appears that MTP plays an essential role in this process.
In conclusion, the studies performed here represent efforts to directly test hypotheses first formulated by phylogenetic and molecular characterization of LLTP family members. The finding that the PL and TG transfer activities of MTP play distinct and separable roles in vertebrate cellular lipid transport may have important therapeutic implications in that selective pharmacologic inhibition of PL transfer activity might reduce conversion of apoB into precursor lipoproteins without disrupting TG trafficking into the ER. As apoB particle number best predicts atherogenesis (29-32) the secretion of fewer molecules of apoB on larger lipoprotein particles could protect the liver from toxic lipid accumulation while simultaneously reducing atherogenesis. This concept has been partially validated in studies in both mice and humans, which demonstrated that antisense oligonucleotide-mediated reduction in apoB expression reduces plasma levels of apoB, cholesterol, and TG with limited adverse effects on liver lipid accumulation (33, 34).
Figure 1. hMTP facilitates the trafficking of TG into the microsomal fraction in COS and CHO cells. A) Either COS-7 or CHO-K1 cells, as indicated, were transiently transfected with hMTP or treated with transfection reagent (FuGENE 6) alone (Mock). Twenty-four hours post-transfection cells were incubated in the presence of 0.8 mM oleate and 10 µCi/mL [³H]-oleate for 24 hours. Cells were then homogenized and microsomes obtained by differential centrifugation. Lipids were extracted from microsomes by the method of Bligh and Dyer, separated by TLC, and TG bands excised and quantified by scintillation counting. DPM were normalized to microsomal protein (n=2). Error bars represent the difference from the mean. B) CHO-K1 cells stably transfected with hMTP were incubated in the presence of 0.8 mM oleate and 10 µCi/ml [³H]oleate for 24 hours. Cells were then homogenized and microsomes obtained by differential centrifugation. Lipids were extracted from microsomes by the Bligh-Dyer method, separated by TLC, and the PL and TG bands were excised and quantified by scintillation. The data is expressed as a ratio of [³H]-PL:[³H]-TG (n=3). Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. * indicates p<0.05
Figure 2. Structure of BMS-212122. BMS-212122 is a benzimidazole-based analogue of the potent MTP inhibitor BMS-201038. Substitutions of small alkyl groups on the benzimidazole ring led to a dramatic increase in potency, both in a cellular assay of apoB secretion and in animal models of cholesterol lowering.
Figure 3. The lipid transfer activity of hMTP is responsible for the trafficking of TG from the cytosol to the microsomal fraction. A) COS-7 cells were transiently transfected with hMTP or treated with transfection reagent (FuGENE HD), as indicated. Twenty-four hours post-transfection cells were treated in the absence (DMSO) or presence of the MTP inhibitor BMS-212122 (5µM). After 2 hours, cells were incubated with 0.8 mM oleate and 10 µCi/ml [³H]-oleate for 4 hours (+) or (-) the inhibitor. Cells were homogenized and microsomes obtained by differential centrifugation. Lipids were extracted from microsomes by the Bligh-Dyer method, separated by TLC, and TG bands excised and quantified by scintillation counting. DPM were normalized to total cell protein (n=3). Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. B) Immunoblot displaying the expression of hMTP in the transiently transfected cells. Twenty µg of total protein was loaded on an 8% SDS-PAGE gel. C) Quantification of immunoblot using ImageJ software (NIH). Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. Different letters indicate a p-value <0.05, nd = not determined.
Figure 4. Analysis of N-terminal FLAG-tagged Human MTP. COS-7 cells were transiently transfected with apoB41. Twenty-four h after transfections cells were labeled for 4 hours with $[^{35}\text{S}]$-Met/Cys (100 µCi/mL). Immunoprecipitation of apoB41 followed by SDS-PAGE and fluorography was used to monitor synthesis and secretion of apoB41. C = cell, M = media. The position of apoB41 (B41) and the MTPs, which co-immunoprecipitate with apoB, are indicated.
Figure 5. Treatment of CHO-K1/F-hMTP inducible cells with 1 µg/mL Dox results in the cellular accumulation of F-hMTP. CHO-K1 cells stably transfected with F-hMTP under the control of a Dox-dependent promoter were treated without (-) or with (+) 1 µg/mL Dox, as indicated. Cell lysates were collected at indicated time points and 50 µg of total cell protein was subjected to SDS-PAGE, electrophoretic transfer to PVDF membrane and Western blotting with anti-FLAG monoclonal antibody M2 (Sigma). Exposure time = 10 sec.
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-: present, +: absent
Figure 6. Induction of hMTP leads to increased TG accumulation in the microsomal fraction. CHO-K1 cells stably transfected with F-hMTP under the control of a Dox-dependent promoter were treated without (-) or with (+) 1 µg/mL Dox, as indicated and, 0.8 mM oleate and 10 µCi/mL [3H]-oleate for 24 hours. Cells were then homogenized and microsomes obtained by differential centrifugation. Lipids were extracted from microsomes by the Bligh-Dyer method, and separated by TLC. The bands corresponding to PL and TG were excised and quantified by scintillation counting. The data are normalized to total cellular protein (DPM [3H]-TG/ µg cell protein) (A) or expressed as a ratio ([3H]-PL: [3H]-TG) (B). C) Immunoblot displaying the expression of hMTP in cells treated without (-) or with (+) 1 µg/mL Dox. Each lane, was loaded with 50 µg total cell protein, represents an individual dish. For A and B, statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. * indicates a p-value <0.05, (n=3).
Figure 7. Induction of hMTP causes an increase in the TG content of both the microsomal lumen and membrane. CHO-K1 wild-type cells were mock transfected (FuGENE HD transfection reagent only) and CHO-K1 cells stably transfected with F-hMTP under the control of a Dox-dependent promoter were treated without (-) or with (+) 1 µg/mL Dox, as indicated. Each group was incubated with 0.8 mM oleate and 10 µCi/mL [3H]-oleate for 24 hours. Cells were then homogenized and microsomes obtained by differential centrifugation. Microsomes were then incubated in 0.1 M sodium carbonate, pH 11.5, to release the contents of the microsomes. After pelleting, A) the pellet (membrane fraction) and B) the supernatant (lumenal contents) were subjected to lipid extraction by the Bligh-Dyer method, separation by TLC and quantification of the excised TG bands by scintillation counting. The data is normalized to total cell protein. Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. Different letters indicates a p-value <0.05 (n=3),
Figure 8. Induction of hMTP with Dox has no impact on total cellular lipid content. CHO-K1 cells stably transfected with F-hMTP under the control of Dox-dependent promoter were treated without (-) or with (+) 1 µg/mL Dox in the presence of 0.8 mM oleate and 10 µCi/mL [3H]-oleate for 48 hours. Total cellular lipids were extracted using hexane/isopropanol (3:2). Lipid classes (CE=cholesterol ester, PL= phospholipid, TG = triglyceride) were separated by TLC, excised and quantified by scintillation. CPMs are reported by dish (n=3). Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. There was no statistical significance in each lipid class with Dox treatment.
Figure 9. The trafficking of TG from the cytosol to the microsomal fraction is dependent on triglyceride transfer activity of MTP. CHO-K1 cells transiently transfected with hMTP, dMTP or transfection reagent alone (Mock) were incubated for 4 hours in the presence of 10 µCi/mL [³H]-oleate and 0.8 mM oleate complexed to BSA. Cells were then homogenized and the microsomal fraction obtained by differential centrifugation. Lipids were extracted from microsomes by the Bligh-Dyer method, separated by TLC, and the TG bands excised and quantified by scintillation counting. DPM were normalized to total cell protein (n=3). Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. Different letters indicate a p-value <0.05.
Figure 10. Comparison of native and amino-terminally FLAG-tagged forms of human and Drosophila MTP COS Cells were transiently transfected with apoB41 and the indicated form of MTP or alkaline phosphatase (AP). Twenty-four hours after transfection, cells were labeled for 4 hours with $^{35}$S-Met/Cys (100 µCi/mL). ApoB41 from cell lysates (C) and media (M) was recovered by immunoprecipitation followed by SDS-PAGE and fluorography. The position of apoB41 (B41) and the MTPs, which co-immunoprecipitate with apoB, are indicated.
Figure 11. Comparison of native and c-terminally FLAG-tagged forms of human and Drosophila MTP. COS cells were transiently transfected with apoB34F (C-terminally FLAG-tagged form of apoB34) and the indicated form of MTP or with transfection reagent alone (None). Twenty-four hours after transfection, cells were labeled for 4 hours with $^{35}\text{S}$-Met/Cys (100 µCi/mL). ApoB34F from cell lysates (C) and media (M) was recovered by immunoprecipitation followed by SDS-PAGE and fluorography. The position of apoB34F and the MTPs, which co-immunoprecipitate with apoB, are indicated.
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- MTP
- apoB34F
- MTPs
Figure 12. The trafficking of TG from the cytosol to the microsomal fraction is dependent on triglyceride transfer activity. CHO-K1 cells were transiently transfected with hMTP-F, dMTP-F, or transfection reagent alone (Mock). Twenty-four hours after transfection, cells were incubated for 4 hours in the presence of 10 µCi/mL [3H]-oleate and 0.8mM oleate complexed to BSA. Cells were then homogenized and microsomes obtained by differential centrifugation. Lipids were extracted from microsomes by the Bligh-Dyer method, separated by TLC, and the TG bands were excised and quantified by scintillation counting. DPM were normalized to total cell protein. Statistical analysis was performed using an unpaired Student’s t-test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. Different letters indicate a p-value <0.05, (n=3). Inset) Immunoblot displaying the expression of HAS-KDEL, hMTP-F, or dMTP-F transfected cells. Each lane, loaded with 20 µg total cell protein, represents an individual dish.
DPM $^3$H-TG/mg cell protein

HSA-KDEL  hMTP-F  dMTP-F

a  b  a
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Chapter III

Human MTP but not *Drosophila* MTP overexpression in mouse liver attenuates hepatic triglyceride accumulation during onset of high fat diet-induced hepatic steatosis

Philip S. MacArthur, Melissa A. Fabritius, Dongmei Cheng, Gregory S. Shelness

Department of Pathology Wake Forest University School of Medicine Winston-Salem, NC 27157
ABSTRACT

Microsomal triglyceride transfer protein (MTP) is a multifunctional protein necessary for first-step conversion of apolipoprotein B (apoB) into precursor lipoproteins and bulk triglyceride (TG) movement into the endoplasmic reticulum (ER) for second-step precursor lipoprotein expansion. Using cell free lipid transfer assays, it was previously demonstrated that vertebrate forms of MTP (e.g., human; hMTP) transfers both phospholipid (PL) and TG between donor and acceptor membrane vesicles, whereas invertebrate forms of MTP (e.g., Drosophila; dMTP) engage only in PL transfer. We hypothesized that PL transfer activity is the primordial function of MTP responsible for conversion of apoB and other large lipid transfer protein (LLTP) family members into small dense precursor lipoproteins and that the TG lipid transfer activity is a vertebrate adaptation required to activate neutral lipid translocation into the ER for second-step particle expansion. To explore this hypothesis we engineered transgenic mice with liver-specific expression of hMTP (PL plus TG transfer) or dMTP (PL transfer only). In this study, 8- to 12-week old dMTP and hMTP transgenic mice were fed a high-saturated fat (45% of calories from lard), high-cholesterol (0.2%) (HFHC) diet for 8 weeks to induce both weight gain and hepatic lipid accumulation. TG transfer activity in hMTP transgenic mouse liver was increased 2-fold as compared to both wild type littermates and dMTP mice. Upon challenge with the HFHC diet, hMTP mice displayed a 50% reduction in liver TG content, relative to wild type controls. Unexpectedly, however, expression of dMTP caused a 2-fold increase in liver TG. Plasma lipids and hepatic TG secretion rates were similar among all three genotypes. These results demonstrate that hMTP overexpression can protect the liver from high-fat, diet-induced steatosis whereas dMTP exacerbates hepatic lipid
deposition. Hence, the different functional profiles of hMTP and dMTP observed in vitro exert distinct effects on liver lipid metabolism, in vivo.
**Introduction**

Hepatic free fatty acids originating from the diet, adipocyte lipolysis, and *de novo* lipogenesis can be utilized for energy via β-oxidation, esterified to form triglyceride (TG) and stored in cytosolic lipid droplets, or packaged as TG into very low-density lipoprotein (VLDL) particles for secretion. An alteration affecting any of these pathways has implications for the etiology of hepatic and plasma lipid and lipoprotein metabolism and the development of metabolic syndrome and its associated comorbidities. For example, reduced TG efflux via the VLDL pathway could lead to cytosolic TG accumulation and eventually, steatosis (1). Conversely, enhanced hepatic production of apoB-containing lipoproteins, which can lead to elevated plasma TG concentrations, is associated with increased body mass index (BMI), metabolic syndrome, and a 3.2 fold increase in 5-year risk of cardiovascular disease (CVD) (2, 3). CVD, including myocardial infarction, stroke and peripheral artery disease, accounts for >50% of the annual mortality rate in the United States of America (4).

Although most of the enzymes responsible for TG formation have been identified and characterized (5), the mechanisms by which lipids are mobilized and secreted via transport into the ER are not well understood. The microsomal triglyceride transfer protein (MTP) was first identified based on its capacity to transfer neutral lipids, including TG and CE, between donor and acceptor membrane vesicles, in vitro. Because MTP is abundant in the liver and the intestine, a possible essential role in apoB-containing lipoprotein formation was suggested. This prediction was validated by the discovery that the absence of apoB-containing lipoproteins in the human genetic disorder abetalipoproteinemia (ABL) is caused by mutations in the gene for MTP (6). The resulting severe lipid and fat soluble vitamin malabsorption phenotype associated with genetic ablation of MTP is due to the absence of chylomicron
formation in the intestine and the absence of apoB-containing lipoproteins in the plasma (7-9). More specifically, convincing data has emerged to suggest that MTP, in addition to directly acting on apoB to initiate lipoprotein formation, also functions in the transfer of TG into the ER and Golgi for second-step apoB assembly, as genetic or pharmacological ablation of MTP activity reduces the neutral lipid content of the microsomal fraction (10-12).

While it is clear that MTP is essential for apoB-mediated lipid transport, efforts to understand how MTP’s disparate properties impact individual steps involved in lipoprotein assembly in vivo have lagged. Previous studies have demonstrated that all orthologs of the microsomal triglyceride transfer protein (MTP), ranging from invertebrate (i.e. Drosophila melanogaster) to vertebrate (i.e. human), have the ability to transfer phospholipid (PL) between donor and acceptor membrane vesicles, in vitro. In contrast, TG transfer activity appears to be unique to human and other vertebrate forms of MTP. Based on a number of theoretical and experimental criteria we hypothesized that the PL transfer activity of MTP is required for the first-step conversion of apoB into precursor lipoprotein particles whereas the TG transfer activity of MTP, which is unique to vertebrate forms, is required for the trafficking of TG into the ER and Golgi for second-step precursor particle maturation (13-15).

Previously, using cell-based approaches, we directly addressed this hypothesis by comparing the TG content of microsomes from hMTP and dMTP expressing cells (Chapter II). It was determined that compared to mock-transfected cells, hMTP increased microsomal TG content by ~ 2.5-fold while cells transfected with dMTP demonstrated no increase in microsomal TG. These data suggest that MTP promotes TG trafficking into the ER and that the TG-transfer activity of MTP is essential for this function. To extend these studies and explore their implications for lipid metabolism, in vivo, we created B6D2F1 mice with liver specific
expression of either hMTP or dMTP. The presence of the different MTP orthologs in mouse liver exerted distinct effects on liver lipid content and morphology, confirming the differential functional potentials of vertebrate and invertebrate MTP orthologs.
EXPERIMENTAL PROCEDURES

Plasmids and generation of transgenic mice

The plasmid pLIV11-Not1 was used for generation of dMTP transgenic mice. The plasmid pLIV-NSR (16), used for the generation of hMTP transgenic mice, was developed in our laboratory from pLIV11-Not1 and contains an engineered splice acceptor site (SAS) within the multiple cloning site of the pLIV11-Not1 plasmid to facilitate proper splicing of the hMTP gene (16). Insertion of dMTP and hMTP cDNA into pLIV11-Not1 and pLIV-NSR, respectively, was achieved as previously described (16) and the resulting plasmids were termed pLN-dMTP and pLN-hMTP.

The entire insert and flanking regions of each plasmid construct was verified by sequence analysis. The pLN-dMTP and -hMTP plasmids were separated from the plasmid backbone by digestion with NotI (5') and SpeI (3'). Following isolation by agarose gel electrophoresis, transgenes were microinjected into pronuclei of fertilized B6D2F1 (Harlan Teklad) eggs by the Transgenic Mouse Core of Wake Forest University School of Medicine. Genotypically positive founder mice were identified using 2-3 mm tail snips digested in DirectPCR lysis reagent containing proteinase K, which was used as a template for PCR with primers specific for checking transgene integrity, the hepatic control region, the human apoE promoter region, and the insert sequences (16). All mice were housed in a pathogen-free animal facility in plastic cages in a temperature-controlled room (22°C) with a 12-hour light/12-hour dark cycle. The mice were fed *ad libitum* a cereal-based rodent chow diet until the onset of the study. Male mice were then fed *ad libitum* a high-saturated fat (45% of calories from lard), high-cholesterol (0.2%) (HFHC) diet for 8 weeks. The HFHC diet was produced by the diet kitchen at
Wake Forest University School of Medicine (diet #1251). The institutional animal care and use committee at Wake Forest University School of Medicine approved all animal procedures.

**Generation of anti-hMTP and anti-dMTP antibodies**

The cDNA corresponding to amino acid residues 772-874 of hMTP was generated by PCR and cloned into the vector pMALc2x (New England Biolabs) for generation of maltose binding protein fusion. Following purification via amylose affinity chromatography, the antigen was injected subcutaneously with incomplete Freund's adjuvant into rabbits (Lampire Biologicals, Pipersville, PA). The resulting antibody is referred to anti-hMTP. Unless indicated, whole serum was used at a 1:1000 dilution for western blot analysis.

Likewise, cDNA corresponding to amino acid residues 500-600 of dMTP was generated by PCR and cloned into the vector pMALc2x (New England Biolabs) for generation of maltose binding protein fusion. Due to the insolubility of the fusion protein, the protein pellet was suspended in 50 mL 25 mM Tris-HCl (pH 7.4) containing 2% Triton X-100. After centrifugation at 14,000 x g for 15 minutes, the pellet was suspended in 8M urea, 25 mM Tris-HCl (pH 7.4) and incubated 30 minutes at room temperature. The protein solution was then subjected to a 20-fold buffer dilution into 25 mM Tris-HCl (pH 7.4) at 4°C. The cloudy mixture was centrifuged at 14,000 x g for 15 minutes and the supernatant, containing soluble fusion protein, was purified by amylose affinity chromatography and injected into rabbits for antibody generation, as described above. The resulting antibody is referred to as anti-dMTP. Unless indicated, whole serum was used at a dilution of 1:1000 for western blot analysis.
Detection of MTP by immunoblotting

Samples containing indicated amount of liver homogenate protein, as determined by BCA assay (Pierce), were resolved by 8% SDS-PAGE. After electrophoretic transfer of protein to a polyvinylidene difluoride (PVDF) membrane, the membrane was blocked in 5% non-fat milk powder dissolved in a solution of 10 mM phosphate, 150 mM NaCl and 0.1% Tween-20 (PBS-T) overnight at 4°C while shaking. The membrane was then incubated for 2 hours at room temperature (~ 25°C) in the polyclonal, rabbit anti-dMTP or rabbit anti-hMTP antibody described above at a dilution of 1:1000 in PBS-T containing 2.5% non-fat milk powder. The membrane was washed in PBS-T for 1 hour then incubated for 1.5 hours with horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:4000 in PBS-T containing 2.5% non-fat milk powder. Following a 1-hour wash in PBS-T before immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and GeneMate Blue Autoradiography Film (BioExpress).

Determination of hepatic TG transfer activity of transgenic mice

TG transfer activity of liver homogenates was measured in the laboratory of Dr. M. Mahmood Hussain, SUNY Downstate Medical Center. Flash frozen liver samples were homogenized in 1 ml hypotonic buffer (1 mM Tris-HCl, 1 mM MgCl₂, and 1 mM EGTA, pH 7.4). The homogenate was then passed through a 27 gauge, ½ inch needle 10 times and subjected to centrifugation at 13,000 rpm for 5 minutes at 4°C. The supernatant was then used for the TG transfer assay using protocols described previously (17). Briefly, each reaction contained 5 µL of donor (with NBD-labeled TG) and acceptor vesicles, 10 µg of total protein, and assay buffer (10 mM Tris-HCl, pH 7.4, 0.1% BSA, and 150 mM NaCl) up to a final volume of 100 µL in a
microtiter plate. The reaction was incubated for 30 minutes at room temperature. The samples were excited at 485 nm and read at 550 nm in a fluorimeter. Specific activity was calculated as 

\[
(\%\text{TG transfer} \times 30 \text{ min})^2 / (\mu g \text{ of total protein})
\]

**Plasma lipid measurements**

Plasma samples were obtained by submandibular vein or heart puncture (at necropsy). TPC and TG were analyzed by enzymatic assay (Roche Diagnostics).

**Determination of hepatic lipid content**

Approximately 100 mg of liver tissue was thawed, minced, weighed, and placed in a glass tube. Lipids were extracted in 5 mL CHCl₃:MeOH (2:1) at room temperature overnight. The lipid extract was transferred to a clean glass tube and dried down at 60°C under a stream of nitrogen. The dried lipids were dissolved in 1 mL CHCl₃:MeOH (2:1) after which 0.626 mL of 0.05% H₂SO₄ was added to acidify the sample. The samples were vortexed and centrifuged at 1,500 rpm (Juon 3.12, tabletop centrifuge) for 10 minutes. The upper aqueous phase was discarded and 200 µL of the bottom phase was dried down at 60°C under a stream of nitrogen. Samples were dissolved in 1 mL 1% Triton X-100 in CHCl₃ and the solvent was again dried down at 60°C under a stream of nitrogen. Deionized water (1 mL) was added to each tube and samples were vortexed until the solution was clear. Triglycerides were quantified enzymatically by applying 25 µL of the 1 mL suspension to the Triglyceride/GB kit (Roche Diagnostics).
Determination of hepatic triglyceride secretion

After a 4-hour fast, animals were weighed and then injected retro-orbitally with 500 mg/kg of Triton WR1339 (Tyloxopol, Sigma, St. Louis, MO) in sterile saline. Prior to injection and at 30 minutes, 1 hour, 2 hours, and 3 hours after injection, ~ 80 µL of blood was drawn by retro-orbital bleed, and plasma was assayed for TG content as described above.

Statistical analysis

Unless noted, statistical analysis was performed by a 1-way ANOVA followed by a Tukey's multiple comparison test using GraphPad Prism (GraphPad Software, San Diego, CA). Results are expressed as mean ± SE. A p-value of less than 0.05 was regarded as statistically significant.
RESULTS

Creation of dMTP and hMTP antibodies

In our previously described inducible cell system (Chapter II), use of the FLAG epitope tag sequence at the 5’ end of hMTP enabled immunoblot detection using anti-FLAG monoclonal antibody M2. However, for our in vivo studies we opted to use the native hMTP and dMTP gene for the creation of transgenic mice, which then required the production of dMTP and hMTP antibodies for protein detection. The initial attempts to generate an antibody to hMTP and dMTP involved the generation of the full length and amino terminal 50% of each protein as a carboxyl-terminal fusion with maltose binding protein (MBP). However, the fusion proteins dMTP/MBP, dMTP50%/MBP, hMTP/MBP, and hMTP50%/MBP were insoluble (data not shown), making purification via affinity chromatography difficult. To maximize the solubility potential, we analyzed the hydropathy of dMTP and hMTP by the method of Kyte and Doolittle (18) and also reduced the length of the MTP protein to be expressed. It was determined that amino acids 500-600 of dMTP (Figure 1, upper panel) and the carboxyl-terminal (C-term) 10% (amino acids 772-874) of hMTP (Figure 1, lower panel) were sufficiently hydrophilic to increase the potential for production of a soluble fusion protein. These sequences were produced by PCR and then cloned into the vector, pMALc2x. The hMTP C-term 10% fusion protein was soluble and was successfully purified via affinity chromatography and then injected into rabbits for antibody production. The antiserum was tested by probing membranes containing cell extracts from COS cells transfected with an irrelevant plasmid (AP) or plasmids encoding FLAG tagged forms of hMTP. Comparison of pre-immune and post-immune serum revealed the presence of immunoreactive bands of
the proper size using only the post-immune serum, indicating generation of an effective anti-hMTP antibody (Fig. 2A).

To generate the antibody for dMTP, we cloned a PCR product of dMTP encompassing amino acids 500-600 into the pMALc2x vector. Unlike the hMTP C-term 10% fusion protein, the dMTP500-600/MBP fusion protein remained ~ 95% insoluble (data not shown). To circumvent this issue, the aggregate fraction was solubilized using 8 M urea. The dMTP500-600/MBP fusion protein was then subjected to buffer exchange via a 20-fold dilution. After the fusion protein was purified via affinity chromatography it was injected into rabbits. Comparison of pre-immune and post-immune serum (Fig. 2B) indicates generation of an effective anti-dMTP antibody. It is also important to note that the anti-hMTP and -dMTP sera do not cross react.

Production of hMTP and dMTP transgenic mice

With the assistance of the Transgenic Core Facility (Wake Forest University School of Medicine), we developed liver-specific dMTP and hMTP transgenic mice. Our construct utilized the vector pLIV.11, which includes the 5' human apoE promoter and a 3' hepatic control region that is designed to limit expression to the hepatocyte (19, 20). After cloning cDNAs into the polylinker region, the gene and regulatory sequences were separated from the plasmid backbone via restriction enzyme digestion and agarose gel purification. The purified constructs were then microinjected into pronuclear B6D2F1 fertilized single-cell mouse embryos. The initial genotyping of mice was performed by PCR analysis of tail DNA using both vector- and MTP-specific primers (16). These tests identified genotypically positive founders containing the intact transgene for both dMTP and hMTP mice (16)
Hepatic expression of the dMTP and hMTP transgene

To test for dMTP or hMTP expression, aliquots of liver extracts from transgenic founders crossed with wild type B6D2F1 mice (50 µg protein) were fractionated by 8% SDS-PAGE, and the proteins were then transferred to a PVDF membrane. The western blot analysis of the liver homogenates with anti-dMTP and -hMTP antisera detected protein expression of dMTP (Figure 3A); however, initially there was no evidence of hMTP protein accumulation among any of the hMTP transgenic founder mice (16). The basis for this failed expression was alternative splicing that removed the initiating codon from the 5' end of the hMTP insert (16). We were able to circumvent this issue by reengineering the pLIV.11 vector to include a potent SAS within the multiple cloning site of the pLIV.11 vector upstream of the gene of interest thus shielding the SAS contained within hMTP and redirecting splice activity to the new SAS (16). Prior to injecting this construct into pronuclear B6D2F1 fertilized single-cell mouse embryos, hMTP protein expression was confirmed by transient transfection into HepG2 cells and immunoblotting the cell lysate (16). After removal of the plasmid backbone sequences, the hMTP construct was microinjected into the pronuclei of fertilized B6D2F1 mouse embryos. Offspring were then screened for transmission of the transgene by PCR and protein expression was analyzed by western blotting (Figure 3B).

To explore whether the expression of the exogenous transgene impacted endogenous mouse MTP expression, blots were stripped and re-probed with the mouse-specific monoclonal antibody (BD Biosciences), which reacts strongly with mouse MTP (mMTP) but shows no immunoreactivity with hMTP and dMTP. These analyses revealed no evidence that transgenic expression of dMTP or hMTP had an effect of endogenous mMTP expression (Figure 3, bottom panels).
Ultimately, 4 founder lines were generated for dMTP (Figure 3A), including 2 founder lines displaying high expression (founders 4 (dMTP/4) and 6 (dMTP/6)) and two with lower expression levels (founders 2 (dMTP/2) and 3 (dMTP/3)). Only one founder line was established for hMTP (hMTP/5) (Figure 3B).

**Triglyceride transfer activity in chow and HFHC diet-fed mice in hMTP and dMTP over-expressing mice**

As a measure of the level of MTP over-expression, we collaborated with Dr. M. Mahmood Hussain to measure triglyceride transfer activity in wild type, dMTP and hMTP transgenic mice. The TG transfer activity in hMTP expressing mice was increased 2-fold compared to dMTP and wild type controls (Figures 4). In addition, the increase in TG transfer activity was seen in mice fed chow, as well as, the HFHC diet. There was no significant increase in TG transfer activity detected in mice expressing dMTP when compared to wild type. These studies indicate that the triglyceride transfer activity was increased by at least two-fold in hMTP when compared to dMTP expressing livers or wild type liver. The absence of an increase in TG transfer in dMTP mice was expected based on the absence of an increase in TG transfer activity seen in dMTP-transfected cells (21).

**Weight and plasma lipid values in HFHC diet-fed hMTP and dMTP expressing mice**

Eight- to 12-week old wild type, dMTP/4, dMTP/6 and hMTP/5 transgenic mice were placed on the HFHC diet. At the beginning of the diet period each genotype displayed similar average weights of ~ 20 grams. Over the 8-week diet time course, wild type, dMTP/4 and
hMTP/5 mice displayed expected increases in weight, to ~ 40 grams (Figure 5). In contrast, dMTP/6 mice increased weight at a significantly slower rate compared to wild type, dMTP/4 and hMTP/5 mice, gaining only ~ 10 g (Figure 5) during the same period.

At the start of the diet and at two-week intervals during the 8-week time course, mice were fasted for 4 hours and blood was drawn by submandibular puncture to determine the total plasma cholesterol (TPC) and TG levels. During the 8 weeks on the HFHC diet there was no difference in TPC (Figure 6A) or plasma TG (Figure 6B) levels compared to wild type mice. In fact, there was a modest, although non-significant, reduction of plasma TG in dMTP/4, dMTP/6, and hMTP/5 mice compared to wild type.

**MTP over-expression and resistance to hepatic steatosis.**

As high fat, high cholesterol diets promote hepatic steatosis, we were able to directly test the hypothesis that MTP over-expression protects the liver from diet-induced hepatic lipid accumulation. For this purpose, the 8-week HFHC dietary period concluded with the mice being anesthetized, exsanguinated, and dissected. A representative liver from each genotype was photographed in situ (Figure 7A). Unexpectedly, the livers from the dMTP transgenic mice appeared to be more pale and larger than the liver from wild type mice. In contrast, the livers from hMTP/5 mice appeared similar in color and size to the wild type mice. When the liver from each animal was removed and weighed there was an increase in raw liver weight of hMTP/5 and dMTP/4 mice compared to wild type and dMTP/6 mice (Figure 7B). However, when the liver weight was expressed as a percentage of body weight, dMTP/4, dMTP/6, and hMTP/5 mice each displayed an ~ 80% increase compared to the wild type mice (Figure 7C).
To further analyze the livers from these mice, a portion of each was placed in 10% formalin for histologic studies and the remainder was flash frozen for lipid analysis. With the assistance of the Histology Core Laboratory at Wake Forest School of Medicine, formalin fixed portions of liver were mounted, sectioned, and stained with hematoxylin and eosin (H &E). H & E staining revealed the presence of numerous vacuoles indicating fatty liver droplets in mice expressing dMTP (Figure 8B and 8C) compared to wild type mice (Figure 8A). Expression of hMTP reduced the number of vacuoles (Figure 8D) compared to wild type mice (Figure 8A). Biochemical analysis of liver TG content confirmed the H & E results as mice expressing dMTP demonstrated an ~ 2-fold increase in hepatic TG compared to wild type mice. In contrast, mice expressing hMTP demonstrated a 50% reduction in hepatic TG compared to wild type mice, although this change was not statistically significant (Figure 9).

**Differential effects of dMTP and hMTP on hepatic TG secretion rates.**

ApoB-containing lipoprotein production is dependent on MTP. Therefore, we hypothesized that hMTP and dMTP could exert different effects on hepatic TG secretion rates. Secretion rates were measured, in vivo, by blocking catabolism of apoB-containing lipoproteins with the surfactant Triton WR1339 (22, 23). After a 4-hour fast, wild type, hMTP and dMTP transgenic mice were injected retro-orbitally with 500 mg/kg body weight Triton WR1339. Retro-orbital blood samples were drawn at 0 hours (immediately prior to Triton WR1339 injection) and at 30 minutes, 1 hour, 2 hours, and 3 hours post injection and were subsequently analyzed for TG content. Despite the increase of hepatic lipid content in dMTP expressing mice, and the decrease of hepatic lipid content in hMTP expressing mice compared to wild type mice (Figure 9), there was no difference in the rate of TG secretion during the 3
hours post-Triton block (Figure 10).
Discussion

Previous kinetic and structural data have established that invertebrate forms of MTP (e.g., dMTP) are capable of PL transfer, whereas vertebrate forms of MTP (e.g., hMTP) engage in both PL and TG transfer. These data suggest that MTP evolved initially as a phospholipid transfer protein and later acquired triglyceride transfer activity, perhaps during vertebrate evolution (24). However, the specific roles of the PL versus TG transfer activities of MTP in lipoprotein assembly, in vivo, remain unknown. Cell based data generated in our laboratory suggest that expression of hMTP (TG transfer competent) but not dMTP (TG transfer incompetent), facilitates the accumulation of TG in the ER (Chapter II). This led to the hypothesis that the PL transfer activity of MTP promotes the conversion of apoB into a precursor lipoprotein, whereas the TG transfer activity is required to translocate neutral lipid into the ER for second-step precursor particle expansion, a process associated only with vertebrate lipoprotein assembly. Although the cell-based studies reported in Chapter II support this prediction, little is known about how the enhanced expression of various MTP orthologs might impact VLDL assembly and secretion and ectopic lipid storage associated with HFHC diet feeding and NAFLD. To explore this question we created mice that express hMTP or dMTP under the control of the apoE 5’ proximal promoter and 3’ hepatic control region in the plasmid pLIV11. We hypothesized that the hepatic over-expression of hMTP, and subsequent increase in TG translocation into the ER, would create an increased efflux of neutral lipids from the liver into plasma, which would render the hMTP-expressing mice resistant to diet-induced steatosis. In contrast, we expected that the ability of dMTP to engage only in precursor lipoprotein assembly, without the capacity to engage in bulk neutral lipid translocation into the ER, would not protect the liver from steatosis.
Upon characterization of hMTP-overexpressing mice a 2-fold increase in TG transfer activity was observed relative to both wild type and dMTP mice (Figure 4). Interestingly, despite this doubling in hepatic TG transfer activity, hMTP mice displayed no significant difference in plasma TPC or TG compared to dMTP and wild type mice (Figure 6). These data are inconsistent with a previous study that utilized adenovirus-mediated expression of hMTP in chow fed C57BL/6 mice, which reported an ~ 86% increase in plasma TG levels (25). It is possible that the magnitude of overexpression achieved in our study was insufficient to provide a clear effect with regards to plasma lipid levels as the aforementioned study reported an ~ 3-fold increase in TG transfer activity (25). In contrast, a more recent study demonstrated that adenovirus-mediated overexpression of mouse MTP (mMTP) in ob/ob mice, which exhibit robust hepatic lipid accumulation, resulted in an ~ 3-fold increase in TG transfer activity, yet no difference in plasma TG compared to control littermates (26). Hence our data and those of Chen et al., suggest that an increase in liver MTP TG transfer activity does not necessarily affect plasma TG levels, even under conditions of hepatic lipid accumulation.

To explore if chronic overexpression of hMTP in our transgenic mice affected TG secretion, we blocked the catabolism of newly secreted apoB-containing lipoproteins with Tyloxapol and monitored plasma TG accumulation rates. We observed no significant difference in newly secreted TG among the groups (Figure 10). One might have speculated that augmenting TG transfer activity by increasing hMTP abundance might reduce co- and post-translational degradation of apoB and thereby increase the secretion of newly synthesized apoB100-containing particles. As a result, a potential increase in apoB availability plus abundant TG substrate, originating from the HFHC diet, could lead hMTP but not dMTP, overexpression to produce an increase in TG secretion. Again, however, previously published
data both support and refute this rationale. First, work from Liao, et al, suggested that adenovirus mediated overexpression of MTP in oleate stimulated HepG2 cells increased the amount of apoB-100 lipoproteins secreted and lowered the density of the particles (27), indicating that in this system MTP is limiting in the secretion of TG. Furthermore, Tietge, et al (25) demonstrated that in vivo administration of an adenovirus encoding human MTP increased VLDL-TG secretion in wild type C57BL/6 mice by >2-fold. In contrast, Chen et al demonstrated that adenovirus mediated overexpression of mMTP in ob/ob mice produced no difference in TG secretion, as compared to control adenovirus; however, when placed in an apoB100 only background, mice overexpressing MTP displayed an ~ 2-fold increase in TG secretion compared to control mice (26). Therefore, our findings from mice chronically overexpressing MTP, as well some of the previous studies cited above, might imply that MTP abundance or activity is not always by itself limiting for hepatic VLDL secretion. Alternatively, it is possible that the level of overexpression observed in our transgenic mouse models is not sufficient to create a detectable change in VLDL-TG secretion rate.

Considering that non-alcoholic fatty liver disease (NAFLD) and NASH have emerged as major comorbidities associated with obesity and the metabolic syndrome (28-30), it is imperative to experimentally define whether enhancing MTP expression can protect the liver from ectopic lipid accumulation. Although previous studies using adenovirus-mediated overexpression have demonstrated that hMTP expression in an MTP null background can reduce liver lipid levels (21) it has not been established whether modest and persistent hMTP overexpression can protect the liver from diet-induced lipid accumulation. In order to address this question, we analyzed H&E stained liver sections and hepatic TG data from the HFHC diet-fed hMTP, dMTP, and wild type mice.
H&E staining revealed a dramatic increase in lipid droplet accumulation in mice expressing dMTP as compared to wild type mice (Figure 8A, B, and C). This was accompanied by an ~ 2-fold increase in hepatic TG levels as assessed by enzymatic assay, which was observed in two separate founder lines of dMTP transgenic mice (Figure 9). It was unexpected that mice expressing dMTP had such a profound impact on liver TG accumulation. It is possible that the dMTP transgene acts in a dominant-negative capacity, resulting in an increase in hepatic TG as a result of inhibition of endogenous mouse MTP activity. However, we have determined that the expression of the dMTP and hMTP transgene does not affect the expression of endogenous mouse MTP (mMTP) (Figure 3) or total TG transfer activity (Figure 4). Additionally, we have already established that any alteration in hepatic TG is not a result of altered secretion (Figure 10). Therefore, it is possible that dMTP overexpression may be affecting the expression of other genes involved in lipogenesis, catabolism, and cellular energy expenditure. Irrespective of the underlying mechanisms involved, our data indicate that the chronic hepatic overexpression of dMTP exacerbates liver lipid accumulation induced by a HFHC diet.

In contrast to the dMTP mice, H&E staining demonstrated a decrease of neutral lipid droplet accumulation in mice expressing hMTP, compared to wild type littermates (Figure 8). This finding was confirmed by the 50% reduction in liver TG content as assessed by enzymatic assay (Figure 9). Hence, it appears that modest but chronic MTP overexpression can protect the liver from high fat diet induced ectopic lipid accumulation. Surprisingly, however, this was observed to occur despite the fact that hMTP overexpression failed to detectably increase the rate of TG secretion, as discussed above. One possible explanation for this apparent discrepancy is that a very small increase in TG secretion, while not detectable experimentally...
over the three hour period of Triton block, is still sufficient to attenuate liver lipid accumulation caused by high fat diet, a process that occurs over many weeks. Alternatively, hMTP overexpression may be impacting other genes that affect hepatic lipid homeostasis beneficially.

The studies performed here are the first to utilize mice engineered to chronically overexpress MTP in liver. Such chronic overexpression may represent a more physiologically relevant method of determining the role of MTP in hepatic lipid metabolism, as compared to acute expression achieved by adenoviral methods. In addition to direct overexpression of MTP, two previous studies have explored how overexpression of specific transcription factors impact MTP expression and other pathways related to hepatic and whole body lipid homeostasis (31, 32). One such study demonstrated that adenoviral co-delivery of the transcriptional co-activators Pgc-1β and Foxa2 increased MTP expression and in turn increased VLDL-TG secretion in ob/ob mice (31). However, these studies also demonstrated that other target genes, including DGAT2, which is known to increase hepatic TG production, were also induced. As a corollary to this study it has been noted previously that overexpression of either diacylglycerol: acyl transferase 1 (DGAT1) or diacylglycerol: acyl transferase 2 (DGAT2) in the liver, which causes a dramatic increase in hepatic TG content, fails to produce an increase in hepatic VLDL secretion (33, 34). The authors of these studies concluded that there is a limiting factor, possibly MTP, which prevents export of pathophysiologic levels of TG under these conditions (33, 34). If that is true, the coordinated overexpression of MTP and DGAT2 could be particularly potent in promoting hepatic VLDL secretion, as observed in Pgc-1β and Foxa2 overexpressing ob/ob mice. Indeed, these
authors propose that insulin mediated inhibition of the Foxa2/Pgc-1β complex may in part underlie the inhibition of VLDL secretion observed in insulin sensitive subjects.

Other studies designed to assess insulin-mediated control of hepatic VLDL production noted that MTP is a FoxO1 target gene. Hence, both adenoviral delivery of FoxO1 to HepG2 cells and transgenic overexpression of a constitutively active form of FoxO1 in mouse liver both increased MTP expression and VLDL-TG secretion (32). However, the impact of FoxO1 on other genes likely to impact VLDL secretion were not extensively explored. Hence, while hepatic insulin signaling diverges at FoxO1 to target genes for controlling both hepatic glucose and VLDL production, whether the regulation of VLDL secretion is mediated only by FoxO1-mediated effects on MTP expression are not full elucidated (32).

Our in vivo model has provided important preliminary results regarding the overexpression of MTP in relation to hepatic TG metabolism and resistance to steatosis. However, to better define the lipid trafficking potential of the TG and PL lipid transfer activity of MTP, we must consider that more beneficial data may result from a mouse model that chronically overexpresses dMTP or hMTP in the absence of endogenous mMTP. It has already been demonstrated by Khatun, et al. that adenovirus-mediated expression of hMTP in liver-specific MTP knockout mice can reverse hepatic steatosis caused by MTP deficiency (35). Additionally, this study demonstrated that the acute expression of dMTP was sufficient to protect mice against hepatic steatosis, suggesting that the phospholipid transfer activity of MTP was sufficient to protect the liver from hepatic steatosis without increasing plasma TG levels (35). Understanding the basis for that phenotype may provide insights into how the specific lipid transfer and other activities of MTP contribute to apoB lipoprotein formation. However, we
believe further studies in hepatic MTP knockout mice chronically expressing dMTP or hMTP are needed to substantiate those results.

In conclusion, the studies here have provided a direct correlation between the specificity of MTP lipid transfer activity on hepatic TG metabolism and hepatic steatosis. Based on the dramatic differences in lipid transfer specificity observed in vitro (Chapter II), we expected robust differences in the phenotype of animals expressing the dMTP and hMTP transgenes. Although we did not establish any changes in plasma TG levels of mice expressing dMTP or hMTP, we were able to observe a vast difference in hepatic TG accumulation. We determined that overexpression of hMTP, unlike dMTP, had a protective effect on liver TG accumulation. Coupled with prior studies indicating that hMTP possess two distinct lipid transfer capabilities, polar and neutral, our results could provide a foundation for the development of pharmacological agents that may favorably alter the hepatic secretion of atherogenic lipoproteins, without causing steatosis.
**Figure 1. Hydropathy analysis of dMTP and hMTP.**

Amino acid sequences for dMTP (top panel) and hMTP (bottom panel) were subjected to hydropathy analysis. The x-axis indicates amino acid residue number and the y-axis indicates hydrophilicity. In this readout, values above 0 indicate spans of residues (window = 7) that are hydrophilic. The red boxes indicate the approximate range of amino acids selected from each protein for antibody development.
Kyte/Doolittle hydrophilicity: Window = 7

Hydropilicity

100 200 300 400 500 600 700 800

Kyte/Doolittle hydrophilicity: Window = 7

Hydropilicity

100 200 300 400 500 600 700 800

dMTP

hMTP
Figure 2. Characterization of dMTP and hMTP antibodies.

COS-1 cells (100 mm dishes) were transfected with human placental alkaline phosphatase (AP) or N-terminal epitope tagged forms of dMTP (F-dMTP) and hMTP (F-hMTP), as indicated, using the Fugene6 transfection reagent (Roche). Forty-eight hours post-transfection cells were lysed with 1 mL of 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF. After centrifugation at 14,000 x g for 10 minutes, 50 µl of the supernatant was precipitated with an equal volume of 20% TCA, fractionated by 12.5% SDS-PAGE and transferred to PVDF membrane. Membranes were probed with hMTP pre-immune and post-immune serum (Panel A) and dMTP pre and post-immune serum (Panel B). In both cases, anti-FLAG monoclonal antibody M2 (Sigma) was used to confirm presence of the epitope tagged forms of each MTP ortholog. Pre-immune, anti-dMTP, and anti-hMTP serum was used at dilution of 1:1000; anti-FLAG monoclonal antibody M2 (Sigma) was used a concentration of 2.5 µg/ml. Note that the anti-dMTP and hMTP sera do not cross react.
Figure 3. dMTP and hMTP expression in transgenic mice.

A) Immunoblot of dMTP transgenic mouse liver homogenates (upper panel lanes 3-10) using anti-dMTP antibody. Lower Panel: Immunoblot from upper panel was stripped and re-probed with anti-mouse MTP (BD Transduction Laboratories) to confirm endogenous expression and to provide a load control. B) Immunoblot of hMTP transgenic mouse liver homogenates (upper panel lanes 3-4) using anti-hMTP antibody. Lower Panel: Immunoblot from upper panel was stripped and re-probed with anti-mouse MTP as described above. Each sample was run in duplicate.
Figure 4. hMTP transgenic mice display a two-fold increase in TG transfer.

Liver samples were homogenized in 1 mL hypotonic buffer (1mM Tris-HCl, 1mM MgCl₂, and 1 mM EGTA pH 7.4). The homogenate was then passed through a 27 gauge ½ inch needle 10 times and subjected to centrifugation at 13,000 RPM for 5 minutes at 4°C. The supernatant was then used for the TG transfer assay. Statistical analysis was performed using a 1 way ANOVA. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. Chow diet: n=3 per group, HFHC diet: n=6 per group.
% TG Transfer/hr/mg of Total Protein

- Chow Diet
- High Fat Diet

P<0.001 (***)
P=0.525
Figure 5. Body weight increases of dMTP and hMTP transgenic mice fed a HFHC diet for 8-weeks. Eight- to 12-week old hMTP and dMTP transgenic mice, along with wild type littermates, were placed on a HFHC diet for 8 weeks. Mice were weighed at 2-week intervals for the duration of the study. Statistical analysis was performed using a 1-way ANOVA followed by a Tukey’s multiple comparison test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. * indicates a p-value < 0.0001. WT (n=13), dMTP/4 (n=6), dMTP/6 (n=9), hMTP/5 (n=6).
Figure 6. Total plasma cholesterol and TG levels after 8-weeks of HFHC diet. Mice were fasted for 4 hours prior to collecting blood by submandibular vein puncture. 50-100 µL of blood was collected in a 1.5 mL microcentrifuge tube containing 1:10 protease inhibitor cocktail. Samples were then centrifuged at 6,000rpm at 4°C for 10 minutes. Plasma TPC and TG concentrations were determined by enzymatic assay. Statistical analysis was performed using a 1-way ANOVA followed by a Tukey’s multiple comparison test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. ns = not significant. WT (n=13), dMTP/4 (n=6), dMTP/6 (n=9), hMTP/5 (n=6).
Figure 7. Characterization of wild type, dMTP, and hMTP transgenic mouse livers after 8 weeks on a HFHC diet. A) Photographic image of livers; B) Liver weight; C) liver weight normalized to body weight. Statistical analysis was performed using a 1-way ANOVA followed by a Tukey’s multiple comparison test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. * indicates a p-value < 0.003. WT (n=13), dMTP/4 (n=6), dMTP/6 (n=9), hMTP/5 (n=6)
Figure 8. Microscopic examination of liver sections from wild type, dMTP, and hMTP transgenic mice. At time of necropsy, the liver of each animal was removed and a portion (approx. 100 mg) was placed in 10% formalin. Fixed samples were mounted and stained with H&E to visualize lipid droplet accumulation. The figure shows a representative image from wild type, dMTP/4, dMTP/6 and hMTP/5 transgenic mice at 40X magnification.
Figure 9. hMTP transgenic mice trend toward a reduction in liver triglycerides while dMTP transgenic mice show a marked increase compared to wild type mice. Lipids were extracted from approximately 100 mg of liver and TG was quantified by enzymatic assay (Roche). Statistical analysis was performed using a 1-way ANOVA followed by a Tukey’s multiple comparison test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. * indicates a p-value < 0.004. WT (n=13), dMTP/4 (n=6), dMTP/6 (n=9), hMTP/5 (n=4).
Figure 10. Hepatic TG secretion rates in dMTP, hMTP, and wild type mice.

Mice were fasted for 4 hours prior to retro-orbital injection with 500 mg/kg body weight of Triton WR1339. Blood samples were taken at 0, 30, 60, 120, and 180 min post-injection. Plasma TG concentrations were determined by enzymatic assay. Statistical analysis was performed using a 2-way ANOVA followed by a Tukey’s multiple comparison test. Results are expressed as the mean ± SE. A p-value of less than 0.05 was regarded as statistically significant. WT (n=5), dMTP/4 (n=3), dMTP/6 (n=5), hMTP/5 (n=5)
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CHAPTER IV

Conclusions and Future Directions
Hepatic free fatty acids (FFA) originating from the diet, de novo lipogenesis, and adipocyte lipolysis can be utilized for energy via β-oxidation, esterified to triglyceride (TG) and stored as cytosolic lipid droplets, or packaged as TG into very low-density lipoprotein (VLDL) particles for secretion. An alteration affecting any of these pathways has implications for the etiology of hepatic and plasma lipoprotein metabolism and pathophysiology. For example, attenuation of hepatic VLDL-TG export can lead to cytosolic TG accumulation and eventually, steatosis (1). Conversely, hepatic VLDL overproduction, which is often associated with increased body mass index (BMI), and metabolic syndrome, can result in elevated plasma levels of apolipoprotein B (apoB)-containing lipoproteins. As elevated concentrations of plasma apoB-containing lipoproteins are associated with a 3.2-fold increase in 5-year risk of cardiovascular disease (2, 3), understanding the regulatory mechanisms that control both the production and metabolism of hepatic TG-rich lipoproteins is crucial for the prevention and possible treatment of lipid metabolic disturbances associated with obesity, type-2 diabetes, hepatic steatosis, and cardiovascular disease.

Hepatic over-production of TG-rich VLDL observed in insulin resistant states is attributed to both an increased flux of FFA to the liver, arising from the inability of the adipocyte to respond to insulin by suppressing lipolysis, as well as the ability of insulin, even in the insulin resistant state, to stimulate hepatic de novo lipogenesis (1, 4). It has also been proposed that FoxO1-mediated upregulation of the microsomal triglyceride transfer protein (MTP) and other lipogenic genes are partially responsible for VLDL overproduction under conditions of insulin resistance (5). Increased lipid substrate-driven VLDL overproduction by the liver also leads to the formation of large diameter VLDL₁ (6). These particles, which represent one of the hallmarks of diabetic dyslipidemia, are believed to undergo metabolic conversion in the plasma
compartment to small, dense, low-density lipoprotein (LDL), a class of apoB-containing lipoproteins that is believed to be highly atherogenic and which may in part explain the increased cardiovascular risk associated with type-2 diabetes (6).

Although VLDL production can be positively regulated, particularly in response to increased lipid substrate, the ability of the VLDL pathway to efflux liver lipid is finite. Hence, while the liver overproduces VLDL-TG under conditions of perturbed lipid and glucose metabolism associated with the metabolic syndrome, this pathway can become limiting, leading ultimately to nonalcoholic fatty liver disease (NAFLD). Many alterations in hepatic lipid transport and metabolism can lead to NAFLD, including inhibition of the VLDL assembly pathway caused by genetic lesions in the genes for either MTP or apoB (7, 8). Considering the important role played by apoB lipoprotein assembly and secretion in both protecting the liver from steatosis and in determining apoB particle number and characteristics, understanding the details of this pathway have important pathophysiologic consequences for the etiology of metabolic syndrome and for devising strategies to therapeutically treat conditions, such as NAFLD, without adversely affecting apoB lipoprotein particle number or atherogenicity (9-12).

The basic framework for understanding apoB lipoprotein assembly has surprisingly not changed significantly since the seminal work of Alexander, Hamilton, and Havel in 1976 (13). Antibodies directed against LDL and VLDL showed that apoB was located in the rough endoplasmic reticulum (ER), apparently associated with the cisternal membrane, and that osmiophilic TG-rich emulsions devoid of apoB were present in the lumen of the smooth ER (13). The first colocalization between apoB and lipid droplets was observed at the smooth surfaced ends of the rough ER cisternae, corresponding to the transition zone between the smooth and rough ER (13). This paper led to a commonly cited “two-step model” for VLDL
assembly. In this model, a relatively lipid poor apoB-containing lipoprotein precursor lipoprotein is formed in the rough ER; however, the bulk lipid component of VLDL is formed separately in the smooth ER where many lipogenic enzymes are localized (14). The fusion between these entities at transition zones between the rough and smooth ER then gives rise to mature VLDL. Although this two-step model of VLDL assembly is generally accepted as accurate, the mechanistic details that underlie both the first and second steps of particle assembly are not known in great detail.

One point of debate relates to the specific functions of MTP, a 97-kDa protein complexed with the ER localized folding enzyme, protein disulfide isomerase (PDI) (15). MTP is an essential cofactor for the formation of hepatic VLDL and intestinal chylomicrons as well as bulk neutral lipid movement into the ER for precursor particle expansion (16, 17). The conclusion that MTP forms at least part of the neutral lipid translocation system responsible for hepatic VLDL assembly is based on altered lipid trafficking observed upon pharmacologic or genetic inactivation of MTP in hepatocytes and hepatoma cells (18-20). The overall aim of our studies was to define the role of MTP in the mechanisms underlying neutral lipid translocation into the hepatocyte ER, a process that is essential and likely rate limiting for lipoprotein-mediated lipid efflux.

In chapter II, we assessed human MTP’s (hMTP) ability to promote TG trafficking into the ER of transiently transfected or stable, doxycycline (Dox)-inducible, non-hepatic cell lines. In cell lines that expressed hMTP, a 2.5–5.0-fold increase in microsomal TG content was observed (Chapter 2, Figures 1 and 6). This represents the first demonstration that the expression of MTP in non-hepatic cells leads to an increase in secretion-coupled lipids, suggesting that MTP on its own may be the critical cofactor required for neutral lipid
translocation into the ER. As MTP has been ascribed several distinct functions in the formation of apoB-containing lipoproteins (15, 21-24), we were interested in how the lipid transfer activity of MTP was related to its lipid translocation capacity in living cells. First, we were able to establish that the lipid-transfer activity of MTP was indeed necessary for TG redistribution from the cytosol into the ER because, in the presence of the potent MTP inhibitor, BMS-212122, microsomal TG content was similar to background levels observed in mock-transfected cells (Chapter 2, Figure 3). Hence, we could conclude that the altered TG distribution in MTP-transfected cells was not an indirect effect caused by alterations in the expression of other genes related to lipid metabolism or trafficking. Further, we established that MTP’s non-lipid transfer related functions, such as its putative protein and lipid chaperone activities (21, 25), are also not responsible for its ability to increase microsomal TG content.

Another critical question we addressed was how the specificity of lipid transfer of MTP in vitro, relates to its ability to engage in lipid translocation within cells. For this purpose we took advantage of the known differences in lipid transfer specificity observed for hMTP (PL and TG transfer competent) versus Drosophila MTP (dMTP) (PL transfer competent only). Compared to mock-transfected cells, hMTP expression increased microsomal TG content by ~2.5-fold, as observed previously. In contrast, cells transfected with dMTP demonstrated no increase in microsomal TG content, despite the demonstration that dMTP was expressed and was abundant, based on western blotting (Chapter 2, Figure 12). In sum, these data clearly indicate that MTP promotes TG trafficking into the ER and that the TG-transfer activity is the essential property of MTP that is necessary for this function.

Because our data revealed an MTP-dependent increase in lipid translocation into the microsomal fraction of our inducible, FLAG-tagged (F) F-hMTP/CHO-K1 cells (Chapter 2,
Figure 6), we believe this cell model can be utilized to explore additional mechanistic aspects of second-step VLDL particle assembly. For example, a time course of microsomal TG accumulation achieved after F-hMTP induction could be performed to illuminate the kinetics of TG transfer and to determine whether TG transfer into the ER becomes saturable. The changes in lumenal and membrane TG, as a function of time, could reveal the rate at which TG accumulates within each microsomal fraction and, if and when the accumulation reaches a steady state. As F-hMTP/CHO-K1 cells lack apoB and, hence, a mechanism for TG efflux, it is possible that the microsomes will have a limited capacity to sequester TG, as would be evidenced by a plateau in accumulation occurring at relatively early time points. Alternatively, the ER may undergo an adaptation to accommodate a large influx of TG in which case TG accumulation could either continue over time, perhaps with reduced kinetics, or saturate at relatively late time points.

A time-dependent increase in the distribution of TG in microsomes in response to maximal MTP expression would suggest a direct and autonomous role of MTP in lipid translocation. However, to further explore whether MTP is rate limiting in this process we could perform a dose-response study by altering the amount of Dox (26) used for induction of F-hMTP. Should F-hMTP be the sole rate-limiting factor required to achieve TG translocation, it is anticipated that increasing levels of F-hMTP expression would result in a corresponding increase in microsomal TG. Although this dose-response curve could plateau, either because of saturating MTP mass or other limiting factors, the initial kinetics should display a positive linear relationship between MTP mass and microsomal TG content.

Another question that has not yet been addressed is whether there is a precursor-product relationship between cytosolic and lumenal TG in MTP expressing cells. To explore
this question, cells can be labeled with $[^{3}H]$-oleate for sufficient time to produce maximal cytosolic radiolabeled lipid accumulation. At this point radiolabeled oleate can be removed from the cells and Triacsin C, a competitive inhibitor of long-chain acyl-CoA synthetase (27), can be added. Triacsin C predominantly blocks incorporation of fatty acids into TG and does not inhibit FA esterification of lysosphospholipids (27). Hence, the effect of Triacsin C disproportionately affects neutral lipid synthesis (27). Cells can then be harvested at appropriate chase time points and subjected to subcellular fractionation. Should membrane and/or luminal TG originate from cytosolic lipid droplets, we would expect to observe a time-dependent increase in microsomal TG in MTP expressing cells but not in control cells. Likewise, while control cells will likely display a reduction in cytosolic $[^{3}H]$-oleate over time, it is possible that MTP expressing cells will demonstrate an enhanced rate of turnover, that perhaps can be accounted for by the increase in microsomal content.

An additional issue that could be addressed with our cell-based system relates to the possible intracellular trafficking of intraluminal TG and site(s) of second-step lipoprotein particle assembly. The second-step fusion of apoB-containing precursor particles with luminal lipid droplets to form mature VLDL or chylomicrons has been reported to occur in the Golgi (28-31), even though it is assumed that both cytosolic and luminal TG droplets originate at the ER (32, 33). This would imply progressive ER to Golgi transport of TG achieved either by tubulovesicular transport and/or a process of organelle maturation (34). However, it has also been demonstrated that some MTP resides in the Golgi and that this fraction displays TG transfer activity in cell free assays (35). Because our F-hMTP/CHO-K1 inducible cell line lacks the capacity to traffic TG via apoB, we have a unique opportunity to address the inherent trafficking potential of TG within the secretory pathway. This model can aide in establishing
whether MTP transfers lipid directly into both the ER and Golgi, a reflection of the comparative distribution of MTP in each compartment (35), or whether ER TG is a precursor of the Golgi pool, indicating anterograde trafficking of apoB-free TG within the secretory pathway. Assuming an MTP-dependent increase in Golgi TG is observed, we could then determine whether MTP induces TG accumulation in the ER and Golgi simultaneously, suggesting direct transfer into these organelles, or alternatively, whether there is kinetic evidence for a precursor-product relationship between ER and Golgi TG pools.

Due to the possible presence of MTP in the Golgi and ER there exists the likelihood that these subcellular compartments could acquire neutral lipid with similar initial kinetics. However, one confounding issue is that the diacylglycerol acyltransferase (DGAT) enzymes, which are responsible for the final step in TG synthesis, are predominantly localized to the ER (14). Hence, while it has been demonstrated that MTP recovered from the Golgi is capable of transferring neutral lipid from donor to acceptor membranes in the cell free assay (35), our inducible cell model could be used to establish whether such a Golgi-localized reaction occurs in intact cells. If TG formation within proximity of the membrane is a prerequisite for MTP-mediated membrane translocation, then it is possible that even in the presence of considerable Golgi-localized MTP, little accumulation of TG in the Golgi will be observed.

Our in vivo studies in chapter III attempt to delineate the physiologic role of the polar and the neutral lipid transfer activities of MTP and the possibly unique effects they have on whole body lipid homeostasis as well as on hepatic steatosis. To explore whether the PL and TG transfer activities of MTP play distinct roles in the ability to resist hepatic steatosis, we engineered mice with liver-specific expression of hMTP or dMTP. As expected, TG transfer activity in hMTP transgenic mouse liver was increased 2-fold while dMTP mice failed to display
an increase compared to wild type (Chapter 3, Figure 4). When challenged with a high fat and high cholesterol (HFHC) diet, hMTP mice displayed a mild reduction in liver TG content compared to dMTP and wild type mice (Chapter 3, Figure 9), despite similar plasma lipids (Chapter 3, Figure 6) and hepatic TG secretion rates (Chapter 3, Figure 10) among the three genotypes. Unexpectedly, expression of dMTP caused a 2-fold increase in liver TG, relative to wild type mice (Chapter 3, Figure 6). These results demonstrate that modest hMTP overexpression can protect the liver from diet-induced fatty liver without increasing plasma lipids, a property that may require MTP’s intrinsic TG-transfer activity.

Although our in vivo model has provided important preliminary results regarding the over-expression of MTP and resistance to hepatic steatosis, we expect that more beneficial data may result from a mouse model that lacks endogenous MTP expression. This could be achieved by crossing the transgenic mice into liver specific MTP knockout mice. This would allow for the direct comparison of the lipid trafficking potential of the TG and PL lipid transfer activities of MTP without the confounding influence of the endogenous protein. Because apoB100 requires extensive neutral lipid enrichment to ensure its proper folding and secretion (17), we would expect that the hMTP, but not the dMTP ortholog, would function to maintain plasma apoB100 levels equivalent to those of wild type mice. ApoB48, on the other hand, can be secreted in highly under-lipidated forms (36-38), raising the possibility that dMTP expression could result in a selective increase in plasma apoB48. However, an important consideration is that under-lipidated apoB48 resulting from the dMTP transgene may be rapidly catabolized following secretion, thus negating any distinguishable plasma phenotype. Examination of apoB48 and apoB100 production rates by the Triton block method, as described below, may address this issue.
Although apoB-containing lipoprotein production is dependent on MTP (7), we hypothesize that hMTP and dMTP will exert different effects on apoB and VLDL-TG production rates. These rates can be measured, in vivo, by blocking catabolism of apoB-containing lipoproteins with the surfactant Triton WR-1339. With the expression of hMTP we expect stimulation of TG secretion resulting in a significant increase in apoB48 and apoB100 compared to knockout and dMTP expressing mice. In contrast, we predict that dMTP will have a limited impact on hepatic TG secretion rates due to its inability to promote lipid translocation into the ER, as demonstrated in our cell based assays. However, as discussed above, we may be able to detect an increase in apoB48 secretion in response to dMTP expression due to dMTP acting directly on apoB48 to promote precursor lipoprotein assembly through its PL transfer activity, thus further confirming the hypothesis that dMTP is competent to create apoB lipoprotein precursors.

It is likely that the expression of hMTP and dMTP will also differentially affect the composition of apoB48- and apoB100-containing lipoproteins. We expect that hMTP expression, in contrast to dMTP expression, will produce an increase in VLDL-TG compared to hepatocyte specific knockout mice but at similar levels to wild type mice. However, it is likely that the expression of dMTP will enhance the PL transfer-dependent priming of apoB, causing an increase in the secretion of small, dense, PL-rich apoB48-containing lipoproteins in the high-density lipoprotein (HDL) and LDL size ranges (37, 38). As part of the phenotypic analysis of hMTP and dMTP mice on the MTP hepatocyte specific knockout background, we will employ Fast Protein Liquid Chromatography (FPLC) of plasma to establish apoB48 and apoB100 distribution. We expect that hMTP, in contrast to dMTP expression will produce an increase in VLDL-TG involving an increase in both VLDL-associated apoB48 and apoB100.
Because, as discussed above, apoB48 can be secreted in lipid poor form (7, 39, 40), dMTP may enhance the PL transfer-dependent priming of apoB, causing an increase in the secretion of small, dense apoB48 containing lipoproteins in the HDL and LDL size ranges (39, 40). In addition to lipid distribution among the lipoprotein fractions (TG, PL, Cholesterol), apoB48 and apoB100 from each fraction will be quantified using immunoblot analysis. In a similar experiment we will inject wild type, hMTP, and dMTP mice with Triton WR-1339 to block lipolysis of circulating lipoproteins, enabling direct examination of apoB48 and apoB100 production rates based either on western blot or by coinjection of mice with both Triton WR-1339 and $[^{35}\text{S}]$-Methionine, followed by immunoprecipitation of apoB from plasma samples.

In conclusion, our studies examined the independent lipid trafficking potential of MTP orthologs using both a cell-based and an in vivo approach. The side by side comparisons of neutral lipid trafficking achieved by two distant MTP orthologs has provided direct evidence of an important evolutionary adaptation in vertebrate species, which underlies their capacity to transport neutral lipid via the protein secretory pathway rather than across the plasma membrane, as occurs in invertebrate fat body cells (41). That is to say that MTP possesses separable functions in lipoprotein assembly one of which is to promote precursor lipoprotein formation, which requires MTP’s PL transfer activity and the other of which is to promote translocation of neutral lipid into the ER/Golgi, which requires MTP’s TG transfer activity. These studies have provided important insights into the intracellular lipid trafficking routes that have evolved to support whole body lipid homeostasis as well as potential therapeutic avenues to beneficially impact hepatic lipid metabolism for the prevention and treatment of hepatic steatosis and cardiovascular disease.
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Philip Scott MacArthur  
*Curriculum Vitae*

**Personal Information**

<table>
<thead>
<tr>
<th>Date of Birth:</th>
<th>July 19, 1977</th>
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<tbody>
<tr>
<td>Place of Birth:</td>
<td>Bethesda, Maryland</td>
</tr>
<tr>
<td>Citizenship:</td>
<td>United States of America</td>
</tr>
<tr>
<td>Address:</td>
<td>212 Brooks Edge Drive Winston-Salem, NC 27107</td>
</tr>
<tr>
<td>Phone:</td>
<td>336.306.4370</td>
</tr>
<tr>
<td>Email:</td>
<td><a href="mailto:pmacarth@wakehealth.edu">pmacarth@wakehealth.edu</a></td>
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**Educational Background**

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<td></td>
<td>Department of Pathology, Lipid Sciences</td>
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<tr>
<td></td>
<td>Wake Forest University School of Medicine</td>
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<td></td>
<td>Winston-Salem, NC</td>
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<td>GPA 3.55</td>
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<td>January, 2005-May  2006</td>
<td>Post-Baccalaureate Pre-Medicine Program</td>
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<td>Department of Biochemistry</td>
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<td>University of North Carolina, Greensboro</td>
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<td>GPA 4.0</td>
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<tr>
<td>December, 1999</td>
<td>Bachelor of Arts (BA), Anthropology</td>
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<td>Franklin College of Arts and Sciences</td>
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<td>Athens, GA</td>
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**Laboratory and Professional Experience**

**PhD Candidate (August 2006-May 2013)**

Wake Forest University School of Medicine, Molecular Pathology Graduate Program, Section on Lipid Sciences
Mentor: Gregory S. Shleness, PhD.

- Designed and conducted studies to elucidate the function of a protein, Microsomal Triglyceride Transfer Protein (MTP)
- Molecular cloning of genes into vectors for expression in mammalian cells for experimental procedures
- Molecular cloning of genes into vectors for expression in e-coli for antigen production to develop antibodies
- Generated stable cell lines over-expressing hMTP and dMTP
- Characterized the phenotype of liver specific human MTP (hMTP) and Drosophila MTP (dMTP) transgenic mice
- Received a Pre-doctoral grant from the American Heart Association, Mid-Atlantic affiliate
- Provided an oral presentation of my data at lab meetings or departmental meetings: lab meetings (weekly) and Program Project Grant meetings (~2/year)
- Participated in a weekly journal club and presented a published article ~2/year
- Additional responsibilities included rodent handling, culturing and maintenance of cell lines, assay troubleshooting, data collection and organization, mentoring undergraduate internship students, and general lab cleaning and maintenance

Archaeologist (March 2001-December 2004)
TRC, Inc. Columbia, SC.
Supervisor: William Green, PhD.

- Phase I, II and III archaeological investigations throughout the southeastern US, as well as, Maryland, Pennsylvania, Ohio, and California
- Co-authored technical reports of archaeological investigations (background, environmental setting, cultural overview, research design and methodology, results, summary, recommendations)
- Produced digital maps of newly discovered archaeological sites for technical reports
- Crew Chief, August 2003-December 2004
- Artifact analysis
- Artifact cataloging

Research and Laboratory Skills

- Cell culture aseptic technique
- Culturing of cell lines (CHO-K1, COS-1, COS-7, HepG2, Huh-7, McArdle-RH7777)
- Molecular cloning
• Sterile, endotoxin-free DNA preparation used for sequencing, molecular cloning, or stock supply
• Transfection of mammalian cell lines (lipofection and electroporation)
• Thin layer chromatography
• Gel electrophoresis and immunoblotting
• Immunoprecipitation
• Blood/tissue collection from mice
• *In vitro* enzymatic assays
• PCR
• Lipid extraction from tissues/cells
• RNA extraction/Real-Time PCR
• Handling and use of e-coli cultures
• Handling and use of radioisotopes ($^3$H and $^{35}$S)
• Transmission and scanning electron microscopy
• Computer skills include Microsoft Word, Excel, PowerPoint, MacVector, GraphPad Prism, and Canvas

**Funding**

**July 2010-June 2012.** American Heart Association, Mid-west affiliate Pre-doctoral grant. “*Mechanism of Neutral Lipid Translocation Across the Endoplasmic Reticulum Membrane.*”

**August 2008-June 2010.** The Integrative Lipid Sciences, Inflammation, and Chronic Diseases Training Program. “*Mechanism of Neutral Lipid Translocation Across the ER Membrane.*”

**Manuscripts in Preparation**

**MacArthur PS**, Fabritius MA, Cheng D, Sheliness GS. Human MTP but not *Drosophila* MTP overexpression in mouse liver attenuates hepatic triglyceride accumulation during onset of high fat diet-induced hepatic steatosis. *In preparation.*

**MacArthur PS**, Hou L, and Sheliness GS. The triglyceride transfer activity of microsomal triglyceride transfer protein promotes neutral lipid accumulation within the microsomal fraction of non-hepatic cells. *In preparation.*
Peer Reviewed Scientific Journal Publications

Abstract


Abstract


Abstract


Presentations


December 14, 2011  Oral Presentation  -  The Impact of Microsomal Triglyceride Transfer Protein on Intracellular Hepatic Neutral Lipid Trafficking and Distribution. Molecular Pathology Seminar Series, Wake Forest University School of Medicine, Winston-Salem, NC

March 23, 2010  Poster Presentation  -  Alternative Splicing Attenuates Transgenic Expression Directed by the ApoE Promoter-Enhancer Based Expression Vector, pLIV11. 10th Annual Graduate Student and Postdoc Research Day. Wake Forest University, Winston-Salem, NC

September 25, 2009  Poster Presentation  -  Alternative Splicing Attenuates Transgenic Expression Directed by the ApoE Promoter Enhancer Based
Expression Vector, pLIV11. South East Lipid Research Conference, Pine Mountain, GA

**May 1, 2009 Poster Presentation** - *Mechanism of Neutral Lipid Translocation Across The Endoplasmic Reticulum Membrane.* Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference, Washington, DC

**April 6, 2009 Poster Presentation** - *Mechanism of Neutral Lipid Translocation Across the ER Membrane.* Wake Forest University Graduate Student Research Day; Winston-Salem, NC

**October 4, 2008 Poster Presentation** - *Mechanism of Neutral Lipid Translocation Across the ER Membrane.* South East Lipid Research Conference, Pine Mountain, GA

**May 14, 2008 Oral Presentation** - *Mechanism of Neutral Lipid Translocation Across The ER Membrane.* Molecular and Cellular Pathobiology Seminar Series, Wake Forest University School of Medicine, Winston-Salem, NC

**Awards/Extracurricular**

**July 2010-June 2012.** American Heart Association, Mid-west affiliate Pre-doctoral grant. *Mechanism of Neutral Lipid Translocation Across the Endoplasmic Reticulum Membrane.*

**March 23, 2010 First Runner-Up:** Poster Presentation - *Alternative Splicing Attenuates Transgenic Expression Directed by the ApoE Promoter-Enhancer Based Expression Vector, pLIV11.* 10th Annual Graduate Student and Postdoc Research Day. Wake Forest University, Winston-Salem, NC

**October 2008 Second Runner-Up:** Poster Presentation - *Mechanism of Neutral Lipid Translocation Across the ER Membrane- South East Lipid Research Conference at Callaway Gardens, Pine Mountain, GA.

**August 2008-June 2010. Member:** The Integrative Lipid Sciences, Inflammation, and Chronic Diseases Training Program. *Mechanism of Neutral Lipid Translocation Across the ER Membrane.*

**Chancellor's List (UNCG) Spring 2005**

**Dean's List (UNCG) Spring 2005**

**Presedential Scholar Award (UGA),** Fall 1997, Winter 1998
Dean's List (UGA), Fall 1998, Fall 1999

Captain, Club Soccer Team, The University of Georgia, 1999

Player, Club Soccer Team, The University of Georgia, 1996-1999

Player (on Scholarship), Division I Soccer, Wofford College 1995-1996

Coach, U-19 Athena, Athens YMCA soccer team September, 1996- September, 1999 (Georgia Youth Soccer Association, Division Champion, Fall, 1999)