Characterization of lysosomal acid lipase mutations in the signal peptide and mature polypeptide region causing Wolman disease

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Abstract **Wolman disease results from an inherited deficiency of lysosomal acid lipase (LAL; EC 3.1.1.13). This enzyme is essential for the hydrolysis of cholesteryl esters and triacylglycerols derived from endocytosed lipoproteins. Because of a complete absence of LAL activity, Wolman patients accumulate progressive amounts of cholesteryl esters and triacylglycerols in affected tissues. To investigate the nature of the genetic defects causing this disease, mutations in the LAL gene from three subjects of Moslem-Arab and Russian descent living in Israel were determined. Two homozygotes for a novel 1-bp deletion introducing a premature in-frame termination codon at amino acid position 106 (S106X) were identified. A third subject was a homozygote for a G-5R signal peptide substitution and a G60V missense mutation. The functional significance of these mutations was tested by in vitro expression of single and double mutants in *Spodoptera frugiperda* cells. Single mutants G60V and S106X and double mutant G-5R/G60V displayed a virtual absence of lipase activity in cell extracts and culture medium. Signal peptide mutant G-5R retained lipase activity in cell extracts and showed a drastically reduced enzyme activity in culture supernatant, indicating that the mutation may affect secretion of active enzyme from cells. These results support the notion that Wolman disease is a genetically heterogeneous disorder of lipid metabolism.**—Zschenker, O., N. Jung, J. Rethmeier, S. Trautwein, S. Hertel, M. Zeigler, and D. Ameis. **Characterization of lysosomal acid lipase mutations in the signal peptide and mature polypeptide region causing Wolman disease.** ***J. Lipid Res.*** **2001.** 42: **1033–1040.**

Supplementary key words cholesterol metabolism • lysosome • site-directed mutagenesis • expression cloning

Lysosomal acid lipase (LAL, EC 3.1.1.13) is a key enzyme in the intracellular degradation of neutral lipids derived from plasma lipoproteins (1). The enzyme hydrolyzes cholesteryl esters and triacylglycerols, releasing cholesterol and fatty acids. The released cholesterol contributes to the regulation of endogenous cholesterol synthesis, the uptake of low density lipoprotein, and cholesterol esterification (2). LAL is synthesized by virtually all cells and tissues of the human body, including liver cells, fibroblasts, macrophages, and lymphocytes [reviewed in ref. (3)]. Human fibroblast and liver LAL were purified to apparent homogeneity, full-length LAL cDNA clones were isolated, and their DNA sequence was determined (4, 5). LAL has been mapped on human chromosome 10q23.2-23.3, and the genomic locus was found to consist of 10 exons spread over 36 kb (6, 7). Human LAL purified from various sources showed different molecular sizes. Fibroblasts, for instance, express two molecular forms of 41 and 49 kDa (8), and LAL purified from human liver showed two molecular forms of 41 and 56 kDa (5). LAL expression in insect cells produced two forms of 41 and 46 kDa (9, 10). This heterogeneity results from posttranslational processing involving glycosylation and possibly proteolysis (5, 8, 9). Comparisons of the protein sequence of LAL with those of known lipases revealed significant amino acid homologies with human gastric lipase, rat lingual lipase, and bovine pregastric esterase, establishing the enzymes as members of a gene family of highly conserved acid lipases (4, 11). Structure-function studies of LAL have focused on delineating the roles of serine residues in the two Gly-X-Ser-X-Gly consensus sequences of LAL, demonstrating that S153 is important for hydrolysis of triolein and cholesteryl esters whereas S99 does not affect the enzymatic activity of LAL (9, 12). This has been substantiated by the three-dimensional model building of LAL based on the crystal structure of human gastric lipase (13).

Deficiency of LAL activity leads to two clinically distinguishable phenotypes: Wolman disease (WD) and cholesteryl ester storage disease (CESD). WD is a severe infantile-onset variant, presenting with hepatosplenomegaly, steat-

Abbreviations: CESD, cholesteryl ester storage disease; LAL, lysosomal acid lipase; WD, Wolman disease.

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orrhea, abdominal distention, adrenal calcification, and failure to thrive. Massive intracellular storage of both cholesteryl esters and triacylglycerols is observed in the liver, adrenal gland, and intestine. WD patients usually succumb to hepatic and adrenal failure within the first year of life (14, 15). CESD follows a milder clinical course and frequently remains unrecognized until adulthood. Lipid deposition is widespread although hepatomegaly may be the only clinical manifestation (16, 17). Survival beyond middle age generally occurs with occasional development of premature atherosclerosis (18).

A number of LAL mutations have been identified in WD patients. Various missense mutations [L179P, ref. (19); G321W, ref. (20)] and nonsense mutations [T22X, ref. (21); Q277X, ref. (22); Y303X, ref. (23)] have been found in the LAL gene. Insertion mutations [635insT, ref. (19); 351insA, ref. (24)] and deletion mutations [Δ159–166, ref. (20); Δ435–436, ref. (20); Δexon8, ref. (25)] have also been encountered in pedigrees with WD. In the rat model of WD, a deletion of 4.5 kb and a 60-bp substitution resulted in a premature stop codon at amino acid position 368 and a consecutively truncated LAL protein (26).

In the present study, the molecular basis of LAL deficiency was investigated in three WD subjects found in Israel. In two cases, aborted fetuses were available for tissue culture of skin fibroblasts. In a third case, the subject died after presenting with typical symptoms of WD. We report that a previously undescribed single-base deletion leads to a premature stop codon at amino acid position 106 in two subjects. The third subject was a homozygote for a signal peptide mutation and a missense mutation at amino acid position 60. Interestingly, neither mutation has been found in WD patients but both have previously been associated with CESD. Two mutants, expressed in *Spodoptera frugiperda* cells, lacked detectable residual activity, with the exception of the signal peptide mutation. This mutant had residual activity in cell culture medium and normal activity in cell lysates.

MATERIALS AND METHODS

Subjects

We studied three WD subjects found in Israel (**Table 1**). Subject 1 was a fetus whose parents were cousins of Moslem-Arab origin living in the northern Galilee region. The parents had lost one child at the age of 3 months with clinical features of WD including hepatosplenomegaly, diarrhea, anemia, and calcified adrenals. After prenatal enzymatic analysis of a chorionic villus biopsy had demonstrated homozygous lipase deficiency, the pregnancy was terminated. Subject 2 was a female of Moslem-Arab descent, who died at the age of 3 months after presenting with a typical course of WD. Subject 3 was a fetus of Jewish-Russian parents originating from Uzbekistan and living in Israel. The parents were first-degree cousins who had previously lost a child 3 months after birth. A new pregnancy was terminated after prenatal homozygous lipase deficiency had been demonstrated. Fibroblast cultures of all three subjects were available for study.

Morphological and biochemical analysis of normal and mutant fibroblasts

To directly visualize lipid accumulation in WD fibroblasts, cultured cells were fixed for 10 min in 70% ethanol. Neutral lipids were stained by incubating fixed cells for at least 2 h in 60% isopropanol containing 1-[(4-[xylazol]xylyl)azo]-2-naphthol (oil red O, 3 mg/ml; Sigma, St. Louis, MO). Neutral lipids appeared orange-reddish. Cells were washed with H_2O, and nuclei were lightly counterstained for 5 min with hematoxylin and for 10 min with tap water. For lipid determinations and chromatographic separation of lipids, a control fibroblast line and fibroblasts from WD subjects were cultured in 162-cm^2 flasks. Cells were solubilized by incubation at 4°C for 30 min with 0.2 M NaOH. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL), using bovine serum albumin as a reference. Lipids were extracted with methanol–hexane 1:2 (v/v), dried under nitrogen, and resuspended in ethanol or chloroform for lipid determinations or thin-layer chromatography, respectively (27). Cholesterol and cholesteryl ester concentrations were determined by the cholesterol oxidase method (28) (Boehringer Mannheim, Indianapolis, IN). Triacylglycerol concentrations were enzymatically determined by the glycerol kinase/glycerol phosphate oxidase/

TABLE 1. Clinical and laboratory data of subjects

Variable	Control	Subject 1	Subject 2	Subject 3
Ethnic origin		Moslem-Arab	Moslem-Arab	Jewish-Russian
Consanguinity		+	−	+
Age at death		Fetus	3 months	Fetus
Hepatosplenomegaly[a]		+	+	+
Calcified adrenals[a]		+	+	+
ApoE genotype[b]		3/3	2/3	3/3
LAL 4-MUB hydrolysis[c] (nmol 4-MUB/mg protein per min)	4.3 ± 2.7	0.17	0.33	0.4
LAL trioleoylglycerol hydrolysis[d] (nmol FFA/mg protein per h)	1.0 ± 0.2	n.d.	0	0

Abbreviations: LAL, lysosomal acid lipase; FFA, free fatty acids; 4-MUB, 4 methylumbelliferone; n.d., not determined.

[a] Hepatosplenomegaly and calcified adrenal glands were detected in the index cases of the WD pedigrees, respectively.

[b] Determined according to ref. (47).

[c] Activity determined in cultured skin fibroblasts. Determined as described in ref. (5).

[d] Activity determined in peripheral blood mononuclear cells using tri-[9,10(n)-^{3}H]oleoylglycerol as substrate. Determined as described in ref. (5).

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peroxidase method (Boehringer Mannheim). Concentrations were normalized to milligrams of protein content. For thin-layer chromatography, lipid were spotted onto silica plates (Merck, Rahway, NJ), and separated with a solvent system containing *n*-heptane–diethyl ether–acetic acid 240:60:6 (v/v/v). Lipids were visualized in the vapor phase of iodine.

Detection of mutations

For the preparation of RNA and DNA from cultured cell lines, fibroblasts of a normal individual and three WD patients were cultured in DMEM with 10% FCS and 1 mM HEPES (pH 7.31) containing penicillin and streptomycin at 100 U/ml and 100 μg/ml, respectively, at 37°C in a 5% CO_2 atmosphere. RNA and DNA were isolated from cultured fibroblasts by the guanidinium isothiocyanate or the acid-phenol extraction method, respectively, as described (29). Oligonucleotides were designed on the basis of sequences of human liver LAL (5) and obtained from Genset (Paris, France). LAL cDNAs were synthesized by incubation of 2 μg of fibroblast total RNA with 200 U of SuperScript RNase H^- reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and 100 ng of primer hLAL1600U (**Table 2**). After incubation at 37°C for 1 h, a 1/10 volume of the cDNA was amplified in a 100-μl reaction containing 5 pmol of each oligonucleotide primer; a 2 mM concentration each of dATP, dCTP, dGTP, and dTTP; 4× reaction buffer (80 mM tricine at pH 8.7, 8% DMSO, 32% glycerol, and 0.34 M potassium acetate); and a mixture of 2 U of r*Tth* polymerase and 2 U of Vent DNA polymerase (Boehringer Mannheim) and 1 mM magnesium chloride. Thirty-five cycles of denaturation for 1 min at 94°C, annealing for 1 min at 53°C, and extension for 2 min at 72°C were performed. The final extension time was 10 min at 72°C. PCR products were analyzed by electrophoresis on 1% SeaKem agarose gels (FMC, Rockland, ME) in Tris-borate buffer containing ethidium bromide (0.5 μg/ml) and visualized by UV transillumination. Sequencing was performed with a *Taq* cycle sequencing kit (Applied Biosystems, Foster City, CA) with fluorochrome-labeled M13 universal primers, and reaction products were analyzed on an Applied Biosystems 373A DNA sequencer.

Expression and characterization of mutations

Upstream primer hLALfwd and downstream primer hLALrev were designed to contain *Bgl*II and *Not*I recognition sites complementary to pBacPAK8 sites (Table 2). Standard PCRs were performed with Vent polymerase (New England BioLabs, Beverly, MA), using 35 cycles of 1 min at 94°C, 1 min at 53°C, and 2 min at 72°C. Amplified products were gel purified, digested with *Bgl*II and *Not*I, and cloned into the *Bgl*II/*Not*I-precut pBacPAK8 vector. The complete coding region of the obtained plasmid pBacLAL was sequenced to ensure the absence of PCR-generated mutations.

To verify the significance of individual WD mutants, site-directed mutagenesis by the Quick Change strategy was used (Stratagene, La Jolla, CA). On the basis of the double mutant allele of WD subject 3, two mutants, G-5R and G60V, were constructed, using oligonucleotide-directed mutagenesis (Table 2), and introduced into the LAL expression plasmid pBacLAL. For the double mutant G-5R/G60V and the nonsense mutant S106X, the respective patient alleles were reamplified and subcloned into pBacLAL. Standard PCRs were performed with *Pfu* DNA polymerase, with 16 cycles for 30 sec at 95°C, 1 min at 55°C, and 14 min at 68°C. Successful mutagenesis was verified by sequencing the region surrounding the respective mutation.

To express wild-type and mutant LAL protein, *S. frugiperda* cells were propagated as monolayer cultures in TC-100 medium (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FBS containing penicillin and streptomycin at 100 IU/ml and 100 μg/ml, respectively. Cells (10^6) were cotransfected by Lipofectin with 0.5 μg of wild-type or mutant plasmids and *Bsu*36I-digested BacPAK6 DNA. Recombinant viruses were plaque purified to obtain homogeneous virus stocks. Correct recombination was verified by PCR amplification of viral DNA, using LAL- and BacPAK8-specific primers. For expression of recombinant LAL, viral stocks were amplified four times. Concentrated stocks were used to infect *S. frugiperda* cells grown in 150-mm petri dishes. After 72 h, culture medium was stored at 4°C, and cells were harvested by centrifugation at 2,000 rpm for 5 min at 4°C. Cell pellets were suspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA, and again collected by centrifugation. Pellets were dissolved in 200 μl containing 10 mM sodium acetate at pH 5.0, 0.1 mM DTT, and 1% Triton X-100, and sonified on ice with a Branson sonifier. Enzymatic activities using tri-[9,10(*n*)-^{3}H]oleoylglycerol were determined both in detergent extracts and in culture medium as described (30).

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TABLE 2. Synthetic oligonucleotide primers[a] used for cDNA sythesis, PCR, subcloning, and site-directed mutagenesis

Primer	Sequence
I. Primer for cDNA synthesis	
hLAL1600U	5′-CTTAAGTCAGTGACAGATTT-3′
II. Primers for PCR of cDNA and subcloning	
hLALfwd	5′-TTTT**AGATCT**AGACAGCGGCCCGGCAGGACA-3′
hLALrev	5′-ACGT**GCGGCCGC**TGACATAATCATTGACTTGTT-3′
III. Primers for generation of mutant LAL[b]	
hLAL G-5fwd	5′-CATTCTGAGGGGTCT**GGA**GGGAAACTGACAGCT-3′
hLAL G-5rev	5′-AGCTGTCAGTTTCCC**TCC**AGACCCCTCAGAATG-3′
hLAL G60fwd	5′-GTCTTCCTGCAACAT**GGC**TTGCTGGCAGATTCT-3′
hLAL G60rev	5′-AGAATCTGCCAGCAA**GCC**ATGTTGCAGGAAGAC-3′

[a] Nucleotide sequence numbering refers to the 5′ nucleotide of the respective oligonucleotide and is according to human liver LAL (5). Fwd and rev denote forward and reverse strand orientation of the oligonucleotide, respectively. The forward and reverse primers were designed to contain *Bgl*II and *Not*I recognition sites, respectively (indicated in boldface letters).

[b] For the construction of mutant alleles in the expression vector pBacLAL, site-directed mutagenesis was conducted on the cloned mutant allele of WD subject 3. Therefore, the oligonucleotide primers given confer reversion of Arg-5 to Gly-5 and Val60 to Gly60, respectively. The codons imparting the mutations are bold face.

RESULTS

The deficiency of LAL activity was confirmed in lysates of cultured fibroblasts and peripheral blood mononuclear cells of the three subjects of our study, using 4-methylumbelliferone (4-MUB) and tri-[9,10(n)-^{3}H]oleoylglycerol as substrates (Table 1). Compared with wild-type extracts, the LAL activity was 3.9% to 9.3% for 4-MUB. Activities with tri-[9,10(n)-^{3}H]oleoylglycerol were not detectable in cases 2 and 3.

To demonstrate the metabolic sequelae of deficient LAL activity, staining of neutral lipids was conducted in normal and WD fibroblasts. After fixation with ethanol, cells were incubated with oil red O, and nuclei were counterstained with hematoxylin (**Fig. 1**). WD fibroblasts showed a marked intracellular accumulation of orange-red droplets, corresponding to neutral lipids stored in lysosomes. Cell lines of both subjects 1 and 3 (Fig. 1B and C, respectively) displayed a similar cellular lipid overload. In addition, separation of lipids was conducted by high performance thin-layer chromatography to determine which lipids preferentially accumulated in WD fibroblasts (**Fig. 2**). After chromatography on silica plates with a solvent system containing *n*-heptane, diethyl ether, and acetic acid and staining with iodine, the chromatogram demonstrated a significant accumulation of cholesteryl ester in WD fibroblast lines of subject 1 (Fig. 2, lane 3) and subject 3 (Fig. 2, lane 4) in comparison with normal fibroblasts (Fig. 2, lane 2). Triacylglycerol was not detectable. Lipids were quantitated enzymatically in extracts from a control fibroblast line and fibroblasts from WD subjects 1 and 3 (**Table 3**). Both total cholesterol and cholesteryl esters were increased 1.55- to 1.7-fold and 1.9- to 20.3-fold, respectively. Free cholesterol and triacylglycerol levels were unchanged. Therefore, the fibroblast lipid composition was changed such that cholesteryl esters increased significantly (Table 3).

To determine the mutations responsible for the LAL deficiency in the three WD subjects, mRNA was extracted from fibroblast cell lines, and reverse transcription with an LAL-specific oligonucleotide primer in conjunction with PCR amplification (RT-PCR) was performed. PCR products were subcloned into pCR-Script-SK(+). For subsequent DNA sequence analysis, each pair of oligonucleotides consisted of one member with a 5′-biotin modification to facilitate purification of streptavidin-coated Dynabeads. The other oligonucleotide had a 5′ extension of 17 bases complementary to the universal M13 primer recognition site. Using this strategy, the complete LAL cDNA sequence was determined on both DNA strands, using fluorochrome-labeled M13 primers. When compared with a control subject or with the cloned LAL cDNA sequence (5), the LAL cDNA of Wolman subjects 1 and 2 of Moslem-Arab origin contained a 1-bp deletion of nucleotide 398. This resulted in a replacement of a serine residue by a stop codon at position 106, denoted S106X (**Fig. 3A**), truncating the mature lipase protein by 266 amino acids. This S106X mutation was confirmed by determining the DNA sequence of the complete LAL-coding region of 10 clones and solid-state DNA sequencing of genomic DNA of subjects 1 and 2. Both subjects were found to be homozygotes for the nucleotide 398 deletion and the resulting S106X nonsense mutation.

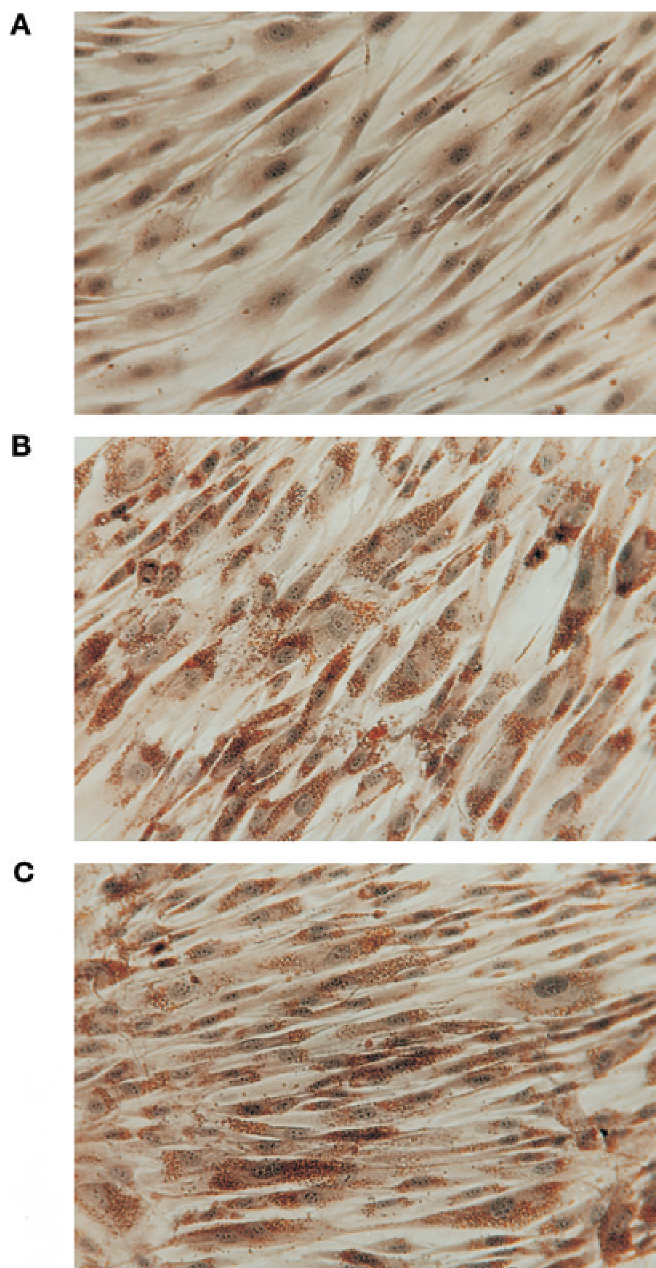

Fig. 1. Lipid accumulation in fibroblasts of WD subjects. A: Monolayer culture of skin fibroblasts of a control individual. Fibroblasts of WD subject 1 (B) and WD subject 3 (C) showing a massive intracellular accumulation of orange-red droplets corresponding to neutral lipids. Staining was performed with oil red O at a concentration of 3 mg/ml for 2 h. Nuclei were lightly counterstained with hematoxylin. Original magnification: ×200.

Mutant alleles responsible for the LAL deficiency in subject 3, originating from Uzbekistan, were assessed by RT-PCR and DNA sequence determination of 10 clones, revealing two mutations in the LAL-coding region. A single nucleotide substitution occurring in the first base of codon −5, changing the wild-type G residue to A, was identified (Fig. 3B). This transition mutation resulted

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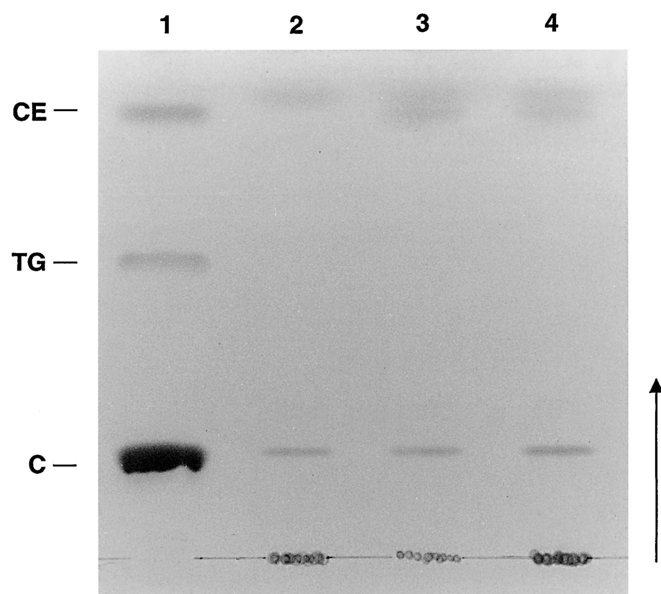

Fig. 2. Thin-layer chromatogram of total lipid extract of fibroblasts from a normal individual and WD subjects 1 and 3. C, Unesterified cholesterol; TG, triacylglycerol; CE, cholesteryl ester. Lane 1, control: 50 μg of cholesterol, 50 μg of CE, and 50 μg of TG; lane 2, lipids equivalent to 8 μg of total cholesterol from a control fibroblast line; lane 3, lipids equivalent to 18 μg of total cholesterol from WD subject 1; lane 4, lipids equivalent to 20 μg of total cholesterol from WD subject 3. Solvent system, *N*-heptane–diethyl ether–acetic acid 240:60:6 (v/v/v). Lipid visualization was performed with iodine vapor.

in a substitution of arginine for glycine in the signal peptide (G-5R). In all sequenced clones, the mutant allele contained a further single-nucleotide substitution, changing a wild-type G residue to A. This mutation occurred in the second base of codon 60, changing the codon from GGC to GTC. This resulted in a substitution of valine for glycine, denoted G60V (Fig. 3C). Subject 3 was thus a compound homozygote for the mutations G-5R and G60V.

The effects of the three LAL mutations on enzyme activities were assessed by constructing expression vectors containing four mutant alleles. The two mutations of the compound homozygous genotype of WD subject 3 were expressed both as G60V allele and as double-mutated allele G-5R/G60V. The respective PCR products were subcloned into pBacLAL, a vector suitable for heterologous expression in the baculovirus system. Wild-type or mutant plasmids were cotransfected into *S. frugiperda* cells, and virus stocks were plaque purified and amplified. Protein expression was conducted for 72 h, a time previously found to allow high level generation of recombinant protein (10). Culture medium was saved, and cells were extracted in a buffer at pH 5.0 containing Triton X-100. Enzymatic activities were determined with tri-[9,10(n)-^{3}H]oleoylglycerol as substrate. LAL activities of mutant clones were standardized to wild-type LAL activities and compared in cell extracts and tissue culture medium (**Fig. 4**). The mutant clone G60V, the double mutant clone G-5R/G60V, and clone S106X had residual activities of 1.1% and less than 0.67% in cell extracts and medium, respectively. For clone G-5R carrying the signal peptide mutation, the LAL activity in cell extracts was virtually wild-type level. In contrast, the LAL activity in culture medium was reduced to 6.3% of wild-type control. Expression experiments were conducted in triplicate. Therefore, the mutations G60V and S106X abolished enzymatic activities in cell extracts and medium, demonstrating that the mutated LAL was inactive. The G-5R mutation resulted in active enzyme that was not secreted properly.

TABLE 3. Lipid determinations in fibroblasts of subjects 1 and 3

Variable	Control	Subject 1	Subject 3
		μg/mg protein	
Total cholesterol	7.20 ± 5.08	12.11 ± 5.34*	11.16 ± 4.40*
Free cholesterol	3.12 ± 1.35	2.89 ± 1.65^{ns}	3.96 ± 1.35^{ns}
Cholesteryl ester	4.08 ± 2.27	9.31 ± 6.35*	7.62 ± 6.10*
Triacylglycerol	4.71 ± 2.91	6.03 ± 2.73^{ns}	5.34 ± 2.52^{ns}

Cholesterol and free cholesterol were determined enzymatically, using cholesterol oxidase. Cholesteryl ester values were determined as the difference between total cholesterol and free cholesterol. Triacylglycerol was determined enzymatically by the glycerol kinase/glycerol phosphate oxidase/peroxidase method. Activities are shown as mean values ± standard deviation. Three separate determinations were performed.
* Significant at $P < 0.05$; ^{ns} Not significant.

DISCUSSION

In the present study, the molecular defects of LAL leading to WD were assessed in three subjects from Israel. Fibroblast lines were analyzed by histochemistry and thin-layer chromatography. When cell lines from subjects 1 and 3 were stained with oil red O, a profound intracellular accumulation of neutral lipids was noted. When separated by thin-layer chromatography, accumulating lipids were identified as cholesteryl esters. This coincides with lipid determinations of liver extracts of WD patients showing a 5- to 160-fold elevation of stored cholesteryl esters (31, 32). Interestingly, triglycerides did not accumulate to any significant amount in cultured WD fibroblasts, contrasting with lipid determinations of liver extracts showing a 2- to 10-fold elevated triglyceride content (31). Whether the lack of triglyceride accumulation in the presence of significant cholesteryl ester accumulation represents a tissue-specific lipid storage pattern or is due to cell culture conditions needs to be explored.

Molecular characterization revealed three different mutations in the LAL gene. In subjects 1 and 2, a novel single-base deletion of nucleotide 398, leading to a premature stop codon at position 106 (S106X), was found. Both subjects were homozygotes for this defect. Although there was no obvious genetic relationship in the subjects, both families were living in neighboring villages in the northern Galilee region of Israel. Because one parent of subject 2 originated from the village of subject 1, some form of consanguinity may be assumed. Another possibility is that of a

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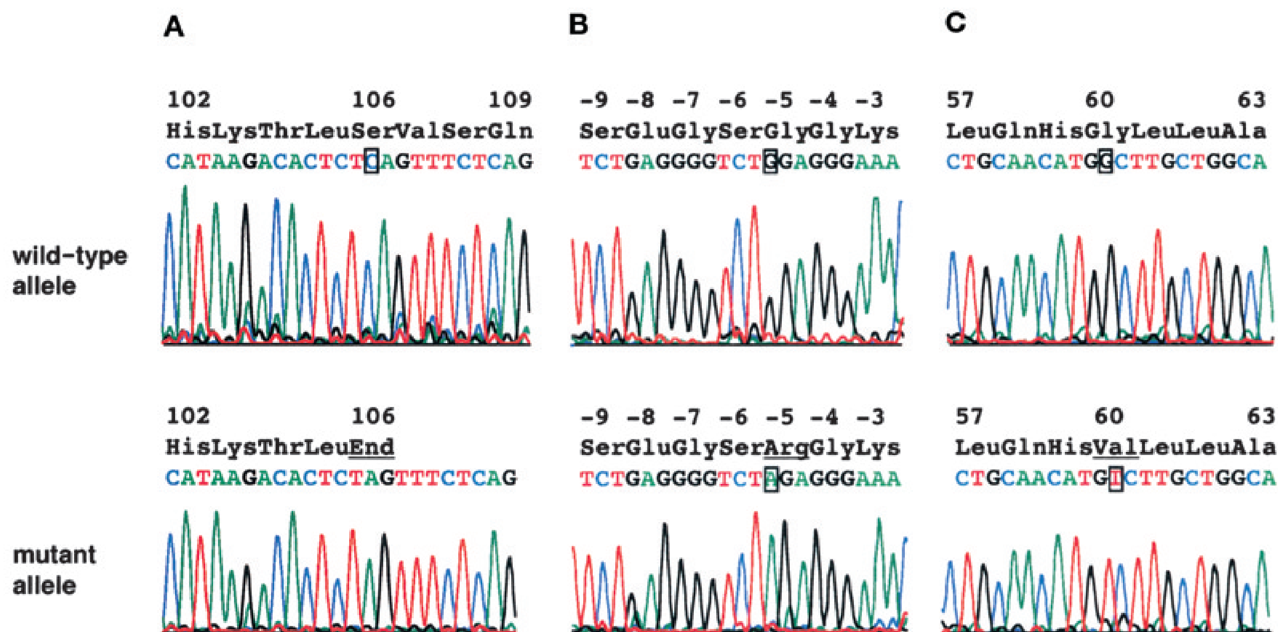

Fig. 3. Comparison of nucleotide sequences of PCR-amplified LAL cDNA from three WD subjects. A: Top, cDNA sequence of a normal individual at codons 102 to 109; bottom, mutation in subjects 1 and 2, showing a C deletion in nucleotide 398, leading to a premature stop codon in position 106 (S106X). Both subjects were homozygous for this nonsense mutation. B: Top, cDNA sequence of a normal subject at codons −9 to −3; bottom, mutation 1 of subject 3, showing a G-to-A transition mutation, leading to a substitution of Gly-5 by Arg (G-5R). C: Top, cDNA sequence of a normal subject at codons 57 to 63; bottom, mutation 2 of subject 3, showing a G-to-T transition, resulting in a Gly-to-Val missense mutation at codon 60 (G60V). Subject 3 was a compound homozygote for the G-5R and G60V mutations. All three mutations were verified in genomic DNA of the WD subjects. The positions of the mutations are boxed, and the amino acid residues generated by them are underlined.

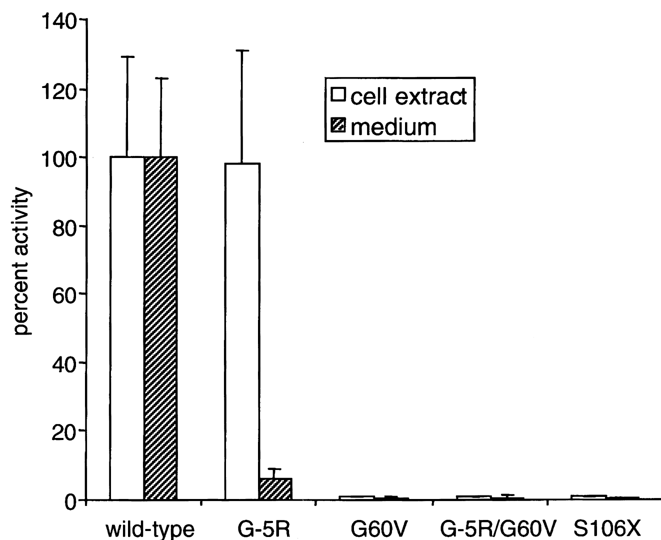

Fig. 4. Effect of mutations of WD subjects on LAL activities. *Spodoptera frugiperda* cells were infected with BacLAL, denoted wild type, or with BacLAL carrying individual mutations. To separate the effects of the G-5R and G60V mutations for which WD subject 3 was a compound homozygote, the mutations were expressed both on one allele and on two separate alleles. Lipolytic activities were determined with tri-[9,10(*n*)-^{3}H]oleoylglycerol in cell extracts (open columns) and cell culture medium (hatched columns). LAL activities are given as a percentage of wild type. Expression experiments were performed in triplicate. Activities are given as mean values ± standard deviation.

recurrent mutation in the LAL gene leading to the S106X nonsense mutation. Expression studies in insect cells showed that the S106X mutation and the resulting truncation of the lipase protein abolished lipolytic activity. These data are consistent with nonsense mutations in other positions of the LAL gene. WD probands of Japanese, German, Turkish, African, and Italian descent were homozygous for nonsense mutations T22X (21), G24X and D116X (20), Q277X (22), and Y303X (23), respectively. All these mutations lead to an enzymatically inactive LAL protein.

In WD subject 3, two mutations were detected for which the subject was homozygous. A single-base transition at nucleotide position 67 of the LAL cDNA resulted in a Gly-to-Arg missense mutation. According to data from peptide microsequencing of human liver LAL (5), the amino acid position of this mutation was assigned to −5, and the mutation was denoted G-5R. On the basis of a different amino terminus of LAL containing six additional amino acids, this mutation has previously been reported in the heterozygous form as G2R (33). In that study, the mutation was associated with the exon 8 splice-site mutation of a CESD proband. The estimated allele frequency was 0.05 for a population of healthy control individuals, suggesting a polymorphism in this amino acid position (33). Unlike individuals in that study, WD subject 3 of this report was homozygous for the G-5R mutation. Expression of the G-5R signal peptide mutation in insect cells re-

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vealed that the mutant enzyme was catalytically active in cell extracts and was thus not responsible for the complete lipase deficiency. However, culture medium of insect cells transfected with the G-5R mutant cDNA contained only 6.3% of wild-type LAL activity, suggesting a reduced secretion of the lipase.

Signal peptides typically consist of three domains, including a positively charged amino terminus, a hydrophobic core, and a polar carboxy-terminal region (34). They target nuclear-encoded proteins to their cellular destinations (35). Signal peptide mutations that cause human disease have thus far been identified only in secretory or endoplasmic reticulum proteins: preprovasopressin (36), preproparathyroid hormone (37), coagulation factor X (38), and bilirubin UDP-glucuronosyltransferase (39). In vasopressin and factor X, mutations were found at the −1 and −3 positions of the carboxyl terminus of the signal peptide, respectively. These mutations block cleavage of signal peptide by signal peptidase. In parathyroid hormone and bilirubin UDP-glucuronosyltransferase, mutations were identified that disrupted the hydrophobic core of the signal peptide and blocked targeting and translocation of the protein across the endoplasmic reticulum membrane. Human manganese-containing superoxide dismutase, a mitochondrial enzyme crucial to controlling oxygen toxicity to the organelle, contained a G/V signal peptide polymorphism at position −9 of a 24-amino acid signal peptide (40). This polymorphism had no effect on the enzymatic activity of superoxide dismutase but was associated with an altered cellular allocation of the enzyme. The G-5R mutation in the carboxy terminus of the LAL signal peptide may exert a similar effect on LAL protein synthesis. This LAL mutation may thus be the first example of a lysosomal hydrolase in which a naturally occurring signal peptide mutation results in a reduced secretion of the enzyme. It remains to be determined whether the mutation is functionally relevant in vivo. Supporting this assumption are data from cultured fibroblasts, where a significant proportion of active lipase was found to be secreted and recaptured from the cell surface by the mannose 6-phospate receptor (41). Because the G-5R mutant was observed as part of a mixed homozygous genotype in subject 3 of this study, it remains speculative whether the mutation per se, when present in the homozygous state, would result in a clinically apparent LAL deficiency.

The relation between LAL genotype and phenotype in WD and CESD individuals is still a matter of debate. Although it has been suggested that a residual lipase activity remains in CESD individuals, resulting in a more benign clinical course than in WD (22, 23, 25), other investigators have presented data on LAL mutants not supportive of this concept (42–44). In this regard, it is interesting to note that the second mutation of WD subject 3, for which subject 3 was homozygous, was a missense mutation at amino acid position 60, substituting Val for Gly (G60V). This mutation has been previously identified in a CESD proband (23). When expressed in a vaccinia system, Pagani and coworkers (23) obtained a residual lipolytic activity of 3.3 ± 1.5% with triacylglycerol as substrate. This residual activity was assumed to prevent excess cholesteryl ester and triglyceride accumulation and thus the severe WD phenotype. In our expression system using insect cells, expression of the G60V mutant resulted in a triacylglycerol hydrolysis of only 1.1% of wild-type LAL, a level of activity most likely not sufficient to prevent the lipid accumulation. It is unclear, however, whether the results obtained in insect cells represent lipolytic activities in human tissues. Determination of the crystal structure of human gastric lipase, a hydrolase with a 59% amino acid homology compared with human LAL, has provided important insights into the molecular mechanisms underlying various LAL mutations (13). For G60V, it was found that the valine side chain interfered with the active-site serine residue and partly blocked access to the presumed triglyceride-binding site of the lipase.

Nonsense mutations of LAL have produced conflicting data with respect to genotype-phenotype correlations. While the T22X mutation (21) and the D124X mutation (45) have been associated with a Wolman phenotype, the R44X genotype was observed in a CESD proband (46). In these nonsense mutations in the amino-terminal region of the lipase, a complete LAL deficiency without any residual activity might be assumed. Nonsense mutations in the carboxy-terminal half of the lipase have also been associated with both phenotypes. G245X, for example, was found in a CESD proband (25). Q277X (22) and Y303X (23), on the other hand, resulted in a Wolman phenotype. Additional mechanisms besides a deficiency of LAL may thus play a role in the production of the two phenotypes, WD and CESD.

In summary, mutations in the LAL gene were identified in three WD subjects. When expressed in insect cells, two of these mutants, G60V and S106X, resulted in abolished enzymatic activity of the lipase. The third mutation, G-5R, led to a catalytically active enzyme that was not secreted appropriately.

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