Identification of a Lipoprotein Lipase Cofactor-binding Site by Chemical Cross-linking and Transfer of Apolipoprotein C-II-responsive Lipolysis from Lipoprotein Lipase to Hepatic Lipase*

Trina L. Mehlhargey‡‡, Yingying Yang‡, Howard Wong§§, and John S. Hill‡ ‡‡

From the ‡University of British Columbia McDonald Research Laboratories/CAPTIVE Centre, Department of Pathology and Laboratory Medicine, St. Paul’s Hospital, and the University of British Columbia, Vancouver, British Columbia V6Z 1Y6, Canada, the ‡Department of Veterans Affairs, Greater Los Angeles Healthcare System, Los Angeles, California 90073, and the ‡Department of Medicine, University of California, Los Angeles, Los Angeles, California 90095

To localize the regions of lipoprotein lipase (LPL) that are responsive to activation by apoC-II, an apoC-II peptide fragment was cross-linked to bovine LPL. Following chemical hydrolysis and peptide separation, a specific fragment of LPL (residues 65–86) was identified to interact with apoC-II. The fragment contains regions of amino acid sequence dissimilarity compared with hepatic lipase (HL), a member of the same gene family that is not responsive to apoC-II. Using site-directed mutagenesis, two sets of chimeras were created in which the two regions of human LPL (residues 65–68 and 73–79) were exchanged with the corresponding human HL sequences. The chimeras consisted of an HL backbone with the suspected LPL regions replacing the corresponding HL sequences either individually (HLPL-(65–68) and HLPL-(73–79)) or together (HLPLLD). Similarly, LPL chimeras were created in which the candidate regions were replaced with the corresponding HL sequences (LPLHL-(77–80), LPLHL-(85–91), and LPLHLD). Using a synthetic triolein substrate, the lipase activity of the purified enzymes was measured in the presence and absence of apoC-II. Addition of apoC-II to HLPL-(65–68) and HLPL-(73–79) did not significantly alter their enzyme activity. However, the activity of HLPLLD increased ~5-fold in the presence of apoC-II compared with an increase in native LPL activity of ~11-fold. Addition of apoC-II to LPLHL-(77–80) resulted in ~10-fold activation, whereas only ~6- and ~4-fold activation of enzyme activity was observed in LPLHL-(65–91) and LPLHLD, respectively. In summary, our results have identified 11 amino acid residues in the N-terminal domain of LPL (residues 65–68 and 73–79) that appear to act cooperatively to enable substantial activation of human LPL by apoC-II.

Hepatic lipase (HL) and lipoprotein lipase (LPL) are members of the same lipase gene family, along with pancreatic lipase, the pancreatic lipase-related lipases, endothelial lipase, and phosphatidylserine-specific phospholipase A₁ (1–6). Through their ability to hydrolyze triglycerides and phospholipids in a variety of circulating plasma lipoproteins, including chylomicrons and very low, intermediate, and high density lipoproteins, HL and LPL greatly influence lipid metabolism (7–9). HL and LPL are associated with cell surfaces through an interaction with heparan sulfate proteoglycans and are thought to possess non-catalytic functions associated with the binding and clearance of various lipoproteins (10–13). HL and LPL share a number of functional domains such as the Ser-Asp-His catalytic triad, heparin-binding domain, lig domain, and lipid- and receptor-binding domains (15). Based on their similarity of lipolytic function, amino acid homology, and conservation of disulfide bridges, it is believed that HL and LPL share a similar structure (16). Despite these similarities, however, differences remain in important enzyme characteristics such as relative heparin affinity, substrate specificity, and cofactor requirements.

Unlike HL, LPL requires a specific cofactor, apoC-II, to hydrolyze triglycerides in chylomicrons (17, 18). The importance of apoC-II for LPL function is emphasized by the observation of a significant accumulation of triglycerides in patients who have an inherited defect of the apoC-II gene (19). Initially, the study of chimeric lipases (20, 21) suggested that a region in the N-terminal domain of LPL was responsible for cofactor activation because enzymes containing the N-terminal domain of LPL and the C-terminal domain of HL were still able to be activated by apoC-II. However, these chimeric enzymes were not activated by apoC-II to the same extent as native LPL. More recently, we reported that the 60 C-terminal amino acids of LPL also participate in apoC-II activation (22), suggesting that regions in the N-terminal domain alone are not sufficient to achieve optimal activation. These results are more easily interpreted in the context of a head-to-tail dimer model (15, 20, 23–25), which supports the hypothesis that apoC-II interacts simultaneously with regions located in the N- and C-terminal domains of opposing subunits that make up an LPL dimer (22).

To identify specific LPL amino acid residues that are responsive to cofactor, chemical cross-linking of apoC-II to LPL was undertaken. Cross-linking experiments identified a region from the N-terminal domain of LPL that interacted with

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†† Scholar of the Heart and Stroke Foundation of Canada and the Michael Smith Foundation for Health Research. To whom correspondence should be addressed: St. Paul’s Hospital, Healthy Heart Program, 1081 Burrard St., Vancouver, BC V6Z 1Y6, Canada, Tel.: 604-806-8618; Fax: 604-806-8590; E-mail: jshill@interchange.ubc.ca.

1 The abbreviations used are: HL, hepatic lipase; LPL, lipoprotein lipase; SASD, sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl 1,3-di-thiopropionate; PBS, phosphate-buffered saline.

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apoC-II and whose role in activation was determined using chimeric lipases. The LPL fragment contains two candidate regions, one composed of 4 amino acids and the other of 7, that differ from HL, a highly related but cofactor-unresponsive lipase. A series of chimeras were constructed with the variable regions exchanged between the two lipases, and apoC-II responsiveness was determined. The results suggest that LPL residues 65–68 and 73–79 cooperate in cofactor activation, and, moreover, that the functional responsiveness imparted by these LPL residues can be translocated to HL.

**EXPERIMENTAL PROCEDURES**

**Cross-linking of ApoC-II and LPL**

**Overall Strategy**—A fragment of human apoC-II spanning residues 44–79 (apoC-II-(44–79)) was chemically cross-linked to purified bovine LPL (Sigma). The mixture was reduced and incubated with o-iodosobenzoic acid at tryptophanyl residues, which were resolved by SDS-PAGE, transferred to membranes, and visualized by autoradiography.

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**Step 1**—The cross-link reagent sulfo-succinimidyl-2-(p-azidosalicyl-amido)ethyL1,3-dithiopropionate (SASD) was iodinated using IODO-GEN (Pierce) under conditions recommended by the manufacturer. IODO-GEN (1 mg) was dried in microcentrifuge tubes, and SASD was added (1 mg in 1 ml of 100 mM NaPO₄, pH 7.2) together with sodium iodide (100 μCi) and mixed briefly (2 min). The reaction mixture was removed from the tube and desalted to separate unbound radioisotope.

**Step 2**—A cofactor-cross-linker complex composed of apoC-II-(44–79) (100 μM in 100 mM NaPO₄, pH 7.2) linked to the iodinated cross-linking reagent SASD was created (Fig. 1). The photolabile azido group in SASD required all steps to be carried out in dimmed room light or within darkened vessels. The cross-linker was used at a 3-fold molar excess over cofactor to maximize linkage via the succinimidyl moiety at neutral pH. Probable sites of apoC-II-(44–79) derivatization included the N terminus and/or basic residues at positions 48, 50, 55, and 76. Excess unbound cross-linker was removed by gel permeation chromatography prior to incubation with LPL.

**Step 3**—The cofactor-cross-linker complex was incubated with bovine LPL (1 mg/ml) in quartz cuvettes at 4°C. The samples were irradiated for 3 min by an ultraviolet light source placed 4 cm from the cuvettes, with a mirror positioned 2 cm behind. Some experiments contained a “dark control” sample, which was wrapped in foil during irradiation to determine the effect of the absence of UV light exposure. Other samples contained a 50-fold molar excess of unlabeled cofactor to evaluate cross-linking specificity.

**Step 4**—Following UV radiation, samples were treated with dithiothreitol to a final concentration of 10 mM. Reducing agent was used to sever the disulfide bond in the cross-linker moiety (Fig. 1) and served to transfer the iodine label, originally on the cofactor, to a region of LPL at or near the site of interaction. The cofactor provided the binding spec-
Primers defining the 5’ and 3’ termini of the construct to allow for directional cloning. Mutagenic primers (forward and reverse) were designed to span the corresponding boxed coding regions (Fig. 2) and to overlap with one another so that two PCR products could be combined together to form the final full-length cDNA in a third PCR. The primers used for each portion of the chimeras are shown in Table I.

DNA Transfection and Expression

Full-length cDNAs were purified, digested, and inserted into the pcDNA3 expression vector (Invitrogen) using the HindIII and BamHI restriction endonuclease sites. The DNA sequence was confirmed prior to transfection. Chinese hamster ovary Pro5 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). To mediate the transfection of Chinese hamster ovary cells, coprecipitates of plasmid DNA and CaPO4 were prepared (28). The calcium phosphate/DNA mixture was incubated at room temperature for 30 min before it was added to a 50% confluent Chinese hamster ovary monolayer. Stably transfected cells were selected by growth in the presence of Geneticin (G418 sulfate; 500 μg/ml), and surviving colonies were selected and expanded. Cell clones expressing maximal quantities of lipase were identified by enzyme activity analysis.

After growth to confluency in T-175 flasks, the medium was replaced with Opti-MEM (Invitrogen) supplemented with 10 units/ml heparin. The medium was harvested and replaced every 24 h for an 8-day period. After centrifugation at 3000 × g for 10 min to remove cell debris, protease inhibitor mixture for mammalian cell and tissue extracts (Sigma) was added to a final concentration of 0.02 mM, and the harvested medium was stored at −80 °C.

Purification of Recombinant Lipases

All purification steps were carried out at 4 °C. Thawed wild-type or chimeric HL medium (1 liter) was mixed with NaCl to a final concentration of 0.5 M and applied to an octyl-Sepharose column (2.6 × 25 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.2, containing 0.35 M NaCl. Following a wash with 500 ml of 50 mM Tris-HCl, 0.5 M NaCl, 20% glycerol, and 0.02 mM protease inhibitor, pH 7.2 (Buffer A), the lipase was eluted with 500 ml of 50 mM Tris-HCl, 0.35 M NaCl, 20% glycerol, and 0.02 mM protease inhibitor, pH 7.2 containing 1.2% Igepal CA-630 (Sigma) onto a heparin-Sepharose column (2.6 × 25 cm). This column was washed with 500 ml of Buffer A prior to elution with 250 ml of 50 mM Tris-HCl, pH 7.2, 2 M NaCl, 20% glycerol, and protease inhibitor (0.02 mM), onto a 1 × 10-cm metal affinity column (QUIGEN Inc.). The column was washed with 25 ml of Buffer A before elution with 26 ml of 50 mM Tris-HCl, 0.5 M NaCl, and 250 mM imidazole, pH 7.2. The eluent was collected in eight fractions, the first one being 5 ml and the rest 3 ml. Each fraction was assayed for activity, and the active fractions were concentrated in a Millipore filtration unit (molecular mass cutoff of 100,000 Da) to a final volume of ~1 ml and stored at 80 °C. Wild-type LPL and the LPL chimeras were purified in the same manner with two exceptions. 1) The octyl-Sepharose step was omitted; therefore, the thawed medium was loaded directly onto the heparin-Sepharose column with no NaCl added. 2) Buffer A contained NaCl at 0.75 M, not 0.5 M. The purity of the enzyme preparation was determined by densitometry of silver-stained SDS-polyacrylamide gels.

Enzyme Assays

Trioleinase activity was measured using a triolein emulsion containing radiolabeled triolein as described previously (22). ApoC-II-dependent lipase activity was determined by performing the assay in the presence of an apoC-II fragment spanning residues 44–79. This apoC-II fragment has been shown to have the same activating potential as full-length apoC-II (29). Protein concentration was measured by a colorimetric assay developed by Smith et al. (30) using a Pierce micro-BCA protein assay reagent kit. Kinetic constants were determined using GraphPAD Prism Version 3.02 for Windows.

Silver Staining

Gels were fixed in 100 ml of 30% ethanol and 10% glacial acetic acid for 30 min and then washed twice with 10% ethanol and three times with deionized water for 5 min/wash. The gels were soaked in 50 ml of SilverSNAP stain solution with 1 ml of SilverSNAP enhancer solution (Pierce) for 30 min with gentle shaking. The developer was removed, and the gels were washed with deionized water for 30 s. The gels were transferred to 50 ml of SilverSNAP developer with 1 ml of SilverSNAP enhancer for developing until bands appeared.
RESULTS

Electrophoresis and Immunoblotting

Samples were mixed with 0.5% volume of buffer containing 2% SDS, 0.1 M Tris-HCl, pH 6.8, 50% glycerol, 10% β-mercaptoethanol, and 0.05% bromphenol blue. The mixture was placed in boiling water for 5 min prior to loading onto a 10% acrylamide gel. Gels were electrophoresed onto a polyvinylidene difluoride hydrophobic membrane that was treated with 100% methanol for 10 s. The membrane was placed on filter paper and air-dried for 15 min. The blot was placed in 15 ml of 1% casein and 0.04% Tween 20 (antibody buffer) containing either a monoclonal antibody specific for human HL (22) or a chicken polyclonal antibody raised against bovine LPL (a kind gift from Dr. John D. Brunzell) (31) was added to each well of a Costar high binding enzyme immunoassay/radioimmunoassay plate at a dilution of 1:4000. This antibody mixture was then incubated in the dark at room temperature for 10 min. The blot was rinsed with PBS and washed for 5 min with fresh PBS, which was then repeated twice. Immunoblotting with the monoclonal or polyclonal antibody was detected with either anti-mouse IgG or anti-chicken IgG conjugated to horseradish peroxidase in PBS with 0.05% Tween 20; 200 μl of 5D2 peroxidase was added per well; and the plate was covered and incubated in the dark at room temperature for 4 h. The plate was washed three times with PBS and incubated with streptavidin conjugated to horseradish peroxidase in PBS with 0.1% Triton X-100 for 10 min. The blot was developed with chemiluminescent reagents (Pierce) and exposed to chemiluminescent film (Amersham Biosciences).

The buffer was removed from the plate, and standards (purified LPL (36)). Thus, it was concluded that the cofactor-cross-linker complex was suitable for the cofactor-binding site of LPL. LPL was incubated with the cofactor-cross-linker complex and then photoalyzed, reduced, cleaved, and displayed on denaturing gels (Fig. 3). This autoradiograph of an SDS-16% acrylamide gel shows a single radiolabeled peptide fragment, which migrated below the 3.5-kDa standard, but above the dye front. Lane 2 shows the pattern of another sample run under identical conditions, except for the inclusion of a 50-fold excess of unlabeled apoC-II, and the reduction step was omitted. In this case (as for dark control samples), a 3.5-kDa band was seen; no band was detected corresponding to the band in lane 1. The band migrating at 3.5 kDa corresponded to the size of the apoC-I apoC-II cross-linker complex, whose identity was confirmed by microsequence analyses (data not shown). Thus, the 3.5-kDa band in lane 2 is the unbound cofactor-cross-linker complex; and significantly, inclusion of excess unlabeled apoC-II completely eliminated the lower band (Fig. 3, compare lanes 1 and 2), suggesting specific interaction between apoC-II and this portion of LPL.

The mass of the labeled peptide (Fig. 3, lane 1) was determined to be 2.2 kDa by comparison with the migration positions of proteins with known molecular masses. Based on the locations of the 8 tryptophan residues in bovine LPL, the molecular mass of the labeled LPL peptide most closely corresponds to that of peptide 3, from residues 65 to 86 (Table II). This conclusion was supported by the determination of a valine residue at the N terminus of the labeled peptide (data not shown); only the sequence of peptide 3 begins with a valine residue.

Construction of ApoC-II Activation Site Chimeras—Upon comparison of residues 65–86 of LPL with the corresponding region in HL, two regions of dissimilarity were identified (Fig. 2). To determine whether these sequences are associated with the ability of LPL to be activated by apoC-II, chimeras were created in which the candidate regions of LPL were exchanged with the corresponding HL sequences. The first set of chimeras, designated the HL chimeras, consisted of the HL backbone with the suspected LPL regions replacing the corresponding molecular modeling

Molecular Modeling

The model of human LPL was generated using as a template, the 2.46-Å resolution structure of the human pancreatic lipase-collagen complex inhibited by a C11 alkyl phosphonate (Protein Data Bank code 1LPB) (33), which has 30% homology to human LPL. The model was created using the 3D-JIGSAW algorithm (34) (amino acids 1–434 of the mature LPL sequence were modeled) and viewed/analyzed using Swiss-PdbViewer (35).

Table 1

| Name           | Sequence                  |
|----------------|---------------------------|
| HIND5PKHL      | ACT TAA GCT TGC CAC CAT  |
| 1LPLC2HLFOR    | AAC TGG GTG CCA AAA CTT  |
| 1LPC2HLREV     | AGG TCG ATC CAA GTA AGA  |
| 2LPC2HLFOR     | ACG AGA GAA CCA TGC ATT  |
| 2LPLC2HLREV    | TGC TTT TCT CTT CTT CTT |
| D1LPC2HLFOR    | ACG GGA GTG TCC TCT CTT  |
| D2C2HLFOR      | GGA CTC ATC CAA GTA AGA  |
| D1HLC2PLFOR    | TGC TCT CTT CTT CTT CTT |
| HIND5PKLPL     | AGC TAA GCT TGC CAC CAT  |
| 1HLCL2PLFOR    | AGT TGG ATC TGG CAT AGA  |
| 1HCL2PLREV     | ACG CAT CTC CTG CCA CAT  |
| BAM3FLPL       | ACG TGG ATC CAA CAT CCA  |
| 2HLCL2PLFOR    | AAG TCT CAG GGC CAG CCA  |
| 2HLC2PLREV     | ATT TGG GTG CCA AAA CTT  |
| D1HLC2PLFOR    | AGT TGG ATC TGG CAT AGA  |

Identification of a Lipoprotein Lipase Cofactor-binding Site

Samples were mixed with 0.5% volume of buffer containing 2% SDS, 0.1 M Tris-HCl, pH 6.8, 50% glycerol, 10% β-mercaptoethanol, and 0.05% bromphenol blue. The mixture was placed in boiling water for 5 min prior to loading onto a 10% acrylamide gel. Gels were electrophoresed onto a polyvinylidene difluoride hydrophobic membrane that was treated with 100% methanol for 10 s. The membrane was placed on filter paper and air-dried for 15 min. The blot was placed in 15 ml of 1% casein and 0.04% Tween 20 (antibody buffer) containing either a monoclonal antibody specific for human HL (22) or a chicken polyclonal antibody raised against bovine LPL (a kind gift from Dr. John D. Brunzell) (31) was added to each well of a Costar high binding enzyme immunoassay/radioimmunoassay plate at a dilution of 1:4000. The plate was sealed and incubated at room temperature for 4 h. The plate was washed three times with PBS and incubated with streptavidin conjugated to horseradish peroxidase in PBS with 0.1% Triton X-100 for 10 min. The blot was developed with chemiluminescent reagents (Pierce) and exposed to chemiluminescent film (Amersham Biosciences).
the reduction step was eliminated.

product of a sample identical to that in lane 1 cleavage of LPL and the cofactor cross-linker complex; /H18528 show on the left. Migration positions of molecular mass markers and the dye front are amide and 3% bisacrylamide denaturing gel, transferred to HL sequences. These regions were exchanged individually and LPLHLD (Fig. 2).

enzymes with the designations LPLHL-(77-80),—D— and LPLHLD (Fig. 2)). The second set of chimeras, the LPL chimeras and together (HLLPLD, where “D” is double chimera). The second set of chimeras, the LPL chimeras, is essentially the opposite of the first set. The LPL chimeras have an LPL backbone with the candidate regions of LPL being replaced with the corresponding HL sequences. Again, this was done individually and together, resulting in enzymes with the designations LPLHL-(77-80), LPLHL-(85-91) and LPLHLD (Fig. 2).

purification of each enzyme, a concentrated sample was run on a polyacrylamide gel (along with samples taken throughout the purification procedure) and silver-stained to determine the purity of the sample. In the concentrated sample, each HL chimera and wild-type HL showed an intense band at 65 kDa that was not visible in the starting culture medium (data not shown). Similar results were seen for the LPL chimeras and wild-type LPL, with an intense band that was visible at 55 kDa in the concentrated samples (data not shown). Purified samples were determined to be >90% pure by scanning densitometry. There was no discernible difference in molecular mass between the chimeras and their respective parental enzymes.

Western blot analysis of all enzymes resulted in a single band at 55 kDa for the LPL chimeras and wild-type LPL (data not shown). Exchanging the 4- and/or 7-amino acid region in any of the chimeric lipases appears to have no effect on the molecular masses of the chimeras compared with the parental enzymes.

Specific Activity of Chimeras—The specific activity of all eight enzymes was determined in conditioned medium and following purification (Table III). With the exception of an ~8-fold difference between HL and HLLPLD, only modest differences were observed for specific activities among the chimeric enzymes measured in conditioned medium. By contrast, much greater differences were seen following purification. Although the specific activity of HL was 4.10 nmol/min/μg, the specific activity of the HL chimeras appeared to decrease as more amino acids were substituted. HL, and HLHL-(79-79) had specific activities of 0.78 and 0.32 nmol/min/μg, respectively, whereas the specific activity of HLLPL was 0.08 nmol/min/μg. The purified LPL chimeras followed a similar trend, where LPL specific activity was 5.81 nmol/min/μg, and LPLHL-(77-80), and LPLHL-(85-91) specific activities were 0.41 and 0.01 nmol/min/μg, respectively. The specific activity of LPLHLD was unable to be determined due to low protein concentration. A similar pattern of triglyceride hydrolytic activity for all enzymes was observed using the natural substrate very low density lipoprotein (data not shown). To determine whether the differences in specific activity were due to changes in enzyme catalytic activity or possible instability, we measured enzyme activity in medium over a 24-h time course. Initially, the HL chimeras had ~70% the activity of wild-type HL, but lost activity at a faster rate than wild-type HL, with only 8% activity remaining compared with 70% for wild-type HL (data not shown). This suggests that differences in specific activity following purification may be due to the potential instability of the chimeric HL structure. By contrast, the LPL chimeras had similar initial activities in comparison with wild-type LPL and were more stable than their chimeric HL counterparts, with a minimum of 40% activity remaining after 24 h (data not shown). These results suggest that changes in the catalytic potential of the LPL chimeras, in addition to decreased stability, may have caused the observed reductions in their specific activity. To more accurately determine the specific activity of LPL in the culture medium, an enzyme-linked immunosorbent assay method for LPL was applied (Table III). The specific activity of wild-type LPL was greatest at 35.64 nmol/min/μg. The LPL chimeras have specific activities as follows: LPLHL-(77-80) 7.31 nmol/min/μg; LPLHL-(85-91) 23.68 nmol/min/μg; and LPLHLD 5.99 nmol/min/μg.

Kinetic Analysis—Due to the decreased stability of the chimeric enzymes, kinetic analyses were performed using conditioned medium. The kinetic data and apparent Kₘ and V_max values are shown in Fig. 4 and Table IV, respectively. There was very little variation in the apparent Kₘ values for both the
parental and chimeric enzymes, with values ranging from 176 to 211 nM (Table IV). The low variability in enzyme affinity for substrate suggested a minor alteration of key structures involved in substrate binding, despite the substitution of residues in close proximity to catalytic residues and loop structures (38–41). By contrast, greater variability was observed in the apparent \( V_{\max} \) values. The greatest \( V_{\max} \) value was observed for HL at 14.91 nmol/min/mg, but decreased in the HL chimeras as amino acid substitutions were made. The \( V_{\max} \) values for HL and HL PL-(65–68) and HL PL-(73–79) were 6.03 and 13.40 nmol/min/mg, respectively. The HL PL LD chimera was associated with a substantial decrease in \( V_{\max} \), with a value of 1.81 nmol/min/mg.

As with wild-type HL, wild-type LPL had the largest \( V_{\max} \) of the LPL enzymes at 7.92 nmol/min/mg. Differences in the \( V_{\max} \) for the LPL chimeras were relatively modest, with LPL HL-(77–80) at 6.41 nmol/min/mg, LPL HL-(85–91) at 2.70 nmol/min/mg, and LPL HL LD at 5.14 nmol/min/mg.

**ApoC-II Activation of Chimeras**—To assess the lipolytic activity of the enzymes, lipase assays using synthetic triolein substrate were conducted. Lipase activity was measured under two separate conditions: low salt (0.15 M NaCl) with and without apoC-II (Table V). Although chimeras were lower in specific activity compared with wild-type enzymes, it was still possible to determine an effect by apoC-II. Whereas wild-type HL and HL PL-(65–68) were not activated by apoC-II, HL PL-(73–79) demonstrated a modest increase in activity of ~1.7-fold. However, HL PL LD was activated 5-fold in the presence of apoC-II. Under the same conditions, wild-type LPL had an ~11-fold increase in activation in the presence of apoC-II, and LPL HL-(77–80) was activated to nearly the same extent. However, LPL HL-(85–91) was activated only ~6-fold, about half the activation of its parental enzyme. Even more compelling data were obtained from LPL HL LD. In the presence of apoC-II, the -fold activation of LPL HL LD was reduced by two-thirds compared with wild-type LPL.

**Molecular Modeling**—To understand the relationship of the
identified residues with the catalytic triad of LPL, a three-dimensional molecular model was created (Fig. 5) based on the known structure of human pancreatic lipase (33). Secondary structure prediction indicated that residues 65–68 and 73–75 (7 of the 11 residues of the apoC-II activation domain) are contained in an α-helix structure (residues 64–75), whereas residues 76–79 were assigned to a random coil. The helix is equivalent to the α-helix in the terminology of pancreatic lipase (37). Tertiary protein modeling places this α-helical region in close proximity to the catalytic pocket (Ser132, Asp156, and His241) and its associated loop structures such as the lid domain (residues 217–238) and the β6-loop (residues 54–63).

**DISCUSSION**

ApoC-II has been recognized as the principal activator of LPL for some time (17, 18), but the mechanism by which this activation takes place remains poorly understood. To gain further insight into this mechanism, we utilized cofactor cross-linking and the domain exchange approach to determine residues in the N-terminal domain of LPL that are responsible for the ability to be activated by apoC-II.

Our previous studies utilizing chimeric lipases placed one of the LPL apoC-II-binding sites within the first 312 amino acids (the N-terminal domain) (20, 38) and a second site in the last half of the C-terminal domain (residues 389–448) (22). From the expected sizes of LPL fragments generated by o-iodosobenzoic acid cleavage (Table III), it is clear that the second half of the N-terminal domain and the first half of the C-terminal domain are contained in peptide 5, with a molecular mass of ~30 kDa. Labeling was not seen in that sized fragment, thereby excluding those regions in cofactor interaction. The labeled peptide observed in Fig. 1 has a molecular mass consistent with that of peptide 3 (residues 65–86). The identity of the labeled peptide as residues 65–86 was confirmed by sequencing analysis, which identified an N-terminal valine residue; and only peptide 3 begins with that amino acid. Additional labeled peptides were not detected, but other sites of cofactor interaction cannot be ruled out. In fact, it is expected that apoC-II does interact with elements of the C-terminal domain based on previous studies on chimeras (22); but for technical reasons, these peptides may not have been resolved under the current experimental conditions. For example, peptides 6–8 are too small to distinguish from the dye front; thus, it cannot be excluded that apoC-II also interacts with these regions of the molecule. Peptide 3 (residues 65–86) occupies a highly conserved region of LPL (3). Its amino acid sequence is identical in six species, except for a single substitution of aspartate for alanine in chicken LPL. More importantly, comparison of this region between LPL and the highly related enzyme HL revealed significant differences. Because HL activity is not stimulated by apoC-II, the longest contiguous stretch of amino acids in this region of the molecule that differed between the

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**TABLE IV**

Apparent kinetic constants of wild-type HL and LPL and their chimeric lipases

|          | $K_m$ (nM) | $V_{max}$ (nmol/min) |
|----------|------------|---------------------|
| HL       | 176.0      | 14.91               |
| HL<sub>LPL-(65-68)</sub> | 204.3  | 6.03                |
| HL<sub>LPL-(73-79)</sub> | 181.0  | 13.40               |
| HL<sub>LPLD</sub> | 201.9  | 1.81                |
| LPL      | 211.7      | 7.92                |
| LPL<sub>HL-(77-90)</sub> | 196.8  | 6.41                |
| LPL<sub>HL-(85-91)</sub> | 203.5  | 2.70                |
| LPL<sub>LPLD</sub> | 185.0  | 5.14                |

**TABLE V**

Relative trioleinase activity of wild-type HL and LPL and their chimeric lipases

|          | $K_m$ (nM) | $V_{max}$ (nmol/min) |
|----------|------------|---------------------|
| HL       | 1.0        | 1.0 ± 0.1           |
| HL<sub>LPL-(65-68)</sub> | 1.0 | 1.1 ± 0.1         |
| HL<sub>LPL-(73-79)</sub> | 1.0 | 1.7 ± 1.1        |
| HL<sub>LPLD</sub> | 1.0 | 5.0 ± 1.6         |
| LPL      | 1.0        | 10.9 ± 2.2         |
| LPL<sub>HL-(77-90)</sub> | 1.0 | 10.1 ± 2.3       |
| LPL<sub>HL-(85-91)</sub> | 1.0 | 5.7 ± 3.2        |
| LPL<sub>LPLD</sub> | 1.0 | 3.6 ± 0.2         |
two enzymes suggested specific candidate residues participating in LPL-cotfactor interaction. A 4-amino acid (LPL residues 65–68) and 7-amino acid (LPL residues 73–79) region of dissimilarity in sequence homology was identified, and a series of HL and LPL chimeras were constructed in which these regions were exchanged either alone (HL\textsubscript{LPL}(65–68), HL\textsubscript{LPL}(73–79), LPL\textsubscript{HL}(77–80), and LPL\textsubscript{HL}(85–91)) or together (HL\textsubscript{LPLL} and LPL\textsubscript{HL}). The first set of chimeras consisted of an HL backbone with the LPL candidate segments replacing the corresponding HL sequences, whereas the second set of chimeras consisted of an LPL backbone with the LPL candidate segments replaced with HL sequences.

Comparison of the specific activity measurements of the enzymes indicated that the substitution of analogous residues into either lipase resulted in decreased activities (Table III). The lowest activity among the LPL chimeras was associated with the simultaneous substitution of both LPL candidate amino acid regions (−6-fold difference compared with wild-type LPL). A similar result was observed for the HL chimeras. This effect was amplified following purification as result of an inherent decreased stability associated with the chimeric enzymes, particularly apparent in the HL chimeras. Despite the reduced specific activities of the chimeras following purification, it was reasoned that apoC-II responsiveness of the remaining active species could be an accurate measure of the part played by the substituted residues. Kinetic analyses of these lipases (Table IV) indicated very little change in $k_{\text{cat}}$, consistent with previous findings indicating that the primary effect of apoC-II is on the $V_{\text{max}}$ of the reaction (39). The greatest difference in $V_{\text{max}}$ values was observed between wild-type HL and HL\textsubscript{LPL} (−8-fold), suggesting that the presence of LPL residues in this chimera impaired catalysis compared with wild-type HL. However, the presence of both LPL candidate regions in HL resulted in a reduction in lipolysis, which was not due to altered affinity of the enzyme for the substrate, but to other factors such as stability and the inability to bind substrate productively. Significantly, the level of remaining activity was stimulated 5-fold by apoC-II, the first report of the transference to HL of LPL cofactor-dependent lipolysis.

Measurement of the enzyme activity of the chimeras in the presence and absence of apoC-II indicated that LPL residues 65–68 alone were not able to confer apoC-II reactivity to HL (Table V). Similarly, the exchange of these same residues for corresponding HL residues in LPL did not appreciably change the -fold activation in comparison with wild-type LPL. However, the presence of both LPL candidate regions in HL resulted in a reduction in lipolysis, which was not due to altered affinity of the enzyme for the substrate, but to other factors such as stability and the inability to bind substrate productively. Significantly, the level of remaining activity was stimulated 5-fold by apoC-II, the first report of the transference to HL of LPL cofactor-dependent lipolysis.

Our previous study indicating that, in addition to a region in the N-terminal domain, an apoC-II-responsive region also exists in the C-terminal domain of LPL (22).

Evidence has been provided that apoC-II and LPL participate in a protein-protein interaction that involves two molecules of apoC-II for each LPL dimer (40). The portion of apoC-II thought to activate LPL hydrolysis was initially localized to the C-terminal third of the sequence (41). Furthermore, apoC-II peptide inhibition studies have identified the 4 terminal amino acids (residues 76–79) as important for the initial binding of apoC-II to LPL, but not directly for activation (42). More specifically, site-directed mutagenesis studies have implicated Tyr\textsuperscript{63} in apoC-II as a key residue in the activation mechanism, but no single amino acid appears to be essential for activation (43, 44). NMR structural studies of apoC-II have described a number of helical domains thought to associate with lipid (36, 45). Two of these domains in apoC-II (residues 50–58 and 66–75) are located in the C-terminal region, and the latter has been suggested to represent one of the major lipid-binding domains (36). It has been suggested that this helix, together with a helix located in the N-terminal domain (14), may anchor apoC-II to the lipoprotein surface, whereas the interhelical region formed by residues 59–65 may represent the primary activator domain of apoC-II (36).

The LPL model presented in this study (Fig. 5) indicates that residues 65–68 and 73–79 are found in close proximity to the catalytic pocket (Ser\textsuperscript{132}, Asp\textsuperscript{156}, and His\textsuperscript{241}) and both loop domains (residues 54–63 and 217–238). In addition, secondary structure prediction indicates that residues 64–75 constitute an α-helical domain that may enable this region to interact with lipid moieties. However, because models of HL also predict α-helix structure in this region, secondary structure alone cannot explain apoC-II activation. Consequently, helical wheel diagrams of this region for LPL and HL were compared (Fig. 6), and differences in the number of charged residues and amphipathicity are readily apparent. The specified LPL region contains Lys\textsuperscript{67}, Lys\textsuperscript{74}, Arg\textsuperscript{75}, Glu\textsuperscript{76}, and Asp\textsuperscript{78}, whereas the corresponding HL region contains only a single Lys at position 85. The charged and hydrophobic residues in an LPL helix are arranged in a highly amphipathic manner, whereas the corresponding HL region lacks that character. As a result, we suggest that electrostatic interactions may contribute to the interaction of apoC-II with LPL, permitting substrate access to the active site. We speculate that, in LPL, this helix (residues 64–75) either directly or indirectly prevents substrate access to the active site in the absence of apoC-II. Furthermore, because HL has obvious compositional differences at this site, this helix in HL may not normally interfere with substrate access, thus

\[ a \text{ J. S. Hill, unpublished data.} \]
Identification of a Lipoprotein Lipase Cofactor-binding Site

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