Site-directed Mutagenesis of a Putative Heparin Binding Domain of Avian Lipoprotein Lipase

(Received for publication, October 8, 1992)

Darlene Evans Berryman and André Bensadoun
From the Division of Nutritional Sciences and Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Lipoprotein lipase (LPL) binds to heparin and heparan sulfate proteoglycans. We have employed site-directed mutagenesis to dissect one of the proposed heparin binding domains of avian LPL, which contains the sequence Arg-Lys-Asn-Arg (amino acids 281–284). Various single, double, and triple mutants of chicken LPL were constructed in order to alter the positive charge of this region. The mutants and wild-type cDNA were subcloned into an expression vector, pRC/CMV, and expressed in Chinese hamster ovary cells. In general, the LPL mutants with a decrease in regional positive charge showed a decrease in affinity for heparin and heparan sulfate proteoglycans. The greatest effect was seen with the triple mutant, LPL 5G, in which all of the positively charged amino acids were altered to neutral residues. On a heparin-Sepharose column, LPL 5G eluted at 0.96 M NaCl compared with 1.35 M for wild-type LPL. This mutant also had the lowest specific activity with 1.5 μg fatty acid/mg/h for the cell-associated pool and with no detectable activity in the media. Wild-type cells, however, produced a lipase with a specific activity of 12.4 and 13.1 μg fatty acid/mg/h for cell-associated and media lipase pools, respectively. LPL 5G also showed a decrease in affinity for the heparan sulfate proteoglycans on the cell surface of Chinese hamster ovary cells. In conclusion, the region of avian LPL between Arg and Arg does appear to be involved in heparin-binding; however, additional regions must be involved since binding was not completely abolished. In addition, specific activity of the cell-associated and secreted LPL is correlated to affinity of the enzyme for heparan sulfate chains.

Lipoprotein lipase (LPL) plays an essential role in the hydrolysis of very low density lipoproteins and chylomicron triglycerides (Ref. 1; for review, see Refs. 2–5). The catalytically active form of the enzyme is a noncovalent homodimer (6–8) that requires binding of apolipoprotein CII for maximal activity. LPL also binds with high affinity to heparin and heparan sulfate proteoglycans (HSPG) (Refs. 9 and 10; reviewed in Ref. 11). These features suggest that the enzyme contains several structural domains, including a region responsible for triglyceride hydrolysis, an apolipoprotein CII binding site, a heparin-binding site, and a region responsible for dimer formation. The only structural domain that has been clearly identified is the Ser-Asp-His triad responsible for catalysis (12, 13).

The major focus of this study was to identify regions of LPL that interact with heparin and heparan sulfate proteoglycans. Two lines of evidence imply that the interaction of LPL with HSPG is ionic. 1) High ionic strength is necessary to elute LPL from heparin matrixes (14, 15). 2) A decrease in sulfate density of the heparan sulfate chains leads to a decreased affinity of the enzyme for HSPG (16). The heparin binding domain of LPL was localized to the COOH-terminal portion of the molecule by a ligand blot assay using LPL proteolytic fragments and 125I-heparin (17). These results suggest that positively charged residues within LPL’s carboxyl terminus interact with the negatively charged sulfate of HSPG. By comparison of known heparin-binding sequences of other proteins, two consensus sequences for glycosaminoglycan recognition have been postulated as -B-B-X-B- and -B-B-B-X-B-, where B represents a basic residue and X a hydrophobic residue (18). Two regions of avian LPL contain these exact consensus sequences: amino acid residues 148–153 and residues 281–284. An additional cluster of basic amino acids, between residues 294–299 with the sequence -B-X-B-B-X-B-, has also been attributed to LPL’s binding to heparin. The five published cDNA sequences for various species of LPL show that only two of these three regions, 281–284 and 294–299, are highly conserved (19).

We have employed site-directed mutagenesis to dissect the proposed heparin binding domain with the consensus sequence -B-B-X-B- from amino acid residues 281–284 in avian lipase. Various mutants were designed to alter the overall positive charge of the region. Our results indicate that this region does play a role in the LPL/glycosaminoglycan interaction. We also found that this interaction is critical for stabilizing the active form of the lipase.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression Vector Construction—The OBC clone for the chicken LPL cDNA was previously described by Cooper et al. (19). This LPL clone contains 130 nucleotides of 5’-untranslated sequence, 1439 nucleotides of the protein coding region, and 688 nucleotides of 3’-untranslated sequence. All site-directed mutagenesis was performed using the T7-GEN™ In Vitro Mutagenesis Kit (United States Biochemical Corp.) according to the manufacturer’s instructions. Oligonucleotides, synthesized and 5’-phosphorylated by Cornell Biotechnology Facility, had the following sequences (bases different than those of wild type LPL are underlined).
For most experiments, cells and units/ml heparin (Sigma) was added. After 10 min of shaking at 4°C, the heparin was then subcloned into the BstXI sites of the expression vector pRc/policeman in 0.25 ml/dish modified lysis buffer A containing 5% fetal bovine serum, 2 mM glutamine, and 10 mM HEPES, pH 7.2. Cell extracts were collected by scraping the dish with a rubber policeman in 0.25 ml/dish modified lysis buffer A (4 mM CHAPS, 50 mM NH₄OH, pH 8.1). Cell extracts were then sonicated at 50 watts for 30 s using a Braun-sonic 1510 probe sonicator equipped with a 4-mm microprobe. For the equilibrium binding studies with secreted LPL, cells containing their 5-h incubation media were placed at 4°C for 2 h prior to sample collection. This was assumed to be an adequate amount of time to reach equilibrium between cell surface-bound and medium LPL.

**Heparin-Sepharose Chromatography**—A 0.6 × 5.5-cm column containing 1.2 ml of heparin-Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.) was equilibrated in buffer A (0.1 M NaCl, 0.5 mM bovine serum albumin, 50% glycerol, 10 mM sodium phosphate, pH 7.0). For each incubation medium from 4-75 cm² flasks was centrifuged at 40,000 rpm in a Beckman Ti 60 rotor for 25 min at 4°C. The medium was loaded onto the column at a flow rate of 1 ml/min. The column was then washed with 6 ml of Buffer A. LPL was eluted with a linear NaCl gradient (0.25 ml/min) from 0.1 M NaCl (Buffer A) to 2.0 M NaCl (Buffer B) using a total volume of 25 ml. The fractions were assayed for chicken LPL protein by ELISA and for conductivity using a CDM2 conductivity meter (Radiometer, Copenhagen). For selected media samples, pooled fractions were concentrated with a Centricon™ microconcentrator and were assayed immediately for lipase catalytic activity.

**Lipase Activity Measurements**—LPL activity was determined as described (23) with a H-labeled triolein substrate. Because of the small amount of LPL protein in the samples, a high specific activity substrate, 500,000 cpm/μeq fatty acid, was used. A total of 35 μl of cell-associated LPL or 310 μl of media was assayed per tube. To monitor chicken LPL activity as opposed to the endogenous triglyceride lipase activity of CHO cells, samples were preincubated for 60 min, 4°C, with 2.5 μl of either control goat serum or anti-chick lipoprotein lipase goat serum. The titer of the avian LPL antiserum was 9,600 μeq fatty acid/h/ml of antiserum (1). The difference in activity between the two serum treatments was the hydrolytic activity attributed to the transfected lipase. The anti-chick LPL goat serum did not interfere with the endogenous lipolytic activity of CHO cells as demonstrated with nontransfected cells.

**RESULTS**

**Site-specific Mutagenesis**—Eight mutants of chicken LPL were made to investigate whether charged amino acids between residues 281 and 284 are involved in heparin binding (Table I). For single substitutions, each basic residue was changed to an acidic residue. For double and triple mutants, basic amino acids were substituted with the neutral residue glutamine. To determine whether the symmetry of the basic residues was important, the neutral residue at position 283 was altered separately to an acidic and to an alternative neutral residue, glycine. Since basic residues are highly polar, they are most likely exposed at the protein surface; therefore, in this study basic residues were only substituted with other polar residues to keep structural disturbances to a minimum. Finally, the mutagenesis system employed made it necessary to construct an additional mutant, LPL 9B. Since this mutant has the same amino acid sequence as wild-type LPL, LPL 9B is essentially an additional control.

Aliquots of each mutant and wild-type LPL were cloned into an expression vector, pRC/CMV, and the LPL proteins were stably expressed in CHO-K1 cells. Both confluent and growing CHO cells are known to produce and secrete endogenous lipoprotein lipase (22). Our data do not reflect the fate of this endogenous LPL. We were able to specifically follow the transfected lipase, because the polyclonal antibody to chicken LPL did not cross-react with the CHO lipase.

**Heparin-Sepharose Affinity Chromatography**—The mutant and wild-type LPLs expressed by CHO cells were examined by heparin-Sepharose affinity chromatography. The lipases were eluted from the column by a linear salt gradient from 0.1 to 2.0 M NaCl. For every mutant and wild-type run, LPL protein eluted at two separate salt concentrations (Table II). The first peak eluted at approximately 0.5 M NaCl for every sample. The mean coefficient of variation for these measure-
ments was 3.7 ± 2.3%. Using selected samples, we detected no lipolytic activity associated with this first peak. This low salt LPL peak has been reported previously (22, 24, 25) and has been attributed to a conformationally altered lipase probably due to dissociation of dimers to monomers or to formation of oligomers. Unlike the first peak, the second peak for wild-type and mutant LPL eluted at various NaCl concentrations. For a given mutant, the mean coefficient of variation for these values was 18 ± 8.5%. The second peak for wild-type LPL, LPL 9B, and LPL 7D eluted around 1.36 M NaCl, whereas LPL 5G eluted at the lowest salt concentration of 0.96 M NaCl (Fig. 1). The remaining mutants eluted at intermediate salt concentrations. Furthermore, this second peak contained all of the detectable lipolytic activity, which presumably corresponds to the dimeric form of the enzyme.

The proportion of LPL protein that eluted in the two peaks varied with the different samples. This proportion was reproducible and is presented in Table II as a ratio of areas of Peak 2 to Peak 1. Interestingly, the salt molarity necessary for elution of the second peak was directly correlated with this ratio of areas (r = 0.893).

**LPL Activity Measurements**—If the stability of the active homodimer is affected by the amino acid substitutions, one would expect to also see a decrease in the specific activity of these mutants. Lipolytic activity assays were performed on LPL from the media and cell-associated pools, and lipase protein was quantitated by ELISA (Table III). For a given mutant, the specific activity was similar in the cellular extracts and media. The controls, LPL 9B, and wild-type LPL from transfected cells gave the highest specific activity. Lipase from LPL 7D also had a specific activity similar to controls. In the remaining mutants, the less positive the proposed heparin binding region became, the lower the specific activity. For example, LPL 5G cells produced an LPL molecule with the lowest regional charge and with the lowest specific activity. These results followed the same trend as the ratio of Peak 2 to Peak 1 for heparin-Sepharose chromatography. This supports the hypothesis that the first peak is the inactive form of the lipase and the second peak is the active dimeric form.

![Fig. 1. Heparin-Sepharose elution profiles of wild-type LPL and LPL 5G. Mutant and wild-type LPL were eluted from a heparin-Sepharose column with a linear NaCl gradient from 0.1 to 2.0 M NaCl as described under “Experimental Procedures”. NaCl molarity was determined by comparing conductivity measurements of the fractions with buffers of known NaCl molarity. LPL is expressed as a percent of the total mass recovered from the column.](image-url)
Affinity of Mutant LPLs for the CHO Cell Plasma Membrane—Recent experiments with adipocytes (21, 26) demonstrate that the increase in LPL secretion observed upon addition of heparin in the medium is due solely to an inhibition of enzyme degradation. In the absence of heparin, newly synthesized LPL associates with cell surface HSPGs; a fraction of the bound enzyme is released in the medium, and the balance is internalized and degraded in the lysosomes (21).

Exogenous heparin in the medium is thought to compete with cell surface HSPG, thereby inhibiting internalization and degradation. Utilizing chlorate treatment of adipocytes to modulate sulfation density on heparan sulfate chains, Hoggewerf et al. (16) showed that the strength of the interaction between HSPG and lipase is a major determinant of degradation rate. If the results obtained with adipocytes apply to CHO cells, one would expect that the stimulatory effect of heparin in CHO cells transfected with various LPL mutants would be proportional to the affinity of the enzyme for heparin. CHO cells transfected with mutant and wild-type LPL were incubated with or without 100 units/ml heparin for 5 h and the medium analyzed for LPL protein by ELISA. Most media samples showed an increase in LPL protein with addition of heparin in the media (Table IV). Wild-type LPL, LPL 9B, and LPL 7D cells had over twice as much LPL in the media containing heparin versus media without heparin. LPL 5G cells, which secreted mutant lipase with the lowest affinity for heparin-Sepharose, showed no significant stimulation of lipase secretion with heparin treatment. The remaining mutants had intermediate increases in LPL secretion with heparin in the medium.

Another cell experiment was performed to assess whether interaction of the various LPL mutants with heparin-Sepharose paralleled affinity for cell surface HSPGs. CHO cells transfected with various LPL mutants were incubated at 37 °C for 5 h after a medium change; the dishes were then placed at 4 °C for 2 h to allow for equilibrium binding of the secreted LPL with binding sites on the plasma membrane. With the experimental protocol described, endogenous LPL concentrations in the medium are 3 orders of magnitude lower than the expected K₂ (21). Therefore, binding of LPL is described adequately by the equation: B = nKS, where B is the amount of LPL bound per dish, n is the maximum binding per dish, K is the affinity constant, and S is the LPL concentration in the medium. The maximum binding n is expected to be the same for all CHO cell mutants, since the same CHO-K1 strain was utilized for all transfections. Consequently, the B/S ratio for the various mutants (Table V) gives a set of numbers proportional to their affinity constants. As would be expected, the B/S ratios were highly correlated with the salt molarity needed for elution of the active LPL from heparin-Sepharose columns (r = 0.937).

### DISCUSSION

In the present work we have characterized the heparin-binding properties of several avian LPL mutants. All of the mutants with a decrease in local charge between amino acid residues 281 and 284 exhibited a decrease in affinity for both heparin and cell surface heparan sulfate proteoglycans. The one mutant with no change in local positive charge, LPL 7D, behaved as wild-type LPL both in the capacity to interact with these proteoglycans and in its enzyme-specific activity. From these results we conclude that this proposed heparin binding region does play a role in LPL’s binding to heparan sulfate proteoglycans. These results also support the proposal that the interaction is an ionic one. The best support for this claim is offered by LPL 5G, which lacks all the positively charged amino acids in this region. This mutant associates poorly with cell surface HSPG and elutes from heparin-Sepharose at a lower salt concentration than the wild-type enzyme. In addition, the mutants lacking 1 or 2 basic residues have intermediate levels of affinity for heparin. On the other hand, the symmetry of the domain, due to the hydropathic amino acid at position 283, is less restrictive. Note that changing this amino acid to a smaller neutral residue (LPL 7D) does not alter the enzyme’s heparin binding, whereas substituting to an acidic residue (LPL 6E) does decrease its affinity for HSPG.

Another issue that this investigation addresses is the role that heparan sulfate binding plays in stabilizing the active form of the enzyme. A reduction in the capacity to interact with heparan sulfate chains was accompanied by a reduction in the specific activity of secreted enzyme. These results, obtained with LPL mutants with varying affinities for heparan sulfate, confirm those of other investigators reporting the stabilizing effects of heparin (25, 27). A decrease in the mutant’s specific activity was correlated with a decrease in the fraction of the enzyme eluting at high salt concentrations from heparin-Sepharose. The enzyme protein eluting at high salt concentrations was active, whereas that eluting at 0.5 M NaCl was inactive. This is consistent with the observation of Bengtsson-Olivecrona and Olivecrona (25) that the monomeric inactive form binds to heparin with a lower affinity than the dimer catalytically active form. A recent report utilizing monoclonal antibody probes and human enzyme demonstrated that LPL activity and dimer coeluted at 1.1 M NaCl, whereas inactive enzyme eluted at 0.75 M NaCl in

### Table IV

<table>
<thead>
<tr>
<th>Mutant notation</th>
<th>Fold increase in media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type LPL</td>
<td>B-X-B 2.5 ± 0.35</td>
</tr>
<tr>
<td>LPL 2M</td>
<td>B-X-A 1.6 ± 0.15</td>
</tr>
<tr>
<td>LPL 3B</td>
<td>A-X-B 1.7 ± 0.24</td>
</tr>
<tr>
<td>LPL 4G</td>
<td>N-X-B 1.2 ± 0.17</td>
</tr>
<tr>
<td>LPL 5G</td>
<td>N-N-X-N 1.7 ± 0.47</td>
</tr>
<tr>
<td>LPL 6E</td>
<td>B-A-B 2.3 ± 0.52</td>
</tr>
<tr>
<td>LPL 7D</td>
<td>B-N-B 1.7 ± 0.59</td>
</tr>
<tr>
<td>LPL 8K</td>
<td>B-A-X-B 2.4 ± 0.61</td>
</tr>
<tr>
<td>LPL 9B</td>
<td>B-X-B 2.35 ± 0.68</td>
</tr>
</tbody>
</table>

### Table V

<table>
<thead>
<tr>
<th>Mutant notation</th>
<th>B/S (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type LPL</td>
<td>B-X-B 2.03 ± 0.99</td>
</tr>
<tr>
<td>LPL 2M</td>
<td>B-X-A 0.92 ± 0.16</td>
</tr>
<tr>
<td>LPL 3B</td>
<td>A-X-B 1.12 ± 0.22</td>
</tr>
<tr>
<td>LPL 4G</td>
<td>N-X-B 0.88 ± 0.18</td>
</tr>
<tr>
<td>LPL 5G</td>
<td>N-N-X-N 0.35 ± 0.07</td>
</tr>
<tr>
<td>LPL 6E</td>
<td>B-A-B 1.43 ± 0.41</td>
</tr>
<tr>
<td>LPL 7D</td>
<td>B-N-B 2.63 ± 1.00</td>
</tr>
<tr>
<td>LPL 8K</td>
<td>B-A-X-B 0.61 ± 0.10</td>
</tr>
<tr>
<td>LPL 9B</td>
<td>B-X-B 2.35 ± 0.68</td>
</tr>
</tbody>
</table>
heparin-Sepharose elution profiles (24). Thus, we propose that the mutant LPLs in this paper are defective primarily in heparin binding which in turn resulted in their reduction in catalytic activity.

The defects that we observed in heparin binding could potentially be due to other defects in the LPL mutant proteins. Other possibilities include production of mutant proteins that are unable or less efficient at dimer formation, that have a less stable tertiary structure, or that are altered at a site close to a heparin binding domain. Although we cannot conclusively eliminate any of these possibilities, our results support the hypothesis of a defective heparin binding domain. If dimer formation was less efficient in the mutants, one would expect, as observed, a higher proportion of protein in the inactive monomer peak. However, one would not expect the dimer to have altered affinity for heparin-Sepharose. A conformational change is also unlikely to account for the observed reduction in heparin binding of the various mutants. First of all, polar amino acids were substituted by other polar amino acids which would minimize the risk of gross structural abnormalities. Furthermore, only those amino acid substitutions resulting in a decrease of local positive charge caused a reduction in heparin affinity. LPL 7D (Asn283 → Gla) had no change in its regional charge nor heparin binding capacity. If dimer formation was less efficient in the mutants, this mutant gave only one LPL peak, which again could potentially be involved in this interaction include the basic residues between 294-299 and 148-153. Our future efforts will include mutating these regions to assess their role in heparin binding.

Acknowledgments—We thank Kristan Melford and Barry Hughes for expert technical assistance.

REFERENCES