Mutational analysis of human lipoprotein lipase by carboxy-terminal truncation

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Abstract We have previously reported a Trp^{382} (TGG) → stop (TGA) mutation that causes familial lipoprotein lipase (LPL) deficiency. Expression study of the Trp^{382} → stop mutant showed that the truncated LPL was catalytically inactive with a marked reduction in the expressed mass. To investigate the minimal amino-terminal sequence of human LPL required for an active enzyme, a series of carboxy-terminal (C-terminal) truncated LPLs were expressed in vitro, and the enzyme activity, mass, and LPL mRNA levels were analyzed. The lipolytic activity showed a stepwise reduction between LPL-437 (Cys^{438} → stop; 68% of normal LPL activity in medium) and LPL-434 (Phe^{435} → stop; 3%). Without affecting LPL mRNA levels, LPL mass was reduced with the mutants not larger than LPL-437. In terms of specific activity, a significant difference was observed between LPL-436 (Lys^{437} → stop; 88% of that of normal LPL in medium) and LPL-435 (Val^{436} → stop; 22%), implying the importance of the role of Val^{436} in LPL action. Furthermore, our results unexpectedly showed that LPL-446 (Ser^{447} → stop), which is considered to be a common polymorphic form of LPL, exhibited higher activity than normal LPL (185% in medium). These results demonstrate that the C-terminal region of human LPL is closely associated with the expression of enzyme mass and catalytic activity.—**Kozaki, K., T. Gotoda, M. Kawamura, H. Shimano, Y. Yazaki, Y. Ouchi, H. Orimo, and N. Yamada.** Mutational analysis of human lipoprotein lipase by carboxy-terminal truncation. *J. Lipid Res.* 1993. **34:** 1765–1772.

Supplementary key words C-terminal deletion mutant • expression study • disulfide bond • hydrophobic amino acids

Lipoprotein lipase (LPL) plays an important role in plasma lipoprotein metabolism, by hydrolyzing triglyceride-rich lipoproteins such as chylomicrons and very low density lipoproteins. The congenital defect of LPL is known as familial LPL deficiency, which manifests type I hyperlipoproteinemia (1). Recently, various mutations have been identified in the human LPL gene from patients with this disease (2, 3). Most of them are missense mutations, but apart from the deletional and insertional mutations, there are six one-base substitutions resulting in the introduction of premature stop codons at Tyr^{61} (4), Trp^{64} (5), Gln^{106} (6), Tyr^{262} (7), Trp^{382} (4), and Ser^{447} (8) (**Fig. 1**). Human LPL comprises 448 amino acids in its mature form (9). Except for the Ser^{447} → stop mutation, which is commonly found in the normal population (8, 10, 11), the Trp^{382} → stop mutation presents the largest molecule among the nonsense mutations reported so far.

Recent X-ray crystallographic analysis of human pancreatic lipase (PL) provided an informative three-dimensional structure of this protein (12). The polypeptide chain of PL is divided into two folding units, a larger spherical amino-terminal (N-terminal) domain and a smaller flattish carboxy-terminal (C-terminal) domain. This structure may well be applied to LPL and hepatic lipase (HL), because of the high homology in the predicted amino acid sequences among these three lipases (13–18). Based on the structural information of human PL, interesting models to study the function of the two domains were generated, lipase with the N-terminal domain of rat HL linked to the C-terminal domain of human LPL (HL/LPL) (19) and vice versa (LPL/HL) (20). These two chimeric lipases were functionally active with a catalytic character of the lipase comprising the N-terminal domain. The N-terminal domain also determines apolipoprotein C-II (apoC-II) interaction and salt sensitivity (20). On the other hand, interestingly enough, the authors emphasized that the C-terminal domain is responsible for substrate specificity and heparin binding (19, 20).

We tried to demonstrate the significance of the C-terminal domain of human LPL by a different approach. First, in order to confirm that the Trp^{382} → stop mutation is truly a cause of familial LPL deficiency, expression study of the Trp^{382} → stop mutant was performed. The

Abbreviations: LPL, lipoprotein lipase; PL, pancreatic lipase; N-terminal, amino-terminal; C-terminal, carboxy-terminal; HL, hepatic lipase; apoC-II, apolipoprotein C-II; FFA, free fatty acid; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

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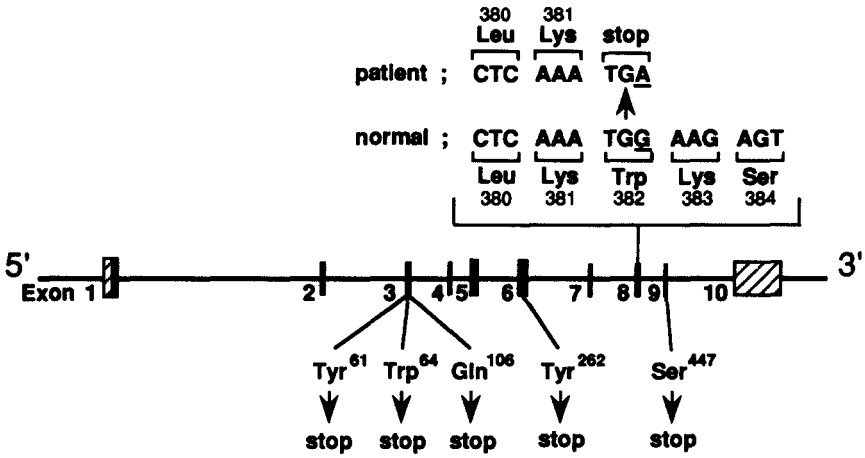

Fig. 1. Schematic representation of the nonsense mutations identified in the human LPL gene. The case described in this study is shown in the upper part of the figure to highlight the nucleotide substitution (underlined). The other reported nonsense mutations are shown at the bottom.

expressed LPL was catalytically inactive and was very low in amount both in medium and in cells. This result raised an important question: what is the minimal N-terminal sequence of human LPL required for an active enzyme? To address this question, 13 additional C-terminal truncated LPLs were expressed. As a result, we found that the C-terminal region of human LPL is closely associated with the expression of enzyme mass and activity.

MATERIALS AND METHODS

Subject

The patient was a 6-month-old infant with parents of a consanguineous marriage. She had the manifestations of eruptive xanthoma, lipemia retinalis, and hepatosplenomegaly. Her fasting serum triglyceride concentration was 6,010 mg/dl (normal, 40–150), and total cholesterol concentration was 632 mg/dl (normal, 130–220). Postheparin plasma LPL activity, as measured at 10 min after a bolus injection of heparin (10 units/kg) after an overnight fast (21), was 1.0 μmol free fatty acid (FFA)/ml per h (normal, 6.4 $\pm$ 2.1). The concentration of apoC-II was 14.8 mg/dl (normal, 3.6 $\pm$ 1.0). LPL mass in the postheparin plasma was 22 ng/ml (normal, 410 $\pm$ 96). Based on direct sequencing of the amplified DNA from the patient's genome, it was discovered that the patient was a true homozygote of familial LPL deficiency. There was a G $\rightarrow$ A substitution at nucleotide position 1401 (9) in exon 8, resulting in the creation of a premature stop codon, Trp^{382} (TGG) $\rightarrow$ stop (TGA) (4) (Fig. 1).

Site-directed mutagenesis

To construct the normal expression plasmid (pCMV-LPL), human LPL cDNA was placed under the control of CMV promoter. A 1,581 base-pair cDNA fragment (22) encompassing the entire coding sequence was cloned in the sense orientation into the *Eco*RI site of the Bluescript II KS+ vector (Stratagene, La Jolla, CA). The insert was re-excised with *Hind*III and *Xba*I, and transferred to the *Hind*III/*Xba*I sites of the Rc/CMV vector (Invitrogen, San Diego, CA). To construct each mutant expression plasmid, DNA segment was generated by polymerase chain reaction (PCR) using the 5' primer containing an *Eco*47III site, and the 3' primer including both an *Eco*RI site and an appropriate stop codon. After proteinase K treatment (23), the DNA segment was digested with *Eco*RI and *Eco*47III, and was used to replace the corresponding segment of the original pCMV-LPL vector (**Fig. 2**). After construction, the plasmids were amplified in competent DH5 cells and purified by CsCl density ultracentrifugation. Before transfection, nucleotide sequences of the introduced inserts were verified by direct sequencing.

In vitro expression study

COS-1 cells were plated on a 60-mm dish (0.8–1.2 $\times$ 10^5/dish) 48 h before transfection. Each transfection was performed by lipofection, following the technique provided by Research Products Division Life Technologies, Inc. (Gaithersburg, MD). Normal or mutant plasmid DNA (10 μg) was mixed with 30 μl of Lipofectin™ Reagent (Bethesda Research Laboratories, Gaithersburg, MD). After 15 min of incubation at room temperature to form lipid-DNA complex, the mixture was added to the culture medium of Opti-MEM I Reduced Serum Medium (Gibco Laboratories, Gaithersburg, MD), which was exchanged just before transfection. After 4 h, the culture medium was replaced by DMEM (Gibco Laboratories, Gaithersburg, MD) containing 10% fetal

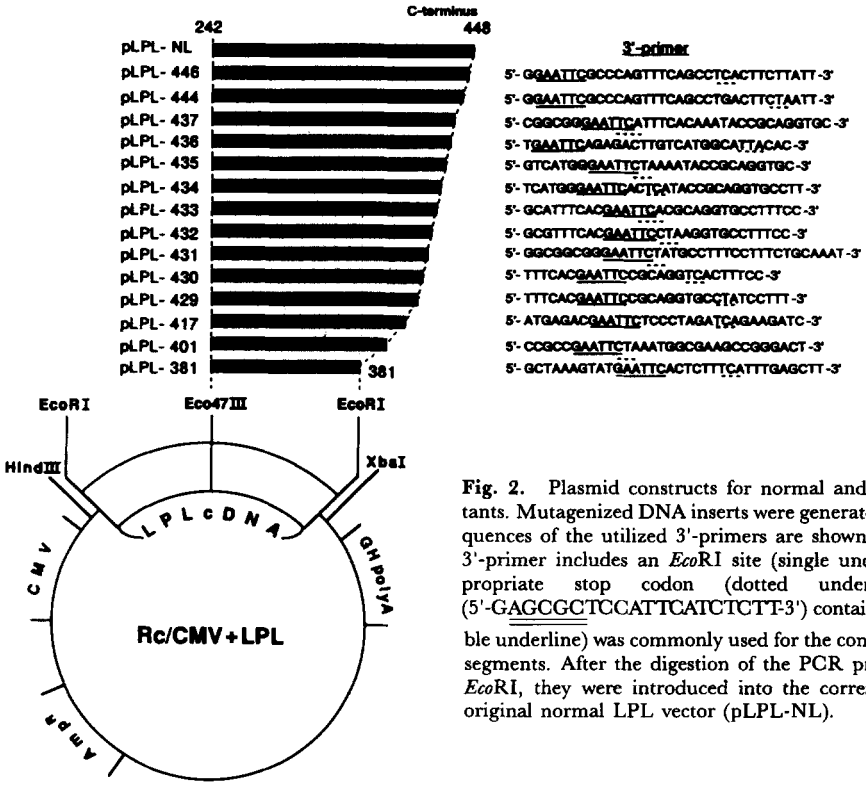

Fig. 2. Plasmid constructs for normal and C-terminal deletion mutants. Mutagenized DNA inserts were generated by PCR. Nucleotide sequences of the utilized 3'-primers are shown in the upper right. Each 3'-primer includes an *Eco*RI site (single underline) as well as an appropriate stop codon (dotted underline). The 5'-primer (5'-GAGCGCTCCATTCATCTCTT-3') containing an *Eco*47III site (double underline) was commonly used for the construction of mutated DNA segments. After the digestion of the PCR products with *Eco*47III and *Eco*RI, they were introduced into the corresponding segments of the original normal LPL vector (pLPL-NL).

calf serum. After 26 h, the medium was supplemented with 5 U/ml of heparin and was incubated subsequently for 18 h. Then, the medium and cell extracts were collected, flash frozen, and stored at −70°C.

Expression studies were carried out ten times in total (ten times for LPL-NL,-437,-436,-435, nine times for LPL-446,-429,-381 and five times for LPL-434). In each of the expression studies, deletion mutant plasmids were transfected separately along with the transfection of pCMV-LPL as a positive control and the transfection of pCMV only (plasmid without LPL cDNA insert) and the transfection without plasmid (incubation of only COS-1 cells) as negative controls.

Northern blot analysis of LPL mRNA

After collecting culture medium, cells were washed three times with phosphate-buffered saline. Total RNA was extracted by the acid guanidium thiocyanate–phenol–chloroform method (24). Each 15 μg of total RNA was electrophoresed on 1.5% agarose gel with 2% formaldehyde, followed by transfer to a nylon membrane, and hybridized with ^{32}P-labeled human LPL cDNA probe (22) at 65°C overnight as described previously (25). The blot was washed with 1 × SSC (150 mM NaCl and 15 mM sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 65°C for 15 min, and exposed on X-ray film. The same membrane was washed in 0.1% SDS at 95°C for 20 min, and rehybridized with human β-actin cDNA probe similarly.

Measurement of LPL activity and mass

LPL activity in culture medium and cell extracts were determined by using tri-[9,10 (n)-^{3}H]oleoyl glycerol (Amersham Life Sciences, Buckinghamshire, England) as substrate (26). LPL mass was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) (4). The ELISA uses three different monoclonal antibodies as the capturing antibody and a polyclonal antibody raised against purified bovine milk LPL as the detecting antibody. Purified human milk LPL was used as a standard to calculate the enzyme mass. In each of the expression studies, LPL activity and mass in the expressed medium and cells for each mutant were determined through triplicate (activity) and quadruplicate (mass) measurements, respectively. These measurements were represented in percent as compared with the activity and mass of LPL-NL.

Statistical analysis

After the ten sets of expression studies were carried out, mean value of the %activity and %mass were calculated for each mutant. The statistical significance of the differences among mutant and normal LPLs was determined by a two-way analysis of variance. Individual means were compared, where appropriate, by the Neuman-Keuls multiple comparison test. Differences were considered to be significant when $P < 0.05$.

RESULTS

The present patient was a true homozygote for a $Trp^{382} \rightarrow$ stop mutation. In order to prove that this nonsense mutation is actually the cause of LPL deficiency in this patient, the $Trp^{382} \rightarrow$ stop mutant was expressed in vitro. After preparing the normal LPL expression plasmid (pLPL-NL), the mutant plasmid lacking the C-terminal 67-amino acid coding region in human LPL cDNA was constructed (Fig. 2). The resulting truncated LPL was produced by COS-1 cells by transfecting the cells with the constructed mutant plasmid. As shown in **Fig. 3** (LPL-381), there was a marked reduction in the expressed mass and no measurable level of lipolytic activity either in medium or in cells.

Next, to determine the minimal N-terminal sequence of human LPL required for an active enzyme, 13 additional truncated LPLs lacking the C-terminal 2-, to 47-amino acids were expressed and the activity and mass in medium and cells were measured. The results are shown in Fig. 3 with regard to the seven representative mutants including LPL-381. The activity in medium showed a stepwise decrease as the size of the molecule became smaller from LPL-437 ($Cys^{438} \rightarrow$ stop; 68% of normal) to LPL-434 ($Phe^{435} \rightarrow$ stop; 3%). LPL-434,-433,-432,-431,-430,-429,-417,-401, exhibited no detectable activity (only LPL-434 and LPL-429 are represented). Almost full activity (91.7% in medium) was detected with LPL-444 ($Lys^{445} \rightarrow$ stop) (data not shown). On the other hand, LPL-446 ($Ser^{447} \rightarrow$ stop) exhibited higher activity than LPL-NL (185% in medium), which was always found in nine expression studies (150 to 265%, data not shown).

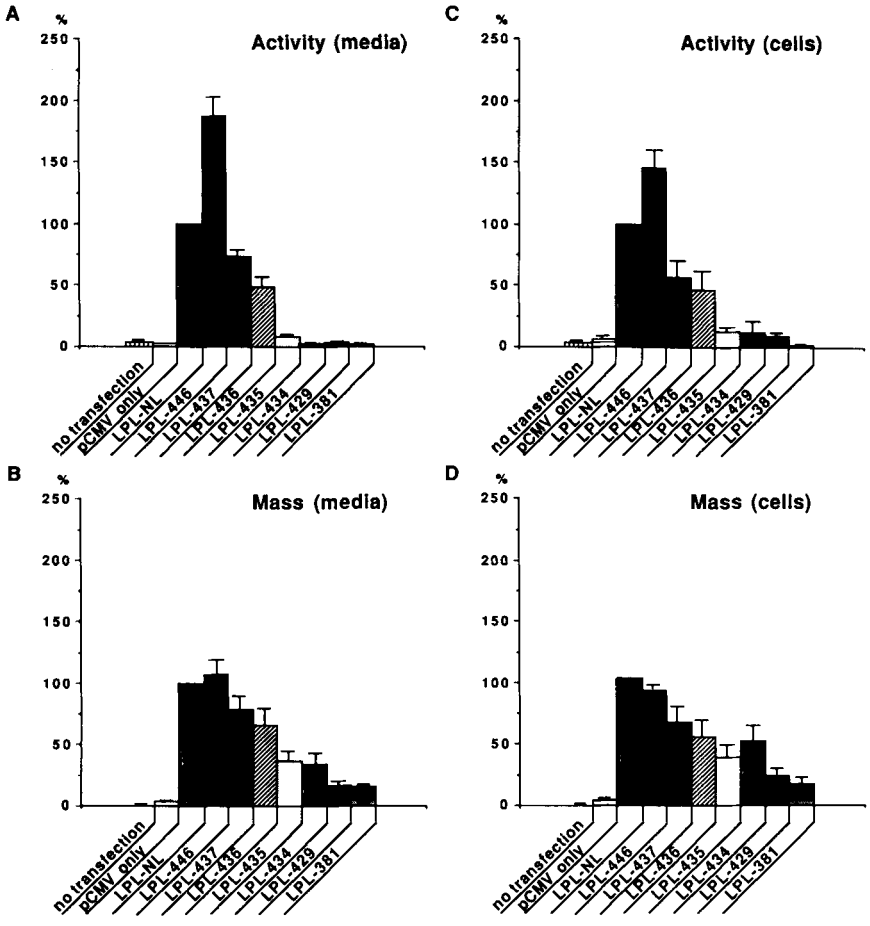

Fig. 3. LPL activity and mass expressed in media and cells. Expression studies were performed by lipofection (see Materials and Methods). LPL activity in medium and cells were determined as described previously (26). LPL mass in medium and cells were measured by ELISA. Expression studies were carried out ten times in total (ten times for LPL-NL,-437,-436,-435, nine times for LPL-446,-429,-381 and five times for LPL-434). In each of the expression studies, %activity and %mass were calculated for each mutant (100% indicates the activity and mass with LPL-NL). All data are expressed as mean ± SE. In the ten expression studies, the activities in medium and cells with LPL-NL were 85.2 ± 19.0 and 15.7 ± 6.8 nmol FFA/ml per h, respectively (mean ± SE), and the masses in medium and cells with LPL-NL were 315 ± 38 and 203 ± 53 ng/ml, respectively (mean ± SE).

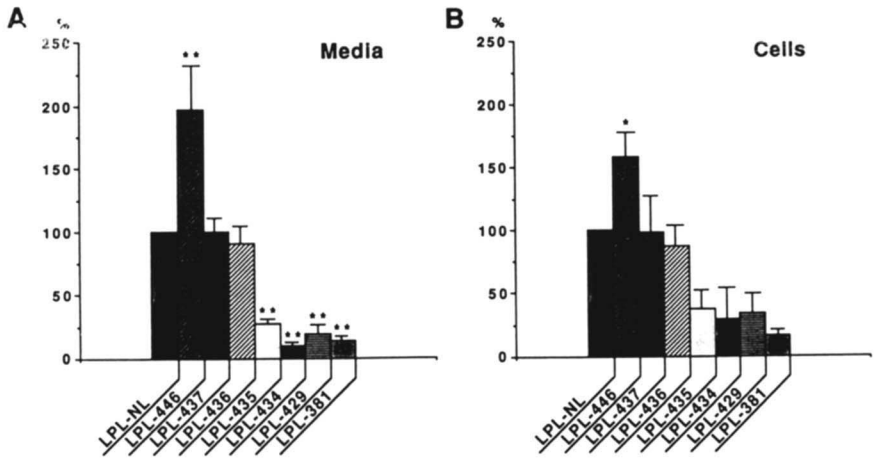

Fig. 4. Specific activity in media and cells. Specific activity was calculated based on the expressed LPL activity and mass. Percent specific activity as compared with that of LPL-NL (A; media, B; cells) are shown as mean ± SE. Specific activities expressed in medium and cells with LPL-NL in the ten expression studies were 220 ± 56 and 72.5 ± 15.5 nmol FFA/min/μg (mean ± SE), respectively. Compared with LPL-NL; $^{*}P < 0.05$, $^{**}P < 0.01$.

Protein mass of the trucation mutants was measured by ELISA. In this assay, LPL was sandwiched by three different monoclonal antibodies and a polyclonal antibody. In another experiment, it was shown that none of the three monoclonal antibodies failed to recognize the truncation mutants (data not shown). In the case of the mutants not larger than LPL-437, they were reduced in amount as compared with LPL-NL, and the reduction was greater as the size of the molecule became smaller.

Because the truncated LPLs not larger than LPL-437 were reduced both in the expressed activity and mass, specific activity was calculated based on the expressed activity and mass for each mutant. As a result, a great difference was discovered between LPL-436 (Lys^{437} → stop; 88% of normal LPL) and LPL-435 (Val^{436} → stop; 22%) in medium (**Fig. 4**). In contrast, the specific activity of LPL-446 was much higher than that of LPL-NL in medium (197%).

The lipolytic activity, mass, and the resulting specific activity in cells showed tendencies similar to those found in media. But the specific activities showed more variation in cells than in media and varied depending on the location of the mutant, whether in media or in cells. This may reflect the technical difficulties in measuring the low levels of activity and mass from cell homogenates.

Northern blot analysis showed no major differences in the abundance of expressed mRNA for normal and mutant LPLs (**Fig. 5**). The fact that the mRNA migrated faster as it became smaller in size, and that no signal was detected in the left two lanes for negative controls, convinced us that the signals truly represent LPL mRNAs. Hybridization with β-actin cDNA probe demonstrated that the amounts of the electrophoresed total RNAs were nearly equal. Reproducibility of the results was confirmed in quadruplicate experiments.

DISCUSSION

In vitro expression of the Trp^{382} → stop mutant

Gene mutations causing familial LPL deficiency are quite heterogeneous, probably reflecting the presence of multiple intact domains necessary for normal LPL function. Apart from the deletional and insertional mutations, six distinct point mutations are known to create premature stop codons; Tyr^{61} (4), Trp^{64} (5), Gln^{106} (6), Tyr^{262} (7), Trp^{382} (4), and Ser^{447} (8) to stop (Fig. 1). Expression study of the Trp^{382} → stop mutant revealed that the trun-

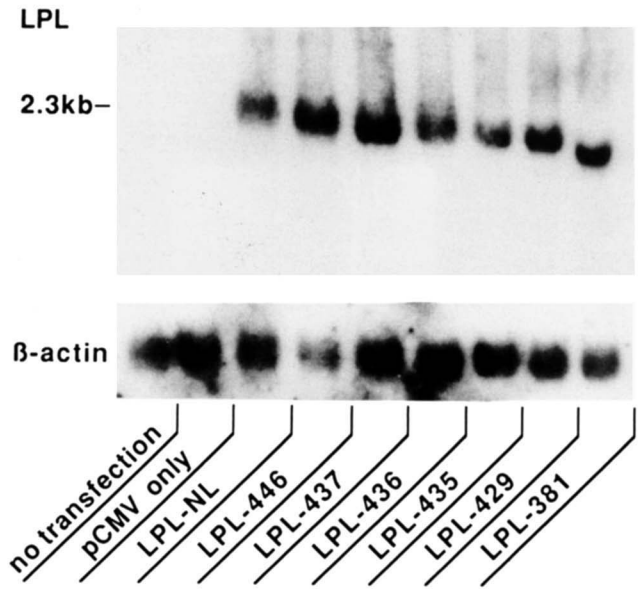

Fig. 5. Northern blot analysis of LPL mRNA isolated from COS-1 cells transfected with expression plasmids encoding normal and C-terminal deletion mutant LPLs. Hybridization was carried out with human LPL cDNA or β-actin cDNA probe.

cated LPL was catalytically inactive and was markedly reduced in amount. In the postheparin plasma, the patient had a trace amount of LPL activity at similar levels to the background with LPL mass nearly 1/20 of normal levels. Thus, the results of our in vitro experiment are consistent with the in vivo data of the patient and establish the etiological significance of the Trp^{382} → stop mutation.

Protein mass reduction with the mutants not larger than LPL-437

In the case of the mutants not larger than LPL-437, the amount of the immunoreactive mass was always reduced, whether in medium or in cells. Furthermore, as the mutant molecule became smaller in size, the expressed mass as well as the activity decreased more drastically. However, the mass reduction was apparently independent of the mRNA levels (Fig. 5), implying that the low levels of expressed mass with the mutants not larger than LPL-437 are independent of the variation at mRNA levels.

Comparative analysis of amino acid sequences showed that Cys^{438} is completely conserved among LPL, PL, and HL across species (27) (**Fig. 6**). Cys^{438} is assumed to be covalently linked with Cys^{418} by a disulfide bond by analogy to the bridging formation of the cystein residues in bovine LPL (28). Therefore, it is conceivable that the decrease in the mutant LPL mass results from loss of the last disulfide linkage, which would affect the stability of LPL molecule.

Decrease in the specific activity with the mutants not larger than LPL-435

The truncated LPLs that are not larger than LPL-435 appear to have some functional impairment, judging from the decrease of the specific activity between LPL-436 (88% of normal in medium) and LPL-435 (22%). This indicates that Val^{436} plays an important role in the action of human LPL. Recent studies using chimeric lipases of HL/LPL (19) and LPL/HL (20) demonstrated that the C-terminal domain of LPL influences substrate specificity and directs heparin binding. When we look at the amino acid alignment around Val^{436}, interestingly, there is a line of six hydrophobic amino acid residues (APAVFV) including Val^{436} in human LPL (Fig. 6). This hydrophobic feature is also found in the corresponding region of LPLs from other species (PHO1 region: indicating the first hydrophobic region). As hydrophobicity is a requirement for lipases to bind to insoluble substrates, this hydrophobic region may contribute to interact with insoluble substrates. It is also noteworthy that there are three consecutive hydrophobic amino acids (PHO2 region: indicating the second hydrophobic region) aligned just before Cys^{418} in human LPL. As Cys^{418} and Cys^{438} are supposed to be linked by a disulfide bond, PHO1 and PHO2 regions would be in close proximity to each other. Taken together, it is possible to envisage that the PHO1 region, together with the PHO2 region, interacts with insoluble substrates.

Sequence comparison in the PHO1 and PHO2 regions between human HL and five species of LPLs shows that highly hydrophobic amino acids (IFV) are conserved in the PHO1 region, while low homology and relatively low hydrophobicity is found in the PHO2 region in human HL. In the case of human PL, amino acid sequences of both PHO1 and PHO2 regions are quite different from those of LPL and HL. This sequence diversity among LPL, HL, and PL may be related to the modulation of the functional regions of the three lipases.

Ser^{447} → stop mutation

Two separate groups performed expression studies of LPL which lacked the last two amino acids at the C-terminal end (Ser-Gly) (10, 29). However, the results in the two studies were quite different. Being the same mu-

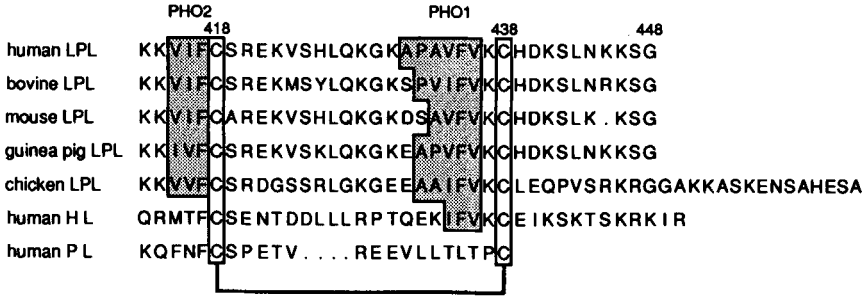

Fig. 6. Comparative alignment of amino acid sequences in the C-terminal region of LPLs (five species), human HL, and human PL. Amino acid sequences are derived from human (9), bovine (30), mouse (31), guinea pig (32), and chicken (33) LPL; human HL (27); and human PL (34). Comparative alignment was referred to the data presented by Hide, Chan, and Li (16). Dots are inserted to give maximum homology of amino acid alignment. Amino acid numbers are represented based on the alignment of human LPL. Shaded-boxed residues indicate the two hydrophobic regions, named PHO1 and PHO2 (see Discussion), common to all LPLs of five species and human HL. Two conserved cysteine residues are boxed and connected by a line.

tant, one kept a full catalytic activity (10), and the other had 45% of normal lipolytic activity (29). Therefore, the former group placed little importance on these two residues, while the latter emphasized the last two amino acids. Unexpectedly, our data differed from each of the two results. The overall and specific activities of the $Ser^{447} \rightarrow$ stop mutant were about twice as high as those of normal LPL in medium (Figs. 3 and 4), and the results were faithfully reproducible in nine experiments. Previously, Hata et al. (8) reported higher frequency of the $Ser^{447} \rightarrow$ stop mutation in control subjects (33%) than in hypertriglyceridemic subjects (9%). Recently, Stocks, Thorn, and Galton (11) also showed a similar difference (19% in normolipidemic controls vs. 10% in hypertriglyceridemic subjects) and suggested the possibility that the $Ser^{447} \rightarrow$ stop mutation may have some protective effect against the development of hypertriglyceridemia. Our results that the $Ser^{447} \rightarrow$ stop mutant had higher specific activity than normal LPL at least in vitro may provide an explanation of the statistical differences observed by Hata et al. (8) and Stocks et al. (11). But further studies are required to know the effect of the $Ser^{447} \rightarrow$ stop mutation.

In the current study, we demonstrated the importance of the C-terminal region of human LPL in the expression of enzyme activity and mass, especially suggesting the importance of Val^{436} and Cys^{438}. Further mutagenic studies and other enzymatic assays will help understand the more precise role of the C-terminal region of human LPL.

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