The Ile128Thr polymorphism influences stability and ligand binding properties of the microsomal triglyceride transfer protein

H. Ledmyr,* L. Ottosson,† M. Sunnerhagen,§ and E. Ehrenborg[1,*]

Atherosclerosis Research Unit,* King Gustaf V Research Institute, Karolinska University Hospital, Stockholm, Sweden; Rheumatology Unit,† Department of Medicine, Karolinska Institutet, Stockholm, Sweden; and Department of Chemistry,§ Division of Molecular Biotechnology, Linköping University, Linköping, Sweden

Abstract The microsomal triglyceride transfer protein (MTTP) is essential for the assembly of VLDLs. We recently observed that a polymorphism in the MTTP promoter (−493G>T), which is in allelic association with an isoleucine-to-theronine substitution at position 128 (Ile128Thr) in the expressed protein, confers an increased risk of coronary heart disease. Two variant proteins comprising amino acids 16–297 of intact MTTP, $MTTP_N$-Ile128 and $MTTP_N$-Thr128, had similar native secondary structure content, as judged by circular dichroism. However, the thermal stability of $MTTP_N$-Thr128 was greatly reduced, and this protein was also more extensively cleaved in limited proteolysis experiments compared with $MTTP_N$-Ile128; both of these findings support a less compact fold. On adding LDL, which includes natively folded apolipoprotein B (apoB), decreased stability of the $MTTP_N$-Thr128-LDL complex was observed compared with that of the $MTTP_N$-Ile128-LDL complex. In a refined model of the N-terminal domain of MTTP, residue 128 is located in a surface-exposed position, in the same region as an identified MTTP binding site in the homologous apoB protein. Thus, the Ile128Thr polymorphism confers reduced structural stability, leading to decreased binding of MTTP to LDL particles. Because the major MTTP binding target on LDL is apoB, the Ile128Thr polymorphism could target the MTTP-apoB interaction.—Ledmyr, H., L. Ottosson, M. Sunnerhagen, and E. Ehrenborg. **The Ile128Thr polymorphism influences stability and ligand binding properties of the microsomal triglyceride transfer protein. *J. Lipid Res.* 2006.** 47: **1378–1385.**

Supplementary key words missense polymorphism • protein function • limited proteolysis • circular dichroism

The microsomal triglyceride transfer protein (MTTP) is essential for the assembly of VLDLs in hepatocytes and chylomicrons in enterocytes (1). VLDLs are large particles consisting of apolipoprotein B (apoB) and bulk triglycerides and are needed in the circulating plasma as energy deposits for muscle tissue. MTTP is also expressed in the heart; its function there is not completely understood, but it has been hypothesized that MTTP could facilitate the export of excess triglycerides from the heart muscle (2, 3). MTTP is functional as a heterodimer of the large and unique 97 kDa subunit and the multifunctional 55 kDa protein disulfide isomerase. The MTTP subunit confers lipid transfer activity, whereas protein disulfide isomerase holds the complex in a stable, active conformation (4). Protein disulfide isomerase possesses a C-terminal endoplasmic reticulum retention signal (lysine-aspartate-glutamate-leucine or KDEL sequence) that is essential for the endoplasmic reticulum localization of the complex (5). MTTP binds to the apoB polypeptide as it is being translated and loads the growing apoB particle with bulk triglycerides to form a mature VLDL particle (6, 7).

Because it is known that lipid metabolism is affected by individual variations in the gene heritage, a deeper analysis of the effects of polymorphisms in the MTTP gene could give important functional information. Three polymorphisms in the MTTP gene have been described to be in almost complete linkage disequilibrium (8). Two are promoter polymorphisms and can be found at positions −493 (a G-to-T substitution) and −164 (a T-to-C substitution). The −493G>T polymorphism has been shown to affect the transcriptional activity of the MTTP promoter in HepG2 cells (9). Furthermore, we recently showed that individuals homozygous for the −493T allele have a markedly increased risk of coronary heart disease (10). These results were somewhat surprising, because the same genotype confers a small decrease of LDL cholesterol levels, and one would expect the opposite effect on coronary heart disease risk under these circumstances. The second promoter polymorphism, −164T>C, is located in

Manuscript received 10 February 2006 and in revised form 10 April 2006.

Published, JLR Papers in Press, April 14, 2006.
DOI 10.1194/jlr.M600072-JLR200

[1] To whom correspondence should be addressed.
e-mail: ewa.ehrenborg@ki.se

a putative sterol response element. The rare allele gives a sterol response element that is more homologous to the low density lipoprotein receptor sterol response element, which could lead to cholesterol-dependent transcription. However, more studies of the promoter polymorphic variants are needed to establish their combined effect on the transcriptional activity of the MTTP gene.

The third polymorphism, which is located in exon 3, leads to an amino acid difference in the expressed protein. The common variant has isoleucine (Ile) and the rare variant has threonine (Thr) in amino acid position 128 (11). Although no physiological polymorphisms in the expressed MTTP protein have yet been functionally analyzed, several amino acid substitutions that are associated with changes in the structure and/or function of proteins involved in lipoprotein metabolism have been reported. One of the best-known cases is the polymorphisms in the apoE gene, which result in three major functional isoforms of the apoE protein (12). In the apoB protein, substitution of an arginine with a tryptophan at position 3,500 has been shown to diminish the receptor-mediated uptake of LDL and to cause familial defective apoB (13). A third example is the alanine-threonine substitution at position 176 in the lipoprotein lipase protein. Individuals who have this genetic variant show abnormal heparin binding and loss of enzymatic activity (14).

A general idea of the structural location of the isoleucine-to-theronine substitution at position 128 (Ile128Thr) polymorphism can be derived from the common ancestry of apoB and MTTP with vitellogenin (15). The vitellogenins are ancient lipid transport and storage proteins that are ligands to proteins of the LDL receptor superfamily and deliver nutrients to the egg yolk (16, 17). Although no structures of apoB or MTTP are available, a low-resolution structure of lipovitellin, which is the mature form of vitellogenin, was used to provide structural models of both apoB and MTTP (15). From these models, it is clear that the third polymorphism (Ile128Thr) is located in an N-terminal well-conserved β-barrel-type subdomain (15).

To elucidate possible functional implications of the Ile128Thr polymorphism located in the MTTP N-terminal domain, it should be noted that these regions in both MTTP and apoB appear to have interactive properties. The N-terminal region of apoB (residues 1–264) has been reported to bind to the N-terminal region of MTTP (residues 22–303) (15). Furthermore, a proteoglycan binding site on apoB has been mapped to amino acid residues 84–94 (18), also in the N-terminal domain (15). Because the position of the Ile128Thr polymorphism is confined to this region, the genetic variation could influence MTTP interactions, presumably leading to the observed patient phenotypes.

In this study, we aimed to evaluate whether the Ile128Thr polymorphism might have any impact on the structure and/or function on the N-terminal domain of MTTP. A combined strategy using limited proteolysis, circular dichroism (CD) spectroscopy, and modeling using the recently refined lipovitellin coordinates (19) was used.

METHODS

Cloning

The sequence corresponding to amino acids 16–297 of the MTTP gene was amplified from cDNA obtained from HepG2 cells. Primers were designed with overhangs corresponding to the recognition sequence of the restriction enzymes *Nde*I and *Eco*RI. The forward primer was MTTPSer16 (5′-CATATGTTCAGCTTCTGTTAAAGGTC-3′), and the reverse primer was MTTPHis297 (5′-GAATTCGTGGCTCTGGAAGACC-3′). Amplification was performed in a 25 μl reaction mix containing 100 ng of cDNA, 0.8 μM of each primer, 2 mM of each deoxynucleoside triphosphate (Boehringer Mannheim), 1 unit of Taq polymerase (Promega, Madison, WI), 50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, and 0.1% Triton X-100. Fresh PCR product was ligated into pGEM Easy II vector according to the manufacturer's protocol (Promega).

The expression vector pETMCSIII (20) and the pGEM Easy II vector containing the MTTP gene fragment were digested with *Nde*I and *Eco*RI (New England Biolabs) in 50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, and 0.025% Triton X-100, pH 7.5, at 37°C. The PCR-amplified MTTP gene fragment was purified and subsequently ligated into the pETMCSIII vector with a vector-insert ratio of 5:1. Successful cloning of the MTTP insert into the pETMCSIII vector was verified by ABI sequencing according to the manufacturer's protocol.

To obtain the rare Thr128 variant, a site-directed mutagenesis kit (Stratagene) was used together with primers MTTP128Thr-1 (5′-GCAAAGACCTACGCTCCTTCATCTAACCCATGGAAAGGTC-3′) and MTTP128Thr-2 (5′-CTCTTTGACCTTTCCATGGGTTAGATGAAGGAGCGTAGG-3′) according to the manufacturer's protocol. Successful mutagenesis was verified by ABI sequencing.

Protein expression and purification

Plasmids containing the correct MTTP sequence were transformed into the competent *Escherichia coli* strain BL21(DE3)plys-s gold and grown at 37°C in Luria-Bertani medium containing carbenicillin and cloramphenicol. Cultures were induced with isopropylthio-β-galactoside (0.1 mg/ml) and harvested after 4 h. Pellets were resuspended in 50 mM NaH_2PO_4, 100 mM NaCl, and 10 mM Tris at pH 8.0 and sonicated on ice. The pellet was finally resuspended in 100 mM NaH_2PO_4, 10 mM Tris, and 8 M urea at pH 8.0, sonicated on ice, and spun down at 20,000 *g* and 4°C for 30 min.

Protein purification was performed on a nickel-nitrilotriacetic acid agarose affinity column according to the manufacturer's protocol (Qiagen). Fractions of 60 drops each were collected and frozen. Samples were run on a 15% (w/v) SDS gel to verify successful purification. Refolding of the denatured protein was performed by dialysis in 50 mM NaH_2PO_4, 100 mM NaCl, 10 mM Tris, 5 mM EDTA, 2 mM DTT, 1 mM benzamidine, and 1 mM β-mercaptoethanol using number 6 dialysis membrane tubing (Spectrum Medical Industries, Inc.). To verify the presence of disulfide bonds, we estimated the total number of free sulfhydryl groups (21). No free thiols were observed in any of the variants, which indicates the formation of a suggested disulfide bond in the folded structure, as suggested in previous MTP structural modeling (15). Proper folding of the protein was confirmed by CD spectroscopy (see below).

Proteolysis experiments

Before limited proteolysis, the protein was dialyzed into 50 mM NaH_2PO_4, 100 mM NaCl, 10 mM Tris, and 5 mM EDTA

to a concentration of 3 pmol/μl ($MTTP_N$-Ile128) or 1.6 pmol/μl ($MTTP_N$-Thr128). Aliquots of the protein (30 μl) were digested with 10^{-2}-fold dilutions of chymotrypsin stock solutions of 1.6 mg/ml for 1, 2, 5, 10, 20, 30, 50, and 100 min. The proteolysis reaction was stopped by adding trichloroacetic acid. Samples were then incubated on ice for 30 min and spun at 15,000 *g* for 30 min, the supernatant was discarded, and the precipitated protein was dissolved in 12 μl of SDS-PAGE loading buffer (80 μM Tris-HCl, pH 6.8, 35% glycerol, 3% SDS, 0.02 M 2-mercaptoethanol, and 0.002% bromphenol blue) before visualization on a 15% (w/v) SDS-polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue R-250.

The identities of fragments produced by proteolysis were determined by N-terminal sequencing (Protein Analysis Centre, Karolinska Institutet). The protein fragments were blotted onto a polyvinylidene difluoride (Tropiflour™) membrane and stained with Coomassie Brilliant Blue R-250, and the bands of interest were cut out and submitted to five cycles of Edman degradation.

Ligand binding experiments

Ligand binding experiments were carried out with human LDL cholesterol particles that were prepared the day before the assays. Plasma LDL cholesterol was isolated by density gradient ultracentrifugation (22). After a 16 h spin at 40,000 rpm and 15°C (Beckman SW40), the top 0.5 ml layer was aspirated (VLDL). The tube was then sliced 57 mm from the top to harvest the fraction (density = 1.006–1.061 kg/l) containing LDL. The protein concentration of the isolated fraction was determined according to the method of Lowry et al. (23) after the addition of SDS to the reagent mixture to clear turbidity. Previous studies have shown a stimulating effect of oleic acid on the assembly of VLDL (24); therefore, the ligand binding assays in this study were performed with 1 mM oleic acid (Sigma).

CD measurements

Wavelength scans and denaturation experiments were performed on an AVIV Associates model 62DS CD spectrometer. All wavelength-scanned spectra were recorded in a 0.1 cm cuvette at 25°C over the wavelength range of 195–250 nm with a step size of 1 nm, a bandwidth of 1.5 nm, an average collection time of 2 s per point, and an equilibration time of 1 min. The CD spectra were averaged from four wavelength scans and blanked against the used buffer (10 mM sodium phosphate buffer, pH 7.5). Denaturation experiments were performed in a 0.1 cm cuvette positioned in a thermostat-equipped sample holder, recording the ellipticity at a single wavelength (218 nm) while increasing the temperature at a rate of 2°C/min. The resulting denaturation curve was fitted to linear predenaturation and postdenaturation curves representing the temperature dependence of the ellipticity in the folded and unfolded states, respectively.

Sequence analysis and domain structure modeling

The N-terminal domain of the native MTTP sequence, residues 28–265 (SWISSPROT number P55157), was aligned toward the common MTTP and apoB ancestor protein vitellogenin (SWISSPROT number Q91062) using ClustalW (25) and 3D-PSSM (26). The refined coordinates of vitellogenin (15) were used as a template for the structural modeling. The final model was derived using Modeller4 (27), in which the initial alignment was optimized to relieve local energetic stress and to optimize the alignment in regions of low similarity. Results were visualized with PyMOL (28). The $MTTP_N$-Ile128 variant was used for the model.

RESULTS

Subcloning, expression, and purification of $MTTP_N$-Ile128 and $MTTP_N$-Thr128

To investigate whether the Ile128Thr polymorphism could influence the functional properties of the expressed gene, an MTTP subregion comprising the N-terminal domain (residues 16–297) was subcloned into a histidine-tagged expression vector (see Methods). Both the $MTTP_N$-Ile128 and $MTTP_N$-Thr128 proteins were expressed as inclusion bodies; therefore, a refolding protocol was designed to regain the native fold (see Methods). The $MTTP_N$-Thr128 protein, representing the less common allele, had to be refolded in a 2-fold more dilute concentration compared with the $MTTP_N$-Ile128 variant to avoid precipitation and to attain a proper fold, as judged by CD spectroscopy (see below). The $MTTP_N$-Thr128 protein also showed a higher tendency to adhere to surfaces (plastic, glass); thus, all analyses had to be performed at low micromolar concentrations.

Analysis of folding and stability by CD spectroscopy

The secondary structure of the two variants of the N-terminal domain was evaluated by CD spectroscopy in near-ultraviolet light. The CD spectra of $MTTP_N$-Ile128 and $MTTP_N$-Thr128 were similar. Both spectra show typical features of a mixed β-sheet/random coil structure at room temperature (**Fig. 1**). Although buffer conditions prevented the recording of spectra below 200 nm, a rough estimate of the secondary structure content by K2d (29) of $MTTP_N$-Ile128 suggests 40% β-sheet, 48% random coil, and 12% α-helix. A K2d fit of the $MTTP_N$-Thr128 spectrum was less reliable, but judging from the similar spectral features compared with the Ile128 variant, the two $MTTP_N$ variants appear to be similarly structured at room temperature.

The stability of the two protein domains was investigated by thermal denaturation monitored by CD (**Fig. 2**). Surprisingly, the stabilities of $MTTP_N$-Ile128 and $MTTP_N$-

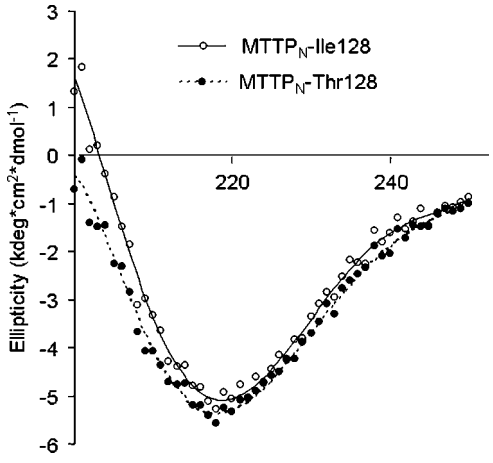

Fig. 1. Circular dichroism (CD) spectra of the two variants of the N-terminal domain of microsomal triglyceride transfer protein (MTTP). $MTTP_N$-Ile128 is denoted by open circles, and $MTTP_N$-Thr128 is denoted by closed circles.

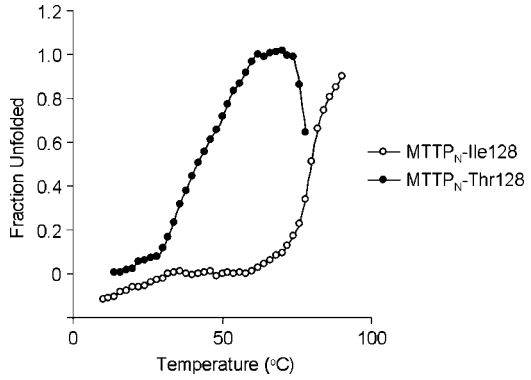

Fig. 2. CD spectra of the thermal denaturation of the two variants of the N-terminal domain of MTTP. MTTP$_N$-Ile128 is denoted by open circles, and MTTP$_N$-Thr128 is denoted by closed circles.

Thr128 are significantly different. After a plateau of stability at physiological temperatures and above, and with even higher stability at lower temperatures, the MTTP$_N$-Ile128 variant is stable up to 60°C, at which it unfolds rapidly and cooperatively. The MTTP$_N$-Thr128 variant, on the other hand, starts to unfold already at 30°C, with low cooperativity, both of which suggest a much less compact fold (Fig. 2). After complete denaturation, the MTTP$_N$-Thr128 variant precipitates, disabling measurements at temperatures of >70°C. The MTTP$_N$-Ile128 variant, on the other hand, is still not completely denatured at 90°C.

Limited proteolysis with chymotrypsin

The extent and nature of the hydrophobic core in a folded protein domain can be assayed by proteolysis, because the hydrophobic core is proteolytically protected in a stable fold. To probe for the extent of exposed hydrophobic residues, chymotrypsin, which cleaves preferentially at tyrosine, phenylalanine, isoleucine, leucine, and valine, was chosen for a time-resolved proteolysis experiment. To probe whether the lower stability of the MTTP$_N$-Thr128 variant was attributable to a more exposed hydrophobic core, both MTTP $_N$-Ile128 and MTTP$_N$-Thr128 were subjected to chymotrypsin cleavage (**Fig. 3**). The same proteolytic fragments are produced for both variants, which indicates that MTTP$_N$-Ile128 and MTTP$_N$-Thr128 share a common fold. However, three distinct fragments (I, II, and III) were observed at a significantly earlier time point for the MTTP$_N$-Thr128 protein compared with the MTTP$_N$-Ile128 protein (Fig. 3). N-terminal sequencing of these three fragments corresponded to (H)LIGH at residue 127 (I), the N terminus (II), and (Y)KLT at residue 35 (III) (residues in parentheses correspond to the residue N terminal to the cleavage site). Although the fragment comprising the N terminus does not give any information regarding structure because its C-terminal end was not identified, the increased cleavage rates at residues 127 and 35 suggest that these regions are more exposed in MTTP$_N$-Thr128 compared with MTTP$_N$-Ile128. Such exposure could be attributable to a less compact folding of the MTTP$_N$-Thr128 variant or a more dynamic assembly of secondary structure elements, both of which would allow for increased accessibility to proteases.

Ligand binding experiments

Ligand binding experiments were carried out by limited proteolysis to examine whether the change from an isoleucine to a threonine at position 128 would interfere with the binding of MTTP to its natural ligand apoB. ApoB is the main lipoprotein present on the LDL particle, but apoB precipitates after delipidation. Therefore, we chose the intact LDL particle as a ligand in this experiment to enable interactions with a fully folded apoB. MTTP$_N$-Ile128 and MTTP$_N$-Thr128 were incubated with human LDL cholesterol particles and 1 mM oleic acid before digestion with chymotrypsin. The oleic acid was added to facilitate the interaction between MTTP and apoB. Results show that both the MTTP and the apoB components of the presumed MTTP$_N$-Thr128-LDL complex were proteolytically cleaved at a faster rate than those in the presumed MTTP$_N$-Ile128-LDL complex (**Fig. 4**). Although the MTTP$_N$-Thr128 protein is completely digested after 50 min, the MTTP$_N$-Ile128 protein is still present after 100 min. ApoB is cleaved into two distinct fragments already after 5 min in the complex containing the MTTP$_N$-Thr128 variant, whereas apoB together with MTTP$_N$-Ile128 appears to be completely protected at 20 min. Thus, the apoB-MTTP$_N$-Thr128 complex appears less stable than the apoB-MTTP$_N$-Ile128 complex on LDL particles. The rapid accumulation of additional

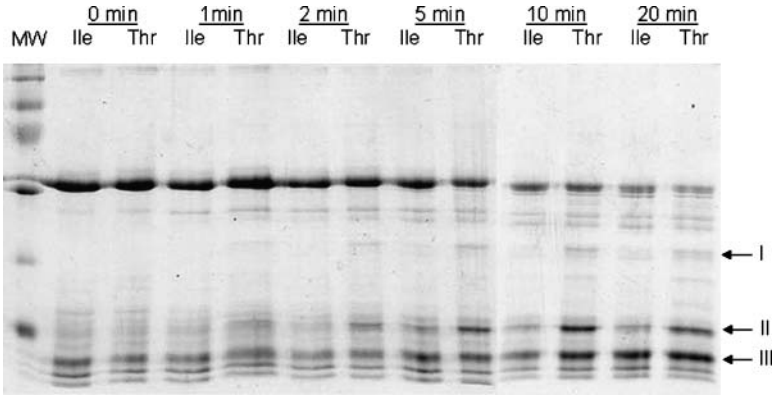

Fig. 3. Proteolysis by chymotrypsin of the MTTP$_N$-Ile128 (Ile) and MTTP$_N$-Thr128 (Thr) variants of MTTP. Time points in minutes are given at the top of each lane. The molecular weight marker is shown at left (MW). The arrows denoted I, II, and III show the fragments formed during proteolysis that were subsequently sequenced.

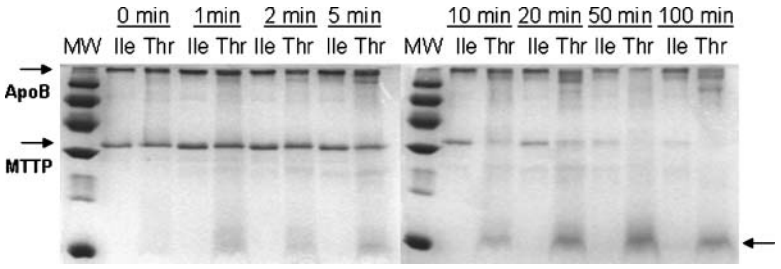

Fig. 4. The common Ile128 and the less common Thr128 variants of the N-terminal domain of MTTP were incubated with human LDL cholesterol particles at room temperature and subjected to time-resolved proteolysis by chymotrypsin. The $MTTP_N$-Ile128-LDL and $MTTP_N$-Thr128-LDL complexes are indicated as Ile and Thr, respectively. Time points in minutes are given at the top of each lane. The apolipoprotein B (ApoB) and MTTP complexes, as well as an unknown complex, are denoted by an arrow. MW, molecular weight.

small cleavage products in the less stable complex (Fig. 4) supports this conclusion.

Structural modeling of the N-terminal domain of MTTP

The experimental CD data on the two N-terminal MTTP domains ($MTTP_N$-Ile128 and $MTTP_N$-Thr128) show predominant β-sheet structure, which agrees with previous modeling attempts, in which a less resolved crystal structure of vitellogenin was used as a template (15). To further define the $MTTP_N$ domain model, the refined vitellogenin crystal structure (Protein Data Bank deposit 1LSH, (19) was used as a template to model the structure of the N-terminal MTTP domain. Only residues 28–265 were modeled, because they correspond to the N-terminal domain of vitellogenin (**Fig. 5**). The MTTP model consists of 13 β-strands and 2 α-helices, forming a β-barrel (**Fig. 6**). The secondary structure content of $MTTP_N$ derived from CD spectra (see above) is in good agreement with that in the model (43% random coil, 47% β-sheet, and 9% α-helix). In this model, Ile128 is located in a loop protruding from the top of the β-barrel, which faces the N terminus of the domain. This loop does not take part in forming the hydrophobic protein core; rather, it protrudes away from the protein core with a high degree of surface exposure. Thus, Ile128, despite its hydrophobic character, is also exposed, and no candidates for hydrophobic side chain interactions to this residue were identified.

DISCUSSION

In this study, we aimed to evaluate possible functional and structural differences at the protein level between two variants of MTTP resulting from a Ile128Thr polymorphism in exon 3, which is in almost complete linkage disequilibrium with two promoter polymorphisms, −493G>T and −164T>C (8). Our interest was prompted by previous observation that healthy individuals homozygous for the rare alleles have a slight decrease in LDL cholesterol as well as a slight increase in body mass index and insulin levels (8) and our own observation that individuals homozygous for the rare −493T allele have a markedly increased risk of death from coronary heart disease as well as a slight increase of electrocardiogram abnormalities (10).

Analysis by CD spectroscopy and limited proteolysis both strongly support the idea that although the two variants have similar folds at room temperature, the rare variant $MTTP_N$-Thr128 is significantly less stable. CD spectroscopy of $MTTP_N$-Ile128 and $MTTP_N$-Thr128 showed similar secondary structure content at room temperature but significantly decreased thermal stability of the Thr128 variant. In agreement with this, identical limited proteolysis products at room temperature suggested a common fold of the two variants, but a locally more rapid degradation of the Thr128 variant, reflected in the increased intensity of three proteolytic products, suggested lower structural stability. Together, these findings suggest

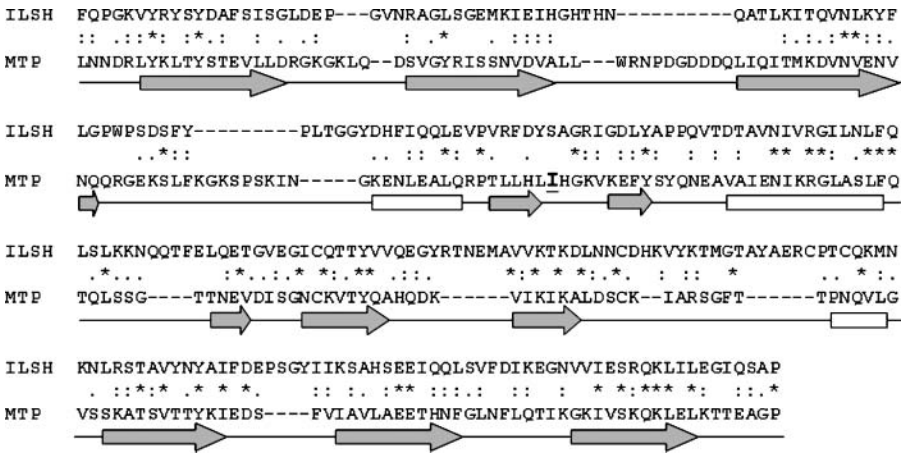

Fig. 5. Alignment of lamprey vitellogenin (1LSH) and human MTTP (MTP) sequences. The notation identifies identical (asterisks), conserved (colons), and semiconserved (dots) amino acids. The polymorphic residue 128 is boldfaced and underlined. The secondary structure, as in the derived model, is shown below the sequence alignment. β-sheets are shown as gray arrows, and amphipathic α-helices are shown as white boxes.

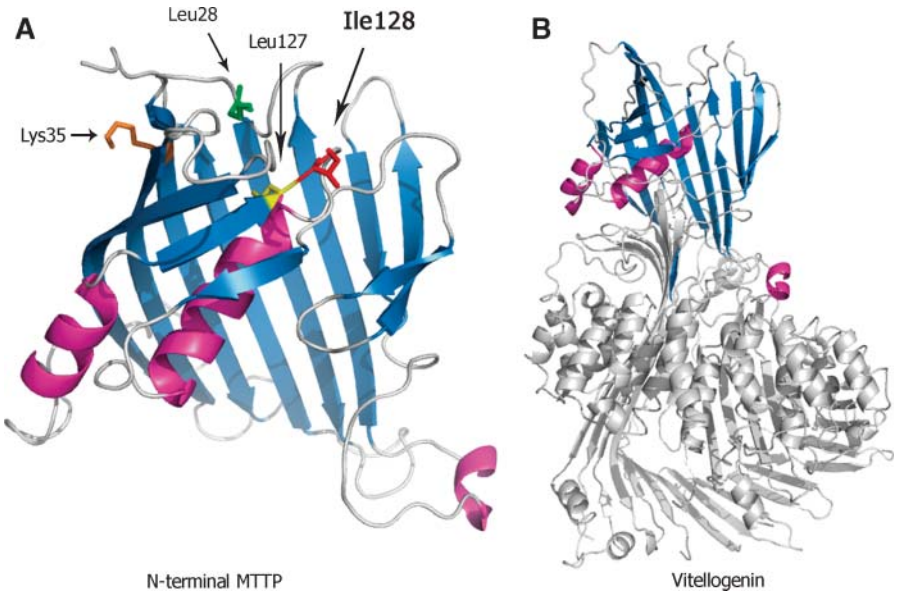

Fig. 6. Cartoon representation of the predicted model of the N-terminal domain of MTTP (left) according to the alignment with lamprey vitellogenin in Fig. 5, and model of the complete vitellogenin (right). The corresponding N-terminal domain of vitellogenin is highlighted in the same colors as the N-terminal domain of MTTP. Stick-represented residues in MTTP are Ile128 (depicted in red), Lys35 (orange), Leu127 (yellow), and the N-terminal Leu28 (green).

that the Ile128Thr polymorphism results in a more open assembly of secondary structure elements in the rare protein variant, reflected both in the increased accessibility to cleavage in a limited part of the protein domain and in decreased overall stability, as indicated by the decreased melting temperature in the CD thermal denaturation experiments.

In the present $MTTP_N$ model, the Ile128Thr polymorphic site is located at the barrel opening facing the domain N terminus. Interestingly, the N-terminal proteolytic cleavage sites corresponding to fragments I and III (Figs. 3, 6) that show increased cleavage rates in the $MTTP_N$-Thr128 variant are both located on the same side of the barrel. One site is directly adjacent to the polymorphic site, suggesting increased protease accessibility in this region as a direct result of the polymorphism (Fig. 6). This is in agreement with the decreased stability of the $MTTP_N$-Thr128 variant. Furthermore, the MTTP model revealed residue 128 to be located in an exposed loop position in the N-terminal region, pointing out from the core, which is unexpected for such a hydrophobic residue. No tertiary stabilizing contacts to residue 128 were observed in the model that would explain its exposed position, such as contacts with exposed neighboring secondary structure elements. Although sequence similarity is limited between MTTP and vitellogenin in the region surrounding the polymorphism, it is less likely that this sequence segment would be folded back and buried into the core, because this would disrupt the otherwise conserved general fold that is in experimental agreement with CD data. Rather, the low sequence similarity surrounding residue 128 is in agreement with the idea that exposed loops are generally less conserved than buried and/or structure-defining motifs. The lack of sequence homology could also indicate that this loop contains MTTP sequence-specific patterns. Thus, the exposed positioning of Ile128 may have functional relevance, for example, as part of a protein interaction surface. Indeed, the top of the β-barrel, the "portal region," is a motif that is well known for being able to participate in fatty acid binding (30). Disorder-order transitions in the portal region of intestinal fatty acid binding protein have been suggested to be pivotal for its ligand binding, and a polymorphism in this region is suggested to be linked to metabolic consequences (31, 32).

The common ancestry of apoB and MTTP with vitellogenin suggest that the two proteins have similarly folded N-terminal domains (15). Furthermore, it is well known that apoB and MTTP interact. Indeed, the N-terminal domain of MTTP (amino acids 22–297) has been shown to bind to a site on apoB, which includes apoB residues 1–152 (15). If so, the Ile128Thr polymorphism maps to the same domain of MTTP that was identified on apoB as an interacting region. Furthermore, a proteoglycan binding site on apoB has been mapped to a similar region, amino acid residues 84–94 (18). Together, these findings suggest a highly interactive property of this surface region in the $MTTP_N/apoB_N$ fold. The Ile128Thr polymorphism confers a change from an uncharged to a polar amino acid, which, although structurally minor, could influence electrostatic properties involved in stability-enhancing interactions and interactive properties.

Our results indicate that there is indeed a negative effect of the polymorphism on MTTP's binding to LDL, and possibly also to apoB. To investigate whether the Ile128Thr polymorphism affects the binding to apoB,

human LDL particles were used in the ligand binding experiments. Whole LDL particles were used instead of pure apoB protein because of the insolubility of apoB when it is delipidated. Furthermore, apoB is the only lipoprotein harbored by LDL. In the presence of LDL, increased cleavage propensity, suggesting a lower degree of protection, was observed for both apoB and MTTP with the $MTTP_N$-Thr128 variant compared with the breakdown pattern observed when the $MTTP_N$-Ile128 variant was used. This indicates that there is indeed a negative effect of the polymorphism on MTTP's binding to LDL, and possibly also to apoB. Further experiments would be necessary to evaluate the full implications of the Ile128Thr polymorphism on VLDL assembly and secretion.

How, then, could the decreased stability in the MTTP domain in the less common variant relate to decreased apoB affinity? Indeed, the three more sensitive proteolysis cleavage sites observed for the $MTTP_N$-Thr128 protein map to the same face of the MTTP domain where the polymorphism is located. Thus, it is highly suggestive that the polymorphic variant leads to the partial unfolding of this region, opening it up for additional proteolytic cleavage and simultaneously decreasing protein stability. Also, if this surface is involved in protein interactions, the higher entropy obtained by the destabilization would act unfavorably in the interaction with target proteins.

Together, these results indicate that the Thr128 allele confers a decreased stability of the domain as well as a decreased ability of MTTP to bind to apoB. It can be hypothesized that this could be a contributing factor to the phenotype previously seen in the West of Scotland Coronary Prevention Study cohort (10). MTTP is expressed in the heart; its function there is not completely understood, but it has been hypothesized that MTTP would facilitate the export of excess triglycerides from the heart muscle (2, 3). The absence of functional MTTP in the heart leads to increased cardiac triglyceride stores in mice, which in turn is associated with heart failure and sudden cardiac death (33); additionally, Unger and Orci (34) have proposed that overloading the myocardium with triglycerides causes lipotoxic heart disease. If the Thr128 variant of the MTTP is less stable and binds less effectively to apoB than the Ile128 variant, it could be speculated that the export of the excess triglycerides from the heart muscle is impaired, and this in turn could render the myocardium more susceptible to ischemic damage.

The authors thank Dr. A. Vlamis-Gardikas for assistance with the determination of free thiols and Malin Fladvad for generous technical advise. Professor M. M. Hussain (State University of New York Downstate Medical Center) is acknowledged for helpful discussions. The present study was supported by grants from the Heart-Lung Foundation, the Swedish Research Council (12659 and 621-2003-6475), the Swedish Medical Society, and the following foundations: Carl Trygger, Harald and Greta Jeansson, Sigurd and Elsa Golje Memorial, Fredrik and Ingrid Thuring, Magn. Bergwall, Professor Nanna Svartz, Old Servants, and Lars Hierta Memorial. H.L. was supported by a PhD studentship from the National Network for Cardiovascular Research.

REFERENCES

1. Wetterau, J. R., and D. B. Zilversmit. 1986. Localization of intracellular triacylglycerol and cholesteryl ester transfer activity in rat tissues. *Biochim. Biophys. Acta.* **875:** 610–617.
2. Nielsen, L. B., M. Veniant, J. Boren, M. Raabe, J. S. Wong, C. Tam, L. Flynn, T. Vanni-Reyes, M. D. Gunn, I. J. Goldberg, et al. 1998. Genes for apolipoprotein B and microsomal triglyceride transfer protein are expressed in the heart: evidence that the heart has the capacity to synthesize and secrete lipoproteins. *Circulation.* **98:** 13–16.
3. Boren, J., M. M. Veniant, and S. G. Young. 1998. Apo B100-containing lipoproteins are secreted by the heart. *J. Clin. Invest.* **101:** 1197–1202.
4. Wetterau, J., K. Combs, S. Spinner, and B. Joiner. 1990. Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. *J. Biol. Chem.* **265:** 9801–9807.
5. Gordon, D., H. Jamil, D. Sharp, D. Mullaney, Z. Yao, R. Gregg, and J. Wetterau. 1994. Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc. Natl. Acad. Sci. USA.* **91:** 7628–7632.
6. Mitchell, D. M., M. Zhou, R. Pariyarath, H. Wang, J. D. Aitchison, H. N. Ginsberg, and E. A. Fisher. 1998. Apoprotein B100 has a prolonged interaction with the translocon during which its lipidation and translocation change from dependence on the microsomal triglyceride transfer protein to independence. *Proc. Natl. Acad. Sci. USA.* **95:** 14733–14738.
7. Liang, J-S., and H. N. Ginsberg. 2001. Microsomal triglyceride transfer protein binding and lipid transfer activities are independent of each other, but both are required for secretion of apolipoprotein B lipoproteins from liver cells. *J. Biol. Chem.* **276:** 28606–28612.
8. Ledmyr, H., F. Karpe, B. Lundahl, M. McKinnon, C. Skoglund-Andersson, and E. Ehrenborg. 2002. Variants of the microsomal triglyceride transfer protein gene are associated with plasma cholesterol levels and body mass index. *J. Lipid Res.* **43:** 51–58.
9. Karpe, F., B. Lundahl, E. Ehrenborg, P. Eriksson, and A. Hamsten. 1998. A common functional polymorphism in the promoter region of the microsomal triglyceride transfer protein gene influences plasma LDL levels. *Arterioscler. Thromb. Vasc. Biol.* **18:** 756–761.
10. Ledmyr, H., A. D. McMahon, E. Ehrenborg, L. B. Nielsen, M. Neville, H. Lithell, P. W. MacFarlane, C. J. Packard, and F. Karpe on behalf of the WOSCOPS Executive. 2004. The microsomal triglyceride transfer protein gene -493T variant lowers cholesterol but increases the risk of coronary heart disease. *Circulation.* **109:** 2279–2284.
11. Narcisi, T. M., C. C. Shoulders, S. A. Chester, J. Read, G. B. Brett, G. B. Harrison, T. T. Grantham, M. F. Fox, S. Povey, T. W. de Bruin, et al. 1995. Mutations of the microsomal triglyceride-transfer-protein gene in abetalipoproteinemia. *Am. J. Hum. Genet.* **57:** 1298–1310.
12. Hixson, J., and D. Vernier. 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J. Lipid Res.* **31:** 545–548.
13. Fisher, E., H. Scharnagl, M. M. Hoffmann, K. Kusterer, D. Wittmann, H. Wieland, W. Gross, and W. Marz. 1999. Mutations in the apolipoprotein (apo) B-100 receptor-binding region: detection of apo B-100 (Arg3500->Trp) associated with two new haplotypes and evidence that apo B-100 (Glu3405->Gln) diminishes receptor-mediated uptake of LDL. *Clin. Chem.* **45:** 1026–1038.
14. Beg, O. U., M. S. Meng, S. I. Skarlatos, L. Previato, J. D. Brunzell, H. B. Brewer, Jr., and S. S. Fojo. 1990. Lipoprotein lipaseBethesda: a single amino acid substitution (Ala-176-Thr) leads to abnormal heparin binding and loss of enzymic activity. *Proc. Natl. Acad. Sci. USA.* **87:** 3474–3478.
15. Mann, C. J., T. A. Anderson, J. Read, S. A. Chester, G. B. Harrison, S. Kochl, P. J. Ritchie, P. Bradbury, F. S. Hussain, J. Amey, B. Vanloo, et al. 1999. The structure of vitellogenin provides a molecular model for the assembly and secretion of atherogenic lipoproteins. *J. Mol. Biol.* **285:** 391–408.
16. Byrne, B. M., M. Gruber, and G. Ab. 1989. The evolution of egg yolk proteins. *Prog. Biophys. Mol. Biol.* **53:** 33–69.
17. Brown, M. S., J. Herz, and J. L. Goldstein. 1997. LDL-receptor structure. Calcium cages, acid baths and recycling receptors. *Nature.* **388:** 629–630.

18. Flood, C., M. Gustafsson, P. E. Richardson, S. C. Harvey, J. P. Segrest, and J. Boren. 2002. Identification of the proteoglycan binding site in apolipoprotein B48. *J. Biol. Chem.* **277:** 32228–32233.
19. Thompson, J. R., and L. J. Banaszak. 2002. Lipid-protein interactions in lipovitellin. *Biochemistry.* **41:** 9398–9409.
20. Neylon, C., S. E. Brown, A. V. Kralicek, C. S. Miles, C. A. Love, and N. E. Dixon. 2000. Interaction of the *Escherichia coli* replication terminator protein (Tus) with DNA: a model derived from DNA-binding studies of mutant proteins by surface plasmon resonance. *Biochemistry.* **39:** 11989–11999.
21. Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82:** 70–77.
22. Karpe, F., and A. Hamsten. 1994. Determination of apolipoproteins B-48 and B-100 in triglyceride-rich lipoproteins by analytical SDS-PAGE. *J. Lipid Res.* **35:** 1311–1317.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
24. Pan, M., J-S. Liang, E. A. Fisher, and H. N. Ginsberg. 2002. The late addition of core lipids to nascent apolipoprotein B100, resulting in the assembly and secretion of triglyceride-rich lipoproteins, is independent of both microsomal triglyceride transfer protein activity and new triglyceride synthesis. *J. Biol. Chem.* **277:** 4413–4421.
25. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.
26. Kelley, L. A., R. M. MacCallum, and M. J. Sternberg. 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.* **299:** 499–520.
27. Sali, A., and T. L. Blundell. 1993. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234:** 779–815.
28. DeLano, W. L. 2002. The PyMOL molecular graphics system. Accessed February 6, 2006, at http://www.pymol.org.
29. Andrade, M. A., P. Chacon, J. J. Merelo, and F. Moran. 1993. Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. *Protein Eng.* **6:** 383–390.
30. Hamilton, J. A. 2004. Fatty acid interactions with proteins: what X-ray crystal and NMR solution structures tell us. *Prog. Lipid Res.* **43:** 177–199.
31. Baier, L. J., J. C. Sacchettini, W. C. Knowler, J. Eads, G. Paolisso, P. A. Tataranni, H. Mochizuki, P. H. Bennett, C. Bogardus, and M. Prochazka. 1995. An amino acid substitution in the human intestinal fatty acid binding protein is associated with increased fatty acid binding, increased fat oxidation, and insulin resistance. *J. Clin. Invest.* **95:** 1281–1287.
32. Hodsdon, M. E., and D. P. Cistola. 1997. Discrete backbone disorder in the nuclear magnetic resonance structure of Apo intestinal fatty acid-binding protein: implications for the mechanism of ligand entry. *Biochemistry.* **36:** 1450–1460.
33. Bjorkegren, J., M. Veniant, S. K. Kim, S. K. Withycombe, P. A. Wood, M. K. Hellerstein, R. A. Neese, and S. G. Young. 2001. Lipoprotein secretion and triglyceride stores in the heart. *J. Biol. Chem.* **276:** 38511–38517.
34. Unger, R. H., and L. Orci. 2000. Lipotoxic diseases of nonadipose tissues in obesity. *Int. J. Obes. Relat. Metab. Disord.* **24(Suppl 4):** 28–32.