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A naturally occurring mutation at the second base of codon Asparagine 43 in the proposed N-linked glycosylation site of human lipoprotein lipase: In vivo evidence that Asparagine 43 is essential for catalysis and secretion

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The patient was a 20-year-old male. His fasting plasma triglyceride and cholesterol levels were 1258 mg/dl and 138 mg/dl, respectively. The lipoprotein lipase(LPL) activity and mass from postheparin plasma of the patient were 0.00 μmol/ml/h (normal range: 5.51±1.12) and 23 ng/ml (normal range: 220±42), respectively. DNA sequence analysis of the LPL gene from the patient revealed a homozygous nucleotide change: a A → G transition at nucleotide position 383, resulting in an amino acid substitution of Ser for Asn[43], which is believed to be an N-linked glycosylation site of the LPL mature protein. Expression studies of this mutant LPL cDNA produced an inactive LPL protein which was not secreted into the media.

Lipoprotein lipase (LPL) is an endothelial enzyme responsible for the hydrolysis of the triglyceride components of circulating chylomicron and very low density lipoproteins(VLDL)(1).

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The enzyme is glycosylated, a post-translational modification which may be critical for normal activity (2). Patients with lipoprotein lipase(LPL) deficiency usually have fasting plasma triglyceride levels above 1500 mg/dl. They usually present in childhood with abdominal pain, recurrect attacks of pancreatitis, hepatosplenomegaly, lipemia retinalis and eruptive xanthomas. The diagnosis of LPL deficiency is established by the absense of LPL enzymic activity in postheparin plasma in the presence of an exogenous source of apolipoprotein CII. Recently using molecular biological techniques (3-14), many LPL deficient patients have been shown to have various types of mutations.

Among these alterations, missense mutations appear to yield important information concerning structure-function relationships in the LPL molecule. Because LPL is a glycoprotein, carbohydrate seems to be required for the production of active LPL (15).

A number of reports suggest that the N-linked glycosylation is essential for activation and secretion of lipoprotein lipase(2,16,17). Among them, Semenkovich et al.(17), using in vitro expression of site specific human LPL mutant in Cos cells, found that the loss of Asn^{43}, which is believed to be N-linked glycosylation(18), results in production of inactive LPL enzyme protein which is not secreted into the media.

In this manuscript, we present the first report of a natural mutation occurring in the potential N-linked glycosylation site of the LPL, a missense mutation (Asn^{43} to Ser) in the LPL gene from a LPL deficient patient, contributing to LPL deficiency.

Materials and Methods

Clinical data

The patient is a 20-year-old man who was diagnosed as an infant with chylomicronemia. Throughout childhood, the patient had recurrent abdominal pain and pancreatitis. Since infancy, the proband has been given a low fat diet and medium chain triglyceride (MCT) as treatment for hypertriglyceridemia. Upon

evaluation, the patient's fasting plasma triglyceride and cholesterol levels were 1258 mg/dl and 138 mg/dl, respectively. His plasma apolipoprotein CII level was 5.2 mg/dl. Diagnosis of LPL deficiency was established by the virtual absence of postheparin plasma lipoprotein lipase activity in the proband's plasma and the presence of normal plasma level of apolipoprotein CII. Conventional biochemical indices of renal, thyroid and hepatic function were normal.

Quantitation of lipoprotein lipase activity and mass

Plasma postheparin total lipase activity was measured 15 min after a heparin bolus injection(30IU/kg) after an overnight fast, using Triton X-100-emulsified triolein as previously reported (19). LPL activity was calculated as the activity in whole plasma inhibited by an anti-LPL monoclonal antibody(5D2) added to plasma (20). LPL mass was determined by a sandwich enzyme immunoassay(21).

Preparation of DNA and oligonucleotide primers

DNA was extracted from peripheral blood lymphocyte mononuclear cells. Buffy coat leukocytes were isolated from blood collected into tubes containing EDTA. Chromosomal DNA was released by lysis of cells in 10mM Tris-HCl,pH 8.0,150μM $MgCl_2$, 0.5% Triton X-100 and 1% SDS containing 500 μg/ml of proteinase K. Oligonucleotide primers were synthesized by the phosphoramidite method in a DNA synthesizer (model 380B; Applied Biosystem Inc.,Foster City,CA)

PCR amplification and direct sequencing determination of amplified DNA

Target DNA sequences were amplified (22) in a 100 μl reaction volume containing 0.5 μg of chromosomal DNA; 50 pmol of each oligonucleotide primer; 200μM each of dATP, dCTP, dGTP and dTTP; 1 x reaction buffer(50 mM KCl, 10 mM Tris-HCl, pH 8.4, 3 mM $MgCl_2$, 0.05% Tween 20, 0.05% Triton X-100, 200 μg/ml of gelatin); and 2 U of AmpliTaq DNA polymerase(Perkin-Elmer Cetus CA). Amplified DNA was desalted and excess oligonucleotide primers and didexynucleotide triphosphates were removed by electroelution using 1% agarose gels. Sequencing analysis was performed with gel-purified PCR products using the dideoxychain termination method(23).

Site-directed mutagenesis

Site-directed mutagenesis was performed by the overlap extension method in combination with the polymerase chain reaction(22). The mutant DNA generated by this method was then subcloned into pCMV-NL, thus replacing the normal sequence.
All constructs were characterized by sequence analysis of the complete LPL cDNA insert using the dideoxynucleotide termination methods of Sanger et al (23).

Transfection of the mammalian cells

The parent plasmid utilized in the transfection studies has been previously described(11). It contains a CMV promoter and transcriptional start site as well as the polyadenylation signal from SV 40 cloned into pUC18(24)(Fig. 1). A 1536 bp fragment of LPLc DNA that spanned the signal peptide through the termination codon (TGA) was cloned into HpaI and XbaI sites of the pCMV vector placing it under the control of pCMV promoter.
The constructs pCMV-NL and pCMV-43 containing the normal and the mutated LPL cDNA were characterized by DNA sequencing before transfection into human embryonal kidney-293 cells.
DNA transfections were performed as described (25), using a calcium phosphate coprecipitation method (26) modified by supplementing the medium with 2 U/ml heparin sulfate.

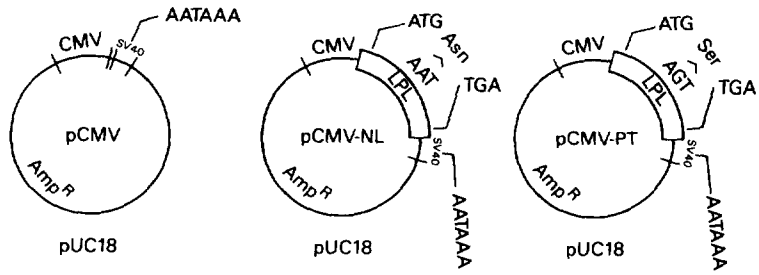

Figure 1. The plasmid constructs used for transient expression of normal and mutant LPL cDNA in human embryonal kidney-293 cells are illustrated. The normal LPL expression vector (pCMV-NL) contains a 1536-base-pair cDNA fragment of LPL inserted between the XbaI and HpaI cloning sites, placed under the control of the cytomegalovirus(CMV) promotor.

Results

LPL activity and mass from postheparin plasma of the proband (Table I)

The results of the quantification of LPL mass and activity from the postheparin plasma of the proband are shown in Table I. The LPL activity and mass from postheparin plasma of the proband were 0.00 μmol/ml/h (normal range: 5.51±1.12) and 23 ng/ml (normal range: 220±42),respectively, indicating that the patient has a functionally defective LPL which is not secrected.

Mutation analysis

To amplify LPL exons, we synthesized a pair of oligonucleotide primers specific for each exon. These primers are complementary

Table I

LPL activity and mass in the postheparin plasma of control subjects and the proband

	Activity (μmolFFA/ml/h)		Mass(ng/ml)
	HL	LPL	LPL
proband	7.52	N.D.	15.8
control	5.92±1.55	5.51±1.12	220±42

Data are mean ± SD, n=11.
LPL, lipoprotein lipase; HL, hepatic lipase.

to intron sequences flanking the individual exons. Using this approach, the coding exons of LPL and exon/intron junctions were amplified and sequenced. When compared to a control subject and to the LPL cDNA (18), no difference in nucleotide sequence was found in the DNA of the proband and control subject, except in exons 2 and 8. Figure 2 shows the result of the direct sequencing analysis for exon 2 in the LPL gene from the proband. The proband was found to have a homozygous point mutation in exon 2: an A to G transition at nucleotide position 383, resulting in an amino acid substitution of Ser for Asn^{43}, which is believed to be the potential N-linked glycosylation site of LPL. The proband was also found to have a heterozygous nucleotide change in exon 8: a

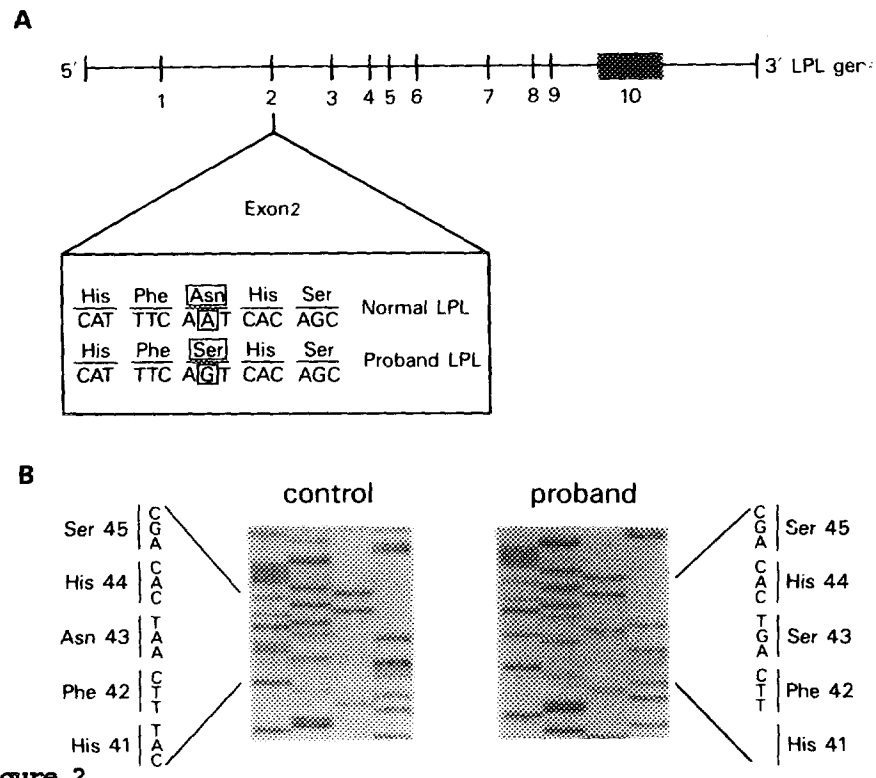

Figure 2.
A. Schematic representation of the LPL gene. Exons are illustrated by solid bars interrupted by lines that represent introns.
The A → G substitution in the patient's LPL sequence is highlighted by a box.
B. Autoradiograms of sequencing gels of DNA from a normal subject and the proband. The order of lanes of the corresponding autoradiogram is A, C, G and T.

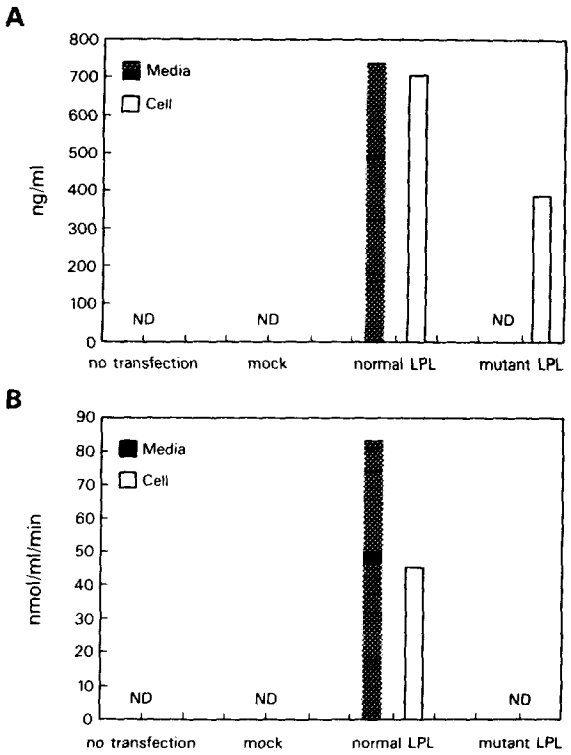

Figure 3.
Effect of substitution of serine for the potential N-linked glycosylation site asparagine-43 on LPL expression in human embryonic kidney-293 cells. Cells were transfected with pCMV vector, denoted mock, or with pCMV vector carrying the normal LPL cDNA, indicated as normal LPL, or with LPL cDNA containing the $Asn^{43} \rightarrow Ser$ mutation, denoted mutant LPL.
A. LPL mass in nanograms per milliliter as measured by an enzyme-linked immunosorbent assay.
B. LPL activity determination in nmoles/ml/min.
In both panels, LPL activity and mass were determined in cell homogenates (open bars) and tissue culture media (solid bars).
ND: not detectable.

C to A transversion at nucleotide position 1338 (the third letter of Thr 361) causing no amino acid change (data not shown).

Expression of and functional analysis of the mutant LPL (Fig.3)

To determine whether this amino acid substitution could contribute to the formation of a defective LPL, we expressed LPL cDNA with a A to G transition at nucleotide position 383 causing the amino acid substitution of Ser for Asn^{43} (Fig. 3) by site-directed mutagenesis. The activity and mass of the expressed LPL

were investigated in culture media and in cell extracts in embryonic kidney 293 cells transfected with LPL cDNA constructs. LPL activity was not detectable in these cells either with no transfection or with mock transfection.

However, cells transfected with wild type LPL contained significant amounts of LPL enzyme activity and LPL mass both intracellularly and in the culture medium. Substitution of Ser for the potential N-linked glycosylation site Asn^{43} completely abolished LPL enzyme activity. Considerable amount of LPL mass, on the other hand, was detected intracellularly but not detected in the culture medium.

Thus, as a model for this particular LPL deficient phenotype, we have shown by in vitro expression studies that substitution of Ser for the N-linked glycosylation site, Asn^{43} of human LPL produces an aberrant lipase which is catalytically inactive and is not secreted.

Discussion

We have studied the underlying molecular defects that lead to lipoprotein lipase (LPL) deficiency in a 20-year-old male. Analysis of LPL in the proband's postheparin plasma demonstrated markedly decreased LPL mass and no detectable LPL activity, indicating the presence of a catalytically inactive enzyme which is not secreted efficiently.

Sequencing analysis of the patient's LPL DNA demonstrated a homozygous missense mutation: an A → G transition at a nucleotide position 383 that results in the amino acid substitution of Ser for Asn^{43}. Transient expression of the mutant LPL cDNA in human embryonic kidney 293 cells resulted in the synthesis of an inactive enzyme, which was not secreted into the media. The Asn^{43} is believed to be a part of N-linked glycosylation site: Asn-His-

Ser (residue 43-45) of the human LPL (18). The functional significance of the N-linked glycosylation site, however, is still controversial. Hansson et al.(27) report that a glycosylation site is not essential for catalytic activity of human milk bile salt-stimulated lipase, the sequence of which is identical to that of pancreatic carboxylic ester hydrolase and shows only little homology to lipoprotein lipase. Stahnke et al.(28) report that N-linked glycosylation is essential for secretion but not needed for activation of the enzyme. They used site specific mutants involving the two N-linked glycosylation sites of rat hepatic lipase, which shows high homology with lipoprotein lipase. Recently, however, Wölle et al.(29) have reported that mutation of Asn^{56} to either Gln or Ala in human hepatic lipase, which is homologous to Asn^{43} in human LPL, led to the production of a totally inactive enzyme that accumulated intracellularly but was not secreted into the medium, strongly supporting the essential role of this conserved N-linked glycosylation site in human hepatic lipase, which is evolutionally related to LPL.

This result seems to be inconsistent with the result obtained by Stahnke et al. The use of different methods in the transfection and the estimation of enzyme activity would partially explain the discrepancy between those two studies.

Abouakil et al.(30) report that N-linked glycosylation is essential for secretion and expression of a fully active enzyme of bile salt dependent lipase in rat pancreas.

Several laboratories have suggested that in LPL, N-linked glycosylation is essential for the lipase activity by showing that LPL synthesized in the presence of tunicamycin, which blocks N-linked glycosylation, in rat preadipocytes(2),ob/ob adipocytes(31), or 3T3 adipocytes(16) is inactive. Olivecrona et

al. have further shown that the inactive LPL in tunicamycin-treated 3T3-L1 adipocytes accumulates intracellularly and is not secreted to the medium. Ong et al.(32) have found that cultured rat adipocytes deprived of glucose synthesized a non-glycosylated form of LPL that was not secreted and had no lipolytic activity. Ben-Zeev et al.(15) described glucose trimming of lipoprotein lipase as essential for enzyme activation. Using in vitro expression of a site specific human LPL mutant in Cos cells, Semencovich et al.(17) found that the loss of Asn^{43}, which is believed to be N-linked glycosylated, results in the production of an inactive LPL enzyme protein which is not secreted into the media. Those studies are consistent with the idea that in LPL, N-linked glycosylation is responsible for activation of the enzyme as well as its secretion.

In summary, the $Asn^{43} \rightarrow Ser$ mutation of the LPL gene in familial LPL deficiency described in this study is the first natural mutation involving the N-linked glycosylation site of LPL. The loss of both activation and secretion of the LPL strongly suggests that this site is important both for activation and secretion of this enzyme, resulting in LPL deficiency.

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