# Human lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: site-directed mutagenesis of $Cys_{227}$ and $Cys_{236}$ results in substrate-dependent reduction of enzymatic activity

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**Abstract** Chemical modification studies and site-directed mutagenesis experiments have provided evidence that human lysosomal acid lipase/cholesteryl ester hydrolase (HLAL), human gastric lipase (HGL), and rat lingual lipase (RLL) are serine esterases. Loss of HLAL and HGL activity was also observed in the presence of sulfhydryl-reactive substances, suggesting that cysteines are likewise essential for substrate hydrolysis. To study the functional role of the HLAL and HGL cysteine residues, we replaced these amino acids with alanine by site-directed mutagenesis. Substitutions at positions 227 and 236, alone or together, drastically reduced hydrolytic activity in a substrate-dependent manner while the other mutants were not affected to any great extent. HLAL($Cys_{227} \rightarrow$ Ala), HLAL($Cys_{236} \rightarrow$ Ala), and HLAL($Cys_{227} \rightarrow$ Ala, $Cys_{236} \rightarrow$ Ala) were essentially inactive against cholesteryl oleate, but retained about 23–39%, 28–37%, and 13–17% of catalytic activity for both triolein and tributyrin, respectively. The data obtained with the corresponding HGL mutants confirmed the importance of residues 227 and 236 in maintaining enzymatic activity towards long- and short-chain triglycerides. In order to assess the contribution of the eight amino acids delimited by $Cys_{227}$ and $Cys_{236}$ to lipolysis, we generated HLAL replacement mutants containing the corresponding residues 228–235 of HGL or RLL. Both HLAL chimeras were catalytically active towards all three substrates analyzed, indicating that these amino acids do not determine HLAL substrate specificity. Deletion of the eight-amino acid α-helix as well as disruption of its hydrophobic surface, in contrast, abolished enzymatic activity. Our studies suggest that $Cys_{227}$, $Cys_{236}$, and the amphipathic helix formed by residues 228-235 are essential for HLAL- and HGL-mediated neutral lipid catabolism.—**Lohse, P., P. Lohse. S. Chahrokh-Zadeh, and D. Seidel.** Human lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: site-directed mutagenesis of $Cys_{227}$ and $Cys_{236}$ results in substrate-dependent reduction of enzymatic activity. *J. Lipid Res.* 1997. **38:** 1896–1905.

**Supplementary key words** lipid metabolism • serine hydrolase • sulfhydryl enzyme • structure–function relationships • catalytic site • lipid–enzyme interaction

Human lysosomal acid lipase/cholesteryl ester hydrolase (HLAL; EC 3.1.1.13), a 43,000-Dalton protein, plays a central role in lipoprotein and intracellular lipid metabolism (for review see refs. 1 and 2). The enzyme is synthesized by virtually all cells except erythrocytes and hydrolyzes cholesteryl esters and triglycerides contained within endocytosed lipoprotein particles at acid pH to cholesterol, di- and monoglycerides, and free fatty acids. The latter are transferred from the lysosomal compartment to the cytoplasm and used for energy production and biosynthetic processes, or they may become reesterified for storage. An inherited deficiency or low activity of the enzyme results in the intralysosomal storage of the lipid substrates, known as fatal Wolman's disease in infants and as more benign cholesteryl ester storage disease (CESD) in adults.

During the initial characterization of HLAL, it was noted that the protein was sensitive to sulfhydryl-blocking reagents and that thiols were required for stabilization of enzyme activity (3), indicating that cysteine residues may be important for substrate catalysis.

Subsequent cloning of the HLAL cDNA (4) revealed that the protein is a member of a gene family of mammalian acid lipases, the 378 amino acid sequence being 58% and 57% identical with the deduced sequence of human gastric lipase (HGL; 379 residues; 5) and rat lingual lipase (RLL; 377 residues; 6), respectively. In all three enzymes, a cluster of conserved cysteines is found at amino acid positions 227, 236, and 244. RLL has an additional cysteine at residue 27 and HLAL contains six cysteines within the mature protein and another one in the signal peptide.

The three enzymes also share the presence of two so-

Abbreviations: BSSL, bile salt-stimulated lipase; HGL, human gastric lipase; HLAL, human lysosomal acid lipase; RGL, rabbit gastric lipase; RLL, rat lingual lipase.

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called lipase consensus sequences -Gly-Xaa-Ser-Xaa-Gly- (where Xaa represents any amino acid) at amino acid positions 97–101 and 151–155. These motifs contain the catalytically active serine residue in almost all the known lipases. Chemical modification of HLAL (3, 4) and HGL (7) with boronic acids and diethyl-*p*-nitrophenyl phosphate (E600) as well as recent studies using mutagenized HLAL (8, 9) and HGL cDNA constructs (9) have shown that, despite the importance of one or more sulfhydryl groups for catalytic activity, these enzymes are serine hydrolases, $Ser_{153}$ of HLAL and HGL being the nucleophilic residue essential for substrate hydrolysis as part of the classical Ser-Asp-His triad of mammalian lipases (for review see refs. 10–13).

The purpose of this study was to determine the involvement of cysteine residues in the enzymatic reactions catalyzed by HLAL and HGL. Using the site-directed mutagenesis approach, we replaced the six cysteines of HLAL and the three cysteines of HGL with alanine, expressed the mutant lipases in Cos-7 kidney and human embryonal kidney-293 cells as well as $Ltk^-$ mouse fibroblasts, and assessed enzyme activity by measuring cholesteryl oleate, triolein, and tributyrin hydrolysis in the transfected cell extracts and, in the case of the HGL constructs, in the media.

## MATERIALS AND METHODS

### Isolation of HLAL cDNA

Human leukocytes were lysed in guanidinium isothiocyanate (14) and total RNA was recovered by ultracentrifugation through a CsCl gradient. A complementary copy of HLAL mRNA was obtained with Superscript reverse transcriptase (Bethesda Research Laboratories) using an oligonucleotide primer with an artificial Hpa I restriction site complementary to the 5′-end (5′-CATT TTCACATGACATAATCGTTAACTTGGTGGTACACA GC-3′) of the 3′-nontranslated region contained in HLAL exon 10. This cDNA was further amplified by the polymerase chain reaction (PCR) technique (15) using a sense primer with an incorporated restriction enzyme site for Nhe I (5′-ACTGCGACTCGAGCTAGCGGCCCG GCAGGACAG-3′) located in exon 1.

### Isolation of HGL cDNA

Total RNA was prepared from human stomach tissue. Ten μg was reverse transcribed to cDNA using an oligo$(dT)_{18}$ primer that contained, in addition, artificial restriction sites for Sma I, BamH I, and Hpa I. One-tenth of the reaction mixture was subsequently amplified by PCR using an exon 1 sense primer with an artificial Xba I site (5′-GAAACAGAATTCTAGATATTTC TG-3′) and an oligonucleotide with an incorporated Hpa I site complementary to the 3′-nontranslated region (5′-CAAACGGTTAACTCTTTAAATCC-3′) or a primer identical to the restriction enzyme sites incorporated into the oligo $(dT)_{18}$.

### Site-directed mutagenesis

A 1258 bp fragment of HLAL cDNA and a HGL cDNA of 1239 bp, which spanned the signal peptide through the termination codon, were cloned into the Xba I and Hpa I restriction sites of the pUC 18-derived eukaryotic expression vector pCMV (16) and thereby placed under the control of the cytomegalovirus (CMV) immediate early promoter. These constructs, designated as pCMV-HLAL and pCMV-HGL, were subsequently used as templates for oligonucleotide-mediated site-directed mutagenesis by the overlap extension PCR method (17). The mutant cDNAs were subcloned into the pCMV vector and all inserts were characterized by the dideoxy chain termination method (18) using T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH).

### In vitro expression of HLAL cDNA constructs

Recombinant plasmid DNA was purified by the cesium chloride double banding method (19). At least six individual transfections of Cos-7 cells and $Ltk^-$ mouse fibroblasts (American Type Culture Collection) were performed with 10 μg of wild-type or mutagenized plasmid DNA and 1 μg of the reporter construct pRSV-Luciferase (20) per 10-cm dish, using the calcium phosphate–DNA coprecipitation protocol (21). After a glycerol shock and removal of plasmid DNA, cells were maintained for 72 h at 37°C in a humidified $CO_2$ incubator to obtain high level expression of HLAL (4).

### HLAL enzyme assay

Cells were rinsed twice with phosphate-buffered saline, harvested by scraping, and sonified in 0.5% Triton X-100. Protein content of the cell extracts was determined by an automated Lowry method (22) on a Cobas Fara analyzer (Hoffmann-LaRoche AG, FRG). HLAL activity was assayed at pH 4 using the radiolabeled substrates cholesteryl[1-$^{14}$C]oleate (sp act 50–60 mCi/mM; Amersham, Arlington Heights, IL), glycerol-tri-[1-$^{14}$C]oleate (triolein; sp act 50–60 mCi/mM; Amersham, Arlington Heights, IL), and glycerol-tri-[1-$^{14}$C]butyrate (tributyrin; sp act 39 mCi/mM; Sigma Chemical Co., St. Louis, MO). Reaction mixtures contained, in a volume of 100 μl, 45–75 μg protein as enzyme source, 63 μM 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, 0.05% Triton X-100, 5 mM β-mercaptoethanol, and 1 mM EDTA. After incubation for 3 h at 37°C, the reactions

were terminated by the addition of 0.1 mM cholesteryl oleate, triolein, or tributyrin in chloroform–heptane–methanol 1.3:1:1.4 and 0.1 M NaOH (23). The upper phase containing the radiolabeled free fatty acids was dissolved in scintillation fluid and counted.

### In vitro expression of HGL cDNA constructs

Wild-type and mutant plasmids were purified on QIAGEN-tips (QIAGEN Inc., Chatsworth, CA). Transfections of human embryonal kidney-293 cells (American Type Culture Collection) were performed using the calcium phosphate coprecipitation method (21) by adding 10 μg of plasmid DNA and 1 μg of the reporter construct pRSV-Luciferase (20) to each 10-cm plate.

### HGL enzyme assay

Twenty-four h after glycerol shock, the media were harvested and cell extracts were prepared after washing by sonification in 0.5% Triton X-100. Triglyceride hydrolysis was determined at pH 5.4 with 90 μg of protein as enzyme source using glycerol-tri-[1-$^{14}$C]oleate (triolein) and glycerol-tri-[1-$^{14}$C]butyrate (tributyrin). Triolein assay mixtures contained 63 μmoles 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 in a volume of 200 μl. Tributyrin hydrolysis was assayed in 200 μl 150 mM sodium phosphate buffer, pH 5.4, containing 138 μM unlabeled substrate, 2 mM sodium taurodeoxycholate, and 0.75 mg/ml (w/v) albumin. Samples were incubated for 1 h at 37°C and the reaction was terminated by the addition of 0.1 mM triolein or tributyrin in chloroform–heptane–methanol 1.3:1:1.4 and alkaline buffer (23).

### Determination of enzymatic activity

In each series of experiments, a parallel cell culture dish was subjected to the transfection protocol without plasmid DNA. These "mock"-transfected cells as well as cells transfected with the insert-free pCMV vector served as controls for basal HLAL- as well as non-HLAL- or non-HGL-related hydrolysis of cholesteryl oleate, triolein, and tributyrin which averaged 5%, 34%, and 35%, respectively, of wild-type HLAL and HGL levels in the three cell lines studied. These negative control values, which are similar to the average basal activities of 8–47% reported for Cos-1 and Ltk$^-$ cells (4), were subtracted from those of the HLAL- and HGL-overexpressing cells prior to the calculation of the percentage of activity. In addition, we measured luciferase expression in the cotransfected cell extracts to normalize for differences in the efficiency of transfection. Data are reported as mean ± SD.

## RESULTS

The cDNA-derived amino acid sequence of HLAL (4) predicts six cysteines at positions 41, 188, 227, 236, 240, and 244 of the mature protein with another one in the signal peptide. The enzyme thus has the potential to form three S–S bonds by the disulfide–sulfhydryl interchange reaction to increase the stability of the three-dimensional structure and to prevent the lipase from unfolding at the lipid–water interface. Of the seven cysteine residues, $Cys_{227}$, $Cys_{236}$, and $Cys_{244}$ are completely conserved within the acid lipase gene family. HGL contains no additional cysteines, while a fourth residue is present in RLL at position 27. As functional domains of a protein are often encoded by a single exon in eukaryotic genes, it is interesting to note that the codons for the conserved cysteines are located in exon 7 in both the HLAL gene (24–26) and the nearly identical HGL/RLL gene (26).

To analyze the role of the conserved and non-conserved cysteines in the HLAL-mediated catabolism of cholesteryl esters and triglycerides, we have used oligonucleotide-mediated site-directed mutagenesis to replace the individual cysteine residues with alanine, an equally small, nonpolar amino acid incapable of forming S–S bridges. Mutant cDNA constructs were expressed in the Cos-7 kidney cell line and in Ltk$^-$ mouse fibroblasts, and enzymic hydrolysis was measured in the cell extracts with two radiolabeled natural substrates, cholesteryl oleate and glycerol-tri-oleate (triolein). Relative specific activities were determined after correction for endogenous fatty acyl hydrolase activities and for transfection efficiency by comparison with values obtained from extracts transfected with the wild-type HLAL cDNA (pCMV-HLAL).

As shown in **Fig. 1** and **Tables 1 and 2,** replacement of the conserved cysteines at amino acid positions 227 and 236 with alanine either separately or in combination resulted in a dramatic 98% reduction of cholesteryl oleate hydrolysis in both cell lines. Triolein cleavage, however, was less affected. HLAL($Cys_{227} \rightarrow$ Ala) retained 23% and 39% of wild-type hydrolytic activity in Ltk$^-$ and Cos-7 cells, respectively, while the mutagenized construct HLAL($Cys_{236} \rightarrow$ Ala) hydrolyzed 28% and 32% of the lipid substrate in these cell lines. Substitution of both cysteines with alanine led to a reduction of triolein hydrolysis to 13% of wild-type levels in Cos-7 cell extracts.

Mutagenesis of the third conserved residue, $Cys_{244}$, in

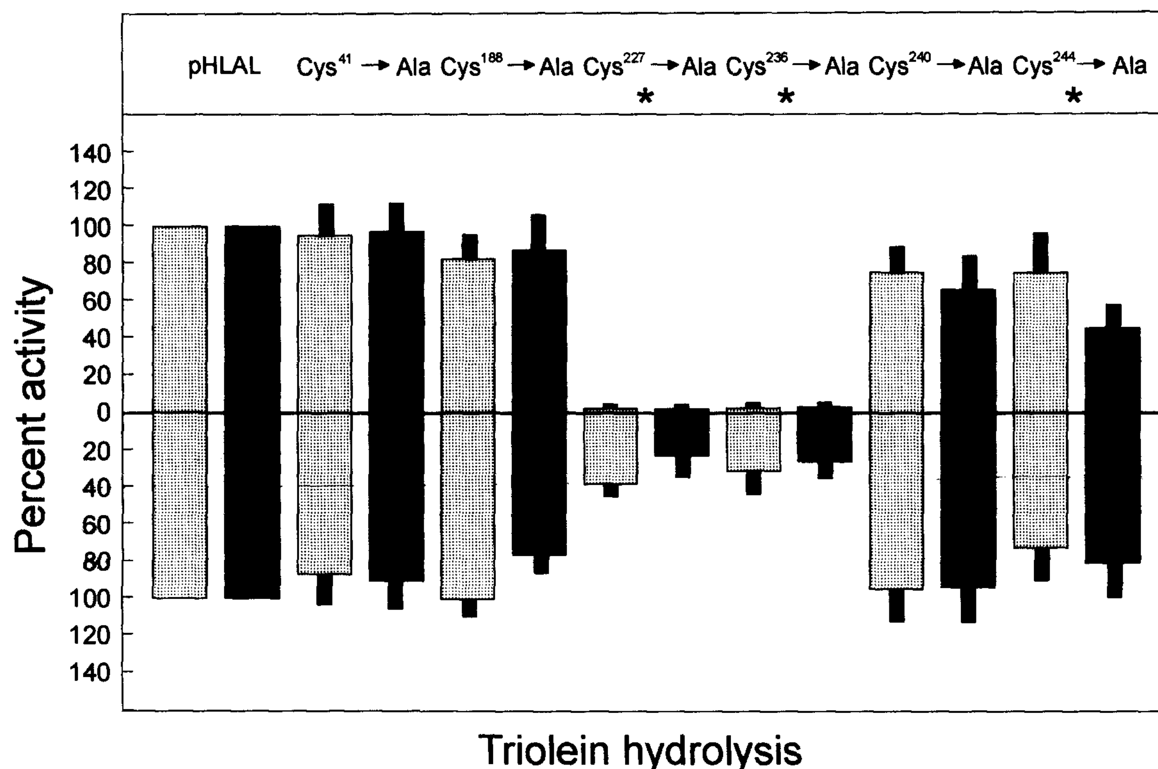


**Fig. 1.** Hydrolysis of cholesteryl oleate (top panel) and triolein (bottom panel) in Cos-7 (hatched boxes) and Ltk$^-$ (solid boxes) cells transfected with a wild-type HLAL cDNA construct (pCMV-HLAL) and six mutagenized cDNAs coding for alanine instead of cysteine at positions 41, 188, 227, 236, 240, and 244, respectively. Enzyme activities are expressed as percent of wild-type HLAL level (defined as 100%) and represent the mean values of at least six independent transfections (see Table 1). Results were normalized for transfection efficiency by measuring luciferase expression in the same cell homogenates. Asterisks designate cysteine residues conserved within the acid lipase gene family.

contrast, resulted in an enzyme that exhibited catalytic activities of 75% and 44% for cholesteryl oleate and 73% and 81% for triolein in Cos-7 and Ltk$^-$ cell lines, respectively. Replacement of non-conserved cysteine residues also yielded functional enzymes. HLAL($Cys_{41}$ → Ala) displayed 95% and 97% cholesteryl oleate and 87% and 91% triolein esterase activities in Cos-7 cells and Ltk$^-$ mouse fibroblasts, respectively. The mutagenized HLAL with alanine at position 188 hydrolyzed 87% and 83% of the cholesteryl oleate and 77% and 101% of the triolein when extracts of Ltk$^-$ and Cos-7 cells were assayed for enzyme activity. Substitution of residue 240

TABLE 1. Cholesteryl oleate, triolein, and tributyrin hydrolysis in Cos-7 and Ltk$^-$ cell homogenates transfected with wild-type and mutant HLAL cDNA constructs

| | % of Activity Relative to pCMV-HLAL | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cholesteryl Oleate | | | | Triolein | | | | Tributyrin | | | |
| Plasmid | Cos-7 | Ltk$^-$ | n | | Cos-7 | Ltk$^-$ | n | | Cos-7 | Ltk$^-$ | n | |
| pCMV-HLAL | 100 | 100 | | | 100 | 100 | | | 100 | 100 | | |
| pCMV-HLAL($Cys_{41}$ → Ala) | 95 ± 17 | 97 ± 15 | 7 | 9 | 87 ± 17 | 91 ± 15 | 8 | 9 | 89 ± 16 | 91 ± 20 | 5 | 5 |
| pCMV-HLAL($Cys_{188}$ → Ala) | 83 ± 13 | 87 ± 19 | 9 | 7 | 101 ± 9 | 77 ± 9 | 6 | 7 | 91 ± 20 | 118 ± 14 | 5 | 5 |
| pCMV-HLAL($Cys_{227}$ → Ala) | 2 ± 2 | 2 ± 1 | 9 | 10 | 39 ± 7 | 23 ± 11 | 9 | 6 | 38 ± 12 | 33 ± 10 | 4 | 4 |
| pCMV-HLAL($Cys_{236}$ → Ala) | 2 ± 3 | 2 ± 2 | 9 | 9 | 32 ± 12 | 28 ± 8 | 7 | 9 | 37 ± 12 | 35 ± 6 | 4 | 4 |
| pCMV-HLAL($Cys_{240}$ → Ala) | 75 ± 14 | 65 ± 18 | 9 | 8 | 96 ± 17 | 95 ± 18 | 6 | 8 | 125 ± 25 | 138 ± 32 | 5 | 5 |
| pCMV-HLAL($Cys_{244}$ → Ala) | 75 ± 21 | 44 ± 13 | 7 | 6 | 73 ± 17 | 81 ± 18 | 8 | 6 | 119 ± 20 | 99 ± 9 | 5 | 4 |

Levels of esterase activity are shown as mean values ± standard deviation relative to the normal cDNA construct pCMV-HLAL (defined as 100%) after subtraction of endogenous hydrolase activities and normalization for transfection efficiency. The number (n) of independent transfection experiments used for the calculations is given.

TABLE 2. Cholesteryl oleate, triolein, and tributyrin hydrolysis in Cos-7 cell homogenates transfected with wild-type and mutant HLAL cDNA constructs

| | % of Activity Relative to pCMV-HLAL | | | | | |
|---|---|---|---|---|---|---|
| | Cholesteryl Oleate | | Triolein | | Tributyrin | |
| Plasmid | Cos-7 Cells | n | Cos-7 Cells | n | Cos-7 Cells | n |
| pCMV-HLAL | 100 | | 100 | | 100 | |
| pCMV-HLAL($Cys_{227} \rightarrow$ Ala, $Cys_{236} \rightarrow$ Ala) | 3 ± 2 | 10 | 13 ±6 | 9 | 17 ± 6 | 7 |
| pCMV-HLAL-cHGL | 80 ± 16 | 7 | 93 ± 10 | 8 | 94 ± 34 | 8 |
| pCMV-HLAL-cRLL | 102 ± 18 | 7 | 104 ± 13 | 7 | 98 ± 21 | 7 |
| pCMV-HLAL-cBSSL | 6 ± 4 | 8 | 14 ± 9 | 7 | 34 ± 15 | 7 |
| pCMV-HLAL($Lys_{233} \rightarrow$ Ile) | 5 ± 2 | 10 | 29 ± 8 | 9 | 24 ± 7 | 7 |
| pCMV-HLAL($Leu_{235} \rightarrow$ Glu) | 0 ± 1 | 8 | 1 ± 1 | 8 | 4 ± 4 | 8 |
| pCMV-HLAL(Del. aa 228–235) | 1 ± 2 | 6 | 3 ± 2 | 6 | 3 ± 3 | 6 |

reduced the breakdown of cholesteryl oleate to 75% in Cos-7 cells and to 65% in Ltk$^-$ mouse fibroblasts, while triolein catalysis was unimpaired.

To determine whether the cysteine residues play a role in the hydrolysis of monomeric, water-soluble fatty acid esters independent of interfacial lipid binding, we also measured the esterase activities of the mutant constructs using the short chain fatty acid triglyceride glycerol-tri-butyrate (tributyrin; C4:0) which, below a concentration of 0.25 mM, does not form micelles (27). Tables 1 and 2 summarize the results of these assays. Esterolysis was significantly lower only in Cos-7 and Ltk$^-$ cell extracts transfected with pCMV–HLAL($Cys_{227} \rightarrow$ Ala), pCMV–HLAL($Cys_{236} \rightarrow$ Ala), and pCMV–HLAL ($Cys_{227} \rightarrow$ Ala, $Cys_{236} \rightarrow$ Ala). Replacement of either one of the two conserved cysteines resulted in a 64% reduction of tributyrin hydrolysis, while substitution of both Cys residues led to a 83% decrease in substrate catalysis. Esterase activities of HLAL($Cys_{41} \rightarrow$ Ala), HLAL($Cys_{188} \rightarrow$ Ala), and HLAL($Cys_{244} \rightarrow$ Ala), in contrast, were essentially unchanged when compared to the wild-type enzyme, while HLAL($Cys_{240} \rightarrow$ Ala) showed an enhanced ability to hydrolyze the monodisperse substrate, averaging 125% and 138% in Cos-7 and Ltk$^-$ cells, respectively.

To confirm the results obtained with the HLAL mutants, identical experiments were then performed using wild-type and mutagenized HGL cDNA constructs transiently expressed in vitro in human embryonal kidney-293 cells. Because HGL is secreted by the gastric mucosa of the stomach and hydrolyzes the ester bonds of triglycerides under acidic pH conditions in the gastrointestinal tract, while cholesteryl esters are not attacked, we determined HGL enzymic activity in the cell extracts and in the media using the water-insoluble triolein and the water-soluble tributyrin as substrates.

Consistent with the important role of cysteines 227 and 236 in long- and short-chain triglyceride hydrolysis, the mutagenized enzymes HGL($Cys_{227} \rightarrow$ Ala) and HGL($Cys_{236} \rightarrow$ Ala) displayed 22% and 17% of normal HGL activity towards triolein in the kidney-293 cell extracts, while tributyrin esterase activity was reduced to 31% and 35% (**Fig. 2** and **Table 3**). In the media, however, only 2% and 5% of the triolein and 24% of the tributyrin substrate was hydrolyzed to free fatty acids. Replacement of the HGL $Cys_{227}/Cys_{236}$ pair with an Ala pair resulted in a significantly lower activity towards triolein both intracellularly and in the media, averaging 3% of wild-type HGL levels. Also in accordance with our HLAL studies was the insignificant reduction of enzymatic activity observed in the cell extracts and media of kidney-293 cells transfected with pCMV–HGL($Cys_{244} \rightarrow$ Ala). The mutant lipase exhibited on the average 82% and 93% of wild-type activity against triolein and tributyrin, respectively.

Furthermore, we investigated the potential role of residues 228–235 in HLAL- and HGL-catalyzed neutral lipid hydrolysis. Secondary structure analysis (PC/GENE, IntelliGenetics, Mountain View, CA) using the method of Garnier, Osguthorpe, and Robson (28) and the Gascuel and Golmard Basic Statistical Method (GGBSM; 29) predicted the most probable conformation of the eight residues as helical in HGL and RLL and as extended (aa 228–230/231) and helical (aa 230/231–235) in HLAL. Helical wheel presentation (30) of HLAL, HGL, and RLL residues 228–235 demonstrated the presence of both a predominantly polar and a nonpolar side (**Fig. 3**). The mean hydrophobic moment (μH) of the segments was determined to be 0.45 (HLAL), 0.62 (HGL), and 0.69 (RLL), indicating that the α-helices are highly amphipathic (31).

In a first step, we replaced HLAL amino acids 228–235 with the corresponding HGL and RLL sequences. Comparison of the respective HLAL, HGL, and RLL amino acid sequences (**Table 4**) revealed that six out of the eight residues are identical in HGL and RLL, while in HLAL only two hydrophobic leucines (positions 232 and 235) are strictly conserved. In addition,

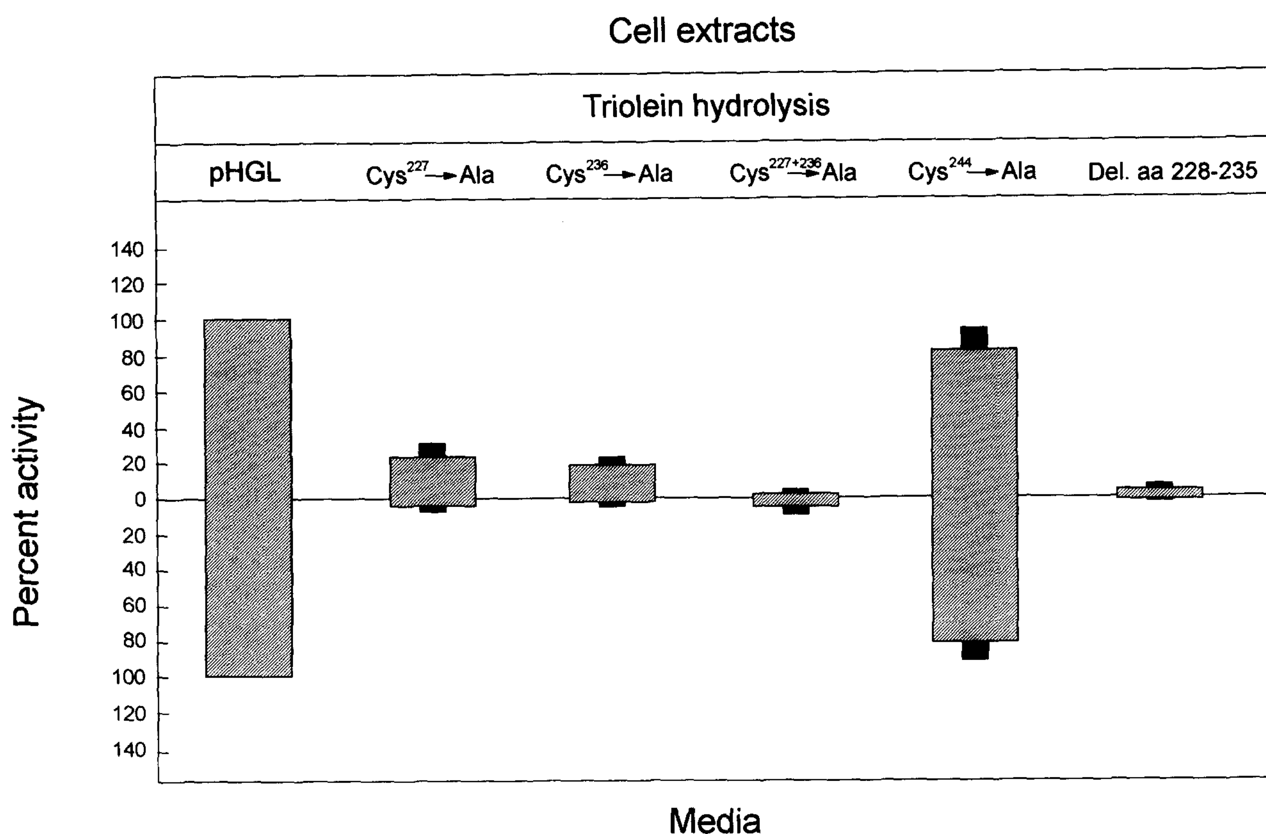


**Fig. 2.** Hydrolysis of triolein in the cell extracts (top panel) and media (bottom panel) of human kidney-293 cells transfected with a wild-type HGL cDNA construct, four mutagenized cDNAs coding for $Ala_{227}$, $Ala_{236}$, $Ala_{227}$ + $Ala_{236}$, and $Ala_{244}$ instead of cysteine, and an amino acid 228–235 deletion mutant. Enzyme activities were normalized according to luciferase expression in the cell extracts. Values 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 seven independent experiments (see Table 3).

residue 231 is always a hydrophobic amino acid. This high degree of sequence similarity between HGL and RLL on the one hand, and the poor homology of HLAL on the other hand, may correlate with differences in substrate specificity. We also created a HLAL chimeric enzyme containing residues 247–256 of human bile salt-activated lipase/cholesteryl ester hydrolase (BSSL). This decapeptide was chosen for the following reasons. *1*) In BSSL it is also flanked by two conserved cysteines at positions 246 and 257 which form an intra-chain di-

TABLE 3. Triolein and tributyrin hydrolysis in the extracts and media of human kidney-293 cells transfected with wild-type and mutant HGL cDNA constructs

| | % of Activity Relative to pCMV-HGL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Triolein | | | | Tributyrin | | | |
| Plasmid | 293 Cells | Media | n | | 293 Cells | Media | n | |
| pCMV-HGL | 100 | 100 | | | 100 | 100 | | |
| pCMV-HGL($Cys_{227}$ → Ala) | 22 ± 8 | 5 ± 3 | 7 | 9 | 31 ± 12 | 24 ± 9 | 8 | 7 |
| pCMV-HGL($Cys_{236}$ → Ala) | 17 ± 5 | 2 ± 2 | 8 | 9 | 35 ± 11 | 24 ± 7 | 6 | 7 |
| pCMV-HGL($Cys_{227}$ → Ala, $Cys_{236}$ → Ala) | 1 ± 2 | 5 ± 4 | 9 | 8 | 28 ± 8 | 31 ± 7 | 9 | 6 |
| pCMV-HGL($Cys_{244}$ → Ala) | 82 ± 11 | 81 ± 12 | 7 | 8 | 89 ± 10 | 97 ± 11 | 8 | 10 |
| pCMV-HGL(Del. aa 228–235) | 3 ± 3 | 1 ± 1 | 7 | 8 | 3 ± 4 | 4 ± 3 | 7 | 8 |

Percent of activity relative to normal HGL (defined as 100%) was determined after subtraction of basal esterase activities and normalization for luciferase expression in the cell homogenates. The number (n) of independent transfections performed to obtain the main values ± standard deviation is illustrated.

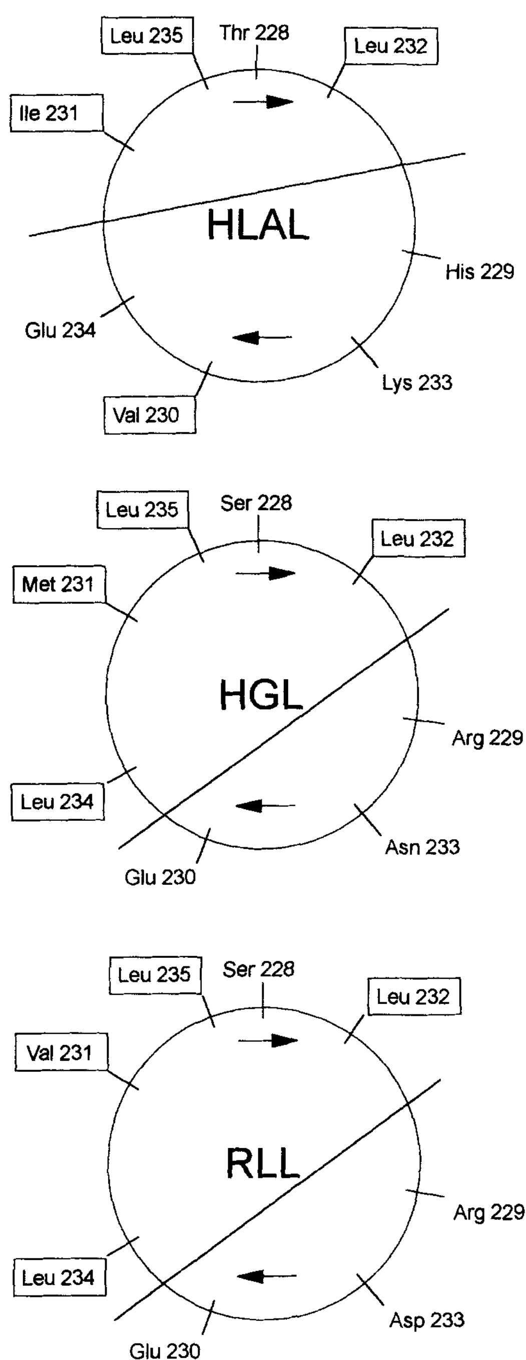


**Fig. 3.** Helical wheel representation of HLAL, HGL, and RLL residues 228–235. The "HELWHEEL" program (PC/GENE; 30) creates a helical wheel diagram of HLAL, HGL, and RLL amino acids 228–235 arranged as an ideal α-helix seen down the long axis, the direction of the wheel in this two-dimensional projection being clockwise. Hydrophobic residues are boxed, and the boundary between the polar and non-polar faces is indicated by a straight line.

sulfide bridge (32; for review see ref. 33); *2)* the method of Garnier et al. (28) predicts its secondary structure to be α-helical (except for $Pro_{247}$); *3)* the α-helix displays alternating hydrophobic and polar faces with a hydrophobic moment of 0.53 μH; *4)* the enzyme has an even wider substrate specificity than HLAL, hydrolyzing cholesteryl esters, tri-, di-, and monoglycerides, phospholipids, and fat soluble vitamin esters; *5)* based on the topological position of the catalytic triad residues, the HLAL and BSSL gene structures resemble each other (9).

Table 2 illustrates that despite the helix exchange the lipolytic and esterolytic properties of the chimeras HLAL-cHGL and HLAL-cRLL were preserved, the activities ranging from 73 to 91% in $Ltk^-$ mouse fibroblast extracts (data not shown) and from 80 to 104% in Cos-7 cell homogenates. Substitution with the BSSL decapeptide, in contrast, resulted in a 94% and 97% reduction of cholesteryl oleate hydrolysis in Cos-7 and $Ltk^-$ cells, respectively. Catalysis of long- and short-chain triglycerides was less impaired, the remaining activity being 14% with triolein and 34% with tributyrin in Cos-7 cells.

Next, we altered the amphiphilicity of the α-helix in HLAL by replacing the completely conserved leucine at position 235 with glutamic acid and by substituting the non-conserved lysine, residue 233, with isoleucine. This resulted in the hydrophobic moment decreasing from 0.45 μH to 0.23 μH (position 235) and 0.1 μH (residue 233). Disruption of the hydrophobic face in HLAL ($Leu_{235} \rightarrow$ Glu) abolished the catalysis of both water-insoluble and water-soluble lipids (Table 2). Almost complete loss of cholesteryl esterase activity was also observed with HLAL ($Lys_{233} \rightarrow$ Ile). The mutant enzyme retained, however, 29% and 24% of wild-type HLAL levels using triolein and tributyrin as substrates.

In a third step, we created mutant HLAL and HGL enzymes in which amino acids 228–235 had been removed. These deletions did not affect the predicted protein secondary structures except for minor changes in the conformation of HLAL residues 224, 238, and 239 and HGL amino acids 226, 227, 236, and 240. Both mutants were, however, almost completely devoid of lipase and esterase activity, hydrolyzing on the average 1% of the cholesteryl oleate and 3% of the triolein and tributyrin substrates (Fig. 2 and Tables 2 and 3).

## DISCUSSION

The acid lipase family members HLAL and HGL were initially proposed to use a cysteine instead of a serine in the hydrolytic reaction, based on their chemical inac-

TABLE 4. Alignment of the cysteine-rich amino acid sequences of HLAL, HGL, and RLL

| | * | | | | | | | | | * | | | | | | | | * |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| aa | 227 | 228 | 229 | 230 | 231 | 232 | 233 | 234 | 235 | 236 | 237 | 238 | 239 | 240 | 241 | 242 | 243 | 244 |
| HLAL | C | T | H | V | I | L | K | E | L | C | G | N | L | C | F | L | L | C |
| HGL | C | S | R | E | M | L | N | L | L | C | S | N | A | L | F | I | I | C |
| RLL | C | S | R | E | V | L | D | L | L | C | S | N | T | L | F | I | F | C |

The single-letter amino acid code was used for comparison. Asterisks designate cysteine residues conserved in HLAL, HGL, and RLL. Vertical bars indicate sequence identity, and hydrophobic residues are boxed.

tivation by hydrophobic disulfides which were shown to react with a single free sulfhydryl group in HGL and rabbit gastric lipase (RGL; 34–36; for review see ref. 37). The titration experiments also led to the conclusion that HGL possesses one disulfide bridge (34). Subsequent characterization of HGL and RGL peptide fragments by SDS-PAGE in the absence of reducing agents strongly suggested that $Cys_{236}$ forms an intra-chain disulfide bond with $Cys_{244}$, while the free SH-group corresponds to $Cys_{227}$ (38).

The identity of the catalytic nucleophile began to unravel with the crystallographic analysis of two unrelated lipases from the fungus *Rhizomucor miehei* (39) and from human pancreas (40). The X-ray studies unexpectedly revealed that the enzymes are remarkably similar in structure. Their central catalytic domain consists of a predominantly parallel β-sheet structure connected by loops of varying length, termed the $\alpha/\beta$ hydrolase fold (41), which contains, in sequential order, the catalytic triad residues serine (as part of the nearly ubiquitous lipase consensus sequence motif -Gly-Xaa-Ser-Xaa-Gly-), aspartic acid, and histidine (for review see refs. 10–13). Assisted by the dipolar oxyanion hole, these three amino acids accelerate the hydrolysis of the ester bond by stabilizing the charge distribution of the transition states during substrate catalysis (42).

The conservation of the lipase core structures, despite undetectable sequence homology beyond the lipase consensus sequence, and the identification of a Ser-Asp/Glu-His triad as the catalytic unit of lipases necessitated a reexamination of the enzymatic mechanism of HLAL and HGL. Incubation with the serine reagent diethyl *p*-nitrophenyl phosphate (E600) inactivated the enzymes (4, 7, 8), while the essential free sulfhydryl group remained unmodified and binding to the lipid–water interface was not impaired (7). Additional studies using the specific lipase inhibitor tetrahydrolipstatin (8, 43–45) as well as recent site-directed mutagenesis experiments (8, 9) also demonstrated that, despite their sensitivity towards sulfhydryl-modifying substances, HLAL and HGL are serine hydrolases, $Ser_{153}$ within the second lipase consensus sequence of the acid lipase family being the catalytic nucleophile (9).

Based on the results of the chemical modification studies, Derewenda (13) proposed that the free thiol group is close to the active site residues $Ser_{153}$, $Asp_{324}$, and $His_{353}$ (9) and that pretreatment with sulfhydryl reagents may distort the environment of the catalytic triad or of the oxyanion hole. As the three Cys residues of HGL are completely conserved in HLAL and RLL, the same argument holds for HLAL and RLL (13).

To examine the functional importance of the conserved cysteines at HLAL and HGL amino acid positions 227, 236, and 244 and of the three non-conserved cysteine residues present only in HLAL, we created alanine-replacement mutants by site-directed mutagenesis and assessed their catalytic properties by short-term expression in cultured eukaryotic cell lines. Our combined data provide strong evidence that $Cys_{227}$ and $Cys_{236}$ are essential for the hydrolysis of cholesteryl esters and to a lesser extent of triolein and tributyrin. The conserved $Cys_{244}$ and the three additional HLAL cysteines, in contrast, appear to play no important role in substrate catalysis.

These results are surprising in light of the HGL and RGL peptide analyses which indicate that, at least in gastric lipases, $Cys_{236}$ and $Cys_{244}$ are disulfide-bonded with $Cys_{227}$ representing the free thiol group (38). As intra-chain disulfide bridges are generally felt to be important for protein folding, one would expect their removal to have a deleterious effect on cholesteryl ester hydrolysis, lipolysis, and esterolysis. However, this is clearly not the case. Substitution of $Cys_{244}$ by alanine reduced substrate catalysis on average by only 16%, strongly suggesting that elimination of the disulfide bond is not detrimental for HLAL and HGL activity. It remains, however, possible that the assignment of the residues involved in the formation of the disulfide bridge is wrong and that, in fact, $Cys_{227}$ and $Cys_{236}$ are linked by an intra-chain S–S bond in order to stabilize the fold, while $Cys_{244}$ is the single free sulfhydryl. Our hypothesis is supported by the observation that bovine

pregastric esterase, another preduodenal lipase with 82% and 76% nucleotide and 75% and 71% amino acid sequence homology to HGL and RLL, contains only two cysteines at positions 226 and 235 while $Cys_{244}$ is replaced by threonine (46).

In order to determine whether the amphipathic α-helical structure containing residues 228–235 and delimited by $Cys_{227}$ and $Cys_{236}$ plays a role in conferring substrate specificity, we performed additional experiments with three helix exchange mutants. The results demonstrate that the HLAL amino acids can be replaced with the corresponding residues of HGL and RLL with only minor or no impairment of lipase function, indicating that the octamer does not confer HLAL cholesteryl ester specificity. This is in contrast to a chimeric construct consisting of a 10-amino acid amphipatic α-helical region of BSSL grafted onto the HLAL core that yielded a mutant enzyme with a 94%, 86%, and 66% reduction, respectively, in the ability to hydrolyze cholesteryl oleate, triolein, and tributyrin. Our data strongly suggest that the amino acid sequences of the helix are interchangeable between closely related enzymes as long as the engrafted residues do not interfere with proper folding of the protein and make correct contact with the rest of the enzyme structure and/or the lipid substrate. Site-directed mutagenesis of two individual amino acids comprising the amphipathic α-helix supports this conclusion. Disruption of the hydrophobic surface by substitution of the completely conserved leucine residue at position 235 with glutamic acid almost completely abolished the ability of HLAL to hydrolyze cholesteryl esters as well as triglycerides. A dramatic 95% reduction of cholesteryl oleate hydrolysis while retaining 29% and 24% of catalytic activity towards triolein and tributyrin was also observed when the non-conserved, positively charged lysine, amino acid 233, in the center of the polar face was replaced with isoleucine. The hydrophobic face of the α-helix therefore appears to be crucial for HLAL function, while destruction of the predominantly hydrophilic side results in a substrate-dependent reduction of hydrolytic activity remarkably similar to that observed with the $Cys_{227}$- and $Cys_{236}$-substituted mutants.

Not surprisingly, removal of the helix in HLAL and HGL almost completely inactivated the enzymes, again demonstrating that structural integrity of the amphipathic α-helix as well as of the flanking cysteine residues is of prime importance for HLAL- and HGL-mediated substrate catalysis.

In summary, site-directed mutagenesis was used to replace cysteines in HLAL and HGL, one of which is believed to be relevant for neutral lipid hydrolysis as deduced from previous experiments with sulfhydryl-modifying reagents. Expression studies established the essential role of the conserved cysteine residues at amino acid positions 227 and 236 for the hydrolysis of cholesteryl esters and, to a lesser extent, of aggregated and water-soluble triglycerides whereas the other cysteines appear to play no major role in catalysis. Substitution of the amphipathic, α-helical region formed by HLAL amino acids 228–235 with the corresponding residues of HGL and RLL demonstrated that the primary amino acid sequence of the helix is relatively unimportant and does not confer substrate specificity. Its amphiphilicity, especially the integrity of its hydrophobic face, however, appears to be a prerequisite for acid lipase activity.

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