Homozygosity for two point mutations in the lipoprotein lipase (LPL) gene in a patient with familial LPL deficiency: LPL(Asp^{9} → Asn, Tyr^{262} → His)

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Abstract Familial lipoprotein lipase (LPL) deficiency is an inherited disorder of lipoprotein metabolism characterized by hypertriglyceridemia and recurrent episodes of abdominal pain and pancreatitis. We have studied the genetic basis of LPL deficiency in a 62-year-old black male with undetectable pre- and post-heparin plasma LPL mass and activity. DNA sequence analysis of the patient's LPL cDNA and gene as well as digestion with Bcl I and Asu I revealed that the proband is a homozygote for two separate gene defects. One mutation changed a G to an A, resulting in the conversion of amino acid 9 of the mature protein, aspartic acid (GAC), to asparagine (AAC). The second substitution, a C for a T, replaced tyrosine (TAC) at residue 262 with histidine (CAC). Northern blot analysis of monocyte-derived macrophage RNA demonstrated the presence of LPL mRNA of approximately normal size and quantity when compared to control. Expression of both mutations separately (pCMV-9 and pCMV-262) or in combination (pCMV-9+262) in human embryonal kidney-293 cells demonstrated that LPL-9 had approximately 80% the specific activity of wild type LPL, but LPL-262 and LPL-9+262 had no enzymic activity, thus establishing the functional significance of the LPL-262 defect. Despite an absolute deficiency of LPL mass and activity demonstrated by analysis of patient post-heparin plasma, in vitro expression of both LPL mutants was normal, suggesting that the absence of LPL in patient post-heparin plasma was a result of altered in vivo processing. Analysis of the heparin binding properties of the mutant enzymes by heparin-Sepharose affinity chromatography indicated that most of the LPL-262 mass was present in an inactive peak, which like the normal LPL monomer, eluted at 0.8 M NaCl. Thus, the Tyr^{262} → His mutation may alter the stability of the LPL dimer, leading to the formation of inactive LPL-262 monomer which exhibits reduced heparin affinity. Based on these results, we propose that, in vivo, enhanced formation of LPL-9+262 monomer leads to abnormal binding of the mutant lipase to endothelial glycosaminoglycans ultimately resulting in enhanced catabolism of the mutant enzyme and lower enzyme mass in post-heparin plasma.—**Rouis, M., P. Lohse, K. A. Dugi, P. Lohse, O. U. Beg, R. Ronan, G. D. Talley, J. D. Brunzell, and S. Santamarina-Fojo.** Homozygosity for two point mutations in the lipoprotein lipase (LPL) gene in a patient with familial LPL deficiency: LPL (Asp^{9} → Asn, Tyr^{262} → His). *J. Lipid Res.* 1996. **37:** 651–661.

Supplementary key words glycosaminoglycans • heparin binding • hypertriglyceridemia • lipoprotein lipase deficiency • LPL monomer • pancreatitis

Lipoprotein lipase (LPL) is a 55-kD glycoprotein that plays a major role in the hydrolysis of triglycerides present in plasma chylomicrons and very low density lipoproteins (VLDL) (1). LPL is synthesized by parenchymal cells of a number of tissues including adipocytes, heart and skeletal muscle, lung, kidney, mammary gland, and monocyte-derived macrophages (2–5). LPL is released into the bloodstream, where it becomes bound to the endothelium of the capillary wall by glycosaminoglycans such as heparan sulfate (6). In the presence of its cofactor apoC-II (7, 8), LPL hydrolyzes dietary and endogenous triglycerides to mono- and diglycerides as well as free fatty acids, which can be either further oxidized to provide energy or stored in adipose tissue after re-esterification.

The important role that LPL plays in triglyceride metabolism has been established by the identification of patients with LPL deficiency. Familial LPL deficiency, an autosomal recessive disorder of lipoprotein metabolism, is characterized by profound fasting hyper-

Abbreviations: apo, apolipoprotein; bp, base pairs; HL, hepatic lipase; LPL, lipoprotein lipase; PCR, polymerase chain reaction; PL, pancreatic lipase.

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triglyceridemia and massive accumulation of chylomicrons due to their delayed plasma clearance, as well as markedly reduced cholesterol concentrations of low and high density lipoproteins. Patients with LPL deficiency often present in childhood with recurrent episodes of abdominal pain and pancreatitis, as well as hepatosplenomegaly, eruptive xanthomas, and lipemia retinalis. Despite the very high levels of plasma triglycerides and often cholesterol, these patients do not appear to be at an increased risk of developing premature cardiovascular disease (1, 9).

The underlying molecular defects that lead to a deficiency of LPL have been investigated (10, 11). Although several major gene rearrangements (12–14) as well as single point mutations resulting in abnormal splicing (15–17) or the introduction of premature stop codons (18–21) have been identified, most LPL gene defects are missense mutations that are located in the amino-terminal region of the enzyme and lead to the synthesis of an inactive LPL (10, 22–25). Interestingly, the post-heparin plasma LPL concentrations in a subset of patients with LPL gene defects appear to be markedly reduced or undetectable even though many of these mutant enzymes appear to be secreted normally by mammalian cells in vitro (16, 18, 26–28).

In the present study we characterize the underlying molecular defect in a 62-year-old black male with familial LPL deficiency with undetectable LPL mass and activity in pre- and post-heparin plasma. DNA sequence analysis and digestion of patient DNA with Bcl I and Asu I identified the patient as a homozygote for two point mutations in the LPL gene. Functional analysis of both mutations, using an in vitro expression system, revealed normal expression of all mutant lipases and demonstrated that substitution of $Tyr^{262} \rightarrow$ His but not $Asp^{9} \rightarrow$ Asn results in markedly reduced LPL dimer stability, leading to impaired lipolytic activity of the mutant enzyme. Thus, replacement of $Tyr^{262} \rightarrow$ His may enhance the formation of inactive monomer with reduced heparin binding properties, ultimately leading to enhanced catabolism of the mutant lipase in vivo.

MATERIAL AND METHODS

Clinical data

The patient is a 62-year-old black male who suffered from recurrent episodes of abdominal pain as a child. Evaluation at the age of 22 revealed lipemia retinalis, profound hepatosplenomegaly, hypertriglyceridemia, and chylomicronemia. The absence of plasma post-heparin LPL activity in the presence of a functional apoC-II established the diagnosis of LPL deficiency. Since his initial diagnosis, the patient has continued to have recurrent episodes of pancreatitis, which have resulted in the development of pancreatic insufficiency and insulin-requiring diabetes. The patient is currently receiving 10 units of NPH insulin in the morning and, if needed, additional insulin in the evening according to blood glucose levels. The last documented episode of pancreatitis occurred 2 years ago and the triglyceride level at that time was 4,860 mg/dl. Although triglyceride levels as high as 10,200 mg/dl have been documented in the past, the current values are around 1,000 mg/dl. The patient's low BMI of 18.5 could be an indication that the currently lower levels are caused by wasting due to pancreatic insufficiency.

Quantification of post-heparin plasma HL activity and LPL activity and mass

Blood was collected in EDTA tubes before and 10 min after an intravenous injection of heparin of 60 units/kg body weight in the proband and control subjects. Total post-heparin plasma lipolytic activity was quantitated as previously reported (29) using glycerol tri-[^{14}C]oleate (Amersham, Arlington Heights, IL) as substrate. HL and LPL activities were determined in triplicate by selectively blocking LPL with 1 M NaCl. LPL mass was measured either by a sandwich ELISA using the monoclonal antibody 5D2 for capture and measurement as previously described (30) or an ELISA using the 5D2 antibody for capture and a polyclonal LPL antibody from chicken for measurement.

Reverse transcription and PCR amplification of LPL cDNA

LPL cDNAs from control and the LPL-deficient subjects were synthesized by incubating 1 μg of monocyte-derived macrophage total RNA with 15 units of Molony murine leukemia virus reverse transcriptase (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and 0.2 μM each of two primers that spanned bases 129–147 and 1636–1665 of the LPL cDNA as previously described (23). After incubation at 37°C for 2 h, the newly generated cDNA was amplified by the automated PCR technique (31) for 30 cycles using *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) and two different, internal primers that spanned bases 141–170 and 1621–1650 of LPL (according to ref. 3) and contained the restriction enzyme sites for Xba I and Hpa I. The PCR reactions were performed with 1 min denaturations at 94°C, 1 min primer annealing at 55°C, and 2 min extension at 72°C. DNA was identified on a 1% agarose gel by staining with ethidium bromide.

DNA sequencing

Amplified DNA was digested with the appropriate restriction enzymes and subcloned into pCMV vector

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DNA (23) for both sequencing and expression studies. Sequencing of cloned double-stranded LPL cDNA was performed by the dideoxynucleotide chain-termination method of Sanger, Nicklen, and Coulson (32). Six independent clones obtained from PCR amplification of the patient's LPL cDNA were sequenced. Oligonucleotide primers were synthesized by the phosphoramidite method in a DNA synthesizer (model 380B; Applied Biosystems Inc., Foster City, CA).

LPL cDNA expression vectors

The normal LPL cDNA construct, designated pCMV-NL, has been previously described (23). It contains the cytomegalovirus early promoter driving the expression of a 1470 bp fragment of the LPL cDNA spanning the signal peptide through the termination codon. The mutant LPL cDNAs, pCMV-9, pCMV-262, and pCMV-9+262, were generated from the pCMV-NL construct by site directed mutagenesis using the overlap extension method (33). DNA sequence analysis of the constructs was performed by the dideoxynucleotide chain termination method (32). Large scale preparation of the normal and mutant LPL constructs was performed by double equilibrium centrifugation in CsCl (34).

In vitro expression

Human embryonal kidney 293 cells (ATCC, Rockville, MD), cultured in DMEM medium (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (Biofluids, Rockville, MD), were transfected in triplicate with 40 μg of plasmid DNA per 100 mm dish using the calcium phosphate co-precipitation method (35). After 24 h of incubation at 37°C and 3% CO_2, the cells were washed with DPBS w/o calcium and magnesium (Biofluids) and 6 ml of fresh media containing two units/ml of sodium heparin (Lipho Med, Melrose Park, IL) were added to each dish. The cells were then incubated for an additional 14–16 h at 37°C and 5% CO_2. The media were harvested and mixed with glycerol at a final concentration of 30% (v/v). LPL mass was quantitated in quadruplicate (30) and tributyrin (36) as well as triolein (29) activities were assayed in duplicate as previously described. The cells were washed, scraped in phosphate-buffered saline, lysed, and sonicated in 0.22 M Tris-HCl, pH 8.5, 0.25 M sucrose, 1% BSA, 0.005% (g/g) heparin, 0.2% sodium deoxycholate, and 0.008% (v/v) Nonidet P40. The cell extracts were centrifuged, aliquoted, flash frozen, and stored at -70°C for determination of LPL mass.

Northern blot analysis of monocyte-derived macrophage RNA

Monocyte-derived macrophages obtained from the patient and a control subject were cultivated in vitro and total RNA was prepared by the guanidine isothiocynate method (37) at day 9. Samples of 10 μg were subjected to electrophoresis in a 1% formaldehyde/agarose gel (38) and blotted onto a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH) following the protocols recommended by the manufacturer. The Northern blot was hybridized with a uniformly ^{32}P-labeled human LPL cDNA probe, washed, and scanned with a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA). To correct for differences in the amount of total RNA loaded, the blot was rehybridized with a uniformly ^{32}P-labeled 412 bp Dra I/Rsa I restriction fragment of the human β-actin 3′-untranslated region (39), kindly provided by Dr. Larry Kedes (Stanford University School of Medicine and Veterans Administration Medical Center, Palo Alto, CA). The membrane was then re-scanned and exposed to X-ray films.

Heparin-Sepharose affinity chromatography

Affinity chromatography of media from cells transfected with normal LPL cDNA (pCMV-LPL) and mutated LPL (pCMV-9, pCMV-262, and pCMV-9+262) was performed as described by Emi et al. (40) with some modifications. A 1-ml bed volume of heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology, Inc. Piscataway, NJ) was packed in each 0.7 × 4 cm Flex Column (Kontes, Vineland, NJ) at 5°C. About 15 ml of transfected media was applied to each column at 15.6 ml/cm^2 per h with a peristaltic pump. The unabsorbed protein was eluted with 17–20 ml of the equilibration buffer and 15 ml of 2 N NaCl in 0.01 M sodium phosphate (pH 7.4), 0.1% CHAPS, and 30% glycerol. One ml fractions were collected in polypropylene tubes containing 2 units of heparin. Each fraction was monitored with a Radiometer conductivity meter at 5°C. The conductivities were then related to a linear curve of standard NaCl solutions.

RESULTS

Table 1 summarizes the lipid and lipoprotein profile as well as lipase activities in the proband's plasma. The patient was markedly hypertriglyceridemic and had decreased levels of LDL and HDL. LPL activity and mass in patient pre- as well as post-heparin plasma was undetectable, consistent with an LPL class I defect (41). Hepatic lipase activity, on the other hand, was present but at a lower level than normal. Although most patients with LPL deficiency have normal HL activity, patients with low HL activity have been previously described (42, 43). The cause for the decreased hepatic lipase activity in this subset of patients is unknown.

Figure 1 illustrates the autoradiogram of DNA sequencing gels of normal and patient LPL cDNA. Se-

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TABLE 1. Plasma lipids, lipoproteins, and lipase activity in the LPL-deficient patient

	Lipids and Lipoproteins					Lipase Activity		LPL Mass	
	TC	TG	VLDL-C	LDL-C	HDL-C	LPL	HL	Pre	Post
			mg/dl			nmol/ml/min		*ng/ml*	
Patient	243	1295	202	31	10	0	52	0	0
Controls[a]	196 ± 27	105 ± 48	16 ± 11	121 ± 23	54 ± 18	153 ± 41	212 ± 60	0-.50	179 ± 50

TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.
[a]Values are mean ± standard deviation.

quence analysis of the patient's LPL cDNA revealed two point mutations that were present on both strands in all clones sequenced. One mutation replaced a G by an A (Fig. 1 A/B), replacing a negatively charged aspartic acid (GAC), residue 9 of the mature protein, with a neutral asparagine (AAC). The second base substitution replaced a T with a C (Fig. 1 C/D), changing residue 262 of the mature protein, a neutral tyrosine (TAC), to a basic histidine (CAC). Both substitutions, inherited on the same allele, result in the expression of a mutant protein with a two charge unit more basic isoelectric point (pI 8.14) than normal LPL (pI 7.97) (data not shown).

Analysis of the deduced amino acid sequences for human (3), bovine (44), mouse (4), and guinea pig (5) LPL are highly homologous with 85–94% of the residues being identical (45). Comparison of these sequences revealed that residues 9 and 262, aspartic acid and tyrosine, are completely conserved in these species. Alignment with the only non-mammalian LPL sequence reported thus far demonstrated that amino acid 262 is also conserved in chicken LPL, while aspartic acid,

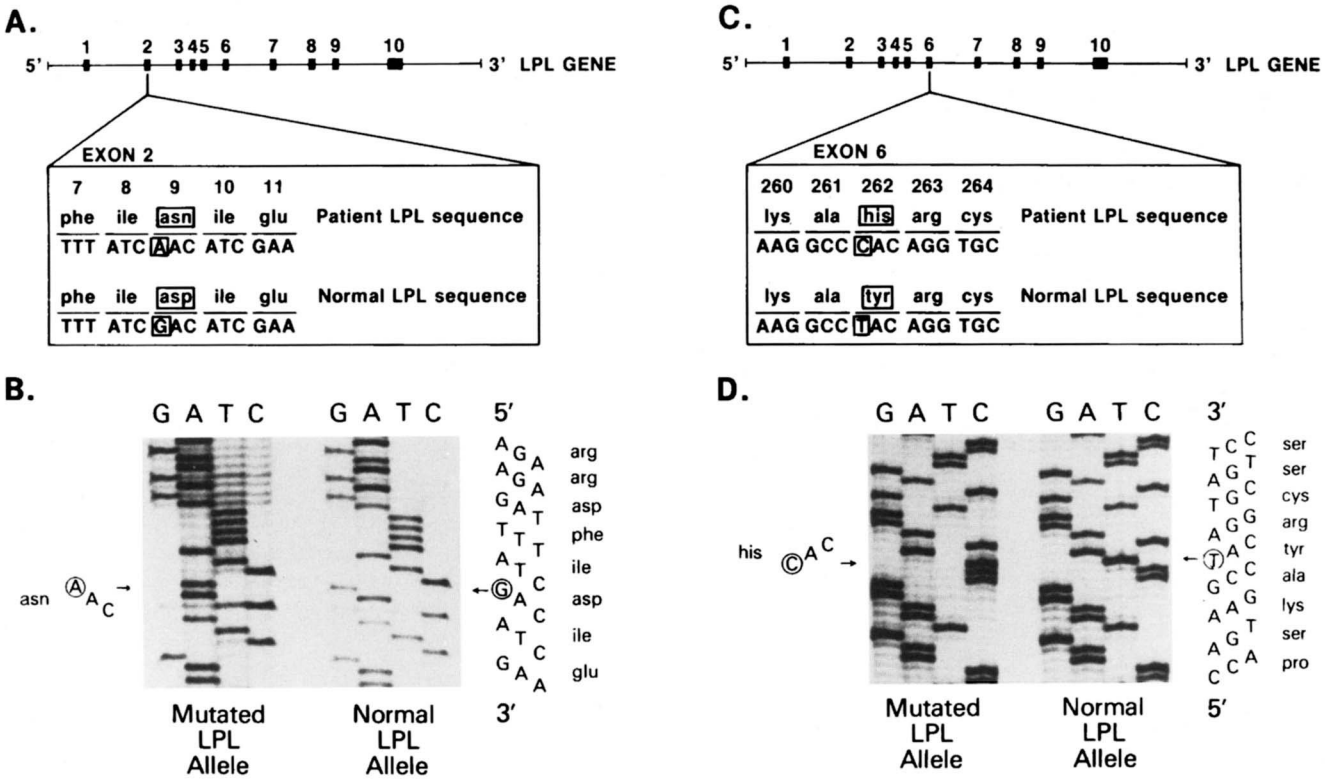

Fig. 1. DNA sequence of the LPL cDNA in a normal subject and in the patient with familial LPL deficiency. The autoradiogram of a sequencing gel illustrates the G to A substitution identified in exon 2 of the LPL-deficient patient, as indicated by the arrows (panel B). This mutation replaces the negatively charged aspartic acid, residue 9 of the mature LPL, with a neutral asparagine (panels A and B). The DNA sequence of normal and mutant LPL cDNA in the region of the second base substitution is illustrated in panels C and D. As shown in this autoradiogram, the normal LPL cDNA contains the sequence TAC, coding for tyrosine at position 262 of mature LPL. In the patient with LPL deficiency, this triplet codon is changed to CAC, representing the amino acid histidine, and thus adding one positive charge unit to the protein.

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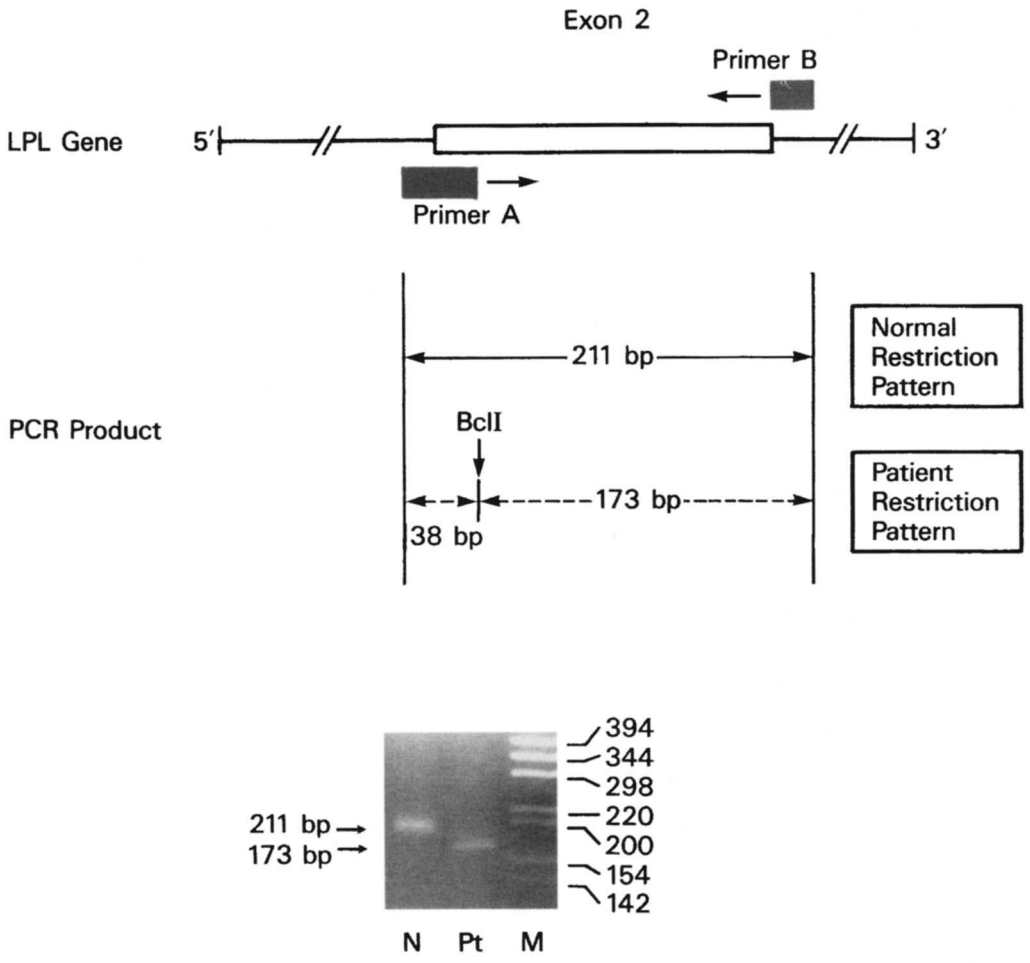

Fig. 2. Restriction enzyme digestion of PCR-amplified genomic DNA isolated from the patient and a control subject. Primers A and B used to amplify the region of the mutation within exon 2 of the human LPL gene are shown in the upper panel. The resulting product of 211 bp was then digested with restriction enzyme Bcl I, which cuts the patient's DNA into two fragments of 173 and 38 bp, respectively. The lower panel illustrates the electrophoretic analysis of PCR-amplified DNA from a normal subject (N) and the patient (Pt) after restriction digestion with Bcl I. Size standards are illustrated (M) and the sizes of restriction fragments are indicated on the right. The 38 bp fragment comigrates with the PCR primers.

residue 9, is replaced by glycine (46). Thus, tyrosine 262 but not aspartic acid 9 appears to be a highly conserved residue in different species.

Computer analysis (PC/GENE, IntelliGenetics, Mountain View, CA) of the patient's LPL cDNA revealed that the G to A substitution results in the loss of a cleavage site for the restriction enzyme Taq I (5′-T/CGA-3′). The second base mutation of a T to a C creates a new restriction site for the enzyme Asu I (5′-G/GCCC-3′), while recognition sites for the enzymes Hae I (5′-AGG/CCT-3′) and Stu I (5′-AGG/CCT-3′) are eliminated. The Taq I site, however, is ill-suited for haplotype analysis due to a second cleavage site of the enzyme six nucleotides further downstream in the LPL cDNA sequence. We therefore PCR-amplified from genomic DNA of the patient and of a control subject 211 bp spanning the mutation in exon 2 using a 42 bp sense primer that had been engineered to create a Bcl I restriction site with this point mutation. The PCR products were digested with the polymorphic enzyme Bcl I and analytical gel electrophoresis demonstrated that the uncleaved product was present only in the normal control, while the patient's DNA was cut into two fragments, 173 and 38 bp in length (**Fig. 2**). This established that the patient is a homozygote for the T to C mutation.

To confirm that the patient is also a homozygote for the second point mutation, we PCR-amplified 238 bp in exon 6 from genomic DNA of the patient and of a control subject. After gel purification of the PCR products, the DNA was digested with the restriction enzyme Asu I. Analysis of the DNA fragments on agarose gel electrophoresis demonstrated that the uncleaved product was present only in the normal control, while the patient's DNA was cut into two fragments, 81 and 157

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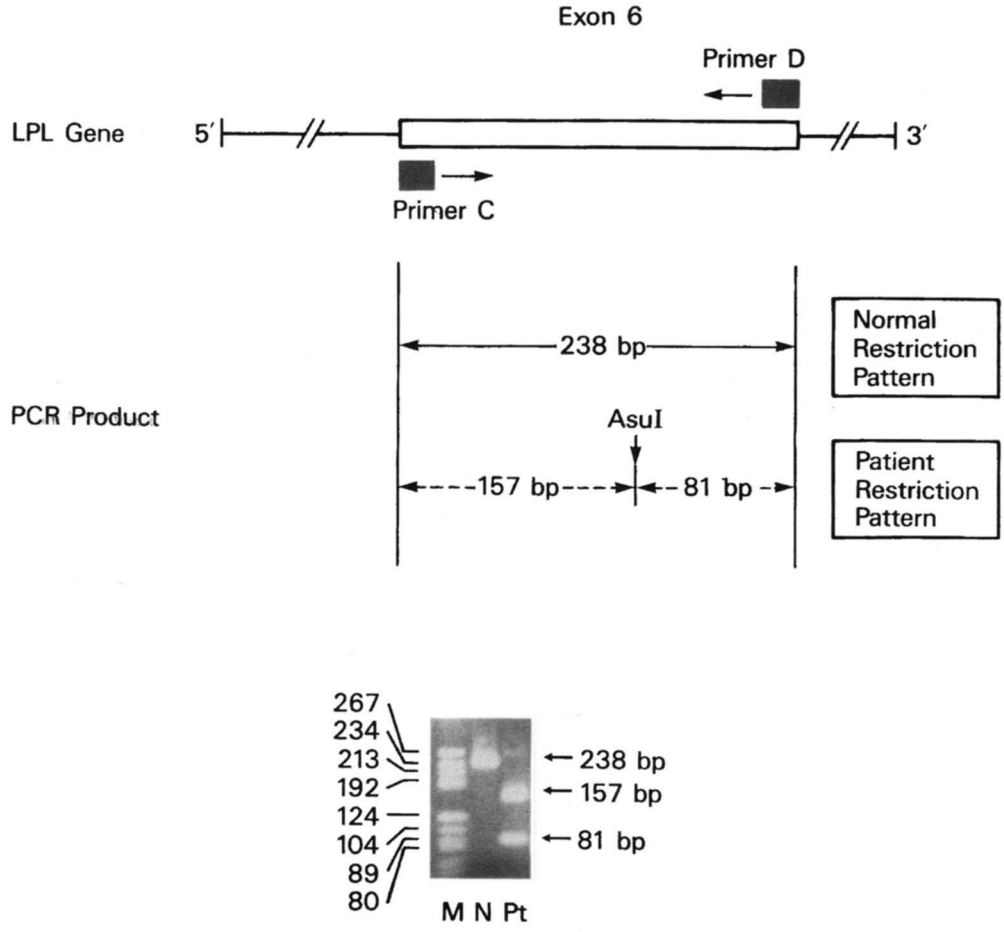

Fig. 3. Restriction enzyme digestion of PCR-amplified genomic DNA from the patient and a control subject. Primers C and D were used to amplify the region of the mutation within exon 6 of the human LPL gene as shown in the upper panel. The resulting product of 238 bp was then digested with restriction enzyme Asu I, which cuts the patient's DNA into two fragments of 157 and 81 bp, respectively. The lower panel illustrates the electrophoretic analysis of PCR-amplified DNA from a normal subject (N) and the patient (Pt) after restriction digestion with Asu I. Size standards are illustrated (M) and the sizes of restriction fragments are indicated on the right.

bp in length (**Fig. 3**), thus establishing that the patient was a homozygote for two separate gene defects.

To evaluate LPL mRNA size and levels, total RNA was isolated from cultured monocyte-derived macrophages obtained from the patient and a control subject. Due to alternative sites of 3′ polyadenylation, LPL mRNA of two distinct sizes is usually detected. Results shown in **Fig. 4** indicate that the size and quantity of LPL mRNA synthesized in the patient's macrophages were similar to that isolated from normal control macrophages.

In order to establish the functional significance of the two mutations, four different expression vectors were constructed containing either normal LPL cDNA (pCMV-NL), the mutation in exon 2 (pCMV-9), the mutation in exon 6 (pCMV-262), or both base changes (pCMV-9+262) as shown in **Fig. 5.** The constructs were then transfected into human embryonal kidney 293 cells. Results of these studies, summarized in **Table 2,** indicate that the conversion of tyrosine to histidine at position 262 leads to the synthesis of a completely inactive enzyme which is unable to hydrolyze triolein or tributyrin substrates, whereas the substitution of aspartic acid to asparagine at position 9 results in the synthesis of an enzyme with approximately 80% the specific activity of normal LPL. Like LPL-262, the combined LPL mutant (LPL-9+262) was functionally inactive. The same results were obtained when analyzing the triolein-hydrolyzing activity of intracellular protein. Compared to cells transfected with wild type LPL (90.2 ± 9.6 nmol/ml per min), cells transfected with LPL-9 had only a slight reduction of triolein activity to 81.6% that of normal (73.6 ± 6.4 nmol/ml per min), while cells transfected with LPL-262 or LPL-9+262 showed no triolein activity (< 0.2% of wild type). Interestingly, the concentration of

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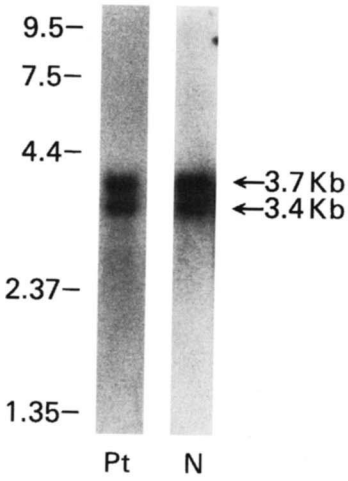

Fig. 4. Northern blot analysis of total macrophage RNA from a normal subject (N) and the lipoprotein lipase-deficient patient (Pt). Arrows identify the positions of the 3.4 and 3.7 kb LPL mRNAs present in both subjects. The sizes of the RNA markers in kb are indicated on the left.

mutant enzymes (LPL-9, LPL-262 and LPL-9+262) detected in the expression media was similar to that of native LPL, suggesting normal secretion of the mutant lipases by human embryonal kidney 293 cells.

In order to investigate the effect of the two mutations on the heparin binding properties of the mutant enzymes, heparin-Sepharose elution chromatography was performed on in vitro synthesized normal LPL, LPL($Asp^9 \rightarrow Asn$), LPL($Tyr^{262} \rightarrow His$), and LPL($Asp^9 \rightarrow Asn$, $Tyr^{262} \rightarrow His$). LPL mass and activity were measured in the elution fractions of the different lipases after chromatography on heparin-Sepharose (**Fig. 6**). As previously described (26, 40), native LPL present in the media of cells transfected with normal vector (pCMV-LPL) eluted in two separate immunoreactive peaks that represent inactive LPL monomer (0.8 M) and active LPL homodimer (1.2 M). The elution pattern of LPL-9 (Fig. 6, second panel) was similar to that of normal LPL. However, for mutant LPL-262 and LPL-9+262, all of the immunoreactive protein eluted with the low affinity, 0.8 M NaCl peak (Fig. 6, two bottom panels) which exhibited no measurable lipolytic activity.

DISCUSSION

In the present manuscript we describe the underlying molecular defect in a patient presenting with the chylomicronemia syndrome and undetectable LPL mass and activity in post-heparin plasma, previously described as LPL deficiency class I (41). Sequence analysis of the LPL cDNA and gene as well as digestion with restriction enzymes Bcl I and Asu I identified the 62-year-old black male as being homozygous for two point mutations which resulted in the replacement of the negatively charged aspartic acid (GAC), residue 9 of the mature LPL, with a neutral asparagine (AAC), and substitution of the neutral amino acid 262, tyrosine (TAC), with a positively charged histidine (CAC).

The high degree of conservation of tyrosine 262 indicates that this residue may be essential for lipase function. Interestingly, tyrosine 262 is in the vicinity of a 24-residue segment spanning amino acids 264–287, which contains four cysteines and one glycine completely conserved among lipoprotein, hepatic, and pancreatic lipases, as well as the steroid and thyroid hormone receptor superfamily (45). In addition, Tyr^{262} is directly adjacent to Arg^{263}, which, as a result of X-ray crystallography studies, has been suggested to be involved in heparin binding (47). Furthermore, Tyr^{262} is located close to the heparin/polyanion binding region spanning amino acids 279 to 282 in human LPL which has been shown by site-directed mutagenesis to play an important role in mediating lipase–heparin interaction (48–50). Aspartic acid 9, on the other hand, does not appear to be absolutely conserved in all LPLs from different species, indicating that it may not be essential for lipase activity.

Analysis of culture media from 293 cells transfected with plasmids containing the mutant LPL-9 demonstrated expression of an enzyme with approximately 80% the specific activity of wild type LPL using either tributyrin or triolein substrates. This slight reduction in specific activity confirms the in vitro data of a recent report which might support a suggested link between the $Asp^9 \rightarrow Asn$ mutation and the development of hypertriglyceridemia (51). Transient transfection of plasmids containing mutant LPL-262 or LPL-9+262 resulted in the synthesis of mutant enzymes that were catalytically inactive against tributyrin as well as triolein substrates. These studies demonstrate that the LPL-262 defect is mainly responsible for the phenotypic expression of the chylomicronemia syndrome in this patient.

A striking finding in this report is the complete absence of LPL mass in the post-heparin plasma of the patient, although the mutant lipases were normally secreted in vitro. To ensure the validity of this result, we repeated the mass measurements in plasma and media that had both been originally done with the 5D2 sandwich ELISA (30) with a different LPL ELISA. This ELISA has been shown to detect LPL monomer as well as dimer (26). Like the sandwich ELISA, this assay was able to detect LPL mass in pooled control post-heparin plasma (330 mg/dl) as well as transfection media, but failed to detect any LPL mass in the patient's post-heparin plasma, thus confirming the absence of immunodetectable LPL mass in the patient. A potential mechanism

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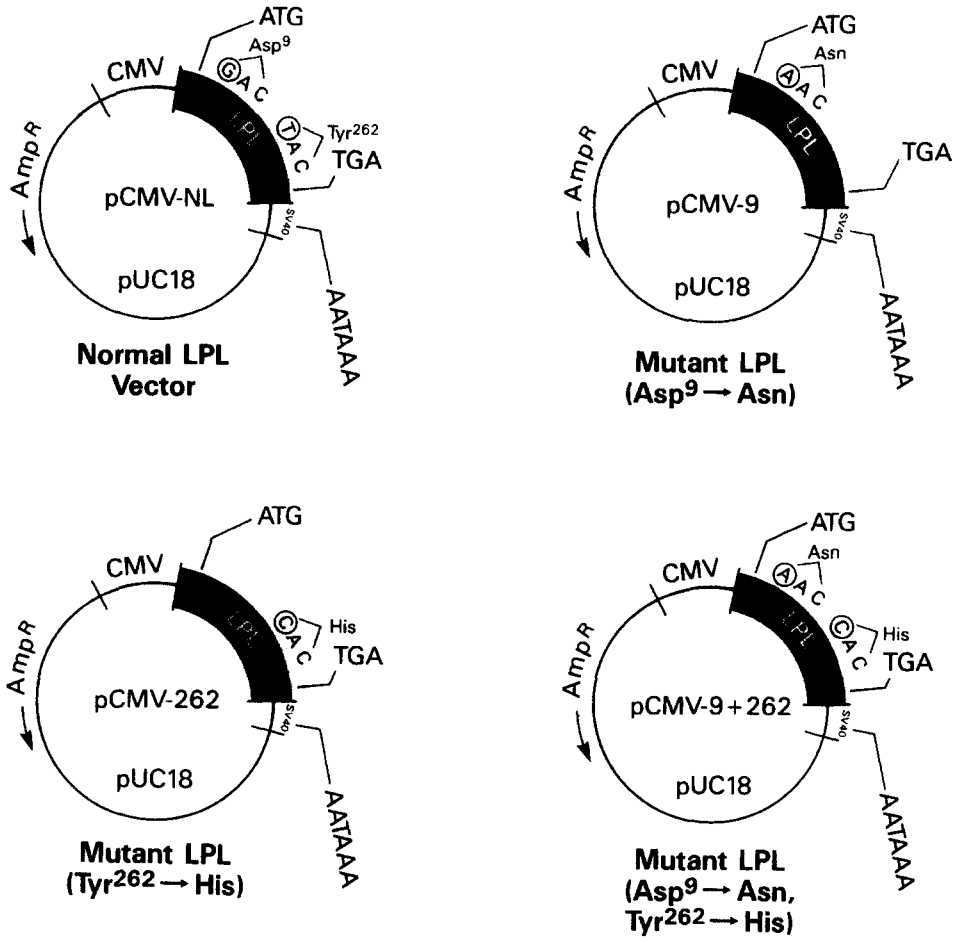

Fig. 5. pCMV expression vectors used for the transfection experiments of normal and mutant LPL. Each of the plasmids, pCMV-NL, pCMV-9, pCMV-262, and pCMV-9+262, contains a 1470-bp cDNA fragment of LPL inserted between the Xba I and Hpa I cloning sites of the parent plasmid.

explaining the absence of LPL mass in the patient's post-heparin plasma could involve alterations in the heparin binding properties of LPL induced by the amino acid substitution in the mutant enzyme. This substitution might ultimately affect lipase interaction with cell wall glycosaminoglycans and lead to enhanced catabolism of unbound enzyme, as the liver avidly takes up monomeric LPL via heparin-insensitive sites (52, 53).

Heparin-Sepharose affinity chromatography of LPL-9 demonstrated that this mutant lipase, similar to normal LPL, eluted in two separate peaks corresponding to the inactive monomer and active dimer, respectively (26,

TABLE 2. In vitro expression of normal and mutant LPL

Construct	Media Mass[a]	Intracellular Mass[a]	Secretion	Tributyrin Activity in Media	Specific Tributyrin Activity in Media	Triolein Activity in Media	Specific Triolein Activity in Media
	ng/ml		%	*nmol/ml/min*	*nmol/μg/min*	*nmol/ml/min*	*nmol/ng/min*
LPL-NL	760 ± 10	410 ± 79	96	0.70 ± 0.08	0.93 ± 0.10	70.9 ± 14.1	0.094 ± 0.019
LPL-9	803 ± 116	537 ± 126	94	0.60 ± 0.09	0.75 ± 0.03	59.9 ± 8.2	0.075 ± 0.001
LPL-262	1010 ± 234	650 ± 156	95	0	0	0.06 ± 0.09	0
LPL-9+262	907 ± 107	542 ± 3	95	0	0	0	0

The standard deviations are calculated from the results of triplicate transfection experiments.
[a]Intracellular and media volumes per plate were 0.5 and 6.0 ml, respectively.

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40), indicating that the Asp^{9} → Asn substitution did not affect the heparin binding properties of the mutant enzyme. In contrast to LPL-9, the substitution of Tyr^{262} → His had a dramatic effect on the ability of the mutant enzymes to bind to heparin. Most of the immunoreactive protein in LPL-262 and LPL-9+262 eluted with the low affinity, inactive 0.8 M NaCl peak in a position similar to the elution fractions for the normal LPL monomer and no immunoreactive protein could be detected in the 1.2 M NaCl fractions. Based on these data, the substitution of residue 262 but not residue 9 appears to be primarily responsible for the alteration in the heparin binding properties of the combined LPL mutant. Although X-ray crystallography and site-directed mutagenesis studies indicate that residues near tyrosine 262 may be essential for normal lipase–heparin interaction (47–50), it appears unlikely that the mutation identified in our LPL-deficient patient would directly lead to reduced heparin binding as the replacement of the neutral amino acid, tyrosine 262, by the more basic residue, histidine, should enhance rather than reduce the heparin binding properties of the mutant enzyme when compared to normal LPL. Thus, it appears that other mechanisms may be responsible for the altered heparin binding properties of LPL-262 and LPL-9+262.

Other studies have demonstrated that the loss of activity observed in a subset of mutants with missense mutations in exons 3, 5, and 8 (18, 26, 27) of LPL may result from disruption of the active LPL dimer. These mutant lipases, like LPL-262, demonstrate an increase in the inactive, immunoreactive protein fraction which elutes in the location of the inactive LPL monomer. Thus, mutations resulting in a shift of the normal LPL monomer–dimer equilibrium may lead to the accumulation of monomers that exhibit reduced heparin binding affinity both in vitro and in vivo and, in addition, may be more susceptible to degradation than the LPL dimer (54). Interestingly, despite virtually undetectable plasma levels in patient post-heparin plasma (18, 26–28), these LPL mutants, like LPL-262, appear to be normally expressed in vitro. We thus propose that a subset of mutations in the LPL gene, including Tyr^{262} → His, lead to enhanced in vivo formation of a monomer which, compared to the active dimeric LPL, has a reduced ability to interact with endothelial cell wall glycosaminoglycans. This decreased binding, together with increased degradation, would result in enhanced catabolism of the mutant enzymes by heparin-insensitive sites in the liver and consequently to low LPL mass in post-heparin plasma.

In summary, we have identified two separate mutations in the LPL gene of a patient presenting with the familial chylomicronemia syndrome. Substitution of Asp by Asn at residue 9 results in the expression of an

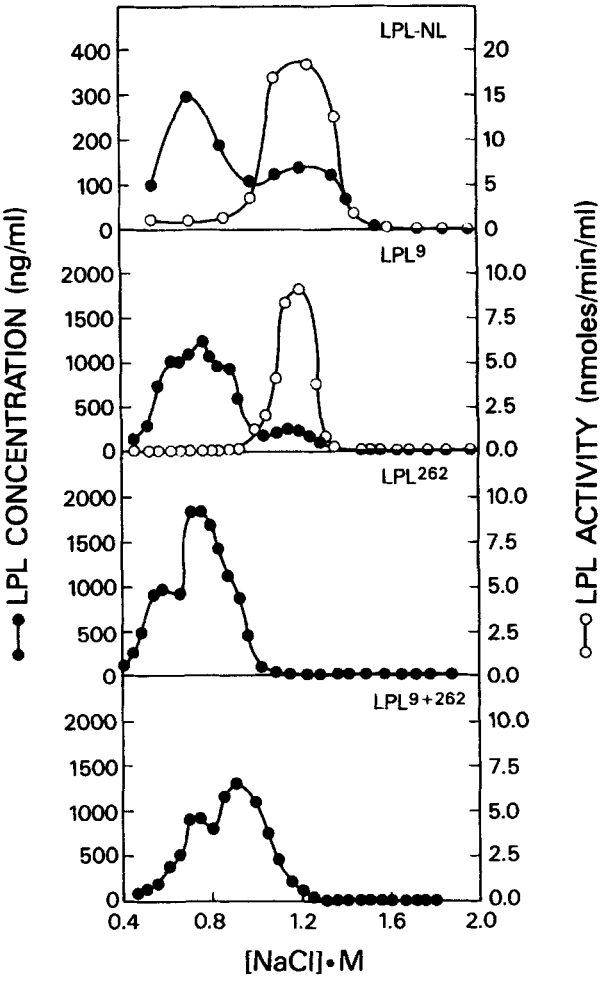

Fig. 6. Heparin-Sepharose elution profiles of in vitro synthesized normal LPL (LPL-NL), Asp^{9} → Asn (LPL^{9}), Tyr^{262} → His (LPL^{262}), and Asp^{9} → Asn, Tyr^{262} → His (LPL^{9+262}). Media from transfected human embryonal kidney 293 cells was loaded on a heparin-Sepharose affinity column. The increasing NaCl concentration of the elution buffer is indicated on the x-axis. The column fractions were analyzed for LPL mass (open circles) by ELISA and for LPL activity (solid circles) by using tri-[1-^{14}C]oleate as substrate (29).

enzyme with only slightly reduced specific activity, indicating that despite the charge substitution, this region is not crucial for LPL function. The Tyr^{262} → His substitution, in contrast, results in a shift of the LPL monomer–dimer equilibrium in favor of the formation of the LPL monomer and the synthesis of a catalytically inactive mutant enzyme, establishing the functional significance of this gene defect. We propose that LPL gene mutations that result in the synthesis of an enzyme with altered monomer–dimer equilibrium may exhibit abnormal in vivo binding to endothelial glycosaminoglycans. Thus, the underlying mechanism resulting in ab-

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sence of LPL in these patients may relate to defective interaction of the mutant lipase with the vascular endothelium resulting in enhanced catabolism of the mutant LPL monomers.

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