Characterization of Lysosomal Acid Lipase by Site-directed Mutagenesis and Heterologous Expression*

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Lysosomal acid lipase (LAL) is essential for the hydrolysis of cholesterol esters and triglycerides that are delivered to the lysosomes via the low density lipoprotein receptor system. The deficiency of LAL is associated with cholesteryl ester storage disease (CESD) and Wolman's disease (WD). We cloned the human LAL cDNA and expressed the active enzyme in the baculovirus system. Two molecular forms (M, ~41,000 and ~46,000) with different glycosylation were found intracellularly, and ~24% of the M, ~46,000 form was secreted into the medium. Tunicamycin treatment produced only an inactive M, ~41,000 form. This result implicates glycosylation occupancy in the proper folding for active-site function. Catalytic activity was greater toward cis- than trans-unsaturated fatty acid esters of 4-methylumbelliferyl and toward esters with 7-carbon length acyl chains. LAL cleaved cholesterol esters and mono-, tri-, and diglycerides. Heparin had a biphasic effect on enzymatic activity with initial activation followed by inhibition. Inhibition of LAL activity by tetrahydro lipstatin and diethyl p-nitrophenyl phosphate suggested the presence of active serine in binding/catalytic domain(s) of the protein. Site-directed mutagenesis at two putative active centers, GXSXG, showed that Ser153 was important to catalytic activity, whereas Ser99 was not and neither was the catalytic nucleophile. Three reported mutations (L179P, L336P, and ΔAG302 deletion) from CESD patients were created and expressed in the SF9 cell system. None cleaved cholesterol esters, and L179P and L336P cleaved only triolein at ~4% of wild-type levels. These results suggest that mechanisms, in addition to LAL defects, may operate in the selective accumulation of cholesterol esters or triglycerides in CESD and WD patients.

Lysosomal acid lipase (LAL) hydrolyzes cholesteryl esters and triglycerides that are delivered to the lysosomes by low density lipoprotein receptor-mediated endocytosis. LAL is important to the regulation of cholesterol synthesis and homeostasis since it liberates free cholesterol for negative feedback of hydroxymethylglutaryl-CoA reductase (1). Defective human LAL (hLAL) activity has been associated with two rare autosomal recessive traits, Wolman’s disease (WD) and cholesteryl ester storage disease (CESD). WD is lethal within the first year of life due to hepatosplenomegaly, adrenal calcification, and massive accumulation of triglycerides and cholesterol esters in these organs as well as macrophages and blood vessels. CESD is a less severe disorder with longer survival, hepatomegaly, premature atherosclerosis, and dyslipoproteinemias. Residual LAL activity has been detected in CESD, but not in WD (2).

The hLAL cDNA and chromosomal gene have been characterized, and the locus maps to human chromosome 10q23.2-23.3 (3, 4). The gene has 10 exons spread over ~36 kilobase pairs (5, 6). Several hLAL mutations have been detected in cDNAs derived from mRNAs of CESD and WD patient cells. In CESD, a splice donor site G to A transition leads to aberrant splicing of exon 8 and a 72-base pair (24-amino acid) deletion (7). An AG deletion leads to frameshift at amino acid 302 (ΔAG302) and a truncated lipase with a 34-amino acid C-terminal deletion (8). Two proline to leucine substitutions (L179P and L336P) have been detected in different CESD patients (5, 9). In WD, a T insertion at nucleotide 635 results in a frameshift (fs177) and premature translation termination at amino acid 189 (5).

hLAL is a member of a highly conserved lipase family that includes gastric and lingual lipases (3). The hLAL cDNA encodes a 372-amino acid mature protein and a 27-amino acid signal sequence (10). The predicted mature polypeptide contains six cysteines. Three of these cysteines are conserved among members of the lipase family at positions 227, 236, and 244. This glycoprotein has six glycosylation consensus sequences (Asn-X-Ser/Thr), and three at Asn13, Asn80, and Asn252 are conserved among members of the lipase gene family (3). The enzyme is trafficked to the lysosomes via the mannose 6-phosphate receptor system (11, 12). However, the dependence of catalytic activity on glycosylation is unknown. All the members of the lipase gene family have conserved GXXG pentapeptide sequences that contain the active-site serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles (13-15). hLAL has two such sequenc
Role of Ser^{99} and Ser^{153} and Glycosylation in Enzymatic Activity

(19). hLALs from liver (10) or expressed in COS cells have activity toward triglyceride and cholesteryl esters (3). The rabbit enzyme also degrades 4-methylumbelliferyl fatty acid esters of various chain lengths with preference for medium (7-carbon) size chain esters (20, 21). Sulfonic acid and boronic acid derivatives, diethyl pyrocarbonates, and heparin inhibit hLAL (22).

We have cloned and expressed normal and mutant hLAL cDNAs to develop a more detailed understanding of the properties of hLAL. Specifically, we explored the roles of G, diethyl pyrocarbonates, and heparin inhibit hLAL (22).

Methods—The following were from commercial sources: [1-14C]cholesterol (40–60 mCi/mmol), tri(1-14C)oleoylglycerol (80–120 mCi/mmole), [1-14C]oleic acid (40–60 mCi/mmol), [32P]ATP (3000 Ci/mmole), and [32P]dATP (1500 Ci/mmol) (DuPont,NEN); 2-31-14C-ATP (55 mCi/mmol) and 3-14C-monomethionine (55 mCi/mmol) (American Radiolabeled Chemicals, St. Louis, MO); Triton X-100, heparin, 4-methylumbelliferyl acetate, 4-methylumbelliferyl elaidate, 4-methylumbelliferyl heptanate, 4-methylumbelliferyl deate (4-MUO), 4-methylumbelliferyl palmitate, 4-methylumbelliferyl propionate, 4-methylumbelliferyl stearamine, 4-methylumbelliferyl phenylacetate, 4-methylumbelliferyl (3′) (the asterisk indicates the site of a frameshift), 9-taurolithocholate, sodium taurodeoxycholate, and L-α-phosphatidylcholine (CalBiochem); cholesterol oleate, monolein, didecyl, and oleic acid (Nuchek Prep, Elmsyn, ENSIA); precoated silica gel TLC plates (Whatman and Fisher); PET21a (+) expression vector and Ni2+ column (Novagen, Madison, WI); λ-ZAP cDNA library and phage R408 (Stratagene, La Jolla, CA); Geneclean kit (BIO 101, Inc., Vista, CA); baculovirus transfection kit and Escherichia coli CJ 236 (Invitrogen, San Diego, CA); restriction endonucleases and Vent R polymerase (Stratagene, La Jolla, CA); Geneclean kit; Nunc tissue culture reagents, and isopropyl-1-thio-

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Antibody Production and Immunoblotting—For antibody production, the hLAL mature coding region was cloned in frame into pET21a (+) vector to enable synthesis of hLAL containing a 3-His tag. The entire coding region for the hLAL fusion protein was sequenced to ensure fidelity of the polymerase chain reaction. Following transformation of E. coli with pET21a (+), plasmid expression of hLAL was induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 4 h in BL21(DE3) cells. The recombinant hLAL protein was accumulated as inclusion bodies that were solubilized in 7 M urea and purified on a Ni2+ column. The eluate from this affinity column had single band at 41 kDa by silver staining on 12.5% SDS-polyacrylamide gel.

Immunoblotting of insect and bacterial cell-expressed recombinant enzymes was performed using polyclonal anti-hLAL antibody as described for anti-human β-glucosidase (27) with slight modifications. Briefly, Sf9 cells were infected with wild-type or pure recombinant AcNPV; 1 h later, the virus-containing medium was aspirated, and cells were washed and changed to serum-free and protein-free Grace's medium. Three days after infection, the medium and cells were collected separately by centrifugation (525 g × 10 min). The cell pellet was further washed three times in phosphate-buffered saline and solubilized in 0.1 M phosphate, pH 6.8, containing 10 mM β-mercaptoethanol, 0.2% Triton X-100, and 0.2% sodium deoxycholate in 0.5 M NaCl. The soluble fraction was further purified by ultrasonic irradiation at 4 °C using a cup sonicator (30, 20, and 10 s; 4 °C). The sonicates were centrifuged (875 g × 20 min), and the supernatant was used for immunoblot analysis. Immunoblotting was done with the polyclonal anti-hLAL antibody (1:2000 dilution) and alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody. The antibody used in this study had a titer of 1:10^6 using 2 ng of purified protein. The specificity of the antibody was evaluated by immunoblotting using recombinant hLAL and lyases from human fibroblasts and HepG2 cells. A single band with Mass ~46,000 in lysates of bacteria expressing HLA and Mass ~46,000 in proteins in human fibroblasts were detected by this antibody.

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and medium or from human fibroblast cell lysate or partially purified enzyme from the medium was added (0.1-25 µl) to initiate the reactions.

Briefly, 25 µmol of 4-MU fatty acid in hexane and 40 µmol of L-α-phosphatidylcholine in chloroform were evaporated under N₂. The residue was resuspended in 2.4 mM sodium taurodeoxycholate and sonicated at 4°C (using a cup sonicator for 3-5 min at 50 watts). The resultant translucent and homogeneous dispersions were used as the substrates. Under the standard assay conditions, 50 µl of 4-MUO-L-α-phosphatidylcholine liposomes were added to the reaction mixture. Enzyme assays were in 0.2 M sodium acetate, pH 5.5 (400 µl). Assays were stopped with 1.6 ml of 0.1 M Tris-HCl, pH 7.6, and the fluorescence intensity was quantified (Aminco Bowman spectrophotometer).

For the lipid substrates, cholesterol ester or tri-, di-, or monoglycerides were emulsified in individual mixtures of 0.05% Triton X-100, 1% sodium cholate (fatty acid-free), 3.13% sodium taurocholate, and 0.15 M acetic acid (40:1, v/v). To extract the lipids, 2 ml of H₂O and 5 ml of hexane were added to the mixture. After vigorous shaking, the hexane phase was collected and evaporated to dryness under N₂, and the residues were solubilized in chloroform (500 µl). The various lipids in 25-µl aliquots were resolved by TLC in 1:2-dichloroethane/methanol (98:2, v/v). Autoradiograms were developed from TLC plates, and the bands were visualized by fluorography and/or quantitated in a scintillation spectrometer. All assays were conducted in duplicate, and the results are the means of three experiments. Assays were linear in the time frame used, and ~10% of the substrates were cleaved during these reactions.

To evaluate the effect of enzyme modifiers on hLAL activity, the required amounts of DNP, THL, heparin sulfate, and tunicamycin were added to the enzyme solution and incubated for 15 min at ambient temperature prior to the addition of substrate. DNP and THL were added in acetate/EtOH, and heparin sulfate and tunicamycin in sodium acetate, pH 5.5. A blank solution of an acetate/EtOH mixture had no effect on enzymatic activity. Enzyme kinetic data were analyzed using Kaleidograph™ version 3.0 (Abelbeck Software).

RESULTS

Active hLAL was present in cell lysates and spent medium of SF9 cells infected with recombinant baculovirus containing the hLAL cDNA. Using the 4-MUO substrate, the apparent Km values for LAL from lysates and medium were 20.3 ± 3.9 and 37.7 ± 5.2 µM, respectively. The respective Vmax values for these cell lysates were 501.4 ± 20.2 nmol/min/mg of protein and 386.0 ± 14.53 nmol/min/10 ml of medium (Fig. 1). On immunoblotting, equal amounts of enzymatic activity from cell lysates and medium gave about equal densitometric signals (data not shown), indicating similar Kcat values from these sources. The pH activity profile was bell-shaped, with a pH optimum for 4-MUO of 5.5 and ~7-10-fold decrease in activity at pH 4.5 and 6.5 (data not shown). The Km values were unchanged in this pH range. Using hLAL from spent medium, the substrate specificity was evaluated with 4-MU fatty acid esters (250 µM) of varying acyl chain lengths (Fig. 2). Substrates with acyl chains less than C₁₀ were poorly hydrolyzed (~17% of C₁₀ rates). The maximal hydrolytic rates were obtained with the C₁₀ alkyl esters. The C₁₀ Δ⁹-ds-4-MU (4-MUO) had 56% of the rate achieved with the heptanone derivative. The C₁₀ Δ⁹-trans-ε-laidenate ester was hydrolyzed at 21% of the rate observed with 4-MUO. The stearate (C₁₇) and palmitate (C₁₆) derivatives were very poor substrates.

hLAL also hydrolyzed cholesteryl esters and tri-, di-, and monoglycerides, but with ~30-100-fold lower rates than obtained with 4-MUO (Tables I and II). By using sn-1 or sn-3 randomly radiolabeled trioleins, we monitored the rates of di- to mono-olein conversion and oleic acid appearance. These analyses showed that the accumulation of diolein was in excess of that for monolein, whereas the appearance of free oleic acid was rapid (Fig. 3). The ratio for production of diolein versus mono-
Heparin had a biphasic effect on hLAL: at low concentrations (0–2.5 μM), the activity was enhanced 2-fold (Fig. 5). In comparison, at higher concentrations (>2.5 μM/ml), the activity was inhibited with an IC_{50} of ~25 μM/ml.

To evaluate the similarities of active-site function between hLAL and other lipases, the active center-directed inhibitors DNP and THL were used (30, 31). DNP inhibition of hLAL and other lipases, the active center-directed inhibitors DNP and THL were used (30, 31). DNP inhibition of hLAL was more complex and potent. The inhibition curve with an IC_{50} of 2.0 μM and THL (B), Different concentrations of DNP or THL were incubated with 5 μl of spent medium from Sf9 cells at room temperature for 30 min. Substrate (250 μM 4-MUO)-containing liposomes were added, and the enzymatic activity was determined. Control (○) and S99A (■) refer to wild-type hLAL and mutant LAL with a Ser^{99} → Ala substitution, respectively.

TABLE I
Hydrolysis of 4-MUO, cholesteryl oleate, and triolein by hLALs expressed in Sf9 cells
Data represent the means ± S.E. of three independent experiments done in duplicate.

| Cell lysate | Enzyme activity | 4-MUO | Cholesteryl oleate | Triolein |
|-------------|-----------------|-------|--------------------|---------|
| hLAL        | nmol/min/mg protein | 459.4 ± 22 | 4.93 ± 0.53 | 14.41 ± 2.1 |
| S99A        | 434.7 ± 2.7 | 4.06 ± 0.34 | 14.13 ± 0.22 |
| S153A       | 17.3 ± 1.1 | 0.07 ± 0.01 | 0.59 ± 0.06 |
| L179P       | 14.1 ± 0.8 | 0.04 ± 0.002 | 0.72 ± 0.15 |
| L336P       | 15.2 ± 0.8 | 0.05 ± 0.003 | 0.23 ± 0.07 |
| AcNPV       | 2.2 ± 0.9 | 0.01 ± 0.001 | 0.18 ± 0.02 |

FIG. 3. Cleavage of triolein by hLAL from Sf9 lysates (lanes 2, 5, and 8) and normal cultured skin fibroblasts (lanes 1, 4, and 7). Lanes 3, 4, and 9 are controls from Sf9 cells infected with nonrecombinant baculovirus. The reactions were conducted for 15 min (lanes 1–3), 30 min (lanes 4–6), and 60 min (lanes 7–9). The various lipids were resolved on polyester-supported silica gel plates with dichloroethane/methanol (50:1, v/v) as the running solvent.

FIG. 4. Inhibition of recombinant hLAL activity by DNP (A) and THL (B). Different concentrations of DNP or THL were incubated with 5 μl of spent medium from Sf9 cells at room temperature for 30 min. Substrate (250 μM 4-MUO)-containing liposomes were added, and the enzymatic activity was determined. Control (○) and S99A (■) refer to wild-type hLAL and mutant LAL with a Ser^{99} → Ala substitution, respectively.

TABLE II
Hydrolysis of mono-, di-, and triacylglycerols by expressed recombinants
Data represent the means ± S.E. of three different experiments done in duplicate.

| Cell lysate | Enzyme activity | 1,3-Monoolein | 2,3-Diolein | Triolein |
|-------------|-----------------|---------------|-------------|---------|
| hLAL        | nmol/min/mg protein | 19.2 ± 2.4 | 12.9 ± 1.4 | 14.41 ± 2.1 |
| S99A        | 42.1 ± 1.9 | 17.5 ± 2.3 | 14.13 ± 0.22 |
| S153A       | 0.63 ± 0.34 | 0.14 ± 0.06 | 0.59 ± 0.06 |
| L179P       | 0.34 ± 0.13 | 0.17 ± 0.07 | 0.57 ± 0.05 |
| L336P       | 0.63 ± 0.19 | 0.44 ± 0.07 | 0.72 ± 0.15 |
| ΔAG302      | 0.75 ± 0.20 | 0.32 ± 0.06 | 0.23 ± 0.07 |
| AcNPV       | 0.73 ± 0.30 | 0.15 ± 0.06 | 0.18 ± 0.02 |

THL and DNP were shown to bind to putative catalytic nucleophilic serines of other lipases in the conserved sequence (GXSXG). hLAL has two such putative active-site sequence surrounding Ser^{99} and Ser^{153}. To determine if either of these serines participates in the catalytic process, each was individually substituted with alanines that cannot participate as nucleophiles. The S99A (but not S153A) enzyme was active to a similar degree as the wild-type enzyme (Table I). The S153A enzyme was completely inactive to the 4-MUO substrate, even though similar amounts of hLAL protein were detected by immunoblotting of Sf9 cells expressing the normal, S99A, or S153A cDNA (Fig. 6A). The S99A enzyme also was inhibited by DNP and THL with respective IC_{50} values of 2.0 ± 0.1 μM and 34.0 ± 4.7 μM. S99A also had essentially normal enzymatic activity toward cholesteryl ester and tri-, di-, and monooleins (Tables I and II).

Three additional mutant hLALs were expressed in the Sf9...
system, including L179P, L336P, and ΔAG302. These mutations in hLALs had been identified homozygously or as compound heterozygotes in patients with CESD and WD (5, 8, 9). In lysates from Sf9 cells infected with the various mutant recombinant viruses, no enzymatic activity toward 4-MUO and cholesterol esters and only very low activity toward triolein could be detected (L179P and L336P) (Tables I and II). Immunoblots demonstrated the presence of normal size hLAL in Sf9 cells infected with the L179P and L336P recombinant viruses (Fig. 6). In comparison, the ΔAG302 protein had a smaller molecular weight, indicating the premature termination of translation.

Although hLAL is targeted to the lysosomes via the mannose 6-phosphate receptor system, it is unknown if glycosylation is needed to preserve or to develop catalytically competent conformers. The single molecular species of normal or mutant hLALs could not be obtained in an active form in E. coli. In Sf9 cells, two molecular weight forms, M, ~41,000 and ~46,000, were detected at a ratio of ~5:1 (Fig. 7). The M, ~46,000 form was the predominant form in spent medium. To determine the relationship of these forms, hLALs from cellular lysates and spent medium were deglycosylated with N-glycanase. Only the M, ~41,000 form was detected following deglycosylation. This indicates that the larger form was glycosylated and the smaller form was either unglycosylated or severely underglycosylated. Similarly, when Sf9 cells were treated with tunicamycin prior to infection with the hLAL recombinant virus, only a single band at M, ~41,000 was detected (Fig. 7). No enzyme was detected in the medium. By immunofluorescence, the generalized intracellular distribution of hLAL in Sf9 cells was unaltered by tunicamycin treatment (data not shown). hLAL for tunicamycin-treated cells was catalytically inactive (Table III).

**DISCUSSION**

Although hLAL plays an essential role in the lysosomal catabolism of cholesterol esters and triglycerides, its low level of expression in most tissues has limited biochemical studies of this enzyme. We and others (3) have recently cloned the cDNA for hLAL, and except for minor polymorphic variants (10), the coding sequences are identical. This facilitated the heterologous expression of hLAL in mammalian and insect cell systems at high levels for further characterization. The pVT plasmid vector was used in these studies since it contained an insect-derived leader sequence from honeybee that has been shown in other systems to facilitate the expression of some mammalian
proteins in insect cells (23). Our results show that this expression system provides sufficient amounts of normal and mutant hLALs for biochemical analyses of mutations associated with WD and CESD and to examine the role of particular residues in the catalytic function. These results provide insights into the roles of glycosylation and two putative nucleophilic serines (Ser<sup>99</sup> and Ser<sup>153</sup>) in the development of catalytically active hLAL as well as an appreciation for the role of hLAL in WD and CESD.

N-Glycosylation, particularly the presence of a mannose 6-phosphate residue, is important to the lysosomal localization of many hydrolases (32). However, the potential role of glycosylation in the formation or maintenance of catalytically active formers of lysosomal enzymes has not been fully explored. Acid β-glucosidase, the enzyme deficient in Gaucher’s disease, is known to require glycosylation at a site near the N terminus for the development of a catalytically active conformer (33). Based on deglycosylation of hLAL with either endoglycosidase H or F, investigators concluded that glycosylation was not important for the catalytic function of hLAL (10, 22). Indeed, a preliminary report suggested that active hLAL could be expressed in bacterial systems that completely lack the glycosylation apparatus (9). We could not achieve expression of active hLAL in E. coli using our pET vector system. In addition, tunicamycin treatment of SF9 cells infected with the recombinant virus containing LAL resulted in the production of a catalytically inactive hLAL. By immunofluorescence, no difference in the distribution of hLAL between untreated and tunicamycin-treated SF9 cells was observed; all unglycosylated hLAL was retained intracellularly. In the absence of tunicamycin, two major molecular weight forms of hLAL were detected by immunoblotting of cell lysates. The lower molecular weight form (M<sub>r</sub> = 41,000) appeared to be unglycosylated or severely underglycosylated, i.e. retained only the core N-acetylglucosamine residues or was totally unglycosylated. The higher molecular weight species in cells or in spent medium were glycosylated as demonstrated by mobility shifts following N-glycanase treatment or treatment of cells with tunicamycin. Also, equal amounts of high molecular weight (M<sub>r</sub> = 46,000) cross-reacting immunologic material from hLAL within cells or in spent medium provided about the same level of enzymatic activity. These results suggest that the lower molecular weight form was unglycosylated and inactive. We conclude from these above studies that cotranslational glycosylation of hLAL is required for the formation of a catalytically active conformer, similar to the results with acid β-glucosidase (33). The preservation of enzymatic activity following deglycosylation indicates that occupancy of glycosylation sites is unnecessary for the maintenance of a catalytically active conformer. These results suggest that glycosylation may be involved in the global folding of hLAL and/or in directing disulfide bond formation. We have no explanation for the presence of large amounts of unglycosylated LAL in insect cells. This has been noted with other proteins, herpes simplex virus-1 and -2 glycoprotein, and tissue plasminogen activator (34, 35), expressed in this system, but the basis for this is not known.

Recombinant hLAL had similar catalytic properties to those reported for lysosomal acid lipases from human fibroblasts and liver and rabbit aorta and liver (10, 20–22). These properties include a pH optimum of ~5.3, a broad substrate specificity, a preference for intermediate fatty acid acyl chain length substrates and cis-unsaturated fatty acid acyl esters, K<sub>m</sub> values of 38 μM for 4-MUO, and stabilization by bovine serum albumin and β-mercaptoethanol. Despite the broad substrate hydrolysis, our studies show a clear selectivity and structural requirements for recognition of substrates by the enzyme. The kink at the ninth carbon in the oleate chain, due to its cis-unsaturation, creates a linear 9-carbon chain for potential interaction with hLAL. The similar kinetic properties for the 4-MU oleate and heptanole substrates raise the interesting possibility that 7–9-carbon fatty acid acyl chain lengths may be the ideal size for enzyme binding and catalysis by accommodation in the active site. The absence of the double bond and the rigidity of the long fatty acid chain in the stearate and palmitate substrates may impair substrate interaction. Similarly, the 4-MU elaidate, which has a Δ<sub>9</sub>-trans-unsaturation, was catalyzed 80%less efficiently by LAL than the corresponding oleate. These preferences were further indicated by the cleavage of triglyceride (triolein) to its di- and monoacylglycerols and fatty acid derivatives. The accumulation of diolein during cleavage of the triolein shows that diacylglycerols are poorer substrates for hLAL than either triacylglycerols or monoacylglycerols. The studies with tri-, di-, and monoacylglycerols as pure substrates also indicated a potentially different binding mechanism, i.e. no differences were found in the level of enzymatic activity or preference since the same catalytic activities were obtained with each of the substrates. This is in contrast to the clear preference for triolein or monoolein substrates when triolein was used as the initial substrate. One explanation for these results would be the inability of LAL to release the diolein rapidly after removal of the first fatty acid acyl chain. This would then require a rebinding and possibly reorientation so that a preferential (sn-1 or sn-3) bond could be cleaved. In contrast, when the diolein is supplied as a pure substrate, its orientation for preferential bond cleavage in the active site would be determined during the initial binding step. An equally plausible explanation for this effect would be a slow reconfiguration of LAL that was required for the cleavage of fatty acid from diolein compared with the triolein or monoolein without release of the substrate. In either case, there appears to be a preference for the sn-1- or sn-3-primary ester group compared with the secondary ester on carbon 2. This is consistent with the lack of cleavage of 2-monooleylglycerol by rabbit LAL (18). Although additional studies will be required to elucidate the absolute substrate preference using more highly purified enzymes, these results are consistent with previous data of rabbit and human LAL substrates preferences. These results are similar to those on other mammalian lipases that show preference for primary ester bonds in substrates such as lipoprotein, pancreatic, hepatic, and lingual lipases (36–38). In comparison, bile salt-activable lipase and monoglycerol lipase lack positional specificities (39, 40).

The presence of two putative active-site sequences (GXSGXG) in hLAL suggested the possibility that either one of these could determine the substrate specificity for cholesteryl ester and/or triacylglycerols. Since serine has been identified as an active-site nucleophile in several lipases and we demonstrated DNP and THL inhibition of hLAL, a serine was likely involved as the catalytic nucleophile for hLAL. By mutating serine 99 or
153 to alanine, we obliterated the nucleophilic capacity of these residues. The results demonstrate that serine 99 is uninvolved in the catalytic activity of hLAL, whereas serine 153 is important, but not necessary, for the activity. Our results do not support the complete separability of cholesteryl ester and triglyceride activities as determined by serine 99 or 153. In addition, serine 153 appears to be very important for the cleavage of 4-MUO, cholesteryl oleate, and triolein as well as mono- and diacylglycerols. However, the S153A mutant retains some activity toward triolein (−4% of the wild type). This very low level of activity is reproducible, whereas cleavage of diolein and monolein as well as cholesteryl ester could not be demonstrated by this mutant enzyme. We conclude that serine 153 is very important to the catalytic mechanism of LAL, but is not necessarily the catalytic nucleophile. In comparison, S99A mutant LAL had normal activity toward all substrates, except the monoglycerol, with which activity was increased by −2-fold. This suggests that although Ser99 is not important as a catalytic nucleophile, conformational changes near Ser99 or its direct participation may influence catalytic activity or entrance to the active site. In either event, it is not essential to catalysis.

To explore the pathogenesis of WD or CESD, we created the three identified mutants. These two autosomal recessively inherited disorders result in the accumulation preferentially of cholesteryl esters in CESD or cholesteryl esters and triglycerides in Wolman’s disease. Wolman’s disease is a much more severe disorder, leading to death in the first year of life, whereas CESD may be compatible with long-term survival (2). Several investigators have suggested that the primary differences in phenotype in WD and CESD relate to the level of residual LAL activity present in various tissues (29, 41, 42).

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Expression of each of the mutant enzymes in Sf9 cells indicated exceedingly low to absent cholesteryl ester cleavage activity for any of the enzymes. In comparison, the L179P and L336P enzymes showed reproducible cleavage (−4% level) of triglyceride, whereas the LAG302 enzyme was inactive. In addition, the L336P mutant had very low levels of activity toward the diacylglycerol substrate. The overall conclusion is that cholesterol esterase activity is absent in all three mutant enzymes, whereas low level triglyceride activity is present in L179P and L336P. It is instructive to examine the genotypes and phenotypes of the patients who have these mutant alleles. For example, a L336P homozygote had CESD, whereas L179P was found in CESD and WD in the presence of null alleles, i.e., an exon 8 splice junction and frameshift mutations, respectively. Thus, from the heteroallelic CESD and WD variants, no enzymatic basis is evident for distinguishing between CESD and WD. Although it is possible that our in vitro studies do not adequately reflect the in situ residual enzymatic activity, these findings support the hypothesis of Burke and Schubert (42) that other factors or mechanisms besides LAL must be operative to account for the selective accumulation of cholesteryl esters and triglycerides in CESD and WD.

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