Mutagenesis in four candidate heparin binding regions (residues 279–282, 291–304, 390–393, and 439–448) and identification of residues affecting heparin binding of human lipoprotein lipase

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Abstract Lipoprotein lipase (LPL) interaction with membrane-associated polyanions is a critical component of normal catalytic function. Two strong candidate binding regions, rich in arginine and lysine residues, have been defined in the N-terminal domain (aa279–282 and aa292–304) that show homology to the heparin-binding consensus sequences -X-B-B-X-B-X- and -X-B-B-B-X-X-B-X-, respectively. Additional candidate regions appear in the C-terminal domain, (residues 390–393), which are homologous to the thrombospondin heparin-binding repeat, and the positively charged terminal decapeptide (residues 439–448). To determine residues and domains critical to heparin binding, we have generated different LPL mutants that have alanine substitutions of single arginine and lysine residues and sequence interchanges with the homologous hepatic (HL) and pancreatic (PL) lipases. The mutant cDNAs were expressed in COS-1 cells and catalytically active mutants were assessed for binding to heparin-Sepharose. All the alanine substitutions within the two regions homologous to the heparin-binding consensus sequences in the N-terminal domain either abolished activity or produced a lowering of heparin binding affinity. None of the mutants in the C-terminal domain of LPL showed a loss of activity or a reduction in heparin binding affinity. These data demonstrate that charged residues at positions 279–282 and 292–304 of LPL are important for heparin binding affinity whereas the residues 390–393 and 439–448 in the C-terminal domain are not involved in heparin binding.—**Ma, Y., H. E. Henderson, M-S. Liu, H. Zhang, I. J. Forsythe, I. Clarke-Lewis, M. R. Hayden, and J. D. Brunzell.** Mutagenesis in four candidate heparin binding regions (residues 279–282, 291–304, 390–393, and 439–448) and identification of residues affecting heparin binding of human lipoprotein lipase. *J. Lipid Res.* 1994. **35:** 2049–2059.

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The binding of LPL to the polyanionic surfaces of membrane-associated proteoglycans is likely to be a critical element in the normal functioning of this enzyme whose major role is the hydrolysis of lipoprotein triacylglycerols in the capillary vasculature of tissues that have a metabolic requirement for free fatty acids (1). The retention of LPL on the capillary endothelial surface is mediated through the binding of LPL to the heparan sulfate side chains of cell surface proteoglycans (2). This interaction also likely stabilizes the active dimer form of the enzyme which is known to dissociate rapidly into inactive monomer and aggregated forms when free in solution (3). Another role for LPL in lipoprotein metabolism has recently been postulated and it is possible that the polyanionic binding site is also involved. This role derives from in vitro studies that show that LPL can function as a ligand for the low density lipoprotein receptor-related protein (LRP) and facilitate the uptake of chylomicron remnants (4–6). Also, LPL has been shown to enhance the binding of other lipoproteins such as VLDL, IDL, LDL, and lipoprotein [a] to cultured fibroblasts and HepG2 cells (7–10). This LPL-mediated lipoprotein binding is dependent on cell surface heparan sulfate as it is greatly decreased or abolished when the cell surface is treated with heparinase. These findings indicate that the heparin-binding domain of LPL is likely to be a critical element in the binding of these LPL/lipoprotein complexes. It remains to be determined whether the heparin-binding domain of LPL also serves as a site of interaction with LRP.

Abbreviations: LPL, lipoprotein lipase; HL, hepatic lipase; PL, pancreatic lipase; LRP, low density lipoprotein receptor-related protein; FFA, free fatty acids.

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A recent study examining the physical properties of chimeric lipases, constructed by interchanging functional domains of LPL with rat HL, which has a lower affinity for heparin, has suggested that the C-terminal domain of LPL (aa313–448) may also participate in heparin binding (15). Several candidate regions occur in this domain. One of these is the positively charged terminal decapeptide of LPL which contains three lysine residues. The candidacy of this region is supported by the observation that a similar extension is not found in the homologous PL which has poor heparin-binding properties. Another candidate region is defined by the studies on heparin-binding peptides demonstrating that interaction of proteins with sulfated proteoglycans does not always involve charged residues. Type I repeats of thrombospondin do not require positively charged residues for heparin binding but a non-charged tetrapeptide, Trp-Ser-X-Trp (consensus sequence, WSXW) (16, 17). LPL carries this sequence at residues aa390–393, this being -W-S-D-W-. In HL, with a lower affinity for heparin, this sequence has altered to -W-S-T-G-, while for PL which interacts poorly with heparin, this sequence is entirely absent.

In this study, we report on the generation of different LPL mutants with alterations in the charge profile of four putative heparin-binding regions. We have additionally attempted to determine the limits of one of these regions by substituting flanking charged residues. Our data suggest that both charge and possibly conformation in two (positions 279–282 and 292–304) of the four regions are important for the interaction of LPL with heparin.

EXPERIMENTAL PROCEDURES

Different approaches to mutagenesis could be undertaken to determine the contribution of positively charged residues to the ionic interaction between LPL and heparin. We initially chose to substitute lysines and arginines on an individual basis, thereby generating mutant species with one charge difference in the putative heparin-binding regions. We expected this approach to minimize the conformational change induced by the amino acid substitution, and to give a graded effect on heparin affinity as some residues were likely to be more critically positioned than others. We later sought to neutralize or alter the charge profile of the entire region by interchanging them with the homologous regions from PL and HL which have lower binding affinities for heparin.

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 (CDM8) for both mutagenesis and expression as previously described (18). A gapped-duplex DNA template containing a single-stranded LPL cDNA gap for mutagenesis was annealed with individual mutagenic primers and mutagenic reactions were performed (18). Twenty mutant constructs were generated and are listed in **Tables 1A and 1B.** Mutagenesis primers for the single codon alterations contained centrally positioned 1 or 2 bp mismatches, generating one of the codons for alanine (GCA, GCG, GCT) and the TAA stop codon for the 439 truncation mutant. The mismatched bases in these primers were flanked by 9 or 10 bp of complimentary sequence. Mutagenesis primers for the chimeric mutants contained the sequence to be exchanged and 9 bp of flanking complimentary sequence. Primers for the deletion mutants comprised 9 to 12 bp of sequence flanking the site of deletion (Table 1A and B).

Mutant clones were identified by hybridization with individual mutagenic primers and confirmed by DNA sequencing. Alanine was selected to replace the charged residues because of its short nonpolar methyl side chain which is unlikely to impose severe constraints on the normal folding of the protein as it has been found both in buried hydrophobic environments and in solvent-exposed positions (19, 20).

Twenty mutant constructs were generated. Nine of these involved alanine substitutions of basic residues in the putative heparin-binding sequences of the N-terminal domain. To assess the role of the thrombospondin-like heparin binding sequence WSDW in the C-terminal domain (aa390–393), we separately deleted this tetrapeptide or replaced it with the WSTG sequence of HL. The role of the terminal decapeptide (aa439–448) in heparin binding was investigated by deleting this section through the introduction of a premature termination codon at position 439. Heparin binding activity could only be assessed for nine mutants that eluted in active dimer form on heparin Sepharose chromatography. These nine mutants are listed in Table 1A while the other mutants that revealed absent LPL catalytic activity or did not elute in active dimeric form on heparin-Sepharose columns, are listed in Table 1B.

LPL, HL, and PL are members of a single gene family and show significant amino acid sequence homology, especially in exons 3, 4, 5, and 6 in the N-terminal domain (21). This homology, however, is weak in the two proposed heparin-binding sites of LPL located between aa279–282 and 291–304 in exon 6 and may be invoked to explain the difference in heparin binding affinity observed among these lipases particularly as HL binds to heparin-Sepharose with a lower affinity than LPL, while in vitro studies have shown that PL binds poorly to heparin (14, 22). To explore this further, we generated chimeric mutants in which the putative heparin-binding sequences of LPL were replaced with the homologous regions from either HL or PL (Table 1B).

TABLE 1. A: I. Charge to alanine substitutions of basic residues in candidate heparin binding regions that resulted in a catalytically active LPL assessed for heparin binding

N-terminal mutants

Site #1

```
                          aa279  282
                            |     |
hLPL                        C-R-K-N-R-C
K280A                       ----A------
```

Site #2

```
                        aa292      297    300      304
                          |          |      |        |
hLPL                    I-N-K-V-R-A-K-R-S-S-K-M-Y-L-K-T
K292A                   ----A-------------------------
R294A                   --------A---------------------
R297A                   ------------A-----------------
K300A                   ----------------A-------------
```

Flanking region

```
                      aa289                  306
                        |                     |
hLPL                  Y-E-I..(291-304)..T-R-S..
E289A                 --A--..(291-304)..-----..
```

II. C-terminal domain active chimeric mutants generated by substitution of LPL residues with homologous resididues from PL and HL

Site #1

```
                          aa390   393
                            |     |
hLPL                        F-S-W-S-D-W-W-S
LPL-(del390-393)-PL         F-S-W-S
LPL-(390-393)-HL            F-S-W-S-T-G-W-S
```

Site #2

```
                        aa439                 448
                          |                    |
hLPL                    C-H-D-K-S-L-N-K-K-S-G-Stop
LPL-(His439Stop)-PL     C-Stop
```

B: In vitro mutants that resulted in a catalytically inactive LPL protein or that did not elute in active dimeric form

N-terminal domain candidate regions

Site #1

```
                          aa279   282
                            |     |
hLPL                        C-R-K-N-R-C
LPL-(279-282)-PL            --P-S-G-G--
R279A                       --A--------
R282A                       --------A--
```

Site #2

```
                        aa291          297 299       304
                          |              |   |         |
hLPL                    I-N-K-V-R-A-K-R-S-S-K-M-Y-L-K-T
LPL-(291-297)-HL        --K-Q-E-P-G-S-K----------------
LPL-(292-297)-PL        ----R-Y-P-G-K-T----------------
LPL-(299-304)-HL        ------------------K-R-L-F-L-V--
LPL-(300-304)-PL        --------------------K-F-Y-L-D--
LPL-(Ins299)-PL         I-N-K-V-R-A-K-R-S-S-(V-G-Q)-K-M-Y-L-K-T
K296A                   ----------A-------------------
K304A                   ----------------------------A
```

Flanking region

```
                      aa289                  306
                        |                     |
hLPL                  Y-E-I..(291-304)..T-R-S..
R306A                 -----..(291-304)..--A--..
```

Transient expression of mutant LPL in COS-1 cells

Phagemid DNA carrying the desired mutations was purified and introduced into COS-1 cells by electroporation as previously described (23). The transfected cells were plated in either 90- or 175-mm culture dishes in 10–15 ml of DME medium with 5% fetal calf serum (FCS) and incubated for 24 h. At this point, the medium was replaced with an equal volume of fresh DME medium containing 5% FCS and 40 μg/ml of heparin. The cells were cultured for a further 72 h with medium collected every 24 h. These samples were snap-frozen in dry ice–ethanol and stored at −120°C. Transfection experiments for each mutant were performed 2–3 times to determine reproducibility. For mutants that showed reduced affinity for heparin-Sepharose, transfection experiments were repeated under similar conditions but with 40 ng/ml of heparin to ensure that the reduction in affinity was not due to the relatively high concentration of heparin (40 μg/ml) used in the initial experiments.

Measurements of mutant LPL activity and mass

LPL lipolytic activity in the COS-1 cell medium was determined using a radiolabeled tri[1-^{14}C]oleoylglycerol-lecithin emulsion as substrate (24). The released fatty acids were extracted and counted in a liquid scintillation counter. The LPL activity in COS-1 cell medium was expressed as nanomoles free fatty acids released per minute per ml (nm FFA/min per ml) while activity in fractions collected from the heparin-Sepharose column was expressed as free fatty acids released per minute by a 50-μl aliquot.

LPL dimer and monomer mass was determined by ELISA using the monoclonal antibodies 5D2 and 5F9, either separately or in combination as previously described (25). We have previously shown that residue 400 of mature LPL is a critical component of the epitope recognized by the 5D2 antibody (26). The epitope for the 5F9 antibody is unknown at present. Both these epitopes remained intact in each of the mutants examined in this study. Specific activities of the LPL mutants were estimated by division of lipase activity in the medium by the dimer mass.

Heparin-Sepharose chromatography

Heparin binding affinity was assessed on a Bio-Rad Econo-Column (0.5 cm ID by 10 cm length) packed with 1 ml heparin-Sepharose CL-6B (Pharmacia). The column was equilibrated with 10 mM phosphate buffer (pH 7.5) containing 30% glycerol (v/v), 0.1% CHAPS (v/v), and 0.4 M NaCl. Prior to application, 3 ml postheparin plasma was adjusted to 0.1% (w/v) with respect to deoxycholate while the transfected COS-1 cell medium was treated with an appropriate volume of glycerol, 10% deoxycholate, and solid NaCl to a final concentration of 30% glycerol, 0.1% deoxycholate, and 0.4 M NaCl. Aliquots of COS cell media containing 300–400 ng dimer mass were applied to the column at a flow rate of 0.25 ml/min and eluted, without an intermediate washing step, with a linear NaCl gradient from 0.4 M to 1.8 M. The total volume of the gradient buffer was 36 ml with a single fraction size of 1.5 ml (24 fractions). We found that >80% of LPL activity was bound to the column when less than 400 ng of wildtype or mutant LPL was loaded. LPL mass and activity, and eluate conductivity were determined in each fraction. Recovery of lipase activity from the column loaded with control LPL transfection medium was 60–80%.

Computer modeling

The crystallographic coordinates of human PL were kindly supplied by Dr. F. Winkler (Central Research Unit, F. Hoffman-La Roche, Basle). Molecular modeling was carried out using Biosym Insight II and Discover software on a silicon Graphics W-4D-35G workstation. Comparative analyses of the primary sequence of PL with that of LPL and HL were carried out using the sequence alignments reported previously (21, 27).

RESULTS

Mass and activity measurements

All LPL mutants yielded detectable immunoreactive mass in the medium from the transfected COS-1 cells (**Tables 2A and 2B**). The majority were tested on more than one occasion and showed the expected scatter of LPL mass levels from one experiment to another. This is due to variation in transfection efficiencies which we found to be 10–20% using a reporter vector (data not shown). Many of the charge-to-alanine single mutants were capable of producing mutant LPL at levels comparable with normal LPL, although the general tendency was towards lesser amounts (Table 2A). However, the LPL mass levels for most of the chimeric mutants were low and some of them were as low as 10–20% of normal (Table 2B).

The LPL catalytic activity in the COS-1 medium for five mutants was very low or absent including the K296A sample, which was an alanine substitution mutant (Table 2A), and the other four were chimeras (Table 2B).

LPL specific activities

Specific activities are reported herein as apparent specific activities as they reflect the relative amounts of active LPL dimer and inactive form of LPL present in the medium from the transfected COS-1 cells. The apparent specific activity of normal LPL was found to be relatively consistent at 0.30 ± 0.07 nmol/min per ng (n = 9). Specific activities of the individual mutants, however,

TABLE 2. A: LPL mass and activity levels of charge to alanine and chimeric mutants that were catalytically active in cos cell medium

	Mass	Activity	Specific Activity
	ng/ml	*nmol/min/ml*	*nmol/min/ng*
hLPL(1)	344 + 12.8	86 + 2.8	0.25
hLPL(2)	794 + 5.6	284 + 5.7	0.36
hLPL(3)	279 + 12.7	97 + 4.2	0.35
Charge-to-alanine mutants			
K280A(1)	307	98	0.32
K280A(2)	500	85	0.17
K292A(1)	116	6	0.06
K292A(2)	318	21	0.07
K292A(3)	350	47	0.13
R294A(1)	181	59	0.33
R294A(2)	140	ND	ND
R294A(3)	80	ND	ND
R297A(1)	114	53	0.46
R297A(3)	110	ND	ND
K300A(1)	337	24	0.07
K300A(2)	350	59	0.17
Flanking region			
E289A	375	47	0.13
C-terminal mutants			
LPL-(del390-393)-PL	40	49	1.23
LPL-(390-393)-HL	70	106	1.51
LPL-(His439Stop)-PL	273	57	0.21

B: LPL mass and activity levels of charge to alanine and chimeric mutants that were catalytically inactive or did not elute in active dimer form

	Mass	Activity	Specific Activity
	ng/ml	*nmol/min/ml*	*nmol/min/ng*
Charge-to-alanine mutants			
R279A(1)	89	11	0.13
R279A(2)	206	33	0.16
R279A(3)	108	47	0.43
R282A(1)	305	53	0.17
R282A(2)	672	69	0.10
K296A(1)	64	0.2	0
K296A(2)	220	0	0
K296A(3)	120	0	0
K304A(1)	247	6.6	0.03
K304A(2)	300	32.0	0.11
K304A(3)	1000	95.0	0.10
LPL-(279-282)-PL(1)	19	0	0
LPL-(279-282)-PL(2)	32	0	0
LPL-(279-282)-PL(3)	36	0	0
LPL-(291-297)-HL	144	3	0.02
LPL-(292-297)-PL(1)	54	21	0.39
LPL-(292-297)-PL(2)	88	28	0.32
LPL-(299-304)-HL(1)	25	1	0.04
LPL-(299-304)-HL(2)	52	1	0.02
LPL-(299-304)-PL(1)	35	1	0.03
LPL-(299-304)-PL(2)	55	0	0
LPL-(Ins299)-PL(1)	275	39	0.14
LPL-(Ins299)-PL(2)	137	47	0.34
Flanking region			
R306A(1)	570	161	0.28
R306A(2)	1050	220	0.21

ND, measurements not done.

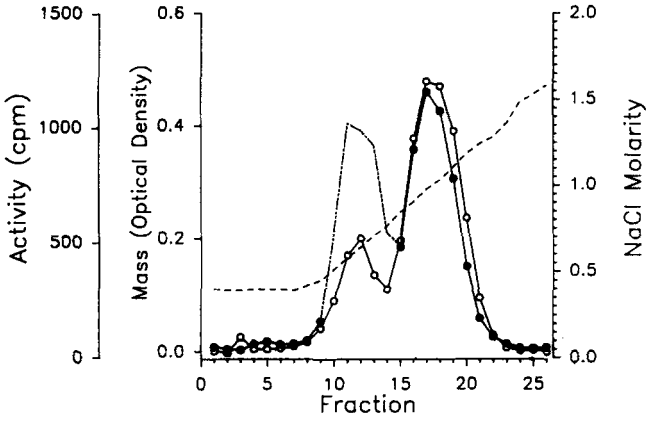

Fig. 1. Chromatography of postheparin plasma on heparin-Sepharose. Pooled plasma (3 ml) was adjusted to 0.1% deoxycholate, loaded on to a 1-ml column of heparin-Sepharose and eluted by a linear salt gradient from 0.4 to 1.8 M NaCl. LPL activity and mass were determined in each fraction as was the conductivity (see Experimental Procedures). LPL activity (-●-) eluted as a single peak and was centered on fractions 17–18 at a NaCl molarity of 1.1. LPL mass (-O-) eluted as two peaks with the major peak underlying that of the activity profile and representing LPL dimer. The earlier eluting monomer form of LPL was centered on fraction 12 and a NaCl concentration of 0.8 M. Hepatic lipase activity elutes earlier than LPL activity from heparin-Sepharose (24) and is shown by the dotted and dashed line (----).

varied greatly ranging from approximately 0.1 to 1.5 but with the general trend being towards lower values than that of normal LPL (Tables 2A and 2B).

Heparin-Sepharose chromatography of normal LPL

Catalytically active LPL in postheparin plasma exists predominately as a homodimer. Peak activity was found to elute from the heparin-Sepharose column in fractions 18–20 at a peak NaCl concentration of 1.1 M (**Fig. 1**). This is consistent with our previous observation (24) and with reports by others (28). LPL dissociates readily into its constituent monomers and this is coupled to a loss of catalytic activity plus reduced affinity for heparin. This inactive LPL monomer was found to elute in fractions 11–13 at 0.8 M NaCl (Fig. 1). The activity and mass elution profiles of normal LPL expressed in COS-1 medium was similar to that of postheparin plasma with homodimer activity and mass eluting in fractions 19–20 at 1.1 M and inactive monomer mass eluting in fractions 11–14 at 0.8 M (**Fig. 2A**). The minor activity and the mass peak eluting in the void volume represents unbound LPL.

LPL mass and activity elution profiles of in vitro expressed LPL mutants

Heparin binding affinity could only be assessed for the nine mutants giving activity profiles by retaining their active dimeric forms upon exposure to the inhibitory effects of the high NaCl concentrations of the elution gradient (Table 2A), while five (R296A, LPL-(279–282)PL, LPL-(291–297)-HL, LPL-(299–304)-HL, LPL-(299–304)-PL) of the original 20 mutants were catalytically inactive in

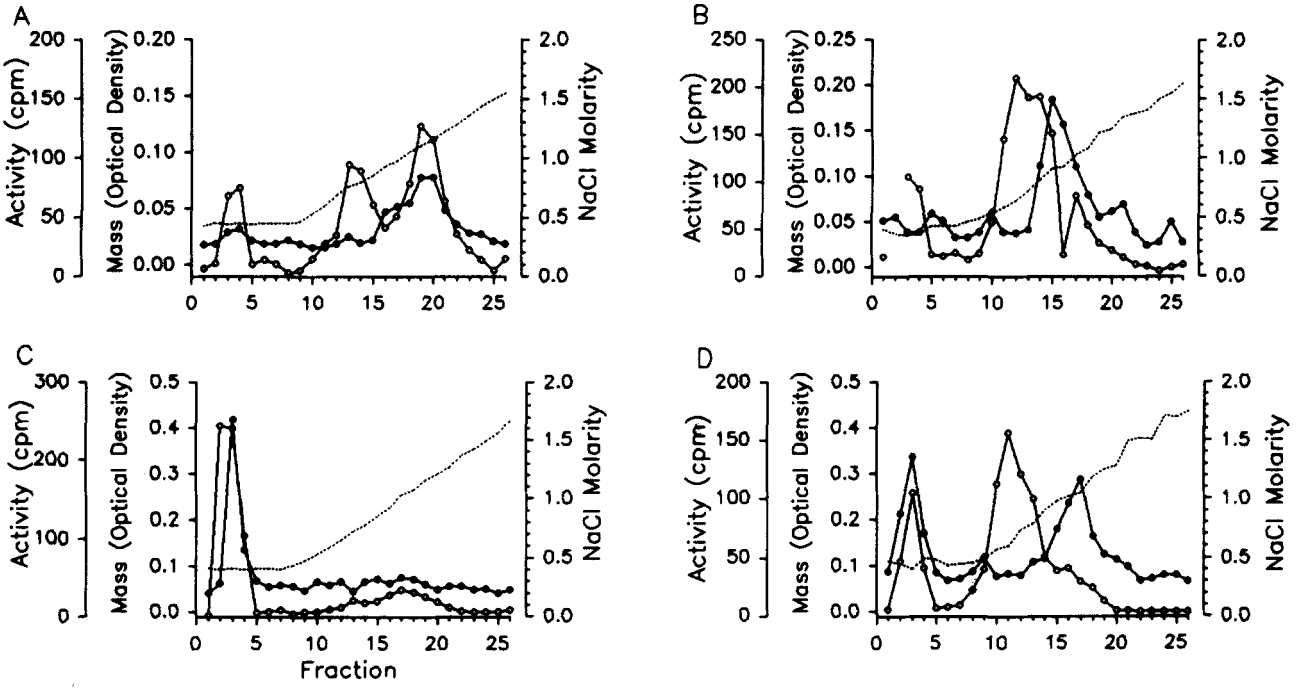

Fig. 2. Chromatography of LPL mutants on heparin-Sepharose. Medium from transfected COS-1 cells was adjusted to 30% glycerol, 0.1% CHAPS, and 0.4 M NaCl and chromatographed on a 1-ml column of heparin-Sepharose. LPL activity and mass levels were determined as described (see Experimental Procedures). Four sets of profiles are shown in this figure where (-●-) depicts activity while (-O-) depicts mass. A) Mass and activity profiles of normal LPL secreted by the transfected cells. B) Profile of mutant (K280A) showing a moderate decrease in affinity for heparin. C) Profile of mutant R294A with a major decrease in affinity for heparin. D) Profile of LPL-(390–393)-HL chimera showing normal affinity for heparin.

the COS-1 medium, and six (R279A, R282A, K304A, R306A, LPL-(292-297)-PL, LPL(Ins299)-PL) proved to be unstable on the column and did not elute in the active dimer form (Table 2B).

Two peaks of LPL mass were typically seen in the mass profiles of the nine mutants that retained activity on elution from the affinity column. Some of these profiles are shown in Fig. 2A, B, C and D. The inactive component of these profiles consistently eluted in fractions 11–14, irrespective of the affinity and elution behavior of the active dimer form. Only mutant R294A deviated from this pattern with all measured mass and activity appearing in the void volume (Fig. 2C). For the remaining six mutants that lost activity on the column, all LPL mass eluted in fraction 12 (0.8 M NaCl).

N-terminal domain single base substitutions

Three mutants showed moderate to slight reductions in binding affinity, eluting between 0.84 and 0.90 M NaCl (R280A, R297A, K300A) (**Table 3**). One mutant in this domain, K292A, demonstrated normal heparin binding, while another, R294A, demonstrated a major loss in binding affinity by failing to bind to the column at the 0.4 M NaCl concentration of the loading buffer (Fig. 2C). This finding was confirmed by duplicate runs from different transfection experiments. The mass elution profile of K294A also differed from that of the other mutants with altered affinities. Whereas all the four others showed a prominent inactive mass peak at 0.8 M NaCl no such peak was seen in the profile of R294A (Fig. 2C). To further investigate this mutant we ruled out the possibility of a random cloning error introducing a second mutation in the cDNA by sequencing the entire cDNA and showed no change of sequence in this clone from normal (data not shown).

The COS cell medium used in the transfection experiments contained heparin at 40 μg/ml. To determine whether heparin at this concentration had any effect on the elution behavior of the various mutants, we repeated the transfections and lowered the heparin concentration of the culture medium 1000-fold (40 ng/ml). All the profiles determined were identical to those shown by the mutants at the higher concentration (Table 2B).

Two charged residues were substituted in the sequence immediately flanking the second heparin-binding consensus region in the N-terminal domain (Table 1A). Both these mutants were catalytically active in the COS cell medium (Table 2A). One of these, E289A, showed mildly reduced heparin affinity and eluted at 0.88 M of NaCl while the other, R306A, proved to be unstable and did not elute from the column in an active form.

TABLE 3. Heparin-Sepharose elution data for active LPL mutants

	Fraction #	NaCl (M)
hLPL	18–20	1.10; 1.14
N-terminal mutants		
Site #1		
K280A	15	0.84
Site #2		
K292A	17	0.98
R294A	3	<0.4
R297A	16	0.88
K300A	16	0.88
Flanking region		
E289A	16	0.88
C-terminal mutants		
LPL-(del390-393)-PL	18	1.1
LPL-(390-393)-HL	17	1.05
LPL-(His439Stop)-PL	17	1.01

C-terminal domain mutants

Both the mutants carrying alterations in the WSDW (390–393), thrombospondin-like heparin-binding sequence were found to have normal affinity for heparin. Deletion or substitution of this tetrapeptide, therefore, does not affect the heparin binding sites of LPL (Fig. 2D). Deletion of the terminal decapeptide of LPL also did not influence heparin binding as this mutant was found to elute with normal LPL at a molarity of 1.0 M NaCl (Table 3).

None of the chimeric mutants could be assessed for heparin binding defects as they were all poorly expressed by the transfected COS cells and additionally showed a rapid loss of activity, either in the medium or on the column (Table 2B).

DISCUSSION

In this study we report on in vitro mutagenesis in four putative heparin-binding regions of LPL. Two of these occur in the N-terminal domain, spanning residues 279–282 and 291–304 and which show strong homology to the consensus interactive regions of other heparin-binding proteins (11–13). The other two occur in the C-terminal domain and include the noncharged heparin-binding thrombospondin-like sequence from residues 390 to 393 and the positively charged terminal decapeptide of residues 439–448. Of twenty mutant constructs that were produced and expressed in COS-1 cells, only nine were catalytically active and eluted in active form. The secreted mutants were harvested from the medium and assessed for heparin-binding properties by affinity chromatography on heparin-Sepharose.

Involvement of the N-terminal domain in heparin binding

The first of the strong candidate regions in LPL contains three positively charged residues -RKNR-

(aa279–282) which matches the heparin-binding consensus sequence -BBXB-. We found that the substitution of the second basic residue at position 280 (K280A) produced a mutant lipase eluting earlier than normal LPL from the affinity column (Fig. 2). The binding affinity of the other two mutants in this area, R279A and R282A, could not be assessed as they both proved to be unstable.

The second candidate binding region (aa291–304) is 9 amino acid residues downstream and contains 6 positively charged residues and shows homology with the consensus sequence -XBBBXXBX-. We similarly found that substitution of 5 of the 6 positively charged residues by alanine altered the properties of the enzyme by either lowering the binding affinity for heparin or by destabilizing the active homodimer form. Mutants R297A and K300A showed a slight shift in the elution profile, both eluting earlier than normal LPL. Mutant R294A, in contrast, showed a dramatic reduction in binding affinity, failing to bind to the column on application. In contrast the heparin binding affinity for mutant K292A was not significantly different from the normal.

The two heparin-binding segments (aa279–282 and aa291–304) are separated by a segment of 9 residues containing a negatively charged glutamate at residue 289. We investigated the contribution of this residue to heparin binding by introducing an alanine residue. This E289A mutant manifested slightly reduced affinity for heparin similar to the R297A and K300A mutants. This might suggest that the charge distribution of this intermediate region between the two heparin-binding segments is of some importance in maintaining the proper conformation of the two heparin-binding segments.

Two other research groups have recently reported on mutagenesis experiments in both of these N-terminal candidate regions and in some cases identical mutants have been studied (29, 30). While our findings for these regions generally concur, some of the individual mutants have given conflicting results. In region aa291–304, for example, our report and that from Hata et al. (30) both show a decrease in the affinity of mutants R297A and K300A for heparin. In contrast, Hata et al. (30) report normal binding for the R294A mutant while we determined a significant reduction in the binding affinity for heparin despite normal activity. Other discrepancies involve mutants K296A where we found no catalytic activity in the medium while Hata et al. (30) report on an active mutant with a reduced affinity for heparin. We have ruled out the possibility of a random mutagenesis error introducing a second mutation in the LPL cDNA in mutant K296A by sequencing the entire cDNA and confirming that the K296A substitution was the only mutation.

We have not established the reason for the different behavior of some of these mutants but presume that it is related to differences in experimental detail such as the nature of the heparin-linked matrix of the affinity column plus the different heparin and FCS concentrations in the harvesting medium. These differences do not detract, however, from the general conclusion of both manuscripts that alteration of charged residues in both of these N-terminal candidate regions lowers heparin-binding affinity of LPL. These data do not however distinguish between a reduction in heparin binding affinity due to a specific loss of ionic binding in those regions or the loss of ionic interactions as a result of conformational changes induced by the substituted residues.

An interesting observation in our study, however, is the significant reduction in the affinity for heparin introduced by neutralization of the single basic charge at residue 294. Although we regard R294A as a true heparin-binding mutant, we suspect that substitution of this particular residue also causes a significant conformational change that distorts the alignment of all other residues critical to a stable interaction with heparin but not with the residues involved in the dimerization of LPL as this mutant retains catalytic activity. The activity and inherent stability of this mutant suggest that it may yield valuable data in transfection and transgenic systems determining the importance of heparin sulfate interactions in the normal processing, transport, binding, and lipolytic functions of LPL.

In an attempt to better understand the difference in heparin affinity between LPL, HL, and PL, we generated chimeric mutants by interchanging HL and PL sequences with LPL. Each of these mutants proved to be unstable and we assume that the degree of conformational change induced by the multiple residue substitution was incompatible with normal catalytic function (31).

The high homology of primary and secondary sequence between LPL, PL, and HL strongly suggests that the three-dimensional structures of these enzymes are very similar (31, 33). Predictions of common structure/function relationships and the identification of critical residues are thus possible after the crystallization of PL and the construction of molecular models of the monomer subunit either free in solution or as a complex with its activator polypeptide colipase, and lipid substrate (33, 35). The residues of PL that are homologous to those of the consensus heparin binding regions of LPL are carried on the N-terminal domain and lie close to the junction with the C-terminal domain (**Fig. 3**). These peptides are located distal to the lid of the active site and catalytic channel and are positioned on a different face of the monomer. The first binding region forms a short 4-residue loop which is stabilized by a conserved disulfide bridge, and is highly exposed to solvent. The second is 10 residues further along and consists of regions of random coil and partial beta-strand with the former exposed to solvent while the latter is partially buried. These regions become more exposed to solvent when the N and C-terminal domains bend inwards at the lipid/water inter-

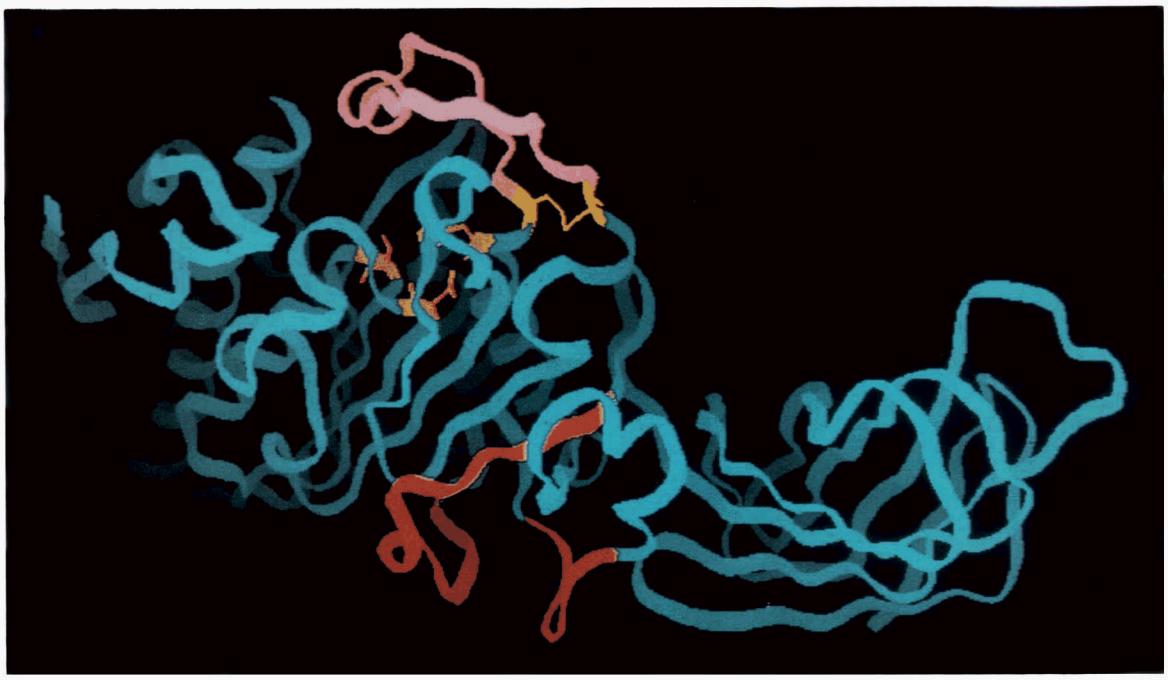

Fig. 3. A ribbon diagram of the alpha-carbon backbone of human pancreatic lipase as determined by Winkler, D'Arcy, and Hunziker (33). Regions homologous to the heparin binding regions of LPL are colored in red. Region 1 is a short disulphide bridged loop comprising residues 301–304 while region 2 involves residues 314–329 which show sections of exposed random coil and partially buried beta-strand. The loop masking the active site is given in pink while the active site residues are colored in orange.

face as part of the conformational change that ensues when PL complexes with colipase and the phospholipid substrate to form a hydrophobic surface comprised of residues of the catalytic channel, the displaced active site lid, and the loop regions of colipase (34). While these data must be interpreted with caution when considering structure/function relationships of LPL, the location of residues of the interfacial binding region and the heparin-binding region on different faces of the LPL molecule is to be expected as this enzyme is known to interact simultaneously with lipoprotein substrates and cell surface proteoglycans (36). This dual binding affinity is assumed to contribute to the molecular interactions that result in the retention of lipoprotein particles on the capillary endothelium. No data are available on the arrangement of the individual monomers in the LPL dimer or the residues involved but it is likely that the adjacent monomers will show parallel axes with the interfacial binding regions in the same plane. This would leave the heparin binding regions of both monomers free to complex with polyanions.

Evidence against particular regions of the C terminal domain being involved in heparin binding

No prior studies have investigated the charge residues in the C terminal domain for their involvement in heparin binding. We find no evidence for a significant disruption in heparin-binding affinity after alteration of the charged residues in the two C-terminal regions examined, (aa390–393 and aa439–448). All mutants generated gave elution profiles similar to normal LPL. We therefore conclude that the consensus heparin-binding sequence (WSXW) previously reported in type I repeats of thrombospondin is not involved in mediating heparin binding of human LPL nor is the terminal charged decapeptide of LPL. Our data are consistent with a recent study by Lookene and Bengtsson-Olivecrona (12) where chymotryptic fragment of bovine LPL showed that heparin-binding affinity resided in the N-terminal domain between aa220–390 while the C-terminal fragment of LPL, residues 390–448, was unable to bind to heparin at the same affinity as the intact LPL. However, our own studies have only addressed two particular regions in the C terminal domain, and the potential involvement in heparin binding of other regions in the C terminal domain remains to be investigated.

The LPL gene has been extensively analyzed for sequence alterations in patients who present with chylomicronemia. While over 30 mutations have now been described (37) to the best of our knowledge, none have been reported to involve residues of the two heparin-binding domains. We interpret this to indicate that binding-defective LPL may either not result in modulation of LPL activity or may only result in partial loss of activity and present with a lipoprotein phenotype other than Type I hyperlipoproteinemia.

In this study, we have provided further evidence for the localization of the heparin-binding domain to particular residues in the N-terminal region of the LPL protein and have further excluded the participation of specific residues in the C-terminal domain in this function.

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