A Novel Abetalipoproteinemina Genotype

IDENTIFICATION OF A MISSENSE MUTATION IN THE 97-kDa SUBUNIT OF THE MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN THAT PREVENTS COMPLEX FORMATION WITH PROTEIN DISULFIDE ISOMERASE*

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The microsomal triglyceride transfer protein (MTP) is a heterodimer composed of the ubiquitous multifunctional protein, protein disulfide isomerase, and a unique 97-kDa subunit. Mutations that lead to the absence of a functional 97-kDa subunit cause abetalipoproteinemia, an autosomal recessive disease characterized by a defect in the assembly and secretion of apolipoprotein B (apoB) containing lipoproteins. Previous studies of abetalipoproteinemic patient, C.L., showed that the 97-kDa subunit was undetectable. In this report, [35S]methionine labeling showed that this tissue was capable of synthesizing the 97-kDa MTP subunit. Electrophoretic analysis showed two bands, one with a molecular mass of the wild type 97-kDa subunit and the other with a slightly lower molecular weight. Sequence analysis of cDNAs from additional intestinal biopsies showed this patient to be a compound heterozygote. One allele contained a perfect in-frame deletion of exon 10, explaining the lower molecular weight band. cDNAs of the second allele were found to contain 3 missense mutations: His297 → Gln, Asp384 → Ala, and Arg540 → His. Transient expression of each mutant showed that only the Arg540 → His mutant was non-functional based upon its inability to reconstitute apoB secretion in a cell culture system. The other amino acid changes are silent polymorphisms. High level coexpression in a baculovirus system of the wild type 97-kDa subunit or the Arg540 → His mutant along with human protein disulfide isomerase showed that the wild type was capable of forming an active MTP complex while the mutant was not. Biochemical analysis of lysates from these cells showed that the Arg to His conversion interrupted the interaction between the 97-kDa subunit and protein disulfide isomerase. Replacement of Arg540 with a lysine residue maintained the ability of the 97-kDa subunit to complex with protein disulfide isomerase and form the active MTP holoprotein. These results indicate that a positively charged amino acid at position 540 in the 97-kDa subunit is critical for the productive association with protein disulfide isomerase. Of the 13 mutant MTP 97-kDa subunit alleles described to date, this is the first encoding a missense mutation.

The microsomal triglyceride transfer protein (MTP)1 is an endoplasmic reticulum resident protein that catalyzes the transfer of lipids between phospholipid surfaces. Although MTP can transfer most classes of lipids, it shows a distinct preference for triglyceride and cholesteryl esters. MTP is a heterodimer composed of the multifunctional protein, protein disulfide isomerase (PDI) and a unique large subunit of 97 kDa (1). PDI is a ubiquitous protein found at high levels in many different tissues (2). The 97-kDa subunit of MTP is expressed primarily in hepatocytes and intestinal enterocytes (3). Thus, the active MTP complex is found predominantly in the liver and small intestine. The subcellular localization, tissue distribution, and activity of MTP all suggest a role for this protein in the assembly and secretion of apolipoprotein B (apoB) containing lipoproteins.

Abetalipoproteinemia is an autosomal recessive disease characterized as a defect in the assembly and/or secretion of the apolipoprotein B containing lipoprotein particles: very low density lipoproteins and chylomicrons. It has been shown that an absence of a functional MTP complex is the proximal cause of abetalipoproteinemia (4, 5). In four subjects that were studied, the lack of MTP activity in intestinal biopsies from abetalipoproteinemic patients was associated with undetectable levels of the 97-kDa subunit (4). Studies of the genes encoding the MTP 97-kDa subunit from two patients showed the presence of a homozygous frameshift mutation in one and a homozygous nonsense mutation in the other (6). In a third case, a splice site mutation was associated with exon skipping (5). In these three cases the mutations lead to severely truncated versions of the 97-kDa subunit. More recently, additional truncations caused by novel nonsense mutations or large deletions caused by splicing defects have been reported (7, 8). These studies clearly showed that a lack of functional MTP is the proximal cause of abetalipoproteinemia and firmly establish the requirement for MTP in the assembly and secretion of apoB containing lipoproteins. This conclusion was further supported by studies showing that expression of the MTP 97-kDa subunit in non-lipoprotein producing cells yields an active MTP complex that is capable of directing the assembly of co-expressed apoB into

1 The abbreviations used are: MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; apoB, apolipoprotein B; PCR, polymerase chain reaction; CMV, cytomegalovirus.

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secretion competent lipoprotein particles (9, 10). In the absence of MTP, the apolipoprotein B was retained intracellularly and eventually degraded. These results confirm the requirement of MTP for assembly and secretion of apoB containing lipoproteins and suggest that, it alone, may be sufficient to mediate this pathway.

In this report we describe the characterization of the first missense mutation to be reported for the 97-kDa subunit of MTP from a patient with abetalipoproteinemia. Earlier studies of this patient showed that intestinal biopsies had undetectable levels of MTP activity and 97-kDa subunit protein (4). Herein we report that via \(^{35}\)S)methionine labeling of intestinal biopsies, we observed that this abetalipoproteinemic tissue was indeed capable of synthesizing the MTP 97-kDa subunit. Furthermore, SDS-polyacrylamide gel electrophoresis showed that two forms of the protein were present. To determine the genetic basis of this phenotype, RNA was extracted from intestinal biopsy samples and cDNA clones encoding the MTP 97-kDa subunit were isolated and sequenced. This patient was found to be a compound heterozygote possessing a large deletion mutation in one allele and three amino acid substitutions in the other. Characterization of these amino acid substitutions and their effects on the structure and function of the active MTP complex are described.

**EXPERIMENTAL PROCEDURES**

**Materials**

All cell culture media, fetal bovine serum, and cell culture reagents were from Life Technologies (Grand Island, NY). HeLa-229 cells were supplied by the American Type Culture Collection (Rockville, MD). All other chemicals and reagents were from Sigma.

**Methods**

**Tissue Biopsies and Organ Cultures**—Intestinal biopsies were obtained from the abetalipoproteinemic patient after explaining the nature of the study and its possible consequences to the guardians and obtaining informed consents. A normal control intestinal biopsy was from a normolipidemic individual and was part of material obtained for diagnostic purposes. It was grossly and microscopically normal. The procedures for obtaining biopsy and blood samples and the experimental methods employed in their analysis are part of a biomedical project (Projet de Recherche Biomédicale, number 94002) for the study of hereditary disorders of malabsorption and lipoprotein assembly and secretion. This project has been approved by INSERM (Institut National de la Santé et de la Recherche Médicale) and the bioethics committee of the Bichat Hospital, Paris, France. The intestinal biopsies were placed into organ culture and metabolically labeled with \(^{35}\)S)methionine as described previously (11, 12). The labeled intestinal tissue was homogenized and solubilized. Solubilized material was incubated with protein A-Sepharose beads to which polyclonal anti-serum to MTP (4) had been absorbed. Immunoprecipitated proteins were eluted and analyzed under reducing conditions on SDS-polyacrylamide (12%) gels (13). Radioactivity in the immunoprecipitated bands was quantitated with a Molecular Dynamics PhosphorImager model 425 (Sunnyvale, CA).

**Preparation of RNA from Intestinal Biopsy Samples and Polymerase Chain Reaction Amplification**—Tissue samples weighing 5–10 mg were stored frozen at 80 °C. RNA was isolated from the biopsy using the RNAzol reagent as described previously (6). The method used to amplify segments of the mRNA by polymerase chain reaction (PCR) technology is a modification of the procedure originally described by Kawasaki (14). The primers and enzymes were supplied as a kit by Perkin-Elmer and were applied as follows in a Perkin-Elmer GeneAmp model 9600 thermal cycler. The mRNA was reverse transcribed into first strand cDNA in a reaction containing 1 mM random hexamer primers, 0.5 mM MgCl\(_2\), 1 mM of each deoxynucleotide triphosphate, 1 unit of placentals ribonuclease inhibitor, and 2.5 units of Moloney murine leukemia virus reverse transcriptase. The reaction was incubated at 22 °C for 10 min to allow for primer annealing, increased to 42 °C for 30 min to permit primer extension, and terminated by incubation for 5 min at 99 °C followed by cooling to 4 °C. First strand cDNA was added to a PCR reaction containing 0.15 mM upstream and downstream primers (Fig. 2, top), 2.0 mM MgCl\(_2\), 0.2 mM of each deoxynucleotide triphosphate, and 2.5 units of Taq polymerase in 1.25 × PCR buffer. The amplification reaction profile consisted of 94 °C for 30 s, 42 °C for 30 s, and 72 °C for 1.0 min. Clones from the independent PCR reactions were sequenced as described (6).

**Site-directed Mutagenesis**—A 3.2-kilobase pair fragment containing the entire coding sequence of the 97-kDa subunit of human MTP extending from nucleotide 64 to +3138 was subcloned into plasmid pRc/CMV to yield pRc/MTP (6). Transcription of the 97-kDa subunit of MTP in this plasmid is under the control of the CMV immediate early promoter. Site-directed mutagenesis was performed on pRc/MTP by the method of Marotti and Tomich (15). Phosphorylated 30-base oligonucleotides containing the mutation of interest were annealed to pRc/MTP, previously denatured with alkali. The mixture was neutralized and the oligonucleotide extended with the Klenow fragment of E. coli DNA polymerase I in the presence of T4 DNA polymerase and T4 gene 32 protein. The reaction mixture was extracted with phenol/chloroform (1:1) and ethanol precipitated. The precipitate was dissolved in water and used to transform competent E. coli strain AG1. The resultant colonies were screened by hybridization to a shorter 15-base, oligonucleotide containing the desired base change(s). The following are the oligonucleotides used in the mutagenesis step for producing the mutations at amino acid positions 297, 384, and 540, respectively (bases in bold face comprised the probes used to screen for the mutants): His\(^{297}\) → Gin, 5′-GTCCTTCACAGGACGTTGCAAGGGGATGCTC-3′; Asp\(^{384}\) → Ala, 5′-GAATTTCAAAAGGTGCTAGTAGTTATTCCTA-3′; and Arg\(^{540}\) → His, 5′-TGAAGACTGTGGCTACAGGCGCGCTGCCG-3′.

**Stable Expression of Apolipoprotein B in HeLa Cells**—Cells previously described (9) plasmid, pB53, containing the amino-terminal 53% of human apolipoprotein B (apoB-53) was stably expressed in HeLa-229 cells as follows. HeLa cells at ~50% of confluency in 100-mm dishes were transfected with 50 μg of pB53 in the presence of 120 μl of Lipofectam reagent. Twenty hours later, the transfection mixture was removed and lipid rich medium (RPMI 1640, 3% fatty acid-free bovine serum albumin, 0.8 mM sodium oleate, and 1.0 mM glycerol) was added. After an additional 24 h, stable transfectants were selected for resistance to 1.5 mg/ml geneticin for 2 weeks after which time the concentration was reduced to 1 mg/ml. Resistant colonies were isolated and cell extracts were analyzed for the presence of apoB-53 by immunoblot as described (9). One clone was selected for further experimentation which was designated HLB-34.

**Transient Expression of MTP 97-kDa Subunit Mutants in HLB-34 Cells**—Transient expression of the mutants as well as wild type MTP 97-kDa subunit and the control plasmid pRc/CMV was accomplished by transfecting 25 μg of each expression plasmid per 100-mm dish in the presence of 120 μl of Lipofectam reagent. Twenty hours later, the transfection mixture was removed and lipid rich medium (RPMI 1640, 3% fatty acid-free bovine serum albumin, 0.8 mM sodium oleate, and 1.0 mM glycerol) was added. After an additional 24 h, media and cells were harvested and analyzed as described previously (9) for apoB and MTP 97-kDa subunit, respectively.

To assess transfection efficiency, a plasmid was constructed containing a secretable form of human placental alkaline phosphatase under the control of the cytomegalovirus immediate early promoter. This plasmid, designated pHPLAP-543 (9) was co-transfected with the mutant expression plasmids and in the control plates at a level of 1.0 μg/100-mm dish. Alkaline phosphatase activity was measured in media as described (9).
Expression of Recombinant Proteins in Sf9 Cells—Recombinant baculovirus transfer vectors were co-transfected into Sf9 cells with wild type Autographa californiae linear DNA by cationic liposome-mediated transfection. Transfection, screening, plaque purification, and the production of high-titer viral stocks of recombinant virus were all carried out as described by the manufacturer of the MaxBac 2.0 Baculovirus Expression System (Invitrogen). The recombinant virus designated VL-PDI containing the coding sequence of human PDI has been previously described (8).

Expression of Recombinant Proteins in Sf9 Cells—Sf9 cells were cultured in suspension at 25°C in ExCell 401 medium supplemented with 10% heat-inactivated fetal bovine serum. To produce recombinant proteins, 10-ml suspension cultures at a density of 2.5 x 10^6 cells/ml were infected with recombinant virus coding for wild type or mutant 97-kDa subunit at a multiplicity of infection of 0.5. A multiplicity of infection of 25 was used when expression of protein disulfide isomerase was desired. Cells were harvested for analysis approximately 68 h post-infection. Analyses of MTP 97-kDa subunit and PDI expression (8) and MTP-mediated triglyceride transfer activity were carried out as described (4). Additional details are presented in the figure legends.

**RESULTS**

Tissue Biopsy Organ Cultures—We previously reported that intestinal biopsies from an abetalipoproteinemic individual (C.L.) had undetectable levels of MTP activity and 97-kDa subunit protein (4). In order to further investigate the molecular basis for the lack of MTP, freshly isolated intestinal biopsies from an abetalipoproteinemic individual were homogenized and immunoprecipitated with antiserum against MTP. After a 2-h pulse and 1-h chase, the protein immunoprecipitated from the normal subject appeared as a single band on autoradiographs of SDS-polyacrylamide gels with an apparent molecular mass of 97 kDa, consistent with the molecular mass of the 97-kDa subunit of MTP (Fig. 1, lane 1). In contrast, material immunoprecipitated from the abetalipoproteinemic subject revealed two protein bands, one with an apparent molecular mass identical to that obtained from the normal subject and one of approximately 3,000 Da less (Fig. 1, lane 2). A high resolution scan of the radioactivity present in the gel with the PhosphorImager confirmed that there was only one band present in the sample from the normal individual whereas two bands were present in the sample from the abetalipoproteinemic subject (Fig. 1, panel B). Quantitation showed that there was less radioactivity present in the sum of the two MTP bands of the abetalipoproteinemic subjects compared to the single MTP band of the normal subject when normalized for total trichloroacetic acid precipitable radioactivity. These observations indicate that the intestinal biopsies from the abetalipoproteinemic subject synthesized two forms of MTP, one that was normal size and one that was smaller by approximately 3,000 Da. Based upon these results we hypothesized that the previously reported (4) failure to detect MTP activity or 97-kDa subunit protein in this subject was due to a compound heterozygous genotype with one allele generating a large deletion and the other containing either a small deletion or a missense mutation. Furthermore, since both of these mutant forms of the 97-kDa subunit could be synthesized, the failure to detect them in the previous study (4) was probably due to a loss in stability within the ER lumen.

Amplification and Sequence Analysis of the cDNAs Encoding the 97-kDa Subunit of MTP—Total cellular RNA was purified and reverse transcribed from a 10-mg intestinal biopsy from an abetalipoproteinemic patient C.L. After first strand cDNA synthesis was performed, sequences encoding the 97-kDa subunit of MTP were amplified by PCR with primer sets 1 and 2 (Fig. 2). Agarose gel analysis of the amplified products indicated two bands of approximately 1,200 bases and 1,100 bases each for primer set 1 and 1000 bases and 900 bases each for primer set 2 (data not shown). Sequence analysis (Fig. 2) of the smaller bands indicated a deletion of 107 nucleotides from 1237 to 1344. This corresponds to a perfect in-frame deletion of exon 10 of the MTP 97-kDa subunit gene (16). This yields an internal deletion of 36 amino acids stretching from residue 413 to 448, explaining the difference in electrophoretic mobility between this form of the protein and the wild type form, as shown in Fig. 1.

To characterize the second allele we took advantage of the fact that the first allele had a large deletion in the central portion of the coding sequence of the mRNA. By positioning one primer in the area deleted in the first allele (nucleotides 1237 to 1344), it was possible to specifically amplify the second allele by PCR (see Fig. 2). cDNAs from the deleted allele do not contain these sequences and would not be amplified. Primer sets 3, 4, and 5 were employed to amplify the full coding sequence of the second allele. As shown in Fig. 3, sequence analysis revealed the presence of 3 missense mutations. Mutation 1 at nucleotide position 891 resulted in the conversion of the glutamine at amino acid position 297 to a histidine. Mutations 2 and 3 at nucleotide positions 1151 and 1619, respectively, resulted in the conversion of the aspartic acid at amino acid position 384 to an alanine and the arginine at position 540 to a histidine.

Expression of Apolipoprotein B-53 in HLB-34 Cells—In order to test the effects the three missense mutations had on MTP function, a cell line was developed that was dependent upon MTP activity for the secretion of apoB. This cell line was generated by co-transfecting HeLa cells with plasmid pB53...
along with a plasmid carrying the gene for resistance to the antibiotic Geneticin. Selection with Geneticin yielded a number of clonal cell lines which were screened for the presence of apoB-53 in crude cell extracts. One cell line, HLB-34, was selected for further analysis by immunoblot. Fig. 4 shows that these cells contained 20% the level of apoB as compared to HepG2 cells on a molar basis.

Cell culture media were analyzed for apoB by an enzyme-linked immunoassay capable of detecting concentrations as low as 1.25 ng/ml (17). ApoB was undetectable in lipidsupplemented medium exposed to HLB-34 cells for 24 h (data not shown). Thus, as expected, these cells were capable of making apoB-53 protein, but were incapable of secreting it.

Analysis of the Effect of Individual Missense Mutations on the Ability of MTP to Mediate Apolipoprotein B Secretion—In order to determine which missense mutation(s) was responsible for inactivating MTP, site-directed mutagenesis was employed to individually insert each mutation into the coding sequence for wild type MTP 97-kDa subunit in expression vector pRc/hMTP. The individual plasmids were then transiently expressed in separate parallel cultures of HLB-34 cells. Plasmid pRc/hMTP was used as the positive control and pRc/CMV as the negative control. A plasmid expressing a secretable form of human placental alkaline phosphatase was co-transfected with the MTP plasmids in order to monitor transfection efficiency in each culture. Twenty-four hours after initiation of transfection, the cells were fed lipid-rich medium for an additional 24 h to ensure maximal stimulation of apoB secretion. At the end of this incubation period, the media were harvested and analyzed for apoB and alkaline phosphatase as described.

Extracts of the cells (10,000×g supernatants) were analyzed for expression of the MTP 97-kDa subunit. Fig. 5, panel A, shows that transfection of the plasmid carrying the wild type cDNA for the MTP 97-kDa subunit strongly stimulated apoB secretion from HLB-34 cells as evidenced by the presence of apoB-53 in crude cell extracts. One cell line, HLB-34, was selected for further analysis by immunoblot. Fig. 4 shows that these cells contained 20% the level of apoB as compared to HepG2 cells on a molar basis. Cell culture media were analyzed for apoB by an enzyme-linked immunoassay capable of detecting concentrations as low as 1.25 ng/ml (17). ApoB was undetectable in lipidsupplemented medium exposed to HLB-34 cells for 24 h (data not shown). Thus, as expected, these cells were capable of making apoB-53 protein, but were incapable of secreting it.

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Fig. 5, panel A, shows that transfection of the plasmid carrying the wild type cDNA for the MTP 97-kDa subunit strongly stimulated apoB secretion from HLB-34 cells as evidenced by the presence of apoB-53 in the medium. Expression of the plasmids carrying the His<sup>297</sup> → Gln mutation or the Asp<sup>384</sup> → Ala mutation were also capable of stimulating apoB-53 secretion from HLB-34 cells to a similar extent. In contrast, the plasmid carrying the Arg<sup>540</sup> → His amino acid change was responsible for inactivating MTP leading to the abetalipoproteinemic phenotype.

The results of the immunoblot analysis of the cell extracts for expression of the MTP 97-kDa subunit show (Fig. 5, panel B) that similar levels of the protein were present in the cells transfected with a plasmid encoding the wild type 97-kDa

**Fig. 2. Primers for PCR amplification of MTP 97-kDa subunit cDNAs and sequence of the deleted exon in the first allele from an abetalipoproteinemic subject.** RNA was isolated from an intestinal biopsy taken from an abetalipoproteinemic subject and first strand cDNA synthesis was carried out. The cDNAs were amplified using two different primer sets yielding overlapping products covering the central region of the coding sequence. The primers for the fragment spanning nucleotides 853-2072 (primer set 1) were as follows: forward primer, 5'-ACGGCCATTCCCATATTGCGGCGAGG-3'; reverse primer, 5'-CCACCATAGGAGTCAAGGTTCT-3'. For the fragment spanning nucleotides 530-1542 (primer set 2): forward primer, 5'-CCTACCAAGGCTCATCAAAGAAAG-3'; reverse primer, 5'-AGGGAGATCATCATCTGAGAGCAATG-3'. Both primer sets yielded two products, one of the expected size and one that was approximately 100 base pairs shorter. After subcloning these products, sequence analysis revealed the shorter product had a perfect in-frame deletion of exon 10. The sequence of this segment is shown spanning 108 nucleotides from 1237 to 1344. In order to amplify the second allele only, three primer sets were designed as shown such that one of the primers annealed to sequences deleted from the first allele. These primers are as follows: fragment −30–1299 (primer set 3), forward primer, 5'-TGGATGCAAGTTGAGAGATGC-3', reverse primer, 5'-CCCAGTGATCATACACAC-3'; fragment 1240–2719 (primer set 4), forward primer, 5'-AACGTTCAAAGGCTTCTATGG-3', reverse primer, 5'-GAGAAAATCTGCAAATG-3'; fragment 30–1299 (primer set 5), forward primer, 5'-CTCATTCTCATATTCGACG-3', reverse primer, 5'-CCACATGATCATACACAC-3'.
subunit, the His297→Gln polymorphism, or the Asp384→Ala polymorphism. However, only very low levels were observed in the cells transfected with the plasmid encoding the Arg540→His mutation. The alkaline phosphatase data show that a difference in expression efficiencies could not be responsible for the low level of MTP in the cells transfected with the plasmid encoding the Arg540→His mutation (wild type; 0.105±0.001 OD/min/mg protein, versus Arg540→His; 0.111±0.001 OD/min/mg protein).

These data were consistent with the studies of the intestinal biopsies that showed that this mutant MTP 97-kDa subunit could be synthesized (see Fig. 1) and immunoprecipitated, but was not detectable by immunoblot using the same antiserum (4). However, the possibility, albeit unlikely, remained that the site-directed mutagenesis protocol may have caused additional defects in the noncoding portion of the plasmid (the coding sequences were all verified before transfection), leading to decreased levels of expression. In order to eliminate this as a possible explanation for the results shown in Fig. 5, the plasmid carrying the Arg540→His mutant was reverted back to wild type and expressed in HLB-34 cells. Fig. 6 shows that while the Arg540→His mutant (lane 3, panels A and B) could not stimulate apoB synthesis and was not detectable in cell extracts, the revertant (lane 4, panels A and B) was capable of stimulating apoB synthesis and was present in cell extracts at levels similar to the wild type (lane 2, panels A and B). Thus, the low levels of expression of the 97-kDa subunit of MTP observed with this plasmid are not an artifact of the site-directed mutagenesis but are the biological consequence of the mutation.

In order to determine the structural basis for this observation, the previously described strategy was employed (8). Wild type 97-kDa subunit, the Arg540→His mutant, and PDI were inserted into a baculovirus expression vector. In addition, site-directed mutagenesis was utilized to convert Arg540 to a lysine residue. High titer viral stocks of each clone were produced and used to infect Sf9 cells. At the end of the infection period, total cell homogenates were treated with deoxycholate to release the contents of the microsomes. The soluble components were separated from the membranes and insoluble proteins by centrifugation at 100,000×g. Total cell homogenates and 100,000×g pellets were also analyzed for 97-kDa subunit protein (data not shown). Fig. 7, panel A, shows that large amounts of the 97-kDa subunit were expressed in Sf9 cells regardless of the amino acid identity at position 540 and regardless of whether or not PDI was also expressed. The lower amount of 97-kDa subunit recovered from the cells coexpressing PDI has been previously observed (8) and likely reflects the upper limit of Sf9 cells to...
express multiple exogenous proteins. Fig. 7, panel B, shows that human PDI is also highly expressed in these cells under the described experimental conditions. Furthermore, PDI behaves as a typical soluble microsomal protein in that it is readily observable in the 100,000 × g supernatant. Fig. 7, panel C, shows that when the 97-kDa subunit is expressed in the absence of PDI (lanes 3, 4, and 5) it is not observed in the 100,000 × g supernatant regardless of the amino acid at position 540. In fact, nearly all of the 97-kDa protein was observed in the 100,000 × g pellets (data not shown). As expected, triglyceride transfer activity was undetectable in these samples (Fig. 8). On the other hand, coexpression of the wild type 97-kDa subunit along with PDI generates a soluble MTP complex (Fig. 7, panel C, lane 6) capable of transferring triglyceride (Fig. 8). In contrast, coexpression of the Arg540 ⇒ His mutant generates a profile similar to expression of the wild type in the absence of PDI; the 97-kDa subunit was not observed in the 100,000 × g supernatant even when 8-fold more sample was analyzed (Fig. 7, panel D). It was observed in the 100,000 × g pellet (data not shown) and no triglyceride transfer activity was detected. Interestingly, conversion of Arg540 to a lysine does not cause a change in the behavior of the protein compared to the wild type (Fig. 7, panel C, lane 8). The Lys540 variant forms a soluble MTP complex that is capable of transferring triglyceride to the same extent as the wild type.

**DISCUSSION**

While much is known about the physiological role of MTP in lipoprotein assembly (18), information about the relationship between its structure and function has been difficult to obtain due to several characteristics of the 97-kDa subunit. First, the 97-kDa subunit does not share strong sequence homology with any other protein. It has been reported that some weak structural similarities to the egg yolk lipid carrier protein vitellogenin exist (19), however, this has yet to be confirmed by physical methods. Second, analysis of sequence conservation across the three species sequenced to date have been uninformative because the 97-kDa subunit of MTP is highly conserved throughout the length of the polypeptide (6, 20). Thus, this potentially powerful technique for determining the putative functional domains of a protein could not be utilized. This in turn makes the use of site-directed mutagenesis to define the function of conserved domains much more difficult.

Studies of naturally occurring mutations in patients with inherited diseases have also been useful in defining the structure-function relationships of many proteins. In the case of the MTP 97-kDa subunit, demonstration that defects in this protein were the proximal cause of abetalipoproteinemia opened the possibility that this approach could provide important information concerning the functional domains of the protein. However, nucleotide sequence analysis of the genes and/or cDNAs representing 10 separate alleles showed only either major truncations or large deletions (5–7). More recently, Ricci et al. (8) have described an 11th allele that contains a more subtle 30-amino acid truncation of the carboxyl terminus of the 97-kDa subunit of MTP. Biochemical studies showed that this deletion disrupts interaction of the 97-kDa subunit with PDI, indicating that the carboxyl-terminal domain of the 97-kDa subunit plays a role in the formation of the active quaternary structure of the MTP heterodimer. Subsequent studies on this region of the protein have shown that Cys878 is critical for formation of an active 97 kDa-PDI complex (7). While this study provides insight into the role of the carboxyl-terminal domain, nothing is known about the remainder of the protein.

The limited physical data we have comes from biochemical and molecular biological studies. Using these approaches, it has been shown that while neither subunit alone can bind and/or transfer lipid, it is clear that the 97-kDa subunit confers the lipid transfer activity upon the complex (21). The role of PDI in forming and maintaining an active heterodimer is unclear. However, when the 97-kDa subunit is separated from PDI in vitro, it irreversibly aggregates (21). This indicates that PDI is essential in holding the 97-kDa subunit in a stable, active conformation. In addition, since the 97-kDa subunit lacks a carboxyl-terminal KDEL endoplasmic reticulum retention signal (5, 6), PDI, which possesses this signal (2), is most...
of the microsomal fraction from cells coexpressing the Arg540 His mutant, or the Arg540 Lys variant, respectively; and lane 6, 20% of the microsomal fraction from cells coexpressing the Arg540→His mutant and PDI.

The Arg540 His mutant was undetectable in the 100,000 g aggregate and sediment at 100,000 g. Asp384 do not play important structural or functional roles in the MTP. In the case of Arg540, while histidine and arginine are conservative. Since the other two polymorphisms did not inactive MTP, it is possible that the regions around His297 and Asp384 do not play important structural or functional roles in MTP. In the case of Arg540, while histidine and arginine are likely responsible for the endoplasmic reticulum localization of the complex.

In the present study, we describe the identification of two novel mutant alleles encoding the 97-kDa subunit of MTP from a compound heterozygous abetalipoproteinemic individual. Analysis of cDNAs from intestinal biopsies showed that the novel mutant alleles encoding the 97-kDa subunit of MTP from the complex. Likely responsible for the endoplasmic reticulum localization of the complex.

In the present study, we describe the identification of two novel mutant alleles encoding the 97-kDa subunit of MTP from a compound heterozygous abetalipoproteinemic individual. Analysis of cDNAs from intestinal biopsies showed that the first allele contains an in-frame deletion of exon 10 which removes 36 amino acids from the center portion of the protein. Interestingly, Narcisi et al. (7) described an abetalipoproteinemic patient with a microdeletion at positions 5–11 of the 5’ end of intron 10. This changes the sequence such that the guanine residue at position 5 is converted to an adenine. A substitution of this type is known to generate various types of aberrant splicing, including exon skipping which would delete exon 10 from the mRNA. While no analysis of the size of the mRNA or protein was reported in the previous study, it can be speculated that a similar or identical mutation might be present in patient C.L. even though these two individuals are of completely different ethnic backgrounds.

The second allele described herein contains three amino acid changes as follows: His297→Gln, Asp384→Ala, and Arg540→His. Site-directed mutagenesis was used to individually insert each mutation into an expression vector carrying the full coding sequence for wild type human MTP 97-kDa subunit. Each vector was subsequently tested for the ability to direct apoB secretion in a non-MTP producing cell line stably expressing apoB-53. The first two mutations, His297→Gln and Asp384→Ala, were capable of directing apoB-53 secretion indicating that these mutations are silent polymorphisms. The His297→Gln polymorphism has been previously observed in the Caucasian population of United Kingdom (7), while the Asp384→Ala amino acid change is novel. The last mutation, Arg540→His was incapable of efficiently stimulating apoB secretion. Thus, this amino acid change is responsible for inactivating this allele of the MTP 97-kDa subunit.

The possibility was thus presented that this single amino acid mutation could be informative as to the function of this domain of the protein. In order to take advantage of this opportunity, an expression system capable of producing high levels of the protein was required. This was especially the case with the Arg540→His mutant since repeated attempts at expression in the HeLa cells failed to generate significant steady state levels of the protein (Figs. 5 and 6). Thus, the wild type, Arg540→His mutant and the Arg540→Lys variant of the 97-kDa subunit were inserted into a baculovirus expression vector. Previous experience showed that SF9 cells expressing the 97-kDa subunit accumulated large amounts of the protein whether human PDI was coexpressed or not (8). This was also observed in the current study (Fig. 7, panel A). Also, as previously shown, the wild type 97-kDa subunit only forms a soluble active MTP complex when PDI is coexpressed. In contrast, the Arg540→His mutant was undetectable in the 100,000 g supernatant even when coexpressed with PDI and even when 8-fold more sample was analyzed (Fig. 7, panel D). Likewise, triglyceride transfer activity was undetectable in this sample (Fig. 8). Given the known propensity of the 97-kDa subunit to aggregate and sediment at 100,000 g in the absence of PDI (21), we conclude from these results that the conversion of Arg540 to His prohibits the association of the 97-kDa subunit and PDI. This causes the mutant 97-kDa subunit to aggregate, rendering it insoluble at a centrifugal force of 100,000 g. It is interesting to note that of the three amino acid changes identified, the Arg540→His could be considered the most conservative. Since the other two polymorphisms did not inactive MTP, it is possible that the regions around His297 and Asp384 do not play important structural or functional roles in MTP. In the case of Arg540, while histidine and arginine are
both basic amino acids, the $pK_a$ for the ionizable guanidino nitrogen of arginine is 12.5 while that of the imidazole nitrogen of histidine is 6.0. At physiological pH, the side chain of nearly all of the arginines will be positively charged, while only 10% of the histidines will be positively charged. This strongly suggests that a positively charged residue is required at amino acid position 540. To test this hypothesis, $Arg^{540}$ was converted to a lysine. The $pK_a$ of the side chain nitrogen of lysine is 10.5 and thus would be positively charged at physiological pH. Expression of the Lys$^{540}$ form of the 97-kDa subunit along with PDI in the baculovirus system led to formation of a soluble MTP complex with a triglyceride transfer activity similar to that of the wild type.

Thus we conclude that a positively charged residue is required at amino acid position 540, suggesting a salt bridge is formed with a negatively charged center on PDI. Alternatively, $Arg^{540}$ may be involved in an intramolecular salt bridge with a negatively charged center at a distal site on the 97-kDa subunit polypeptide. Disruption of this salt bridge could alter the tertiary structure of the protein such that it is no longer capable of complexing with PDI. While it is known that sequences in the carboxyl terminus of the 97-kDa subunit of MTP mediate association with PDI, data indicating a similar function for the carboxyl terminus of the 97-kDa subunit of MTP mediate association with PDI, data indicating a similar function for the carboxyl terminus of the 97-kDa subunit with PDI, data indicating a similar function for the carboxyl terminus of the 97-kDa subunit with PDI, data indicating a similar function for the carboxyl terminus of the 97-kDa subunit with PDI, data indicating a similar function for the carboxyl terminus of the 97-kDa subunit with PDI, data indicating a similar function for the carboxyl terminus of the 97-kDa subunit with PDI.

This observation has an important implication regarding the structure of MTP and the appearance of naturally occurring missense mutations. It appears that all of the structural features required for association of the 97-kDa subunit with PDI must be intact in order for the active complex to form. Mutations in either the carboxyl terminus or at amino acid 540 destroy this interaction. Furthermore, we have observed that expression of the human 97-kDa subunit in mouse- or hamster-derived cell lines does not generate active MTP even though amino acid conservation is 95% or greater. Similarly, expression of human 97-kDa subunit in monkey cells yields inefficient formation of active MTP. These observations suggest that the sequence requirements for recognition of the 97-kDa subunit by PDI are quite rigid. However, this is inconsistent with the observation that with the exception of the missense mutation described herein, all the other mutations described to date have involved deletions of mid to large sized regions of the primary amino acid sequence. Furthermore, many additional silent amino acid changes have been observed (7). It would be expected that a protein as highly conserved as the 97-kDa subunit of MTP would be particularly susceptible to inactivation by single amino acid changes. As a result we should observe a higher incidence of these types of mutations as opposed to silent polymorphisms and large deletions. It would also be expected that we should observe a higher incidence of patients with abetalipoproteinemia. That this appears not to be the case begs the question of what the evolutionary pressure is that has maintained a high level of conservation from humans through hamsters and bovines to birds. Perhaps this is simply due to the fact that an insufficient number of alleles have been identified and that as more are described, additional missense mutations will be observed.

The analysis described herein of the first point mutation in the 97-kDa subunit of MTP has shown that amino acid Arg$^{540}$ is critical for the association of this protein with protein disulfide isomerase. Thus, this site, along with the carboxyl-terminal 30 amino acids have defined functional roles in the maintenance of the active quaternary structure of this protein. Remaining to be identified are the lipid binding, putative membrane binding and perhaps apoB-binding domains.

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