Two Naturally Occurring Mutations at the First and Second Bases of Codon Aspartic Acid 156 in the Proposed Catalytic Triad of Human Lipoprotein Lipase

IN VIVO EVIDENCE THAT ASPARTIC ACID 156 IS ESSENTIAL FOR CATALYSIS*

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We are studying naturally occurring mutations in the gene for lipoprotein lipase (LPL) to advance our knowledge about the structure/function relationships for this enzyme. We and others have previously described 11 mutations in human LPL gene and until now none of these directly involves any of the residues in the proposed Asp[156]-His[241]-Ser[132] catalytic triad. Here we report two separate probands who are deficient in LPL activity and have three different LPL gene haplotypes, suggesting three distinct mutations. Using polymerase chain reaction cloning and DNA sequencing we have identified that proband 1 is a compound heterozygote for a G → A transition at nucleotide 721, resulting in a substitution of asparagine for aspartic acid at residue 156, and a T → A transversion, resulting in a substitution of serine for cysteine at residue 216. Proband 2 is homozygous for an A → G base change at nucleotide 722, leading to a substitution of glycine for aspartic acid at residue 156. The presence of these mutations in the patients and available family members was confirmed by restriction analysis of polymerase chain reaction-amplified DNA. In vitro site-directed mutagenesis and subsequent expression in COS cells have confirmed that all three mutations result in catalytically defective LPL. The two naturally occurring mutations, which both alter the same aspartic acid residue in the proposed Asp[156]-His[241]-Ser[132] catalytic triad of human LPL, indicate that Asp[156] plays a significant role in LPL catalysis. The Cys[216] → Ser mutation destroys a conserved disulfide bridge that is apparently critical for maintaining LPL structure and function.

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Lipoprotein lipase (LPL) is a member of a highly homologous gene family of serine esterases including hepatic lipase (HL) and pancreatic lipase (PL) (1). LPL also exhibits significant sequence similarity to the Drosophila yolk proteins which apparently show no functional relationship to the serine esterases (1). The complementary DNAs (cDNAs) for these lipases from several species have been cloned and sequenced (2–8). Considerable overall homology in amino acid sequence has been identified, and the degree of sequence identity is especially high in a segment corresponding to exons 4, 5, and 6 of human LPL gene (2–8).

The major function of LPL is to hydrolyze the triglyceride core of circulating chylomicrons and very low density lipoproteins, resulting in the release of free fatty acids (9). This has a major modulating effect on the levels and lipid composition of low and high density lipoproteins. However, the structural determinants of the catalytic function of LPL are not well understood. Recently, the three-dimensional structure of human PL has been determined by x-ray crystallography, and an Asp-His-Ser triad has been proposed as the catalytic site of this lipase which is similar to that seen in trypsin like serine proteases and is highly conserved among lipases of various species (10). For human LPL, the corresponding residues for the catalytic triad are Asp[156]-His[241]-Ser[132]. Recent results of in vitro site-directed mutagenesis studies on 8 completely conserved serine residues including the Ser[132] strongly support the central role of Ser[132] in LPL catalysis (11).

We have chosen to study the structure/function relationship of human LPL by analyzing gene mutations in patients who present with LPL deficiency. Interestingly, six of the eight missense mutations that have been reported to cause a catalytically defective LPL protein are located at completely conserved residues in exons 4, 5, and 6, providing in vivo evidence that these residues are important for LPL catalysis (12–20). Until now, however, none of the reported mutations directly involves any of the three key residues in the proposed catalytic triad. Here we report two missense mutations at the first and second nucleotides of the codon for Asp[156] which result in a substitution of asparagine for aspartic acid and glycine for aspartic acid, respectively. In vitro mutagenesis studies have confirmed that these mutations cause catalytically defective LPL and support the important role of Asp[156].

1 The abbreviations used are: LPL, lipoprotein lipase; HP, hepatic lipase; PL, pancreatic lipase; PCR, polymerase chain reaction; bp, base pair(s).
in catalysis. In addition, we have identified a substitution of serine for cysteine at residue 216 on one of the patients' other mutant allele and confirmed that this mutation causes an inactive LPL protein. The Cys<sup>216</sup> residue is involved in a disulfide bridge that is completely conserved in LPL, HL, and PL, suggesting important functional significance.

**MATERIALS AND METHODS**

**Subjects**—The proband (II-5) of the Italian kindred (see Family 1 in Fig. 1) presented at age 25 with recurrent episodes of pancreatitis and chylomicronemia. Diagnosis of LPL deficiency was based on an extremely low level of postheparin LPL activity and the presence of normal apolipoprotein CII. The parents of the proband, although coming from the same rural area in Italy, are apparently unrelated.

The proband (II-1) in the Turkish kindred presented with abdominal pain resulting in laparotomy at age 17. His brother (II-3) presented with pancreatitis at about 1 year of age and had chylomicronemia (see Family 2 in Fig. 1). The parents of these affected siblings are second cousins.

**Measurement of LPL Mass and Catalytic Activity**—Blood samples were collected from patients and family members after an overnight fast following heparin injection of heparin (60 units/kg of body mass), and the postheparin blood samples were obtained 10 min after the injection. Plasma was separated after centrifugation at 3,000 x g for 10 min at 4 °C. LPL mass in pre- and postheparin plasma samples was measured by an enzyme-linked immunosorbent assay using purified bovine milk LPL as a standard (21). LPL and HL lipolytic activities were measured using a radiolabeled [1-tri-14C]oleate emulsion as described previously (22).

**DNA Analysis**—Genomic DNA was isolated from white blood cells of the patients and some of their family members as described previously (23). DNA haplotypes of mutant LPL alleles were constructed using the HindIII, BamHI, and PvuII (24, 25) restriction fragment length polymorphisms at the LPL locus.

Each of the 10 coding exons of LPL was individually amplified from 0.5-1 µg of genomic DNA from the probands using the polymerase chain reaction (PCR) as described previously (15, 17). The amplified exons were then purified and sequenced either directly or after cloning into a pUC18 vector or TA cloning vector (In Vitrogen Inc.).

**In Vitro Site-directed Mutagenesis of Human LPL cDNA and Transient Expression in COS Cells**—A 1.6-kilobase Dral/EcoRI fragment containing the entire coding sequence was prepared from the full-length LPL cDNA clone (pLPL35). This fragment was cloned into the EcoRV/EcoRI sites of the Bluescript KSII+ vector (Stratagene), reexcised using the flanking restriction sites for HindIII, BamHI, and PvuII (24, 25) restriction fragment length polymorphisms at the LPL locus.

Each of the 10 coding exons of LPL was individually amplified from 0.5-1 µg of genomic DNA from the probands using the polymerase chain reaction (PCR) as described previously (15, 17). The amplified exons were then purified and sequenced either directly or after cloning into a pUC18 vector or TA cloning vector (Invitrogen Inc.).

**RESULTS**

The results of lipoprotein and LPL activity for the Italian proband and family members (Family 1) are presented in Fig. 1. The proband, II-5, presented with clinical features of LPL deficiency, and one of his brothers, II-2, also showed fasting chylomicronemia with plasma triglycerides of 78.7 mmol/liter (7,000 mg/dl). The patient's postheparin LPL activity was essentially absent (7 nmol of free fatty acid/min/ml) despite incremental LPL mass of 370 ng/ml. HL activity was normal.

DNA haplotyping indicated that this Italian patient has LPL haplotype H1 and H2, suggesting two separate mutations. The nomenclature for the haplotypes (total of eight) has been described previously (15, 17). Sequence analysis of exon 5 DNA from this patient revealed heterozygosity for a G → A substitution at nucleotide 721 (first nucleotide of codon Asp<sup>156</sup>) (Fig. 2a) and a T → A alteration at nucleotide 901 (Fig. 2b). The first mutation results in an amino acid substitution of asparagine (AAT) for aspartic acid (GAT) at residue 156, and the second mutation results in a substitution of serine (AGT) for cysteine (TGT) at residue 216. Several independent PCR amplifications of exon 5 and subsequent DNA sequencing for both the coding and noncoding strand were performed to confirm the presence of these two mutations. The DNA sequence of the 9 remaining exons and the exon-intron junctions of the LPL gene were found to be normal.

The Asp<sup>156</sup> → Asn substitution abolishes a TaqI and an MboI restriction site which normally excises 29 (TaqI) or 30 (MboI) base pairs from the 5' end of the PCR amplified exon 5 (Fig. 3). Therefore, normal exon 5 DNA digested with MboI generates two fragments of 30 and 225 bp, respectively, whereas exon 5 DNA from a mutant allele remains intact as a 255-bp fragment after digestion. To identify additional patients who might carry this Asp<sup>156</sup> → Asn substitution, exon 5 of the LPL gene from 25 LPL-deficient patients of different ancestries for whom no mutation was known was amplified by PCR. Ten microliters of the amplified DNA were mixed with 10 units of MboI restriction enzyme (Bethesda Research Laboratories) and incubated at 37 °C (MboI) for 2 h. The digested DNA fragments were then separated on a 2% agarose gel. A Turkish proband and his affected brother were found to be homozygous for the large normal fragment, suggesting that they may carry the same mutation as the Italian proband (Fig. 3). Lipoprotein and lipase data for the Turkish kindred (Family 2) are presented in Fig. 1. However, the Turkish proband and his affected brother are homozygotes for haplotype H5, which is different from that of the Italian proband (H1 and H2), suggesting different mutations.

The MboI enzyme recognizes a 4-base DNA sequence, 5'-GATC-3', and mutations in any of the 4 bases can destroy the cutting site, producing the large abnormal band (255 bp) seen on the agarose gel (Fig. 3). However, by using both TaqI (recognizing 5'-TCGA-3') and MboI (recognizing 5'-GATC-3') digestion, one can narrow the range of possible substitutions to the 2-base GA sequence shared by the two enzymes. Therefore, changes in the GA sequence destroy the cutting sites for both enzymes whereas substitutions in the remainder of the recognition sequence abolish only one of the two cutting sites. TaqI digestion was performed using exon 5 DNA from the Italian proband and the two Turkish patients. The Turkish patients revealed homozygosity for the absence of the TaqI site (data not shown), suggesting that their mutation, if different from the Italian patient, is located at the second nucleotide of codon Asp<sup>156</sup>. DNA sequence analysis of exon 5 of the Turkish proband revealed homozygosity for an A → G
FIG. 1. Plasma triglyceride and lipoprotein concentrations, LPL catalytic activity and mass in members of two LPL-deficient families. Filled and open symbols indicate the presence and absence of LPL deficiency, respectively, determined by DNA analysis. Half-filled symbols represent heterozygotes who carry one mutant and one normal LPL allele. The proband in each family is identified by an arrow. Age refers to year at the time of sampling. Fasting plasma total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and very low density lipoprotein cholesterol (VLDL-C) are given in mmol/liter. LPL and HL activity, and incremental LPL mass after heparin injection, are expressed in nmol of free fatty acid/min/ml and ng/ml, respectively.

FIG. 2. DNA sequences of the normal and the mutant exon 5 spanning residues Asp156 and Cys216. Exon 5 DNA from the Italian proband was PCR amplified and cloned into a pUC18 vector. Plasmid DNA was individually purified from 16 positive clones and sequenced for both coding and noncoding strands. These clones were found to carry either the Asp156 or the Cys216 mutation, indicating that the patient is a compound heterozygote for these two mutations. a, nucleotide sequence of the coding strand from exon 5 of the Italian proband (Family 1, Fig. 1) and a control normal subject. A G → A substitution resulting in a substitution of asparagine for aspartic acid at residue 156 is highlighted in the boxes. b, DNA sequence of the noncoding strand from exon 5 of the same patient. A T → A alteration resulting in a substitution of serine for cysteine at residue 216 is indicated in the boxes.

substitution at nucleotide 722 (the second nucleotide of codon Asp156), which results in a substitution of glycine for aspartic acid (Fig. 4a). Both the coding and noncoding strand of exon 5 DNA from several independent PCR reactions of exon 5 of were sequenced to confirm the presence of the Asp156 → Gly mutation.

Another C → G DNA alteration in exon 9 was also identified (Fig. 4b), resulting in a premature stop codon occurring on the same alleles as the Asp156 → Gly substitution in the Turkish family. This alteration has been identified previously as a normal variant of LPL gene (27). It results in the generation of a new MnlI site which cosegregates with the Asp156 → Gly mutant LPL gene (Fig. 4c) in this family. No other alteration was identified in the remaining 8 exons or intron-exon boundaries.

To determine whether the missense substitutions at the first and second nucleotides of codon Asp156 and Cys216 are responsible for the loss of catalytic activity of LPL in these patients, we introduced these mutations into cloned LPL cDNA by site-directed mutagenesis using a dual-function mutagenesis expression vector. The sequences of the mutant oligonucleotides for mutagenesis were: mutation 156A
LPL protein was present in the medium of COS cells transfected with mutant Asp<sup>156</sup> → Asn cDNA at an equivalent but slightly higher level than that seen in the medium of cells transfected with the normal LPL cDNA (Fig. 5a) and similar LPL mass results were seen in the cell homogenate samples. Interestingly, however, the medium from cells transfected with cDNA carrying the Asp<sup>156</sup> → Gly alteration had an LPL protein level lower than that seen in the medium of the cells transfected with the normal LPL cDNA. Similarly, the LPL mass level in the cell homogenate of cells transfected with the mutant Asp<sup>156</sup> → Gly cDNA was also lower than that seen in the normal control.

LPL enzymatic activity in the medium of cells transfected with both mutant Asp<sup>156</sup> → Asn and mutant Asp<sup>156</sup> → Gly cDNA was essentially absent (0.2 and 0.4 nmol of free fatty acid/min/ml, respectively) as compared with the activity of the normal LPL (36.6 nmol of free fatty acid/min/ml) (Fig. 5b). These data demonstrate that the secreted LPL from both Asp<sup>156</sup> → Asn and Asp<sup>156</sup> → Gly mutants is catalytically inactive.

**Fig. 4.** DNA sequences of the normal and mutant exon 5 spanning residue Asp<sup>156</sup> and exon 9 surrounding residue 447. Exon 5 and exon 9 DNA from the Turkish proband were PCR amplified and sequenced directly as described previously (15). a, nucleotide sequence of the coding strand of exon 5 of the Turkish proband and a control. An A → G transition resulting in a substitution of glycine for aspartic acid at residue 156 is indicated in the boxes. The proband is a homozygote for Asp<sup>156</sup> → Gly alteration. b, DNA sequence of the coding strand of PCR amplified exon 9 from the same proband and a control. A C → G transition results in a Ser<sup>447</sup> → Stop substitution. This premature stop codon is located 2 residues from the normal stop codon at 449 and has previously been reported as a normal variant of LPL (27). c, the C → G alteration at Ser<sup>447</sup> results in the generation of an MnlI site. PCR amplified exon 9 DNA from members of the Turkish family was digested with MnlI and separated on a 2% agarose gel. The presence of the MnlI site cosegregates with the Asp<sup>156</sup> → Gly mutation in this family. L, 123-bp ladder (as a DNA size marker). C, control exon 9 (PCR-amplified exon 9 before enzyme digestion).

Phagemid DNA from these mutant clones and a normal clone containing wild-type LPL cDNA (positive control) was purified and used to transfect COS-7 cells. LPL mass and activity were assayed in the transfected COS cell medium and the cell homogenate for each set of control and mutant cDNA. (first nucleotide): 5'-ACTGGCCTCAATCCAGCT-3'; mutation 156B (second nucleotide): 5'-ACTGCGCGCTGTTCCA GCT-3'; mutation 216: 5'-TCAGGAGGAAGTAACATT GG-3'. Mutant specific clones were identified by oligonucleotide hybridization. DNA from positive clones from each mutagenesis were purified, and the substitutions were confirmed by sequencing.

Phagemid DNA from these mutant clones and a normal clone containing wild-type LPL cDNA (positive control) was purified and used to transfect COS-7 cells. LPL mass and activity were assayed in the transfected COS cell medium and the cell homogenate for each set of control and mutant cDNA.
The results for LPL mass and activity in the medium and cell homogenate of Cys\(^{216}\)→ Ser mutant are shown in Fig. 5, c and d. Despite the presence of a significant amount of mutant LPL immunoreactive mass in both medium and cell homogenate (Fig. 5c), LPL activity was zero (Fig. 5d). This indicates that the Cys\(^{216}\)→ Ser substitution causes a catalytically inactive enzyme.

**DISCUSSION**

The amino acid sequences of pancreatic, hepatic, and lipoprotein lipase show considerable homology, and the alignment of these sequences from various species reveals several “clusters” of completely homologous residues that are located in a segment encoded by exons 4, 5, and 6 of human LPL. Six naturally occurring missense mutations at residues 142, 157, 176, 207, 243, and 244 have been identified in the residues that are completely conserved, providing in vivo evidence that they are crucial for LPL catalysis (12–20). In this report, we describe two naturally occurring mutations at the first and second bases of codon Asp\(^{156}\) which, together with serine at 132 and histidine at 241, have been proposed previously as the catalytic triad for human LPL (10) (Fig. 6).

The three-dimensional structures of pancreatic, hepatic, and lipoprotein lipase are likely to be very similar because of the overall homology, and particularly because of the homology in the active site region in which many residues in addition to the catalytic triad are conserved or conservatively replaced. The known crystal structure of human pancreatic lipase (10) has therefore been used to examine the possible structural consequences of the Asp\(^{156}\) (Asp\(^{176}\) in hPL) and Cys\(^{16}\) (Cys\(^{176}\) in hPL) mutants. As Asp\(^{156}\) is part of the proposed catalytic triad, its mutation to glycine and asparagine can be expected to affect the catalytic rate substantially. For serine protease subtilisin, mutations in any of the 3 residues of the catalytic triad to alanine have been shown to result in a dramatic loss of activity (29). In serine proteases, the essential aspartic acid is believed to keep the essential histidine properly oriented and in the proper tautomeric form to act as a general base during catalysis. Moreover, the negative charged aspartic acid may stabilize the positively charged histidine that forms during the reaction (30). Apart from electrostatic stabilization, an asparagine, instead of an aspartic acid, could function similarly provided that its carbonyl oxygen is directed toward the histidine and accepts a hydrogen bond. However, for trypsin the Asp\(^{176}\)→ Asn mutant is more than 4 orders less active (31). Analysis of its three-dimensional structure excludes that this could be caused by a significant structural change (30). The arrangement of hydrogen bond donor and acceptor atoms around Asp\(^{156}\) of trypsin suggests that an asparagine would preferably orient in this environment such as to donate a hydrogen bond to the histidine through its NH₂ group and thereby stabilize the wrong tautomeric form of the histidine side chain. A similar situation occurs in the structure of human pancreatic lipase in which optimal hydrogen bonding of an asparagine side chain at position 176 of hPL (Asp\(^{156}\) in hLPL) would also require the histidine to be present in the wrong tautomer form. The loss of enzymatic activity of the two Asp\(^{156}\) mutants of human LPL supports the suspected essential role of this residue in catalysis.

The Ser-His-Asp triad has been observed directly in the catalytic sites of two lipases (pancreatic and fungal (Mucor miehei)) whose three-dimensional structures have been determined (10, 32). More recently the atomic structure of a lipase from Geotrichum candidum has been determined, but unlike other lipases and serine proteases, the catalytic triad is formed by Ser-His-Glu with glutamic acid replacing the usual aspartic acid even though the three-dimensional structure in the region of the catalytic triad is similar to other lipases (33). This highlights the possibility that other residues might comprise the catalytic triad of LPL. The crystallographic structure of the human pancreatic lipase has been the model for the structure of LPL. The fidelity of this model awaits the elucidation of the atomic structure of LPL.

Cysteine is another highly conserved residue among LPL, PL, and HL. There are 10 completely conserved cysteine residues each in LPL and HL in all species studied, and they are all linked by disulfide bridges (4, 28, 34). These data strongly suggest that the cysteine residues are important in maintaining the secondary structure required for proper function of the lipases. Cys\(^{216}\) is involved in one of the disulfide bridges that is linked to Cys\(^{176}\) (Fig. 6). In human PL this disulfide bridge (Cys\(^{37}\) to Cys\(^{26}\)) holds the loop (called flap) that covers the active site. No substrate can reach the active site unless this flap is detached in some way, and it is reasonable to assume that this occurs when the enzyme binds to the lipid-water interface. Although the effect of the Cys\(^{216}\)→ Ser mutation could not have been predicted from the structural knowledge, the resulting inactivation of the enzyme suggests that the precise conformation or conformational flexibility of this loop is important for the proper function of the enzyme.

Each of the two mutations at the first and second bases of codon Asp\(^{156}\) abolishes both TagI and MboI restriction sites (Fig. 3), and this provides a convenient means for the detection of these mutations. However, the two mutations cannot be distinguished by a restriction digestion using either or both enzymes. Therefore, one might wrongly assume that a change in restriction pattern with TagI and/or MboI digestion is caused by an identical mutation. In this instance, haplotype analysis showed differences and suggested two separate mu-
tations which were confirmed by DNA sequence analysis. However, even with the same haplotype, particularly if it is common in the general population, it would be inappropriate to assume that similar results on digestion of PCR amplified exon 5 DNA were caused by an identical mutation.

In summary, the crucial role of Ser146 has recently been supported through in vitro mutagenesis studies (11). We have now provided evidence for the central role of Asp146 in LPL catalysis by analysis of two independent naturally occurring but different mutations involving this residue.

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Note Added in Proof—The Asp146 → Gly mutation has recently been reported by Faustinella et al. (35).

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