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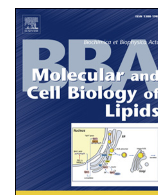

Structure-function analyses of microsomal triglyceride transfer protein missense mutations in abetalipoproteinemia and hypobetalipoproteinemia subjects

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ABSTRACT

We describe two new hypolipidemic patients with very low plasma triglyceride and apolipoprotein B (apoB) levels with plasma lipid profiles similar to abetalipoproteinemia (ABL) patients. In these patients, we identified two previously uncharacterized missense mutations in the microsomal triglyceride transfer protein (MTP) gene, R46G and D361Y, and studied their functional effects. We also characterized three missense mutations (H297Q, D384A, and G661A) reported earlier in a familial hypobetalipoproteinemia patient. R46G had no effect on MTP expression or function and supported apoB secretion. H297Q, D384A, and G661A mutants also supported apoB secretion similarly to WT MTP. Contrary to these four missense mutations, D361Y was unable to support apoB secretion. Functional analysis revealed that this mutant was unable to bind protein disulfide isomerase (PDI) or transfer lipids. The negative charge at residue 361 was critical for MTP function as D361E was able to support apoB secretion and transfer lipids. D361Y most likely disrupts the tightly packed middle α-helical region of MTP, mitigates PDI binding, abolishes lipid transfer activity, and causes ABL. On the other hand, the hypolipidemia in the other two patients was not due to MTP dysfunction. Thus, in this study of five missense mutations spread throughout MTP's three structural domains found in three hypolipidemic patients, we found that four of the mutations did not affect MTP function. Thus, novel mutations that cause severe hypolipidemia probably exist in other genes in these patients, and their recognition may identify novel proteins involved in the synthesis and/or catabolism of plasma lipoproteins.

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1. Introduction

Apolipoprotein B (apoB)-containing lipoproteins (Blps) are triglyceride (TG)-rich particles that transport the bulk of plasma lipids from the liver and intestine to peripheral tissues. The liver synthesizes and secretes very low density lipoproteins (VLDL), while the intestine secretes chylomicrons (CMs) [1]. Lipoproteins are assembled in the endoplasmic reticulum (ER) of liver and intestinal cells. Microsomal triglyceride transfer protein (MTP) physically interacts with apoB, which may help it translocate across the ER membrane [2–4]. It most likely also transfers different lipids to translating apoB to form secretion competent primordial lipoproteins. In the absence of MTP, apoB is insufficiently lipidated and intracellularly degraded. Primordial lipoproteins are assumed to fuse with lumenal lipid droplets to form mature lipoproteins, which are secreted into the circulation. In the bloodstream, lipoproteins are continuously remodeled by lipid transfer proteins and lipases before they are cleared from the plasma via the LDL receptor (LDLR), LDL receptor-related protein, or proteoglycans [5,6]. Decreased secretion or increased catabolism of Blps causes low plasma apoB and LDL-cholesterol (LDL-C) levels in abetalipoproteinemia (ABL) and familial hypobetalipoproteinemia (FHBL) subjects.

ABL (OMIM # 200100) is characterized by the absence of plasma Blps. Patients often present with growth retardation, malnutrition, diarrhea, and failure to thrive. A closer examination shows hepatic and intestinal steatosis, steatorrhea, elevated transaminases, and acanthocytosis. Due to the complete absence of Blps, patients have undetectable plasma TG and apoB, low plasma cholesterol, and fat soluble vitamin deficiency leading to detrimental neurological and ophthalmological effects. Early

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detection and a low fat diet with fat soluble vitamin supplementation can prevent the neurological and ophthalmological complications. Finally, since lipolysis of VLDL and CM results in the generation of HDL, the plasma levels of HDL-C and apoA-I are sometimes reduced in patients with ABL.

ABL is a rare (<1 in 1,000,000), autosomal recessive, monogenic disorder caused by loss-of-function mutations in MTP [7–9]. MTP is a heterodimer composed of the active M subunit and protein disulfide isomerase (PDI). PDI retains MTP in the ER, maintains MTP's solubility, and is required for the M subunit's lipid transfer activity [10–12]. A predictive tertiary structure for the M subunit was generated based on homology modeling with lipovitellin, an ancient lipid transfer protein [12]. The M subunit is divided into three structural domains: 1) the N-terminal domain has 12 β-strands arranged in a β-barrel, 2) the middle α-helical domain has 17 tightly packed α-helices, and 3) the C-terminal domain contains 2 β-sheets that form a hydrophobic pocket with two amphiphatic α-helices (AH1 and AH2) at the entrance. The N-terminal region binds apoB and PDI and possibly contains the slow phospholipid transfer site. The middle α-helical and N-terminal regions bind PDI. The hydrophobic pocket in the C-terminal domain is assumed to be involved in the bulk transfer of phospholipid, TG, cholesteryl ester, ceramide, and sphingomyelin [13–16]. Finally, helix AH1 and AH2 may be involved in acquiring lipids from the ER membrane for subsequent transfer to apoB [17].

Biochemical characterization of missense mutations in ABL subjects have corroborated and refined the predicted model of MTP. A mutation in the N-terminal region, D169V, abolishes PDI binding most likely due to disruption of an internal salt bridge [18]. Another ABL missense mutation, R540H, in the middle α-helical region also disrupts an internal salt bridge and abolishes PDI binding and lipid transfer activities [11]. Other mutations in this region (Y528H, L435H, and S590I) do not affect PDI binding, but still abrogate lipid transfer activities, suggesting that the importance of the middle α-helical region in lipid transfer activity extends beyond the PDI binding site [19,20]. Mutations in the C-terminal pocket (G746E, N649S) or in helices AH1 or AH2 (N780Y) also affect lipid transfer activity independently of PDI binding [9,17,21,22].

FHBL, an autosomal co-dominant disorder, is also characterized by severe hypolipidemia. It is subdivided into two categories: FHBL1 (OMIM # 107730) and FHBL2 (OMIM # 605019). Classical cases of FHBL1 are caused by mutations in the *APOB* gene resulting in the synthesis of various truncated apoB peptides. Truncated apoBs are more likely to be degraded than full length apoBs due to insufficient lipidation of the translated protein [23–25]. If assembled and secreted, Blps with truncated apoBs are catabolized quicker [23–25]. FHBL1 subjects have plasma apoB and LDL-C levels in the bottom 5th percentile (<30% of normal values). Heterozygous FHBL1 subjects are asymptomatic or have hepatic steatosis of variable severity. FHBL1 subjects carrying two mutant apoB alleles present with many of the same clinical symptoms as ABL, such as fatty liver, fat malabsorption, and ataxic neuropathy. Recently, dominant negative mutations and loss-of-function mutations in proprotein convertase subtilisin kexin 9 (PCSK9) have also been implicated in FHBL1 [26–28]. PCSK9 is a secreted serine protease that binds to LDLR at the plasma membrane, targets it to the lysosome for degradation, and prevents LDLR recycling to the plasma membrane. Loss-of-function mutations in PCSK9 increase LDLR recycling on the cell surface, enhance catabolism of Blps, and reduce plasma lipids [27,28].

FHBL2, also known as familial combined hypolipidemia, is characterized by a marked reduction in TG, LDL-C, and high density lipoprotein (HDL)-C. It has been linked to loss-of-function mutations in Angiopoietin-like 3 (*ANGPTL3*) [29–33]. ANGPTL3 inhibits lipoprotein lipase and endothelial lipase, which are involved in the lipolysis and catabolism of both VLDLs and HDLs. Plasma from patients with FHBL2 have reduced circulating ANGPTL3, leading to increased lipoprotein lipase and endothelial lipase activity, increased clearance of LDL and HDL, and low plasma lipids.

Although patients with ABL, FHBL1, and FHBL2 present with decreased plasma LDL-C and apoB, the mode of transmission and severity of symptoms differ. Patients with loss of function mutations in *MTTP* (ABL) or with two mutant alleles of the *APOB* gene (homozygous FHBL1) have a more severe phenotype (hepatic steatosis and intestinal lipid malabsorption) than patients with loss of function mutations in the *PCSK9* or *ANGPTL3* genes. FHBL1 patients that express shorter apoB peptides (<apoB20) have a more severe phenotype than homozygous and compound heterozygous individuals for longer peptides (>apoB80). Such genotype-phenotype diversity has not yet been reported for ABL subjects. Further, obligate FHBL1 heterozygotes have 1/2–1/3 normal levels of plasma apoB and LDL-cholesterol (LDL-C), while obligate ABL heterozygotes usually have normal lipid phenotypes. In FHBL2, the reduction in plasma TG is inherited as a co-dominant trait, while conflicting reports indicate that LDL-C and HDL-cholesterol (HDL-C) might be inherited either as co-dominant or recessive traits [32,34,35]. Molecular diagnosis of ABL, FHBL1, or FHBL2 is confirmed by genetic sequencing of the *MTTP*, *APOB*, *PCSK9*, and *ANGPTL3* genes.

Missense mutations in MTP usually have an "all or none" effect on apoB secretion; they either completely abolish apoB secretion (causing ABL) or have no effect on apoB secretion (polymorphisms). MTP had been implicated in one FHBL patient, but the functional effects of these mutations were not studied [36]. In this FHBL1 patient, we had identified three missense mutations in the *MTTP* gene and had speculated that these mutations might contribute to hypolipidemia [36]. Here, we characterized these three missense mutations individually and in combination. Further, we identified two new ABL patients with novel homozygous missense mutations in the *MTTP* gene and characterized their effects on MTP function.

2. Materials and methods

The Ethics Committees of each participating institution approved this study. It is in accordance with ethical standards as formulated in the Helsinki Declaration of 1975 (revised in 1983). Informed consent was obtained from the parents of all three probands.

2.1. Analysis of plasma lipids, lipoproteins, and apolipoproteins

Plasma lipids were measured after an overnight fast as described before [36,37]. LDL-C was measured directly. The IMMAGE immunochemistry system (Beckman, Fullerton, CA, USA) was used to measure apoA-I and apoB.

2.2. Genetic analysis

Isolation of genomic DNA, polymerase chain reaction (PCR) amplification of exons, and Sanger sequencing of *MTTP*, *APOB*, *PCSK9* and *ANGPTL3* genes were performed as previously described [28,30,36–38]. PCR fragments were purified with a commercial kit (High Pure PCR purification kit (Roche Diagnostics GmbH, Germany) and sequenced directly in both directions using the BigDye Terminator Cycle sequencing kit (Applied Biosystems, Warrington, UK) in a 3100 ABI PRISM automated DNA Sequencer (Applied Biosystems, Warrington, UK). For all genes, all exons and at least 40 bp of intronic sequence at each intron–exon boundary as well as the promoter (5′-flanking regions) region (up to −700 bp for *MTTP* gene, up to −900 bp for *APOB* gene; up to −500 bp for *PCSK9* gene) were sequenced. The results were analyzed with ABI PRISM SeqScape software (Applied Biosystems, Warrington, UK). The *MTTP* mutations found in the probands were screened by direct sequencing in all family members available for study.

Bioinformatics analyses of the missense mutations on MTP function was performed using PolyPhen (http://genetics.bwh.harvard.edu), Panther (http://www.pantherdb.org) and SIFT (http://sift.jcvi.org/) algorithms. Computational modeling of the tertiary structure of the M

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