Identification of the epitope of a monoclonal antibody that inhibits heparin binding of lipoprotein lipase: new evidence for a carboxyl-terminal heparin-binding domain

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Abstract A panel of 13 monoclonal antibodies to avian lipoprotein lipase (LPL) was screened for inhibition of LPL binding to primary avian adipocytes. One monoclonal antibody, designated xCAL (monoclonal antibody to chicken adipose lipoprotein lipase) 3-6a, was found to inhibit the binding of LPL to primary avian adipocytes. In solid phase assays, xCAL 3-6a inhibited the binding of LPL to both heparan sulfate and heparin. XCAL 3-6a did not inhibit the catalytic activity of the avian enzyme. The monoclonal antibody was not found to cross-react significantly with bovine lipoprotein lipase. In order to determine the location of the epitope of xCAL 3-6a on lipoprotein lipase, several avian lipoprotein lipase deletion mutants were constructed and produced as glutathione S-transferase (GST) fusion proteins in *E. coli*. These mutants were screened for their ability to react with xCAL 3-6a using Western blotting. The minimum continuous fragment of lipoprotein lipase that was required for reactivity contained the amino acids 310 to 450. Site-directed mutagenesis of basic residues 321, 405, 407, 409, 415, and 416 revealed that Arg 405 is necessary for the interaction of LPL with xCAL 3-6a. Additional deletions of either the amino- or carboxyl-terminal portion of the fragment containing residues 310–450 resulted in loss of antibody binding, suggesting that the epitope is a discontinuous one that is formed when the termini are brought together through protein folding. Heparin-Sepharose chromatography of wild-type LPL and a mutant LPL in which the well-characterized heparin-binding sequence (Arg 281–Lys 282–Arg 284) has been mutated was carried out in the presence and absence of xCAL 3-6a. These experiments indicate that lipoprotein lipase contains a heparin-binding domain, in addition to Arg 281–Arg 284, that can be blocked by xCAL 3-6a.**—Sendak, R. A., K. Melford, A. Kao, and A. Bensadoun.** Identification of the epitope of a monoclonal antibody that inhibits heparin binding of lipoprotein lipase: new evidence for a carboxyl-terminal heparin-binding domain. *J. Lipid Res.* 1998. **39:** 633–646.

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Lipoprotein lipase (LPL), a key enzyme in lipid metabolism, catalyzes the hydrolysis of triglycerides in very low density lipoproteins and chylomicrons (1, 2). This catalysis takes place at the luminal surface of the capillary endothelium where LPL is anchored by heparan sulfate chains (3, 4). The interaction of LPL with cultured endothelial cells has been studied in vitro (5–7). Extracellular-matrix-bound LPL dissociates, in a catalytically active form, from the cell surface into the surrounding medium in the presence of triolein emulsion and/or serum (7). Although LPL is active in this bound state in vitro, it is more effective in solution (8). The mechanism by which lipoproteins contact the capillary endothelium and initiate lipolysis, in vivo, is not yet clear (9).

The interaction of LPL with heparan sulfate is not only important in localizing the enzyme at its site of action, but appears to play a broader role in lipid metabolism. Binding of LPL to heparan sulfate on the surface of liver parenchymal cells appears to be necessary for the normal clearance of LPL from the circulation (10). In addition, the interaction of LPL with heparan sulfate may facilitate the uptake of lipoprotein particles (11).

Identification of the region(s) of LPL involved in heparan sulfate binding will lead to an increased understanding of how the interaction of LPL with the capillary endothelium and liver parenchymal cells affects the biological activity and metabolism of the enzyme. Heparan sulfate is highly negatively charged, suggest-

Abbreviations: LPL, lipoprotein lipase; xCAL, monoclonal antibody to chicken adipose lipase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl-sulfate; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; LB, Luria-Bertani; IPTG, isopropyl β-thiogalactoside; GdnHCl, guanidine hydrochloride; LRP, low density lipoprotein receptor-related protein.

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ing that basic residues most likely constitute heparan sulfate-binding regions of proteins. This is in agreement with the glycosaminoglycan binding sequences postulated by Cardin and Weintraub (12) based on known heparin-binding regions of other proteins. LPL contains several positive charge clusters in its primary sequence. Studies using site-directed mutagenesis of residues 281–284 of avian LPL (13) and the corresponding residues, 279–282, of human LPL (14, 15) have demonstrated that this region plays a role in heparin binding. Mutagenesis of residues 390–393 and 439–448 of human LPL indicated that these residues were not involved in heparin binding (15). Evidence for the region of human LPL that contains residues 292–304 is conflicting (14, 15).

The production of monoclonal antibodies against proteins of interest has provided a unique way of studying structural characteristics of these proteins (16, 17). The heparin binding site(s) of LPL are, by necessity, present on the surface of the enzyme and may provide an epitope for the production of monoclonal antibodies. In this study we have characterized an antibody, xCAL 3-6a, that disrupts the binding of LPL to primary avian adipocytes, heparin, and heparan sulfate. We have identified the region of LPL that contains the epitope of the antibody. Interestingly, we find that the epitope is present in the carboxyl-terminus of the protein; a region that is distinct from the well-characterized heparin-binding sequences previously identified in human and avian LPL.

MATERIALS AND METHODS

Preparation of xCAL 3-6a

Monoclonal antibodies to avian LPL were generated by the same general procedures described previously (18). xCALs were purified from ascites fluid by chromatography on a 5-ml protein G-Sepharose column (Pharmacia). The immunoglobins were bound in the presence of 20 mm sodium phosphate, pH 7.0; the column was washed with ten bed volumes of loading buffer and eluted with 0.1 m glycine/HCl, pH 2.7. The eluted immunoglobin fraction was collected in tubes containing 0.2 ml of 1 m Tris-HCl, pH 9.0, per 3-ml fraction and dialyzed against 0.2% sodium azide, 0.15 m sodium chloride, 10 mm sodium phosphate, pH 7.0.

A Mouse Isotyping Kit (Boehringer Mannheim) was used to characterize immunoglobins.

Screening for disruption of the binding of lipoprotein lipase to primary avian adipocytes

Avian primary adipocytes were cultured in 60-mm dishes as described (19). The adipocytes were washed twice with 2 ml of phosphate-buffered saline (PBS; 10 mm phosphate, 150 mm sodium chloride, pH 7.4) containing 0.5% bovine serum albumin. Highly purified avian LPL (4 μg) was pre-incubated with 400 μg of each of the xCALs for 3 h at 4°C in 2 ml of 1× Roswell Park Memorial Institute (RPMI) 1640 medium containing 0.5% bovine serum albumin. RPMI medium (1.5 ml) was added to each of the dishes followed by the addition of 500 μl of each of the pre-incubated xCAL mixtures, in triplicate. The adipocytes were incubated for 2 h at 4°C with shaking. The dishes were then washed twice with 2 ml of PBS containing 0.5% bovine serum albumin. Two ml of PBS (0.5% bovine serum albumin) containing 100 units of heparin was added to each dish. The adipocytes were incubated at 4°C with shaking for 15 min. The heparin wash was then collected and spun in a microfuge at 12,000 rpm for 5 min. One ml of the supernatant was added to 1 ml of 60% glycerol, 2 m NaCl, 1% bovine serum albumin. As a control for non-specific binding, dishes containing no cells were incubated with LPL and were then treated with the heparin wash. The presence of LPL was detected using a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) previously described by Cisar et al. (19). The optical density at 490 nm was read with a Dynatech MR7000 plate reader and the data were processed using the Biolinx version 2.21 software. The standard curve ranged from 0.002 to 1.0 ng of LPL with an R^2 value of 0.999. At 1.0 ng the average optical density at 490 nm with *o*-phenylenediamine substrate solution (25 mm citric acid, 50 mm sodium phosphate, 0.4 mg/ml *o*-phenylenediamine (Sigma), 0.012% hydrogen peroxide) was 0.568 ± 0.024 (n = 6). The LPL protein standard was determined by direct amino acid analysis of a concentrated LPL sample. The concentrated sample (0.645 mg/ml) was diluted and aliquoted in 1 m NaCl, 0.3% bovine serum albumin, 0.02% sodium azide, 30% glycerol, 10 mm phosphate, pH 7.4, and stored at −80°C.

Effect of Fab fragments of xCAL 3-6a on the binding of lipoprotein lipase to adipocytes

Fab fragments were generated by digestion of xCAL 3-6a with immobilized papain (Pierce) according to the protocol provided by the manufacturer. The digestion appeared to be complete after 16 h at 37°C as judged by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The Fab fragments were separated from the Fc region and undigested material by protein-A (Pharmacia) chromatography (20).

Solutions of xCAL 3-6a Fab fragments (0–340 μg) and LPL (3.4 μg) were made up to 1.7 ml with 0.5% bovine serum albumin in RPMI. Mixtures containing

mouse IgG (Sigma) in place of Fab fragments were prepared as controls. The mixtures were incubated on ice for 3 h. Avian adipocytes were cultured on 60-mm dishes. Adipocytes were washed twice with 2 ml of 0.5% bovine serum albumin in PBS. Five hundred microliters of each antibody/LPL mixture was diluted with 1.5 ml of RPMI containing 0.5% bovine serum albumin and added to the dishes in triplicate. The adipocytes were incubated at 4°C on ice for 2 h with shaking. The medium was aspirated and the dishes were washed twice with 0.5% bovine serum albumin in PBS. Heparin wash (100 units in 2 ml of 0.5% bovine serum albumin in PBS) was added to each dish followed by a 15-min incubation on ice with mild lateral rotation. The heparin wash was removed and a sandwich ELISA (19) was used to detect the presence of LPL. The experiment was repeated with the whole antibody.

Disruption of LPL binding to heparin and heparan sulfate

Heparin (Sigma H-3393) and heparan sulfate (Sigma H-9902) (25 μg/well) were bound to 96-well microtiter plates (Corning) overnight at 4°C in the presence of 50 mm sodium bicarbonate, 0.02% sodium azide, pH 9.6. Excess binding sites were blocked with 3% bovine serum albumin, 0.02% Tween-20 in PBS for 1 h at 37°C. The wells were washed with PBS containing 0.02% Tween-20, 3% bovine serum albumin. LPL (0.02 μg/ml) was incubated with 0–25 μg/ml of xCAL 3-6a in PBS containing 1% bovine serum albumin on ice for 1 h and 15 min. Subsequently, 200 μl of each LPL/xCAL 3-6a mixture was added, in duplicate, to the wells containing heparin and those containing heparan sulfate. After an overnight incubation at 4°C, the wells were washed with PBS containing 0.02% Tween-20, 3% bovine serum albumin. A horseradish peroxidase (HRP)-conjugated antibody to avian LPL was added to the plate which was then incubated overnight at 4°C. After washing, *o*-phenylenediamine substrate solution was added. After a 10-min incubation in the dark, the reaction was stopped with 2.5 m sulfuric acid and the absorbance was measured at 490 nm.

Effect of xCAL 3-6a on lipoprotein lipase activity

xCAL 3-6a (0–150 μg) and mouse IgG were diluted into PBS to produce mixtures containing a total of 150 μg of immunoglobin in 75 μl. These antibody mixtures were pre-incubated with 17 ng of LPL (in 40 μl of 0.15 m sodium chloride, 10 mm phosphate buffer, 30% glycerol, 1% bovine serum albumin, pH 6.5) at 4°C. After 1 h, the LPL activity was determined as described (21) using a ^{3}H-labeled triolein substrate. Briefly, 175 μl of assay mix (0.367 g radio-labeled triolein, 1.88 g triolein, 2.5 ml gum arabic, 2.5 ml of 10% bovine serum albumin, 2.5 ml of 0.1 m calcium chloride, and 1.25 ml of rat serum) was added and the mixture was incubated at 30°C with shaking for 15 min. This procedure was repeated using the monoclonal antibody xCAL 1-11d as a control. The xCAL 1-11d was previously shown to inhibit catalytic activity of LPL (18).

Affinity of xCAL 3-6a for avian and bovine lipoprotein lipases

xCAL 3-6a or mouse IgG were used to coat (50 ng/well) 96-well microtiter plates (Corning) overnight at 4°C. Excess protein binding sites in the wells were blocked with 1% bovine serum albumin, 0.05% Tween-20 in PBS for 1 h at 37°C. The wells were washed with PBS containing 0.05% Tween-20. Subsequently, 200-μl samples containing 0–4 ng of avian LPL or 0–100 ng of bovine LPL in PBS containing 1% bovine serum albumin and 0.05% Tween-20 were added to each well, in duplicate. After an overnight incubation at 4°C, the wells were washed with PBS containing 0.05% Tween-20. HRP-conjugated anti-avian LPL antibody and HRP-conjugated anti-bovine LPL antibody were added to the wells containing avian LPL and bovine LPL, respectively. The plates were incubated overnight at 4°C, the wells were washed with PBS containing 0.05% Tween-20, and after washing, *o*-phenylenediamine substrate solution was added. After a 30-min incubation in the dark, the reaction was stopped with 2.5 m sulfuric acid and the absorbance was measured at 490 nm. Standard curves in which the presence of avian LPL and bovine LPL were detected were included in the assay; 0–0.3 ng of avian LPL gave a linear range with a maximum OD_{490} of 1.03. The data from wells coated with mouse IgG were subtracted from those obtained for the xCAL 3-6a-coated wells to account for nonspecific binding. After correction for nonspecific binding, bovine LPL exhibited no signal indicating that it has essentially no affinity for xCAL 3-6a. The data for avian LPL were fit to equation 1 using SigmaPlot® version 2.0 (Jandel Scientific):

$$v = nx/(K_d + x) \qquad \textit{Eq. 1})$$

where v is bound LPL, n is the maximum amount of bound LPL, K_d is the dissociation constant, and x is the free LPL.

LPL mutant analysis

Construction of expression plasmids for GST/LPL fusion proteins. The OBC clone for the avian LPL cDNA was previously described by Cooper et al. (22). The cDNA was excised from pGEM-3Z (Promega) using the *Cfr*10 I site at the start of the coding region and the *Sal* I site in the multiple cloning region of the vector. The cDNA was cloned into the *Xma* I and *Xho* I sites within the

multiple cloning region of pGEX-4T-2 (Pharmacia). LPL(Δ399–465) was prepared from the full-length construct by removing a section of the cDNA using restriction digestion, creating blunt ended DNA, and ligating. The DNA was digested with *Not* I, treated with Klenow (GIBCO/BRL), then digested with *Bsm* I, and treated with mung bean nuclease (New England Biolabs). LPL(Δ1–333) was prepared from the original pGEM/OBC construct; the DNA was digested with *Acc* I, treated with mung bean nuclease, and then cut with *Eco*R I. The fragment encoding the carboxyl-terminal residues of LPL was cloned into pGEX-4T-2 that had been digested with *Bam*H I, treated with mung bean nuclease, and then digested with *Eco*R I. LPL(Δ1–309) was prepared from pGEM/OBC; the DNA was digested with *Bsi*HKA I, treated with mung bean nuclease, and then digested with *Not* I. The appropriate fragment was cloned into pGEX-4T-2 that had been digested with *Eco*R I, treated with Klenow, and then digested with *Not* I. In order to prepare LPL(Δ1–309, Δ334–399), LPL(Δ1–309) was digested with *Eco*R I and the fragment encoding for LPL was cloned into the *Eco*R I site of pGEX-2T. This construct LPL(Δ1–309)/pGEX-2T was digested with *Acc* I, treated with Klenow, digested with *Bsm* I, treated with mung bean nuclease, and was then re-ligated. LPL(Δ1–309, Δ451–465) was prepared using LPL(Δ1–309)/pGEX-2T. The DNA was digested with *Ban* I, treated with mung bean nuclease, then digested with *Eco*N I. The appropriate fragment was cloned into pGEX-2T that had been digested with *Eco*R I, treated with mung bean nuclease, and then digested with *Eco*N I. LPL(Δ1–309, Δ436–465) was prepared using LPL(Δ1–309)/pGEX-2T; the DNA was digested with *Eco*R I and *Ssp* I. The appropriate fragment was cloned into pGEX-4T-2 that had been digested with *Not* I, treated with Klenow, and then digested with *Eco*R I. LPL(Δ1–325) was constructed using the polymerase chain reaction (23). LPL(Δ1–309)/pGEX-4T-2 was used as a template. The mutagenesis primer was designed to anneal to the multiple cloning region of pGEX-4T-2 as well as the codons of amino acids 326 through 332 of LPL, thus omitting the intervening DNA encoding for residues 310 through 325. The PCR fragment was cloned using the *Bam*H I site in the mutagenesis primer and the *Bsm* I site in LPL. After ligation of the deletion mutants, the DNA was transformed into the *E. coli* strain DH5α. The resulting clones were confirmed by sequencing.

Site-directed mutagenesis of candidate antibody binding regions of lipoprotein lipase. Nucleotide substitutions were introduced into the sequence of LPL using the overlap extension method in combination with the polymerase chain reaction (24). The full-length LPL construct in pGEX-4T-2 described above was used as the template. Mutagenesis primers (**Table 1**) were synthesized by the Cornell Biotechnology Facility. LPL 1A, 1B, 1F, and 1H were created using the overlap extension method. LPL 1G was synthesized in one step of the PCR reaction by taking advantage of the *Xba* I site within the mutant oligonucleotide. LPL 1C, 1D, and 1E were also synthesized in one step by utilizing the *Bsm* I site within the oligonucleotide. LPL 1G was created in one step of the PCR reaction and cloned using the *Xba* I site within the mutant oligonucleotide.

The reactivity of a previously described mutant (13), 5G, in which the basic residues in the region Arg 281–Lys 282–Asn 283–Arg 284 have been changed to glutamine was investigated. The mutant LPL was removed from pRc/CMV (Invitrogen) by cutting with *Cfr*10 I and *Not* I and introduced into the *Xma* I and *Not* I site of pGEX-4T-2.

Expression and purification of LPL mutants. An overnight culture of DH5α containing the appropriate plasmid was grown in 10 ml of Luria-Bertani (LB) medium containing 50 μg of ampicillin per ml at 37°C with shaking. The overnight culture was used to inoculate 1 liter of LB medium which was grown with ampicillin at 37°C until the absorbance (600 nm) reached 0.5–0.6. Isopro-

TABLE 1. Primers used in PCR generation of site-directed mutants of avian lipoprotein lipase[a]

Mutant	Mutagenesis Primer
LPL 1A[b]	^{1151}TTCCATTATCAGGTCGACATACATTTCTTTGGA^{1183}
LPL 1B[b]	^{1403}GCATTCACCATTCAGAACGTCAACGTGAACTCAGGCGAAACTCAG^{1447}
LPL 1C[c]	^{1388}TGGTGGACTCCATTTGCATTCACCATTCAGAACGTTAGAGTGAAGTCAGGCGAAACTCAGAAA^{1450}
LPL 1D[c]	^{1388}TGGTGGACTCCATTTGCATTACCCATTCAGAGAGTCAACGTGAAGTCAGGCGAAACTCAGAAAAAG^{1453}
LPL 1E[c]	^{1388}TGGTGGACTCCATTTGCATTCACCATTCAGAGAGTCAGAGTGAATTCAGGCGAAACTCAGAAAAAGGTGGTA^{1459}
LPL 1F[b]	^{1430}AAGTCAGGCGAAACTCAGAATAATGTGGTATTCTGTTCT^{1468}
LPL 1G[c]	^{1249}ACTCTCGTCTAGAGTGCCATACAGAGAGATCAGGAATGGCTGGTTTGTAGGTATTAGATTCCGTT CCAGAGAAATG^{1172}
LPL 1H[b]	^{1388}TGGTGGAGCAGCCCAGGATTCGACATTGGAAGAGTCAGAGTGAAGTCAGGC^{1438}

[a] Primers are written in the 5′ to 3′ orientation and mutated nucleotides are underlined.
[b] The overlap extension method was used to generate these mutations: a primer that contained the reverse complementary sequence was also used.
[c] These mutants were generated in a single step of the polymerase chain reaction: cloning utilized restriction sites within the primers.

pyl β-thiogalactoside (IPTG) was added to a final concentration of 0.24 mg/ml and the bacteria were grown for 2.5–3.5 h. The cells were then pelleted by centrifugation and subsequently resuspended in 30 ml of PBS containing 0.02% sodium azide. Guanidine hydrochloride (GdnHCl) (23.9 g) was added to the cell suspension (giving a final GdnHCl concentration of 5 m) and the mixture was shaken for approximately 20 min at room temperature to lyse the cells and solubilize the fusion protein. The solution was diluted, using constant mechanical stirring, with 450 ml of PBS containing 0.02% azide. The cell debris was pelleted by centrifugation and the supernatant was loaded onto a column containing 3 ml of glutathione-agarose (Sigma) at a rate of 0.4 ml/min at 4°C. The column was then washed with approximately 25 ml of 0.5 m GdnHCl in PBS and eluted using 25 mm glutathione (reduced form, Sigma) in the same buffer used for washing. The majority of the protein was found to elute in the first 10 ml. The protein was precipitated by dialyzing the solution against 3.6 m ammonium sulfate in 10 mm phosphate buffer, pH 7.2, at 4°C and then pelleted by ultracentrifugation at 41,000 rpm for 30 min. The protein was separated from its degradation products on a preparative SDS PAGE gel (10% or 12.5%) and the band corresponding to the fusion protein with the highest molecular weight was cut out and electro-eluted using an S&S Elutrap electro separation system (Schleicher & Schuell). The protein was eluted into SDS running buffer (25 mm Tris, 192 mm glycine, 0.1% SDS, pH 8.3) at 100 volts for 8 h at room temperature. The protein concentration was determined by a modified Lowry assay (25). The protein was checked for purity by SDS PAGE followed by staining with Coomassie blue. The reactivity of each mutant to a polyclonal antibody made against avian LPL was checked by Western blotting using the procedure described below (the polyclonal antibody concentration for the primary incubation was 0.9 μg/ml).

Thrombin cleavage of GST fusion protein. Seven micrograms of the fusion protein was digested with 0.075 NIH units of thrombin (Sigma) in 40 μl of PBS at 37°C for 1 h. The resulting LPL fragment was analyzed by Western blot for reactivity towards the polyclonal antibody.

Reactivity of xCAL 3-6a to GST/LPL mutants. One microgram of the purified full-length GST/LPL construct and equimolar equivalents of all other deletion mutants were run on a 10% SDS PAGE mini-gel. Equimolar amounts of purified wild-type LPL and purified recombinant GST were also run as controls. The proteins were transferred to an Immobilon-P membrane (Millipore) using a semi-dry transfer apparatus. A current of 0.8 mA/cm^2 for 1 h was found to be sufficient for the transfer of the majority of the protein to the membrane. The membrane was blocked in 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 2 h at room temperature with shaking. XCAL 3-6a (17 μg/ml) in 10 ml of blocking solution was added to the membrane followed by an overnight incubation at 4°C with shaking. The membrane was washed extensively with PBS containing 0.1% Tween-20 and then incubated at room temperature for 1 h with HRP-conjugated anti-mouse antibody (Amersham) diluted 1:3000 in 10 ml of blocking solution. The membrane was washed and the bands were detected using the Amersham ECL Western blotting analysis system.

Heparin-Sepharose chromatography of wild-type LPL and mutant 5G LPL

Production and media collection of wild-type and mutant 5G LPL. Chinese hamster ovary cells (American Type Culture Collection) that were previously stably transfected with the cDNA of wild-type or mutant 5G LPL in the vector pRc/CMV (Invitrogen) were cultured and the medium was collected as described (13). After the medium was collected, either xCAL 3-6a or control mouse IgG (Sigma) was added to a final concentration of 50 μg/ml, followed by a 30-min incubation at 4°C with shaking. The medium was centrifuged at 15,000 rpm in a Sorvall SS-34 rotor for 30 min at 4°C. The medium was then loaded on a heparin-Sepharose column.

Production of monomer LPL. Purified avian LPL (1 μg) was incubated in 0.5 ml of 3.5 m GdnHCl, 0.5 m NaCl, 1% BSA, 20 mm Tris, pH 7.4, for 12 h at 4°C. The solution was dialyzed against 0.15 m NaCl, 1% BSA, 20 mm Tris, pH 7.4, for 2 h and then diluted with 0.5 ml of buffer A (see below) containing 50 μg of either control mouse IgG (Sigma) or xCAL 3-6a. This was preincubated at 4°C for 30 min, microfuged at 12,000 rpm for 10 min, and then loaded on a heparin-Sepharose column.

Heparin-Sepharose chromatography of wild-type and mutant 5G LPL. Heparin-Sepharose chromatography was carried out using an FPLC (Pharmacia). A 1-ml prepacked HiTrap heparin-Sepharose column (Pharmacia) was equilibrated in buffer A (0.15 m NaCl, 1% BSA, 30% glycerol, 10 mm phosphate, pH 6.5) containing 50 μg/ml of xCAL 3-6a or control mouse IgG. The LPL samples were then loaded onto the column; media samples were loaded at a flow rate of 0.5 ml/min, purified avian LPL monomer samples were loaded at a flow rate of 0.25 ml/min. The column was washed with 5 ml of buffer A (containing 50 μg/ml xCAL 3-6a or control IgG) and then eluted with a linear salt gradient (0.5 ml/min) from 0.15 m NaCl to 2.0 m NaCl (buffer A with 2.0 m NaCl and 50 μg/ml xCAL 3-6a or control IgG) with a total volume of 40 ml. The fractions were assayed for LPL by ELISA. The salt concentration was

determined by conductivity using a CDM2e conductivity meter (Radiometer, Copenhagen).

Other methods

Avian LPL was purified by previously described methods (26). Bovine LPL was prepared by the procedure of Socorro, Green, and Jackson (27).

RESULTS

Characterization of the binding of xCAL 3-6a to LPL

Thirteen monoclonal antibodies were tested for their ability to interfere with the binding of LPL to primary adipocytes. Only one monoclonal antibody, xCAL 3-6a, significantly disrupted the binding of LPL to the cell surface (**Fig. 1**). Only 6% of the binding of LPL to adipocytes, as compared to mouse IgG, remained after incubation of LPL with xCAL 3-6a. Other xCALs, such as 3-3a, 3-2a, 1-14, 1-13, 1-11d, and 3-8a, affected binding of LPL to adipocytes to a lesser degree than xCAL 3-6a (62–69% of the control). The monoclonal antibody, xCAL 3-6a, was identified as an IgG_1 isotype.

The ability of Fab fragments of xCAL 3-6a to interfere with the binding of LPL to primary avian adipocytes is illustrated in **Fig. 2**. Binding of LPL to adipocytes at 4°C dropped off very rapidly as the concentration of the Fab fragments increased. Above 5 μg of 3-6a per ml, maximal inhibition of binding to adipocytes was observed. Only 5% of the heparin-releasable binding remained. The inhibition of adipocyte binding by intact xCAL 3-6a closely resembled that of the xCAL 3-6a Fab fragments (data not shown).

In solid phase assays with heparan sulfate and heparin immobilized on ELISA plates, xCAL 3-6a inhibited the binding of LPL to both heparin and heparan sulfate (**Fig. 3**). The binding curves are identical; above 7.5 μg/ml of xCAL 3-6a maximal inhibition of binding is seen. In the experiment, the wells were coated with equal amounts of heparin and heparan sulfate (25 μg); however, the maximum amount of LPL that was bound in the absence of xCAL 3-6a differed: 1.77 ng and 0.48 ng of LPL were bound to heparan sulfate and heparin, respectively. Although heparin is more negatively

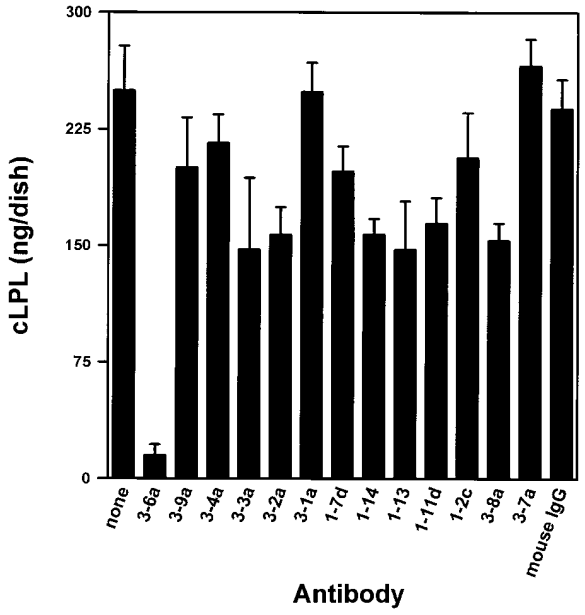

Fig. 1. Screening of xCALs for inhibition of LPL binding to avian adipocytes. Avian LPL was pre-incubated with each of the thirteen monoclonal antibodies and then added to cultured avian adipocytes. After incubation with the LPL–antibody mixtures, the adipocytes were washed and the bound LPL was eluted with a heparin wash (see Materials and Methods for experimental details). The eluted LPL was detected by ELISA and is expressed as the total amount detected per dish. Dishes containing no adipocytes were incubated with LPL alone followed by the heparin elution to give an indication of non-specific binding; 32.5 ng of LPL was recovered and this was subtracted from each value obtained for the xCALs. The values are the mean of triplicate dishes and the standard deviations are indicated by the error bars. This experiment was repeated three times with essentially identical results; results from one experiment are shown.

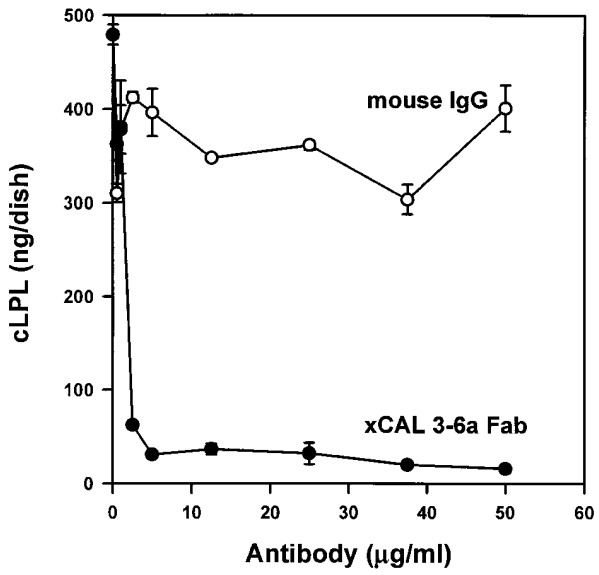

Fig. 2. Disruption of LPL binding to adipocytes by xCAL 3-6a Fab fragments. Avian LPL was pre-incubated with varying amounts of xCAL 3-6a and then added to cultured avian adipocytes. The adipocytes were incubated with the LPL–antibody mixtures, washed, and the bound LPL was eluted with a heparin wash (see Materials and Methods for experimental details). The eluted LPL was detected by ELISA and is expressed as the total amount detected per dish. Values are the average of triplicate measurements and the error bars indicate the standard deviation.

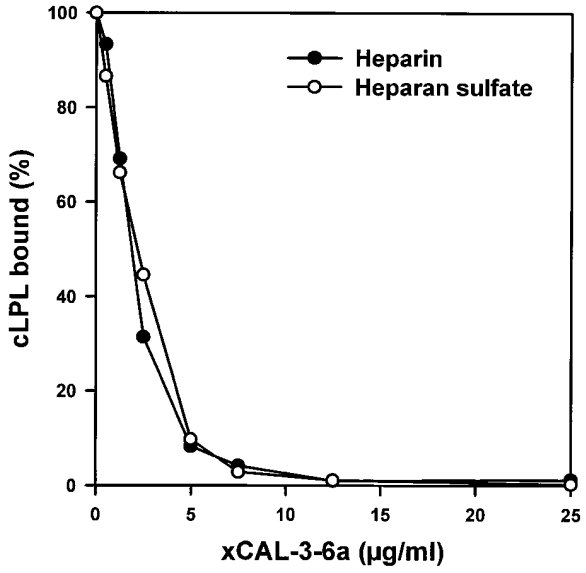

Fig. 3. Disruption of LPL binding to heparan sulfate and heparin by xCAL 3-6a. Microtiter plates were coated overnight at 4°C with heparan sulfate or heparin (25 μg/well). The wells were blocked with 3% bovine serum albumin, 0.05% Tween-20 in PBS for 1 h at 37°C. Mixtures of LPL (0.02 μg/ml) and xCAL 3-6a (0–25 μg/ml) were incubated on ice for 1 h and 15 min. The mixtures (200 μl/well) were added to the wells coated with heparin or heparan sulfate. After an overnight incubation at 4°C, the wells were washed and an HRP-conjugated antibody to avian LPL was added. After an overnight incubation at 4°C, the wells were washed and *o*-phenylenediamine substrate solution (see Materials and Methods) was added. The reaction was stopped with 2.5 m sulfuric acid after a 10-min incubation and the absorbance at 490 nm was measured. The values are expressed as a percentage of the maximum binding in the absence of xCAL 3-6a. The values are the average of two measurements.

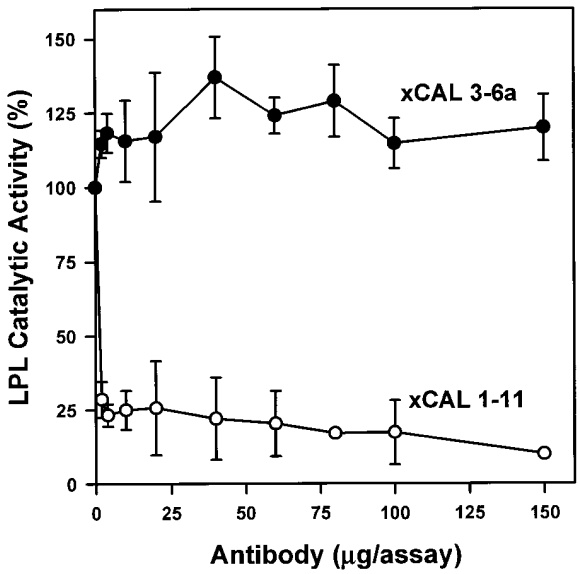

Fig. 4. Effect of xCAL 3-6a and xCAL 1-11d on the catalytic activity of LPL. Avian LPL (17 ng) was incubated at 4°C for 1 h with 0–150 μg of xCAL 1-11, which was previously found to inhibit catalytic activity, and xCAL 3-6a. The lipolytic activity was determined using ^{3}H-labeled triolein substrate. The activity is expressed as a percentage of the activity of LPL in the absence of xCALs. The assay was performed in triplicate and the error bars indicate the standard deviation. The results from one experiment are shown with similar results obtained in two other experiments.

charged, it had a lower apparent binding capacity for LPL than heparan sulfate. This may be due to differences in either the amount of heparin and heparan sulfate that bound to the well in the coating process or in the conformation of the material that did bind.

The effect of xCAL 3-6a and xCAL 1-11 on the catalytic activity of avian LPL is shown in **Fig. 4**. In contrast to xCAL 1-11, xCAL 3-6a did not inhibit the activity of LPL.

The affinity of avian LPL for xCAL 3-6a is shown in **Fig. 5**. The calculated K_d was found to be 1.2×10^{-11} m. Bovine LPL was assayed in a similar manner and did not exhibit significant reactivity towards xCAL 3-6a. This suggests that at least part of the region of LPL that contains the epitope of 3-6a is not conserved between the two species.

LPL mutant analysis: requirements for binding to xCAL 3-6a

All of the deletion mutants showed reactivity towards a polyclonal antibody to avian LPL with the exception of LPL(Δ1–309, Δ436–465). Two possibilities exist: the LPL fragment may contain few, or low affinity epitopes for the polyclonal antibody, or within the fusion protein, the GST fragment (26.3 kD) conceals the epitopes of the smaller LPL fragment (14.8 kD). In order to eliminate the second possibility, thrombin was used to cleave the fusion protein, releasing the LPL deletion fragment. The truncated protein exhibited the same low reactivity towards the polyclonal antibody, suggesting that this region of the protein contains few epitopes for this antibody.

The ability of the deletion mutants to interact with xCAL 3-6a was evaluated by Western blot (**Fig. 6**). The shortest deletion mutant that retained reactivity towards xCAL 3-6a was LPL(Δ1–309, Δ451–465). This indicates that the region important in xCAL 3-6a binding is between residues 310–450. The fact that LPL(Δ1–325) did not have reactivity towards xCAL 3-6a suggests that a region between 310 and 325 is involved in xCAL 3-6a binding. xCAL 3-6a did not react with either the GST fusion of LPL(Δ1–309, Δ436–465) (Fig. 6) or the LPL fragment produced by thrombin cleavage (data not shown). The reactivity of LPL(Δ1–309, Δ451–465), but not LPL(Δ1–309, Δ436–465) suggests that the epitope of xCAL 3-6a may also contain a region between 436 and 450.

Site-directed mutants were designed and constructed based on the result that antibody binding requires

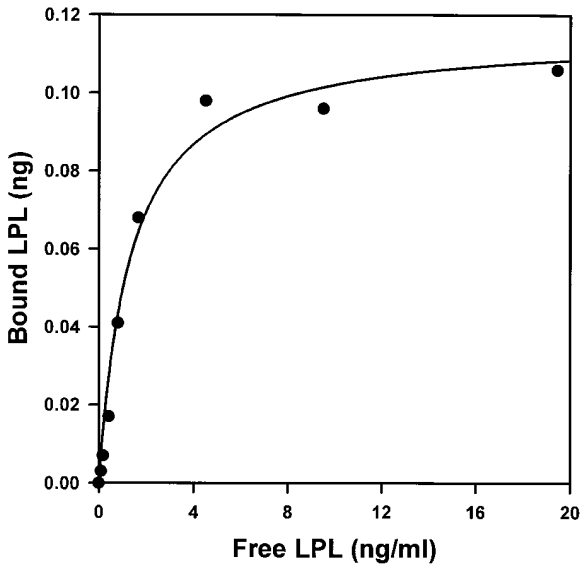

Fig. 5. Affinity of xCAL 3-6a for LPL. Microtiter plates were coated with xCAL 3-6a or mouse IgG (50 ng/well) at 4°C overnight. Nonspecific binding sites were blocked with 1% bovine serum albumin in PBS containing 0.05% Tween-20. LPL (0–4 ng) in 200 μl of blocking solution was added to the wells, in duplicate for each concentration. After an overnight incubation at 4°C, the plate was washed and an HRP-conjugated antibody to avian LPL was added to the wells. After an overnight incubation at 4°C, the plate was washed and *o*-phenylenediamine substrate solution (see Materials and Methods section) was added. The OD at 490 nm was measured after a 30-min incubation in the dark. The bound mass of LPL was determined using a standard curve for LPL. The values obtained for wells coated with mouse IgG were subtracted from those obtained for xCAL 3-6a-coated wells to correct for nonspecific binding. The K_d was determined to be 1.2×10^{-11} m.

amino acids between residues Gln 310 and Gly 450. Because the epitope of xCAL 3-6a may have considerable overlap with a heparin-binding region, basic residues were selected for mutagenesis. Analysis of the known sequences of lipoprotein lipase from many species showed that within this region of avian LPL twelve basic residues are highly conserved; Lys 314, Lys 321, Lys 381, Arg 405, Arg 407, Lys 409, Lys 415, Lys 416, Arg 422, Lys 430, Lys 439, and Lys 448. Several of these residues (Lys 321, Arg 405, Arg 407, Lys 409, Lys 415, and Lys 416) have been postulated to form a charge cluster in the folded structure of LPL (28). These residues were chosen as candidates for mutagenesis to acidic (Asp) or neutral (Asn) amino acids to determine whether the removal of positive charge at these sites results in the disruption of xCAL 3-6a binding to LPL. The site-directed mutants that were constructed and their reactivity towards xCAL 3-6a are summarized in **Fig. 7**. LPL 1A (Lys 321 to Asp) retained its ability to react with xCAL 3-6a. LPL 1B (Arg 405 to Asn, Arg 407 to Asn, and Lys 409 to Asn) and LPL 1C (Arg 405 to Asn) did not react with xCAL 3-6a. Mutagenesis of Arg 407 to Asn (LPL 1D) and Lys 409 to Asn (LPL 1E) failed to disrupt the binding of LPL to xCAL 3-6a. LPL 1F (Lys 415, Lys 416 to Asn) showed reactivity towards xCAL 3-6a. In summary, of the basic residues mutated, Arg 405 appears to be critical to xCAL 3-6a binding while mutation of the others had no effect on the binding of the antibody.

The region of avian LPL from Phe 325 to Asp 334 shows little sequence homology (20%) to bovine LPL, an enzyme that has very low affinity for xCAL 3-6a. We postulated that this region may account for the difference in affinities of bovine and avian LPL for xCAL 3-6a. Construction of LPL 1G involved replacing 10 residues of avian LPL with the corresponding residues of bovine LPL. LPL 1G showed reactivity towards xCAL 3-6a, indicating that sequence Phe 325 to Asp 334 is not the basis for the difference in the affinities of bovine and avian LPL for the antibody. By similar analysis, the region of avian LPL from Thr 397 to Gln 404 has low sequence identity (25%) to bovine LPL. Mutation of the residues in the avian LPL sequence to those found in the bovine sequence (LPL 1H) resulted in the loss of reactivity towards xCAL 3-6a. This indicates that the region Thr 397 to Gln 404 is, at least partially, responsible for the difference in the affinities of bovine and avian LPL for xCAL 3-6a.

Heparin-Sepharose chromatography of wild-type and 5G LPL

Wild-type and mutant 5G LPL expressed in Chinese hamster ovary cells eluted at two separate salt concentrations (**Fig. 8A**) in the absence of xCAL 3-6a. The first peak for both proteins eluted at 0.49 m NaCl and the second peak eluted at 1.31 m and 0.98 m NaCl for wild-type and mutant 5G LPL, respectively. This is in agreement with data obtained previously (13). In the presence of xCAL 3-6a (Fig. 8B), 5G LPL eluted in one peak at 0.49 m NaCl and wild-type eluted at 0.83 m NaCl.

Monomer LPL in the absence of xCAL 3-6a, eluted in a broad peak from about 0.5 m NaCl to 1.0 m NaCl (Fig. 8C). In the presence of xCAL 3-6a, the monomer eluted in a sharp peak at 0.5 m NaCl.

DISCUSSION

We have generated a monoclonal antibody against avian LPL that inhibits binding of LPL to adipocytes (Fig. 1 and Fig. 2). In solid phase assays with heparan sulfate and heparin immobilized on ELISA plates, xCAL 3-6a inhibited the binding of LPL to both hep-

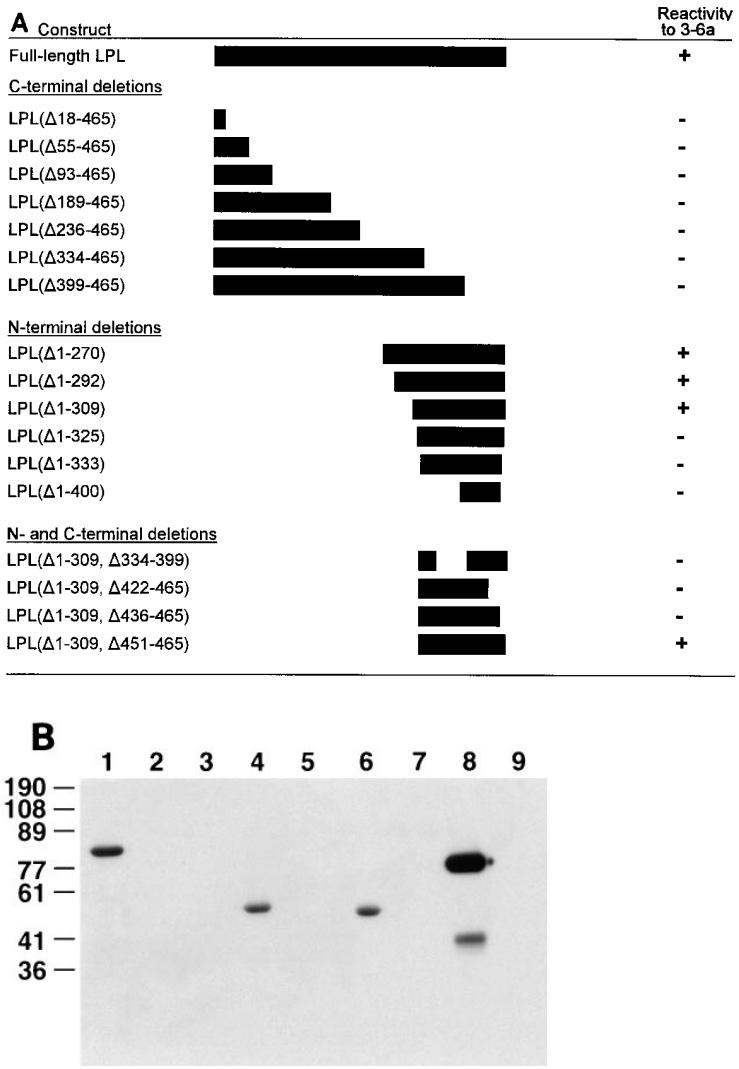

Fig. 6. Identification of the minimum continuous sequence of avian LPL that is required for binding of xCAL 3-6a; deletion mutants of avian LPL. The deletion mutants of LPL, and whether they react (+) or not (−) with xCAL 3-6a, are shown (A). One microgram of the full length GST/LPL construct (lane 1), along with equimolar equivalents of each deletion mutant, purified avian LPL (lane 8), and recombinant GST (lane 9), was electrophoresed on a 12.5% SDS PAGE gel and electrophoretically transferred to an Immobilon-P Membrane for Western blotting (B). The following deletion mutants are shown; lane 2, LPL(Δ399–465); lane 3, LPL(Δ1–333); lane 4, LPL(Δ1–309); lane 5, LPL(Δ1–309, Δ334–399); lane 6, LPL(Δ1–309, Δ451–465); lane 7, LPL(Δ1–309, Δ436–465). The membrane was blocked in 5% non-fat dry milk for 2 h at room temperature, incubated with xCAL 3-6a (17 μg/ml) in blocking solution at 4°C overnight, then washed with PBS. The blot was then incubated with HRP-conjugated anti-mouse antibody (Amersham) and detected with the Amersham ECL Western blotting analysis system. Molecular weight standards (Sigma) were pre-stained.

arin and heparan sulfate (Fig. 3). Most of the binding of LPL to CHO cells (29) and adipocytes (9) is due to interactions with cell surface heparan sulfate chains. These previous studies and our solid phase assays suggest that xCAL 3-6a disrupts the binding of LPL to adipocytes by interfering with the binding of LPL to heparan sulfate chains on the surface of these adipocytes. The inhibition of the binding of LPL to heparan sulfate chains suggests that xCAL 3-6a may be used as a tool to study receptor-mediated endocytosis of lipoproteins involving the low density lipoprotein receptor-related protein (LRP). LPL stimulates the binding of lipoproteins to LRP on the cell surface; however, treatment of heparan sulfate chains by heparitinase inhibits this stimulatory function (9). The mechanism by which LPL and heparan sulfate chains affect LRP function is unknown (11). The antibody may be used to block the LPL/heparan sulfate association while allowing the apoE and apoB/heparan sulfate interaction to occur. The ability to isolate individual effects (i.e., LPL/heparan sulfate and lipoprotein/heparan sulfate interactions) in the endocytosis of lipoprotein particles may provide a more detailed picture as to how these interactions affect the system.

A limitation of the use of a monoclonal antibody to identify structural features of a protein is the possibility that the antibody binds to a region that is adjacent to the area of interest and masks the area by steric hindrance. This possibility can be reduced by producing Fab fragments and determining whether the fragments retain the activity of the whole antibody. xCAL 3-6a Fab fragments inhibit the binding of LPL to adipocytes, as does the whole antibody, suggesting that xCAL 3-6a binds in close proximity to the heparin binding region.

The affinity of xCAL 3-6a for avian LPL was found to be much higher than that for bovine LPL. Two chimeric mutants were constructed to try to identify the residues responsible for the difference in affinity. The mutant in

A

construct	wild-type amino acid sequence	mutant amino acid sequence	reactivity to 3-6a
LPL 5G	Arg 281-Lys 282-Asn 283-Arg 284	Gln 281-Gln 282-Asn 283-Gln 284	+
LPL 1A	Lys 321	Asp 321	+
LPL 1B	Arg 405-Val 406-Arg 407-Val 408-Lys 409	Asn 405-Val 406-Asn 407-Val 408-Asn 409	-
LPL 1C	Arg 405	Asn 405	-
LPL 1D	Arg 407	Asn 407	+
LPL 1E	Lys 409	Asn 409	+
LPL 1F	Lys 415-Lys 416	Asn 415-Asn 416	+
LPL 1G	Phe 325-Gly-Lys-Thr-Asn-Val-Thr-Lys-Val-Asp 334	Ser 325-Gly-Thr-Glu-Ser-Asn-Thr-Tyr-Thr-Asn 334	+
LPL 1H	Thr 397-Pro-Phe-Ala-Phe-Thr-Ile-Gln 404	Ser 397-Ser-Pro-Gly-Phe-Asp-Ile-Gly 404	-

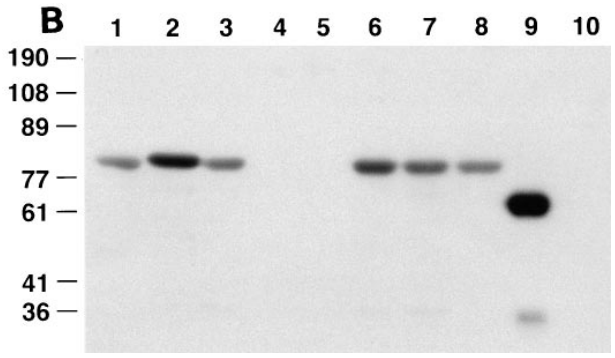

Fig. 7. Identification of residues of avian LPL required for the binding of xCAL 3-6a; amino acid substitution mutants of avian LPL. The mutants of LPL, and whether they react (+) or not (−) with xCAL 3-6a, are shown (A). One microgram of the full-length GST/LPL construct (lane 1) and each of the mutants, along with equimolar amounts of purified avian LPL (lane 9), and recombinant GST (lane 10), was electrophoresed on a 12.5% SDS PAGE gel and electrophoretically transferred to an Immobilon-P membrane for Western blotting (B). The following mutants are shown; lane 2, 1A; lane 3, 1G; lane 4, 1B; lane 5, 1C; lane 6, 1D; lane 7, 1E; lane 8, 1F. Conditions were identical to those described in Fig. 6B.

which the bovine sequence between residues 325 and 334 was substituted for the avian sequence (LPL 1G) retained its reactivity towards xCAL 3-6a (Fig. 7). This suggests that residues 325–334 are not required for antibody binding; however, two residues of the ten are conserved between the two lipases, so this cannot be stated with certainty. LPL 1H, a mutant in which the bovine LPL sequence between residues 397 and 404 was substituted for the avian sequence, showed no detectable reactivity towards xCAL 3-6a (Fig. 7). This indicates that the epitope of xCAL 3-6a contains residues within this region. Six residues of the eight are not conserved between the bovine and avian sequence suggesting that this portion of the epitope is responsible for the difference in affinity of xCAL 3-6a for the two lipases. It is possible that other non-conserved residues within the epitope may contribute to the difference in affinity as well.

The minimum continuous sequence required for xCAL 3-6a binding contains the residues 310–465. The binding sequence was further refined by making minor deletions in the termini; these results indicated that residues 310–325 and 436–450 are necessary for antibody binding. The epitope also contains residues within the region 397–405. Residues in the intervening sequences may or may not be required for antibody binding. These results are summarized in **Fig. 9**.

The binding of xCAL 3-6a appears to require residues within the regions 310–325, 397–405, and 436–450, indicating that the epitope is discontinuous. This is not unusual for a globular protein; a theoretical study that involved calculating the probability of finding "continuous patches" of primary sequence within the surface of a folded protein predicted that even for a low-affinity antibody ($K_A = 10^4\ m^{-1}$) with an epitope of 300 $Å^2$, less than 10% of the possible epitopes would be continuous (30). The regions 310–325, 397–405, and 436–450 are relatively far apart in the primary sequence of LPL with respect to the typical dimensions of an antibody–antigen recognition site. The dimensions of one antibody epitope ($K_A = 2–4 \times 10^7$) were found to be 20 × 25 Å (31), while the intervening sequence between residues 326 and 400 of LPL, in an extended

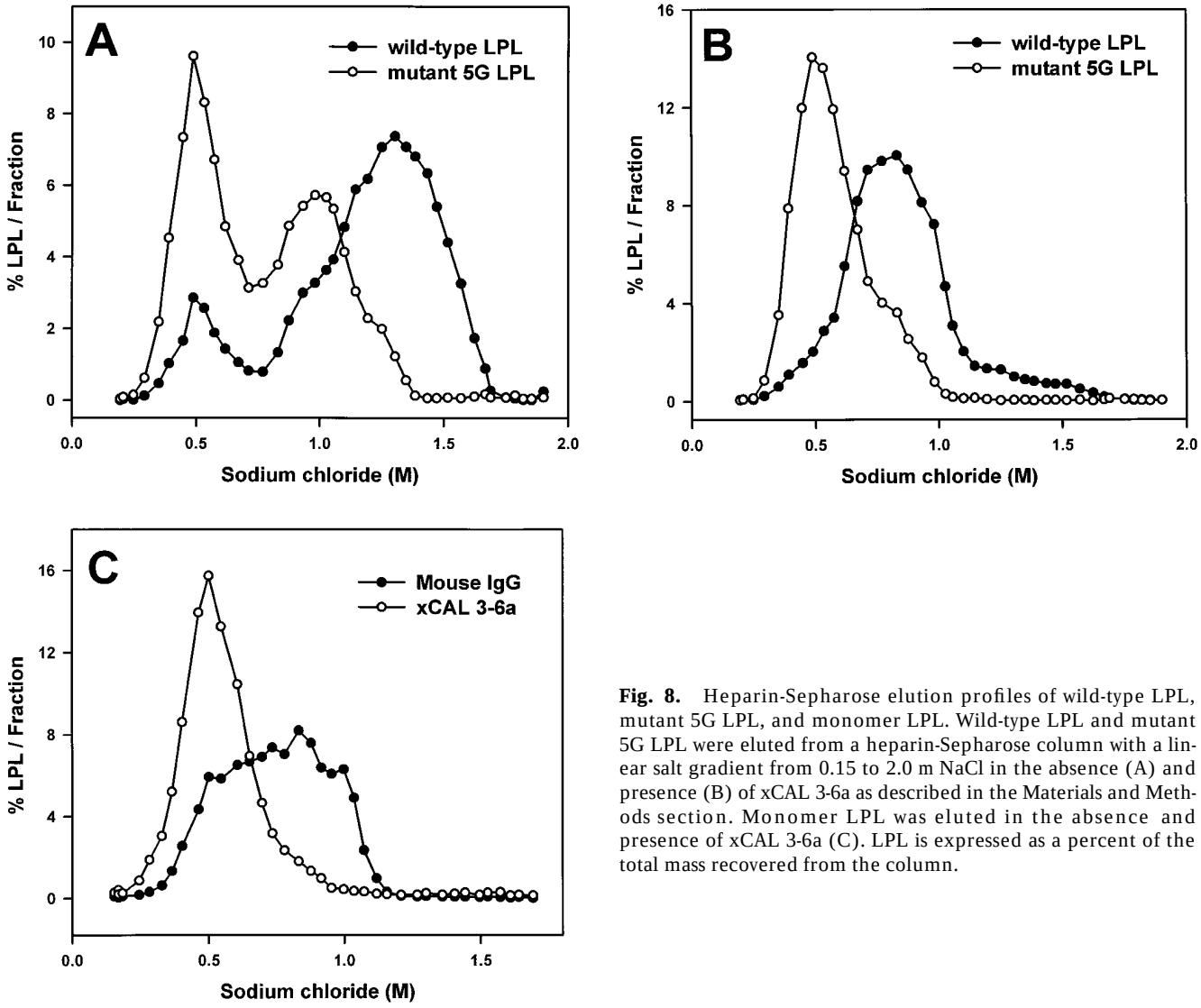

Fig. 8. Heparin-Sepharose elution profiles of wild-type LPL, mutant 5G LPL, and monomer LPL. Wild-type LPL and mutant 5G LPL were eluted from a heparin-Sepharose column with a linear salt gradient from 0.15 to 2.0 m NaCl in the absence (A) and presence (B) of xCAL 3-6a as described in the Materials and Methods section. Monomer LPL was eluted in the absence and presence of xCAL 3-6a (C). LPL is expressed as a percent of the total mass recovered from the column.

conformation, is 209 Å, assuming a length of 2.82 Å/residue (32). Therefore, in the Western blotting experiments, LPL must exist in a "collapsed state" in which the individual elements of the epitope are spatially much closer than they would be in the extended conformation. The fact that xCAL 3-6a interacts with LPL in this state indicates that it is essentially a native-like conformation with respect to this region of the protein. It is not unlikely that this conformation exists in the Western blotting procedure; although the deletion mutants were denatured during purification and electrophoresis, the Immobilon membrane is subsequently handled under renaturing conditions.

Based on the results of the Western blots of the deletion mutants, the epitope of the antibody appears to be contained within the residues 310–450. These data suggest that amino acids in this region of LPL are involved in heparin binding. A recent paper by van Tilbeurgh et al. (28) predicted the three-dimensional structure of LPL based on the determined X-ray crystal structure of pancreatic lipase (30% sequence identity to LPL). Four charge clusters are predicted to exist in human LPL: residues 279–282, 294–300, 148–152, and 319, 403, 405, 407, 414 and 415. The corresponding residues in avian LPL are conserved: 281–284, 296–302, 149–153, and 321, 405, 407, 409, 415 and 416. The first three clusters represent continuous sequences, while the last does not. Site-directed mutagenesis studies of the first three clusters have shown that residues 279–282 are involved in heparin binding (13–15), residues 148–152 are not (14), while the evidence for the residues in the second cluster (residues 294–300) is conflicting (14, 15). In our experiments, the shortest mutant that reacts with xCAL 3-6a, LPL(Δ1–309, Δ451–465), contains all of the

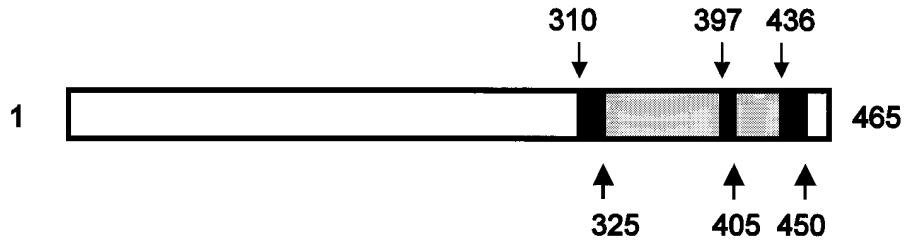

Fig. 9. The region of LPL required for the binding of xCAL 3-6a as determined by LPL mutant analysis. The diagram represents the sequence of avian LPL; black represents sequences that were found to be necessary for binding to xCAL 3-6a, white represents unnecessary regions, and grey indicates sequences that may or may not be involved in xCAL 3-6a binding.

residues in the fourth predicted charge cluster. In addition, LPL(Δ1–309, Δ451–465) does not contain any of the residues that have previously been shown to be necessary for heparin binding, suggesting that xCAL 3-6a blocks a unique heparin binding epitope. The data from the site-directed mutants (Fig. 7) show that residues Lys 321, Arg 407, Lys 409, Lys 415, and Lys 416 are not important in the binding of xCAL 3-6a to LPL, but that Arg 405 appears to be critical. This supports the proposal that xCAL 3-6a blocks the putative heparin-binding region of the fourth predicted charge cluster.

Elution of both wild-type and 5G LPL from a heparin-Sepharose column in the absence of xCAL 3-6a yielded two peaks, a low salt peak at 0.49 m and a high salt peak at 1.31 and 0.98, respectively (Fig. 8A). These peaks have been observed previously for wild-type LPL (13, 33, 34); the low salt peak has been attributed to partially denatured LPL and the high salt peak constitutes active dimer LPL. The low-affinity binding is most likely due to ionic interactions between LPL and heparin and/or LPL and Sepharose. In the partially denatured state, the high-affinity heparin-binding sites within LPL are in a conformation that does not permit the specific interaction of these sites with heparin-Sepharose to occur; however, the primary sequence of LPL contains many regions of basic residues that most likely interact with lower affinity. In addition, it has been shown previously (35) and confirmed in our laboratory that LPL interacts with unsubstituted Sepharose and elutes at low salt. These two types of low-affinity ionic interactions most likely give rise to the first elution peak.

Mutation of the well-characterized heparin-binding sequence between Arg 281 and Arg 284 resulted in a reduced affinity of dimer LPL for heparin (Fig. 8) (13). The loss in affinity is not complete, however, suggesting that another high-affinity heparin-binding epitope exists in LPL. In the presence of xCAL 3-6a, mutant 5G LPL eluted at 0.49 m NaCl (Fig. 8B) indicating that all of the high-affinity heparin binding of LPL has been removed. Interestingly, all of the high-affinity binding not attributable to the region Arg 281–Arg 284 is blocked by the antibody.

Wild-type LPL dimer eluted at 0.83 m NaCl in the presence of xCAL 3-6a (Fig. 8B). This suggests that xCAL 3-6a does not block the region Arg 281–Arg 284; if this site was neutralized by the antibody, the LPL–xCAL 3-6a complex would have eluted at 0.49 m NaCl, as mutant 5G did. This appears to contradict the adipocyte and solid-phase heparin-binding results that imply almost no binding of LPL in the presence of xCAL 3-6a to either adipocytes or heparin coated to microtiter plates. It is difficult, however, to make a direct comparison of the equilibrium binding experiments and the heparin-Sepharose chromatography experiments. Characterization of the binding of LPL to glycosaminoglycan-substituted Sepharose provides a measure of the affinity as reflected by the salt concentration needed to elute LPL from the affinity matrix. This salt concentration, however, is dependent on the level of substitution of glycosaminoglycan on the Sepharose (35) and the nature of the glycosaminoglycan (36). In addition, the relationship between the salt concentration required for elution of a protein from a heparin-Sepharose column and the affinity constant of that protein for heparin is not an obvious one. A recent study in which the binding of LPL to heparin was characterized reported a 6000-fold difference in affinity constants between the monomer and dimer (37): these species elute at 0.5 m and 1.3 m NaCl, respectively (Fig. 8A) (13). For this reason, and because the LPL-binding moiety in the individual experiments differs in local concentration, orientation, and exact nature, a direct comparison of the experiments is not feasible.

The LPL monomer eluted as a broad peak between 0.5 m NaCl and 1.0 m NaCl in the absence of xCAL 3-6a (Fig. 8C). This peak most likely represents a mixture of partially denatured forms of LPL; after unfolding in Gdn HCl the enzyme can regain some structure (38). In the presence of xCAL 3-6a, monomer LPL elutes in a sharp peak at 0.5 m NaCl indicating that any "high affinity"

sites that may have been partially or fully formed during the partial refolding of LPL are susceptible to neutralization by xCAL 3-6a.

Our results are in agreement with several studies that indicate the presence of a heparin binding region in the carboxyl-terminus of LPL. A chimera experiment in which the carboxyl-terminus of hepatic lipase was joined with the amino-terminus of LPL was reported (39). The chimera possessed heparin binding affinity similar to that of hepatic lipase and not LPL, suggesting that binding of heparin is mediated by the carboxyl-terminus of LPL. Another study (40), which demonstrated that the carboxyl-terminal residues of bovine LPL (380–425) are responsible for binding the low density lipoprotein receptor-related protein (LRP), also showed that heparin was capable of blocking the LRP binding site of LPL. In experiments involving the proteolytic digestion of lipoprotein lipase (41), the carboxyl-terminal domain was found to be sufficient for binding to heparin.

In summary, we have produced a monoclonal antibody to avian lipoprotein lipase that inhibits the binding of LPL to primary adipocytes, heparan sulfate, and heparin without affecting catalytic activity. Through the production of amino- and carboxyl-terminal deletion mutants of LPL we have identified the minimum continuous protein sequence required for binding to the antibody. This deletion mutant, LPL(Δ1–309, Δ451–465), does not contain any of the regions that have previously been shown by site-directed mutagenesis to be involved in heparin binding; it does, however, contain residues that are homologous to those that have been postulated to form a discontinuous charge cluster in folded human LPL. This charge cluster (residues 321, 405, 407, 409, 415, and 416) contains one residue, Arg 405, of the epitope of the heparin-binding inhibitor xCAL 3-6a. Although only one residue is necessary for xCAL 3-6a binding, it is likely that a larger subset of the cluster may be required for heparin binding.

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REFERENCES

1. Havel, R. J., and R. S. Gordon, Jr. 1960. Idiopathic hyperlipemia: metabolic studies in an affected family. *J. Clin. Invest.* **39:** 1777–1790.
2. Kompiang, I. P., A. Bensadoun, and M-W. W. Yang. 1976. Effect of an anti-lipoprotein lipase serum on plasma triglyceride removal. *J. Lipid Res.* **17:** 498–505.
3. Cheng, C-F., G. M. Oosta, A. Bensadoun, and R. D. Rosenberg. 1981. Binding of lipoprotein lipase to endothelial cells in culture. *J. Biol. Chem.* **256:** 12893–12898.
4. Olivecrona, T., G. Bengtsson, S. E. Marklund, U. Lindahl, and M. Höök. 1977. Heparin–lipoprotein lipase interactions. *Fed. Proc.* **36:** 60–65.
5. Shimada, K., P. J. Gill, J. E. Silbert, W. J. Douglas, and B. L. Fanburg. 1981. Involvement of cell surface heparin sulfate in the binding of lipoprotein lipase to cultured bovine endothelial cells. *J. Clin. Invest.* **68:** 995–1002.
6. Williams, M. P., H. B. Streeter, F. S. Wusteman, and A. Cryer. 1983. Heparan sulphate and the binding of lipoprotein lipase to porcine thoracic aorta endothelium. *Biochim. Biophys. Acta.* **756:** 83–91.
7. Chajek-Shaul, T., G. Friedman, G. Bengtsson-Olivecrona, I. Vlodavsky, and R. Bar-Shavit. 1990. Interaction of lipoprotein lipase with subendothelial extracellular matrix. *Biochim. Biophys. Acta.* **1042:** 168–175.
8. Saxena, U., L. D. Witte, and I. J. Goldberg. 1989. Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acid. *J. Biol. Chem.* **264:** 4349–4355.
9. Goldberg, I. J. 1996. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J. Lipid Res.* **37:** 693–706.
10. Sehayek, E., T. Olivecrona, G. Bengtsson-Olivecrona, I. Vlodavsky, H. Levkovitz, R. Avner, and S. Eisenberg. 1995. Binding to heparan sulfate is a major event during catabolism of lipoprotein lipase by HepG2 and other cell cultures. *Atherosclerosis.* **114:** 1–8.
11. Santamarina-Fojo, S., and K. A. Dugi. 1994. Structure, function and role of lipoprotein lipase in lipoprotein metabolism. *Curr. Opin. Lipidol.* **5:** 117–125.
12. Cardin, A. D., and H. J. R. Weintraub. 1989. Molecular modeling of protein–glycosaminoglycan interactions. *Arteriosclerosis.* **9:** 21–32.
13. Berryman, D. E., and A. Bensadoun. 1992. Site-directed mutagenesis of a putative heparin binding domain of avian lipoprotein lipase. *J. Biol. Chem.* **268:** 3272–3276.
14. Hata, A., D. N. Ridinger, S. Sutherland, M. Emi, Z. Shuhua, R. L. Meyers, K. Ren, T. Cheng, I. Inoue, D. E. Wilson, P-H. Iverius, and J-M. Lalouel. 1993. Binding of lipoprotein lipase to heparin. Identification of five critical residues in two distinct segments of the amino-terminal domain. *J. Biol. Chem.* **268:** 8447–8457.
15. Ma, Y., H. E. Henderson, M-S. Liu, H. Zhang, I. J. Forsythe, I. Clark-Lewis, M. R. Hayden, and J. D. Brunzell. 1994. 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.* **35:** 2049–2059.
16. Raffai, R., R. Maurice, K. Weisgraber, T. Innerarity, X. Wang, R. MacKenzie, T. Hirama, D. Watson, E. Rassart, and R. Milne. 1995. Molecular characterization of two monoclonal antibodies specific for the LDL receptor-binding site of human apolipoprotein E. *J. Lipid Res.* **36:** 1905–1917.
17. Speziale, P., D. Joh, L. Visai, S. Bozzini, K. House-Pompeo, M. Lindberg, and M. Hook. 1996. A monoclonal antibody enhances ligand binding of fibronectin MSCRAMM (adhesin) from *Streptococcus dysgalactiae.* *J. Biol. Chem.* **271:** 1371–1378.
18. Gershenwald, J. E., A. Bensadoun, and A. Saluja. 1985. Monoclonal antibodies to avian lipoprotein lipase. Purification of the enzyme by immunoaffinity chromatography. *Biochim. Biophys. Acta.* **836:** 286–295.

19. Cisar, L. A., A. J. Hoogewerf, M. Cupp, A. Rapport, and A. Bensadoun. 1989. Secretion and degradation of lipoprotein lipase in cultured adipocytes. *J. Biol. Chem.* **264:** 1767–1744.
20. Goding, J. W. 1983. Monoclonal Antibodies: Principles and Practice. Academic Press, New York, NY. 120–121.
21. Bensadoun, A., and R. A. Marita. 1986. Dibutyryl cyclic AMP decreases the rate of lipoprotein lipase synthesis in cultured adipocytes. *Biochim. Biophys. Acta.* **879:** 253–263.
22. Cooper, D. A., J. C. Stein, P. J. Strieleman, and A. Bensadoun. 1989. Avian adipose lipoprotein lipase: cDNA sequence and reciprocal regulation of mRNA levels in adipose and heart. *Biochim. Biophys. Acta.* **1008:** 92–101.
23. Mullis, K. B., and F. A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155:** 335–350.
24. Ho, S. N., H. D. Hunt, R. M. Horton, J. J. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* **77:** 51–59.
25. Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70:** 241–250.
26. Cheung, A. H., A. Bensadoun, and C-F. Cheng. 1979. Direct solid phase radioimmunoassay for chicken lipoprotein lipase. *Anal. Biochem.* **94:** 346.
27. Socorro, L., C. C. Green, and R. L. Jackson. 1985. Preparation of a homogeneous and stable form of bovine milk lipoprotein lipase. *Prep. Biochem.* **15:** 133–143.
28. van Tilbeurgh, H., A. Roussel, J-M. Lalouel, and C. Cambillau. 1994. Lipoprotein lipase. Molecular model based on the pancreatic lipase X-ray structure: consequences for heparin binding and catalysis. *J. Biol. Chem.* **269:** 4626–4633.
29. Berryman, D. E., and Å. Bensadoun. 1995. Heparan sulfate proteoglycans are primarily responsible for the maintenance of enzymatic activity, binding, and degradation of lipoprotein lipase in Chinese hamster ovary cells. *J. Biol. Chem.* **270:** 24525–24531.
30. Barlow, D. J., M. S. Edwards, and J. M. Thornton. 1986. Continuous and discontinuous protein antigenic determinants. *Nature.* **322:** 747–748.
31. Amit, A. G., R. A. Mariuzza, S. E. Philips, and R. J. Poljak. 1985. Three-dimensional structure of an antigen-antibody complex at 6 Å resolution. *Nature.* **313:** 156–158.
32. Marsh, R. E., and J. Donohue. 1967. Crystal structure studies of amino acids and peptides. *Adv. Protein Chem.* **22:** 235–256.
33. Peterson, J., W. Y. Fujimoto, and J. D. Brunzell. 1992. Human lipoprotein lipase: relationship of activity, heparin affinity, and conformation as studied with monoclonal antibodies. *J. Lipid Res.* **33:** 1165–1170.
34. Bengtsson-Olivecrona, G., and T. Olivecrona. 1985. Binding of active and inactive forms of lipoprotein lipase to heparin: effects of pH. *Biochem. J.* **226:** 409–413.
35. Bengtsson, G., and T. Olivecrona. 1977. Interaction of lipoprotein lipase with heparin-Sepharose: evaluation of conditions for affinity binding. *Biochem. J.* **167:** 109–119.
36. Bengtsson, G., and T. Olivecrona. 1980. Interaction of lipoprotein lipase with native and modified heparin-like polysaccharides. *Biochem. J.* **189:** 625–633.
37. Lookene, A., O. Chevreuil, P. Østergaard, and G. Olivecrona. 1996. Interaction of lipoprotein lipase with heparin fragments and with heparan sulfate: stoichiometry, stabilization, and kinetics. *Biochemistry.* **35:** 12155–12163.
38. Olivecrona, T., and G. Bengtsson. 1984. Lipases in milk. *In* Lipases. B. Borgström and H. L. Brockman, editors. Elsevier, Amsterdam. 205–261.
39. Wong, C. H., J. Nikazy, K. Wang, Q. Han, and M. C. Schotz. 1992. Chimeras of hepatic lipase and lipoprotein lipase. *J. Biol. Chem.* **267:** 21499–21504.
40. Nykjær, A., M. Nielsen, A. Looken, N. Meyer, H. Røigaard, M. Etzerodt, U. Beisiegel, G. Olivecrona, and J. Gliemann. 1994. A carboxyl-terminal fragment of lipoprotein lipase binds to the low density lipoprotein receptor-related protein and inhibits lipase-mediated uptake of lipoprotein in cells. *J. Biol. Chem.* **269:** 31747–31755.
41. Bengtsson-Olivecrona, G., T. Olivecrona, and H. Jörnvall. 1986. Lipoprotein lipases from cow, guinea-pig and man. Structural characterization and identification of protease-sensitive internal regions. *Eur. J. Biochem.* **161:** 281–188.