Human lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: identification of the catalytically active serine, aspartic acid, and histidine residues

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Abstract Human lysosomal acid lipase/cholesteryl ester hydrolase (HLAL), human gastric lipase (HGL), and rat lingual lipase (RLL) constitute a family of mammalian lipases characterized by an acidic pH optimum. HGL and RLL are secreted by the chief cells of the stomach and by the serous von Ebner's glands of the tongue, respectively, and hydrolyze dietary long-chain triglycerides in the gastrointestinal tract. HLAL, in contrast, catalyzes the intralysosomal degradation of both triglycerides and cholesteryl esters in virtually all cells except erythrocytes. All three enzymes are proposed to be serine esterases with a catalytic Ser-Asp-His triad similar to other lipases, despite their sensitivity towards sulfhydryl modifying reagents. To investigate the role of conserved serine, aspartic acid, and histidine residues in HLAL and HGL, we constructed 24 mutant lipases with single amino acid substitutions using the site-directed mutagenesis approach. Our combined data strongly support the conclusion that Ser_{153}, Asp_{324}, and His_{353} are components of the catalytic triad of HLAL and HGL. Structural integrity of the conserved His-Gly dipeptide of lipases also appears to be important for neutral lipid hydrolysis, as replacement of His_{65} by glutamine abolished HLAL and HGL enzymic activity. Substitution of HLAL residues Asp_{93}, Asp_{130}, and Asp_{328} with glycine, in contrast, had a more pronounced impact on cholesteryl oleate hydrolysis than on triglyceride hydrolysis. These results provide new insights into the structural basis of HLAL and HGL function.—**Lohse, P., S. Chahrokh-Zadeh, P. Lohse, and D. Seidel.** Human lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: identification of the catalytically active serine, aspartic acid, and histidine residues. *J. Lipid Res.* 1997. **38:** 892–903.

Supplementary key words lipid metabolism • serine hydrolase • esterase activity • α/β hydrolase fold • lipase consensus sequence • catalytic triad • site-directed mutagenesis • in vitro expression

Lipases and cholesteryl esterases catalyze the hydrolysis of ester bonds in neutral lipids (for review see refs. 1–4). The nucleophilic attack on the ester carbon of the scissile bond is facilitated by three amino acids which are referred to as the catalytic triad. A negatively charged residue, a histidine, and a serine form a hydrogen-bonding network that leads to the activation of the serine hydroxyl. In concert with the dipoles of the oxyanion hole, these residues provide an environment that stabilizes the transition states of the catalytic reaction by complementing the changes in charge distribution through electrostatic free energy (5).

With the exception of a lipase/acyltransferase from *Aeromonas hydrophila* (6, 7), the active site serine is always part of the conserved pentapeptide motif –Gly–Xaa–Ser–Xaa–Gly–. This lipase consensus sequence forms a loop structure with the serine residue connecting a β-strand to an α-helix (8). This β–ϵSer–α conformation, also known as the "nucleophilic elbow" (9), facilitates interaction of the nucleophile with the histidine and the ester carbon in a precisely oriented complex. The completely conserved histidine is part of a loop located in the carboxyl-terminal domain of the enzymes. The carboxylic amino acid, in contrast, can be either aspartic acid or glutamic acid and its topological position within the common α/β hydrolase fold appears to vary (10). The linear order Ser-Asp/Glu-His in the polypeptide sequence, however, is absolutely preserved.

The catalytic triad is covered by an α-helical surface loop. This lid creates a hydrophobic pocket and makes the catalytic residues inaccessible to the surrounding solvent. In order to hydrolyze the substrate, the lid has to rotate away to expose the active site, thereby creating

Abbreviations: BSSL, bile salt-stimulated lipase; HGL, human gastric lipase; HL, hepatic lipase; HLAL, human lysosomal acid lipase; LPL, lipoprotein lipase; PL, pancreatic lipase; RLL, rat lingual lipase.

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a larger hydrophobic surface believed to be necessary for adsorption of the enzyme at the lipid–water interface.

The discovery of a serine protease-like catalytic triad and the similarity of the three-dimensional core structures have stimulated interest in the structural characterization of other lipases. Human lysosomal acid lipase/cholesteryl ester hydrolase (HLAL), human gastric lipase (HGL), and rat lingual lipase (RLL) constitute a family of mammalian enzymes with acidic pH optimum and little sequence homology to other lipases, in which the catalytic residues remain to be determined. HLAL is a key enzyme for the intralysosomal degradation of cholesteryl esters and triglycerides taken up by receptor-mediated endocytosis (for review see refs. 11, 12), whereas HGL and RLL are preduodenal enzymes critical for the hydrolysis of ingested triglycerides in the gastrointestinal tract (for review see refs. 13–15).

In order to identify the amino acids involved in the catalytic breakdown of ester bonds by HLAL and HGL, we replaced serine, aspartic acid, and histidine residues completely conserved within this lipase family with threonine, glycine, and glutamine, respectively. In this manuscript, we provide evidence that Ser_{153}, Asp_{324}, and His_{353} are essential residues for the lipolytic and esterolytic properties of HLAL and HGL and are most likely part of the catalytic center of this enzyme family.

MATERIALS AND METHODS

Isolation of HLAL cDNA

Total cellular RNA was isolated from human leukocytes by the guanidinium isothiocyanate method (16) followed by ultracentrifugation through a CsCl cushion. First strand synthesis was performed with SuperScript reverse transcriptase (Bethesda Research Laboratories) using anti-sense primers with incorporated Hpa I restriction sites located at the 5′-end (5′-CATTTTCACAT GACATAATCGTTAACTTGGTGGTACACAGC-3′) or at the 3′-end (5′-TTGGGCCGTTAACTCTCACTCAATT TTATTG-3′) of the 3′-untranslated region of the published HLAL cDNA sequence (17). The newly generated HLAL cDNA was further amplified by the PCR technique (18) using a sense primer in exon 1 containing an artificial restriction enzyme site for Nhe I (5′-ACT GCGACTCGAGCTAGCGGCCCGGCAGGACAG-3′).

Isolation of HGL cDNA

An oligo $(dT)_{18}$ primer that contained, in addition, restriction sites for Sma I, BamH I, and Hpa I was used to prime the reverse transcriptase reaction of 10 μg of total RNA from human stomach tissue. One-tenth of the reaction was subjected to PCR amplification using a sense primer in exon 1 with an incorporated Xba I site (5′-GAAACAGAATTCTAGATATTTCTG-3′) and an anti-sense oligonucleotide located in the 3′-nontranslated region containing an artificial Hpa I site (5′-CAAA CGGTTAACTCTTTAAATCC-3′) or a primer identical to the restriction sites incorporated into the oligo $(dT)_{18}$ primer.

Site-directed mutagenesis

A wild-type HGL cDNA of 1239 bp extending from nucleotides +18 to +1256 (19), a 1258 bp fragment of normal HLAL cDNA spanning the signal peptide through the termination codon and a 2472 bp fragment containing in addition the complete 3′-untranslated region of the HLAL cDNA (17) were cloned into the Xba I and Hpa I sites of the eukaryotic expression vector pCMV (20) containing the cytomegalovirus (CMV) immediate early promoter and the polyadenylation signal of simian virus 40 (SV40). The resulting constructs were designated as pCMV-HGL and pCMV-HLAL 1 and 2, respectively. Synthesis of mutant cDNAs was performed by the overlap extension PCR method (21) using the plasmids pCMV-HLAL 1 and pCMV-HGL as templates. The mutagenized fragments were subcloned into the pCMV vector and all constructs were characterized by sequence analysis (22) of the complete cDNA insert.

In vitro expression of HLAL cDNA constructs

Ten μg of wild-type or mutant plasmid DNA and 3μg of DNA of the reporter construct pRSV-luciferase (23) per 10-cm dish, prepared by the cesium chloride double banding method (24), were used to transfect Ltk^- mouse fibroblasts, a subclone of the L-cell line, and Cos-7 cells, derived from the established African green monkey kidney cell line CV-1 (American Type Culture Collection), by the calcium phosphate coprecipitation method (25). Cells were maintained for 72 h after glycerol shock at 37°C to allow high level expression of acid lipase (17).

HLAL enzyme assay

Acid lipase activity was determined at pH 4 with 45–75 μg protein as enzyme source using the radiolabeled substrates cholesteryl-[1-^{14}C]oleate (specific activity 50–60 mCi/mM; Amersham, Arlington Heights, IL), glycerol-tri-[1-^{14}C]oleate (triolein; specific activity 50–60 mCi/mM; Amersham), and glycerol-tri-[1-^{14}C]butyrate (tributyrin; specific activity 39 mCi/mM; Sigma Chemical Co., St. Louis, MO), dispersed in a solution of 100 μl volume containing 63 μmol of the unlabeled substrate, 0.05% Triton X-100, 2 mM sodium taurocholate, 30 mM sodium citrate phosphate buffer, pH 3.5, 1.5 mg/ml

(w/v) albumin, 5 mM β-mercaptoethanol, 1 mM EDTA, and 25 mM sodium chloride. Reactions were terminated after a 3-h incubation at 37°C by the addition of 0.1 mM cholesteryl oleate, triolein or tributyrin in chloroform–heptane–methanol 1.3:1:1.4 and alkaline buffer to form a two-phase partition system (26). Liberated fatty acids were dissolved in scintillation fluid and counted.

In vitro expression of HGL cDNA constructs

Cos-7 cells and human embryonal kidney-293 cells (American Type Culture Collection) were plated on 10-cm diameter culture dishes and transfected with 10 μg of wild-type or mutant plasmid DNA and 3 μg of DNA of the reporter construct pRSV-luciferase per dish purified on QIAGEN-tips (QIAGEN Inc., Chatsworth, CA).

HGL enzyme assay

Media were harvested 24 h after glycerol shock and cell extracts were prepared after washing by sonification in 0.5% Triton X-100. Protein content was determined on a Cobas Fara analyzer (Hoffmann-LaRoche AG, FRG) with an automated Lowry method (27). Enzymic activities were quantified at pH 5.4 with 90 μg protein as enzyme source using glycerol-tri-[1-^{14}C]oleate (triolein) and glycerol-tri-[1-^{14}C]butyrate (tributyrin) as substrates. Triolein hydrolysis was assayed in a 200 μl reaction containing 63 μmol of the unlabeled substrate, 30 mM sodium citrate phosphate buffer, pH 3.5, 25 mM sodium chloride, 2 mM sodium taurocholate, 1.5 mg/ml (w/v) albumin, 5 mM β-mercaptoethanol, 1 mM EDTA, and 0.05% Triton X-100. The tributyrin assay was performed in a solution of 200 μl volume containing 138 μm unlabeled substrate, 150 mM sodium phosphate buffer, pH 5.4, 2 mM sodium taurodeoxycholate, and 0.75 mg/ml (w/v) albumin.

After an incubation at 37°C for 1 h, enzyme reactions were terminated by the addition of 0.1 mM triolein or tributyrin in chloroform–heptane–methanol 1.3:1:1.4 and alkaline buffer (26).

In each experiment, a parallel cell culture dish was subjected to the transfection protocol without plasmid DNA. These mock-transfected cells served as controls to reflect both basal HLAL as well as non-HLAL- or non-HGL-related enzymic activities. The values obtained were subtracted from those of HLAL- and HGL-overexpressing cells prior to the calculation of the percentage of esterase activity. In addition, hydrolytic activities measured in the media and extracts were normalized with respect to luciferase expression in the cell homogenates to correct for differences in transfection efficiency.

Northern blot analysis

Total RNA was extracted from Ltk$^-$ mouse fibroblasts and Cos-7 cells transfected with wild-type and mutant HLAL and HGL constructs, respectively. Hybridization with the corresponding cDNA probes revealed no major differences in the cellular mRNA levels between the various constructs (data not shown).

RESULTS

Active site serine

Both enzymes contain two conserved –Gly–Xaa–Ser–Xaa–Gly– lipase consensus sequences around Ser_{99} and Ser_{153}. In addition, there are nine absolutely conserved serine residues in HLAL, HGL, and RLL. However, none of these serines is flanked by two glycines and only Ser_{268} is preceeded by a glycine in position 266. Because, in most lipases, the active site serine residue is surrounded by two invariant glycines in order to maintain the tight bend between the β-strand and the α-helix (8, 9), it appears highly unlikely that one of these amino acids is the nucleophile of the catalytic triad. We therefore decided to replace only Ser_{99} and Ser_{153} with threonine, thereby theoretically not precluding the formation of an acyl intermediate.

Site-directed mutagenesis of Ser_{153} eliminated HLAL activity against both glycerol trioleate and cholesteryl oleate (**Fig. 1** and **Table 1**). Replacement of Ser_{99} with threonine, in contrast, was not completely detrimental for enzymic activity. The transiently expressed mutant enzyme retained about 39–41% (in the case of Cos-7 cells) and 63–66% (in the case of Ltk$^-$ cells) of catalytic activity for both substrates.

To analyze whether the amino acid substitutions lead to an impaired catalysis or to an inhibition of lipid binding at the interface, we also measured the esterolytic activities independent of lipid interaction using the water-soluble short-chain triglyceride substrate tributyrin (C4:0). Unlike triolein, tributyrin does not produce a lipid interface at or below a concentration of 0.25 mM (28). The results paralleled those obtained with triolein as substrate (Table 1). In contrast to HLAL(Ser_{99} → Thr) which exhibited 28% and 34% esterase activity in transfected Ltk$^-$ mouse fibroblasts and Cos-7 cells, respectively, the mutant HLAL(Ser_{153} → Thr) had lost almost all activity towards the tributyrin substrate. Thus, Ser_{153} appears to be necessary for the hydrolysis of both emulsified and monodisperse lipids by HLAL.

These results were confirmed by transient in vitro HGL expression studies. Consistent with a catalytic role for Ser_{153}, the mutant enzyme HGL(Ser_{153} → Thr) had on average less than 4% and 6% of wild-type activity in the extracts and media, respectively, of both cell lines (**Fig. 2** and **Table 2**). Alteration of Ser_{99}, in contrast, yielded a partially functional enzyme that hydrolyzed 41% and 45% of triolein in the Cos-7 cell extracts and

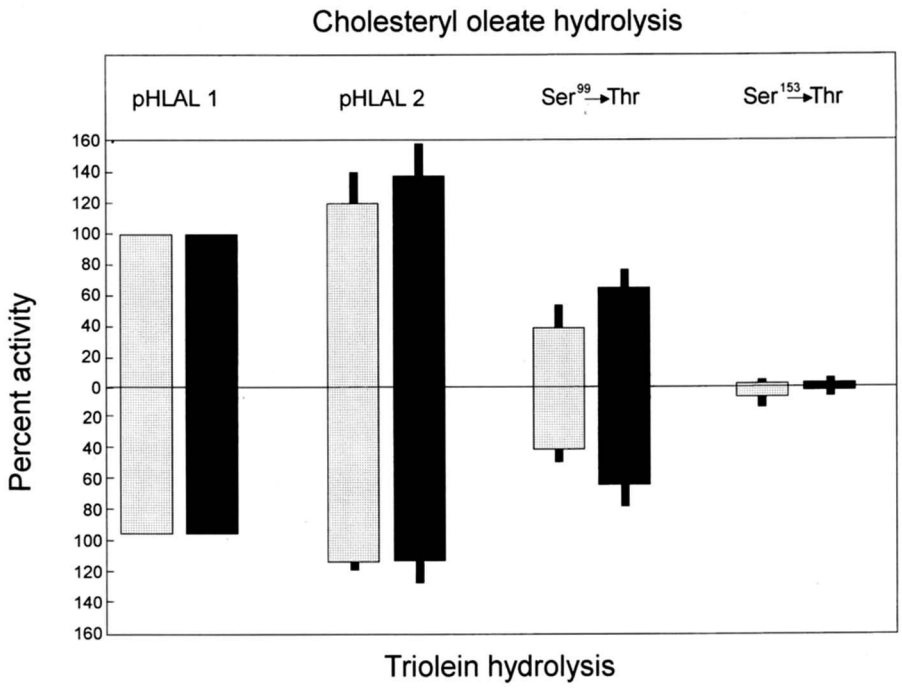

Fig. 1. Hydrolysis of cholesteryl oleate (top panel) and triolein (bottom panel) in Cos-7 (hatched boxes) and Ltk⁻ cells (solid boxes) transfected with two wild-type HLAL cDNA constructs and two mutagenized cDNAs coding for Thr_{99} and Thr_{153} instead of serine. Data are expressed as percent of activity relative to the normal construct pCMV-HLAL 1 (defined as 100%) and were normalized for transfection efficiency with respect to the luciferase activity in the same cell lysates. The error bars indicate standard deviations calculated from the results of at least four independent transfections (see Table 1).

media, respectively, and 35% and 34% of triolein in the kidney-293 cell homogenates and media, respectively.

To assess whether the amino acid substitutions at HGL residues 99 and 153 affected the ability of the enzyme to bind lipid at the oil–water interface, we also measured the rate of hydrolysis of the monodisperse substrate tributyrin. HGL(Ser_{153} → Thr) was virtually inactive as an esterase (Fig. 2 and Table 2), consistent with the hypothesis that Ser_{153} is one of the residues of the catalytic triad of HGL. Substitution of threonine for serine at position 99, in contrast, resulted in a mutant enzyme that retained 76% and 57% of activity against tributyrin in the extracts and 97% and 75% of hydrolytic activity in the media of Cos-7 and human kidney-293 cells, respectively.

Active site histidine

Six conserved histidines were changed to glutamine because of the loss of the imidazole ring which normally links the active site serine to an acidic residue. Substitution of His_{262}, His_{298}, and His_{345}, although absolutely conserved in HLAL, HGL, and RLL, resulted in mutant

TABLE 1. Cholesteryl oleate, triolein, and tributyrin hydrolysis in Cos-7 cells and Ltk⁻ mouse fibroblasts transfected with wild-type and mutant HLAL(Ser → Thr) cDNA constructs

	% of Activity Relative to pCMV-HLAL1									
	Cholesteryl Oleate				Triolein				Tributyrin			
Plasmid	Cos-7	Ltk⁻	n		Cos-7	Ltk⁻	n		Cos-7	Ltk⁻	n	
pCMV-HLAL1	100	100			100	100			100	100		
pCMV-HLAL2	121 ± 21	139 ± 23	4	4	120 ± 7	120 ± 14	4	4				
pCMV-HLAL(Ser_{99} → Thr)	39 ± 12	63 ± 14	6	5	41 ± 8	66 ± 13	5	6	34 ± 8	28 ± 8	6	7
pCMV-HLAL(Ser_{153} → Thr)	1 ± 1	1 ± 2	9	7	3 ± 3	1 ± 2	8	7	7 ± 6	8 ± 7	6	8

Levels of esterase activity of the mutagenized enzymes were normalized for transfection efficiency by luciferase expression and are shown as mean values ± SD relative to wild-type HLAL (defined as 100%). The number (n) of independent transfection experiments used for the calculations is given. Specific activities of recombinant normal HLAL for cholesteryl oleate, triolein, and tributyrin averaged 108, 121, and 85 pmol of free fatty acids released per min per mg protein in transfected Ltk⁻ mouse fibroblasts and 160, 189, and 115 pmol FFA per min per mg protein in Cos-7 cells, respectively.

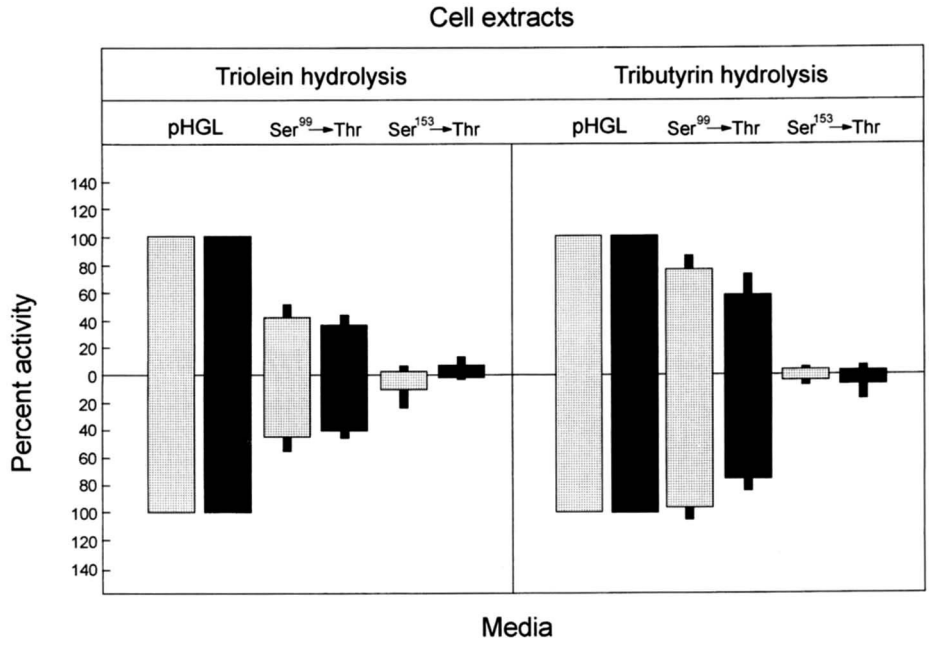

Fig. 2. Hydrolysis of triolein and tributyrin in the cell extracts (top panel) and media (bottom panel) of Cos-7 (hatched boxes) and human kidney-293 cells (solid boxes) transfected with a wild-type HGL cDNA construct and two mutagenized cDNAs coding for Thr_{99} and Thr_{153} instead of serine. Enzyme activities were measured and normalized according to luciferase expression in the cell extracts. Data are shown as percent of activity relative to the normal construct pCMV-HGL (defined as 100%) and represent the mean values ± standard deviation of at least six independent transfections (see Table 2).

HLAL enzymes with significant lipolytic activities in both cell lines (**Fig. 3** and **Table 3**). Replacement of the remaining three conserved residues His_{65}, His_{274}, and His_{353}, in contrast, dramatically reduced HLAL activity, virtually eliminating both cholesteryl oleate and triolein hydrolysis.

Based on these observations, we introduced three analogous amino acid substitutions into the HGL cDNA by the PCR overlap extension method. In accordance with the results demonstrating an essential role of HLAL residues His_{65} and His_{353} for the breakdown of neutral lipids, the mutagenized enzymes HGL(His_{65} → Gln) and HGL(His_{353} → Gln) had 3% and 1% of normal HGL activity against triolein in kidney-293 cell extracts, respectively, whereas, in the media, substrate hydrolysis was even lower (**Fig. 4** and **Table 4**). Cos-7 cell homogenates and media transfected with the HGL(His_{353} → Gln) cDNA converted about 5% of the long-chain triglyceride substrate, while expression of HGL(His_{65} → Gln) was accompanied by a 99% and 98% reduction of lipolytic activity in the Cos-7 cell extracts and supernatants, respectively. The two mutant lipases were also vir-

TABLE 2. Triolein and tributyrin hydrolysis in the extracts and media of Cos-7 and human kidney-293 cells transfected with wild-type and mutant HGL(Ser → Thr) cDNA constructs

	% of Activity Relative to pCMV-HGL															
	Triolein								Tributyrin							
	Cos-7		293						Cos-7		293					
Plasmid	Cells	Media	Cells	Media	n				Cells	Media	Cells	Media	n			
pCMV-HGL	100	100	100	100					100	100	100	100				
pCMV-HGL(Ser_{99} → Thr)	41 ± 8	45 ± 10	35 ± 10	34 ± 6	7	6	6	8	76 ± 9	97 ± 6	57 ± 15	75 ± 8	8	8	7	9
pCMV-HGL(Ser_{153} → Thr)	1 ± 2	10 ± 11	6 ± 6	2 ± 1	8	9	8	8	1 ± 1	4 ± 4	2 ± 1	6 ± 7	9	8	8	8

Percent of activity relative to normal HGL (defined as 100%) was determined after normalization for luciferase expression in the cell homogenates. The number (n) of independent transfections performed to obtain the mean values ± SD is illustrated. Native HGL specific activities for triolein and tributyrin were on average 130 and 406 pmol FFA per min per mg protein in Cos-7 cell extracts and 95 and 310 pmol FFA per min per mg protein in kidney-293 cell homogenates, respectively. In the media, triolein and tributyrin hydrolyzing HGL activities were distinctly higher, averaging 242 and 735 pmol FFA per min per mg protein in Cos-7 cell supernatants and 236 and 629 pmol FFA per min per mg protein in kidney-293 cell supernatants, respectively.

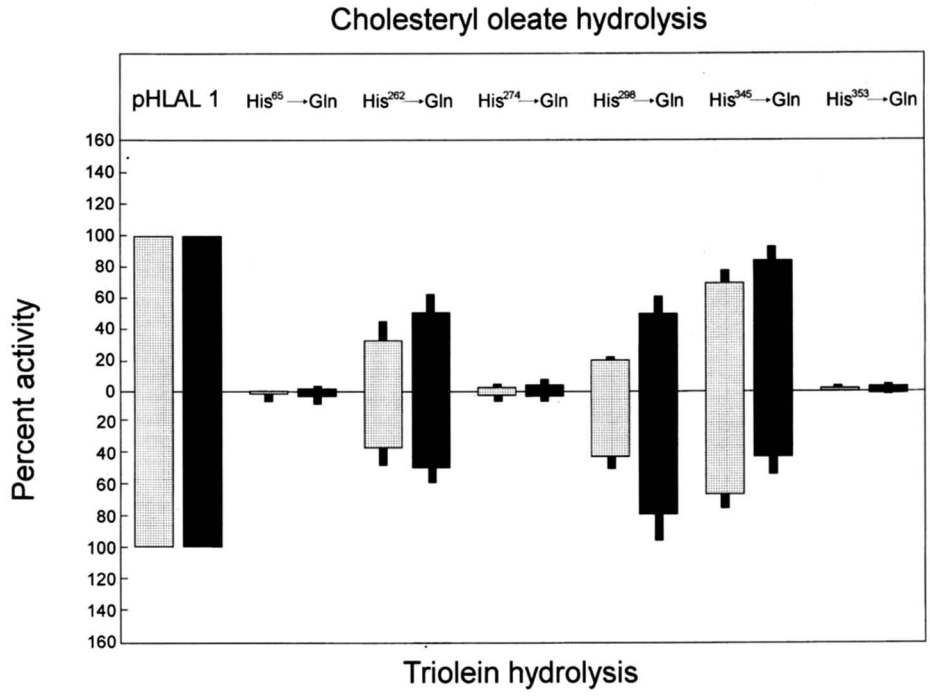

Fig. 3. Hydrolysis of cholesteryl oleate (top panel) and triolein (bottom panel) in Cos-7 (hatched boxes) and Ltk$^-$ cells (solid boxes) transfected with a wild-type HLAL cDNA construct and six mutagenized cDNAs coding for glutamine instead of histidine at amino acid position 65, 262, 274, 298, 345, and 353. The error bars indicate standard deviations calculated from the results of at least four independent transfections (see Table 3).

tually inactive as esterases, hydrolyzing on average about 1% to 2% of the monodisperse substrate tributyrin in the homogenates and in the media of transfected kidney-293 and Cos-7 cells.

In contrast to the corresponding HLAL mutant, the HGL enzyme with glutamine instead of histidine at position 274 was partially functional, hydrolyzing approximately 24% and 22% of the triolein and 38% and 21% of the tributyrin in kidney-293 and Cos-7 cell extracts, respectively. In the media of kidney-293 and Cos-7 cells, however, only 2% and 5%, respectively, of the triolein was converted to free fatty acids. The activity towards the water-soluble tributyrin was also lower, averaging 30% and 16%, respectively (Fig. 4 and Table 4).

Active site aspartic acid

The carboxylate of the catalytic triad is contributed by either Asp or Glu. The amino acid sequences of HLAL, HGL, and RLL contain eight conserved aspartic acids between His_{65} and His_{353} at amino acid positions 89, 93, 124, 130, 257, 324, 328, and 331. Four of these (residues 257, 324, 328, and 331) are the most likely carboxylic acid candidates, assuming that the linear sequence of the active site residues is Ser_{153}–Asp–His_{353}. There are also five glutamic acids absolutely conserved within this lipase family at positions 11, 29, 35, 117, and 125. However, because of their amino-terminal location with respect to Ser_{153}, it appears highly unlikely that one of these Glu residues is part of the catalytic center.

TABLE 3. Cholesteryl oleate, triolein, and tributyrin hydrolysis in Cos-7 cells and Ltk$^-$ mouse fibroblasts transfected with wild-type and mutant HLAL(His → Gln) cDNA constructs

	% of Activity Relative to pCMV-HLAL1											
	Cholesteryl Oleate				Triolein				Tributyrin			
Plasmid	Cos-7	Ltk$^-$	n		Cos-7	Ltk$^-$	n		Cos-7	Ltk$^-$	n	
pCMV-HLAL1	100	100			100	100			100	100		
pCMV-HLAL(His_{65} → Gln)	0 ± 0	1 ± 1	5	4	2 ± 5	4 ± 5	7	7	10 ± 8	3 ± 7	4	6
pCMV-HLAL(His_{262} → Gln)	33 ± 13	52 ± 12	5	5	38 ± 12	51 ± 10	6	4				
pCMV-HLAL(His_{274} → Gln)	2 ± 2	3 ± 3	10	10	3 ± 4	4 ± 4	8	11	5 ± 12	10 ± 10	5	7
pCMV-HLAL(His_{298} → Gln)	20 ± 2	51 ± 12	4	4	44 ± 8	79 ± 18	4	4				
pCMV-HLAL(His_{345} → Gln)	72 ± 8	88 ± 19	6	7	66 ± 9	41 ± 14	7	5				
pCMV-HLAL(His_{353} → Gln)	1 ± 2	2 ± 2	8	8	0 ± 0	2 ± 3	8	8	7 ± 9	3 ± 6	6	5

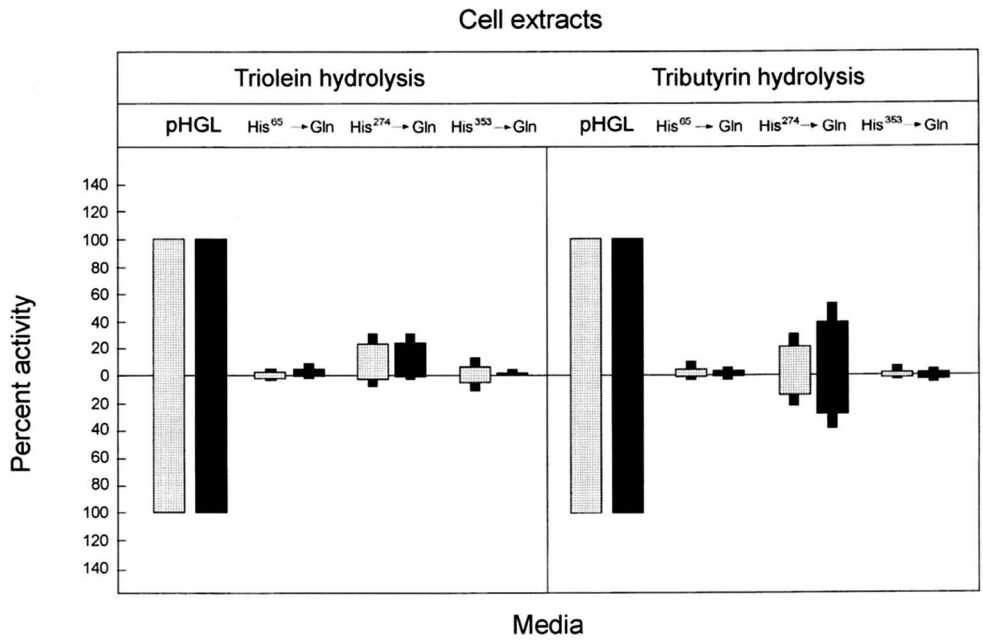

Fig. 4. Hydrolysis of triolein and tributyrin in the cell extracts (top panel) and media (bottom panel) of Cos-7 (hatched boxes) and human kidney-293 cells (solid boxes) transfected with a wild-type HGL cDNA construct and three cDNA mutants coding for Gln_{65}, Gln_{274}, and Gln_{353} instead of histidine. Data represent the mean values ± standard deviation of at least four independent transfections (see Table 4).

In order to investigate the functional role of the conserved aspartates, we created eight additional mutant enzymes with the standard glycine substitution. HLAL(Asp_{89} → Gly), HLAL(Asp_{124} → Gly), and HLAL(Asp_{257} → Gly) were all found to be catalytically active towards cholesteryl oleate as well as triolein in both cell lines, although to a lesser degree than the wild-type enzyme (**Fig. 5** and **Table 5**). Substitution of Asp_{93}, Asp_{130}, and Asp_{328} by glycine resulted in lipases that retained 22–31%, 15–19%, and 36–43% of hydrolytic activity towards triolein, respectively, in Cos-7 cells and Ltk⁻ mouse fibroblasts, but converted only 3% (in the case of HLAL(Asp_{93} → Gly) and HLAL(Asp_{130} → Gly)) and 5–11% (in the case of HLAL(Asp_{328} → Gly)) of the cholesteryl oleate, suggesting that these residues are especially important for the hydrolysis of cholesteryl esters. Glycine at amino acid positions 324 or 331, in contrast, completely abolished HLAL enzymic activity with both substrates, indicating that one of these residues may be part of the catalytic triad. Hydrolysis of the water-soluble tributyrin was also severely impaired. HLAL(Asp_{331} → Gly) demonstrated 1–3% and HLAL(Asp_{324} → Gly) 3–5% of wild-type activity (Table 5).

To unambiguously identify the active site aspartic acid residue, three more mutants were made by replacing Asp_{324}, Asp_{328}, and Asp_{331} in HGL. In the lysates and media from Cos-7 and kidney-293 cells transfected with the construct pCMV-HGL(Asp_{328} → Gly), normal enzymatic activity towards triolein and tributyrin could be detected (**Fig. 6** and **Table 6**). HGL(Asp_{331} → Gly) was also active in the cell lysates, hydrolyzing 79–88% of the

TABLE 4. Triolein and tributyrin hydrolysis in the extracts and media of Cos-7 and human kidney-293 cells transfected with wild-type and mutant HGL(His → Gln) cDNA constructs

	% of Activity Relative to pCMV-HGL															
	Triolein								Tributyrin							
	Cos-7		293						Cos-7		293					
Plasmid	Cells	Media	Cells	Media	n				Cells	Media	Cells	Media	n			
pCMV-HGL	100	100	100	100					100	100	100	100				
pCMV-HGL(His_{65} → Gln)	1 ± 1	2 ± 2	3 ± 3	1 ± 1	4	5	6	8	4 ± 5	2 ± 2	2 ± 1	1 ± 2	4	4	8	9
pCMV-HGL(His_{274} → Gln)	22 ± 10	5 ± 5	24 ± 8	2 ± 1	7	8	8	11	21 ± 8	16 ± 7	38 ± 13	30 ± 10	6	7	8	6
pCMV-HGL(His_{353} → Gln)	5 ± 4	5 ± 6	1 ± 1	0 ± 0	10	7	7	8	1 ± 3	1 ± 1	1 ± 1	2 ± 2	8	5	8	8

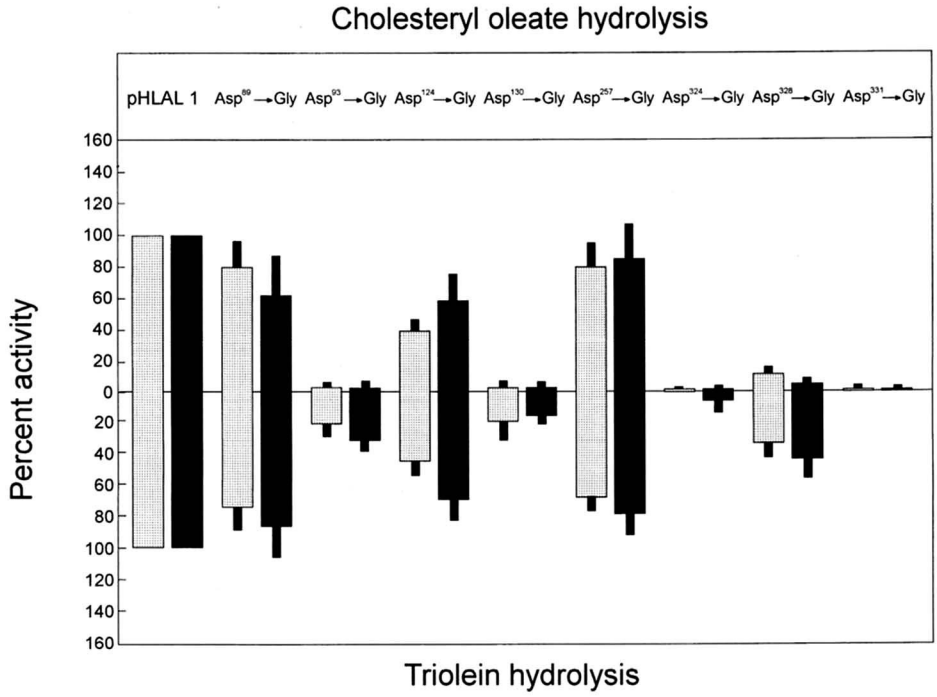

Fig. 5. Hydrolysis of cholesteryl oleate (top panel) and triolein (bottom panel) in Cos-7 (hatched boxes) and Ltk$^-$ cells (solid boxes) transfected with a wild-type HLAL cDNA and eight mutagenized cDNAs coding for glycine instead of aspartic acid at residue 89, 93, 124, 130, 257, 324, 328, and 331. Data are reported as mean ± SD of at least five independent transfections (see Table 5).

triolein and 55–79% of the tributyrin, which proved that Asp_{331} is not part of the active site. Enzymic activity in the media, however, was reduced to 2–26% of wild-type levels. Replacement of Asp_{324}, in contrast, almost completely abolished triolein and tributyrin hydrolysis in the cell lysates and media from Cos-7 and kidney-293 cells, the remaining activity averaging 3% of normal values. These data demonstrate that Asp_{324} contributes to enzyme catalysis probably by stabilizing the charges of the His^+ t^- transition state (5).

DISCUSSION

In spite of our increased knowledge concerning the molecular basis of interfacial activation and lipid hydrolysis, many questions remain unanswered. In the case of the acid lipase family, for example, a catalytic triad has not yet been defined experimentally and it is still unknown whether or not these enzymes are in fact true lipases. Earlier attempts to identify the amino acids involved in substrate catalysis have produced conflicting

TABLE 5. Cholesteryl oleate, triolein, and tributyrin hydrolysis in Cos-7 cells and Ltk$^-$ mouse fibroblasts transfected with wild-type and mutant HLAL(Asp → Gly) cDNA constructs

	% of Activity Relative to pCMV-HLAL1											
	Cholesteryl Oleate				Triolein				Tributyrin			
Plasmid	Cos-7	Ltk$^-$	n		Cos-7	Ltk$^-$	n		Cos-7	Ltk$^-$	n	
pCMV-HLAL1	100	100			100	100			100	100		
pCMV-HLAL(Asp_{89} → Gly)	79 ± 17	61 ± 25	8	7	73 ± 14	85 ± 20	8	7				
pCMV-HLAL(Asp_{93} → Gly)	3 ± 3	3 ± 4	8	9	22 ± 8	31 ± 7	7	5				
pCMV-HLAL(Asp_{124} → Gly)	39 ± 7	58 ± 17	5	5	46 ± 9	68 ± 14	7	7				
pCMV-HLAL(Asp_{130} → Gly)	3 ± 4	3 ± 3	8	9	19 ± 12	15 ± 5	6	6				
pCMV-HLAL(Asp_{257} → Gly)	79 ± 15	84 ± 22	6	6	67 ± 9	77 ± 14	8	5				
pCMV-HLAL(Asp_{324} → Gly)	1 ± 1	1 ± 2	10	10	0 ± 0	5 ± 7	8	10	5 ± 7	3 ± 7	6	7
pCMV-HLAL(Asp_{328} → Gly)	11 ± 4	5 ± 3	8	9	36 ± 8	43 ± 12	10	5	7 ± 7	2 ± 5	8	8
pCMV-HLAL(Asp_{331} → Gly)	1 ± 2	1 ± 1	8	8	0 ± 0	0 ± 0	6	6	1 ± 3	3 ± 6	5	5

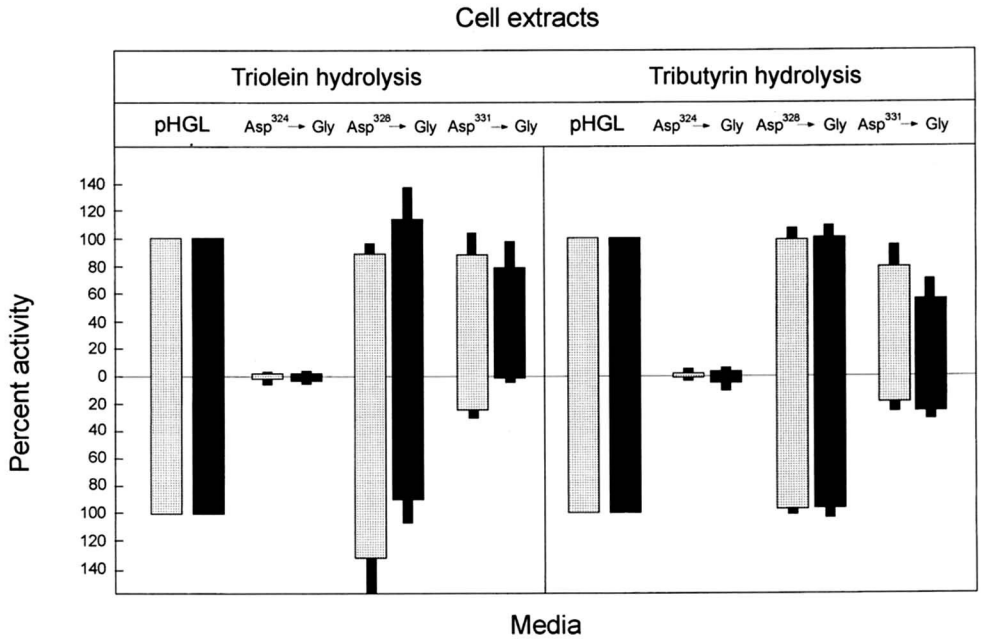

Fig. 6. Hydrolysis of triolein and tributyrin in the cell extracts (top panel) and media (bottom panel) of Cos-7 (hatched boxes) and human kidney-293 cells (solid boxes) transfected with a wild-type HGL cDNA construct and three cDNA mutants coding for Gly_{324}, Gly_{328}, and Gly_{331} instead of aspartate. Each bar represents the mean ± SD of at least four independent transfections (see Table 6).

results. For many years, acid lipases were thought to be sulfhydryl enzymes because of their sensitivity towards SH-reactive substances (14, 29–31). Recent studies, however, have shown that both human and rabbit gastric lipases (32) as well as HLAL (17) were stoichiometrically inactivated by chemical modification with diethyl *p*-nitrophenyl phosphate (E600), indicating that a serine residue may be important for enzymatic activity. Further support for a serine as the catalytic nucleophile came from experiments using boronic acid (29) as well as the specific lipase inhibitor tetrahydrolipstatin (33–35).

We have chosen another approach to determine the catalytic amino acids by using site-directed mutagenesis to genetically modify serine, aspartic acid, and histidine residues absolutely conserved within the acid lipase family. Extensive evaluation of the hydrolytic properties of 16 HLAL and 8 HGL mutants demonstrates that His_{65}, Ser_{153}, Asp_{324}, and His_{353} are essential amino acids for the catabolism of cholesteryl esters as well as long- and short-chain triglycerides. The study also gives strong support to the view that the two enzymes are very similar in structure and function.

The identification of Ser_{153} as one of the three triad residues is in agreement with sequence comparisons that demonstrate that the pentapeptide around Ser_{153} has a higher degree of similarity to other lipase consensus sequences than the motif containing Ser_{99} and is more likely part of the catalytic triad (1). Our data are also in line with a recently published study expressing HLAL(Ser_{153} → Ala) in a heterologous baculovirus system (35).

TABLE 6. Triolein and tributyrin hydrolysis in the extracts and media of Cos-7 and human kidney-293 cells transfected with wild-type and mutant HGL(Asp → Gly) cDNA constructs

	% of Activity Relative to pCMV-HGL															
	Triolein								Tributyrin							
	Cos-7		293						Cos-7		293					
Plasmid	Cells	Media	Cells	Media	n				Cells	Media	Cells	Media	n			
pCMV-HGL	100	100	100	100					100	100	100	100				
pCMV-HGL(Asp_{324} → Gly)	2 ± 3	4 ± 4	2 ± 4	3 ± 2	8	6	8	9	2 ± 3	3 ± 2	1 ± 2	5 ± 6	10	8	9	9
pCMV-HGL(Asp_{328} → Gly)	89 ± 7	131 ± 19	115 ± 23	90 ± 17	6	7	6	7	99 ± 8	97 ± 4	101 ± 8	97 ± 6	8	8	8	8
pCMV-HGL(Asp_{331} → Gly)	88 ± 17	24 ± 6	79 ± 19	2 ± 2	6	4	6	8	79 ± 16	19 ± 7	55 ± 15	26 ± 5	6	6	7	7

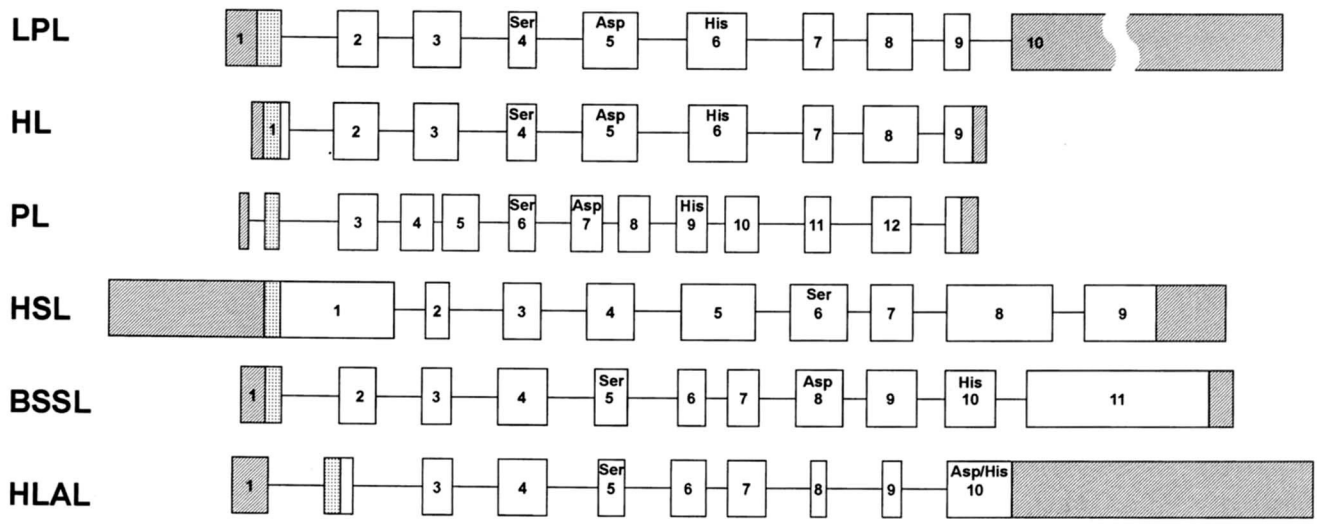

Fig. 7. Comparison of the human LPL, human HL, human PL, human HSL, human BSSL, and HLAL gene organizations. A schematic diagram of the exon/intron structures of six mammalian lipases is shown. Signal peptide, mature protein, and noncoding sequences appear as stippled, open, and hatched boxes, respectively. Introns (not drawn to scale) are indicated by lines. The known or putative exon location of the catalytic triad residues serine, aspartic acid, and histidine is illustrated within the open boxes. Note that exon 10 of LPL is also not drawn to scale.

Substitution of HLAL and HGL residues His_{65} and His_{353} also resulted in an almost complete loss of the esterolytic properties of the enzymes. His_{65} is a component of the His–Gly dipeptide of lipases which in most cases is located near the amino-terminus of the enzymes and preceded by hydrophobic residues. The high degree of sequence conservation led to the assumption that this dipeptide marks the active site histidine (36). However, this is contradicted by the absence of the HG motif in some of the lipases (e.g., from *Candida rugosa* (FG), *Geotrichum candidum* (YG), *Rhizomucor miehei* (RG), and *Rhizopus delamar* (RG); 37) and by the results of the crystallographic analyses. In human pancreatic lipase, the HG dipeptide (residues 75 and 76; nomenclature according to ref. 38) is located at the border of a surface "wing", the β5-loop (amino acids 76–85), which flanks the catalytic site and leans against the closed lid. Opening of the lid in the presence of a lipid substrate at the oil–water interface is accompanied by a structural reorganization of the β5-loop which rolls back upon the core of the enzyme. These loop movements expose the catalytic triad, increase the hydrophobic, solvent-exposed surface area, and create the electrophilic oxyanion hole which stabilizes the reaction transition states (39).

Considering the high degree of sequence conservation in the region between HLAL, HGL, and RLL residues Lys_{58} and Asp_{70} and the essentially identical stereochemistry of lipases, our data also point to an important functional role for the polypeptide chain segment containing His_{65} in acid lipase-mediated substrate catalysis.

His_{353}, on the other hand, is proposed to be a component of the catalytic triad of HLAL, HGL, and RLL. This is based on the results of our site-directed mutagenesis experiments, on the linear order of the active site residues within the protein sequence of lipases, and on comparisons of the tripeptides surrounding the essential histidine (Glu–His–Leu in HLAL and Asn–His–Leu in HGL and RLL) with corresponding regions in human, mouse, pig, and dog PL (Asn–His–Leu), in *Pseudomonas* lipases (Asn/Asp-His-Leu), and in *Rhizomucor miehei* lipase (Asp-His-Leu; 1, 40).

Replacement of His_{274}, in contrast, leads to an inactive HLAL mutant and to a partially active HGL enzyme. Interestingly, an amino acid substitution of His_{274} by tyrosine has been identified in an 11-year-old patient with cholesteryl ester storage disease (41). HLAL activity in skin fibroblasts and in peripheral lymphocytes of the patient was reduced to 3–4% of control values. These data agree with our in vitro studies which demonstrate that replacement of His_{274} by glutamine reduces cholesteryl oleate and triolein hydrolysis by 97–98%.

Homologies to other human lipases

Comparison of the HLAL (42–44) and HGL gene organizations (44) with those of lipoprotein lipase (45, 46), hepatic lipase (47, 48), pancreatic lipase (49), hormone-sensitive lipase (50), and bile salt-stimulated-lipase/pancreatic cholesterol esterase (51) revealed that the HLAL and HGL genes are similar in structure to the BSSL gene, based on the topological position of the putative catalytic triad residues (**Fig. 7**).

Asp_{324} and His_{353} of HLAL and HGL are both encoded by the carboxyl-terminal exon 10, while the co-

don for Ser_{153} is located in exon 5 in both genes (Fig. 7). In the BSSL gene, the catalytic Asp_{320} (52) and His_{435} (53) codons are present in exons 8 and 10, respectively, and Ser_{194} (54) is also encoded by exon 5. In the LPL, HL, and PL genes, in contrast, the residues of the catalytic triad are located in the central portion of the gene structures, in exons 4, 5, and 6 of the LPL and HL genes and in the corresponding exons 6, 7, and 9 of the PL gene (for review see ref. 55).

The structural similarities of the HLAL and BSSL genes are intriguing as both enzymes are lipases with cholesteryl esterase activities. HLAL and BSSL may be either evolutionarily related lipases/cholesteryl ester hydrolases, despite undetectable sequence homology, or the result of convergent evolution due to exon shuffling which combined lipase-like and cholesteryl esterase-like domains, as has been suggested previously for the evolution of the rat BSSL gene (56). HGL, on the other hand, may have lost the ability to hydrolyze cholesteryl esters as a consequence of mutations affecting one or more sites necessary for substrate interaction.

In summary, site-directed mutagenesis was performed to alter serine, histidine, and aspartic acid codons completely conserved among the acid lipases and to produce mutant HLAL and HGL enzymes. In vitro expression and determination of enzymatic activities revealed that mutations of His_{65}, Ser_{153}, Asp_{324}, and His_{353} drastically reduced cholesteryl oleate, triolein, and tributyrin hydrolysis. Based on these results, we conclude that Ser_{153}, Asp_{324}, and His_{353} form the catalytic triad of the acid lipase family. His_{65} of the conserved His–Gly dipeptide of lipases, on the other hand, is proposed to reside at the border of a surface loop similar to the β5-loop of pancreatic lipase. Substitution of His_{65} with glutamine may either interfere with loop movement upon substrate binding or with the formation of the oxyanion hole. Asp_{93}, Asp_{130}, and Asp_{328}, in contrast, appear to be especially critical for HLAL-mediated cholesteryl ester catabolism.

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