Structural features in lipoprotein lipase necessary for the mediation of lipoprotein uptake into cells

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Abstract Lipoprotein lipase (LpL) has been shown to mediate the uptake of lipoproteins into cells. The uptake is initiated by binding of LpL to cell surface proteoglycans and to the low density lipoprotein (LDL) receptor-related protein. This ability of LpL is independent of catalytic activity and depends on the intact dimeric structure of the lipase and functional residues in the C-terminal domain. The goal of this study was to identify structural features in LpL that are essential in the mediation of lipoprotein uptake. Naturally occurring variants and LpL mutants produced by site-directed mutagenesis were cloned and expressed in COS-cells. A combination of immunoassays and separation on heparin-Sepharose columns was used to determine the molar ratio of monomeric to dimeric LpL in the expression media. The mutants were tested for their ability to mediate the uptake of ^{125}I-labeled β-VLDL in cultured Hep3b cells in direct comparison with wild type LpL. We found that the concentration of monomer in the media correlated negatively with the effect on the uptake mediated by the dimeric form of LpL. A mutation affecting the catalytic activity (Asp156Gly) resulted in no significant reduction in the lipase-mediated β-VLDL uptake. Point mutations in the proposed lipid binding region Trp390Ala or Trp393Ala and the substitution of 390–393 with the homologous hepatic lipase (HL) sequence were also normal, while the deletion of 390–393 reduced the ability to mediate the uptake by about 60% in comparison to wild type. A mutation known to impair heparin binding (Arg294Ala) was also less efficient than the wild type in mediating uptake. In conclusion, it is important to determine the monomer/dimer ratio in mutant preparations as the presence of monomers inhibits the uptake mediated by the dimeric LpL. Moreover, sites involved in heparin and lipid binding between residues 390–421 are important for LpL-mediated lipoprotein uptake.—**Krapp, A., H. Zhang, D. Ginzinger, M-S. Liu, A. Lindberg, G. Olivecrona, M. R. Hayden, and U. Beisiegel.** Structural features in lipoprotein lipase necessary for the mediation of lipoprotein uptake into cells. *J. Lipid Res.* 1995. **36:** 2362–2373.

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Lipoprotein Lipase (LpL; triacylglycero-protein acylhydrolase, EC 3.1.1.34) plays a crucial role in the hydrolysis of triglyceride-rich lipoproteins. The chylomicron remnants (CR) generated are rapidly taken up by the liver via receptor-mediated processes (1, 2). Apolipoprotein E has been described as being responsible for the receptor recognition (3, 4). Recent evidence points to the low density lipoprotein receptor-related protein (LRP) as a CR receptor (5–8) in addition to the well-known LDL receptor. LpL has been found to be associated with hydrolyzed lipoproteins (9, 10) particularly CR (11). ApoE (12) and LpL have been described as binding to LRP, and as mediating the binding of CR and VLDL to cells (13–15). Moreover hepatic lipase (HL) can bind to LRP and mediate lipoprotein binding to cells (16, 17). LRP is a multifunctional receptor for which several groups of ligands have been described in addition to the apoE and LpL (18, 19). LRP belongs to the LDL receptor gene family which also includes the LDL receptor, the gp330 surface glycoprotein (20), and the VLDL receptor (21).

LpL is a member of the triglyceride lipase gene family which also contains pancreatic lipase (PL) and HL (22–24). The three-dimensional structure of human pancreatic lipase was defined by X-ray crystallography (25) and has been used to model the LpL structure (26). LpL contains several functional domains (24), including the catalytic center (27) and a surface loop shielding it (28, 29), an apolipoprotein C-II binding site (24), regions responsible for dimer formation (30), and heparin-bind-

Abbreviations: LpL, lipoprotein lipase; HL, hepatic lipase; LRP, LDL receptor-related protein; CR, chylomicron remnants; α_2 MR, α_2-macroglobulin-receptor; cLpL, chymotrypsin cleaved LpL; del, deletion; LDL, low density lipoprotein; FH, familial hypercholesterolemia.

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ing sites (31, 32). The only structures that have been clearly identified in relation to their function in LpL are the catalytic triad (Ser-132, Asp-156, His-241), which is highly conserved (25, 33), and the surface loop between amino acids 217 and 238 (24, 28, 29, 31, 34), which is poorly conserved between LpL, HL, and PL. The active form of LpL is a noncovalent homodimer that requires binding of apolipoprotein C-II (apoC-II) for maximal activity. The binding region of apoC-II was proposed as being located in the N-terminal region (24, 30, 33, 35). Several regions in LpL have been described as being involved in heparin binding (36), including residues Arg^{279}–Arg^{282} (37), Lys^{294}–Arg^{306} (38), and Gln^{402}–Lys^{446} (35). Ma et al. (32) confirmed in their recent mutagenesis studies the importance of residues 279–282 and 292–304 for heparin binding, and excluded certain residues in regions 390–393 and 439–448.

ApoE, LpL, and HL all bind with high affinity to heparan sulfate proteoglycans on the cell surface. This might be the reason for the recently described fact that fast and effective binding of CRs to the proteoglycans represents an important step in the receptor-mediated uptake of these and other lipoproteins (39, 40). For the present report we studied the structural features of LpL that might be responsible for the mediation of lipoprotein uptake into cells. From experiments with chymotrypsin-cleaved LpL (cLpL, 41) we had evidence for a role of the C-terminal domain, as this truncated form of LpL was unable to bind to LRP or to mediate the binding of lipoproteins to human hepatoma cells (14). To refine the residues in LpL important for mediating lipoprotein uptake into cells, both naturally occurring variants and in vitro-generated mutants with mutations in their C-terminal domain were expressed in COS-cells and assessed for their ability to mediate uptake of β-VLDL into Hep3b cells. The LpL effect on lipoprotein uptake was highly dependent on the stability of the dimeric form. LpL monomers inhibited the mediation of uptake, presumably by competition with dimers for binding to LRP. In accordance with earlier studies (14, 42), our data provide further support that the region responsible for the mediation of lipoprotein uptake into cells in LpL is located between Phe^{390} and Glu^{421}, and that heparin and lipid-binding regions are important in the uptake mechanism.

METHODS

Cells

Human hepatoma cells (Hep3b) were plated in 24-well plates (1×10^6 cells per plate) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco) at 37°C in 5% CO_2/95% air for 2 days. The assays were performed on the cells in the indicated assay medium after washing the confluent monolayer with phosphate-buffered saline (PBS) (Gibco). In one experiment human fibroblasts from a patient with familial hypercholesterolemia (FH) were used, which lack the LDL receptor. They were treated exactly as described for the Hep3b cells.

In vitro site-directed mutagenesis

A 1.6 kb cDNA fragment containing the entire coding sequence of human LpL was cloned into a dual function vector (CDM-8) for both mutagenesis and expression as previously described (43, 44). In vitro mutagenesis was performed using the double-primer method (45). A number of mutants were generated and those used in this study are listed in **Table 1.** Mutagenesis primers for single codon alterations contained centrally positioned one or two base pair mismatches. The mismatched bases in these primers were flanked by 9 or 10 base pairs of complementary sequence. The primers for the chimeric and deletion mutants contained a sequence to be exchanged and 9 base pairs of flanking complementary sequence. All mutant clones were identified by hybridization with individual mutagenic primers and confirmed by DNA sequencing.

Transient expression of mutant LpL in COS-1 cells

Phagemid DNAs carrying the desired mutations were purified and introduced into Cos-1 cells by electroporation as previously described (44). Transfected cells were placed on 145-mm culture dishes in 15 ml DMEM medium with 5% fetal calf serum and incubated for 24 h. After exchanging the medium with an equal volume of fresh DMEM containing 5% FCS and 7–70 mU/ml heparin (Liquemin, La Roche), cells were cultured for an additional 72 h, and medium was collected every 24 h. Transfection experiments for each mutant were performed 2–3 times to determine reproducibility.

Measurements of LpL mass

LpL mass was determined by the ELISAs, described by Peterson, Fujimoto, and Brunzell (46), using monoclonal antibodies 5F9 or 5D2 as capturing antibody, and peroxidase-labeled 5D2 for detection. With a combination of these two ELISAs we were able to discriminate between LpL monomers and dimers. The 5D2/5D2 ELISA will only recognize dimers as two identical epitopes are necessary for recognition. 5F9 is directed against another, as yet unknown, epitope and has been described as recognizing only partly denatured LpL. For this ELISA the samples are denatured and all monomers are recognized. For mutants affecting the 5D2 epitope

TABLE 1. Mass of LpL variants and the mediation of β-VLDL uptake

LpL	Mass		Mono/Dimer	β-VLDL	
	Monomer	Dimer		% of bLpL	% of Weight
	ng/ml		*mol/mol*		
Bovine	0	100	0	100	
Wild type (n=22)[a]	106–904	108–530	1.1–3.2	43–71	100
Asp156Gly[a]	1323	376	7.07		23
	426	180	4.75		59
	428	212	4.05		79
(FH)	1086	376	5.81		46
Asp291Ser[a]	408	432	1.90		93
	408	432	1.90		104
Arg294Ala[a]	46	130	0.71		81
	38	186	0.42		85
	60	266	0.45		98
Trp390Ala[b]	177	346	1.0		129
					119
					89
					95
Trp393Ala[a]	243	276	1.78		105
	223	212	2.12		89
	223	212	2.21		104
390–393HL[b]	209	403	1.1		109
					127
					119
					95
del390–393[b]	194	239	1.6		48
					67
					79
					48
del421–430[c]	350				80
					73
stop438[a]	428	210	4.10		77
	372	258	2.90		98
	372	258	2.90		93
	354	222	3.21		85
	354	222	3.21		91

The mass of LpL in the final preparations of bovine LpL and in the COS-cell supernatants for wild type and mutants was determined with the monoclonal ELISAs (*a*) or polyclonal ELISA (*b, c*). The values are given for monomers and dimers when possible. In all uptake experiments, the amount of the different LpLs used was adjusted to 200 ng dimer/ml irrespective of the monomer concentration. The monomer/dimer ratios are given as a range for the wild type from 22 preparations, and for each single experiment in the mutants detectable with the monoclonal ELISAs. For some mutants, the ratio was determined in one representative preparation (*b*) or only the total mass was determined (*c*). The wild type uptake in percent is related to bovine LpL, while the mutants are directly compared to the wild type which was set to 100%.

[a]Monoclonal ELISA.

[b]Polyclonal ELISA following heparin column for one of the preparations.

[c]Polyclonal ELISA with medium - total LpL measured.

(47) a third ELISA using polyclonal chicken anti-bovine LpL antibodies was developed. Affinity-purified IgG from chicken 225 was used for capture and affinity-purified and peroxidase-labeled IgG from chicken 224 was used for detection. The preparations and purification of the antibodies and details of the ELISA procedure have been described elsewhere (10, 48). For comparison, both ELISAs were performed in parallel for two wild

type LpL preparations. The results for total LpL mass were in good agreement between the monoclonal ELISA, 431 ng/ml, and 220 ng/ml, and the polyclonal ELISA, 400 ng/ml, and 205 ng/ml, respectively.

Table 1 gives the results for the individual preparations (supernatant of one dish) for mass determination.

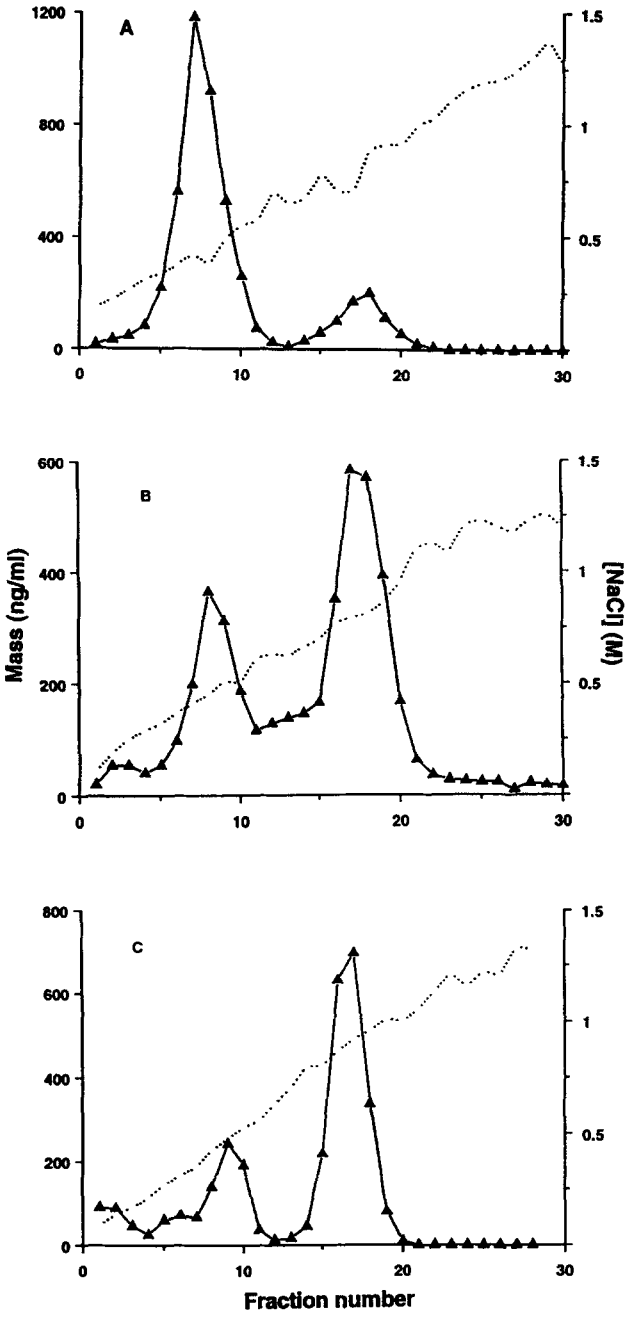

Fig. 1. Heparin-Sepharose chromatography of expressed LpL mutants. A: Asp156Gly; B: del390–393; C: LpL390–393HL. Media were applied to heparin-Sepharose columns and eluted with a gradient from 0.1 to 1.5 M NaCl (....). Monomeric LpL was eluted between fractions 5–12 and dimeric LpL between fractions 15–20. LpL mass (▲) was determined by the polyclonal ELISA.

In the case of mutants that could not be detected by the monoclonal ELISA, monomers and dimers were separated by chromatography on heparin-Sepharose columns and the mass of monomer and dimer was determined separately with the polyclonal ELISA. For this, media from transfected COS-cells were applied to columns containing 4 ml of heparin-Sepharose. The columns were washed with 20 ml 20 mM Tris-HCl (pH 7.4) containing 20% glycerol, 0.1% Triton X-100, 0.1 M NaCl, and BSA (1 mg/ml), and eluted with a salt gradient from 0.1 to 1.5 M NaCl in the same buffer. The total volume of the gradient buffer was 120–140 ml and fractions of 4–5 ml were collected. A flow rate of 1 ml/min was maintained. Conductivity in all fractions was measured and related to standard solutions of known concentration of NaCl made up in the same buffer. The recovery of LpL mass over the columns was > 90%. Control experiments showed that the polyclonal ELISA detected inactive monomer and active dimer of LpL with similar efficiency.

The molar ratio of monomers over dimers was calculated from the respective peaks. Due to the low amounts of LpL in the preparation we could not run columns on all preparations. Therefore, we performed one representative column separation per mutant to determine the relative stability.

Lipid and protein quantification

Cholesterol and triglycerides in the lipoproteins were determined with enzymatic colorimetric assays from Boehringer Mannheim. The protein content of the lipoproteins and the cell proteins was determined by the Lowry technique (49), modified for lipid-containing samples by addition of 0.1% SDS.

Rabbit β-VLDL

The β-VLDL ($d < 1.006$ g/ml) was isolated from the blood of cholesterol-fed rabbits (50). The protein content of the different preparations was 0.89 ± 0.25 mg of protein per ml. For iodination, the ICI method was used (51). The sodium iodide (^{125}I) was purchased from Amersham Buchler. The protein content of the different ^{125}I-labeled preparations was 0.41 ± 0.14 mg of protein per ml. The specific radioactivity in the protein was between 4.7 and 11.5×10^4 cpm/μg.

LpL and cLpL

Bovine LpL was purified from milk as previously described (52). The specific activity of the preparations determined with a phospholipid-stabilized emulsion of soybean oil (Intralipid; Kabi-Nutrition, Stockholm) at pH 8.5 and 25°C, with human serum as source of apoC-II, was 570 units/mg. One unit corresponds to 1 μmol of fatty acid released per min. Protein concentra-

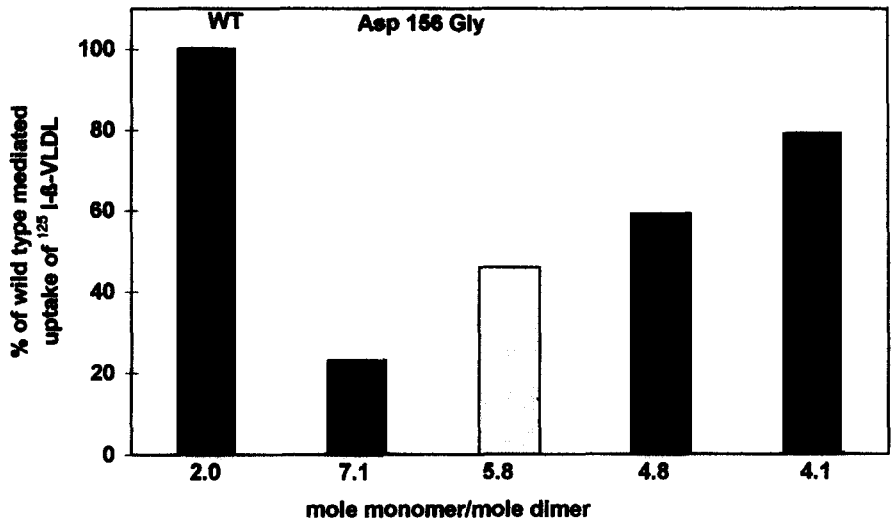

Fig. 2. Specific uptake of β-VLDL into cells mediated by the mutant Asp156Gly compared to wild type LpL. Hep3b cells were used in three experiments (■) and FH fibroblasts were used in one experiment (□). The different molar ratios of monomer/dimer LpL are indicated on the x-axis. The correlation between the molar ratio and the uptake was $r = -0.99$. The conditions of the experiment are described in the Methods section. Nonspecific uptake was evaluated by addition of a 50-fold excess of unlabeled β-VLDL and subtracted from total uptake to obtain the specific value. The specific uptake measured without addition of LpL was considered not to be LpL dependent and therefore it was subtracted from all data. Data are given as % of ^{125}I-labeled β-VLDL uptake mediated by 0.2 μg wild type LpL/ml, and were measured in duplicate.

tions were calculated using the absorbance at 280 nm and the absorption coefficient ($A_{1\%}$) 16.8 cm^{-1}.

The chymotrypsin-truncated LpL (cLpL) was produced and purified as described previously (41). It was obtained in a dimeric active form.

Precipitation of LpL from COS-cell supernatant

The wild type and mutants of LpL expressed in COS-cells were released in the medium by 7–70 mU of heparin (Liquemin, La Roche). The concentration of the different mutants in the medium varied from 0.05 to 0.4 μg/ml. For the assay of cellular uptake, a concentration of 4 μg/ml LpL was necessary and the amount of heparin had to be reduced to below 2 mU/ml, as higher concentrations interfered with the cellular interaction. To achieve these two goals, the mutant LpL was precipitated from COS-cell media by $(NH_4)_2SO_4$. For this a volume of 6–15 ml medium was dialyzed for 65 min at 4°C against 3.4 M $(NH_4)_2SO_4$. To reduce the amount of heparin in the precipitate, the samples were diluted to 50 ml with 2.6 M $(NH_4)_2SO_4$ and the precipitates were isolated by centrifugation. This step was repeated once. The mutant proteins were then dissolved in 100 μl of DMEM or PBS and immediately used for uptake experiments. The recovery following this procedure was 80% for ^{125}I-labeled bovine LpL added to expression media in control experiments. It was not possible to check the recovery of all preparations and therefore all calculations were based on a recovery of 80%. With ^{125}I-labeled dimeric bovine LpL we performed a control precipitation to determine whether this procedure results in the conversion of the enzyme from the dimeric to the monomeric form. We were unable to detect any monomerization of the LpL as a consequence of the precipitation.

Uptake experiments

For the uptake experiments, the Hep3b cells, or in one experiment FH fibroblasts, were grown in 24-well plates (Nunc). The cells (1×10^6) were plated and used as confluent monolayers at day 2. The experiments were performed in 1 ml of DMEM containing 5% BSA (fraction V, Sigma) and 0.02 M HEPES (pH 7.4), with 2 μg of ^{125}I-labeled β-VLDL per ml and 0.2 μg/ml of LpL dimer, irrespective of the monomer concentration. Incubations were performed for 90 min at 37°C. To avoid lipolysis, the active site inhibitor Orlistat (tetrahydrolipstatin, THL; La Roche) was present in the incubation media (25 μg/ml). To determine the fraction of β-VLDL taken up, the cells were washed several times with PBS, pH 7.4, containing 2 mg/ml BSA. The first and last wash was carried out in the absence of BSA. Surface-bound lipoproteins were then released with PBS containing 770 U of heparin/ml (Liquemin). After washing, the cell monolayers were dissolved in 0.1 M NaOH and radioactivity and cell protein were determined. The amount of cell protein per well (34 ± 8 μg) was comparable in all experiments.

The uptake was measured in ng ^{125}I-labeled β-VLDL per mg cell protein. To compare the binding capacity of

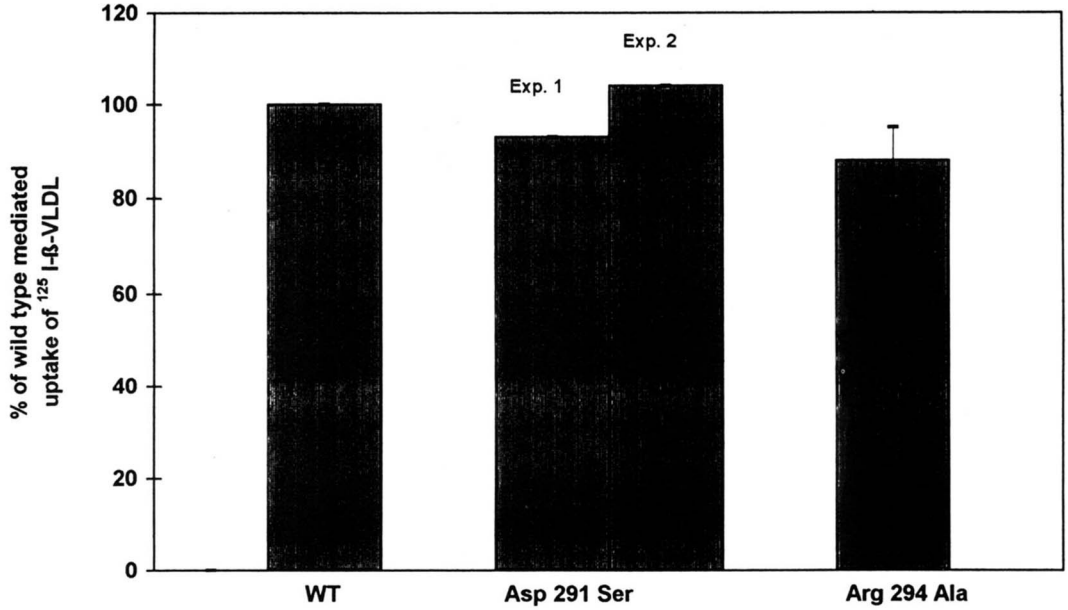

Fig. 3. Specific uptake of β-VLDL mediated by the mutants Asp291Ser and Arg294Ala compared to wild type LpL. Two single experiments for Asp291Ser and the mean of three experiments and standard deviation for Arg294Ala are shown. For Asp291Ser, only one preparation was used with the monomer/dimer molar ratio of 1.9. For Arg294Ala, three experiments were performed with three different preparations. The experimental conditions are described in the legend to Fig. 2.

the LpL mutants directly with the wild type LpL function, all mutants were always analyzed alongside the wild type LpL, e.g., one to three mutants and the wild type in one experiment. In Figs. 2–5 the uptake data for the mutants are presented as a percentage of that for wild type LpL in the respective experiments. The percentage is given in the figures because, due to the biological variation in the ligand and the cells, the different experiments are not comparable in terms of the ng of ligand taken up in the cells. The uptake of β-VLDL alone was determined to be 2–47 ng/mg, while the uptake mediated by the wild type LpL varied between 31 and 221 ng/mg, and that by the mutants between 17 and 202 ng/ml.

RESULTS

Production and measurement of mutant protein

Mutant LpL constructs were obtained by site-directed mutagenesis, and LpL protein was expressed and isolated from COS-1-cell medium. As previously reported (32) the expression media often contained a mixture of LpL monomers and dimers. The molar ratio monomer/dimer was determined with monoclonal ELISAs unless the mutation disturbed the epitope for the detecting antibody, monoclonal 5D2 (47). In these cases, monomers and dimers were separated by heparin-Sepharose chromatography as shown in **Fig. 1** and the masses of both were determined with a polyclonal ELISA. Wild type human LpL isolated from the COS-cell medium was found in the expression media with a monomer/dimer ratio of 1.1–3.2 (mean: 1.95), while the bovine LpL was purified as active dimer and had no detectable monomer. The mutants were rather heterogeneous in their stability. The respective monomer/dimer ratios are given in Table 1.

The mediation of β-VLDL uptake by the wild type human LpL from COS-cells was found to be 43–71% of that of the bovine LpL, based on the same amount of LpL dimer (0.2 μg/ml). As this inhibition was thought to be due to the monomer concentration, we performed the same uptake experiments with 0.2 μg/ml dimeric bovine LpL and added increasing amounts (up to 0.9 μ/ml) of bovine LpL monomers. Uptake was inhibited to 39%.

The uptake experiments required that the LpL concentration in the expression media had to be increased, while the heparin concentration had to be reduced. By precipitation with ammonium sulfate we were able to concentrate the total LpL protein to around 4 μg/ml. In control experiments we showed that this precipitation method did not convert the dimeric into the monomeric form. The precipitation also reduced the heparin concentration in the medium to a level that did not interfere with the assay.

Catalytically defective mutants and a mutant with impaired heparin binding

It has previously been shown that the mutation in the

active site, Asp156Glyn, results in a complete loss of catalytic activity (27, 43). This mutant, here produced in COS-cells, was relatively unstable in its dimeric form as shown in Fig. 1. This was seen as a monomer/dimer ratio of 4.1–7.1 in different preparations, and the effect on lipoprotein uptake was found to be correspondingly reduced to 23–79% of wild type LpL (**Fig. 2**). The decreased uptake of β-VLDL appeared to be a direct consequence of the increased monomer/dimer ratio. When this was taken into account, uptake of β-VLDL mediated by Asp156Gly was essentially normal in comparison to wild type LpL. One experiment was performed on FH fibroblasts, which do not express the LDL receptor. The monomer/dimer ratio for the preparation used in this assay was 5.8. The mediated uptake was 46% of the wild type which corresponds well with the data obtained with Hep3b cells (Fig. 2).

The naturally occurring mutant Asp291Ser has been shown to have reduced enzymatic activity (53) and to cause mild hyperlipidemia in heterozygotes (54). The Asp291Ser mutant was relatively stable with a monomer/dimer ratio of 1.9 and increased the β-VLDL uptake into the cells as efficiently as the wild type (**Fig. 3**). The mutant Arg294Ala, which has a highly reduced heparin binding activity (32), was found to be expressed as a rather stable dimer with a monomer/dimer ratio of 0.4–0.7, which is much lower than the wild type (mean of 1.95). According to this ratio, the function of this mutant in mediation of β-VLDL uptake was expected to exceed that of the wild type. However, the uptake of β-VLDL with Arg294Ala was only 88% of the wild type (mean from three experiments, Fig. 3).

LpL truncated at residue 390 does not mediate β-VLDL uptake into cells

The treatment of bovine LpL with chymotrypsin results in a cleavage between residues Phe390–Ser391 and Trp392–Ser393. The large fragment generated by this cleavage comprises the residues 1–390/1–392, and therefore constitutes an amino-terminal fragment of LpL. This fragment, referred to as 'cLpL', is a dimer (41) and was used in binding experiments to test its ability to mediate the uptake of β-VLDL into cells. No significant increase of the β-VLDL uptake (8 and 12% of the wild type LpL) could be observed (**Fig. 4**).

Analysis of mutations in residues 390–448

Residues 390–448 were shown to be important in the binding of LpL to LRP and for increasing uptake of β-VLDL into cells (14). We therefore performed experiments using mutants in this region. Two point mutations, Trp390Ala and Trp393Ala, did not reduce the mediation of β-VLDL uptake (**Fig. 5**). The mutant Trp393Ala could be detected in the monoclonal ELISA

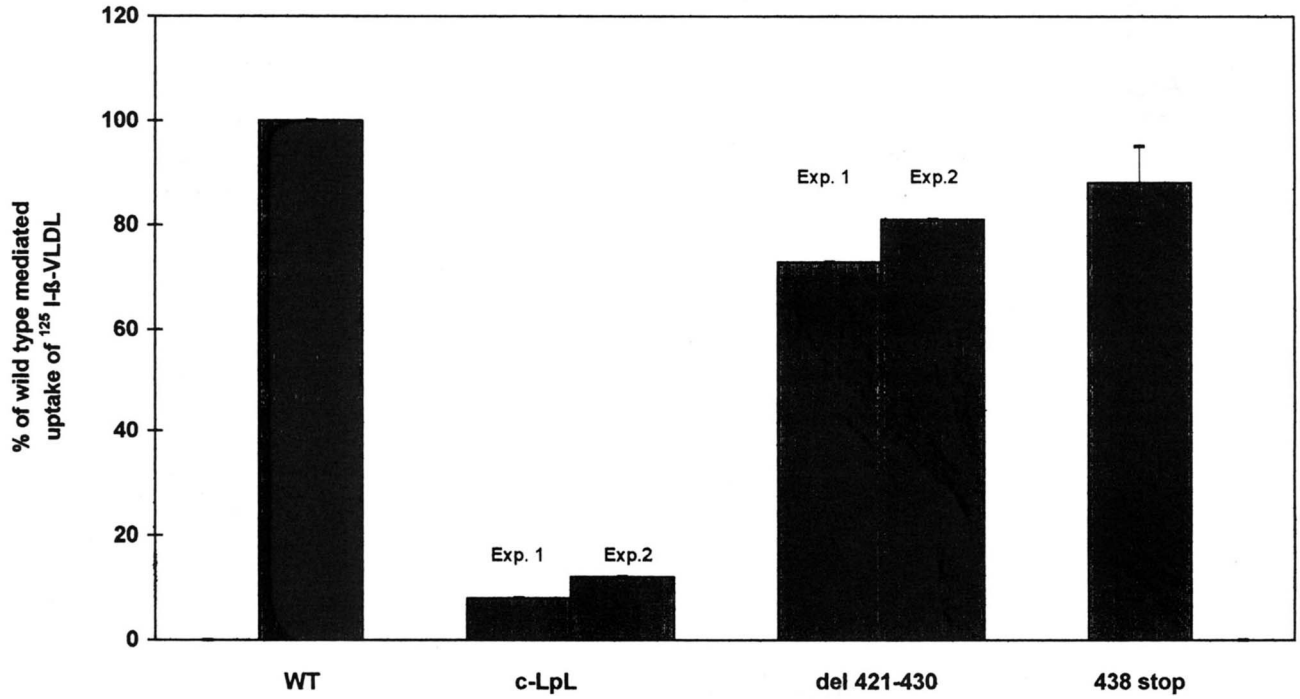

Fig. 4. Specific uptake of β-VLDL mediated by LpL truncated with chymotrypsin (cLpL) or deleted in the C-terminal end and compared to wild type LpL. For cLpL and del421–430, two experiments are shown, respectively, using two different preparations for del421–430. For the 438stop, three preparations were used and five experiments were performed. The mean and standard deviation are shown. The experimental conditions are described in the legend to Fig. 2.

and was found to have a monomer/dimer ratio of 1.78–2.21 which is similar to the wild type (1.95, Table 1). The Trp390Ala could not be measured with the monoclonal ELISA, but after separation of monomers and dimers on a heparin column and measurement with the polyclonal ELISA, the ratio was found to be 1.0. The uptake mediated by this mutant was 89–129% of that of the wild type and therefore can be considered to be normal. In contrast, a deletion of residues 390–393 led to a 40% decrease in β-VLDL uptake compared to the wild type (Fig. 5). This mutant was not recognized by the monoclonal ELISA but after heparin column separation the monomer/dimer molar ratio was found to be 1.6, e.g., it was only slightly higher than that of the wild type preparation used in the same experiment. The defect in mediation of β-VLDL could not, in this case, be due to an excess of monomers. Another mutant in which residues 390–393 were replaced by the corresponding sequence from HL (WS**DW** → WS**TG**) had a slightly greater effect on the uptake of β-VLDL than the wild type LpL (Fig. 5). The monomer/dimer ratio was again estimated after separation on a heparin column and found to be 1:1. We conclude from these data that residues 390–393 are important for the LpL-mediated uptake of lipoproteins and that the corresponding HL sequence has the same functional capacity.

A mutation with a deletion of residues 421–430 was produced and found to be 73 and 80% active in the mediation of β-VLDL uptake (Fig. 4). The polyclonal ELISA revealed 350 ng LpL/ml expression medium, which is comparable with the wild type level. For this mutant we were unable to measure the monomer/dimer ratio as we had no more material. However, due to the rather high efficiency in the mediated uptake, we assume that this region is not of great importance for this function of LpL.

A mutant with a termination codon after residue 438 had a monomer/dimer ratio of 2.9–4.1 which was higher than that of the wild type. This mutant was found to be active in the mediation of uptake of β-VLDL (Fig. 4). The results on the 438 stop and del 421–430 mutants indicate that the final 27 residues in LpL are most probably not involved in mediating β-VLDL uptake.

When all data on uptake mediated by the different LpL variants were plotted against the monomer/dimer molar ratio, a clear negative correlation was seen for most of the preparations (**Fig. 6**). Only two mutants, Arg294Ala and del390–393, were clearly less effective than expected from the wild type data, and therefore they were not included in the correlation.

DISCUSSION

We have investigated structural features of LpL that might be involved in the interaction between LpL and the cell surface by studying the ability of different mutants to mediate the uptake of β-VLDL into cells.

For most previous experiments concerning interaction of LpL with cells or LRP, dimeric bovine LpL was used. It has been shown that both monomers and dimers of LpL bind to LRP and to lipoproteins (14), but only dimers mediate binding of lipoproteins to LRP and the uptake of lipoproteins into cells (14). A suggested explanation was that the binding sites on the monomer for LRP and for lipoproteins partly interfered with each

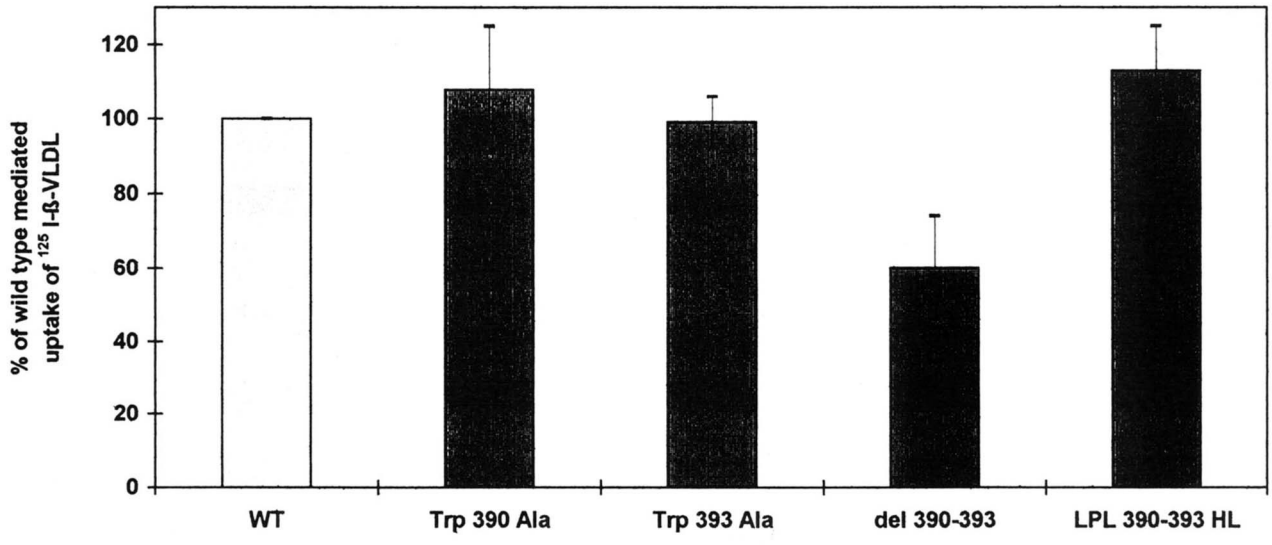

Fig. 5. Specific uptake of β-VLDL mediated by LpL mutated in residues 390–393 compared to wild type LpL. The mutants are Trp390Ala, Trp393Ala, del 390–393, and LpL390–393HL. The mean of three or four separate experiments and the standard deviation are shown for each mutant. The experimental conditions are described in the legend to Fig. 2.

other, and that the bipolar dimer was therefore needed to fulfill the mediating function. Monomers have also been shown to compete with LpL dimers for binding to LRP (17).

Our expression media from the COS-cells contained a mixture of LpL monomers and dimers in molar ratios from 0.4 to 7.1. Some of the monomers were probably formed in the media from active dimers during the 24-h culture period before the media were harvested, and some may also have been directly secreted from the cells. The monomers create a problem in functional analyses of the mutants. Using media with a constant amount of LpL dimers (0.2 μg/ml) and differing amounts of monomers, the efficiency of lipoprotein uptake was a function of the monomer/dimer ratio (Fig. 6). The amounts of LpL protein in the media were, however, not enough to isolate pure dimers in sufficient concentrations for the uptake experiments.

Earlier studies with cLpL showed that this truncated dimeric form of the enzyme is not able to bind to LRP and therefore provided evidence that the C-terminal domain of LpL is responsible for this non-catalytic function of the lipase (14). Recent studies from Nykjaer et al. (17) with C-terminal fragments have demonstrated specific binding of these fragments to LRP; they were, however, not able to mediate lipoprotein binding. As monomeric LpL, the fragments were able to inhibit the effect of dimeric LpL (17). These findings clearly suggest that the domain of LpL primarily involved in mediating the uptake of β-VLDL into cells and the binding of LpL to LRP is located in the C-terminal 58 amino acids of the LpL (residues 390–448). A similar approach was taken by Chappell et al. (55). They used a recombinant fragment of LpL, corresponding to the entire C-terminal domain (residues 318–448), to demonstrate that this part of the molecule could mediate uptake of lipoproteins into cells, and demonstrated that the exchange of residues Lys407Ala reduced this function. These data, and data of Williams et al. (42), proposing that LpL monomers are able to mediate uptake of lipoproteins into cells, are in contradiction to our findings.

From our experiments with the mutants we conclude the following. Mutation in the catalytic triad, Asp156Gly, results in LpL that is less stable than that of the wild type LpL, which was reflected in a high molar ratio of monomers/dimers. The apparent affect on mediation of uptake of β-VLDL was correspondingly low. With this mutant, a clear correlation between the concentration of monomers in the different preparations and the amount of mediated uptake was seen (Fig. 2). Extrapolation from all data, however, indicated that this mutant had a capacity to increase uptake of β-VLDL into cells similar to that of the wild type. With this mutant, one experiment was done using LDL receptor-negative human fibroblasts. The uptake in these cells was similar to that obtained with Hep3b cells. Earlier experiments on normal and FH fibroblasts had also shown that the LDL receptor did not play an important role in LpL-mediated uptake of β-VLDL (56). Several experiments with different ligands performed in our laboratory resulted in a

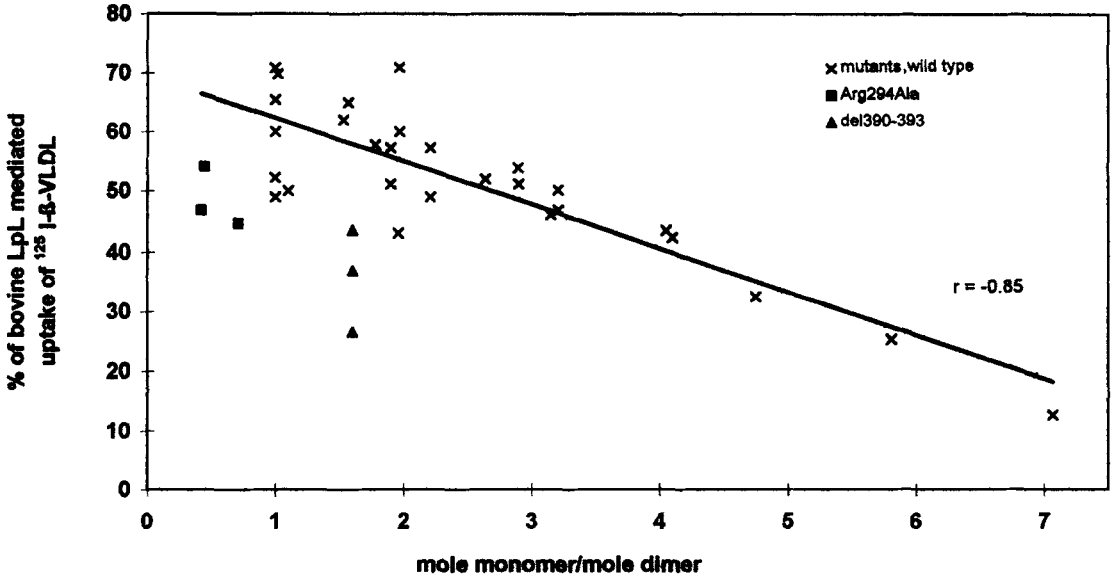

Fig. 6. The correlation between specific uptake of β-VLDL and the monomer/dimer molar ratio is shown for the wild type and all mutants in relation to the function of the bovine lipase. The mean percentage of uptake by the wild type corresponds to 57% of the bovine LpL (see Table 1), and this value was used to recalculate the percentage of the mutants for this figure. Each symbol (see inset) represents one experiment. The correlation curve has been calculated for all mutants (r = -0.85, P < 0.001) except Arg294Ala and del390–393. The uptake by these two mutants clearly differed from the value expected from their monomer/dimer ratio in comparison to the wild type data, and therefore they were not included in the correlation.

slightly higher LpL-mediated uptake into LDL receptor-deficient fibroblasts compared to normal human fibroblasts (A. Krapp and U. Beisiegel, unpublished data).

It has been demonstrated that one important factor in the cellular binding of lipoproteins, mediated either by LpL, HL, or apoE, is interaction with cell surface proteoglycans (39, 40, 57). The suggested mechanism is a rapid, high capacity binding to heparan sulfate proteoglycans, aimed at concentrating the lipoproteins on the cell surface, and followed by interaction with more specific receptors for internalization. We therefore investigated one mutant that had been shown to affect the heparin affinity of LpL, Arg294Ala (32, 37). While Ma et al. (32) found all mutant protein in the void of the heparin column, Hata et al. (37) described only a slightly reduced heparin binding capacity. The monomer/dimer molar ratio for this mutant as used in our experiments was very low (0.4–0.7). This seems to be due to a higher stability of this mutant as also shown by Hata et al. (37). They demonstrated, with heparin column chromatography, that the Arg294Ala had a higher amount of dimer than the wild type. The mediation of lipoprotein uptake expected from the wild type and other mutations for a mutant with this high amount of dimer would be 122% of the wild type, corresponding to 67% of bovine LpL. The mediation found in all experiments was, however, only 88% of the wild type (Table 1), corresponding to 49% of bovine as shown in Fig. 6. This partly reduced function of LpL in mediation of lipoprotein binding could be due to the partly reduced heparin binding as described by Hata et al. (37). These results emphasize the role of LpL dimers with an intact heparin binding site for the initial binding of LpL to the cell surface proteoglycans.

Williams et al. (42) have suggested that Trp393 and Trp394 are involved in binding of LpL to lipoproteins and therefore we created mutations in the 390–393 region (human numbering) and studied the effect on the mediation of uptake of β-VLDL. Exchange of Trp390 and Trp393 for alanine did not change the uptake. In contrast, deletion of residues 390–393 reduced the ability of LpL to increase uptake by 40% as compared to that of the wild type. This function was, however, restored by replacing the deleted amino acids 390–393 with the corresponding residues found in HL, which is also known to influence uptake of lipoproteins (16, 17). All four mutations in the region 390–393 were relatively stable in the dimeric form (monomer/dimer ratio 1.0–2.21). Therefore, the reduced uptake caused by the del390–393 could be due to reduced binding to β-VLDL.

Several other deletions between residues 380 and 430 of human LpL were constructed but none gave rise to significant amounts of LpL protein in the expression media and they could, therefore, not be studied. Such deletion mutants were also found to be unstable by Kozaki et al. (58). One of the deletion mutants (del421–430) was, however, expressed in amounts comparable to the wild type LpL. Its function in mediating lipoprotein uptake was about 80% of that of the wild type. Although we do not know the monomer/dimer ratio of this preparation, it is unlikely that this region is involved in the mediation of uptake.

Pancreatic lipase is shorter than LpL in the C-terminal end by ten residues and does not bind to LRP or mediate binding of lipoproteins to cells. Accordingly, we created a deletion of the last ten amino acids in LpL (438stop) and found that this truncation did not significantly affect the mediating function.

In the present study, we have determined the ability of mutated variants of LpL to influence the uptake of lipoproteins into cells with the longterm goal of defining what structures in LpL are important for the uptake and whether this uptake is dependent on interaction with LRP. A mutant with reduced binding affinity for heparin showed reduced uptake, which underlines the fact that the ability to interact with proteoglycans on the cell surface is of importance for uptake. The del390–393 was reduced in mediating the uptake, probably due to the decreased lipoprotein binding.

In summary, our data indicate that residues 390–421 in the dimeric LpL are relevant for the mediation of lipoprotein uptake into cells. This is in agreement with data from Nykjaer et al. (17) and Williams et al. (42) who defined residues 378–448 and 313–448, respectively, as functional domains. The data presented here underline the concept that dimeric LpL needs three functional domains to mediate lipoprotein uptake. Two of those were also found to be important in our experiments, a proteoglycan binding site and a site for binding to lipoproteins. The third domain, the LRP binding site (17), however, could not be further verified in this study. The main conclusion from our studies is that the most important structural property of LpL for mediation of lipoprotein uptake into cells is its dimeric form. Monomers, even only a 2-fold molar excess over dimers, significantly decreased the uptake. Therefore, any change in the LpL structure that affects the stability of the dimer can be predicted to decrease the mediation of uptake and this should be considered in all studies involving LpL mutants.

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