$Trp^{64} \rightarrow$ nonsense mutation in the lipoprotein lipase gene

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Abstract A lipoprotein lipase (LpL) gene defect has been identified, a G → A transition at nucleotide position 446 of exon 3, resulting in a premature termination codon (Trp → stop) at amino acid residue 64. This defect was identified in a Type I hyperlipoproteinemic subject with an amino acid residue 194 defect in the other allele. Plasma lipoprotein values as well as LpL mass and activity in postheparin plasma were determined in the subjects with the residue 64 defect and in other LpL-deficient heterozygotes. LpL mass levels in both the Type I and the other subject with a 64 LpL defect were markedly reduced. This may be explained by rapid degradation of LpL protein or decreased secretion from the 64 defective allele. Alternatively, the marked reduction or absence of mass associated with the 64 defect may be due to synthesis of a severely truncated protein which escapes immunologic detection.—**Sprecher, D. L., J. Kobayashi, M. Rymaszewski, I. J. Goldberg, B. V. Harris, P. S. Bellet, D. Ameis, R. L. Yunker, D. M. Black, E. A. Stein, M. C. Schotz, and D. A. Wiginton.** $Trp^{64} \rightarrow$ nonsense mutation in the lipoprotein lipase gene. *J. Lipid Res.* 1992. **33:** 859–866.

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Lipoprotein lipase (LpL) deficiency is characterized by hyperchylomicronemia, which is often accompanied by lipemia retinalis, pancreatitis, and eruptive xanthomas (1). A number of genetic defects within the coding region of the LpL gene leading to catalytically inactive protein have been reported (2–15). Many of the defects have been found in the middle portion on the LpL molecule, a highly conserved region among different species and the lipase gene family (16–18).

Mature LpL protein contains 448 amino acid residues, encoded in 10 exons in a gene which is over 30 kb in length (19, 20). The protein is a trypsin-like serine protease, whose catalytic activity is potentially associated with the three-dimensional structure of an Asp-His-Ser triad. A similar arrangement of this catalytic triad is found in the crystalline structure of pancreatic lipase (21). Ser^{123}, in exon 4, has been shown to be the critical residue for LpL catalytic function (22).

In this report, an LpL protein defect resulting from a premature termination codon is presented, and the defects observed in the nuclear family are characterized. LpL mass levels among the two subjects with the 64 defect are quantitated by immunologic assay in postheparin plasma utilizing a monoclonal antibody, and corroborated using a polyclonal assay.

METHODS

Subjects

A Type I subject (MP) and two obligate heterozygotes (MoP and MiP) were previously identified (23, **Fig. 1**) in a family with no known consanguinity. MP was noted to have severe hypertriglyceridemia (> 20,000 mg/dl) prior

Abbreviations: LpL, lipoprotein lipase; PHLA, postheparin lipolytic activity; 64/194, a 64 defect in one allele and a 194 defect in the other allele; 64/normal or 64/N, a 64 defect in one allele and no known defects in the other allele; wt, weight; ht, height; kb, kilobases; bp, base pair; TG, triglyceride; HDL-c, high density lipoprotein cholesterol; PCR, polymerase chain reaction; apo, apolipoprotein; HRP, horseradish peroxidase; PBS-BT, PBS-3% BSA-1% Triton X-100; PBS-Tween, PBS-0.05% Tween; HTGL, hepatic triglyceride lipase; OPD, *o*-phenyldiamine; SD, standard deviation; PHP, postheparin plasma; cpm, counts per minute.

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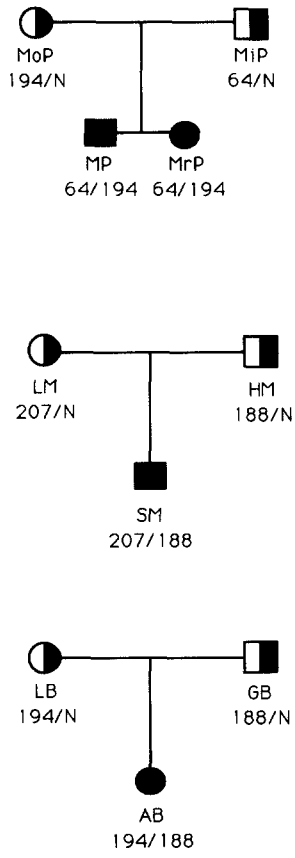


Fig. 1. Pedigree maps indicating the location of the respective defect(s) in the LpL gene for each of the reported families. Filled symbols represent compound heterozygotes and half-filled symbols represent heterozygotes.

to one year of age. The diagnosis of LpL deficiency was confirmed based on the lack of LpL activity (postheparin lipolytic activity [PHLA]), presence of apoC-II, and the typical lipoprotein pattern. MP has had eruptive xanthomas, and recurrent but mild abdominal pain. The mother, MoP, was diagnosed as having systemic lupus erythematosis (SLE) during the past year and placed on systemic corticosteroids. Her renal and liver function tests have remained normal. LpL mass and activity assays were performed with samples obtained before and after the SLE diagnosis with similar results. MiP, the father, was healthy. Another Type I subject, MrP, sister of MP, was also identified but was considered too young to undergo complete evaluation.

In order to compare clinical and biochemical expression of LpL defects, we studied two other families with LpL gene defects. One family, SM (son), LM (mother), and HM (father), was recently reported (23); the other family, AB (daughter), LB (mother), and GB (father), has not been previously reported (Fig. 1). AB is a Type I subject identified at age 5 weeks by her lipemic serum (triglycerides >17,000 mg/dl), presence of apoC-II, and low postheparin plasma LpL activity. LB and GB are healthy adults.

Lipid analyses were performed in an NHLBI-CDC standardized laboratory (23a) using microenzymatic procedures as previously described (24). ApoB-100 and apoA-I analyses were performed by competitive ELISA using monoclonal antibodies (antibody specific for apoB-100) in plasma, with a coefficient of variation of approximately 7% for both (24a–26). Baseline lipid, lipoprotein, and apolipoprotein values for all subjects are given in **Table 1**. Both MP and AB are well controlled on an extremely low fat diet at home, resulting in lower baseline triglyceride values than those characteristic of a Type I patient (Table 1). Among heterozygous subjects, HM has the most elevated triglyceride, total cholesterol, and apoB levels, having been characterized in the past as a type V hyperlipidemic subject. All heterozygotes were in the bottom decile for HDL-c levels according to LRC tables except MoP, who was in the bottom quartile (27).

DNA analysis

LpL gene defects for each compound heterozygote were determined or confirmed by sequencing PCR amplified exons of their genomic DNA. A combination of sequencing, restriction endonuclease digestion, and oligonucleotide hybridization was then used to ascertain the defect in the parents (obligate heterozygote) of each proband.

Sequencing. Direct sequencing was performed on exons 1 through 9 of MP according to the procedures described by Ameis et al. (10). Results for exons 3 and 5 were corroborated according to the M13 cloning and sequencing procedure described below. This procedure was also used for sequencing the alleles of patients AB and SM.

Oligonucleotide primers (9, 10, 20) complementary to the intronic regions flanking each exon were used for amplification. They contained EcoRI and BamHI restriction enzyme recognition sites later used in forced cloning and sequencing. The reaction conditions for PCR were: denaturation at 94°C for 1 min, annealing at 60°C for exon 3 and 64°C for exon 5 for 30 sec, and extension at 72°C for 2 min. This reaction was performed for 30 cycles on a DNA thermal cycler (Perkin-Elmer-Cetus Corp., Norwalk, CT) (28).

Amplified fragments containing exons 3 and 5 were digested with BamHI and EcoRI and forced cloned into BamHI/EcoRI cut M13mp18 and M13mp19. Clones were sequenced by a quasi-end labeling adaptation of the dideoxy-chain termination method (29). Six independent clones of each exon, representing both forced cloned directions of each allele of patients MP, AB, and SM, were isolated and analyzed.

Dot-blot hybridization. To determine whether the heterozygous subjects have the mutation in one allele, dot-blot hybridization was carried out using 50 ng of amplified exon 3 or exon 5 DNA. The DNA was transferred to MA-

TABLE 1. Subject data

Subjects	Age	Sex	Quetelet	TC	TG	LDL	HDL	ApoB	ApoA-I
	yr		*wt/ht² × 1000*			*mg /dl*			
Residue 64-defective									
MiP	23	M	2.52	153	121	97	32	94	91
MP	3	M	1.68	80	234	18	15	76	77
Heterozygotes									
MoP	22	F	2.20	192	91	134	40	120	107
GB	30	M	2.11	199	116	146	30	105	102
LB	28	F	2.03	176	146	115	32	114	93
LM	40	F	2.30	180	163	133	38	81	213
HM	46	M	3.20	243	554	145	25	196	96
Compound heterozygotes									
AB	5	F	1.46	114	429		13	94	74
SM	13	M	2.32	288	1612	20	11	73	84

Abbreviations: TC, total cholesterol; TG, triglycerides; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; ApoB, apolipoprotein B; ApoA-I, apolipoprotein A-I.

GNA nylon (MSI) membranes in duplicate and immobilized using UV crosslink. The DNA was then hybridized with a ^{32}P end-labeled oligomer corresponding to the normal or mutant cDNA. Normal and mutant oligomers, respectively, were 5'-TTTTGGCACCCAACTCTCATA-3' and 5'-TATGAGAGTTAGGTGCCAAAA-3' for the residue 64 substitution; 5' -TGGATTCCAATGCTTCG AC-3' and 5'-GTCGAAGCACTGGAATCCA-3' for the residue 194 substitution; 5'-CATTTACCCGAATGG AG-3' and 5'-CATTTACCTGAATGGAG-3' for the residue 207 substitution.

The membranes were prehybridized for 1 h at 48°C in 5 × Denhardt's solution (0.1% [w/v] Ficoll, 0.1% [w/v] polyvinylpyrrolone, 0.1% [w/v] bovine serum albumin), 0.5% SDS, 5 × SSC (3 M NaCl, 0.3 M Na citrate, pH 7), and 100 μg/ml denatured herring sperm DNA. After removing the prehybridization solution completely, the same hybridization solution was added with a probe concentration of 2 × 10^6 cpm per ml. The samples were hybridized 2 h at 48°C. The membranes were washed under the following conditions: buffer 1 (2× SSPE, 0.5% SDS) for 5 min at room temperature, in buffer 2 (1× SSPE, 0.25% SDS) for 15 min at room temperature, and in buffer 3 (0.1% SSPE, 0.1% SDS) for 30 min at 42°C. The membranes were autoradiographed at −70°C in a cassette with an intensifying screen, and were developed after a 2–4 h exposure (8).

LpL activity and mass

PHLA. After a 12-h fast, blood was drawn just before and 15 min after administration of heparin (50 U/kg) (Elkins-Sinn, Inc., Cherry Hill, NJ). The method described by Baginsky and Brown (30) was used for the assessment of lipase activity. LpL activity was determined by inhibiting hepatic triglyceride lipase (HTGL) activity with goat anti-human HTGL serum. Lipase assays for subject LM and HM were performed by Anh Le, Medlantic Research, Washington, DC, using the same methodology.

Mass assays. LpL mass was assayed in 0 and 15 min postheparin serum aliquots by the enzyme-linked immunosorbent assay (ELISA) method. Ninety-six-well microtiter plates were incubated overnight with 1 μg/ml monoclonal anti-bovine LpL IgG (Oncogene Science, Manhasset, NY) in borate buffer, pH 10. The samples diluted in cold PBS–3% BSA–1% Triton X-100 (Kodak) (PBS-BT) were added and left 18 h at 4°C. After washing with PBS-Tween (0.05% Tween-Sigma) chicken anti-bovine LpL IgG (10 μg/ml) in PBS-BT was added. After a second overnight incubation at 4°C plates were washed, incubated for 2 h at room temperature with a 1:2500 dilution (in PBS-Tween) of goat anti-chicken HRP antibody (Kierkegard & Perry, Gaithersburg, MD) and developed using *o*-phenyldiamine as described previously (31). The standard curves for bovine LpL and human postheparin plasma are shown in **Fig. 2.**

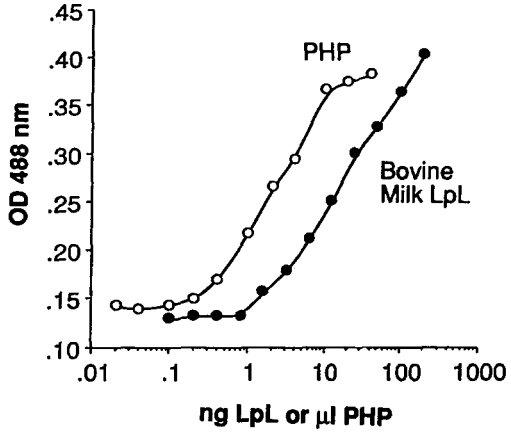


Fig. 2. LpL ELISA. Shown are titration curves for bovine milk LpL and human postheparin plasma (PHP) LpL using the double antibody ELISA described in Methods. Each data point represents the mean of triplicate measures. The horizontal axis represents the mass or volume amount of bovine LpL or standard PHP, respectively, added to the reaction mixture for a final volume of 200 μl. The absorbance was read at a wave length of 488 nm. The data on the parallel portion of the curves were used for obtaining plasma LpL mass from PHP of the subjects.

Normolipidemic male subjects were recruited from a first year medical school class at Columbia University. Postheparin plasma from 20 such subjects were assayed for LpL mass, while postheparin plasma from 51 of the same normolipidemic population was assayed for LpL activity (Table 2).

A truncated LpL molecule would not be detected using the assay presented, since the primary capture-antibody is a monoclonal antibody directed towards the carboxy terminal end of the LpL protein (32). A truncated LpL protein would probably, however, be detected by the double polyclonal ELISA method of Goers et al. (33). Some samples were also measured, therefore, with this polyclonal assay. This was virtually always performed on blood from a separate sampling. Five normal controls were selected from the University of Cincinnati Lipid Research Clinic study population, and evaluated with this alternate polyclonal LpL mass assay.

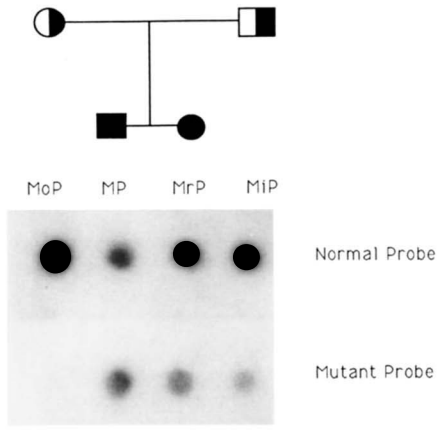
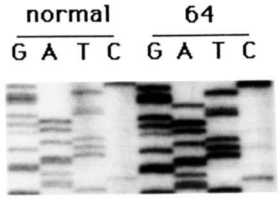


Fig. 4. Dot-blot hybridization of amplified exon 3 DNA for MoP, MP, MrP, and MiP. The upper row of dots was hybridized with normal oligomer, while the bottom row was hybridized with a mutant oligomer, corresponding to a defect within amino acid residue 64 (see Methods). MP, MrP, and MiP are heterozygotes for the 64 defect, while MoP is not.

RESULTS

DNA analysis

Direct sequencing showed a G → A transition at base pair (bp) 446 in exon 3 of MP and MiP which was confirmed with M13 sequencing. This transition was determined to be present in four of six M13-exon 3 clones from MP DNA, corresponding to the substitution of a stop codon for tryptophan (TGG → TAG) at amino acid residue 64 (**Fig. 3**). Direct sequencing of the other allele in MP, the proband, demonstrated a T → C transition at bp 836 in exon 5. This corresponds to the substitution of a threonine for isoleucine (ATT to ACT) at amino acid residue 194 (23). MoP (mother) and MrP (sister) also had the 194 defect established by sequencing M13 clones containing the exon. Direct sequencing did not reveal any further defects in exons 1 through 9 of MP. A dot-blot with amplified exon 3 DNA from MiP, MP, and MrP



64 ···· TAACAGGAATGTATGAGAGTTAGGTGC ····
normal ···· TAACAGGAATGTATGAGAGTTGGGTGC ····

Fig. 3. Sequence analysis, using M13 cloning methodology, of exon 3 of both alleles of the LpL gene in patient MP. Autoradiographic bands representing genomic residues corresponding to LpL cDNA residues 425-451 are shown, with a mutation (*) observed at 446. The TGG to TAG change leads to substitution (amino acid residue 64) of a "stop" codon for tryptophan. The mutation in the other allele was determined to be a substitution of Thr for Iso at residue 194 (not shown) in exon 5.

(MP's Type I sister) showed equivalent hybridization with normal and mutant "64" oligomers, suggesting that one of the two alleles in each case was hybridized. This defines a heterozygote. The dot-blot for MoP hybridized only with the normal oligomer (**Fig. 4**). Therefore, she does not have the defect at amino acid residue 64.

The substitution of Leu for Pro in SM at amino acid residue 207 (bp 875, C → T) is illustrated on an M13 sequencing gel in **Fig. 5.** This was found in three of six clones in SM. The mutation that causes an amino acid substitution at residue 188 in the other allele of SM (23) is also shown in Fig. 5. This substitution of Glu for Gly at amino acid residue 188 (bp 818, A → G) was also present in one allele of AB (**Fig. 6**). The other allele had the 194 defect described above (Fig. 6). Thus, all three Type I subjects represent compound heterozygotes. LB had the 194 defect, and LM the 207 defect, determined by equivalent hybridization to amplified exon 5 DNA with normal and mutant "194" and "207" oligomers (data not shown). Thus, both are heterozygotes. PCR-amplified exon 5 genomic DNA was digested with AvaII. Electrophoretic separation of bands by agarose was used to examine for presence or absence of the published Gly to Glu mutation at amino acid residue 188 (34). The G to A transition causing this mutation eliminates an AvaII site in exon 5. The presence of a 120 bp AvaII fragment in addition to the normal 80 bp fragment for HM and GB was diagnostic of the residue 188 mutation in one allele of these heterozygotes (not shown).

LpL activity and mass

LpL activity and mass for each subject are presented in **Table 2** along with the corresponding LpL defects. LpL mass was low in the subjects with the Trp^{64} → stop substitution (MiP and MP) and those with the Leu → Pro

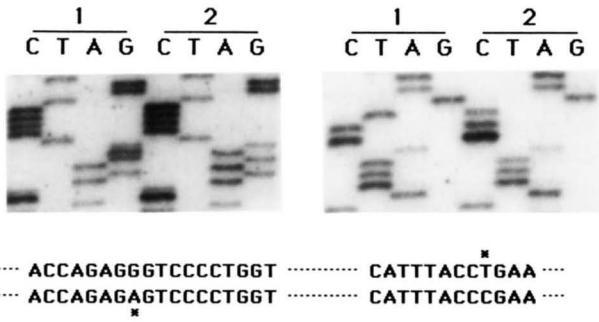


Fig. 5. Sequence analysis of exon 5 of both alleles, 1 and 2, of the LpL gene in patient SM. Identical results were found in a Type I brother, DM. Autoradiograms of sequencing gels derived from M13 clones of both alleles are shown with bands corresponding to genomic residues in two regions in exon 5. The residues correspond to LpL cDNA residues 811–828 (right), with mutations (*) observed at 818 (allele 2) and 875 (allele 1). The GGG to GAG change results in change of glycine to glutamic acid (amino acid residue 188 in three clones) and the CCG to CTG change results in a change of proline to leucine (amino acid residue 207 in three clones).

substitution at amino acid residue 207 (LM and SM). Heterozygotes MoP and LB both have the 194 defect and had high mass levels. GB (188/N) had normal levels, while AB (188/194) mass levels were low-normal. LpL activity was normal to subnormal in all heterozygote subjects. HM and LM LpL activities were 3.5 and 3.2 μmol/ml per h, respectively. HM mass levels were not evaluated with the monoclonal assay system. By definition, the Type I subjects (compound heterozygotes) had little to no in vitro LpL activity.

Postheparin plasma from five normal subjects was evaluated for LpL mass using the polyclonal assay. The mean ± SD was 491 ± 220 ng/ml. With this assay, MiP (64/N) had a mass level of 125 ng/ml, while MP (64/194) had a mass level of 250 ng/ml. LM (207/N) and SM (188/207) had mass levels of 186 and 54 ng/ml, respectively. MoP (194/N) had levels of 1330 ng/ml. The results from the monoclonal assay were very similar to those of the polyclonal assay. Preheparin levels in the monoclonal assay were all less than 50 ng/ml. These low levels were below the reliability of the assay. All LpL mass levels in postheparin plasma were significantly higher than that measured in preheparin plasma.

DISCUSSION

We describe an LpL mutation not previously reported, a G → A (bp 446) transition leading to a premature termination codon at amino acid residue 64. This Type I subject is a compound heterozygote with alterations at amino acid residues 64 and 194. The father is a 64/normal genotype, while the mother has a 194/normal genotype. LpL masses in postheparin plasma from both the 64/normal heterozygote and 64/194 compound heterozygote were lower than in normals or subjects heterozygous for the 194 defect.

As recently reported (12, 15, 34), at least 19 LpL defects have been described. Four of these are located in the third exon, including a splice site modification proximal to residue 57 (gG → aG), two termination codon defects at residues 61 and 106, and a frameshift mutation at amino acid residue 102. Tryptophan, located at amino acid residue 64, is conserved among the three lipases (lipoprotein lipase, hepatic lipase, and pancreatic lipase), as well as among LpL from different species (mouse, bovine, and human cDNA) (16, 19). Exon 3 has the second most frequent number of described defects, with seven defects reported in exon 5. The Trp^{64} → nonsense mutation described herein increases the number of reported exon 3 defects to five. This further establishes exon 3 and the preceding intron as a major site for defects localized in the LpL gene.

Several LpL gene defects have been reported in which the LpL protein may be secreted in substantially truncated form (2, 5, 7, 8, 15). Even though no mass data are available for some (5, 7), mass measurements have been reported for others, with low to absent levels of LpL protein in these cases. Henderson et al. (8) reported on a frameshift mutation at amino acid residue 102 and a resulting nonsense mutation in exon 4, producing a 146 amino acid fragment, 44 residues of which are the result of random DNA sequence. Postheparin plasma LpL mass in this Type I homozygous subject is very low, equivalent to normal preheparin levels. A nonsense mutation at residue 61 (Tyr → stop) in a homozygous subject resulted in absence of LpL mass in postheparin plasma, using a polyclonal antibody (15). The same antibody, when used to analyze plasma from a subject with a defect at residue 382 (Trp → stop) in exon 8, revealed a postheparin mass level of 22 ng/ml, markedly below the reported mean of

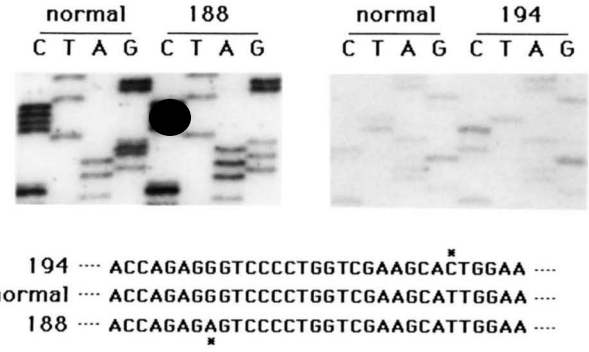


Fig. 6. Sequence analysis of exon 5 of both alleles of the LpL gene in patient AB. Autoradiograms of sequencing gels derived from M13 clones of both alleles are shown with bands corresponding to genomic residues in a similar region in exon 5. The residues correspond to LpL cDNA residues 811–841 with mutations (*) observed at bp 818 and 836, corresponding to amino acid residues 188 (left) and 194 (right), respectively.

TABLE 2. Lipoprotein lipase activity/mass

Subjects	Defect[a]	Lipase Activity	Mass 15 min[b]
		μmol/ml/h	*ng/ml*
Residue 64-defective			
MiP	64/N	5.9	250
MP	64/194	0.0	228
Heterozygotes			
MoP	194/N	5.7	1300
GB	188/N	3.4	650
LB	194/N	2.3	1150
LM	207/N		150
Compound heterozygotes			
AB	188/194	0.0	375
SM	188/207	0.0	195
Controls		9.8 ± 9 (n = 51)	665 ± 363 (n = 20)

[a]Both alleles are represented, N, normal.
[b]All 0 minute mass assays were less than or equal to 50 ng/ml.

410 ± 96 ng/ml (15). As in the case of the splice site defect in the first nucleotide of intron 2, where a reduced message RNA level was detected in macrophages (35), loss of exons or severe truncations may alter protein concentration by reduction in the synthesis of the transcript, rapid intracellular degradation of mRNA, or intracellular degradation of the resulting protein fragment (2, 36). Alternatively, small protein fragments may not be detectable with the antibodies.

The 5D2 monoclonal antibody used in our study (37) is directed at determinant(s) in the carboxy terminal end of LpL, thereby making it incapable of detecting markedly truncated protein (32). LpL mass detected in the 64-defective subjects can therefore only reflect mass derived from the alternate allele. In contrast, a polyclonal assay system should recognize truncated species. However, a postulated 64 amino acid plasma LpL fragment is only 14% of the original protein size. A polyclonal antibody would probably detect, at best, a small proportion of the determinants compared to that of the normal protein, which would result in a low apparent protein concentration. Thus, the similar mass levels for the 64-defective subjects derived from the assay using both the monoclonal as well as the polyclonal antibody may still be consistent with the presence of the foreshortened LpL protein in the plasma. However, the loss of the heparin binding site, the similarity between results obtained from both antibodies, and the reported frequent intracellular degradation of markedly truncated protein argue against a substantial presence of the small fragment in plasma. In vitro expression systems with newly developed antibodies directed at the amino terminal end could further elucidate the processing of truncated proteins.

LpL mass concentrations in postheparin plasma for heterozygous and compound heterozygous subjects with different defects were consistent with levels reported previously by other investigators (6, 9, 11, 13, 14). Subjects with the 194-defect had normal or even high plasma LpL mass (11, 13), while subjects with the 207-defect had lower postheparin LpL mass concentrations (14). Subjects with the 188-defect have low to normal mass levels (6, 9), as observed in the one 188-defective heterozygous subject presented in Table 2.

The LpL-deficient heterozygotes in this current report have lower HDL-c levels, a finding consistent with two previous studies (37, 38). Heterozygous LpL deficiency has been associated with both high TG and low HDL-c (14, 37, 38) as well as with the expression of familial lipid patterns similar to familial combined hyperlipidemia (37). In one report on LpL-deficient heterozygotes, hypertriglyceridemia and low HDL-c were significantly associated with the LpL-defective trait only in subjects over the age of 40 years (38). Postheparin LpL activity (39) as well as DNA polymorphism haplotypes of the LpL gene have been correlated with plasma HDL-c concentrations (40). It currently remains unknown whether a significant subset of hypoalphalipoproteinemic subjects have defects in the LpL gene, and further whether this subset is prone to vascular disease.

In summary, we report an LpL defect not heretofore, described, leading to a premature termination codon at amino acid residue 64. The defect is found in the proband, a Type I compound heterozygote, and his father. This increases the number of exon 3-associated defects to five, and further indicates that this region in the LpL gene is important in screening for LpL structural defects. The LpL postheparin mass recognized by immunologic assay in subjects with the 64-defect is low, probably due to decreased synthesis or lack of immunologic recognition of a truncated LpL protein.

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