Structural and functional consequences of missense mutations in exon 5 of the lipoprotein lipase gene

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Abstract Missense mutations in exon 5 of the LPL gene are the most common reported cause of LPL deficiency. Exon 5 is also the region with the strongest homology to pancreatic and hepatic lipase, and is conserved in LPL from different species. Mutant LPL proteins from post-heparin plasma from patients homozygous for missense mutations at amino acid positions 176, 188, 194, 205, and 207, and from COS cells transiently transfected with the corresponding cDNAs were quantified and characterized, in an attempt to determine which aspect of enzyme function was affected by each specific mutation. All but one of the mutant proteins were present, mainly as partially denatured LPL monomer, rendering further detailed assessment of their catalytic activity, affinity to heparin, and binding to lipoprotein particles difficult. However, the fresh unstable Gly^{188}→Glu LPL and the stable Ile^{194}→Thr LPL, although in native conformation, did not express lipase activity. It is proposed that many of the exon 5 mutant proteins are unable to achieve or maintain native dimer conformation, and that the Ile^{194}→Thr substitution interferes with access of lipid substrate to the catalytic pocket. These results stress the importance of conformational evaluation of mutant LPL. Absence of catalytic activity does not necessarily imply that the substituted amino acid plays a specific direct role in catalysis.—Peterson, J., A. F. Ayyobi, Y. Ma, H. Henderson, M. Reina, S. S. Deeb, S. Santamarina-Fojo, M. R. Hayden, and J. D. Brunzell. **Structural and functional consequences of missense mutations in exon 5 of the lipoprotein lipase gene. *J. Lipid Res.* 2002.** 43: **398–406.**

Supplementary key words LPL deficiency • triglyceride • heparin • LPL gene • LPL mass

LPL hydrolyses TG from TG-rich lipoproteins, thereby making free fatty acids available for tissue uptake and subsequent utilization or storage. Through its action, the TG-rich lipoprotein is converted to LDL, and the redundant surface material is incorporated into HDL. LPL exerts its main function at the capillary endothelium, where it is bound to sulfated proteoglycans. In this position, it can interact with substrate lipoproteins and its activator apolipoprotein C-II (apoC-II). Thus, to function properly, LPL must associate with its substrate, with heparan-sulfate, and with apoC-II (1). Although the 3-D structure of the enzyme is yet to be determined, a predicted molecular model of LPL based on the 3-D structure of pancreatic lipase has been used to identify various functional and structural domains of LPL (2). This proposed structure includes the amino acid residues in the active-site region, the lid domain, heparin binding domain, and the lipid-binding site (2). LPL and hepatic lipase chimeric molecules have been used to assign specific functions to globular N-terminal and the smaller C-terminal domains of the enzyme (3–6). These studies suggest that the N-terminus of the enzyme that includes the catalytic triad regulates the catalytic function of the enzyme, whereas the C-terminal region plays a role in substrate specificity and heparin binding that is localized in the last 60 amino acid residues (6).

LPL deficiency is a rare disorder, which results in severe chylomicronemia, pancreatitis, eruptive xanthomas, and neurological symptoms (1). A number of mutations in the LPL gene have been identified that lead to LPL deficiency (1). The present study characterizes selected mutant LPL proteins to determine what aspect of enzyme overall function has been affected by various mutations, in an attempt to map a certain function to a discrete portion of the molecule. The focus of the present study is on missense mutations in exon 5 of the LPL gene, because this exon is one of the most common locations for mutations leading to LPL deficiency, and because this region is highly conserved in different species and shows strong homology to both hepatic lipase and pancreatic lipase. Previously, it was suggested that the mutations in the exon 5 region may

Abbreviations: DPPC, dipalmitoyl-phosphatidylcholine; GuHCl, guanidine hydrochloride; MAb, monoclonal antibody; PNPB, *p*-nitrophenylbutyrate.

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affect protein folding and stability rather than its catalytic activity directly (7). In the present study we have used a novel approach based on differential epitope recognition by two LPL specific monoclonal antibodies to directly assess the role of exon 5 missense mutations on the conformational changes causing loss of LPL catalytic activity.

MATERIALS AND METHODS

Subjects

The patients were four males and one female, aged 23 to 43 years. All were hypertriglyceridemic and had absent LPL activity in port-heparin plasma (**Table 1**). They had no other diseases and were not on any medications that affect lipoprotein metabolism. Two normal healthy controls were males aged 36 and 32 years. The Human Subjects Review Committee of the University of Washington approved the study protocol. Informed consent was obtained from all participants.

Post-heparin plasma

Ten minutes after an intravenous bolus of heparin (60 IU/kg body weight), blood was collected into lithium-heparin containing tubes on ice. Plasma was separated at 2,500 × *g* for 10 min, snap frozen on dry ice and ethanol, and stored at −70°C.

Monoclonal antibodies

Two previously described monoclonal antibodies (Mabs) were used in this study, 5D2 and 5F9. They were raised against LPL purified from bovine milk (8). Both antibodies react with human LPL. 5D2 inhibits LPL activity toward emulsified substrates (9). 5F9 recognizes only LPL that has undergone a conformational change that occurs concomitant with loss of activity, e.g., during denaturation (10).

Immunoprecipitation and Western blot of LPL

LPL was immunoprecipitated from plasma using 5D2 coupled to a gel-matrix. The precipitate was electrophoresed, blotted onto nitrocellulose membranes, and detected with 5D2 as described (10).

LPL mass

LPL mass was measured in two different ELISAs described in detail elsewhere (9, 10). In the ELISA designated dimer ELISA, immunoplates (Maxisorp, Nunc Inc., Kamstrup, Denmark) were coated with 5D2, samples applied, and the plate incubated overnight at 4°C. Following washes, bound LPL was detected with the same MAb (5D2) conjugated to horseradish peroxidase. Plates were developed by addition of *o*-phenylenediaminedihydrocholoride substrate and read in a microtiter plate reader at dual wavelengths (490–405 nm). By using the same MAb for both binding and detection, the epitope recognized by 5D2 must be present at least in duplicate to be recognized (9). The other ELISA, designated monomer ELISA, utilizes 5F9 to coat the plate and 5D2 to detect the bound antigen. The epitope for 5F9 is not expressed in native LPL, but becomes available for 5F9 binding when LPL loses its activity, an event coupled with dissociation of dimer and a conformational change (10, 11). Purified bovine milk LPL (8) was diluted to obtain a liner standard curve from 0 to 40 ng/ml. The standards in the monomer ELISA were inactivated by guanidine hydrochloride (GuHCl) treatment as described under "Evaluation of conformational state of LPL."

TABLE 1. Study subjects

Amino Acid Substituted	Sex	Age	Ancestry	DNA Results Reported in Ref.
Ala^{176}→Thr	M	39	Afro/American	(14)
Gly^{188}→Glu	M	43	English	(21, 24)
Ile^{194}→Thr	M	23	Dutch/South African	(15)
Ile^{205}→Ser	F	18	Spanish	(25)
Pro^{207}→Leu	M	25	French-Canadian	(16)

LPL activity

LPL activity was routinely measured as hydrolysis of a phosphatidylcholine stabilized triolein emulsion (12). In the presence of other lipase activities, LPL activity was determined as the difference in activity of a sample preincubated in the absence and presence of 5D2. In some experiments LPL activity against the chromogenic, water soluble substrate *p*-nitrophenylbutyrate (PNPB) was measured as described (13), except that Tris-HC1, pH 7.7, was used, and that appearance of product was continuously monitored in a Beckman Ultraspec using the kinetic mode. Preparation of dipalmitoyl-phosphatidylcholine (DPPC) vesicles for enhancement of activity was as described (13).

Evaluation of conformational state of LPL

To determine if wild type and mutant LPL were in the native or partially denatured conformation, as specified by the recognition by 5F9 MAb, a sample was measured in the monomer ELISA with or without prior treatment with GuHCl (6 M GuHCl solution in 10 mM Tris-HC1, pH 10, for concentration of 1 M GuHCl). The sample was incubated on ice for 60 min and diluted at least 10-fold in PBS containing glycerol (15%, v/v), Tween-20 (0.1%, v/v), BSA (50 μg/ml), and heparin (1 mg/ml) (ELISA dilution buffer) before application to the immunoplate. The values obtained after GuHCl treatment were taken as a measure of total LPL. Untreated samples were diluted to the same degree in ELISA dilution buffer only. The values thus obtained represent conformationally altered LPL originally present in the sample. The difference between GuHCl treated and untreated samples is taken as a measurement of LPL initially present in the sample in its native conformation. This measurement has been shown to correlate strongly to LPL activity and to the incremental mass appearing in plasma after heparin injection that is detected by the 5D2 (dimer) ELISA (10).

Heparin-Sepharose column chromatography

Affinity chromatography of LPL on heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology products, Uppsala, Sweden) was carried out as previously described (10). All chromatography was performed at 4°C on 3 or 10 ml columns of heparin-Sepharose equilibrated with 10 mM phosphate buffer, pH 7.5, containing glycerol (30%, v/v), CHAPS (0.1%, w/v), and NaCl (0.4 M). Control experiments did not show any differences between elution concentrations regardless of column size. Prior to application, the sample was made 0.1%, w/v with respect to deoxycholate by addition of 10% deoxycholate. Chylomicronemic plasma samples were centrifuged at 10,000 × *g* for 20 min before the clear infranatant plasma was applied. More than 80% of LPL protein originally in the sample was recovered in the infranatant (data not shown). Solid NaCl was added to give a final concentration of 0.4 M. The sample was applied at a flow rate of 0.2 ml/min, the column was washed with equilibration buffer at a flow rate of 0.4 ml/min, and eluted with a linear NaCl gradient from 0.4 to 1.8 M LPL mass. Lipase activity and conductivity were determined in each fraction. Recovery of lipase activity for control LPL was between 65–85%.

FPLC gel filtration

Preheparin plasma from a normolipidemic donor was mixed with an equal volume of post-heparin plasma LPL purified on

heparin-Sepharose (as described above), incubated on ice for 20 min, and applied to a Superose 6 column (Pharmacia LKB Biotechnology products, Uppsala, Sweden) equilibrated with PBS containing 5 mM EDTA. The sample volume was 500 μl, the flow rate was 0.5 ml/min, and the eluate was collected in 1 ml fractions. The tubes contained glycerol, Tween-20, and heparin to stabilize to eluted enzyme. Absorbance at 280 nm was continuously monitored and the fractions were assayed for LPL activity, 5F9 LPL mass, and total cholesterol (Single Vial Cholesterol, Boehringer Mannheim, Indianapolis, IN). TG was assayed (Kit 33620, Sigma Chemical Co., St. Louis, MO) in separate chromatographic runs without glycerol, because glycerol interfered with the TG assay. LPL concentration in the eluates was not high enough to permit GuHCl denaturation (as this requires a 10-fold dilution prior to application to the microtiter plates); instead, the samples were denatured by heat inactivation at 50°C for 40 min.

In vitro site-directed mutagenesis and expression in COS cells

Site-directed mutagenesis and transient expression in COS cells were performed as previously described (14–16). Briefly, we used a 1.6 kilobase *Dra*I/*Eco*RI cDNA fragment containing expression vector CDM8 which served as a dual function vector for both mutagenesis and expression. The CDM8-LPL double-stranded DNA was transfected into a dut-ung-host, and uracil-containing single-stranded DNA was prepared by superinfecting the host with helper phage. For the Ala^{176}→Thr mutant, a 1536-BP fragment of LPL cDNA that spanned the signal peptide through the termination codon was cloned into the *Hpa*I and *Xba*I sites of the parent plasmid (pCMV) (14) and placed under the control of the CMV promoter. The mutant primer sequences for Ala^{176}→Thr and Pro^{207}→Leu have been previously described (14–16). The sequence of the mutant oligonucleotide for Gly^{188}→ Glu mutation is 5′-CACCAGAGAGTCCCCTG-3′. Mutagenesis was performed by using both the gapped-duplex DNA method (16) and PCR overlap extension method (17). Mutant clones were identified by oligonucleotide hybridization and confirmed by DNA sequencing. Expression phagemid DNA containing mutant cDNA were introduced into COS-1 or COS-7 cells by electroporation or the calcium phosphate co-precipitation method for the human embryonic kidney 293 cells (18). The cells were incubated in DMEM medium supplemented with heparin at 40 μg/ml for 48 h. The medium was removed, replaced with fresh medium containing heparin, and the cells grown for another 12–24 h before the medium was collected.

RESULTS

LPL was immunoprecipitated from plasma from a control and from a patient homozygous for a missense mutation (Gly^{188}→Glu) (**Fig. 1**). The major band detected in both patient and control plasma had the same apparent molecular weight as the LPL activity peak partially purified on heparin-Sepharose, and a slightly higher apparent molecular weight than that of LPL purified from bovine milk. A faint band at this position was also present in pre-heparin plasma. Two bands, presumably immunoglobulin heavy and light chains as seen with the antibody control, were present in all samples. This was expected since the detection method employed anti-mouse antibodies, and the samples can be expected to contain MAb from the preceding immunoprecipitation.

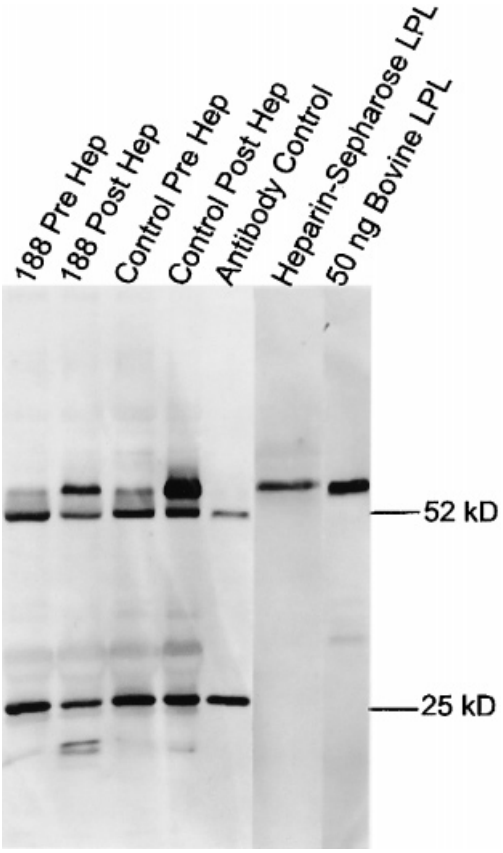

Fig. 1. Western blot of wild-type and mutant LPL immunoprecipitated from postheparin plasma. An amount corresponding to 1 ml of plasma was applied to the gel. LPL was immunoprecipitated from 1 ml of plasma. Lanes 1 and 2: Pre- and post-heparin plasma from a patient homozygote for a missense mutation leading to a Gly^{188}→Gly substitution. Lanes 3 and 4: Pre- and post-heparin plasma from a normal control. Lane 5: Antibody-gel without addition of plasma. Lane 6: Human LPL, partially purified on heparin-Sepharose (~20 ng of LPL). Lane 7: Bovine LPL, purified from milk (~50 ng).

LPL activity was determined in post-heparin plasma from controls and five patients homozygous for missense mutations at 176, 188, 194, 205, and 207 in exon 5 of the LPL gene (**Table 2**). None of the mutant LPL enzymes hydrolyzed the triolein emulsion to any significant extent; however, activities lower than 2 nmol fatty acid (FA) released/min × ml are not accurately measured in the standard plasma activity assay. LPL mass and conformation were studied in the same samples using the 5D2 dimer assay and the conformation dependent 5F9 ELISA (Table 2). LPL from normal controls was predominantly present in a native conformation, i.e., most of their LPL (77% and 79%) was not detected by the 5F9 ELISA, unless the sample had undergone GuHCl treatment. The patient homozygous for the Ile^{194}→Thr substitution also had a major proportion (64%) of his mass present in its native conformation. LPL from the other patients was largely (70% to 90%) present in a conformation readily detected by the 5F9 ELISA without prior GuHCl treatment, suggesting that these LPL forms either never had achieved a native confor-

TABLE 2. LPL mass and activity in post-heparin plasma from LPL deficient subjects

		LPL				
LPL Activity		5D2 (Dimer)	5F9+Gu (Total)	5F9 (Denatured Monomer)	(5F9+Gu)-5F9 (Native, Calculated Dimer)	Native Total
nmol FA/min × ml				*mass ng/ml*		
Control A	317	527	629	138	498	0.79
Control B	389	487	750	175	575	0.77
Ala^{176}→Thr	0	162	294	300	−6	—
Gly^{188}→Glu	0	21	252	228	24	0.10
Ile^{194}→Thr	0	327	405	145	260	0.64
Ile^{205}→Ser	0	4	53	37	16	0.30
Pro^{207}→Leu	0	117	170	132	38	0.22

LPL mass was detected in individual patients. 5D2 ELISA was used as a direct measure of LPL dimer. GuHCl was used to destabilize LPL dimers to convert all to monomeric form and measure total LPL (5F9+Gu). 5F9 assay was used to measure the naturally monomeric LPL. Native LPL (dimer) was calculated by subtraction of naturally monomeric LPL (5F9) from Total LPL (5F9+Gu). The ratio of native to total LPL was determined from the obtained values. The CV of measurement for 5D2 and 5F9 assays are 5.1% and 7.7%, respectively.

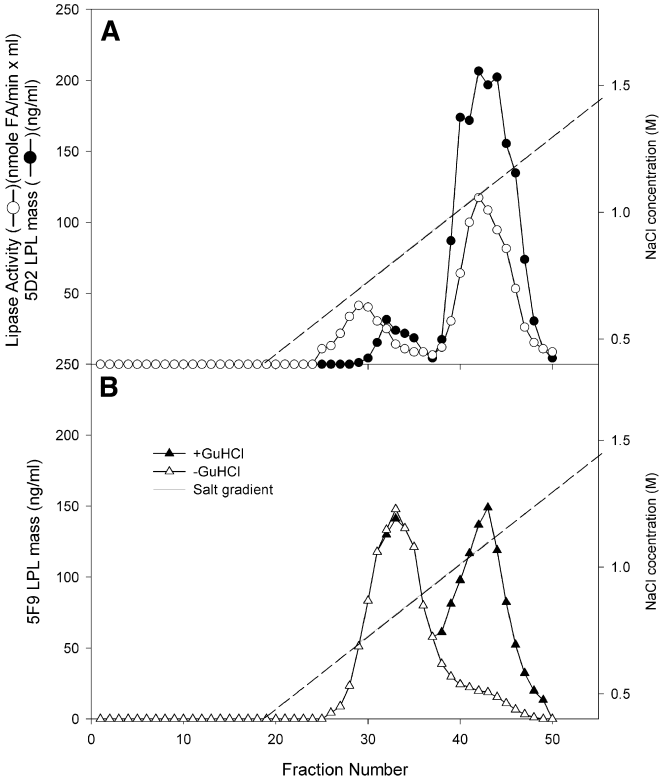

Fig. 2. Heparin-Sepharose chromatography of postheparin plasma from a control. Postheparin plasma (10 ml) was applied to a 10 ml heparin-Sepharose column and washed and eluted with a linear (0.4–1.8 M) NaCl gradient. A: Represents the association of the lipase activity and LPL mass. 5D2 LPL mass (solid circle) represents the LPL dimer that predominantly elutes at 1.1 M NaCl concentration (fraction 42). The lipase activity (open circle) detected at 0.7 M NaCl (fraction 29) corresponds to no LPL dimer and has been shown to correspond to hepatic lipase. Lipase activity at fraction 42 corresponds to LPL activity. B: Represents the 5F9 LPL monomer mass assay. The monomeric LPL mass was detected in the fractions eluted from the column. LPL mass without GuHCl (open triangle) represents the monomeric LPL eluting from the column. The peak monomeric LPL mass was eluted at 0.75 M NaCl (fraction 33). LPL mass with GuHCl (solid triangle) is a measure of total LPL (monomer+dimer). LPL eluted at two peaks, 0.75 M NaCl and 1.1 M NaCl, corresponding to monomeric and dimeric LPL, respectively.

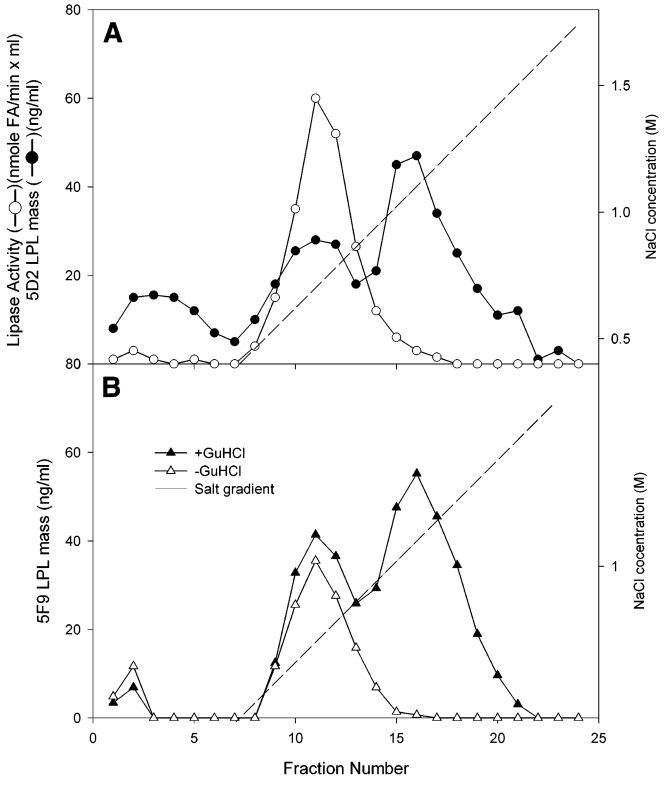

Fig. 3. Heparin-Sepharose chromatography of postheparin plasma (3 ml) from a patient homozygous for a mutation leading to a Ile^{194}→Thr amino acid substitution. Postheparin plasma (10 ml) was applied to a 10 ml heparin-Sepharose column and washed and eluted with a linear (0.4–1.8 M) NaCl gradient. A: Represents the association of the lipase activity and LPL mass. 5D2 LPL mass (solid circle) represents the LPL dimer that predominantly elutes at fraction 16. The lipase activity (open circle) detected at fraction 12 corresponds to some LPL dimmer, but the activity has been shown to correspond to hepatic lipase. B: Represents the 5F9 LPL monomer mass assay. The monomeric LPL mass was detected in the fractions eluted from the column. LPL mass without GuHCl (open triangle) represents the monomeric LPL eluting from the column. The peak monomeric LPL mass was eluted at fraction 12. LPL mass with GuHCl (solid triangle) is a measure of total LPL (monomer+dimer). LPL eluted at two peaks at fractions 12 and 16, corresponding to monomeric and dimeric LPL, respectively.

mation or that they were unable to maintain it (in vivo or in vitro). The 5D2 assay, by design, may detect aggregates of LPL monomer. The apparent high LPL dimer mass for Ala[176]→Thr and Pro[207]→Leu using 5D2 assay may reflect the presence of aggregates.

The heparin affinity of LPL from controls (**Fig. 2**) and the exon 5 mutants (**Figs. 3–5**) was evaluated by heparin-Sepharose chromatography. When post-heparin plasma from a normal donor was applied to the column, lipase activity eluted as two peaks at 0.70 and 1.1 M NaCl. The activity of the former peak, presumably consisting of hepatic lipase, was unaffected by preincubation with 5D2, whereas the second peak disappeared as a result of binding by this antibody (data not shown). Most of LPL mass detected in the 5D2 ELISA co-eluted with LPL activity; however, a small portion eluted at 0.75 M NaCl. LPL mass determined in the 5F9 ELISA eluted at 0.75 M NaCl. When the eluted fractions were treated with GuHCl prior to their assay in the 5F9 ELISA, a peak superimposable with 5D2 mass and LPL activity was detected in addition to the peak at 0.75 M seen prior to denaturation. Thus, the change leading to expression of the 5F9 epitope was coupled to a lower affinity for heparin. When post-heparin plasma from the Ile[194]→Thr mutation was chromatographed (Fig. 3), two peaks of LPL mass were also observed: denatured and native. However, in contrast to the control, the native LPL peak did not show any lipase activity. When post-heparin plasma from a patient homozygous for a missense mutation leading to a Pro[207]→Leu substitution was chromatographed (Fig. 4), all of the LPL mass eluted at 0.75 M NaCl concentration in the non-native conformation. The same was true for LPL from patients homozygous for missense mutations leading to Ala[176]→Thr and Gly[188]→Glu amino acid substitutions (data not shown).

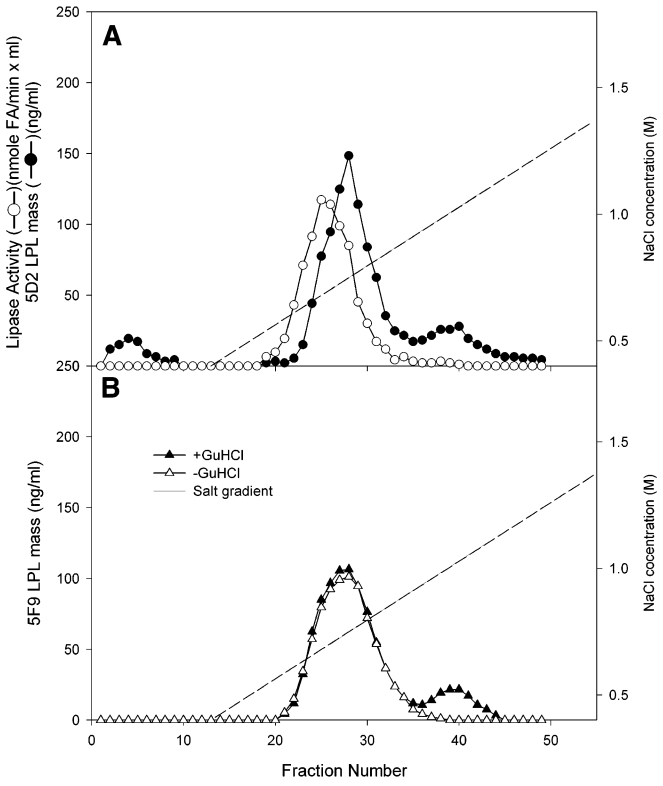

Fig. 4. Heparin-Sepharose chromatography of fresh postheparin plasma (10 ml) from a patient homozygous for a mutation leading to a Gly[188]→Glu amino acid substitution. Postheparin plasma (10 ml) was applied to a 10 ml heparin-Sepharose column and washed and eluted with a linear (0.4–1.8 M) NaCl gradient. A: Represents the association of the lipase activity and LPL mass. 5D2 LPL mass (solid circle) represents the LPL dimer that predominantly elutes at fraction 28. The lipase activity (open circle) detected at fraction 25 corresponds to no LPL dimer and has been shown to correspond to hepatic lipase. B: Represents the 5F9 LPL monomer mass assay. The monomeric LPL mass was detected in the fractions eluted from the column. LPL mass without GuHCl (open triangle) represents the monomeric LPL eluting from the column. The peak monomeric LPL mass was eluted at fraction 28. LPL mass with GuHCl (solid triangle) is a measure of total LPL (monomer+dimer). LPL eluted at two peaks, 0.75 M NaCl and very small amount at 1.1 M NaCl, corresponding to monomeric and dimeric LPL, respectively.

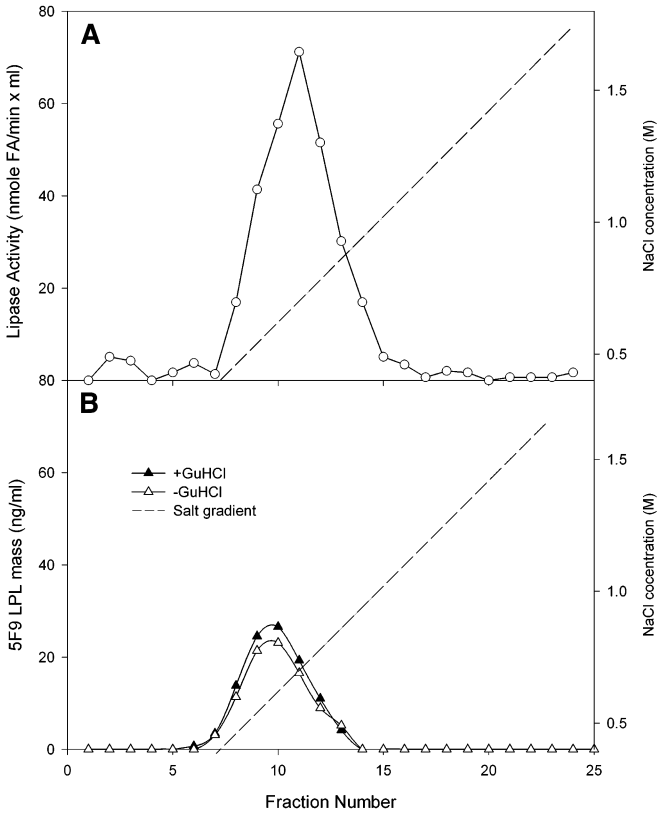

Fig. 5. Heparin-Sepharose chromatography of postheparin plasma (3 ml) from a patient homozygous for a mutation leading to a Pro[207]→Leu amino acid substitution. Postheparin plasma (10 ml) was applied to a 10 ml heparin-Sepharose column and washed and eluted with a linear (0.4–1.8 M) NaCl gradient. A: Represents the elution of lipase activity. 5D2 LPL mass representing LPL dimer was undetectable (data not shown). The peak lipase activity detected (open circle) at fraction 11 corresponds to no LPL dimer and has been shown to correspond to hepatic lipase. B: Represents the 5F9 LPL monomer mass assay. The monomeric LPL mass was detected in the fractions eluted from the column. LPL mass without GuHCl (open triangle) represents the monomeric LPL eluting from the column. The peak monomeric LPL mass was eluted at fraction 10. LPL mass with GuHCl (solid triangle) is a measure of total LPL (monomer+dimer). Total LPL also eluted at fraction 10 corresponding to monomeric LPL.

This is in good agreement with the whole plasma ELISA results (Table 2), suggesting that most of the LPL from the 176, 188, 205, and 207 mutants is present in a non-native conformation at the time of the assay. To test the hypothesis that these mutant proteins were inherently less stable than normal LPL, plasma from a patient with $Gly^{188}\rightarrow$Glu amino acid substitution was chromatographed within 60 min after the blood was drawn and assayed without prior freezing (Fig. 4). Although most of the LPL protein had decreased heparin affinity under these conditions, a small amount of LPL protein in its native conformation was eluted at the position of normal, active LPL. It was not possible to detect any lipase activity in these native LPL fractions, although the concentration of LPL in its native conformation (25 ng/ml) would have been readily detected in the triolein assay had the specific activity of the mutant protein been normal. To further evaluate the stability of the $Gly^{188}\rightarrow$Glu mutant protein, fractions eluting at 1.1 M NaCl were snap frozen, stored at −70°C for 2 days, and assayed again. Unlike the results with fresh samples, LPL was now detected in the 5F9 ELISA without GuHCl treatment, but was no longer detected in the 5D2 ELISA. At this time, treatment with GuHCl did not increase the immunoreactivity, indicating that the native LPL originally present in the fraction had undergone complete spontaneous denaturation. Under identical conditions, control LPL remained in its active native conformation for several months. Fresh post-heparin plasma from the other patients studied was not available.

When the activity, mass, and conformation of wild type LPL, $Gly^{188}\rightarrow$Glu, and $Ile^{194}\rightarrow$Thr mutants were studied in medium from transfected cells (**Table 3**), a pattern similar to that found in patient postheparin plasma was seen, i.e., the total LPL mass present overlapped between mutants and wild type LPL. However, wild type LPL was present in its native conformation to a larger extent (26%, 27%) than any of the mutants (15–19%). In the case of the $Ile^{194}\rightarrow$Thr mutant, this was unexpected, because the same mutant retained its native conformation in the plasma of the patient. The reason for this discrepancy is not clear. Control LPL was present in the medium in a partially denatured form to a greater extent than in control post-heparin plasma (0.74% and 0.22%). This may have been due to instability in the medium at 37°C.

Because the $Ile^{194}\rightarrow$Thr mutant retained its native, dimeric conformation (unlike the other mutants), it was studied in more detail to reveal other enzyme functions that might affect overall catalysis. To evaluate the catalytic site, the activity of the $Ile^{194}\rightarrow$Thr mutant toward PNPB, a water soluble substrate, was compared with that of control LPL (**Table 4**). Heparin-Sepharose $Ile^{194}\rightarrow$Thr LPL expressed significant activity in this assay (Table 4). As PNPB is a substrate for a number of esterases, it was important to establish the identity of this activity. Initially, MAb inhibition was attempted, but although 5D2 completely inhibits LPL activity against an emulsified substrate (9), it did not inhibit activity in the PNPB assay of either control or mutant LPL, suggesting that the antibody somehow interferes with binding or presentation of lipid substrates. However, when 5D2 was coupled to a gel and incubated with the enzyme preparations, sedimentation of the MAb gel removed 30–40% of both $Ile^{194}\rightarrow$Thr mutant and control LPL activity in the PNPB assay, indicating that at least part of the activity was due to LPL mediated hydrolysis. To minimize LPL inactivation, the MAb gel incubation time was kept short (120 min); thus it is possible that a larger proportion than 40% of the activity was due to LPL.

LPL activity toward PNPB has been shown to be stimulated by phospholipid vesicles under certain conditions (13). To assess if this property was intact in the $Ile^{194}\rightarrow$Thr mutant, it was assayed in the PNPB assay in the absence or presence of the phospholipid vesicles. Under the conditions employed (36°C, pH 7.7), the degree of activation was similar for purified bovine LPL (240%), human control LPL (231%), and $Ile^{194}\rightarrow$Thr mutant LPL (201%), suggesting that the substitution did not adversely affect the capacity of the $Ile^{194}\rightarrow$Thr mutant protein to be activated by interfaces.

Because the catalytic site of the $Ile^{194}\rightarrow$Thr mutant presumably was functioning, some other aspect of catalysis seemed to be impaired. To evaluate if the mutant enzyme bound to substrate lipoproteins with normal affinity, mu-

TABLE 3. LPL mass and activity in media from transfected cells

		LPL				
LPL Activity		5D2 (Dimer)	5F9+Gu (Total)	5F9 (Denatured Monomer)	(5F9+Gu)-5F9 (Native, Calculated Dimer)	Native Total
nmol FA/min × ml				*mass ng/ml*		
Wild type	21	167	236	174	62	−0.26
Wild type		183	248	180	68	0.27
$Gly^{188}\rightarrow$Glu	0.2	298	259	220	39	0.15
$Ile^{194}\rightarrow$Thr	0	122	88	72	16	0.18

LPL mass was detected in conditioned media from transfected cells expressing wild-type and mutants of LPL. 5D2 ELISA was used as a direct measure of LPL dimer. GuHCl was used to destabilize LPL dimers to convert all to monomeric form and measure total LPL (5F9+Gu). 5F9 assay was used to measure the naturally monomeric LPL. Native LPL (dimer) was calculated by subtraction of naturally monomeric LPL (5F9) from total LPL (5F9+Gu). The ratio of native to total LPL was determined from the obtained values. The CV of measurement for 5D2 and 5F9 assays are 5.1% and 7.7%, respectively.

TABLE 4. Comparison of activity towards triolein and PNPB for control LPL and Ile^{194}→Thr mutant

	Specific Activity		
	PNPB Assay	PNPB Assay+PL	Triolein Assay
	mol product released/mg LPL × min		
Ile^{194} →Thr	2.1 ± 0.1	4.2	0
Control LPL	6.3 ± 0.2	14.6	660

Post-heparin plasma from a normal control and from a patient homozygous for the mutation was chromatographed on heparin-Sepharose, and the LPL protein peak eluting at 1.1 M NaCl was assayed in the two assays. Equal volumes were assayed. The concentrations of Ile^{194}→Thr LPL and control LPL were 880 and 289 ng/ml respectively. Values are mean (±SD) calculated from five determinations of activity in the PNPB assay and duplicate determinations in the triolein assay. Measurements of LPL mass were performed in triplicate, and represent LPL in its native conformation. For experimental details see Materials and Methods.

tant and control LPL purified on heparin-Sepharose were added to preheparin plasma from a normal donor, and the mixture was chromatographed on a FPLC gel filtration column (**Fig. 6**). LPL 5F9 mass (after heat denaturation), activity, cholesterol, and TG were measured in the eluted fractions. The recovery for mutant and control LPL was similar (88% and 109%). The elution profiles were also similar (Fig. 6A and B). For control LPL, the first two peaks showed catalytic activity, whereas the LPL eluting later was less active. The first peak, co-eluting with the very low density lipoprotein TG, constituted a similar proportion of total LPL for both mutant (12%) and control (11%), and the second peak, similarly co-eluting with LDL and HDL cholesterol, suggested that the mutation did not affect substrate recognition and binding.

DISCUSSION

LPL and pancreatic lipase are highly conserved in the region corresponding to exon 5 of the LPL gene (19), which suggests that their 3-D structures, particularly in this region, are likely to be similar. Based on the known X-ray crystallography structure of pancreatic lipase (20), exon 5 of the LPL gene codes for a portion of a hydrophobic pocket in which the proposed catalytic triad is located (2). In the present study, all but one mutant (Pro^{207}→Leu) represent change to a more hydrophilic amino acid residue. It is conceivable that such substitutions in a region normally not exposed to the aqueous phase could result in a more labile protein. Osborne et al. (11) have shown that for normal LPL, inactivation occurs spontaneously as a result of dissociation of LPL dimer into monomers that then rapidly and irreversibly lose activity and conformation. The results presented in the present study suggest that the spontaneously occurring dissociation of LPL dimer into monomers is accelerated in four of the mutants studied (i.e., 176, 188, 205, and 207). Two previous studies on the Gly^{188}→Glu (21) and Ala^{176}→Thr (14) LPL mutants reported that these defective LPL proteins bound with decreased affinity to heparin-Sepharose, as

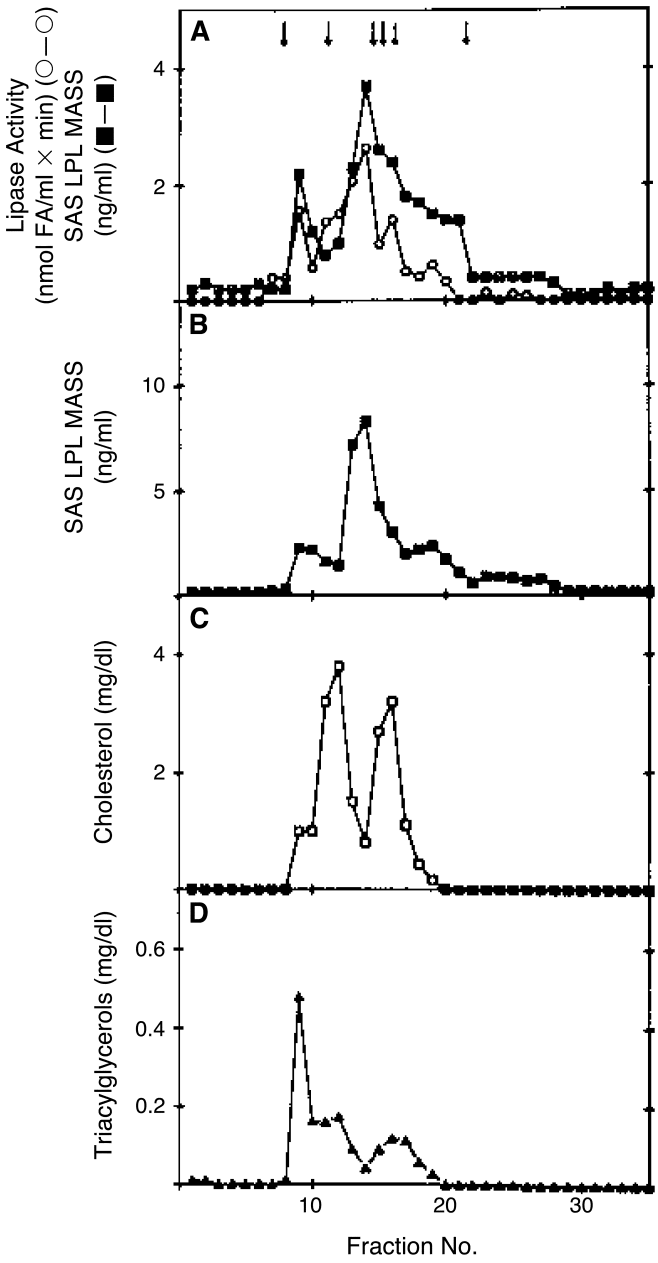

Fig. 6. FPLC gel filtration of the Ile^{194}→Thr and control LPL in the present of plasma. 500 μl of a 1+1 mixture of preheparin plasma and semi-purified control LPL (300 ng/ml) (A) or Ile^{194}→Thr LPL (800 ng/ml) (B) was mixed and chromatographed as described in Materials and Methods. LPL 5F9 mass after denaturation, LPL activity, total cholesterol (C), and TG (D) were assayed. The arrows indicate the retention volumes of (left to right): Blue Dextran, LDL, HDL2, HDL3, HAS, and NaCl.

would be expected if they were present as a partially denatured LPL monomer. Furthermore, the predicted 3-D structure of LPL suggests that some of these residues are located on the hydrophobic surface loops, which can influence substrate binding or dimmer formation and stability (2). The accelerated dissociation of dimer to monomers in these mutants, in agreement with the proposed role of surface loops, might indicate that the region coded by exon 5 plays a role in subunit association; how-

ever, it cannot be ruled out that these substitutions lead to conformational changes in other parts of the molecule, which in turn reduce the monomer/monomer affinity.

Because of the partially denatured state of most of the mutant proteins, it was not possible to evaluate if functions other than subunit association were affected prior to partial denaturation. However, in two of the five mutants (Gly^{188}→Glu and Ile^{194}→Thr), native LPL dimer (as determined by the 5F9 and heparin affinity) could be detected. Despite being in the native dimer conformation, these mutants did not hydrolyze triolein, indicating that a function critical to catalysis was affected. The Gly^{188}→Glu dimer was only marginally stable, but the stability of the Ile^{194}→Thr dimer permitted further characterization. Comparison of the Ile^{194}→Thr mutant to control LPL showed no differences in dimer conformation, heparin binding, esterase activity, surface activation, or lipid binding. Dissociation of lipase and esterase activity for LPL has been reported in another patient with LPL deficiency (22), and for trypsin-treated LPL (23). A possible explanation for the lack of lipase activity in this mutant is that the substitution to a more polar amino acid residue makes the catalytic pocket inaccessible to lipid but not to water-soluble substrate. These results would suggest that substrate binding and phospholipid activation occur at one or more domains separate from the catalytic pocket. The added observation that 5D2 inhibits activity in the lipase assay but not in the esterase assay further supports this notion. ApoC-II is not required for LPL action on water-soluble substrate (13), and it is thus conceivable that LPL with a defect in interaction with its activator could show esterase activity but not lipase activity. However, this seems to be a less likely explanation, since normal LPL has significant lipase activity even in the absence of apoC-II.

Five LPL mutant proteins resulting from missense mutations of exon 5 of the LPL gene were characterized. None of the five hydrolyzed triolein. Four of the mutants were mainly present in a partially denatured monomer form. Of these four, native LPL dimer could only be demonstrated in one, and this dimer present in the fresh sample dissociated rapidly into LPL monomer, demonstrating that a number of mutations in this region affect subunit interaction. One mutant was present as a stable dimer, showed esterase activity and normal binding to lipoproteins. It was activated by phospholipid vesicles and bound to heparin with normal affinity, and was compatible with an impaired access to the catalytic site for lipid substrate.

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