Human Hepatic and Lipoprotein Lipase: The Loop Covering the Catalytic Site Mediates Lipase Substrate Specificity*

(Received for publication, January 20, 1995, and in revised form, August 8, 1995)

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Hepatic lipase (HL) and lipoprotein lipase (LPL) are key enzymes that mediate the hydrolysis of triglycerides (TG) and phospholipids (PL) present in circulating plasma lipoproteins. Relative to triacylglycerol hydrolysis, HL displays higher phospholipase activity than LPL. The structural basis for this difference in substrate specificity has not been definitively established. We recently demonstrated that the 22-amino acid loops ("lids") covering the catalytic sites of LPL and HL are critical for the interaction with lipid substrate (Dugi, K. A., Dichek, H. L., Talley, G. D., Brewer, H. B., J r., and Santamarina-Fojo, S. (1992) J. Biol. Chem. 267, 25086–25091). To determine whether the lid plays a role in conferring the different substrate specificities of HL and LPL, we have generated four chimeric lipases. Characterization of these chimeric enzymes using TG (triolein and tributyrin) or PL (dioleoylphosphatidylcholine (DOPC) vesicles, DOPC proteoliposomes, and DOPC-mixed liposomes) substrates demonstrated marked differences between their relative PL/TG hydrolyzing activities. Chimeric LPL containing the lid of HL had reduced triolein hydrolyzing activity (49% of the wild type), but increased phospholipase activity in DOPC vesicle, DOPC proteoliposome, and DOPC-mixed liposome assay systems (443, 628, and 327% of wild-type LPL, respectively). In contrast, chimeric HL containing the LPL lid was more active against triolein (123% of the wild type) and less active against DOPC (23, 0, and 30%, respectively) than normal HL. Similar results were obtained when the lipase lids were exchanged in chimeric enzymes containing the NH2-terminal end of LPL and the COOH-terminal domain of HL. Exchange of the LPL lid was more active against triolein (123% of the wild type) and less active against DOPC (23, 0, and 30%, respectively) than normal HL. Similar results were obtained when the lipase lids were exchanged in chimeric enzymes containing the NH2-terminal end of LPL and the COOH-terminal domain of HL. Exchange of the LPL lid was more active against triolein (123% of the wild type) and less active against DOPC (23, 0, and 30%, respectively) than normal HL. 

In summary, the lid covering the catalytic domains in LPL and HL plays a crucial role in determining lipase substrate specificity. The lid of LPL confers preferential triglyceride hydrolysis, whereas the lid of HL augments phospholipase activity. This study provides new insight into the structural basis for the observed in vivo differences in LPL and HL function.

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†† The abbreviations used are: HL, hepatic lipase; LPL, lipoprotein lipase; HDL, high density lipoprotein(s); DOPC, dioleoylphosphatidylcholine.
protein fractions, especially HDL (36). Similar changes in plasma phospholipid levels have been observed in some patients with HL deficiency (32), but not with LPL deficiency (37, 38). This difference in relative phospholipid to triacylglycerol hydrolyase activity of the two enzymes may be important for modulating the function of LPL and HL in vivo.

Recent studies involving the analysis of chimeric LPL-HL mutants (39-41) have suggested that the COOH-terminal domains of LPL and HL (Fig. 1) may play a role in determining the preferred substrate of the respective lipase (42). In addition, removal of the COOH-terminal 58 amino acids of LPL by chymotryptic cleavage resulted in the inability of LPL to bind to chylomicrons (43), suggesting that this region of the enzyme may mediate binding to lipoproteins. However, to date, the structural basis for the differences in substrate specificity between the two lipases remains poorly defined. We have recently demonstrated that the lid covering the catalytic domain of the human lipases (Fig. 1) is critically involved in the interaction of the lipases with their lipid substrate (42). In this report, we investigate the role of the lipase lid in determining the substrate specificity of HL and LPL for phospholipids and triglycerides by generating four different chimeric lipases in which the LPL and HL lids have been exchanged. Our studies demonstrate that the LPL lid enhances triglyceride hydrolysis, whereas the HL lid augments phospholipase function, thus establishing the important role of the lid in mediating lipase substrate specificity.

MATERIALS AND METHODS

cDNA Expression Vector—The parent plasmid (pcCMV) used for site-directed mutagenesis and transfection is a pUC18-derived vector containing the cytomegalovirus immediate early promoter and the polyadenylation site of SV40 as described previously (44). A 1473-base pair fragment of normal human LPL cDNA (pcCMV-NLPL) (45) or a 1522-base pair fragment of normal human HL cDNA (pcCMV-NHL) (46-48) was cloned into the XbaI and HpaI restriction sites of pcCMV. The DNA sequence of each fragment, which spanned the signal peptide through the termination codon, was confirmed by DNA sequence analysis using the deoxynucleotide chain termination method (49) and T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.).

Synthesis of Mutant cDNA—The mutant HL and LPL chDNAs were synthesized by the overlap extension polymerase chain reaction (50) using either pcCMV-NLPL or pcCMV-NHL as template. Polymerase chain reaction was performed in an automated DNA thermal cycler (Perkin-Elmer) as described (51) utilizing DNA primer from Pyrococcus furiosus (Stratagene Inc., La Jolla, CA) and 30 cycles with 1-min denaturation at 95°C, 1-min annealing at 50°C, and 2-min extension at 72°C in 1 × buffer 2 (Stratagene Inc.), 200 μM each dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), and 0.5 μM each primer. The exchange of the lids was performed with partially complementary oligonucleotide primers spanning the entire noncomplementary region of the 22-amino acid lid. Generation of chimeric lipases by domain exchange was performed as described previously (40). The mutant cDNAs were subcloned into the pcCMV expression vector and amplified using competent DH5α cells (Life Technologies Inc.). Clones carrying the mutant cDNA were grown overnight at 37°C in LB broth (Biofluidics, Inc., Rockville, MD), and DNA was isolated by one-tube minipreparation (52). All constructs were examined by sequence analysis of the complete cDNA insert. Oligonucleotide primers for overlap extension polymerase chain reaction and sequencing were synthesized by the phosphoramidite method on a DNA synthesizer (Model 380B, Applied Biosystems, Inc., Foster City, CA).

In Vitro Expression of cDNA in Human Embryonal Kidney 293 Cells—Plasmids used for transfection were purified by the cesium chloride double-banding method (53). Transfections were performed using the calcium phosphate coprecipitation method (54) by adding 40 μg of plasmid DNA to each 100-mm plate of subconfluent human embryonal kidney 293 cells (American Type Culture Collection, Rockville, MD). Twenty-four hours after addition of DNA, the cells were washed, and medium containing 10% (v/v) fetal calf serum and 2 units/ml heparin sodium (Eliks-Sinn, Cherry Hill, N. J.) was added. Medium for activity determination was harvested 12–16 h after washing and supplemented with glycerol to a final concentration of 30% (v/v). Intracellular protein was harvested as described by Chait et al. (55). Aliquots of media and intracellular extracts were kept at −70°C until lipid assays were performed. Each plasmid was transfected in triplicate. Wild-type HL and LPL were used as positive controls, and the pcCMV vector without insert was used as negative control.

Determination of HL and LPL Activities—Esterase activity was quantitated in triplicate using [14C]triolein (56), and triglyceride lipase activity was determined in triplicate using [14C]tributyrin (56), and triglyceride lipase activity was determined in triplicate using [14C]triolein as previously published (57). Phospholipase activities were measured in triplicate utilizing three different substrates. Phospholipid vesicles were generated by modifying the synthesis of triolein emulsion (57) as follows: Dioleoylphosphatidylcholine (DOPC) (1 mM; Sigma) was used instead of egg yolk extract. Labeled triolein was substituted with [14C]DOPC (Amersham Corp.) at an activity of 0.1 μCi/ml of substrate. Proteoliposomes were synthesized according to a previously published protocol (58) with the following modifications. DOPC was used as the unlabeled phospholipid, and the proteoliposomes were labeled with 1 μCi of [14C]DOPC. One-hundred microliters of substrate were added to 200 μl of medium from transfected cells in a total volume of 500 μl (0.15 M NaCl, 0.1 M Tris-HCI, pH 8.5, 2.5% bovine serum albumin, 25 μl of human plasma (source of apol-[II], 2 units/ml heparin). Samples were incubated at 37°C in a shaking water bath for 1-4 h, and oleic acid was extracted by the method of Belfrage and Vaughan (59). Mixed liposome substrate was prepared by a modification of a previously published protocol (60) using unlabeled DOPC, unlabeled triolein (Sigma), and 2 μCi of [14C]DOPC. Assay conditions used were the same as in the proteoliposome protocol.

Determination of LPL Mass—LPL mass was determined six times by an enzyme-linked immunosorbent assay using the 52D monoclonal antibody (kindly provided by Dr. J. D. Brunzel, University of Washington, Seattle) for capture and a chicken polyclonal antibody (kindly provided by Dr. I. J. Goldberg, Columbia University, New York) for measurement.

RESULTS

To evaluate a potential role of the lipase lid in modulating the substrate specificities of LPL and HL, we first established...
The importance of the lid region in the hydrolysis of different lipase substrates. We had previously demonstrated that the integrity of the amphipathic helices in the lipase lid is essential for the hydrolysis of water-insoluble long chain fatty acid triglycerides (42). In a similar manner, we now investigated the role of the amphipathic helices in the hydrolysis of water-insoluble phospholipid substrates. Table I summarizes the concentration as well as activity in the medium of cells transfected with wild-type LPL, wild-type HL, or lipase-lid mutant plasmids. Disruption of the amphipathicity of helix 1 or helix 2 of the lid or deletion of the helices destroyed the ability of the lipases to hydrolyze water-insoluble triglycerides, while the esterase activity remained intact. Extensive rearrangement of both helices in HL and LPL markedly reduced esterase activity, possibly by reducing the ability of the lid to change to the open conformation upon interfacial activation (42). The tributyrin activity, however, was still 5 times above background, whereas the more sensitive triolein assay revealed absent activity. These results indicate that the disruption of both helices in HL and LPL selectively prevented the hydrolysis of liposoluble substrate. Similarly, disruption of the amphipathic properties of the lipase lid abolished the ability of all mutant enzymes to hydrolyze phospholipid substrate presented as a DOPC vesicle (Table I). Taken together, these studies demonstrate that the lipase lid is essential for the hydrolysis of not only liposoluble triglycerides, but also of liposoluble phospholipid substrates.

To investigate a potential role of the lid in conferring substrate specificity, we generated several mutant lipases. As illustrated in Fig. 2, in Mut I, the lid of LPL was replaced by the lid of HL, and in the reciprocal mutant (Mut II), the lid of HL was replaced by the lid of LPL. Mut III is a chimeric lipase in which the carboxyl-terminal 134 amino acids of human LPL were replaced by the carboxyl-terminal 146 amino acids of human HL. Mut IV is a chimeric lipase that contains the NH2-terminal domain of LPL and the COOH-terminal domain as well as the lid of HL. In addition to these four chimeric constructs, plasmids containing wild-type LPL and HL cDNAs and a negative control consisting of the parent vector were transfected into 293 cells. Greater than 95% of the total triolein hydrolyzing activity was found in the culture medium of all transfected cells, indicating that the mutant lipases were secreted to a similar extent as wild-type LPL and HL (data not shown).

Table II summarizes the tributyrin, triolein, and phospholipid hydrolyzing activities in the medium of 293 cells transfected with the different plasmids. Tributyrin is water-soluble at the concentrations used in the assay and thus measures the esterase function of the lipases. Table II shows that substitution of the COOH-terminal domain of LPL with that of HL (Mut III) leads to a parallel reduction of triolein and cephalin activities compared with LPL WT possibly due to a destabilization of the active dimer (40). Comparison of Mut I with LPL WT, Mut II with HL WT, and Mut IV with Mut III indicates that despite the exchange of the lids, the catalytic domain and esterase function of the lipases. Table II shows that substitution of the COOH-terminal domain of LPL with that of HL (Mut III) leads to a parallel reduction of triolein and cephalin activities compared with LPL WT possibly due to a destabilization of the active dimer (40). Comparison of Mut I with LPL WT, Mut II with HL WT, and Mut IV with Mut III indicates that despite the exchange of the lids, the catalytic domain and esterase function of the lipases.

**Fig. 2. Schematic representation of wild-type and mutant LPL and HL constructs used for the analysis of substrate specificity.** The name as well as a general description of the constructs utilized in this study are listed. LPL sequences are shown in white, and HL sequences are shown in black. The numbers indicate the amino acid residues of the particular lipase comprising the NH2-terminal domain of the respective construct.
led to a 23% increase in triolein hydrolysis and a reduction of phospholipase activity to <30% of wild-type HL (Fig. 3B).

Previous studies have proposed a role of the COOH-terminal domain of the human lipases in mediating the interaction with lipid substrates (40, 41). We thus analyzed a mutant LPL in which the COOH-terminal domain was replaced by the COOH-terminal domain of HL (Mut III); the lipase in this chimeric enzyme is from LPL. Fig. 3C shows that the presence of the COOH-terminal domain of HL in LPL leads to only a slight decrease in the ability to hydrolyze long chain fatty acid triglycerides. The phospholipase activity of Mut III, however, was significantly reduced using all three different phospholipid substrates. To examine further the role of the lipase lid in modulating lipase substrate specificity, we replaced the lid of LPL in Mut III with the lid of HL to create a double mutant of LPL. In this case, both the lid and the COOH-terminal domain of LPL are replaced by the analogous structures of HL. Fig. 3D demonstrates that compared with wild-type LPL, the double mutation leads to a 50% reduction in the ability of the lipase to hydrolyze water-insoluble triglyceride substrate and up to a >5-fold increase in phospholipase activity, depending on the DOPC substrate used. When the ability of the double mutant LPL Mut IV to hydrolyze different substrates was directly compared with that of the chimera Mut III (Fig. 3E), a 42% reduction of triolein activity but up to a 13-fold increase in phospholipase activity were evident. Thus, the additional presence of the HL lid again resulted in marked enhancement of phospholipase function, stressing the importance of the lipase lid in conferring substrate specificity.

Table III summarizes the DOPC/triolein ratios for wild-type LPL and HL as well as the mutant lipases using either the DOPC vesicle or DOPC-mixed liposome substrate as a measure of phospholipase activity. Compared with native LPL, the ratio for native HL is 5–7-fold greater. To facilitate the evaluation of the role the lipase lid plays in determining substrate specificity, the data were separated into two groups. In the top half of Table III, all lipases containing the LPL lid are listed: wild-type LPL, HL with the LPL lid (Mut II), and LPL with the COOH-

**Table II**

| Construct   | Tributyrin activity | Triolein activity | DOPC vesicle activity | DOPC proteoliposome activity | DOPC-mixed liposome activity |
|-------------|---------------------|-------------------|-----------------------|-------------------------------|------------------------------|
|             | nmol/ml/min         | nmol/ml/h         |                       |                               |                              |
| LPL WT      | 5.2 ± 0.2           | 186.3 ± 9.1       | 11.5 ± 1.5            | 24.1 ± 3.5                    | 762 ± 3.0                    |
| HL WT       | 10.3 ± 0.5          | 32.2 ± 1.3        | 14.2 ± 5.4            | 7.7 ± 0.6                     | 64.2 ± 0.6                   |
| Mut I       | 2.7 ± 0.2           | 46.3 ± 1.8        | 25.7 ± 1.9            | 0 ± 0.2                       | 145.2 ± 2.4                  |
| Mut II      | 11.5 ± 0.7          | 44.1 ± 5.1        | 3.8 ± 0.9             | 0 ± 0.2                       | 18.6 ± 1.2                   |
| Mut III     | 0.9 ± 0.1           | 27.9 ± 1.4        | 0 ± 0.3               | 0 ± 0.3                       | 6.6 ± 0.6                    |
| Mut IV      | 1.1 ± 0.1           | 20.5 ± 1.6        | 4.4 ± 4.2             | 5.8 ± 0.7                     | 96.6 ± 3.6                   |

**Fig. 3.** Triolein and DOPC hydrolyzing activities of wild-type and mutant lipases. Activities are normalized for esterase (tributyrin hydrolyzing) activity and presented as percent of wild-type lipase activity. Wild-type lipase is shown as open bars, and mutant lipase as closed bars. Standard deviations are calculated from three transfection experiments. A, LPL WT and Mut I; B, HL WT and Mut II; C, LPL WT and Mut III; D, LPL WT and Mut IV; E, Mut III and Mut IV. In A, D, and E, hatch marks on the x axis indicate the presence of separate scales for triolein and DOPC activities.
TABLE III

| Construct | DOPC vesicle | DOPC-mixed lipase |
|-----------|-------------|-------------------|
| Lipases containing the lid of LPL | | |
| LPL WT | 1 | 1 |
| Mut II | 1.4 | 1.0 |
| Mut III | 0 | 0.6 |
| Lipases containing the lid of HL | | |
| HL WT | 7.1 | 4.9 |
| Mut I | 9.0 | 7.7 |
| Mut IV | 3.5 | 11.5 |

*Values are normalized to lipoprotein lipase.

terminal domain of HL (Mut III). The constructs containing the lid of hepatic lipase are listed in the bottom half of Table III: wild-type HL, LPL with the HL lid (Mut I), and LPL with the COOH-terminal domain and lid of HL (Mut IV). When normalized for wild-type LPL, all constructs containing the lid of LPL have a DOPC/triolein ratio of 1.4 or less, whereas the constructs with the HL lid have a ratio of 3.5–11.5. Analysis of Mut I and Mut II demonstrates that the exchange of the lipase lid leads to a reversal of DOPC/triolein ratios of LPL and HL, indicating that the lid may be the single most important region in conferring lipase substrate specificity.

**DISCUSSION**

In this study, we have investigated a structural motif in LPL and HL that mediates the different ability of the two enzymes to hydrolyze triglyceride versus phospholipid substrates. Relative to triolein hydrolyzing activity, HL has been shown to be a more potent phospholipase than LPL in vitro (1, 33–35). This difference in substrate specificity may play an important role in the biological function of LPL and HL (35) and becomes apparent in the markedly different phenotype observed in LPL (37, 38) and HL (36) deficiency states. We have recently demonstrated that the 22-amino acid lid that covers the catalytic site (Fig. 1) plays a crucial role in mediating the interaction of the lipases with emulsified triglycerides. Since there is little sequence homology between the LPL and HL lids (5), we postulated that the different primary structures of the lids of LPL and HL may, in part, modulate lipase substrate specificity (42).

The data presented in Table I demonstrate that the lids of LPL and HL are important for phospholipid as well as triglyceride hydrolysis. This is in contrast to guinea pig pancreatic lipase, which contains only a “minilid” of 5 amino acids, but is still able to hydrolyze phospholipid substrate (61). Our mutant in which the 22-amino acid lid was replaced by a 4-amino acid peptide had absent phospholipase activity even though its esterase activity was >2-fold higher than that of wild-type LPL. This, together with the data from the other mutants listed in Table I, indicates that in human LPL and HL, the lid plays a crucial role in triglyceride as well as phospholipid hydrolysis.

Analysis of Mut I and Mut II (Fig. 3, A and B) demonstrates that the simple exchange of the lids between LPL and HL markedly altered the ability of the lipases to hydrolyze certain substrates. The lid of HL increased the phospholipase and reduced the triglyceride hydrolysis activity, whereas the lid of LPL had the opposite effect. Similar findings were obtained in lipase mutants in which the COOH termini alone or both the COOH termini and the lids of LPL and HL were exchanged. Analysis of the chimeric enzyme containing the NH₂-terminal domain of human LPL and the COOH-terminal domain of human HL (Mut III) demonstrated a slight reduction of triolein as well as a significant decrease in DOPC hydrolyzing activity compared with normal LPL (Fig. 3C). This finding is consistent with previous studies (39, 40, 43) that suggest that the COOH-terminal domain of LPL may play a role in mediating the initial interaction of the lipase with lipoproteins and perhaps modulate the major type of particle with which either lipase will interact (42). Davis et al. (41) conclude from their analysis of similar chimeric enzymes in which the COOH-terminal domains of LPL and HL are exchanged that the COOH-terminal domain of HL augments phospholipase function. Based on the results presented here, the lid appears to be the more important determinant of phospholipase hydrolyzing activity. Analysis of the data reported by Davis et al. (41) indicates that replacement of the NH₂-terminal domain of HL with the NH₂-terminal domain and thus the lid of LPL resulted in a 2.5-fold reduction in the dipalmitoylphosphatidylcholine/triolein ratio. Replacement of the NH₂-terminal domain of LPL with that of HL, on the other hand, led to a 20-fold increase in this ratio.

In addition, direct comparison of the activity of our Mut III with that of Mut IV, the latter containing the COOH-terminal domain as well as the lid of HL in the LPL backbone (Fig. 3E), demonstrates that the presence of the HL lid in this chimera leads to a 40% reduction of triolein activity and up to a 13-fold increase in DOPC hydrolyzing activity. Thus, the presence of the HL lid markedly enhanced phospholipase function in Mut IV, indicating that the lid may be more important in determining phospholipase function than the COOH terminus.

Analysis of the DOPC/triolein ratios as an indicator of the relative phospholipase/triglyceride hydrolysis function of normal and mutant lipases demonstrated that this ratio is higher for HL than for LPL (Table III). Our studies clearly demonstrate that whenever the LPL lid was present (Mut II and Mut III) (Table III), the DOPC/triolein ratio was similar to that of LPL (1:4). Conversely, whenever the HL lid was present (Mut I and Mut IV) (Table III), the ratio increased to >3.5, similar to that of normal HL. Thus, regardless of the type of COOH terminus or lipase backbone present, exchange of the lids between the lipases resulted in a reversal of the phospholipase/triglyceride hydrolyse ratios of LPL and HL.

The mechanism by which the 22-amino acid lid mediates the different substrate specificities of LPL and HL may be complex. Previous studies have demonstrated that the lid must be repositioned to allow access of substrate to the catalytic pocket (10). We have suggested that, upon opening of the lid, the hydrophobic sides of its amphipathic helices are exposed (42), thus creating a large hydrophobic area, which may then function as a binding site for lipid substrates. A similar mechanism has been previously demonstrated for an unrelated fungal lipase (62, 63). More recently, studies in which the three-dimensional structure of pancreatic lipase was cocrystallized with mixed micelles of phosphatidylcholine and bile salt (64) have demonstrated that, indeed, one of the two fatty acids of phospholipid binds to the hydrophobic side chains of the amino acid residues in the lipase lid. A molecular model of LPL based on the pancreatic lipase x-ray structure (65) indicates that the lid of LPL is indeed likely to be organized into amphipathic helices and that it may play a role similar to that of the pancreatic lipase lid. Thus, like in pancreatic lipase, the lids of LPL and HL may come into intimate contact with lipase substrates and thus play a major role in mediating lipase substrate specificity.

In this context, the primary, secondary, and tertiary structures of the lid may be important. Although the secondary structural organization of the lid into highly amphipathic helices appears to be very similar in LPL and HL (42), there is only a small degree of primary sequence homology. In fact, 15 out of 22 amino acids of the lid are different between LPL and HL. Further analysis of the lipase lids reveals that the LPL lid...
contains four acidic and three basic amino acids, whereas the lid of HL contains only one acidic but five basic residues. In the investigated group of substrates, substrate specificity is controlled for different fatty acid preferences between LPL and HL (22–25) by using oleic acid as the fatty acid in triglyceride (triolein) and phospholipid (DOPC) substrates. Thus, the observed differences in substrate specificity cannot be ruled out, the reversal of phospholipid/phospholipid (DOPC) substrates. Thus, the observed differences in substrate specificity may, in part, be due to different abilities of the LPL and HL lids to accommodate the polar head group of phospholipids. The differences in the distribution of charged residues in the lid may thus be crucial since a negatively charged polar head group into the catalytic pocket. Therefore, the lid of HL may be more suited for interaction with a phospholipid substrate than the LPL lid.

A molecular model of LPL based on the x-ray structure of hepatic lipase suggests that the lids of LPL and HL are the single most triglyceride-hydrolyzing ratios by the mere exchange of the lids. Nevertheless, the reversal of phospholipid/phospholipid (DOPC) substrates. Thus, the observed differences in substrate specificity may, in part, be due to different abilities of the LPL and HL lids to accommodate the polar head group of phospholipids. The differences in the distribution of charged residues in the lid may thus be crucial since a negatively charged polar head group into the catalytic pocket. Therefore, the lid of HL may be more suited for interaction with a phospholipid substrate than the LPL lid.

In conclusion, characterization of chimeric enzymes in which the LPL and HL lids have been exchanged has demonstrated that the lipase lid plays a crucial role in mediating the different substrate specificities of LPL and HL. The lid of HL augments phospholipase activity, whereas the lid of LPL increases triglyceride hydrolysis. This study provides new insights into the structural basis for the observed in vivo differences in lipid and substrate function.

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