A Surface Loop Covering the Active Site of Human Pancreatic Lipase Influences Interfacial Activation and Lipid Binding*

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The distinguishing feature of lipases is their increased activity at an oil-water interface, termed interfacial activation. X-ray crystallography of lipases suggested a mechanism for interfacial activation by revealing conformational changes in several surface loops that cover the active site. In one conformation, these loops prevented substrate from entering the active site, and, in the other conformation, movement of the loops opened the active site. We tested the role of the major surface loop, the lid domain, in human pancreatic lipase (hPL) function by creating deletions in this region and expressing the mutant proteins in baculovirus-infected insect cells. The mutants were tested for activity against tributyrin and triolein, colipase interaction, interfacial activation, and binding to tributyrin. The purified mutants had decreased activity against both tributyrin and triolein compared to wild-type hPL and did not show a preference for either substrate. Although colipase was required for maximum activity in the presence of bile salts, the mutants had significant activity against tributyrin, but not triolein, in the absence of colipase. Both mutants were active against monomers of tributyrin demonstrating that they did not require an interface for activity. Finally, both mutants had decreased binding to tributyrin particles. These results suggest that the lid domain in hPL mediates interfacial activation and influences interfacial binding.

The absorption and assimilation of dietary fats depends on the actions of multiple lipolytic enzymes (1, 2). Many of these lipases have homologous protein and gene structures suggesting that lipases form a gene family evolved from a common ancestral hydrolase (3, 4). The archetype of this family is pancreatic triglyceride lipase (PL), and information about PL has been extrapolated to other lipase family members. For instance, experiments based on earlier PL studies produced new knowledge about hepatic and lipoprotein lipases (5–8).

Human pancreatic triglyceride lipase (hPL) has the characteristic and intriguing property of preferring water-insoluble substrates that form oil-water interfaces over water-soluble substrates (9). In dilute solutions of short-chain triglycerides where monomers predominate, hPL displays little activity. Once the concentration of triglyceride exceeds its solubility, particles form and the reaction velocity increases dramatically, a property termed interfacial activation.

Three-dimensional structures of lipases have demonstrated that the active sites of hPL and of several fungal lipases are covered by peptide loops that would prevent diffusion of substrate into the active site unless these loops move (10, 11). Movement of the loops was observed in hPL co-crystallized with procolipase and mixed micelles of phospholipid and detergent (12). Similar movements were seen in a fungal lipase crystallized with an irreversible inhibitor and were fortuitously observed in the crystal structure of another fungal lipase (13, 14). These groups proposed that the movements produced the phenomenon of interfacial activation (12, 14, 15).

One of the loops in hPL, a surface helix of 23 amino acids bounded by Cys236 and Cys302, the lid domain, adopted a markedly different conformation when substrate or inhibitor was present in the crystals (12, 16). The new position of the lid opened the active site, created a hydrophobic surface, allowed movement of a second loop, the β5 loop, which interacted with the lid in the closed conformation, and permitted new contacts between the open lid and colipase. The description of a guinea pig lipase with a 4-amino acid lid domain suggested that the lid domain did prevent diffusion substrate into the active site (17). The enzyme was highly active against monomeric substrates and did not exhibit interfacial activation consistent with the hypothesis that movement of the lid domain constitutes interfacial activation.

The participation of the lid domain in interfacial activation has not been tested directly by creating mutations in the lid domain and determining the effects on interfacial activation. Mutations have been introduced into the putative lid domain of lipoprotein lipase (5, 6). The authors demonstrated effects of the mutations on activity and substrate specificity, but did not measure interfacial activation.

We tested the contribution of the hPL lid domain to interfacial activation and lipid binding by introducing deletions into the cDNA encoding hPL and expressing the mutant lipases in COS-1 cells or baculovirus-infected insect cells. The mutants were tested for activity against tributyrin and triolein. Their sensitivity to bile salts and interaction with colipase was determined, and interfacial activation was measured.

EXPERIMENTAL PROCEDURES

Construction of Mutants—The previously described hPL cDNA was the template for mutagenesis (18). The amino acid numbering is based on the hPL amino acid sequence. Two deletions were introduced into the region between Cys236 and Cys302 by the polymerase chain reaction overlap extension method (19, 20). Amino acids Lys240 to Ala280 were deleted in one mutant, and residues Asp446 to Arg557 were deleted in the other mutant. The polymerase chain reaction products were subcloned into pGEM3Z, and the sequence was confirmed by the dideoxynucleotide chain termination method according to the Sequenase protocol. Each mutant was then subcloned into pVL1392 for preparing recombinant baculovirus for infecting insect cells. Plasmid DNA was prepared with Qiagen columns according to the manufacturer’s instructions.

Infection of Insect Cells with Recombinant Baculovirus—Recombinant baculovirus was prepared as described previously, and 392 or Hi-5 insect cells grown in spinner flasks with serum-free medium (Ex-Cell 400) were

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1 The abbreviations used are: PL, pancreatic triglyceride lipase; hPL, human pancreatic triglyceride lipase; PAGE, polyacrylamide gel electrophoresis.
infected with the virus (21). The medium was harvested 3–4 days post infection by centrifugation at 5000 rpm for 10 min in a Beckman J2–21 centrifuge with a JA-20 rotor.

Purification of Recombinant Lipases—Both mutants and hPL were purified from the harvested insect cell medium. Wild-type hPL was purified by immunoaffinity chromatography.1 Preliminary experiments demonstrated that the two mutants bound and eluted from the immunoaffinity column, but were inactivated by the elution buffer. Thus, an alternative purification method was developed. Benzamidine, 2 mM, and phenylmethylsulfonyl fluoride, 0.25 mM, were added to the clarified medium, and the medium was dialyzed overnight against 15 liters of 10 mM Tris-HCl, pH 8.0, 2 mM benzamidine, and 0.25 mM phenylmethylsulfonyl fluoride. The medium was applied to a DEAE-Blue Sepharose column (200 ml bed volume) and washed through with 10 mM Tris-HCl, pH 8.0. Both mutants were in the pass-through. The pH of the pass-through was adjusted to 6.2 with 0.5 mM succinate and loaded onto a CM-Sepharose column equilibrated in 30 mM Tris succinate, pH 6.2. The column was washed with equilibration buffer and eluted with a salt gradient from 0 to 300 mM NaCl in the equilibration buffer. Lipase was located by activity against triolein. The active fractions were pooled and concentrated by ultracentrifugation over an Amicon YM30 membrane.

Analysis of Lipases—SDS-PAGE and immunoblot were accomplished as described previously (22). Lipase activity was measured against [3H]triolein as described or by a pH-STAT technique. For potentiometric measurements, the sample was added to 1 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 2 mM CaCl2, and 4 mM taurodeoxycholate. The assay substrate was prepared by adding 0.5 ml of tributyrin to 15 ml of buffer and sonicating. Determination of interfacial activation was done in 0.3 mM succinate and loaded onto a CM-Sepharose column equilibrated in 30 mM Tris succinate, pH 6.2. The column was washed with equilibration buffer and eluted with a salt gradient from 0 to 300 mM NaCl in the equilibration buffer. Lipase was located by activity against triolein. The active fractions were pooled and concentrated by ultracentrifugation over an Amicon YM30 membrane.

RESULTS

Expression of hPL and the Lid Domain Mutants—Two mutants were created in the region of the lid domain. One, the 240–260 deletion mutant, removed virtually all of the lid domain to test the importance of the domain in lipase function. The second mutation, the 247–258 deletion mutant, was designed to remove the α-helix covering the active site and to minimize disruption of the remaining lid domain residues.

Both lid domain mutants and hPL were expressed in recombinant baculovirus-infected insect cells. Earlier, we and others demonstrated that milligram quantities of wild-type PL could be expressed in this system (21, 23). After infecting insect cells with the recombinant baculovirus, the presence of the lid domain mutants in the medium was demonstrated by SDS-PAGE and immunoblot (data not shown). Larger scale infections were done, and 3 to 5 mg of each protein was isolated from the medium. The isolated proteins were homogeneous by SDS-PAGE analysis and protein staining (Fig. 1). These purified recombinant proteins were used for further characterization of the role of the lid domain in lipolysis.

Activity of the Mutant Lipases—The lipase activity of the recombinant lipases was determined in the presence of colipase and taurodeoxycholate by the pH-STAT technique with tributyrin, a short-chain triglyceride, as substrate (Fig. 2A). Lipolytic activity was readily detectable and linear over at least 5 min demonstrating that the insect cells made functional lipases. With longer incubation times, the reaction rate for the 248–257 deletion decreased (inset, Fig. 2B). Because only 2% of the available fatty acids were released, substrate depletion was not an adequate explanation for the decreasing activity. Alternatively, decreased stability of the lid domain mutants or product inhibition could explain the fall off in activity.

To determine if the lid domain mutants had decreased activity against long-chain triglycerides, we tested the lid domain mutant activity against [3H]triolein. A previous study demonstrated that deleting the lid in lipoprotein lipase increased activity against tributyrin and greatly decreased activity against trilaurin (6). The authors speculated that the lid domain influenced substrate binding and specificity. hPL and both mutants had activity against trilaurin, in contrast to the result with lipoprotein lipase (Fig. 3). Interestingly, the lid deletion mutants, but not wild-type hPL, had a long lag phase with trilaurin that was not seen with tributyrin. The assay system did not influence the lag phase because the mutants also had an exaggerated lag phase with trilaurin (data not shown).

The specific activities of hPL and the two mutants for tributyrin and trilaurin were determined by the pH-STAT method to detect marked differences in activity against either substrate. Regardless of the substrate, the deletion mutants had lower specific activities than wild-type hPL when colipase was in the assay (Table I). The decrease in specific activity was about 8-fold for the 248–257 deletion mutant for both tributyrin and trilaurin. Likewise, the specific activity of the 240–260 deletion mutant decreased about 17-fold for both substrates. Neither mutant had a significant preference for either substrate indicating that the lid domain may not contribute to or influence the substrate binding pocket.

Although maximum activity of the wild-type hPL and the lid deletion mutants required colipase for both substrates, the mutants had significant activity against tributyrin in the absence of colipase (Table I). In contrast, detectable hydrolysis of trilaurin required colipase. When tributyrin was the substrate, colipase increased lipase activity 22-fold for wild-type hPL compared to just 1.5-fold for the lid deletion mutants demonstrating a marked difference in the salt inhibition of tributyrin hydrolysis between the mutants and wild-type hPL.

Interaction with Colipase—One potential explanation for the decreased activity of the lid domain mutants is decreased binding of the mutant lipases to colipase. To test this possibility, we initially attempted to measure colipase binding to the lid domain mutants by several previously published methods and obtained inconsistent results, presumably because of the weak binding of colipase and hPL in free solution. Consequently, we employed a competition assay to determine if the 240–260 lid deletion mutant could inhibit wild-type hPL activity against trilaurin. If wild-type hPL and the 240–260 deletion were added to the same reaction, trilaurin hydrolysis during short incubations would be a result of the wild-type hPL activity and not the mutant because of the mutant’s lag phase. Activity would only be inhibited by molar excesses of the mutant if the mutant bound colipase with the same affinity as the wild-type hPL.
Interfacial Activation of Human Pancreatic Lipase

A

FIG. 2. Activity of the lid deletion mutants against tributyrin. The tracings from a pH-STAT determination of tributyrin activity for the proteins purified from insect cell medium are shown. Panel A: dotted line, 2.0 µg of hPL; dashed line, 2.5 µg of 248–257 deletion mutant; solid line, 2.5 µg of 240–260 deletion mutant. Panel B, longer time course for the 248–257 deletion mutant. All incubations contained 0.8 to 1.0 µg of colipase.

B

FIG. 3. Activity of hPL and the lid deletion mutants against triolein. The lipases purified from Sf9 cell medium were assayed with [3H]triolein in 50-µl volumes. The reaction was stopped at various times, and the free fatty acids were extracted. Each point was done in duplicate in three separate experiments. The standard deviation is shown by the error bars. Open circles, 150 ng of hPL; closed circles, 400 ng of 248–257 deletion mutant; 400 ng of 240–260 deletion mutant.

Table I

Activity of wild-type hPL and the two lid domain mutants expressed in insect cells

| Sample   | Tributyrin | Triolein |
|----------|------------|----------|
|          | No colipase| Colipase |
|          | No colipase| Colipase |
| µmol fatty acid/mg protein |
| Wild-type | 0.37 ± 0.02 | 8.2 ± 0.39 | 0.07 ± 0.01 | 2.8 ± 0.22 |
| 248–257  | 0.80 ± 0.46 | 1.2 ± 0.51 | ND | 0.31 ± 0.35 |
| 240–260  | 0.34 ± 0.11 | 0.5 ± 0.25 | ND | 0.16 ± 0.08 |

Activity would be normal or minimally decreased if the mutant had significantly decreased affinity for colipase. The assay conditions included a large excess of substrate, suboptimal colipase concentrations, and a short incubation period. hPL had about 30% of maximal activity under these conditions. Addition of the 240–260 deletion mutant inhibited lipase activity in a dose-dependent fashion (Fig. 4). Adding a 2-fold molar excess of colipase overcame the inhibition suggesting that the 240–260 deletion mutant inhibited wild-type hPL by competing for colipase binding and that the interaction of the mutant with colipase was not vastly different from the interaction with the wild-type hPL (Fig. 4). Furthermore, the interaction must occur quickly and could not account for the lag phase seen with the lid deletion mutants.

FIG. 4. Inhibition of hPL by the 240–260 deletion mutant. hPL was assayed against [3H]triolein for 15 min with various amounts of the 240–260 deletion mutant. The molar ratio of the mutant to hPL is given on the x axis. Open circles, with limiting amounts of colipase (30% maximal activity); closed circles, with 2-fold molar excess of colipase over the combined amount of hPL and the mutant.

Binding of the Lid Domain Mutants to Substrate—Another possible explanation for decreased activity of the mutant lipases is decreased binding affinity of the mutants for the substrate interface. Tributyrin, which can be dispersed in aqueous solutions more readily than longer-chain triglycerides and easily separated from the aqueous phase by centrifugation, presents an interface that binds native porcine PL (24). This system was used to directly measure binding of the lid deletion mutants to a substrate interface (Table II). Short incubations with tributyrin dispersed in buffer followed by centrifugation removed virtually all of wild-type hPL from the aqueous phase, but most of the lid deletion mutants remained in the aqueous phase. Binding of hPL was inhibited by the presence of taurodeoxycholate and was restored if colipase was added.
percentage of the 248–257 deletion mutant bound to the lipid phase if colipase was present, but the extent of binding was still less than for wild-type. These results demonstrated decreased binding of the lid domain mutants to interfaces and suggested that the lid domain forms all or part of the binding site.

**TABLE II**

| Incubationa | Fraction in aqueous phase | 0.16b | 0.95c | 0.94d | 0.18e |
|--------------|---------------------------|-------|-------|-------|-------|
| No additions | Wild-type | 1.00b | 1.00b | 1.00b | 1.00b |
| TB           | 0.92 ± 0.91b | 0.84d | 0.84d | 0.84d | 0.84d |
| TB + TDC     | 0.86d | 0.84d | 0.84d | 0.84d | 0.84d |
| TB + TDC + colipase | 0.19d | 0.64d | 0.64d | 0.64d | 0.64d |

TB, tributyrin; TDC, taurodeoxycholate.

Bile Salt Inhibition of the Lid Domain Mutants—To characterize the bile salt inhibition of the lid domain mutants, we measured the activity of the lid mutants at various taurodeoxycholate concentrations with tributyrin as the substrate (Fig. 6). The mutants' activity against monomers should have absorbed an effect of bile salts as reported for the guinea pig lipase, but bile salts had clear effects on the mutants' activity. Low concentrations of taurodeoxycholate stimulated the mutants and the wild-type hPL (17). Higher concentrations of bile salts inhibited the lid deletion mutants to a much greater extent than the inhibition of wild-type hPL. The lid deletion mutants had almost 5-fold higher activity at 0.5 mM taurodeoxycholate than at the standard assay concentration of 4 mM taurodeoxycholate. The increased sensitivity of the lid domain mutants to bile salt inhibition suggests that the mutants are more susceptible to denaturation by bile salts or to displacement from substrate by bile salts.

**DISCUSSION**

We constructed and expressed two mutant lipases with deletions in a surface loop between Cys 228 and Cys 229, the lid domain, that covers the active site and blocks access of substrate to the binding pocket. Although infected insect cells secreted both mutants in amounts comparable to the wild-type hPL and both mutants had activity against triglyceride substrates, the lid domain mutants differed from the wild-type hPL in several important properties. The mutants showed decreased activity, decreased binding to an interface, increased sensitivity to bile salt inhibition, and, importantly, did not demonstrate interfacial activation.

The lid domain of lipases has been implicated by other studies in the mechanism of interfacial activation and in lipid binding of lipases (10–17). Our data provide direct evidence that the lid domain participates in the mechanism of interfacial activation. This observation is consistent with the hypothesis that the lid domain blocks access of substrate to the active site and that movement of the lid is required for activity. At least two mechanisms for inducing interfacial activation by lid domain movement are plausible. First, binding to an interface could trigger the conformational change in the lid domain which exposes the active site. Second, lipases may exist in several conformations with only the open form actively binding substrate. Evidence that the conformation of lipases is dynamic came from the three-dimensional structure of a fungal lipase that crystallized in the open conformation even in the absence of an interface (14). In this model, monomer hydrolysis is limited by two factors. The open form must be transient and the closed form must be present in a large excess. Binding to an interface would stabilize the open form and permit diffusion of substrate into the active site and provide a structural correlate for the kinetic phenomena of interfacial activation.

The three-dimensional structure of the procolipase-hPL complex in the open form revealed a continuous hydrophobic plateau formed by procolipase and the lid domain (12). The authors proposed that this hydrophobic surface is the lipid binding site of the complex. The formation of the surface by regions from both procolipase and hPL provides a potential explanation for the role of colipase in potentiating the binding of hPL to lipid interfaces. The decreased binding of the deletion mutants to tributyrin is consistent with the notion that the lid domain forms part of the lipid binding site. The preserved activity against triolein argues that the deletion mutants can bind to interfaces and that other regions of the procolipase-hPL complex participate in binding to interfaces.
Interfacial Activation of Human Pancreatic Lipase

Although the decreased binding of the lid domain mutants to a substrate interface is sufficient to explain the decreased activity, increased bile salt sensitivity, and long lag phase of the mutants, other explanations are also possible. The crystal structure of the open and closed forms of the procolipase-hPL complex demonstrated conformational changes in other regions of hPL, notably in the β5 loop (12). These changes constrain Phe78 in position to form the oxyanion hole and help stabilize the oxyanion intermediate. Because lid domain residues interact with residues in the β5 loop, deletion of lid domain residues may affect the position of the β5 loop and destabilize the oxyanion intermediate. Conformational changes in the β5 loop or in other regions of the active site could negatively affect substrate binding affinity or slow substrate turnover. Finally, the interaction with colipase may be inhibited by deletions in the lid domain.

Analysis of the three-dimensional structure of the procolipase-lipase complex co-crystallized with mixed micelles did reveal interactions of procolipase with lid domain residues, Val247, Ser244, and Asn241 (12). All 3 residues were preserved in the 248–257 deletion mutant and were absent in the 240–260 mutant. Yet, both mutants required colipase for activity against triolein and the 240–260 deletion mutant could effectively compete with hPL for colipase. Although neither of these experiments would detect small differences in the binding of colipase to the mutants, the binding was not critically affected by the deletions. In contrast, the inability of colipase to restore binding of the 248–257 deletion mutant to tributyrin suggested that the interaction of colipase with the mutant differed from the interaction of colipase with wild-type hPL. Thus, altered interactions between colipase and the mutants remains a possible explanation for the decreased activity and long lag phase of the lid domain mutants.

The results presented in this paper demonstrate the role of the lid domain, first postulated from the three-dimensional structures of lipases, in interfacial binding and interfacial activation. Movement of the lid domain away from the active site may be the only structural correlate of interfacial activation, but other structures may play a role. The x-ray data suggested that other loops also moved, perhaps as a direct result of the lid domain movement. The effects of deleting portions of the lid domain on other regions of hPL is not clear from the crystal structures or the present studies. Removing the lid domain residues may induce the other loops to assume the positions they normally occupy in the open conformation or to assume positions that do not normally occur. The molecular basis for the changes induced in the lid deletion mutants requires additional detailed knowledge of the mutants’ structure that can be provided by their crystal structure.

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