# Substrate specificity of lipoprotein lipase and endothelial lipase: studies of lid chimeras

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**Abstract** **The triglyceride (TG) lipase gene subfamily, consisting of LPL, HL, and endothelial lipase (EL), plays a central role in plasma lipoprotein metabolism. Compared with LPL and HL, EL is relatively more active as a phospholipase than as a TG lipase. The amino acid loop or "lid" covering the catalytic site has been implicated as the basis for the difference in substrate specificity between HL and LPL. To determine the role of the lid in the substrate specificity of EL, we studied EL in comparison with LPL by mutating specific residues of the EL lid and exchanging their lids. Mutation studies showed that amphipathic properties of the lid contribute to substrate specificity. Exchanging lids between LPL and EL only partially shifted the substrate specificity of the enzymes. Studies of a double chimera possessing both the lid and the C-terminal domain (C-domain) of EL in the LPL backbone showed that the role of the lid in determining substrate specificity does not depend on the nature of the C-domain of the lipase. Using a kinetic assay, we showed an additive effect of the EL lid on the apparent affinity for $HDL_3$ in the presence of the EL C-domain.—** Griffon, N., E. C. Budreck, C. J. Long, U. C. Broedl, D. H. L. Marchadier, J. M. Glick, and D. J. Rader. **Substrate specificity of lipoprotein lipase and endothelial lipase: studies of lid chimeras.** ***J. Lipid Res.*** **2006.** 47: **1803–1811.**

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Endothelial lipase (EL) is a member of the triglyceride (TG) lipase gene family (1) that is synthesized by endothelial cells and by other cell types such as macrophages and hepatocytes (1–3). EL has a distinct substrate specificity profile and is relatively more active as a phospholipase than as a TG lipase (4). Thus, the ratio of TG lipase to phospholipase activity of EL is markedly less than those of LPL and HL. Relative to phospholipase activity, LPL displays the highest TG lipase activity and EL the lowest TG lipase activity of the three enzymes. EL also has greater preference for HDL, and LPL has the greater preference for VLDL (4, 5). Thus, LPL, HL, and EL seem to have evolved with distinct substrate specificities to accommodate the full spectrum of circulating lipoproteins.

The lid covering the active site has been implicated as a major source of substrate specificity for LPL and HL. Dugi et al. (6) studied the hypothesis that the surface lid covering the catalytic pocket may modulate access of the substrate to the active site of LPL. Characterization of a number of mutants with altered amphipathic properties of the LPL lid showed that the disruption of the lid decreased its ability to hydrolyze an emulsified lipid substrate without affecting the ability to hydrolyze a water-soluble substrate. They proposed that the interaction between the lipoprotein substrates and the lid may in part determine substrate specificity. Chimeric lipases were also generated by exchanging the lid region between LPL and HL (7). The lid of LPL conferred preferential TG hydrolysis, as opposed to augmenting phospholipase activity in the case of the lid of HL. Preferential in vivo hydrolysis of phospholipids (PLs) was demonstrated in HL-deficient mice injected with adenovirus-expressing lipases containing the HL lid (HL or LPL with the lid of HL) compared with lipases containing the LPL lid (LPL or HL with the lid of LPL) (8). These studies identified the lid as a major structural motif responsible for conferring different lipid substrate specificities of LPL and HL, a function that may modulate the distinct physiological roles of these two similar lipolytic enzymes in lipoprotein metabolism (8).

The LPL and HL lids are each 22 amino acids long, whereas the EL lid is only 19 amino acids long. Furthermore, the lid region of EL differs from the lids of LPL and HL not only in size but also in amino acid sequence and polarity. To determine the extent to which the lid plays a role in conferring substrate specificity for EL, we studied EL in comparison with LPL, its polar opposite with respect to the lipase activity spectrum. We created chimeric mole-

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Abbreviations: $appK_m$, apparent affinity of the enzyme for the phospholipid present in $HDL_3$ as substrate; C-domain, C-terminal domain; EL, endothelial lipase; PL, phospholipid; TG, triglyceride; TG/PL ratio, ratio of triglyceride lipase activity to phospholipase activity.

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cules of LPL and EL with exchanged lid regions as well as single mutants of the EL lid region. Based on the potential role of the C-terminal domain (C-domain) of lipases in binding substrate (5), we also addressed whether the C-domain of EL cooperates with the lid in determining substrate specificity by replacing both the C-domain and lid of LPL with the C-domain and lid of EL. Lipolytic activities of chimeras and mutants were determined in the presence of synthetic substrates and native lipoproteins.

## MATERIALS AND METHODS

### Construction of lipase mutants

*EL single-residue mutants of the lid region (EL-G241R, EL-245R, and EL-E250Q).* EL lid mutants include the glycine-241→arginine (EL-G241R) and glutamate-250→glutamine (EL-E250Q) substitutions as well as an inserted arginine at position 245 (EL-245R) to mimic the LPL lid sequence (**Fig. 1**). Sequences of primers used for these site-directed mutagenesis procedures were 5′-GT GGA CTC AAC GAT GTC TTG **CGA** TCA ATT GCA TAT GG-3′ for EL-G241R, 5′-GCA TAT GGA ACA ATC ACA **CAG** GTG GTA AAA TGT GAG C-3′ for EL-E250Q, and 5′-G GGA TCA ATT GCA TAT **CGC** GGA ACA ATC ACA GAG GTG G-3′ for EL-245R. All PCRs, including those for the single-amino acid changes for the lid mutants, were performed according to the Quikchange Site-Directed Mutagenesis Kit Protocol (Stratagene). All of the PCR products were sequenced to confirm accuracy.

*EL and LPL lid chimeras (EL/LPL-lid and LPL/EL-lid).* The EL/LPL-lid chimera consists of wild-type human EL backbone with the LPL lid, and the LPL/EL-lid chimera consists of wild-type human LPL backbone with the EL lid (**Fig. 2**). We engineered the exchange of the lid sequence between EL and LPL by overlap extension PCR (9). The primers were designed so that the original base pair sequence was conserved as much as possible. The primers included the sequences for the respective lid sequences flanked on each side by ~20 bp. The sequences of these antisense primers were as follows: 5′-GCT CGC TCA TGC TCA CAT TTT ACC AGC TGG TCG ACG TCT CCA AGT CCT CTC TCT GCA ATC ACG CGG ATA GCT TCT CCA ATG TTA CAG CCT GGC TGG AAG TCA CC-3′ and 5′-GCG CTC GTG GGA GCA CTT CAC CAC CTC TGT GAT TGT TCC ATA TGC AAT TGA TCC CAA GAC ATC GTT GAG TCC ACA TCC TGG CTG AAA AGT ACC

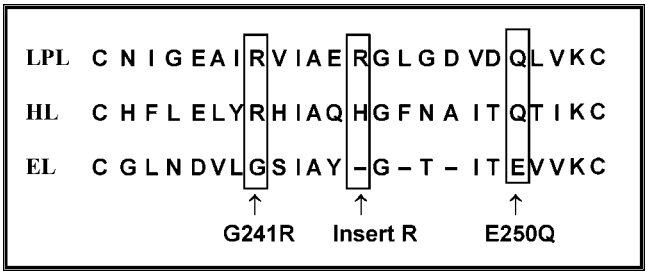


**Fig. 1.** Sequence alignment of the amino acid residues of the lid regions of LPL, HL, and endothelial lipase (EL). This sequence alignment shows that the lid region of EL differs from both HL and LPL lid sequences. Three amino acid residues of the lid of EL that differ from both HL and LPL were mutated to the corresponding residue in LPL. These single-residue mutations are indicated by arrows on the sequence of the EL lid region.

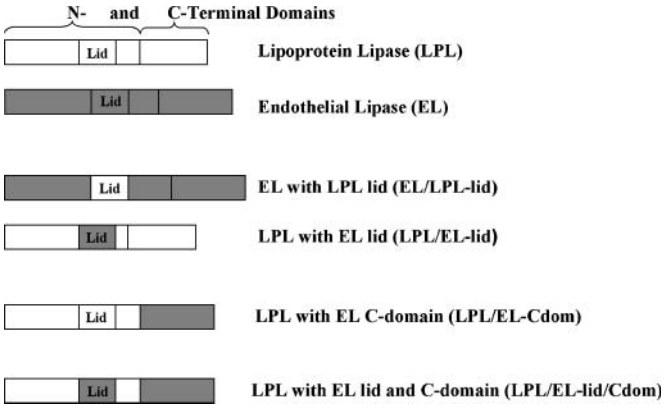


**Fig. 2.** Schematic diagrams of wild-type EL, wild-type LPL, the lid chimeras, and LPL/EL-Cdom and LPL/EL-lid/Cdom chimeras.

TCC-3′. They were used to introduce the LPL lid into the EL backbone and the EL lid into the LPL backbone, respectively. Briefly, the antisense lid primers were used with LPL or EL sense primers for the first PCR. The purified product from the first PCR was then used as a primer in the second PCR. To compare the level of expression of the chimeric and wild-type lipases, we constructed C-terminal myc-His-tagged proteins by inserting the full-length cDNA of each construct and the wild type into the pcDNA3.1/myc-His(−) plasmid expression vector (Invitrogen). Constructs were sequenced to confirm accuracy.

*LPL C-domain chimera (LPL/EL-Cdom) and LPL lid and C-domain double chimera (LPL/EL-lid/Cdom).* The chimeric LPL/EL-Cdom lipase was synthesized by overlap extension polymerase chain reaction as described by Broedl et al. (5). The double chimera (LPL/EL-lid/Cdom) consists of wild-type human LPL backbone with the EL lid and C-domain (Fig. 2). This chimera was generated by double digestion of LPL/EL-Cdom and LPL/EL-lid chimeras with the restriction enzymes *Xba*I and *Eco*47III (all restriction enzymes were purchased from New England Biolabs, Inc.). After digestion of LPL/EL-Cdom, the DNA fragment containing the pcDNA3.1(−) plasmid and the 3′ end of LPL/EL-Cdom just downstream of the lid coding region was purified by gel extraction. Meanwhile, similar digestion was run on the LPL/EL-lid chimera. In this case, the 5′ DNA fragment including the chimeric lid region was purified by gel extraction. These two digested products were then ligated using T4 ligase and a Rapid Ligation Kit (Roche).

### Cell culture and transfections

HEK 293 cells were maintained in DMEM, 10% fetal bovine serum, and 1× antibiotic-antimycotic (Gibco). The cells were transfected in triplicate using Lipofectamine™ reagent (Invitrogen). Twenty-four hours after transfection, the medium was replaced with DMEM containing 10 U/ml heparin (Sigma) and incubated for another 24 h. Heparin was used to release the recombinant lipases that are bound to the cell surface via proteoglycans. Thirty minutes before harvesting this conditioned medium, more heparin was added to the medium, bringing the final concentration of heparin to 20 U/ml. Conditioned media were collected, clarified by low-speed centrifugation, and frozen in aliquots at −80°C.

### Western blotting

Ten microliters of conditioned media was resolved by 10% Bis-Tris SDS-PAGE (Invitrogen) and transferred to Hybond ECL nitrocellu-

lose membranes (Amersham Pharmacia Biotech). Proteins were detected using a monoclonal mouse anti-myc primary antibody (clone 9E10) and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.).

The mass of the chimeras (such as LPL/EL-Cdom and LPL/EL-lid/Cdom) could not be accurately measured with the available LPL and EL ELISAs. Therefore, the level of protein expression of these chimeras was estimated by Western blot.

### TG lipase and phospholipase assays

Emulsions of triolein or dipalmitoylphosphatidylcholine were used to measure TG lipase or phospholipase activity, respectively (4, 10, 11). For the TG lipase assay, the emulsion contained triolein and egg phosphatidylcholine containing glyceryl-tri[9,10(n)-$^{3}$H]oleate stabilized with glycerol. For the phospholipase assay, a similar glycerol-stabilized emulsion was used that contained radiolabeled PLs (dipalmitoylphosphatidylcholine) and cholesteryl oleate as the neutral lipid core. Conditioned medium containing the recombinant proteins was used as an enzyme source. Medium obtained from cells expressing green fluorescent protein was included in all experiments as a negative control, and the resulting background activity was subtracted. Two experimental conditions were necessary, one in the presence of serum (1.33%) as a source of apolipoprotein C-II for LPL activation, and one in the absence of serum as an inhibitory effect of serum on EL activity, which has already been reported (4). Samples were incubated for 15 min at 37°C. All enzyme activities are reported as nanomoles of FFA liberated per hour per milliliter of conditioned medium as a source of enzyme.

The ratio of triglyceride lipase activity to phospholipase activity (TG/PL ratio) reflects the substrate specificity of these lipases. To determine whether the mean of the TG/PL ratio of one mutant and the mean of the TG/PL ratio of the wild-type parental lipase (either EL or LPL, depending on the mutation) were statistically different, statistical analysis were performed using an unpaired *t*-test. The mean values were considered significantly different at $P < 0.05$. The values of the TG/PL ratio were measured across experiments run in triplicate on the conditioned media of independent transfections. The sample size (number of determinations) was at least six or greater.

### Lipid hydrolysis of native lipoproteins

In this assay, two types of lipoprotein particles were used as lipase substrates, VLDL and $HDL_3$. These human lipoproteins were isolated from pooled plasma samples (4) and incubated with medium containing lipases (4, 12). Each reaction tube contained 1.25 mM lipoprotein PLs, 40 or 65.3 μl of conditioned medium containing one of the various enzymes, 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 8 mM $CaCl_2$, and 1% BSA in a final volume of 100 μl. Medium obtained from cells expressing green fluorescent protein was included in all experiments as a negative control, and the resulting background activity was subtracted. The reactions being linear with time for >4 h (data not shown), tubes were incubated for 4 h at 37°C. The released FFAs were measured enzymatically using a commercially available kit (NEFA C; Wako Pure Chemical Industries). Data are presented as nmol FFA produced/ml conditioned medium. The ratio of the enzymatic activity for VLDL to the enzymatic activity for $HDL_3$ reflects the relative preference of each lipase for these two physiologic substrates.

In the case of $HDL_3$, we were also able to optimize the assay to run this experiment varying the concentration of PL present in the reaction. These data were then used to determine the apparent affinity of the enzyme for the PL present in $HDL_3$ as substrate ($appK_m$). These parameters were obtained after fitting the experimental data to the following equation:

$$A = (A_{max} \times [S])/(appK_m + [S])$$

where $A_{max}$ is the maximal activity of the enzyme (in nmol FFA/ml conditioned medium as a source of lipases), [S] is the concentration of PL present in $HDL_3$ as substrate, and $appK_m$ is as defined above.

## RESULTS

### Effects of mutating the EL lid to be more like the LPL lid

The amphipathic properties of the two helices present in the lid have been reported to be important determinants of both LPL and HL substrate specificity (6). Therefore, we analyzed the sequence of the EL lid compared with the lid of LPL (Fig. 1). Three amino acid residues within the lid of EL that differ substantially from LPL were mutated to the corresponding residue in LPL. EL-G241R and EL-E250Q were generated by substitution of a single residue, and EL-245R was generated by inserting an arginine at position 245.

Each mutant (EL-G241R, EL-245R, and EL-E250Q) showed a small increase in the TG/PL ratio compared with wild-type EL (**Table 1**). Table 1 shows the results of one representative experiment. Pooling data collected at least in triplicate across four independent transfections, we

TABLE 1. TG lipase and phospholipase activities of EL and three lid mutants of EL

| Lipases | TG Lipase Activity | Phospholipase Activity | TG/PL Ratio | TG/PL Ratio | | |
|---|---|---|---|---|---|---|
| | | | | Mean ± SD | n | *P* |
| EL | 151 ± 21.9 | 176 ± 39.1 | 0.86 | 1.05 ± 0.19 | 9 | |
| EL-G241R | 84.3 ± 30.6 | 66.5 ± 11.4 | 1.27 | 1.52 ± 0.51[a] | 7 | 0.023 |
| EL-245R | 183 ± 16.5 | 132 ± 23.6 | 1.39 | 1.55 ± 0.33[a] | 9 | 0.001 |
| EL-E250Q | 95.3 ± 11.4 | 55.0 ± 16.6 | 1.73 | 1.90 ± 0.43[a] | 6 | 0.0002 |

EL, endothelial lipase; PL, phospholipid; TG, triglyceride; TG/PL, ratio of triglyceride lipase activity to phospholipase activity. The three lid mutants of EL studied are EL-G241R, EL-245R, and EL-E250Q. The TG lipase activity and phospholipase activity of each conditioned medium were measured in the absence of serum. All enzyme activities are expressed in nanomoles of product (FFA) formed per hour per milliliter of conditioned medium as a source of lipase and are reported are means ± SD. The TG/PL ratio data at left are results of one representative experiment. The TG/PL ratio data at right are means ± SD for EL and the three lid mutants of EL measured in a number (n) of different experiments. The significance of the difference of the mean value of the TG/PL ratio of wild-type EL versus each lid mutant of EL was determined using an unpaired *t*-test.

[a] Statistically significant at $P < 0.05$.

showed the difference of the TG/PL ratio of each mutant to be statistically significant compared with wild-type EL using an unpaired *t*-test. The mean of the TG/PL ratio of EL-G241R, EL-245R, and EL-E250Q increased 1.52-, 1.55-, and 1.90-fold, respectively. This increase of the TG/PL ratio of these mutants compared with wild-type EL was statistically significant in each case (Table 1). Each mutation had the effect of slightly increasing the TG preference of EL, suggesting that the polarity of those residues plays a role in determining the difference in substrate specificity between LPL and EL.

### Effect of exchanging the lid between LPL and EL on their substrate specificity

To determine the role of the lid covering the active site in conferring substrate specificity for EL, we studied EL in comparison with LPL, creating chimeric molecules of LPL and EL with exchanged lid regions (Fig. 2). Western blots of the conditioned media of transfected HEK 293 cells showed high-level expression of wild-type and chimeric lipases (**Fig. 3**). The TG/PL ratio for wild-type EL was 0.44 (**Table 2**). Placing the LPL lid in the EL backbone (EL/LPL-lid) resulted in an increase in the TG/PL ratio to 2.59, indicating a relative increase in substrate specificity for TG. In the presence of apolipoprotein C-II, the TG/PL ratio for wild-type LPL was 110 (Table 2). Placing the EL lid into the LPL backbone (LPL/EL-lid) resulted in a decrease of the TG/PL ratio to 32.

This trend was similar and statistically significant across experiments run on the conditioned media from 10 independent transfections (**Table 3**). The replacement of the EL lid by the LPL lid resulted in a substantial increase of 4.2-fold in the TG/PL ratio compared with wild-type EL but did not fully confer the high degree of preference of LPL for TG substrates (the mean of the TG/PL ratio of LPL across experiments being 83.8). The replacement of the LPL lid by the EL lid consistently generated a decrease in the TG/PL ratio to 32.7% of the ratio of wild-type LPL, indicating an increase of substrate specificity toward PL. However, the LPL/EL-lid chimera remained primarily a TG lipase like wild-type LPL, just as the EL/LPL-lid chimera did not become as active as a TG lipase as wild-type LPL.

We then asked whether the lid influenced the ability of EL to hydrolyze native lipoproteins (**Table 4**). Our results confirmed that wild-type LPL preferentially hydrolyzes the lipids of VLDL over HDL particles and that EL preferentially hydrolyzes the lipids of HDL particles over VLDL, as reported previously (4). When the LPL lid was placed into the EL backbone, there was little effect on the relative ability to hydrolyze $HDL_3$ versus VLDL, and this effect was determined to not be statistically significant (Table 4). Similarly, the ability to hydrolyze lipids of VLDL and $HDL_3$ was not affected with the LPL/EL-lid chimera, indicating that the lid alone is not a determinant of native lipoprotein substrate preference.

Using $HDL_3$ as a substrate, we further studied the kinetics by measuring the amount of FFA generated after incubation of the lipase with different concentrations of $HDL_3$ PLs. By fitting these experimental data, we estimated the apparent affinity of each of these enzymes ($appK_m$) for $HDL_3$ as substrate (**Table 5**). Wild-type EL showed an affinity for $HDL_3$ of 0.18 mM. The apparent affinity for $HDL_3$ of the EL/LPL-lid chimera remained similar to that of wild-type EL. Wild-type LPL showed an apparent affinity for $HDL_3$ lipids of 0.81 mM. This result demonstrates that the affinity of LPL for $HDL_3$ is substantially lower than the affinity of EL in vitro. In the case of the LPL/EL-lid chimera, the affinity for $HDL_3$ was similar to that of wild-type LPL, indicating that the lid alone is not a determinant of affinity for $HDL_3$.

### Effects of replacing the LPL lid with the EL lid and the LPL C-domain with the EL C-domain

We previously reported that the C terminus of EL is a major determinant of its ability to bind and to hydrolyze HDL (5). We hypothesized that there may be an interaction between the lid and the C terminus in determining substrate specificity and affinity. Therefore, we replaced both the C-domain and the lid of LPL with the C-domain

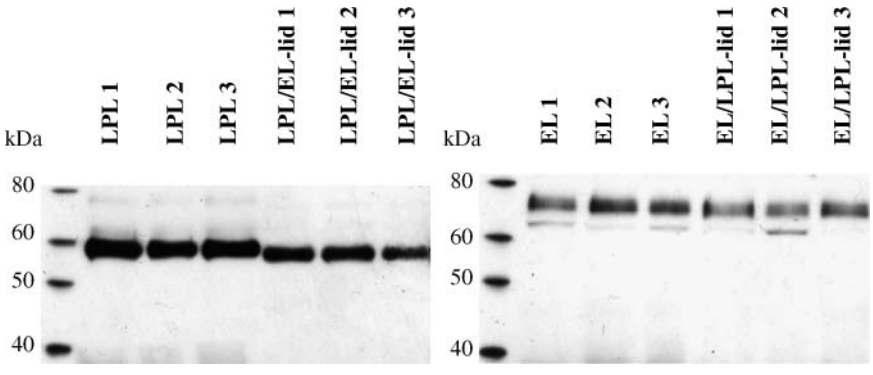


**Fig. 3.** Western blots of LPL, EL, and their lid chimeras. Shown are levels of protein expression in different HEK 293-conditioned media detected by Western blot using a mouse anti-myc monoclonal antibody and HRP-conjugated secondary antibody. The proteins expressed were LPL, LPL/EL-lid, EL, and EL/LPL-lid. The transfection experiment was done in triplicate, and the data shown are representative of the results obtained across independent transfections.

TABLE 2. TG lipase and phospholipase activities of LPL, EL, and their lid chimeras

| Lipases | No Serum | | | Serum | | |
|---|---|---|---|---|---|---|
| | TG Lipase Activity | Phospholipase Activity | TG/PL Ratio | TG Lipase Activity | Phospholipase Activity | TG/PL Ratio |
| EL | 114 ± 7.3 | 257 ± 14.2 | 0.44 | 45.4 ± 8.2 | 4.14 ± 3.2 | 11.0 |
| EL/LPL-lid | 47.1 ± 5.5 | 18.2 ± 2.5 | 2.59 | 14.1 ± 8.0 | 3.7 ± 6.8 | 3.8 |
| LPL | 1,030 ± 28.3 | 2.7 ± 0.7 | 381 | 4,364 ± 269 | 39.7 ± 3.9 | 110 |
| LPL/EL-lid | 346 ± 13.5 | 2.2 ± 1.2 | 157 | 1,940 ± 68.7 | 60.3 ± 3.8 | 32.2 |

The TG lipase activity and phospholipase activity of each conditioned medium were measured both in the presence and absence of serum. All enzyme activities are expressed in nanomoles of product (FFA) formed per hour per milliliter of conditioned medium as a source of lipase and are reported as means ± SD. The values shown are results of 1 representative experiment out of 10 independent experiments.

and the lid of EL in the LPL backbone (LPL/EL-lid/Cdom) (Fig. 2). The reverse double chimera (EL/LPL-lid/Cdom) was not generated because the C-domain chimera (EL/LPL-Cdom) was already shown to have very little activity in vitro (5). Western blots of the conditioned media of transfected HEK 293 cells showed high-level expression of LPL/EL-Cdom and LPL/EL-lid/Cdom (**Fig. 4**). The LPL/EL-Cdom chimera showed much greater TG lipase and phospholipase activities compared with wild-type LPL and a slightly increased preference for TGs, as we reported previously (5) (**Table 6**). The double chimera, LPL/EL-lid/Cdom, had altered substrate specificity. The TG/PL ratio was decreased compared with the LPL/EL-Cdom chimera (Table 6). These results indicate that the presence of the EL lid results in an increased relative preference of LPL for PL in the presence of the EL C-domain. The replacement of the LPL lid by the EL lid in the LPL/EL-Cdom chimera produced only an average of 62.3% of the TG/PL ratio of wild-type LPL and 33% of the ratio of LPL/EL-Cdom. Thus, insertion of the EL lid into either LPL or the LPL/EL-Cdom chimera had the same effect on substrate specificity, reducing the TG/PL ratio to ~33% of that of the parental lipase. These data demonstrate the influence of the EL lid in partially determining the lipid substrate specificity of LPL regardless of the nature of the C terminus.

We then asked whether the EL lid and C-domain influence the ability of LPL to hydrolyze native lipoproteins (**Tables 7, 8**). The C-domain chimera, LPL/EL-Cdom, showed an app$K_m$ of ~0.48 mM (Table 8), a value that is intermediate between those of wild-type LPL and wild-type EL. The replacement of the LPL lid by the EL lid in LPL/EL-Cdom did not drastically modify the preference for VLDL versus $HDL_3$ compared with LPL/EL-Cdom. In addition, the LPL/EL-lid/Cdom showed an app$K_m$ for $HDL_3$ of ~0.16 mM, which is similar to that of wild-type EL (Table 8).

TABLE 3. TG/PL ratios for LPL, EL, and their lid chimeras

| Lipases | TG/PL Ratio | | |
|---|---|---|---|
| | Mean ± SD | n | *P* |
| EL | 0.60 ± 0.38 | 14 | |
| EL/LPL-lid | 2.52 ± 1.52[a] | 14 | 0.0001 |
| LPL | 83.8 ± 40.4 | 22 | |
| LPL/EL-lid | 27.4 ± 13.4[a] | 23 | <0.0001 |

The values shown are means ± SD for LPL, EL, and their lid chimeras (EL/LPL-lid and LPL/EL-lid) measured across a number (n) of experiments. The significance of the difference of the mean value of the TG/PL ratio of each lid chimera compared with its parental lipase (EL or LPL) was determined using an unpaired *t*-test.

[a] Statistically significant at $P < 0.05$.

## DISCUSSION

The ratio of TG lipase activity to phospholipase activity for each lipase reflects its lipid substrate specificity. In the presence of synthetic substrate emulsions, LPL was found to be ~80-fold more active as a TG lipase than phospholipase, whereas the TG lipase and phospholipase activities of EL were almost similar. The lid region of EL differs from the lids of LPL and HL not only in size but also in amino acid sequence and polarity (Fig. 1). Insertion of the LPL lid into the EL backbone resulted on average in a 4.2-fold increase of the TG/PL ratio. The insertion of the EL lid into the LPL backbone decreased the TG/PL ratio to ~33% of that of the parental wild-type LPL. In agreement with previous work done on HL and LPL, we found that the lids altered the substrate specificity (6, 7). However, the alteration was only partial and not a complete conversion of the substrate specificity of EL to the substrate specificity of LPL or vice versa.

Therefore, the lid of EL is not the sole determinant of lipid substrate specificity. This result is in contrast to what Dugi et al. (6, 7) observed in their work on HL and LPL. Although the lid of HL and the lid of LPL are more homologous than the lid of EL and LPL, the lid exchange between HL and LPL was reported to greatly change the substrate specificity of the lid chimeras, whereas we report a much more moderate effect in the case of EL and LPL. Dugi, Dichek, and Santamarina-Fojo (7) concluded that the lid and the hydrophobicity of the lid affect the enzymatic activity. The substrate specificity was reported to be determined by the lid, because the phospholipase to TG lipase ratio of the LPL/HL-lid was more similar to HL than LPL and the ratio of the HL/LPL-lid was more similar to LPL than HL. Although very similar, these ratios were not identical to those of wild-type HL or wild-type LPL, probably indicating that even in the case of HL and LPL, the lid might be the main determinant but not the sole determinant.

Until further investigation, we cannot determine the reason for the difference between the large effect of the lid

TABLE 4. Lipolysis of lipoprotein particles by the lid chimeras

| Lipases | VLDL Lipolytic Activity | $HDL_3$ Lipolytic Activity | VLDL/$HDL_3$ Ratio | VLDL/$HDL_3$ Ratio | | |
|---|---|---|---|---|---|---|
| | | | | Mean ± SD | n | *P* |
| EL | 46.1 ± 7.7 | 55.4 ± 7.8 | 0.83 | 1.05 ± 1.36 | 8 | |
| EL/LPL-lid | 41.0 ± 21.3 | 20.6 ± 4.4 | 1.99 | 2.08 ± 1.35 | 5 | 0.209 (NS) |
| LPL | 12,916 ± 1,868 | 92.2 ± 3.7 | 140 | 104 ± 47.4 | 11 | |
| LPL/EL-lid | 5,240 ± 444 | 32.7 ± 7.4 | 141 | 129 ± 58.5 | 9 | 0.315 (NS) |

Lipase activities of conditioned media containing LPL, EL, or the lid chimeras (EL/LPL-lid and LPL/EL-lid) using isolated lipoprotein fractions (VLDL and $HDL_3$) as substrates were measured as the amount of free fatty acid released after incubation at 37°C for 4 h. The lipolytic activities are expressed in nanomoles of product (FFA) formed per milliliter of conditioned medium as a source of lipase and are reported as means ± SD. The VLDL/$HDL_3$ ratio data at left are results of one representative experiment. The VLDL/$HDL_3$ ratio data at right are means ± SD for LPL, EL, and their lid chimeras (EL/LPL-lid and LPL/EL-lid) measured across a number (n) of experiments. The significance of the difference of the mean value of the VLDL/$HDL_3$ ratio of each lid chimera compared with its parental lipase (EL, LPL, or LPL/EL-Cdom) was determined using an unpaired *t*-test. Two means were determined to be statistically not significant (NS) at $P > 0.05$.

reported for HL and LPL and the partial effect reported in this study. One possibility is to take into consideration the fact that the difference between the TG/PL ratio of wild-type LPL and HL was reported to be only 7-fold (7), whereas the difference was much higher in the case of LPL and EL. Here, we report LPL to have a TG/PL ratio almost 140-fold greater than EL (Table 3). Consequently, it is likely that an effect on substrate specificity could seem almost totally determined by the lid when the difference of substrate specificity is only 7-fold. Meanwhile, when two enzymes (EL and LPL, for instance) have a much greater difference in substrate specificity (140-fold), it appears more clearly that the lid indeed contributes significantly to determine the substrate specificity but is not the sole determinant involved.

Other potential reasons for this difference are the following. *1*) In the case of EL, the role of the lid may be less important than in the case of HL in determining the lipid and lipoprotein substrate specificity of the enzyme. *2*) The role of the lid in the lid replacement experiment could be partially lost as a result of a disruption of the lid-protein interaction. *3*) The interaction of the replaced lid with the substrate might still occur, but other interactions of the substrate with residues in the vicinity of the active site pocket could be disrupted. In any case, our studies clearly indicate that structural determinants other than the lid play a role in the lipid substrate preference differences between LPL and EL.

TABLE 5. Values of the maximal activity and apparent affinity parameters measured for EL, LPL, and the lid chimeras

| Lipases | $appK_m$ |
|---|---|
| | *mM* |
| EL | 0.179 ± 0.02 |
| EL/LPL-lid | 0.216 ± 0.17 |
| LPL | 0.811 ± 0.29 |
| LPL/EL-lid | 0.746 ± 0.59 |

$appK_m$, apparent affinity of the enzyme for the PL present in $HDL_3$ as substrate. The enzymatic activity of the lipases was estimated by measuring the amount of FFA formed after incubation of the conditioned medium as a source of lipases with different concentrations of $HDL_3$. The values shown are means ± SD.

We generated three mutants of the lid region in EL (G241R, 245R, and E250Q) to make the EL lid more similar to LPL. Each mutation had a moderate but significant effect on the substrate specificity, inducing an increase of the TG/PL ratio of 1.5- to 1.9-fold. Considering that the effect of complete replacement of the EL lid by that of LPL in an EL backbone induced an ~4.2-fold increase in substrate specificity, the fact that these single mutations induced an almost 2-fold increase of the substrate specificity shows that the amphipathic properties of the lid are an important factor responsible for the role of the lid in determining the substrate specificity of EL. These results are in agreement with experiments in which altering the amphipathic properties of the lid of LPL and HL decreased the ability of these mutants to hydrolyze emulsified TG lipid substrate without affecting the ability to catalyze the hydrolysis of water-soluble substrate (6).

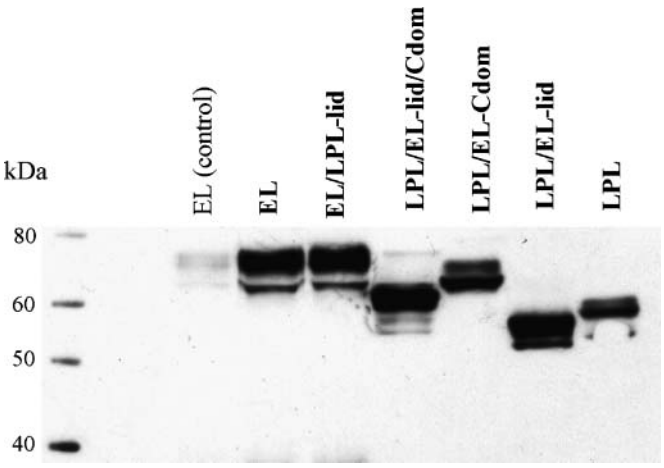


**Fig. 4.** Western blots of LPL, EL, and their chimeras. Shown are levels of protein expression in different HEK 293-conditioned media detected by Western blot using a mouse anti-myc monoclonal antibody and HRP-conjugated secondary antibody. EL from control medium was loaded on the gel as a positive control. The proteins expressed were EL, EL/LPL-lid, LPL/EL-lid/Cdom, LPL/EL-Cdom, LPL/EL-lid, and LPL. The results of this transfection experiment are representative of the results obtained across independent transfections.

TABLE 6. TG lipase and phospholipase activities of LPL, EL, and their chimeras

| Lipases | TG Lipase Activity | Phospholipase Activity | TG/PL Ratio | TG/PL Ratio | | |
|---|---|---|---|---|---|---|
| | | | | Mean ± SD | n | *P* |
| EL | 28.2 ± 2.5 | 45.9 ± 4.8 | 0.61 | 0.60 ± 0.38 | 14 | |
| EL/LPL-lid | 9.1 ± 0.2 | 6.7 ± 1.3 | 1.36 | 2.52 ± 1.52[a] | 14 | 0.0001 |
| LPL | 4,535 ± 897 | 88.6 ± 11.5 | 51.2 | 83.8 ± 40.4 | 22 | |
| LPL/EL-lid | 1,974 ± 179 | 102 ± 14.9 | 19.3 | 27.4 ± 13.4[a] | 23 | <0.0001 |
| LPL/EL-Cdom | 13,920 ± 2,600 | 161 ± 4.4 | 86.5 | 158 ± 79.3 | 6 | |
| LPL/EL-lid/Cdom | 2,626 ± 177 | 111 ± 12.9 | 23.7 | 52.2 ± 28.4[a] | 6 | 0.011 |

The values reported are means ± SD for LPL, EL, and their lid chimeras (EL/LPL-lid, LPL/EL-lid, LPL/EL-Cdom, and LPL/EL-lid/Cdom). The TG lipase activity and phospholipase activity of each conditioned medium were measured in the presence of serum for LPL and LPL chimeras (chimeras possessing LPL backbone) and in the absence of serum for EL and EL chimeras (chimeras possessing EL backbone). All enzyme activities are expressed in nanomoles of product (FFA) formed per hour per milliliter of conditioned medium as a source of lipase. The TG/PL ratio data at left are results of one representative experiment. The TG/PL ratio data at right are means ± SD for LPL, EL, and their chimeras and were measured across a number (n) of experiments. The significance of the difference of the mean value of the TG/PL ratio of each lid chimera compared with its parental lipase (EL vs. EL/LPL-lid, LPL vs. LPL/EL-lid, or LPL/EL-Cdom vs. LPL/EL-lid/Cdom) was determined using an unpaired *t*-test.

[a] Statistically significant at $P < 0.05$.

We also measured the lipolytic activities of the lid chimera constructs in the presence of native lipoproteins. The ability to hydrolyze lipids in VLDL and $HDL_3$ by EL was modestly increased when we replaced the lid with that of LPL. In addition, the LPL lid alone had little effect on the affinity of EL for $HDL_3$. The ability of LPL to hydrolyze lipids in VLDL and $HDL_3$ was not affected by the presence of the EL lid. In addition, the EL lid had little effect on the affinity of LPL for $HDL_3$. Thus, the nature of the lid influences lipoprotein specificity much less than lipid (TG vs. PL) substrate specificity.

Although the exchange of lids resulted in changes in the enzymatic properties of LPL and EL, the conversion of the substrate specificity was not complete. Considering the possibility that EL might be active as a homodimer in a head-to-tail conformation as for HL and LPL (13–18), and to further investigate the reasons for an unexpected partial effect of the EL lid exchange in substrate specificity, we investigated the role of the C-domain on the substrate specificity determined by the lid. To date, only the three-dimensional structure of human pancreatic lipase has been resolved by X-ray diffraction (19). Based on amino acid homology, all other members of the TG lipase gene family, including LPL and EL, are assumed to present a similar three-dimensional structure composed of two domains. The N-terminal domain contains the catalytic site covered by the lid. This N-terminal domain is joined by a short spanning region to a smaller C-domain that reportedly not only has a role in heparin binding (20–22) but also is implicated in lipid binding (5, 23–26).

In the case of the LPL/EL-Cdom chimera, we observed a small but significant change of substrate specificity for synthetic substrates, as reported previously (5). The TG/PL ratio of this chimera increased almost 2-fold in comparison with wild-type LPL, which is in contrast to the observations of Davis et al. (23) with HL and LPL C-domain chimeras. Although the N-terminal domain determined the catalytic efficiency of two C-domain chimeras (LPL/HL and HL/LPL), they reported that the C-domain seemed to unexpectedly influence the substrate specificity of these chimeras, because the PL/TG ratio in both chimeric lipases was enhanced by the presence of heterologous C-domain (23).

We also showed that substituting the C-domain of LPL with the C-domain of EL converted LPL to an enzyme

TABLE 7. Lipolysis of lipoprotein particles by the lid and C-terminal domain chimera of LPL (LPL/EL-lid/Cdom)

| Lipases | VLDL Lipolytic Activity | $HDL_3$ Lipolytic Activity | VLDL/$HDL_3$ Ratio | VLDL/$HDL_3$ Ratio | | |
|---|---|---|---|---|---|---|
| | | | | Mean ± SD | n | *P* |
| LPL | 12,916 ± 1,868 | 92.2 ± 3.7 | 140 | 104 ± 47.4 | 11 | |
| LPL/EL-lid | 5,240 ± 444 | 32.7 ± 7.4 | 141 | 129 ± 58.5 | 9 | 0.315 (NS) |
| LPL/EL-Cdom | 46,880 ± 2,900 | 2,070 ± 107 | 22.7 | 21.0 ± 13.6 | 5 | |
| LPL/EL-lid/Cdom | 11,450 ± 595 | 315 ± 22 | 36.3 | 23.6 ± 14.5 | 5 | 0.778 (NS) |

Lipase activities of conditioned media containing LPL or the chimeric lipases using isolated lipoprotein fractions (VLDL and $HDL_3$) as substrates were measured as the amount of free fatty acid released after incubation at 37°C for 4 h. The lipolytic activities are expressed in nanomoles of product (FFA) formed per milliliter of conditioned medium as a source of lipase and are reported as means ± SD. The VLDL/$HDL_3$ ratio data at left are results of one representative experiment. The VLDL/$HDL_3$ ratio data at right are means ± SD for LPL and its chimeras (LPL/EL-lid, LPL/EL-Cdom, and LPL/EL-lid/Cdom) measured across a number (n) of experiments. The significance of the difference of the mean value of the VLDL/$HDL_3$ ratio of each lid chimera compared with its parental lipase (LPL vs. LPL/EL-lid or LPL/EL-Cdom vs. LPL/EL-lid/Cdom) was determined using an unpaired *t*-test. Two means were determined to be statistically not significant (NS) at $P > 0.05$.

TABLE 8. Values of the apparent affinity measured for the lid and C-terminal domain chimera of LPL (LPL/EL-lid/Cdom)

| Lipases | app$K_m$ |
|---|---|
| | *mM* |
| LPL | 0.81 ± 0.29 |
| LPL/EL-lid | 0.746 ± 0.59 |
| LPL/EL-Cdom | 0.48 ± 0.11 |
| LPL/EL-lid/Cdom | 0.16 ± 0.03 |

The enzymatic activity of the lipases was estimated by measuring the amount of FFA formed after incubation of the conditioned medium as a source of lipases with different concentrations of $HDL_3$. The values shown are means ± SD.

highly capable of binding and hydrolyzing HDL both in vitro and in vivo (5). Here, our kinetic experiments demonstrated that replacing the C-domain in LPL with that of EL resulted in a substantial increase in affinity for HDL. These data strongly indicate that the C-domain mediates the initial substrate binding step before lipid hydrolysis. These data are in agreement with previous reports of C-domain involvement in lipid binding (5, 23–29). Indeed, treatment with antibodies to LPL or HL indicated that the C-domain influences the hydrolysis of triolein (23, 24, 27). The lipase activity of a chimeric lipase composed of the N-terminal domain of HL and the C-domain of LPL (HL/LPL) was inhibited when treated with monoclonal antibodies to LPL, indicating that the C-domain is important for the hydrolysis of long-chain TGs (24). Inhibition studies using anti-HL antibodies were also performed on the reverse C-domain chimera composed of the N-terminal domain of LPL and the C-domain of HL (LPL/HL) (27). These studies demonstrated that the interaction with the HL-derived C-domain of the LPL/HL chimera abolished the ability of the enzyme to hydrolyze the emulsified substrate, triolein, but had no effect on tributyrin hydrolysis. Tributyrin was used to determine the esterase activity of lipases independent of lipid interaction, thereby reflecting the catalytic function of enzyme. Consequently, Dichek et al. (27) hypothesized that the C-domain of both HL and LPL could be involved in the initial interaction of the enzyme with its lipid substrates. Similar studies of domain exchange and antibody inhibition on human pancreatic lipase revealed that the interfacial stability of pancreatic lipase depends on the structure of the C-domain (28). Bezzine et al. (29) showed that a hydrophobic surface loop from the C-domain (β5′) may be involved in the interaction of human pancreatic lipase with the lipid/water interface.

The C-terminal region 415–438 of LPL was shown to play a role in the interaction of LPL with lipid substrates, as mutations in this region were shown to alter the enzyme lipolytic activity toward triolein or both triolein and tributyrin (26). Furthermore, a cluster of three tryptophan residues (Trp390, Trp393, and Trp394) on the LPL C-domain surface appeared to play a role in orienting the enzyme at the lipid/water interface (25). Mutations of this cluster of three tryptophan residues on the LPL C-domain surface were shown to decrease the lipolytic activity of these enzymes against water-insoluble triolein (25). The sequence alignment of the C-domain of EL and LPL shows that the tryptophan cluster present in LPL is only partially conserved in EL (only one tryptophan is still present) and therefore could be one of the regions in the C-domain that plays a role in determining the affinity and binding of these lipases to the different lipoprotein particles. Indeed, Lookene et al. (30) concluded that the C-domain appears important for tethering TG-rich lipoproteins to heparin-bound LPL. Nevertheless, they reported that the deletion of residues 390–393 (WSDW) only slightly decreased the affinity of LPL for lipoproteins (30). Furthermore, truncation of the C-domain of LPL abolished the binding of LPL to chylomicrons (31) and rabbit β-VLDL (32), indicating that the C-domain is important for lipid and lipoprotein binding.

When we replaced both the C-domain and the lid of LPL with the C-domain and the lid of EL, the LPL/EL-lid/Cdom double chimera showed altered substrate specificity compared with the LPL/EL-Cdom chimera. In the presence of synthetic substrate, the replacement of the LPL lid with the EL lid in LPL/EL-Cdom changed the TG/PL ratio to only 33% compared with LPL/EL-Cdom. The ratio decreased to a similar extent when replacing the LPL lid with the EL lid in either LPL or the LPL/EL-Cdom chimera. This result demonstrates that the preference for PL of these two chimeras (LPL/EL-lid and LPL/EL-lid/Cdom) was increased as a result of the presence of the lid of EL. Furthermore, the fact that placement of the EL lid into either LPL or LPL/EL-Cdom had the same effect on the substrate specificity indicates that the partial effect of the lid on the substrate specificity was not influenced by the interaction of the lid with the C-domain.

The ability to hydrolyze lipids in VLDL and $HDL_3$ was not significantly affected when we replaced the LPL lid with that of EL in LPL or LPL/EL-Cdom. Thus, although the lid influences lipid substrate preference, it has no effect on lipoprotein preference, which appears to be in large part determined by the C-domain. Indeed, the kinetic experiments using $HDL_3$ as substrate showed that the lid exchange between EL and LPL generated lid chimeras that have affinity for $HDL_3$ quite similar to their parental lipases. Thus, the lid alone has little effect on the affinity of these lipases for $HDL_3$. However, whereas the C-domain chimera (LPL/EL-Cdom) showed an affinity intermediate between LPL and EL for $HDL_3$, the double chimera (LPL/EL-lid/Cdom) showed an affinity for $HDL_3$ similar to that of wild-type EL. Consequently, in the presence of the C-domain of EL, the EL lid results in an additive effect on the enzyme affinity for $HDL_3$ as substrate.

In conclusion, these data demonstrate the influence of the lids of EL and LPL in only partially determining their substrate specificity for synthetic substrates. Thus, it appears that the lid covering the active site of EL is not the sole determinant of its preference for PL and that other regions of this enzyme also play an important role in substrate specificity. Mutations of key amino acid residues in the lid region of EL showed that the amphipathic properties of the two helices within the lid play a role and are responsible for the contribution of the lid in determining

substrate specificity. In addition, studies of a double chimera possessing both the lid and the C-domain of EL in the LPL backbone (LPL/EL-lid/Cdom) show that the role of the lid in determining the substrate specificity does not depend on the nature of the C-domain of the lipase. Moreover, in the presence of the EL C-domain, the EL lid plays a role in determining the apparent affinity of LPL for $HDL_3$. The mechanism of the lipolysis of lipids in $HDL_3$ by lipases most likely involves the C-domain in the initial binding to the lipoprotein particles, whereas the lid may contribute to the subsequent binding of the lipid substrate required before its hydrolysis. These studies advance our understanding of the structure-function properties of the lipase family and in particular of the ability of EL to bind to and hydrolyze HDL PLs as an important physiologic substrate.

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