Recurrent pancreatitis and chylomicronemia in an extended Dutch kindred is caused by a Gly^{154}→Ser substitution in lipoprotein lipase

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Abstract We report the molecular basis of familial chylomicronemia and recurrent pancreatitis in five members of a large Dutch family. All patients had normal plasma hepatic lipase and apoC-II levels, but absent lipoprotein lipase (LPL) catalytic activity and low LPL mass in postheparin plasma. The mutation in the LPL gene was characterized as a G^{715}→A substitution in the last nucleotide of exon 4, resulting in a substitution of Ser for Gly^{154}. PCR amplification of exons 4 + 5 from the patients' mRNA, followed by direct sequencing, revealed normal splicing of intron 4. The mutation creates a BfaI restriction site that allows rapid screening of family members for the mutation. Reproduction of this mutation in LPL-cDNA by site-directed mutagenesis, followed by transient expression in COS-B cells, revealed production of a catalytically inactive enzyme. The Gly^{154}→Ser substitution appears in a conserved beta-sheet region, in close proximity to Asp^{156}, which is part of the catalytic triad. These studies show that changes to residues close to Asp^{156} can have profound effects on catalytic activity of LPL.—**Bruin, T., S. Tuzgöl, D. E. van Diermen, N. Hoogerbrugge-van der Linden, J. D. Brunzell, M. R. Hayden, and J. J. P. Kastelein.** Recurrent pancreatitis and chylomicronemia in an extended Dutch kindred is caused by a Gly^{154}→Ser substitution in lipoprotein lipase. *J. Lipid Res.* 1993. **34:** 2109–2119.

Supplementary key words splicing • mutagenesis • polymerase chain reaction

Lipoprotein lipase (LPL) (EC 3.1.1.34) catalyzes the hydrolysis of triglycerides to free fatty acids in the capillary beds of adipose and muscle tissue (1). Free fatty acids are subsequently transported to adipocytes for reesterification and storage, or they are used for energy provision through beta-oxidation in myocytes. The main site of action of the enzyme is the vascular endothelium to which it is bound via heparan sulfate proteoglycans (1–3).

LPL is a 55-kDa glycoprotein, which is only active as a dimeric protein and requires apolipoprotein (apo) C-II for optimal activity (3). The enzyme LPL exhibits considerable homology with other serine esterases such as hepatic lipase and pancreatic lipase, which is especially seen in the domains of these enzymes, coded for by exons 4, 5, and 6 (4). Several distinct residues in the LPL-protein have been shown to be essential for catalytic function. The serine at residue 132 forms a proposed catalytic triad with Asp^{156} and His^{241}, by analogy with the catalytic triad of pancreatic lipase (5). The essential role of Ser^{132} is supported by data from site-directed mutagenesis experiments, whereas the importance of Asp^{156} for catalytic function has been emphasized both by the results of the analysis of two naturally occurring mutations and additional in vitro mutagenesis studies (6, 7).

The human LPL gene spans about 30 kilobases and contains 10 exons, of which the 1.9-kb exon 10 only codes for the last nucleotide of the termination codon (8–10). The gene is transcribed into a 3.5-kb mRNA that is predominantly present in adipose tissue and heart muscle (11, 12). Until now, a large number of naturally occurring mutations in the LPL gene have been reported, the vast majority being missense mutations occurring predominantly in exons 4, 5, and 6 (13–28). Only one splicing defect in the LPL-mRNA giving rise to a truncated protein has been reported. In an LPL-deficient patient, the G→A transition in the first nucleotide of the donor splice-site of intron 2 leads to multiple aberrantly spliced mRNAs (23).

Abbreviations: LPL, lipoprotein lipase; TG, triglycerides; PCR, polymerase chain reaction; RT, reverse transcriptase; PL, pancreatic lipase; HL, hepatic lipase.

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In this report we describe the molecular basis of LPL deficiency in a large Dutch kindred with five LPL-deficient probands, which is caused by a nucleotide substitution in the last basepair of exon 4, which could theoretically cause aberrant splicing of LPL-mRNA, or leads to a substitution of Gly^{154} to Ser. Generally, splicing of mRNA precursors requires a consensus sequence at the boundaries of the exons and introns. We present evidence that in these probands the nucleotide substitution does not induce aberrant splicing but substitutes Ser for Gly^{154} in the mature protein. This substitution, which occurs in close proximity to Asp^{156}, is likely to disrupt the tertiary structure of the catalytic triad and lead to complete loss of catalytic function.

MATERIALS AND METHODS

Subjects (Fig. 1)

Our proband (II-7) was a 41-year-old white male who was referred in 1975 because of high serum cholesterol and triglyceride (TG) values. Since 1973 he had suffered from bouts of upper abdominal pain. On some occasions the pain was accompanied by fever and loose stools. During those periods chymotrypsin in his feces was decreased, serum and urine amylase increased, and a diagnosis of chronic pancreatitis was made. Alcohol intake was approximately 3 drinks/day on an ongoing basis. His parents were not related. There was no family history of coronary heart disease. Two sisters and a brother also suffered from abdominal pains. Between 1975 and 1992 the fasting total serum cholesterol varied between 6.8 and 12.1 mmol/l, fasting serum TG varied between 20 and 61 mmol/l, and HDL-cholesterol measured 0.44 to 0.34 mmol/l.

Even on a strict low fat diet after admission to the metabolic ward for 2 weeks, the serum cholesterol remained at 6.3 mmol/l and TG at 16.4 mmol/l. Clofibrate (2 g daily) did not prevent hyperlipidemia. No causes for secondary hyperlipidemia were evident. Fasting blood glucose and serum TSH levels were normal. Lipoprotein lipase activity was determined (Dr. H. Jansen) and was absent after intravenous heparin injection.

Our proband's sister (II-2) was seen at the age of 44 because of a family history of chylomicronemia and pancreatitis in her younger sister and brother. No specific diagnosis was made at the time, although she was noted to have chylomicronemia. At the age of 49 she was diagnosed with maturity-onset diabetes mellitus, type V hyperlipidemia, and hypothyroidism. She was treated with tolbutamide, clofibrate, and thyroid hormone. From the age of 62, control of her diabetes required insulin therapy. At the age of 63 the patient presented with severe abdominal pain and was diagnosed as suffering from pancreatitis. In addition, she was noted to have steatorrhoea and worsening of her diabetes, suggestive of severe pancreatic dysfunction. Fasting total serum cholesterol levels varied between 14.3 and 22.5 mmol/l, with a fasting serum TG from 18 to 80 mmol/l.

Our proband's other sister (II-10) was followed at the

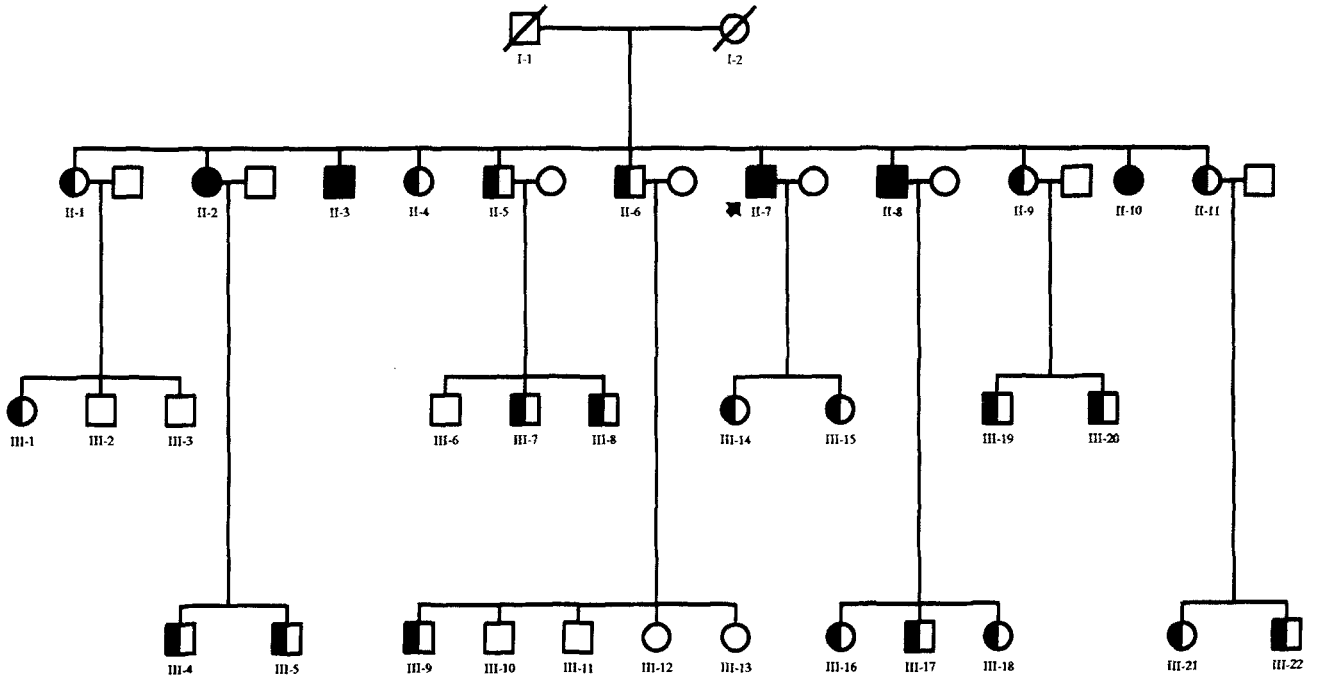

Fig. 1. Family tree showing the presence of the mutant allele. Blackened symbols are homozygotes for LPL deficiency and half-blackened symbols are heterozygotes for the $G^{715} \rightarrow A$ substitution. The arrow indicates the proband.

Department of Gastroenterology of the University Hospital of the University of Utrecht for severe abdominal pain, diagnosed as pancreatitis from the age of 30. The patient consumed no alcohol but did not adhere strictly to the fat-restricted diet. Her physical examination, apart from abdominal tenderness, was otherwise normal. She had normal glucose levels but fat excretion in stools was slightly increased. However, vitamins D and A were normal. Fasting triglycerides varied between 7.7 mmol/l and values above 30 mmol/l.

Our proband's brother (II-8) had suffered from bouts of upper abdominal pain since his twenties and was seen at the Department of Internal Medicine of the University Hospital, Rotterdam. His clinical presentation, however, was complicated by the fact that he started drinking excessively. His triglycerides varied at follow-up visits between 11.2 and 30.7 mmol/l. Adherence to a low fat diet was not strictly followed by this patient.

Another brother (II-3) was healthy and never volunteered any complaints, nor did he show any physical signs or symptoms. He smoked 20 cigarettes a day but abstained from alcohol. He was 1.73 m in height and weighed 70 kg. The heart, lungs, abdominal organs, and peripheral vessels were normal on examination. Blood pressure was 120/85 mm Hg. Fasting total serum cholesterol varied between 4.6 and 9.5 mmol/l, fasting TG from 24 to 51 mmol/l, and HDL from 0.24 to 0.32 mmol/l.

Plasma lipids and apolipoproteins

Total plasma cholesterol and triglycerides were measured in overnight-fasted subjects by enzymatic methods (29–31). High density lipoprotein (HDL)-cholesterol was determined by measuring cholesterol in the supernatant after precipitation of the apoB-containing lipoproteins (VLDL and LDL) from plasma as published previously (32, 33). LDL-cholesterol was then calculated from total plasma cholesterol, triglycerides, and HDL-cholesterol by the formula of Friedewald, Levy, and Fredrickson (34). Comparison with ultracentrifugal methods to determine LDL-cholesterol confirmed the accuracy (35). ApoB was determined on a Behring nephelometer BN100, using standards and references supplied by the manufacturer (Behring, Marburg, Germany). Statistical analysis was performed using the Student's *t*-test.

Measurement of plasma LPL activity and mass

Patients and family members fasted at least 12 h before blood collection. EDTA-anticoagulated blood was drawn before and 15 min after intravenous administration of heparin (50 units/kg body weight). Blood samples were chilled on ice and plasma was obtained after centrifugation at 2,000 *g* for 15 min at 4°C. The plasma was flash-frozen in liquid nitrogen and stored at −70°C. Total postheparin plasma lipolytic activity was measured by using tri-[^{14}C]oleoylglycerol as a substrate in a phosphatidylcholine emulsion. LPL activity was calculated as the activity in whole plasma that was inhibited by a specific monoclonal antibody (5D2) to LPL added to the plasma, and was expressed as nmol of fatty acids released per min per ml of plasma (36). Values for LPL in postheparin plasma in normal controls were 220 ± 59 nmol • min^{-1} • ml^{-1}. LPL mass present in preheparin plasma and released in postheparin plasma was measured by an ELISA. Values in normal controls were 196 ± 59 ng/ml (36).

DNA analysis

Genomic DNA was extracted from EDTA-anticoagulated blood as described previously (37). Genomic DNA of the probands was digested with PstI to screen for large gene rearrangements. DNA haplotype analysis was performed to determine which DNA haplotype of the LPL gene segregated with the phenotype of chylomicronemia. The BamHI polymorphism was determined by digestion of genomic DNA, followed by electrophoresis through an 0.8% agarose gel and transfer to nylon membranes by the Southern blotting technique. The human complementary (c)DNA clone LPL35 (a kind gift from Dr. S. Deeb, University of Washington, Seattle) was labeled with [^{32}P]dCTP, using random oligonucleotide priming according to Feinberg and Vogelstein (38). Autoradiography of the hybridized filters was performed with Kodak XAR-5 films at −70°C.

The HindIII polymorphism was determined as described previously (39). The PvuII, MnlI and BstNI polymorphisms were determined by PCR amplification of the sequences flanking the polymorphic sites using the primer sets Pvu3/Pvu5 for the PvuII polymorphism and BNM3/BNM5 for the BstNI and MnlI polymorphism (40) (**Table 1**). The PCR reaction was followed by digestion with the appropriate restriction endonuclease and electrophoresis on 1.5% agarose.

Each exon of the LPL gene was individually amplified from 500 ng genomic DNA using the PCR reaction and primers as described previously (15). The PCR-amplified double-stranded template was purified on an 1.2%

TABLE 1. Sequences of synthetic oligonucleotides used for PCR reactions and in vitro mutagenesis experiments

A) Primers for amplification of genomic DNA	
Pvu3:	5'-ATG CTG CTT TAG ACT CTT GT-3'
Pvu5:	5'-CTT TCG TGG TAT AGA GGT TG-3'
BNM3:	5'-TCA GCT TTA GCC CAG AAT GC-3'
BNM5:	5'-TAC ACT AGC AAT GTC TAG GTG A-3'
387:	5'-TCC AAG TCC TCT CTC TGC AA-3'
587:	5'-AGG AGT TTA ACT ACC CTC TG-3'
B) Oligonucleotide for site-directed mutagenesis	
LPL154:	5'-GGA TCG AGG CTA GTA ATT CTG-3'

agarose gel using DEAE-cellulose membranes. Direct sequencing of both strands of the template was performed using Sequenase 2.0 as described previously (28).

Amplification of LPL-mRNA from monocytes using PCR

A leucocyte fraction was isolated from 40 ml defibrinated blood using Ficoll-gradient centrifugation. Briefly, the defibrinated blood was mixed 1:1 with phosphate-buffered saline (PBS) and applied on a layer of Ficoll. After centrifugation, the fraction containing the leucocytes was isolated and washed twice with PBS, and finally lymphocytes were pelleted. Usually, the lymphocyte fraction contained 10–20% monocytes. Total RNA was isolated from this fraction using RNA-zol™ (Cinna/Biotecx, Frienswood, TX). RNA showed both 18S and 28S bands (data not shown). For the synthesis of the first strand cDNA, we used 200 ng total monocyte-RNA, 4 mM dNTP, 20 U RNAsin, 5 μM oligo $(dT)_{15}$, and 40 U AMV-reverse transcriptase. The reaction was performed at 42°C for 2 h in a final volume of 20 μl. After denaturation of RNA-DNA hybrids and inactivation of reverse transcriptase at 94°C for 5 min and snap-cooling at 0°C, we performed the PCR reaction. We added 2 μM of a primer set (numbers 587 for the 5'-site of exon 4 and 387 for the 3'-site of exon 5, see Table 1), encompassing exon 4 and exon 5 of the LPL gene, 200 μM dNTP, PCR buffer, 1.0 or 2.5 mM Mg^{2+} for genomic DNA and the cDNA, respectively, and 2.5 U Amplitaq (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 100 μl. The following temperature profile was performed for 30 cycles; 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The amplified products were run on an 1.2% agarose gel followed by staining with ethidium bromide.

Site-directed mutagenesis and transient expression in COS cells

We used the Altered-Sites site-directed mutagenesis kit from Promega, with some modifications. A 2.4 kb PstI/XbaI fragment from an LPL-cDNA clone (a kind gift from Dr. R. Lawn, Genentech Corp., San Francisco, CA) extending from nucleotides 1 to 2413 was cloned into the PstI/XbaI sites of the pSelect-1 phagemid vector (Promega, Madison, WI), and was used as a template for site-directed mutagenesis. Double-stranded pSelect-1 LPL-DNA was prepared in a JM 109 host and subsequently infected with the helper phage R 408 for production of single-stranded phagemid DNA. Site-directed mutagenesis involved annealing of an ampicillin repair oligonucleotide and the mutagenic oligonucleotide LPL154 (Table 1) to the purified pSelect-LPL-DNA, followed by synthesis of the mutant strand with T4 DNA polymerase. The heteroduplex DNA was then transformed into the repair minus host BMH 71-18 mut S. Mutants were selected by overnight growth in the presence of ampicillin. Plasmid DNA was isolated and transformed into the JM-109 host. Mutant, ampicillin-resistant colonies were confirmed by direct sequencing. The PstI/XbaI fragment of mutant LPL-cDNA was then cloned in the sense orientation into the pcDNAI expression vector (Invitrogen, San Diego, CA) and grown in the MC 1061/P3 *E. coli* strain.

Expression phagemids were cesium chloride-purified. Transfection into COS-B cells (a kind gift from Drs. A. J. van Zonneveld and H. Pannekoek, Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands) was done with the DEAE-dextran method. Briefly, COS-B cells were grown in 10-cm^2 wells until subconfluency was reached. After addition of DNA (1 μg/ml) together with DEAE-dextran (300 μg/ml) and chloroquine (52 μg/ml), cells were incubated for 3.5 h at 37°C. The cells were then treated with 10% DMSO for 1 min and subsequently grown for 60–80 h in the presence of heparin (5 U/ml). Aliquots of 1 ml of culture medium were collected, centrifuged for 10 min at 12,000 *g*, and snap-frozen at −70°C. The cells were washed with PBS and removed from the culture dishes using PBS containing 2.5 mM EDTA. Cell extracts and media were assayed for LPL activity by the method of Nilsson-Ehle and Schotz (41) using a glycerol-stabilized [^{3}H]triolein-phosphatidylcholine substrate emulsion. LPL activity was expressed in milliunits, i.e., nmol fatty acid released ml^{-1} min^{-1}. LPL mass was measured by ELISA using the 5D2 antibody against bovine LPL.

RESULTS

LPL and lipoprotein analysis

LPL activity was measured in postheparin plasma of all probands with hypertriglyceridemia and found to be absent in five probands (II-2, II-3, II-7, II-8, and II-10). All probands showed chylomicronemia and hypertriglyceridemia, associated with a low HDL- and LDL-cholesterol, typical for LPL deficiency (**Table 2**). LPL mass was increased after heparin infusion in all affected hyperchylomicronemic probands. Hepatic lipase activity was normal in all these LPL-deficient probands. We also determined the lipoprotein profile in most family members. Lipoprotein analysis revealed that the average triglyceride levels were elevated in heterozygous family members (P = 0.06). Moreover, the HDL-cholesterol concentration was significantly reduced in heterozygotes (P = 0.0083). LDL-cholesterol and apoB did not reveal differences in both groups (**Table 3**).

DNA analysis

After digestion of genomic DNA with PstI, Southern blot analysis did not reveal large rearrangements in the LPL gene. Haplotypes were constructed using the poly-

TABLE 2. LPL activity and mass combined with the lipoprotein profile of the probands

Subject	Age	LPL Activity	LPL Mass t = 0	LPL Mass t = 10	LPL Mass Incr	TG	LDL-C	HDL-C
	yr	*nmol • min⁻¹ • ml⁻¹*		*ng/ml*			*mmol/l*	
II-2	68	0	26	86	60	66	6.0	0.15
II-3	66	0	40	98	58	56	1.5	0.24
II-7	59	−4	3	20	17	48	2.0	0.30
II-8	57	−2	14	26	12	53	1.7	0.25
II-10	54	1	32	70	38	50	1.0	0.34
Contr.		220 ± 59	[a]		196 ± 59	<2	3.0	1.20

Abbreviations: TG, triglycerides; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; t = 0, values before heparin infusion; t = 10, values 10 min after heparin infusion; Incr, incremental increase in LPL mass between t = 0 and t = 10 min.

[a] Immunoreactive material is present in preheparin plasma in some individuals, but not in most.

morphic sites for PvuII, HindIII, BstNI, MnlI, and BamHI. All LPL-deficient probands were homozygous for the haplotype PvuII− −, HindIII++, MnlI− −, BstNI++, and BamHI++. Until now, no mutations have been described with this haplotype. In addition, the identical LPL haplotype in all five probands with LPL deficiency strongly supported a mutation in the LPL gene as the cause for LPL deficiency.

TABLE 3. The lipoprotein profile of the family members

Subject	Age	Het/Unaf	TG	LDL-C	HDL-C	ApoB
	yr			*mmol/l*		
II-1	70	Het	2.57	2.83	0.82	1.31
II-4	63	Het	2.21	2.85	1.41	1.34
II-5	62	Het	1.90	2.81	0.87	1.33
II-6	60	Het	nd	nd	nd	nd
II-9	55	Het	1.77	2.18	1.16	0.98
II-11	46	Het	0.70	2.08	1.37	0.88
III-1	44	Het	0.74	2.15	1.25	1.02
III-2	42	Unaf	0.95	3.60	1.35	0.85
III-3	37	Unaf	1.24	3.71	1.41	1.29
III-4	41	Het	1.08	2.65	1.26	1.12
III-5	38	Het	1.47	2.53	0.77	1.15
III-6	36	Unaf	1.03	2.84	1.08	1.22
III-7	35	Het	3.99	2.19	0.79	1.16
III-8	33	Het	11.69	1.76	0.55	0.90
III-9	32	Het	nd	nd	nd	nd
III-10	30	Unaf	0.53	2.09	1.39	1.89
III-11	28	Unaf	0.47	1.55	1.31	0.80
III-12	26	Unaf	0.50	1.49	1.24	0.71
III-13	19	Unaf	0.45	1.54	1.01	0.78
III-14	31	Het	nd	nd	nd	nd
III-15	29	Het	nd	nd	nd	nd
III-16	25	Het	nd	nd	nd	nd
III-17	24	Het	0.87	2.34	0.85	1.05
III-18	23	Het	nd	nd	nd	nd
III-19	30	Het	1.00	2.47	1.05	1.19
III-20	29	Het	0.34	0.98	1.17	0.42
III-21	23	Het	1.44	3.77	0.80	1.68
III-22	19	Het	1.00	1.98	0.80	0.88

Abbreviations: TG, triglycerides; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; ApoB, apolipoprotein B; Het, heterozygous for the Gly^{154} for Ser substitution; Unaf, unaffected family member; nd, not determined.

Therefore, in order to establish the molecular defect underlying LPL deficiency in these patients, we subjected all 9 exons and intron-exon boundaries from PCR-amplified DNA to direct sequencing. Sequence analysis of exon 4 DNA revealed a G to A transition at position 715 of the published cDNA sequence (**Fig. 2**). The G at position 715 is the last base of exon 4 and codes together with the first 2 bases of exon 5 (GGC) for the glycine at position 154. The G to A substitution predicts a Gly^{154}→Ser substitution. However, the consensus sequence for splicing at the 5'-site of the intron is 5'-CTG!GTAAGT-3' and the G to A substitution in the last base of exon 4, directly adjacent to the splice site, could also possibly result in a splicing defect.

To evaluate the presence of the splicing defect, we developed a technique to analyze LPL-mRNA, using monocytes as the source instead of a biopsy of fat tissue. mRNA from monocytes was isolated, directly followed by reverse transcription using oligo (dT) and PCR with primers designed to specifically amplify exon 4 and exon 5 together. Aberrant splicing of the pre-mRNA would then result in a PCR product also containing the nonexcised intron (**Fig. 3**). Control genomic DNA, containing unspliced exon 4 in conjunction with intron 4 and exon 5, amplified with the primers 587 and 387, revealed a single band of 1168 bp on a 1.2% agarose gel, correlating exactly with the calculated size of exon 4 and 5 (386 bp) added to intron 4 (800 bp) (Fig. 3, lane 2). The products from control mRNA and mRNA from the index patient showed a single band of 386 bp (exon 4 and 5) and not the band of 1186 bp (Fig. 3, lane 3 vs. 4). These data indicate that mRNA of the index patient is correctly spliced. This finding was confirmed by direct sequencing of the RT-PCR product (**Fig. 4**). The transition from exon 4 to exon 5 was not interrupted by intron 4. Furthermore, the missense mutation at position 715 is clearly visible. We, therefore, must conclude that Gly^{154} is indeed substituted by a Ser, and that incorrect splicing had not occurred.

The G to A missense mutation creates a BfaI restriction

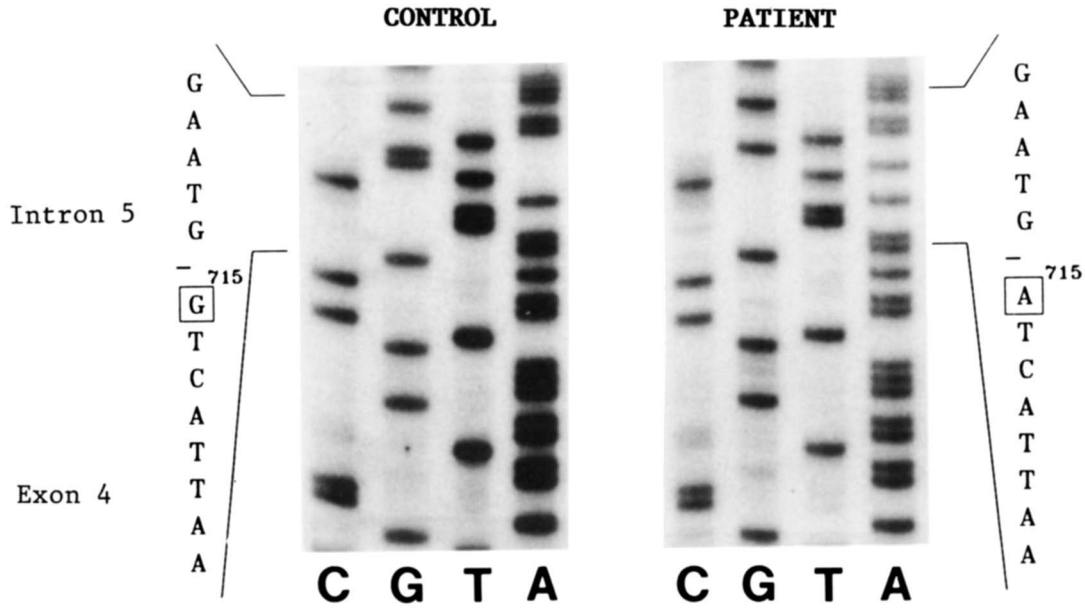

Fig. 2. Direct sequencing of the normal and mutant exon 4-intron 4 boundary showing the $G^{715}\rightarrow A$ substitution, resulting in a substitution of a Ser for Gly. Samples were run on a 6% acrylamide/8 M urea sequencing gel.

site (CTAG), which excises 28 bp from the 3'-end of exon 4 (**Fig. 5**). This enabled fast screening of all family members for the presence of the mutant allele. The results after BfaI digestion of PCR-amplified exon 5 were confirmed by sequencing of the T and A nucleotides (data not shown). We screened 33 family members for the 154 mutation. Five family members were homozygous for the mutation, whereas 21 were heterozygous, and 8 did not possess any mutation (Fig. 1). An additional 48 alleles from LPL-deficient patients of Canadian, Dutch, German, Turkish, Moroccan, Spanish, and Columbian extraction and 100 alleles from normolipemic Dutch patients were screened for the 154 mutation, using the BfaI restriction test, but none was found to have this substitution. All family members that are heterozygous for this substitution show the PvuII−, HindIII+, MnlI−, and BstNI+ allele.

In vitro mutagenesis and transient expression in COS-B cells

In order to investigate whether this substitution of Ser for Gly at amino acid position 154 destroyed catalytic activity of LPL and was the cause of LPL deficiency in this kindred, we reproduced this mutation in vitro. We introduced the G→A substitution at position 715 in the wildtype LPL cDNA and expressed the mutation in COS-B cells. LPL activity and mass were assayed in the medium and cell homogenate of the COS cells transfected with both wild type and mutant cDNA as well as mock-transfected COS-B cells. LPL enzymatic activity was absent in the medium (0.5%) and cell homogenate (1%) of cells transfected with the mutant $Gly^{154}\rightarrow Ser$ cDNA when compared to the activity of the wild type LPL (**Fig. 6A**). LPL protein mass was present in nearly equal amounts in the medium (80%) or cell homogenate (65%) of COS cells transfected with the mutant $Gly^{154}\rightarrow Ser$ cDNA, compared to the medium of cells transfected with the normal LPL cDNA (Fig. 6B). Therefore, these data demonstrate that LPL is synthesized in cells transfected with the mutant $Gly^{154}\rightarrow Ser$ LPL cDNA, and is secreted but is indeed catalytically inactive.

DISCUSSION

We have studied the molecular defect underlying chylomicronemia in five patients from one family who had catalytically defective LPL. Four of these patients presented with clinical signs of chylomicronemia including abdominal pain and recurrent pancreatitis. Interestingly, one patient did not have any symptoms of the disease despite significant lipoprotein abnormalities, in contrast to his siblings. Strict adherence to a fat-restricted diet, abstinence from alcohol, and a lower body mass index than his siblings are likely contributing factors for this difference. In contrast to many of the patients with LPL deficiency who present in childhood, the four patients who had signs or symptoms of chylomicronemia presented in adulthood. This suggests that environmental factors, like a low fat diet and a healthy life style,

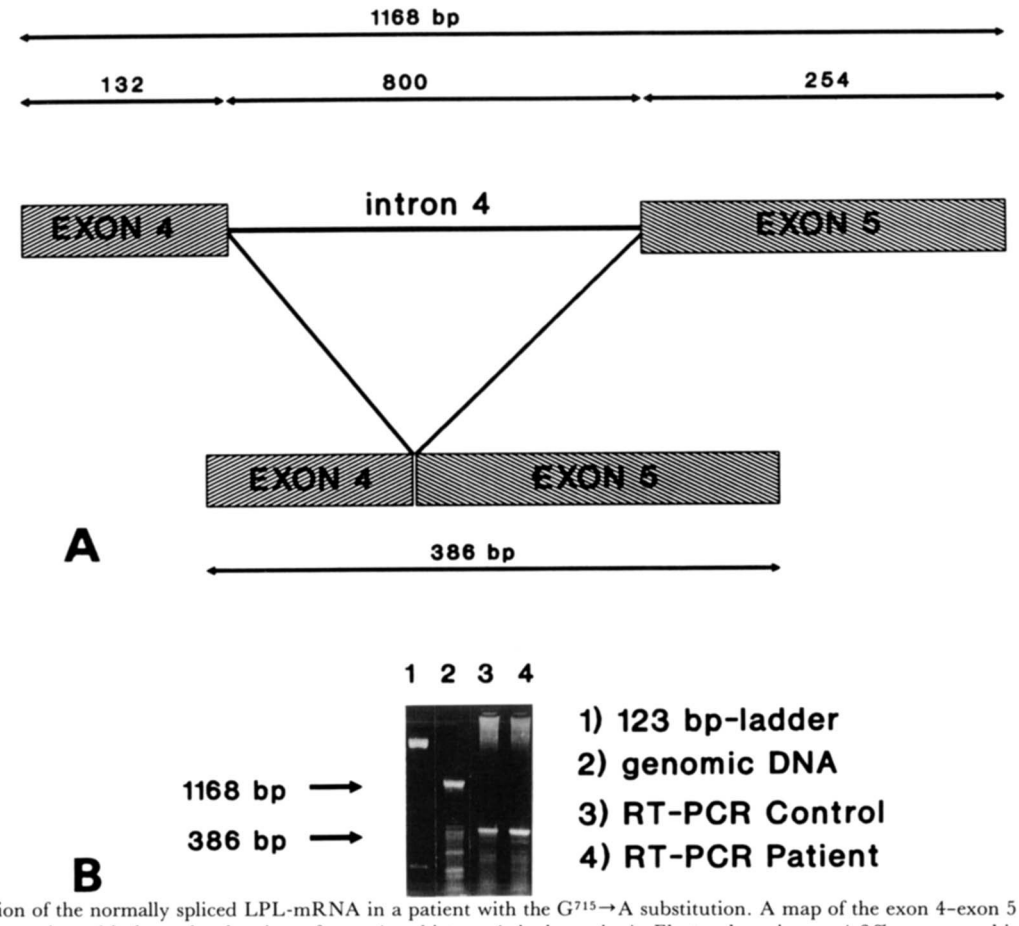

Fig. 3. Detection of the normally spliced LPL-mRNA in a patient with the $G^{715} \rightarrow A$ substitution. A map of the exon 4–exon 5 boundary and the splicing pattern, together with the molecular sizes of exon 4 and intron 4, is shown in A. Electrophoresis on a 1.2% agarose gel is shown in B. Lane 1: 123-bp ladder (BRL); lanes 2–4: PCR products using primers 387 and 587 (Table 1) from, respectively, genomic DNA (lane 2), control cDNA (lane 3), and patients' cDNA (lane 4).

mitigated the expression of the disease in childhood. Recently, data were published suggesting that environmental factors may significantly contribute to the expression of LPL deficiency (42).

No large rearrangements in the LPL gene were detected by the Southern blotting technique. We therefore sequenced all 9 exons from PCR-amplified DNA from the index patient and detected a G to A substitution at position 715. This may result in the substitution of glycine to serine at amino acid residue 154.

However, this G to A substitution occurs at the last basepair of exon 4 which is within the 5'-consensus sequence for splicing (5' CAG!GTAAGT 3') (43, 44). Some mutations in the GTAAGT-nucleotide at the 5' end of intron sequences have been reported to induce the loss of a preceding exon (45, 46). Other mutations in the invariant GT motif of the donor splice site result in creation of cryptic splice sites. Recently, a $G \rightarrow A$ mutation in the first base of the donor splice site of intron 2 of the human LPL gene was described (23). This mutation created multiple cryptic splice sites, including one in exon 2, which was predominantly used and resulted in an 18-bp deletion. Furthermore, there is an example of a mutation in the CAG trinucleotide at the 3' end of an exon, which causes the loss of the 5' splice donor site; a G to A substitution in the last nucleotide of exon 12 of the porphobilinogen deaminase gene led to the loss of exon 12 (47). We therefore investigated whether the nucleotide substitution under study either caused the substitution of Gly^{154} for Ser or leads to an aberrant splicing, which would have resulted in a truncated LPL protein.

The hypothesis that there was, indeed, no defect in splicing was supported by evidence from two experiments. First, we used peripheral blood to isolate monocyte-RNA from the patient and synthesized the first strand of cDNA from mRNA by reverse transcription primed with oligo (dT). We then specifically amplified exons 4 and 5 from the LPL gene by PCR. This experiment did show that normal splicing occurred, because only a single 386 band of the spliced product, and not the 1186 bp band of the unspliced product, was present on an agarose gel. Furthermore, we searched GGT-containing sequences in exon 4

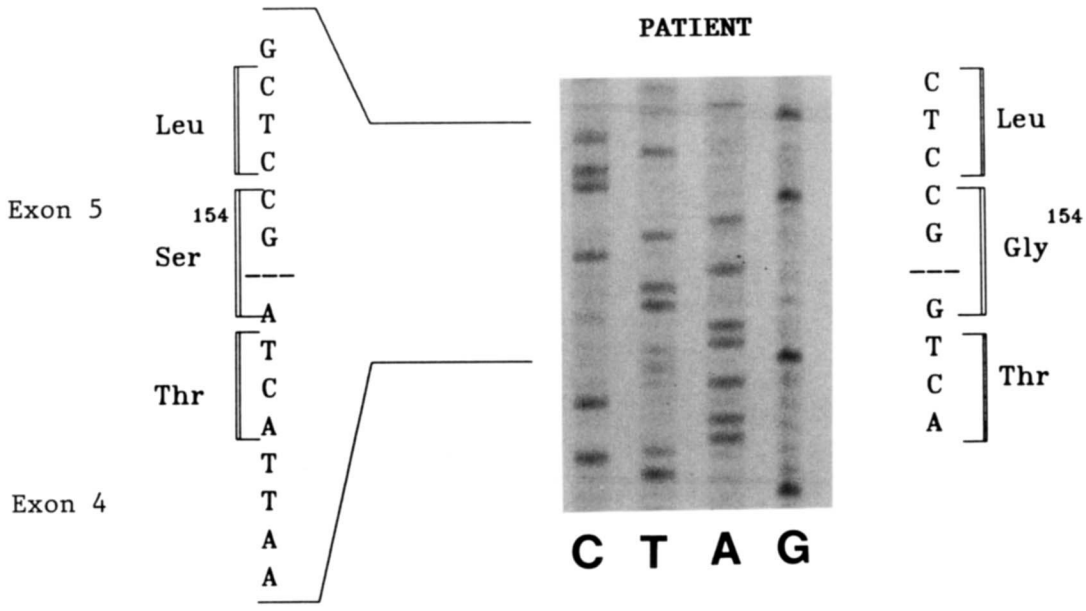

Fig. 4. Direct sequencing of the exon 4–exon 5 boundary of patient (II-7) cDNA. The sequence is run on a 6% acrylamide/8 M urea gel, revealing the substitution of Ser^{154} for Gly.

and the first 560 basepairs of intron 4 for potential cryptic splice sites. This search revealed three sites, respectively −20, −35, and −98 basepairs from the normal donor splice site. On the agarose gel no extra bands between 386 and 1186 bp were present, indicating that no cryptic splice sites are activated. In the second experiment the transition between exons 4 and 5 from the patients cDNA was directly sequenced and showed the missense mutation at position 715 followed by the first nucleotides of exon 5. The missense mutation therefore results in transition of codon AGC to GGC, predicting a Gly to Ser substitution at amino acid position 154.

The G to A substitution creates a BfaI-restriction site, which allowed rapid screening of all family members for the mutant allele, using PCR-amplified exon 4 DNA followed by restriction enzyme digestion and agarose electrophoresis. After screening of 33 family members, 5 homozygotes and 21 heterozygotes for the missense mutation were identified. Lipoprotein analysis in the family members revealed several members with an aberrant lipoprotein profile. Triglycerides were increased and HDL-cholesterol levels were significantly decreased in heterozygotes. The phenotype of the heterozygous subjects was characterized by a mild hypertriglyceridemia. The normal LDL-cholesterol and apoB levels in both groups indicate that heterozygosity for the Gly^{154} to Ser substitution predisposes for familial hypertriglyceridemia rather than familial combined hyperlipidemia.

The substitution of Gly^{154} to Ser, as shown by the site-directed mutagenesis experiments, completely abolished catalytic activity of the LPL protein towards long-chain triacylglycerols, whereas LPL mass was present in normal amounts in the medium as well as the cell homogenate of COS cells transfected with wild-type LPL and the mutant LPL. The incremental LPL mass in cells and medium of

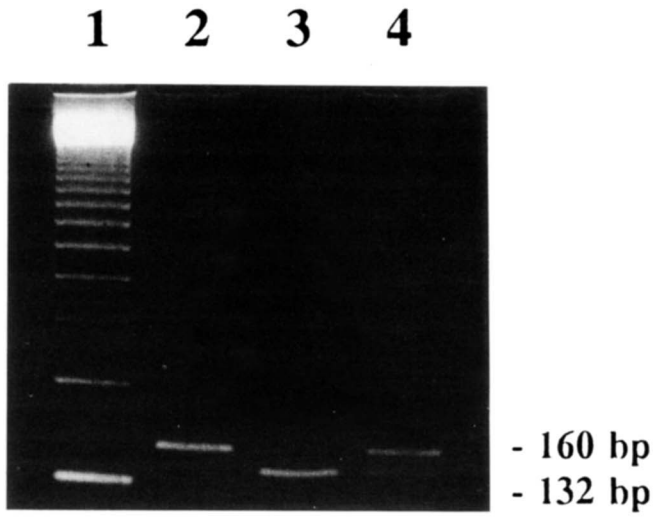

Fig. 5. Analysis of genomic DNA for the Gly^{154}→Ser substitution, using PCR-amplified exon 4 followed by BfaI-digestion. Exon 4 was amplified by PCR and digested with BfaI. PCR-amplified exon 4 normally contains no BfaI site and has a size of 160 bp. The G^{715}→A substitution introduces a BfaI site, giving fragments of 132 and 28 bp, respectively. Lane 1: 123-bp ladder (BRL); lane 2: control DNA; lane 3: homozygote for the Gly^{154}→Ser substitution; lane 4: heterozygote for the Gly^{154}→Ser substitution.

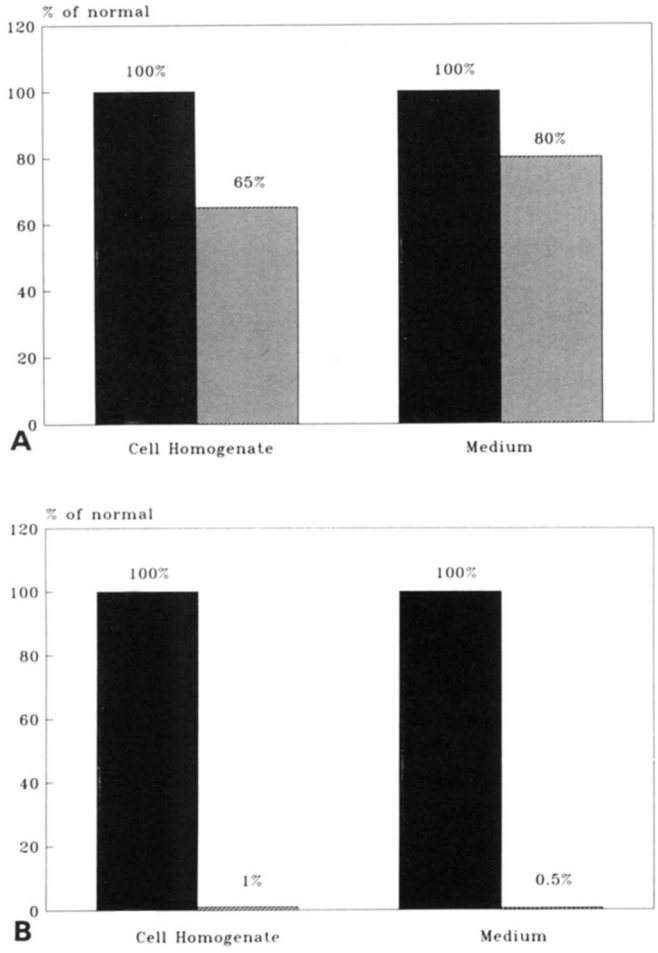

Fig. 6. LPL activity and mass in culture medium and cell homogenate of COS-B cells transfected with normal LPL cDNA and cDNA with the Gly^{154}→Ser substitution. Results are expressed as percentages of the levels determined in the medium and cell homogenate of COS-B cells transfected with the normal LPL cDNA. A) LPL mass of normal LPL (black column) and mutant LPL (striped column). B) LPL activity of normal LPL (black column) and mutant LPL (striped column).

COS cells, transfected with the mutant LPL, were 65% and 80%, respectively compared to wild-type LPL mass (Fig. 6). These data are not consistent with the LPL mass in postheparin plasma from the patient, which is only 30% of control values (Table 1). These seemingly contradictory data are possibly caused by differences in the intra- and extracellular breakdown process of LPL in COS cells and the in vivo situation.

This mutation occurs in an extremely conserved region between the different members of the lipase family (HL and PL), and which also contains the proposed catalytic center, consisting of the three amino acids Ser^{132}-Asp^{156}-His^{241} (4, 5). This is also the domain where more than 90% of the mutations causing LPL deficiency have been identified (48, 49). It is likely that a mutation in the close proximity of one of the amino acids of the catalytic triad will significantly reduce or even completely abolish catalytic activity. In the LPL-deficient patients presented in this report, the substitution at position 154 occurs only two amino acids distant from the proposed catalytic triad Asp^{156}, resulting in a Gly→Ser change.

Gly^{154} is normally part of a conserved beta-sheet region (beta-8 in pancreatic lipase). Human pancreatic lipase contains four of these beta-sheets surrounding the catalytic Ser^{152} (5). These four beta-sheets in hPL (beta-7, -8, -9, and -10) are 90% conserved compared with human LPL and therefore a comparison of LPL with PL in this region can be made. In other lipases, a hydrophobic pocket is formed by these beta-sheets which is accessible for triglycerides after reorientation of the so-called loop or flap by interfacial activation. Triglycerides then can easily reach the catalytic center of the protein. The hydrophobic character of the beta-sheet in LPL, to which Gly^{154} belongs, is lost after the substitution of Ser for Gly, presumably altering accessibility of substrate to the catalytic triad. However, in vitro studies also clearly indicate that this substitution directly results in a catalytically defective protein. Therefore it is likely that this mutation also alters the secondary or tertiary structure of the protein, directly affecting the catalytic triad.

In this report, we have described the use of LPL-mRNA from monocytes to assess gene structure of LPL. LPL-mRNA is present in low amounts in peripheral blood monocytes, but the use of reverse transcriptase PCR technology enabled us to amplify the specific region from the LPL-mRNA without undue difficulty. Furthermore, the use of monocytes from peripheral blood for such studies is better accepted by the patient and also was essential to assess the integrity of mRNA splicing in these patients.

In summary, in this paper we describe an approach to differentiate between a splicing defect and a missense mutation in the LPL gene. Moreover, the missense mutation of Gly^{154}→Ser in the conserved region surrounding the proposed catalytic triad Asp^{156} changes the three-dimensional conformation of the protein, and thus results in a catalytically defective LPL protein. This mutation adds to the emerging consensus of the nonrandom accumulation of missense mutations in the most conserved exons in this gene, namely exons 4 and 5.

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