A compound heterozygote for lipoprotein lipase deficiency, Val^{69}→Leu and Gly^{188}→Glu: correlation between in vitro LPL activity and clinical expression

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Abstract We analyzed the molecular defects in the lipoprotein lipase gene of a patient with type I hyperlipidemia suffering from recurrent pancreatitis, indicative for lipoprotein lipase deficiency. Postheparin lipoprotein lipase activity in the patient was decreased by 70%. Direct genomic sequencing revealed compound heterozygosity for two mutations: the well-known Gly^{188}→Glu and a new Val^{69}→Leu substitution. Val^{69} is situated in a conserved hydrophobic region of the lipoprotein lipase protein, and the substitution with leucine gives rise to a 80% decrease in specific catalytic activity, as supported by site-directed mutagenesis experiments, followed by expression in COS-cells. The combination of both defects in the lipoprotein lipase gene was incidently associated with severe clinical expression of disease, and triglyceride levels of more than 30 mmol/l were measured. In our patient, triglyceride levels were usually below 10 mmol/l. We, therefore, postulate that the residual LPL activity in our patient is usually sufficient to keep the triglyceride level within bounds and expression of disease occurred only when conditions such as alcohol abuse or poor compliance to diet were present.—**Bruin, T., S. Tuzgol, W. J. Mulder, A. E. van den Ende, H. Jansen, M. R. Hayden, and J. J. P. Kastelein.** A compound heterozygote for lipoprotein lipase deficiency, Val^{69}→Leu and Gly^{188}→Glu: correlation between in vitro LPL activity and clinical expression. *J. Lipid Res.* 1994. **35:** 438–445.

Supplementary key words pancreatitis • missense mutation

Lipoprotein lipase ((LPL) (EC 3.1.1.34)) plays a central role in the metabolism of triglyceride-rich lipoproteins (1). It is synthesized in adipose tissue and heart muscle and is secreted to the capillary endothelium, to which the enzyme is anchored by membrane-bound heparan sulfate proteoglycans (HSPGs). LPL hydrolyzes triacylglycerols, present mainly in the core of chylomicrons and VLDL, thereby delivering free fatty acids (FFA) to peripheral tissues. The enzyme is dependent on a cofactor, apolipoprotein (apo) C-II, for catalytic activity (2). LPL is regulated at both transcriptional and posttranscriptional levels, mainly by post-translational glycosylation, while the expression at the luminal surface is augmented by insulin, thyroid hormone, and glucocorticoids (3–8).

LPL protein has been purified from many sources and extensively characterized with regard to structure and activity. It is a glycoprotein, active only in its dimeric form, with a subunit molecular mass of 60–72 kDa (9). The protein consists of 448 amino acids, and has a carbohydrate content of 3–10% by molecular weight. The hydrolytic center consists of a catalytic triad of Ser^{132}, Asp^{156}, and His^{241}, strongly conserved among the different lipases (10).

Recently many different mutations in the LPL gene have been reported that cause hyperchylomicronemia. These mutations include a partial gene duplication, a deletion, an insertion, and a wide variety of point mutations (11, 12). Interestingly, most point mutations cluster around exons 4, 5, and 6, close to the amino acids of the catalytic triad Ser^{132}, Asp^{156}, and His^{241}.

In this study we have characterized the molecular basis for LPL deficiency in a patient of Dutch origin. This patient exhibited only moderate hypertriglyceridemia until adolescence and early adulthood, possibly related to alcohol abuse and chylomicronemia ensued. This suggested

Abbreviations: LPL, lipoprotein lipase; FFA, free fatty acids; HSPG, heparan sulfate proteoglycans; HL, hepatic lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

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that both environmental and genetic factors might have played a part in the expression of his hyperlipidemia.

MATERIALS AND METHODS

Case history

R. W. suffered from recurrent attacks of abdominal pain and diarrhoea of unknown origin from early childhood. At 24 years of age he was admitted to the hospital with a diagnosis of acute pancreatitis, due to excessive alcohol consumption. Subsequently he had five similar admissions. Echography of the upper abdomen and endoscopic retrograde cholangio-pancreaticography (ERCP), performed elsewhere, revealed no abnormalities. At the age of 34, after another admission, physical examination revealed numerous eruptive xanthomas on arms and buttocks. Serum and urine amylase levels were elevated, but all other laboratory parameters, apart from the lipoprotein profile, were normal. During this admission, triglycerides were 32.2 mmol/l, total cholesterol 12.60 mmol/l, LDL-cholesterol 1.86 mmol/l, and HDL-cholesterol 0.50 mmol/l. A diagnosis of LPL deficiency was made and a low-fat (20%) diet was advised. Triglycerides dropped to levels between 5 and 20 mmol/l (see Fig. 1) on outpatient clinic visits. Since his last admission the patient was hospitalized two more times for acute pancreatitis.

Plasma lipids and apolipoproteins

Total plasma cholesterol and triglycerides were measured, after an overnight fast, by enzymatic methods (13–15). High density lipoprotein (HDL)-cholesterol was determined by measuring cholesterol in the supernatant after precipitation of the apoB-containing lipoproteins (VLDL and LDL) as previously described (16, 17). LDL-cholesterol was then calculated from total plasma cholesterol, triglycerides, and HDL-cholesterol by the Friedewald formula (18). When triglycerides were over 4.5 mmol/l, ultracentrifugation was performed to measure LDL-cholesterol. Comparison with ultracentrifugal methods to determine LDL-cholesterol confirmed the accuracy of the calculation (19). 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 unpaired *t*-test.

Measurement of lipase activity

LPL and hepatic lipase (HL) were determined in postheparin plasma after an overnight fast. Blood was collected in heparin-containing tubes 20 min after the intravenous injection of 50 IU heparin/kg bodyweight. Plasma was separated by centrifugation and stored frozen until use. The lipases were determined separately by an immunochemical method essentially as described by Huttunen et al. (20) using a gum acacia-stabilized [^{3}H]trioleoylglycerol substrate. HL activity was determined as the salt-resistant lipase in the presence of 1 M NaCl. LPL activity was determined after inhibition of HL with a goat antibody raised against HL, purified from postheparin human plasma. For the correction of the extraction of fatty acids, [^{14}C]oleate was added to the substrate. To reduce inter-assay variability, pooled plasmas with high and low LPL and HL were included as a reference in each series of determinations.

Southern blot analysis

Genomic DNA was extracted from fresh EDTA-anticoagulated blood as described previously (21). Five μg samples of DNA from the initial proband and his family members were digested with the restriction endonuclease, PstI, which does not recognize restriction fragment length polymorphisms (RFLPs) in the LPL gene, in order to screen for large gene rearrangements. The BamHI RFLP was determined by digestion of genomic DNA with BamHI. DNA fragments were then electrophoresed through a 0.8% agarose gel and transferred to nylon membranes by Southern blotting (22). The human LPL cDNA clone HLPL-26, kindly provided by Dr. S. Deeb, University of Washington, Seattle, was used for hybridization as described by Church and Gilbert (23), with final washing conditions at 65°C in 1× SSC. The cDNA probe was labeled with [^{32}P]dCTP random oligonucleotide priming (24). Nylon membranes were exposed to Kodak XAR-5 films with intensifying screens at −70°C for 72 h.

Polymerase chain reaction and sequence analysis

Genomic DNA from the proband and family members was used for analysis by the polymerase chain reaction (PCR). The reactions were performed in a DNA thermocycler (Perkin-Elmer Cetus). All exons of the LPL gene of the proband were amplified using the PCR reaction under conditions described previously (25, 26) and directly sequenced using the dideoxy method according to Sanger, Nicklen, and Coulson (27). The oligonucleotide primers were synthesized in a DNA synthesizer, model Gene Assembler Plus (Pharmacia, Uppsala, Sweden).

PCR-amplified exon 5 (100 ng) was digested with 10 U AvaII for 2 h at 37°C. The exon 5 fragments were electrophoresed through a 1.2% agarose gel and stained with ethidium bromide for visualization.

Mutagenic PCR was performed on exon 3, using a 5'-exonic mismatch primer (primer 1; 5'-GGAATGTATG-AGAGTTGGGTGCCAAAACAT-3') and a 3'-intronic primer (primer 2; 5'-TAAGTCTCCTTCTCCCAGTC-3'). The mismatch introduces an NlaIII restriction site in control DNA. The 250-bp product was digested with NlaIII and subsequently run on an 6% agarose gel (Boehringer Mannheim, Germany). The HindIII and PvuII RFLPs were determined by PCR amplification of the sequences

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flanking the polymorphic sites, followed by digestion with the appropriate restriction endonuclease (28).

Site-directed mutagenesis and transient expression in COS-cells

A 2.4 kb PstI/XbaI fragment from an LPL-cDNA clone (a kind gift from Dr. R. Lawn, Genentech Corp., San Francisco, CA) was cloned into the pSelect-1 phagemid vector (Promega, Madison, WI) and was used as a template for site-directed mutagenesis using the mutagenic oligonucleotide LPLMUT69 (5'CAGGGCGGCCAGA-AGTTTTGG-3') as previously described (29). Transfection into COS-B cells was performed with the DEAE-dextran method, and LPL activity and mass were measured as described previously (29).

RESULTS

LPL activity and lipoprotein profile

LPL activity in postheparin plasma from the proband was significantly decreased (43 U/ml, normal value 130 ± 28 U/ml). LPL measurement on a second occasion gave an activity of 52 U/ml. Hepatic lipase activity was normal (204 U/ml). ApoC-II was present at a normal concentration and the apoE phenotype was E3/E3. Lipid and lipoprotein data for the index patient, after fat restriction, and his family members are summarized in **Table 1.** The proband exhibited chylomicronemia and low HDL-cholesterol. The mother (I-2) had high cholesterol but normal triglycerides, and one brother (II-2) had both a high triglyceride and low HDL level. All other family members had normal lipoprotein levels. We followed the triglyceride levels of the patient over the last 4 years and found a fluctuating pattern with the lowest triglyceride levels of about 3–4 mmol/l and the highest levels about 60 mmol/l (**Fig. 1**).

DNA analysis

Southern blot analysis, after digestion with PstI, did not reveal large rearrangements in the LPL gene of the proband. The BamHI, PvuII, and HindIII RFLPs of the proband and family members were determined, and the results are depicted in **Fig. 2.** The proband (II-3) was homozygous for the BamHI-, PvuII-, HindIII+ haplotype and therefore the mutations on both alleles of the LPL gene shared the same haplotype. Exon 5 was separately amplified by PCR, in order to screen for the common missense mutation at nucleotide 819 ($Gly^{188}\rightarrow Glu$) (26).

TABLE 1. Lipid and lipoprotein data

Subject	Chol	HDL-C	TG	ApoA-I	ApoB
		mmol/l		*g/l*	
I-1	6.21	1.31	1.04	1.45	1.32
I-2	8.52	1.38	1.80	1.63	2.17
II-1	6.05	1.38	0.85	1.50	1.37
II-2	5.46	0.96	2.73	1.26	1.21
II-3[a]	7.73	0.72	15.67	0.90	0.92
II-4	6.50	1.11	1.64	1.38	1.71

Chol, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride.
[a]Proband.

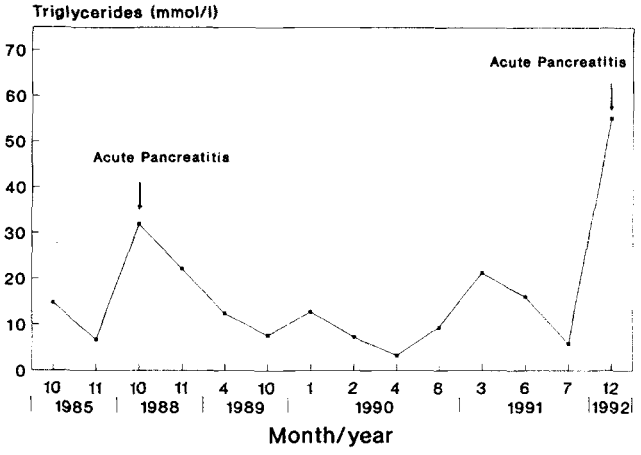

Fig. 1. Triglyceride measurements of the proband during the last 7 years.

PCR-amplified exon 5 from all family members and the proband was digested with AvaII and subjected to electrophoresis on a 1.2% agarose gel (**Fig. 3**). The $Gly^{188}\rightarrow Glu$ substitution results in the loss of one AvaII restriction site in exon 5. As shown in Fig. 3, the index patient was heterozygous for the $G^{819}\rightarrow C$ substitution. The same pattern was observed in the mother (I_2) and the youngest brother (II_4) of the proband.

We then performed direct sequencing of all 9 exons of the LPL gene to find a possible second defect. This revealed heterozygosity for a novel missense mutation in exon 3 and confirmed the presence of the common missense mutation in exon 5. A substitution of G→C was observed in exon 3 at nucleotide position 460, predicting a substitution of Leu for Val at amino acid position 69 (**Fig. 4**), and a G→A substitution was confirmed in exon 5 at nucleotide position 819. The sequence of the other 7 exons was normal compared with the consensus sequence.

Direct sequencing of exon 3 of all family members revealed the presence of the $G^{460}\rightarrow C$ substitution in the father (I_1) and one brother (II_2) (Fig. 2). The novel missense mutation was then confirmed using mutagenic PCR. A mismatch primer was designed which introduces a restriction site for NlaIII in control DNA. However, when the $G^{460}\rightarrow C$ substitution is present, the NlaIII restriction site disappears (**Fig. 5**). Indeed, heterozygosity for the $G^{460}\rightarrow C$ substitution in the proband, the father (I_1), and brother II_2 was confirmed (Fig. 5).

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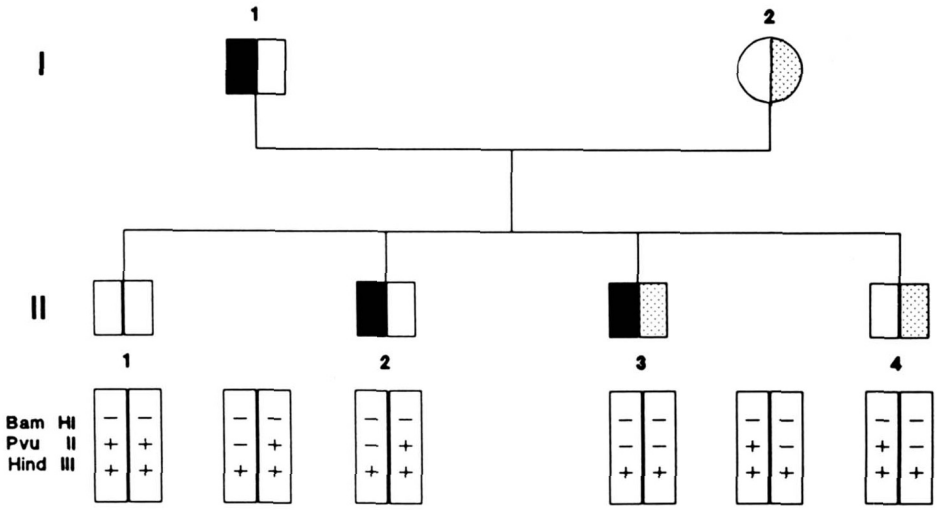

Fig. 2. Family tree combined with the haplotypes for the BamHI, PuvII, and HindIII polymorphisms. Black symbols indicate the presence of the C→G460 substitution, and stippled symbols indicate the presence of the G→C819 substitution.

In vitro expression of wild-type and mutant LPL

The index patient is a compound heterozygote for two different missense mutations; Gly^{188}→Glu and Val^{69}→Leu. The Gly^{188}→Glu substitution has been extensively described in the literature, and the expression of the mutant LPL has been performed on several occasions (26, 30).

We therefore only tested the functional significance of the Val^{69}→Leu substitution by expressing the mutant protein in COS-cells and subsequently testing the produced protein with regard to LPL activity and mass. The results of these experiments are shown in **Fig. 6.** COS-cells transfected with wild-type LPL showed a normal LPL activity in the medium and cell homogenate. The Val^{69}→Leu mutant had a significantly decreased activity, with residual activity approximately 20% of LPL enzyme activity (Fig. 6A). The LPL mass from the mutant in the medium and cell homogenate was comparable to the wild-type protein, indicating that the protein was normally synthesized and secreted (Fig. 6B). We determined that the specific activity of the Val^{69}→Leu mutant in the medium from four consecutive transfections was 26 ± 4% of wild-type LPL. Finally, we established the stability of the Val^{69}→Leu mutant using a method described previously (31). This includes incubation of the growth medium with increasing concentrations of GuHCl, and by a time incubation at 37°C. Both experiments revealed a stability of the mutant virtually identical to the wild-type-expressed LPL (data not shown). We therefore concluded that the mutant protein was synthesized at a normal rate, and had a significantly lower, but not absent, catalytic activity.

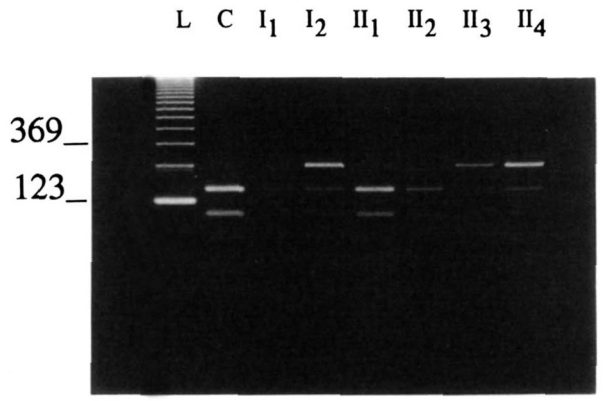

Fig. 3. Detection of the G→C819 substitution with AvaII of PCR-amplified exon 5 of all family members. The expected fragment sizes for control exon 5 are 152, 87, and 35 bp, respectively. The G→C819 substitution results in the loss of an AvaII site, giving a 239 and a 25 bp band. C, control exon 5; L, 123 bp ladder.

DISCUSSION

This study demonstrates compound heterozygosity for a common and a novel missense mutation in the LPL gene as the cause of LPL deficiency and hyperchylomicronemia in a kindred of mixed Indonesian and Dutch descent. The loss of one AvaII restriction endonuclease site in exon 5 of genomic DNA of the proband is caused by a single basepair substitution (G→A) at nucleotide position 819. This missense mutation predicts an amino acid substitution of glycine to glutamic acid (Gly^{188}→Glu) at residue 188. The index patient also possessed a nucleotide substitution in exon 3 (G^{460}→C), predicting an

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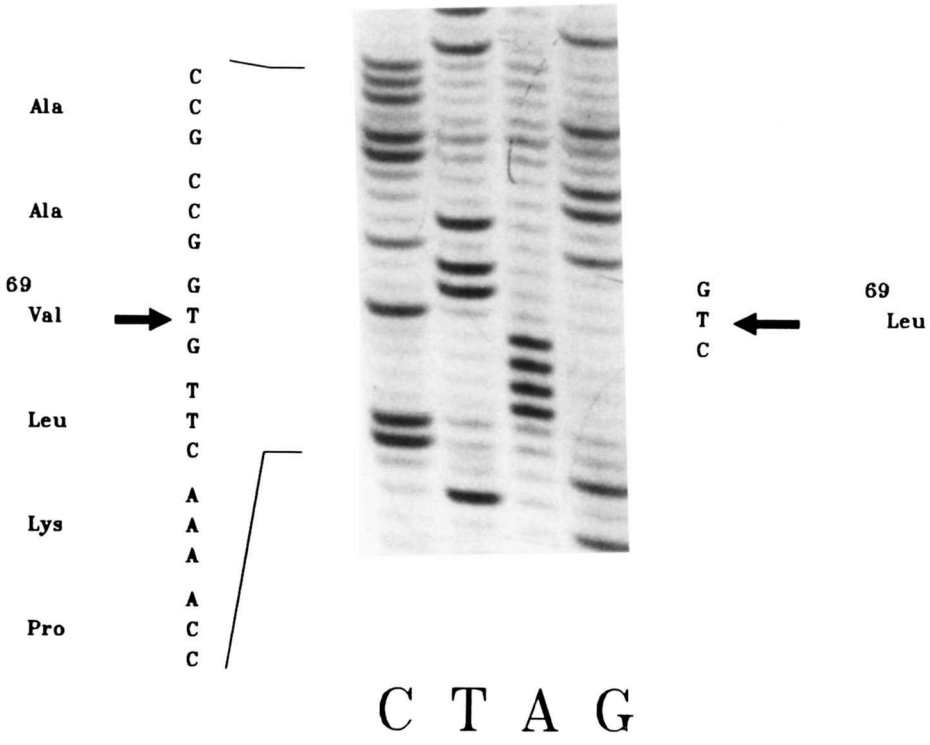

Fig. 4. Sequence ladder of PCR-amplified exon 3 from the proband. The proband is heterozygous for a G to C substitution at position 460, resulting in the Val[69]→Leu substitution. The sequence was run on a 6% acrylamide/8 M urea gel.

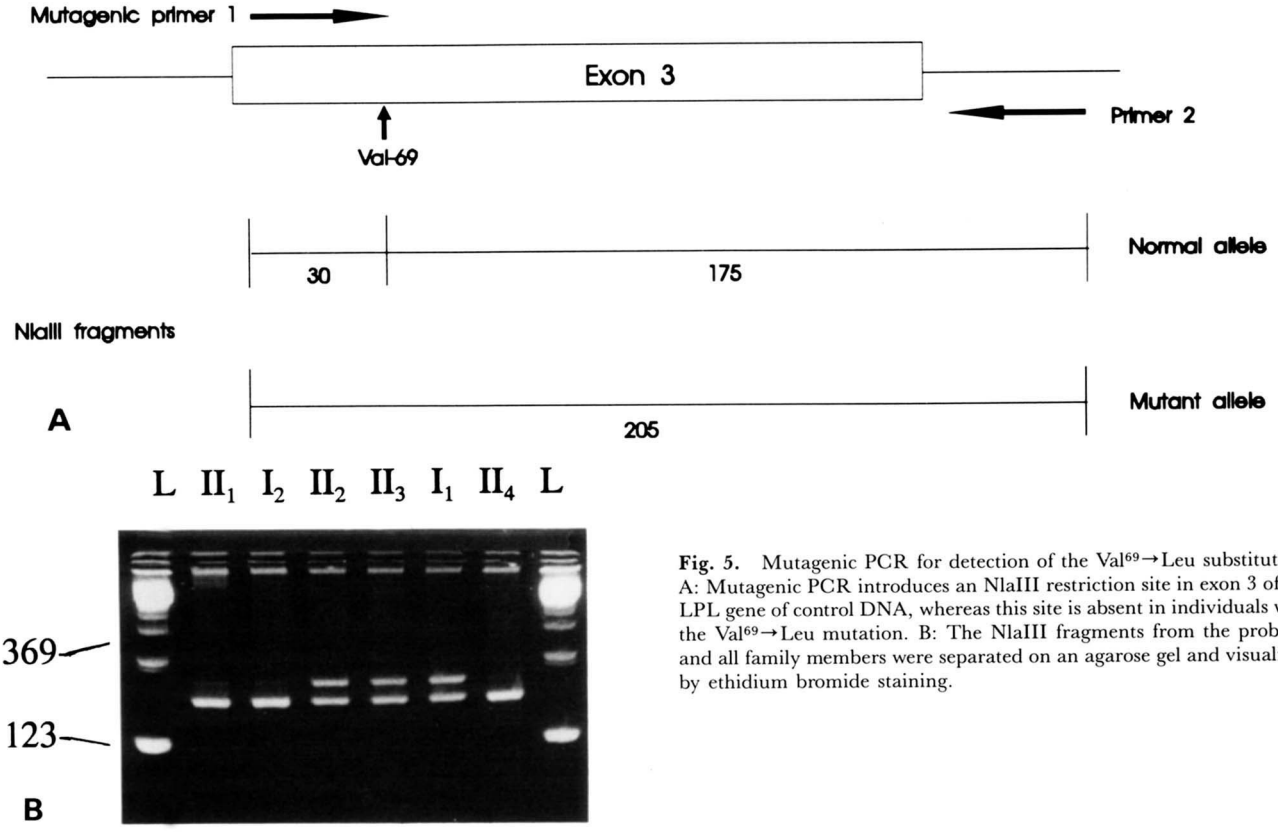

Fig. 5. Mutagenic PCR for detection of the Val[69]→Leu substitution. A: Mutagenic PCR introduces an NlaIII restriction site in exon 3 of the LPL gene of control DNA, whereas this site is absent in individuals with the Val[69]→Leu mutation. B: The NlaIII fragments from the proband and all family members were separated on an agarose gel and visualized by ethidium bromide staining.

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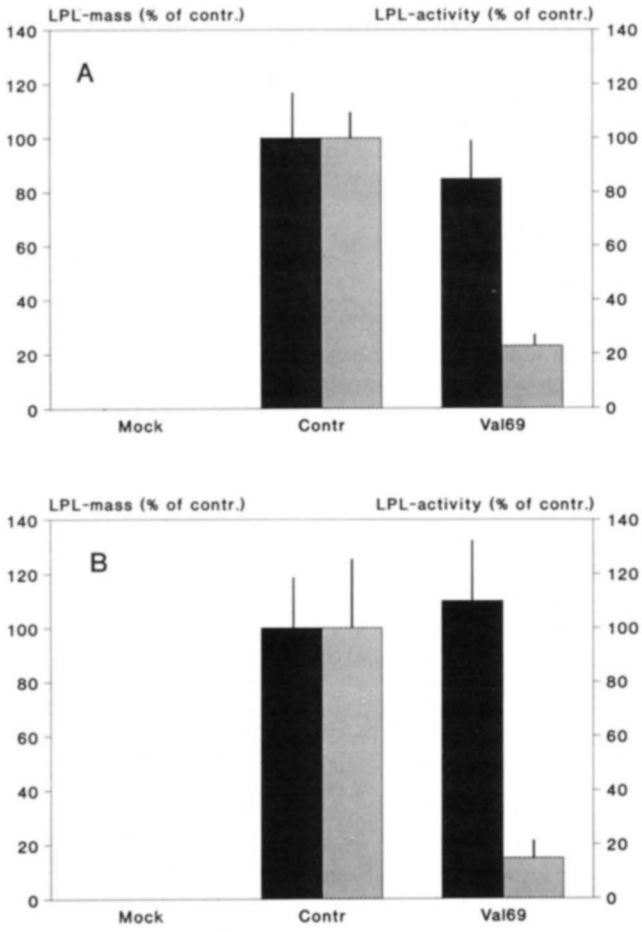

Fig. 6. Transfection of COS-B cells with normal LPL-cDNA and Val^{69}→Leu-cDNA. Results are expressed as percentages of the levels determined in the medium and cell homogenate of COS-B cells transfected with the normal LPL cDNA. Data are averages of four transfection experiments. SD are given by error bars. A: LPL mass (black column) and activity (shaded column) in the growth medium. B: LPL mass (black column) and activity (shaded column) in the cell homogenate.

amino acid substitution of valine to leucine (Val^{69}→Leu) at residue 69.

In our other 16 LPL-deficient kindreds in the Netherlands, we did not observe the Gly^{188}→Glu substitution, revealing that, in contrast with other populations, only a minor proportion of LPL deficiency in our country is caused by this mutation. DNA haplotype analysis of all mutant alleles with the Gly^{188}→Glu substitution revealed the same haplotype as in our patient, i.e., BamH1-, PvuII-, HindIII+ (26). The demonstration of this single haplotype underlying all mutant alleles is suggestive of a common origin. The proband inherited the mutant Glu^{188} from his mother, who is of Indonesian extraction. Until now, the only non-West European people where this mutation has been detected, were of Indian descent. It, therefore, seems likely that the mutation in this Indonesian woman has a Caucasian, possibly Dutch, origin.

The Gly^{188}→Glu substitution in the LPL gene as a cause for LPL deficiency was discovered several years back (26, 30). This mutation lead to a completely catalytically defective enzyme. Heterozygosity for the Gly^{188}→Glu substitution, however, does not lead to LPL deficiency (32). Therefore, the other defect, the Val^{69}→Leu substitution, must also have an effect on the catalytic property of the LPL protein. The latter was confirmed by our in vitro mutagenesis experiments.

Valine^{69} is situated in a conserved region shared by lipoprotein and hepatic lipase (33). As this region, Val^{65} to Leu^{72}, contains mostly hydrophobic amino acids, it might play a role in the hydrophobic interactions within the protein. The difference in molecular structure between a valine and a leucine is relatively small. However, Val^{69} is in the center of an alpha-helix (alpha 2), and substitution for the slightly larger leucine may change the character of this helix. Furthermore, as the region in which the mutation occurred is evolutionary conserved, there are probably strict requirements for its spatial configuration. A valine to leucine substitution, therefore, may change the tertiary structure and subsequently alter the enzyme's function.

LPL activity in postheparin plasma was approximately 30% of control values. We confirmed these data by in vitro expression of the mutant DNA in COS-cells. We found that the in vitro expressed LPL carrying the Val^{69}→Leu substitution had a residual LPL activity of 20%. These data indicate that, unlike most of the amino acid substitutions in LPL, the Val^{69}→Leu mutation will not dramatically alter the tertiary structure of the protein. This is also supported by the finding that the Leu^{69}-mutant had the same stability as wild-type LPL. The residual postheparin plasma activity in our proband will probably only reflect the activity of the Leu^{69}/Leu^{69} dimer, because the Leu^{69}-mutant revealed a stability that is comparable to wild-type LPL. The Glu^{188}/Glu^{188} dimer is instable and has a low heparin binding capacity (30) and will therefore probably be rapidly cleared by the liver. The properties of the Glu^{188}/Leu^{69}-heterodimer cannot be predicted, but it is likely that this dimer also will be unstable.

The medical history of our patient has a few interesting aspects in relation to the molecular basis of his LPL deficiency and to the residual in vivo and in vitro LPL activity. The episodes of acute pancreatitis that occurred before the age of 24 were clearly related to nonadherence to the diet in combination with alcohol abuse. Unfortunately, data about lipid or lipoprotein levels during his childhood or adolescence are lacking. Since the time the patient came to our attention, his triglycerides dropped to unexpectedly low levels at out-patient control visits, sometimes even under 5 mmol/l, which are considered to be only moderately elevated. From 1984 onward, the proband was admitted twice to our hospital, both times with

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a diagnosis of acute pancreatitis. Both episodes occurred around the end of the year, when our patient, who is a professional cook, has to taste and prepare meals very rich in saturated fats and when alcohol is a customary companion at the dinner table. This suggests, therefore, that the clinical expression of the Glu^{188}/Leu^{69} genotype is largely influenced by environmental factors.

Thus, one could postulate that some defects in the LPL gene have a less severe phenotype in terms of triglyceride levels and that in this situation environment is necessary to precipitate severe chylomicronemia and pancreatitis. There are more examples of this phenomenon in patients with molecularly proven LPL deficiency. Recently, a female patient was described, homozygous for a $Ser^{172}\rightarrow$ Cys amino acid substitution. She developed massive chylomicronemia and pancreatitis in pregnancy (34). After gestation, triglyceride levels fell below 265 mg/dl on a fat-restricted diet and all symptoms subsided. Another study, performed in a large family with phenotypic expression of NIDDM and chylomicronemia, showed that diabetic heterozygotes for LPL deficiency are at risk for severe lipemia (35). It is very likely that in the near future more defects in the LPL gene will be discovered that cause moderately elevated triglyceride levels and a less severe clinical picture. At the present time it cannot be excluded that a proportion of hypertriglyceridemia in the general population is caused by defects in the gene coding for lipoprotein lipase. This suggestion is supported by a recent finding that up to 30% of severe hypertriglyceridemic subjects may have functionally defective LPL (36).

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