Lipoprotein lipase^{Bethesda}: A single amino acid substitution (Ala-176 → Thr) leads to abnormal heparin binding and loss of enzymic activity

(lipoprotein lipase deficiency/cDNA mutation/expression vector)

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ABSTRACT The molecular defect that leads to a deficiency of lipoprotein lipase (LPL) activity in the proband from a Bethesda kindred has been identified. The pre- and post-heparin plasma LPL mass in the proband was elevated when compared to controls; however, there was no detectable LPL activity, indicating the presence of a defective enzyme (termed LPL^{Bethesda}). Analysis of the patient's post-heparin plasma by heparin-Sepharose affinity chromatography demonstrated that the mutant LPL had an altered affinity for heparin. Southern blot hybridization of the gene for LPL^{Bethesda} revealed no major rearrangements. Northern blot analysis of LPL^{Bethesda} mRNA from patient monocyte-derived macrophages revealed normal-sized mRNAs (3.4 and 3.7 kilobases) as well as normal cellular mRNA levels when compared to control macrophages. Sequence analysis of polymerase chain reaction-amplified LPL cDNA revealed a G → A substitution at position 781 of the normal LPL gene that resulted in the substitution of an alanine for a threonine at residue 176 and the loss of an *Sfa*NI site present in the normal LPL gene. Amplification of cDNA by the PCR followed by digestion with *Sfa*NI established that the patient was a true homozygote for the mutation. Expression of LPL cDNA in COS-7 cells resulted in the synthesis of a nonfunctional LPL enzyme establishing that the Ala → Thr substitution was the mutation responsible for the inactive LPL. The identification of this mutation in the LPL gene defines a region of the LPL enzyme, at Ala-176, that is essential for normal heparin-binding and catalytic activity. We propose that an amino acid substitution in this critical region of LPL^{Bethesda} results in the synthesis of a nonfunctional enzyme that leads to the chylomicronemia syndrome expressed in this proband.

Lipoprotein lipase (LPL) is a hydrolytic enzyme located on the luminal surface of capillary endothelial cells that plays a central role in triglyceride metabolism. In the presence of its cofactor, apolipoprotein C-II, LPL hydrolyzes triglycerides present in chylomicrons and very low density lipoprotein to monoglycerides, diglycerides, and free fatty acids, thus facilitating the conversion of triglyceride-rich particles to lipoprotein remnants (1).

The importance of LPL as a rate-limiting enzyme in the hydrolysis of lipoprotein triglyceride has been substantiated by the identification of patients with LPL deficiency (2). Patients with this syndrome, which is inherited as an autosomal-recessive trait, have marked alterations of triglyceride metabolism that include elevated fasting triglycerides, chylomicrons, and very low density lipoprotein. Affected individuals often present during childhood or adolescence with abdominal pain and hepatosplenomegaly. Other clinical fea-

tures include the presence of eruptive xanthomas, lipemia retinalis, and recurrent pancreatitis. Diagnosis of LPL deficiency is established by the absence of LPL enzymic activity in post-heparin plasma in the presence of an exogenous source of apolipoprotein C-II.

The structures of LPL from several species have been described (3–8). LPL is a glycoprotein of ≈54 kDa with a carbohydrate content of ≈8% (9). The size of human LPL based on nucleic acid sequence analysis of the cloned LPL cDNA is 475 amino acids of which the 27 amino-terminal residues constitute the signal peptide (3–6). In humans, there are two mRNA species of 3350 and 3750 base pairs (bp) that arise from alternative sites of 3' terminal polyadenylation (7). The human LPL gene is ≈30 kilobases (kb) long and consists of 10 exons interrupted by 9 introns (8, 9). This genomic organization is similar to that of the guinea pig LPL gene except that the guinea pig gene contains only 9 exons (10).

The LPL gene of several LPL-deficient patients was analyzed by Southern blot hybridization (11). Based on these studies, two major rearrangements at the LPL locus were identified that included a 6-kb deletion and a 2-kb insertion detected in the LPL alleles of one patient. The genetic defect underlying the 2-kb insertion has been identified as a partial duplication of exon 6 (12). Screening of 14 other patients from 11 unrelated families for the two defects revealed that three pairs of siblings had the 2-kb insertion, suggesting the possibility that this mutation may be the underlying genetic defect in a significant number of patients with a deficiency of LPL.

In the present manuscript we investigate the molecular defect in the LPL gene of the LPL-deficient proband from a Bethesda kindred. No major rearrangements of the patient's LPL gene were detected. Sequence analysis of the LPL cDNA revealed a single-base change that resulted in an Ala → Thr substitution at residue 176 in the LPL enzyme. This mutation is the basis for the deficiency of LPL activity in the Bethesda kindred (termed LPL^{Bethesda}).

MATERIALS AND METHODS

Clinical Data. The proband is a 39-year-old black male who presented at age 21 years with a history of recurrent episodes of abdominal pain and pancreatitis dating to childhood. Upon evaluation, the patient was found to have lipemia retinalis and splenomegaly. Fasting plasma lipid values of triglycerides and cholesterol were 2880 mg/dl and 210 mg/dl, respec-

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Abbreviations: LPL, lipoprotein lipase; LPL^{Bethesda}, mutant LPL from the Bethesda kindred; HL, hepatic lipase; PCR, polymerase chain reaction; neq, nanoequivalent(s); FFA, free fatty acid; SV40, simian virus 40.

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tively. Diagnosis of LPL deficiency was established by the virtual absence of post-heparin LPL activity in the proband's plasma [proband, 6 nanoequivalents (neq) of free fatty acids (FFA) per min per ml; control, 212 ± 60 neq of FFA per min per ml] and the presence of normal plasma levels of apolipoprotein C-II (13). The patient is presently followed on a low-fat (20 g) diet and medium-chain triglycerides, with recent lipoprotein values as follows: triglycerides, 1595 mg/dl; cholesterol, 230 mg/dl; very low density lipoprotein-cholesterol, 194 mg/dl; low density lipoprotein-cholesterol, 23 mg/dl; high density lipoprotein-cholesterol, 13 mg/dl.

Quantitation of Hepatic Lipase (HL) Activity and LPL Activity and Mass. Heparin (60 units/kg) was injected in the proband and control subjects after a 12-hr fast. Blood was collected in lithium heparin tubes before and 10 min after heparin injection for determination of the enzyme activities of LPL and HL, as well as LPL mass. Total post-heparin plasma lipolytic activity was quantitated as reported (13) using glycerol tri[14 C]oleate (Amersham) as substrate. HL and LPL activities were determined in triplicates by selectively blocking LPL with the monoclonal antibody 5D2 (14). The mean of eight LPL mass measurements was determined by an ELISA using the monoclonal antibody 5D2 as described (14).

Heparin-Sepharose Affinity Chromatography. Affinity chromatographic separation of plasma LPL and HL on heparin-Sepharose was performed as described by Ostlund-Lindqvist and Boberg (15) with the following modifications: 10 ml of post-heparin plasma was adjusted to a final salt concentration of 0.4 M NaCl and fractionated on a 1.2×15 cm heparin-Sepharose 6B column at a flow rate of 21 ml/hr. The column had been equilibrated with 0.4 M NaCl/10 mM sodium phosphate, pH 7.4, containing 0.1% Triton X-100 and 30% (vol/vol) glycerol. A stepwise elution was carried out using phosphate buffer with various NaCl concentrations, as follows: (i) 9 ml of 0.45 M NaCl buffer; (ii) 15 ml of 0.4 M NaCl wash; (iii) 5 ml of 0.75 M NaCl buffer; (iv) 20 ml of 0.75 M NaCl wash; (v) 6 ml of 1.5 M NaCl buffer; (vi) 20 ml of 1.5 M NaCl wash. Aliquots (50 and 200 μ l) from each fraction were used for determination of enzyme activity and mass assays, respectively. Total LPL and HL activities were measured in duplicate samples and their differences were used to calculate the mean LPL activity in each fraction.

DNA Preparation and Southern Blot Hybridization Analysis. DNA was isolated from leukocytes as described (16). Southern blot hybridization analysis was performed after digestion of 20 μ g of DNA with the *Bam*HI, *Eco*RI, *Pst*I, *Nco*I, and *Stu*I (New England Biolabs) as reported (16).

Preparation of Monocyte-Derived Macrophages. Human monocyte-derived macrophages were prepared from 250 ml of acid citrate/dextrose-treated mononuclear cells (2.5 g of dextrose/2.2 g of sodium citrate/0.73 g of citric acid per 100 ml) obtained from healthy donors by monocytopheresis (model CS-3000, Fenwal Laboratories, Deerfield, IL). Mononuclear cells were separated by centrifugation of the mononuclear-enriched plasma in Ficoll/Hypaque and 1×10^7 cells were plated per 30-mm well (Costar) in 3 ml of RPMI 1640 medium containing 10% (vol/vol) human serum and 1% glutamine. After 1–3 weeks, the differentiated macrophages were scrapped using a 4 M guanidinium thiocyanate buffer containing 0.5% sodium *N*-laurylsarcosine (ICN), 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol. Total cellular RNA was then isolated as described (17).

Northern Blot Hybridization Analysis of RNA. Gels for Northern blot analysis were prepared with 1% agarose in the presence of 6% (vol/vol) formaldehyde, electrophoresed at 25 V for 16 hr, transferred to nitrocellulose filters (Schleicher & Schuell), and hybridized with both LPL and β -actin cDNA probes as described (18). Total RNA (8 μ g) was analyzed and gels were stained with ethidium bromide to confirm that

equivalent quantities of RNA were electrophoresed in each lane.

Reverse Transcription and Polymerase Chain Reaction (PCR) Amplification of LPL cDNA. LPL cDNAs from control and LPL-deficient subjects were synthesized by incubation of 1 μ g of monocyte-derived macrophage total RNA with 15 units of Molony murine leukemia virus reverse transcriptase (Pharmacia LKB) and two 32-bp LPL primers (each at 0.2 μ M) containing the restriction enzyme sites for *Hind*III and *Bam*HI, which spanned bases 129–1665 of the LPL cDNA (6). The translated sequence of the LPL cDNA is encoded by bp 175–1602. The buffer and deoxynucleoside triphosphates used for the reaction were performed with the GeneAmp DNA amplification reagent kit (Perkin-Elmer), as recommended for the PCR. After incubation at 37°C for 2 hr the newly generated cDNA was amplified by the automated PCR technique (19) for 30 cycles using *Thermus aquaticus* DNA polymerase (Perkin-Elmer). PCRs were performed with 5-min extensions at 72°C, 30-s denaturations at 95°C, and 1-min primer annealing at 55°C. DNA was identified by staining with ethidium bromide. Amplified DNA was digested with the appropriate restriction enzymes and subcloned into pGEM-3Z vector DNA for sequencing. Synthetic oligonucleotides were synthesized by the phosphoramidite method for oligonucleotide synthesis in a DNA synthesizer (model 380B; Applied Biosystems).

DNA Sequencing. Double-stranded DNA sequencing of LPL cDNA cloned into pGEM-3Z vector DNA was performed by the dideoxynucleotide chain-termination method of Sanger *et al.* (20).

Plasmid Construction and Transfection of Mammalian Cells. The parent plasmid (pCMV) utilized in the transfection studies contains the cytomegalovirus (CMV) promoter and transcriptional start site as well as the polyadenylation signal from the simian virus 40 (SV40) cloned into pUC-18 as described (21). A 1536-bp fragment of LPL cDNA that spanned the signal peptide through the termination codon (TGA) was cloned into the *Hpa*I and *Xba*I sites of the pCMV vector and thus placed under the control of the cytomegalovirus promoter. Standard techniques were used for all cloning procedures and plasmid isolations were performed by the CsCl double-banding method (22). The constructs, pCMV-NL and pCMV-PT, containing the normal and patient's cDNA, respectively, were characterized by DNA sequencing before transfecting into COS-7 cells. DNA transfections were performed as described (23) by the calcium phosphate coprecipitation method (24) with the exception that the medium was supplemented with 2 units of heparin sulfate (Elkins-Sinn, Cherry Hill, NJ) per ml.

Restriction Enzyme Digestion of PCR-Amplified DNA. One-tenth of the total amplified DNA generated by using primers spanning bases 255–287 and 1117–1149 of the LPL cDNA was digested with 2 units of *Sfa*NI (New England Biolabs) for 2 hr at 37°C. The digested fragments were separated on a 4% agarose (FMC) minigel in Tris/acetate/EDTA at 85 V for 4 hr. DNA was identified by staining with ethidium bromide

RESULTS

The results of the quantitation of LPL mass and activity, as well as HL activity, in control and proband pre- and post-heparin plasma are shown in Table 1. Despite higher than normal LPL mass in the post-heparin sample, the patient's plasma had virtually no detectable LPL activity, indicating the presence of an enzymically inactive protein. A significant quantity of LPL mass was present in the proband's pre-heparin plasma sample suggesting defective binding of the mutant LPL^{Bethesda} enzyme to the endothelial vessel wall. HL activity in the patient's post-heparin sample was near normal (Table 1).

Table 1. LPL activity and mass in the plasma of control subjects and the LPL^{Bethesda}-deficient proband

Source	Activity, neq of FFA per min per ml		LPL mass, ng/ml	
	HL	LPL	Pre-heparin	Post-heparin
Proband	66	6	256	393
Controls	153 ± 41*	212 ± 60*	—†	179 ± 50*

*Data are mean ± SD, *n* = 17.

†Usual control values range from 0 to 50 ng/ml.

Analysis of the heparin-binding properties of LPL from control and the patient's post-heparin plasma was performed by stepwise elution on a heparin-Sepharose 6B affinity column (Fig. 1). As expected, LPL mass and activity from control plasma was eluted with 1.5 ml of NaCl containing phosphate buffer. A markedly abnormal elution profile of LPL was observed in the patient's plasma with LPL mass eluting with phosphate buffer containing 0.4 M, 0.75 M, as well as 1.5 M NaCl, indicating abnormal heparin binding by the mutant LPL. No LPL activity was detected in any of the fractions from the patient's plasma. HL activity was eluted in both the control and patient's post-heparin samples with phosphate buffer containing 0.75 M NaCl (data not shown).

Fig. 2 illustrates a Southern blot hybridization analysis of the LPL gene from a normal and the LPL-deficient patient digested with *Bam*HI, *Eco*RI, *Nco*I, *Pst*I, and *Stu*I. No major rearrangements were detected in the LPL gene of the Bethesda proband despite digestion of the DNA with enzymes that identify the 2-kb insertion present in the LPL gene of some LPL-deficient individuals (12). The patient is a homozygote for the 19-kb band obtained by digestion of the LPL gene with the polymorphic enzyme *Bam*HI.

Northern blot hybridization analysis of total RNA isolated from monocyte-derived macrophages from the proband and normal subjects is illustrated in Fig. 3. Approximately normal

FIG. 1. Heparin-Sepharose elution profiles of post-heparin plasma from a control subject (Upper) and the LPL^{Bethesda} proband (Lower) are illustrated. LPL activity and mass are shown by the solid and open bars, respectively. The initial elutions (bars 1, 3, and 5) and subsequent washes (bars 2, 4, and 6) with phosphate buffer containing increasing concentrations of NaCl are illustrated.

FIG. 2. Southern blot analysis of DNA from normal (lanes N) and the LPL-deficient subject (lanes PT) is illustrated. The restriction fragments of the LPL^{Bethesda} proband were identical in size to the polymorphic fragments of control DNA.

levels of both normal-sized LPL mRNA species (3.4 and 3.7 kb) were identified in the patient's sample when compared to the control.

Sequence analysis of six clones obtained from PCR-amplified patient cDNA revealed the presence of a single-base substitution, G → A, at position 781 in the fifth exon, which results in an Ala → Thr substitution at residue 176 of LPL (Fig. 4A). Autoradiographs of sequencing gels from control and LPL-deficient subject illustrating the G → A substitution are shown in Fig. 4B.

Computer analysis (Fristensky-Cornell DNA sequencing analysis program) of the patient's LPL gene cDNA sequence containing the G → A transition revealed the loss of an *Sfa*NI restriction enzyme site present in the normal LPL gene. DNA amplification of the region of the mutation was performed by using the synthetic oligonucleotides indicated and PCR-generated LPL cDNA as template (Fig. 5A). The amplified product was 894 bp long. Digestion of DNA amplified from a normal subject with *Sfa*NI generated two fragments, 513 and 381 kb in size, but digestion of the patient's amplified DNA with the same enzyme resulted in the formation of a single 894-bp band due to the loss of the *Sfa*NI restriction enzyme site. Analysis of the restriction enzyme fragments of the amplified cDNA from the patient and control subjects by agarose gel electrophoresis is illustrated in Fig. 5B. Absence of the normal-sized restriction fragments (513 and 381 bp) in the DNA of the LPL-deficient patient confirmed that the proband is a true homozygote with both LPL alleles having the G → A substitution.

FIG. 3. Northern blot analysis of 8 μ g of monocyte-derived macrophage total RNA from a normal subject (lane N) and the LPL^{Bethesda} proband (lane Pt). The positions of the 3.4- and 3.7-kb LPL mRNA are indicated.

FIG. 4. (A) Schematic representation of the LPL gene. Exons are illustrated by solid bars interrupted by lines that represent introns. The G → A substitution in the patient's LPL sequence is highlighted by a box. (B) Autoradiograms of sequencing gels of DNA from a normal subject and the proband. The G → A substitution is indicated by the arrow.

To establish that the G → A substitution is not a polymorphism but the mutation that results in the expression of an enzymically inactive LPL_{Bethesda} enzyme, expression plasmids encoding for both the normal and mutant LPL were transfected into COS-7 and the medium was assayed for LPL activity and mass. The constructs utilized for these transient expression studies are shown in Fig. 6.

The results of the expression of normal and patient LPL cDNA in COS-7 cells are summarized in Table 2. Medium

FIG. 5. Schematic representation of the portion of the LPL cDNA that was PCR-amplified by using primers A and B is shown in A. The *Sfa*NI site, present in the normal sequence but not in the patient's cDNA, is illustrated by the letter S. Horizontal solid and dashed arrows indicate the size of the restriction fragments generated by *Sfa*NI digestion of amplified DNAs from normal and the LPL_{Bethesda} proband, respectively. (B) Agarose gel electrophoresis analysis of amplified normal (lane N) and patient (lane Pt) DNA after digestion with *Sfa*NI. DNA molecular size standards are indicated in bp (lane M) (11).

FIG. 6. Plasmid constructs used for transient expression of LPL cDNAs in COS-7 cells are illustrated. The parent plasmid (pCMV) contains the cytomegalovirus (CMV) promoter and transcriptional start site as well as the SV40 polyadenylation signal but no LPL insert. Plasmids, pCMV-NL (normal) and pCMV-PT (patient), contain a 1536-bp cDNA fragment of LPL inserted between the *Xba*I and *Hpa*I cloning sites of pCMV. Amp^R, ampicillin resistance.

harvested from untransfected cells and cells transfected with the pCMV vector alone showed similar background levels of both LPL mass and activity, whereas medium obtained from cells transfected with pCMV-NL had a significant quantity of both LPL mass and activity, indicating expression of the normal LPL cDNA. Medium harvested from COS-7 cells transfected with the pCMV-PT construct that contained an LPL concentration (by ELISA) similar to that of the pCMV-NL (normal) construct had no detectable LPL activity. These studies establish that the Ala → Thr substitution is the mutation that results in the loss of enzymic activity in LPL_{Bethesda}.

DISCUSSION

In the present study we have analyzed the LPL DNA, RNA, and protein of the proband with a deficiency of LPL from the Bethesda kindred. Study of the patient's post-heparin plasma revealed the absence of LPL activity despite higher than normal LPL mass concentrations in the pre- and post-heparin samples, indicating the presence of a nonfunctional enzyme. An interesting feature of the mutant LPL_{Bethesda} is that a significant quantity of protein can be detected in the patient's plasma prior to heparin infusion, suggesting defective interaction of the LPL_{Bethesda} enzyme with the endothelial vessel wall, delayed catabolism of the mutant enzyme, or both. *In vitro* analysis of the patient's post-heparin plasma on heparin-Sepharose affinity chromatography documented the elution of LPL at low as well as high NaCl concentrations, demonstrating abnormal heparin binding by LPL_{Bethesda}. This abnormal elution pattern could be due to a relative decrease in the heparin affinity of the mutant LPL. Thus, the LPL_{Bethesda} proband exhibits the class III defect described by Brunzell and coworkers (25), which is associated with both abnormal catalytic activity and defective heparin binding.

LPL mRNA isolated from monocyte-derived macrophages grown *in vitro* was shown to be of normal size and quantity by Northern blot hybridization, and Southern blot analysis

Table 2. LPL activity and mass in the medium of transfected COS-7 cells

Plasmid	LPL mass, ng/ml	LPL activity, neq of FFA per min per ml
Untransfected	1.56 ± 0.29	0.19 ± 0.12
pCMV	1.06 ± 0.55	0.18 ± 0.07
pCMV-NL	26.80 ± 1.22	4.46 ± 0.15
pCMV-PT	24.37 ± 1.91	0.12 ± 0.04

Values are expressed as mean ± SD, *n* = 3. The difference between the mean post-heparin lipolytic activity and HL activities was used to determine the LPL activity (mean ± SD) in each fraction. Plasmid constructs are described in Fig. 6.

revealed no major rearrangements of the patient's LPL gene. Sequence analysis of the LPL^{Bethesda} cDNA revealed the presence of a single-base change in the fifth exon of the LPL gene that resulted in the substitution Ala→Thr at residue 176 of LPL. To exclude the possibility that the amino acid change was a polymorphism and not the mutation that resulted in an inactive LPL enzyme, we analyzed the LPL^{Bethesda} mutation *in vitro*. Transient expression of control and mutant LPL^{Bethesda} cDNA in COS-7 cells established the presence of an enzymically inactive protein in the medium of cells transfected with the mutant cDNA, thus providing definitive evidence that the Ala→Thr substitution was the mutation resulting in the inactive LPL enzyme.

The identification of a single amino acid substitution that results in the synthesis of a molecule that exhibits abnormal elution on heparin-Sepharose affinity chromatography and is enzymically inactive was unexpected. The region near Ala-176, including amino acids 169–179 of LPL, is highly conserved among the mouse, guinea pig, cattle, and human LPLs (3–6), porcine pancreatic lipase (3), as well as rat and human HLs (5). The Ala-176 residue is present in all sequences, suggesting it serves an important role in the maintenance of LPL function. The Ala-176 residue is 44 amino acids away from Ser-132, which, based primarily on homology studies with porcine pancreatic lipase, has been postulated to be the essential serine in LPL needed for the binding of the enzyme to the substrate interface or for charge depolarization during catalysis (4, 6, 26, 27). The interfacial lipid binding region of human LPL has been proposed to be near Ser-132 (6). However, potential heparin-binding regions are located in the carboxyl-terminal half of LPL and not near Ala-176 (4, 27). Thus, it is not clear how a single residue change in LPL could affect both LPL activity and heparin affinity.

Based on previous studies it has been proposed that the LPL monomer is enzymically inactive (3, 28) and has decreased affinity for heparin (28, 29), whereas the dimer is an active enzyme with a greater heparin affinity than the monomeric species. There are several potential mechanisms by which a single amino acid substitution could result in the loss of both normal LPL heparin binding and enzymic activity. One possibility is that the mutation occurs in a region of LPL directly involved in dimer association and thus prevents the formation of a stable dimer. A second alternative is that the mutant residue is distant from the site of dimer interaction but leads to a conformational change in LPL that results in the preferential accumulation of the inactive monomer. In both cases the monomer–dimer equilibrium would be shifted in favor of the inactive monomer that exhibits decreased heparin affinity. The introduction of a threonine at position 176 provides for potential phosphorylation or glycosylation at this site that could mediate this conformational change. Our characterization of LPL^{Bethesda} demonstrates that the mutant protein, like the LPL monomer, is defective in both enzymic activity and heparin binding. Thus, if the Ala→Thr substitution at position 176 alters the dimer–monomer equilibrium in favor of monomer formation, then the mutant LPL^{Bethesda} could exhibit the heterogeneous pattern of elution on heparin-Sepharose affinity chromatography that was observed. A third possibility is that the Ala→Thr mutation could occur in a critical portion of the LPL sequence that does not alter dimer–monomer equilibrium but does result in a conformational change that affects both the enzymic activity and heparin affinity of LPL^{Bethesda}. Further investigations are warranted to resolve this question.

In summary, we have identified a mutation in the LPL^{Bethesda} gene that results in a single amino acid substitu-

tion at residue 176 and the synthesis of a nonfunctional enzyme that also exhibits abnormal heparin affinity. The identification of this mutation defines a region in LPL at Ala-176 that is important for catalytic activity and heparin binding.

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