New lysosomal acid lipase gene mutants explain the phenotype of Wolman disease and cholesteryl ester storage disease

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Abstract **Deficiency of lysosomal acid lipase (LAL) leads to either Wolman disease (WD) or the more benign cholesteryl ester storage disease (CESD). To identify the molecular basis of the different phenotypes we have characterised the LAL gene mutations in three new patients with LAL deficiency. A patient with WD was homozygote for a null allele Y303X. The other two patients, with CESD, presented either homozygosity for T267I or compound heterozygosity consisting of Q64R and an exon 8 donor splice site substitution (G→A in position −1). The mutants T267I and Q64R and the previously reported L273S, G66V, and H274Y CESD substitutions, overexpressed in stable clones, were found to be fully glycosylated and show an enzymatic activity of 3–8% of that of normal LAL. On the other hand, the Δ254–277 mutant protein derived from exon 8 skipping and the Y303X protein were totally inactive. By transient transfection of hybrid minigene constructs, the CESD G→A (−1) substitution resulted in partial exon inclusion, thus allowing the production of a small amount of normal LAL mRNA and hence of a functional enzyme. In contrast, a G→A substitution observed in WD at position +1 of the same exon 8 donor site resulted in complete exon skipping and the sole production of an inactive Δ254–277 protein.▮ In conclusion, LAL genotypes determine the level of residual enzymatic activity, thus explaining the severity of the phenotype.**—Pagani, F., R. Pariyarath, R. Garcia, C. Stuani, A. B. Burlina, G. Ruotolo, M. Rabusin, and F. E. Baralle. **New lysosomal acid lipase gene mutants explain the phenotype of Wolman disease and cholesteryl ester storage disease.** *J. Lipid Res.* 1998. **39:** 1382–1388.

Supplementary key words alternative splicing • mutation • Wolman disease/etiology • recombinant proteins • phenotype

Lysosomal acid lipase (LAL) is a key enzyme involved in the intracellular hydrolysis of cholesteryl esters and triglycerides that have been internalized via receptor-mediated endocytosis of lipoprotein particles (1). During this process, the released free cholesterol regulates its own endogenous synthesis, the uptake of LDL, and cholesterol esterification (1). The importance of LAL in the regulation of intracellular cholesterol flux is further supported by the fact that an altered LAL function has been implicated in the development of atherosclerosis in the population at large (2, 3).

LAL is a 378-amino acid protein containing a 27-amino acid N-terminal signal peptide and six consensus N-glycosylation (N-X-S/T) sites as deduced from the cloned cDNA sequence. Different LAL forms with molecular masses ranging from 41 to 54 kDa correspond to different levels of *N*-glycosylation (4, 5). *N*-linked carbohydrate residues have been shown to be important for optimal catalytic activity (5, 6).

Genetic deficiency of LAL activity results in the accumulation of cholesteryl esters and triglycerides in various tissues of the body and produces two clinically different phenotypes, Wolman disease (WD) and cholesteryl ester storage disease (CESD) (1). Wolman disease is always fatal within the first year of life due to severe hepatomegaly, persistent diarrhea, and failure to thrive. Hyperlipidemia and hepatomegaly are typical features of CESD and can be observed during childhood or develop in adulthood (1). In general, long term survival is observed in patients with CESD, although atherosclerosis related to dyslipidemia and chronic liver disease have been occasionally identified as causes of premature death.

Several mutations of the LAL gene have been found to be associated with disease. A guanine to adenine substitu-

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tion at the −1 position of exon 8 donor site (the 5′ splice site of the corresponding intron) that results in exon skipping and the subsequent loss of 24 amino acids from the protein is the most frequent defect observed in patients with CESD (4, 7–9). Skipping of the same exon has also been observed in a Wolman disease patient. However, in this case, the G to A substitution was in the position +1 of the 5′ splice site of the exon (10). Other splicing defects, point mutations resulting in single amino acid substitutions, and small insertions or deletions that introduce stop codons have also been reported (4, 7, 9–14). These different mutations and/or their heteroallelic combinations could be responsible for the variable phenotypes observed. Although it has been suggested that CESD may be less severe than WD due to a residual level of enzymatic activity (10, 14), direct proof of this hypothesis is still lacking. Furthermore, as the cholesteryl esterase and triacylglycerol lipase activities of LAL can be separated (15, 16), it is also conceivable that some LAL mutants could selectively retain a residual activity toward one of these two substrates. We have been able to show the direct involvement of some LAL mutants in the pathogenesis of acid lipase deficiency in general, but the accuracy of the methodology used (transient expression in mammalian cells) did not allow us to establish firmly whether some of the mutants result in low levels of activity (4) which would be responsible for the benign course of CESD.

In this study we have characterized the LAL gene mutations in three new patients with lysosomal acid lipase deficiency and determined the catalytic activity of different mutant LALs. We expressed them stably in mammalian cells, which allowed us to analyze in detail the catalytic properties and residual activity of the properly glycosylated recombinant LALs. We have also studied the levels of normal LAL mRNA produced by analysis of a transient hybrid minigene system containing the G→A substitutions at position −1 (CESD) or +1 (WD) of the exon 8 donor site. Our results define the biochemical characteristics of the relatively benign CESD as compared to WD.

PATIENTS AND METHODS

Patient presentation

Patient VM was a typical WD case. Hepatomegaly, failure to thrive, persistent diarrhea, and adrenal gland calcifications were observed at 1 month of age. Death occurred at 8 months. His twin developed the same symptoms and died at 3 months of age.

Patient PG was an 8-year-old female with severe CESD. The patient presented persistent diarrhea, failure to thrive, hepatomegaly, adrenal calcifications, and mildly elevated liver function parameters at the age of 8 mo. Severe hypercholesterolemia (8.01 mmol/L) and hypertriglyceridemia (2.47 mmol/L) were reported by that time. A liver biopsy performed at the age of 2 years revealed vacuolization and massive storage of birefringent material in hepatocytes. Diffuse nodular cirrhosis was observed at the age of 6 years. She underwent liver transplantation at 7 years of age and she is well 1 year after the procedure.

Patient PE was an 11-year-old female of Brazilian descent. She was occasionally reported to have liver transaminase values double than normal at the age of 6. Hepatomegaly was noted at 10 yr of age along with hypercholesterolemia (7.8 mmol/L) and hypoalphalipoproteinemia (0.85 mmol/L). A liver biopsy showed mild fibrosis with the presence of microvacuolated histiocytes.

Genetic analysis

Mutant alleles responsible for LAL deficiency in the three new patients were first identified through nucleotide sequencing of single clones derived from RT/PCR of LAL mRNA. The presence of each mutation in genomic DNA and the pattern of inheritance in each family were then analyzed by direct sequencing of DNA amplified fragments. Total RNA was extracted from liver tissue and peripheral blood mononuclear cells by conventional methods (17). Amplification of first strand cDNA was performed using Taq polymerase (Boeringher Manneheim) according to Saiki et al. (18). Ten μl of the first strand cDNA mixture was incubated in a total volume of 100 μl with 50 pm of LALF1 and LALRC1274 primers, which correspond to the coding portion of LAL and 5 U of Taq polymerase for 35 cycles (45 s at 93°C, 60 s at 52°C and 90 s at 72°C). The same amplification conditions were used to amplify genomic DNA. The PCR amplification products (1274 bp fragment) were subcloned and 5 of the clones obtained for each patient were entirely sequenced.

The sequence of the oligonucleotides used in PCRs of genomic DNA and for RT/PCR were as follows.

LAL F1	ACTGCGACTCGAGACAGCGGC
LAL F895	TGCTGGAACTTCTGTGCA
LAL RC1057	ACTGCAGTCGGCACAAGCAT
LAL RC1274	ATAAGCTTGGTGGTACACAGCTCAAGT
ex4F	GAAGCTTGGTGCTACTGCC
ex4R	CTGGAAGCCTGTTGTCTGC
ex8F	TCAATGCCACCTTAATGC
ex8R	GGAAAGGGTTTTGCATGCC
ex10F	GTGCCGGTAATGGCAACTTG

Plasmid DNA, purified on miniprep Spun Columns (Pharmacia Biotech, Sweden), or PCR products, purified on a MicroSpin S400 HR column (Pharmacia Biotech, Sweden), were sequenced according to the dideoxy chain terminating method (19) using [α^{35}S]dATP and modified T7 DNA polymerase (Pharmacia Biotech, Sweden). The mutations detected were numbered according to Anderson and Sando (20). Amino acid sequences are reported in one letter code.

Expression of LALs and biochemical analysis

Normal and mutated LALs were distinguished from endogenous LAL by tagging with an HSV epitope added to the carboxy terminus of the protein. Tagging with an HSV epitope was performed using the PCR-based overlap extension method as previously reported (4). Normal and mutated LALs were cloned in blunt XhoI site of pKG5 vector and stably expressed in COS cells. Selection of the stable recombinant clones was performed with G418 at a concentration of 800 μg/ml. At least two independent clones were analyzed for each mutant. Recombinant LAL proteins were analyzed by Western blotting using an HSV tag monoclonal antibody (Novagen, Madison WI) followed by either alkaline phosphatase-conjugated goat anti-mouse IgG or a goat anti-mouse IgG and ^{125}I-labeled protein A (Amersham). The amount of recombinant protein expressed by the clones was quantified with a phosphoimager (Packard).

Acid lipase activity of cell extracts was measured using either the natural substrates cholesteryl-[^{14}C]oleate or tri-[^{14}C]oleyl-glycerol (4) or 4-methylumbelliferyl-palmitate (MUP) (21). The specific activities of normal LAL clones toward triolein and cholesteryl oleate averaged 1005 and 296 pmol fatty acid released/h per μg protein, respectively. The activity toward MUP for the same clones averaged 546 pmol of methylumbelliferone released/min

per μg protein. Cholesteryl oleate esterase, trioleylglycerol lipase, and acid esterase (MUP) activities of the recombinant clones were normalized for the amount of recombinant protein expressed, and results are expressed as percentages of the activity of normal LAL clones, calculated after subtracting the endogenous background activity (cells transfected with control plasmid alone).

Construction of LAL exon 8 minigenes

The starting material was pSVmFN, a plasmid used to analyze the *cis*-regulatory elements involved in splicing of the fibronectin gene (22). It contains the mouse fibronectin genomic fragment, spanning the extra domain typeA (EDA) exon, its flanking introns and parts of the +1 and −1 exons, cloned in the BstEII site inside the third exon α globin in the pSVα1W vector (23). The pSVmFN NdeI- was created by excision of the NdeI–NdeI fragment that contains the EDA exon and part of the intron–exon junctions (200 bp of the intron −1 and 240 bp of the intron +1). This central part was substituted with the genomic fragment of LAL containing the 72 bp of exon 8, 115 bp of the intron +1 and 135 bp of intron −1. Genomic DNA extracted from a normal individual and from a patient with CESD that is a carrier of the G→A substitution at position −1 of the exon 8 donor site (patient 1 in ref. 4) was amplified with 2 × 8F (ACATACTGAGTTATGGCTCTAG) and 2 × 8R (AACCCAGGACTCTGGGGAAGA) oligonucleotides and the resulting fragment of 322 bp was cloned in the NdeI site of pSVmFN Nde- clone. Sequence analysis of the resulting hybrid minigenes showed clones containing the normal G (LAL wt) or the mutant A (LAL CESD) at position −1 of the exon 8 donor site. An additional clone with A to G substitution at position −2 of the 3′ splice of the exon (LAL Art) was obtained due to a Taq error. As this artificial substitution occurs in a position of the acceptor 3′ splice site that must be maintained unaltered for correct mRNA processing, it was further analyzed as a control. The LAL WD clone with G→A substitution at position +1 of the donor site of exon 8 was created using the PCR-based overlap extension method (24) with the following oligonucleotides (WD1: CACTGGAGCCAGATAGGCATTC and WD2: GGAATGCCTATCTGGCTCCAGT). Sequences of the clones were verified by the dideoxy chain termination method (19) using α^{35}S ATP and modified T7 DNA polymerase (Pharmacia Biotech).

Transfection, RNA preparation, and RT-PCR analysis

COS1 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FCS, 50 μg/ml of gentamycin, and 4 mm l-glutamine. About 1.6 × 10^6 cells were transfected with 5 μg of specific plasmid, and purified by CsCl gradient centrifugation, by means of a modified calcium phosphate precipitation method (25). Cells were harvested 48 h after transfection and RNA was prepared by a single-step extraction method using RNAzol™B (TEL-TEST Inc., Texas). About 2 μg of RNA samples was reverse transcribed in 25 μl reaction mixture containing 50 mm Tris-HCl, pH 7.6, 60 mm KCl, 10 mm DTT, 250 nm dNTPs, 25 pm of random hexanucleotide primer, 10 U of RNAse inhibitor (Stratagene), and 150 U of Moloney leukemia virus reverse transcriptase (GIBCO BRL). LAL exon 8 messenger derived from the transiently transfected constructs was specifically amplified using primer A (CACTGCCTGCTGGTGACGTAC) and B (TGGGCGGCCAGGGTCACGGC). The amplifications were performed in a total volume of 50 μl containing 1× Taq buffer (Boehringer), 200 nm of dNTPs, 40 pm of each of the primers and 2 U of Taq (Boehringer) in a DNA Thermal Cycle 400 (Perkin Elmer). A total of 30 cycles of amplification (30 secs of denaturation at 94°C, 30 secs of annealing at 56°C and 30 secs of extension at 72°C)

were performed and 5 μl of the PCR products was analyzed on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide.

RESULTS

Detection of LAL gene mutations in three patients with LAL deficiency

Mutant alleles responsible for LAL deficiency in the three patients were first identified through nucleotide sequencing of single clones derived form RT/PCR of LAL mRNA. The presence of each mutation in genomic DNA and the pattern of inheritance in each family were then analyzed by direct sequencing of genomic DNA (**Fig. 1**). Genetic analysis of exon 10 showed that the patient with Wolman disease (VM) was homozygote for Y303X due to substitution of A for T at nucleotide position 1012 (Fig. 1a). The truncated protein produced by this allele lacks the last 75 amino acids, a region in which no putative glycosylation sites are located. The parents were heterozygote for the same non-sense mutation. Patient PG, with CESD, was homozygous for a C to T substitution at nucleotide position 903 in exon 8, which leads to the amino acid change T267I (Fig. 1b). Both parents were heterozygote for this substitution (data not shown). Direct sequencing of exon 4 in patient PE showed heterozygosity for an A to G mutation at nucleotide position 294 which produces a Q64R missense substitution (Fig. 1c). The other allele contained the commonly found splice site mutation G→A in position −1 of the exon 8 donor site. Subsequent skipping of the exon 8 produces a LAL mRNA that codes for a protein with a 24 amino acid deletion (Δ254-277).

The other LAL gene defects analyzed in this study have been previously reported (4, 5, 10, 13). The missense substitutions H274Y, L273S, P181L, and G66V have been associated with CESD whereas the G→A substitution at position +1 of the exon 8 donor site has been reported in homozygosity in a WD patient (10).

Expression of fully glycosylated recombinant LAL mutants in stable clones

We have previously demonstrated the direct involvement of mutant LALs in pathogenesis through transient expression in HeLa cells, using the T7 vaccinia system (4). All the mutants analyzed were found to be inactive toward cholesteryl ester and triglycerides within the range of experimental error (<5%) (4). However, the mutant proteins showed a variable proportion of N-glycosylation, which we have recently found to be critical for a full catalytic activity of normal LAL (5). In order to detect more accurately the probably very low residual activity that might be expressed by the different mutants, we generated and selected stable COS clones producing high levels of fully glycosylated recombinant LALs. The mutant LALs detected in the three new patients and the previously reported H274Y, L273S, P181L, and G66V missense substitutions were all tagged with an HSV epitope added to the
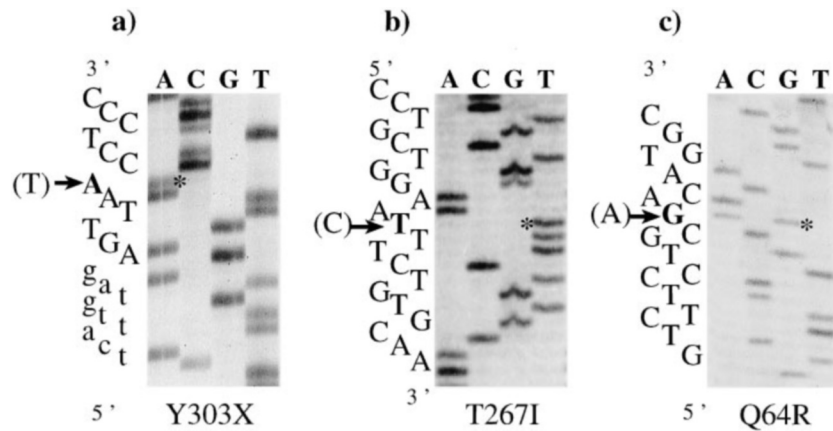
Fig. 1. Detection of LAL gene mutations in patients with LAL deficiency. a) Nucleotide sequencing of intron 9–exon 10 junction of patient VM showing the T→A substitution (asterisk). Genomic DNA was amplified with ex10 F and LAL RC1274 oligonucleotides and the resulting fragment of 355 bp was sequenced with ex10F. Intronic sequences are indicated by lower case letters. The normal T is indicated in parentheses. The patient is homozygote for the Y303X mutation. b) Direct sequencing of exon 8 of patient PG. The missense substitution detected at position 903 changes a C to T (T267I). Genomic DNA was amplified with ex8F and ex8R primers and the resulting 203 bp fragment was sequenced with ex8R. The normal C is indicated in parentheses. The patient is homozygote for the mutation. c) Direct sequencing of exon 4 of patient PE. The genomic DNA was amplified with ex4F and ex4R primers and the resulting 311 bp fragment was sequenced with ex4F. The patient is heterozygote for a A to G substitution at nucleotide position 294 (Q64R). The other allele codes for the G→A splice site mutation in position −1 of the exon 8 donor site.

carboxy-terminus (4). Immunoblotting using an anti HSV antibody showed a single molecular form of M_r corresponding to fully glycosylated LAL in all cases (**Fig. 2**), as opposed to the transient expression of LAL in the T7 vaccinia system, where partially glycosylated and totally deglycosylated forms were observed (Fig. 2, lane LALts). The higher molecular mass of the recombinant L273S protein is due to the presence of an additional oligosaccharide chain at a newly created N-glycosylation site (5). The Δ254–277 and Y303X mutants showed a reduced molecular weight compatible with the deletion of 24 and 75 amino acids, respectively.

Missense substitutions in CESD produce partially active LAL proteins

Measurement of the enzymatic activity of T267Y, Q64R, H274Y, L273S, P181L, and G66V recombinant proteins showed a low but clearly detectable activity of about 3–5% of that of normal LAL toward the natural substrates cholesteryl ester and triglyceride, or 7–8% of normal LAL when using the artificial substrate MUP (**Table 1**). On the contrary, no activity was detected in the case of the stable clone expressing the recombinant deleted form of LAL derived from exon 8 skipping (Δ254–277) or from the Y303X allele.
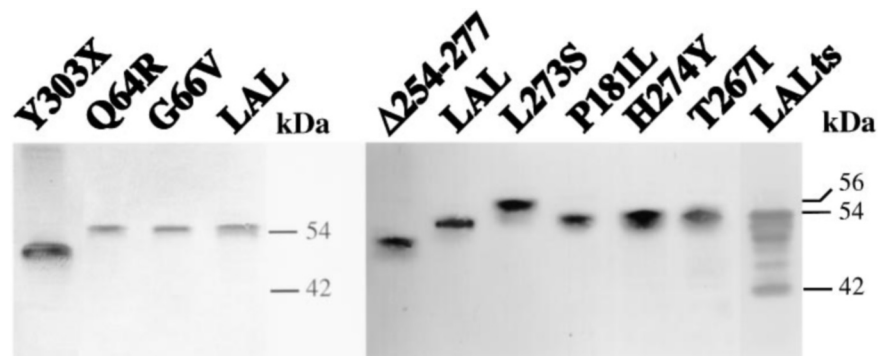

Fig. 2. Immunoblots of normal and mutant LALs. Normal LAL was transiently expressed in HeLa cells with the T7 vaccinia system (LALts) as previously reported (4) or stably expressed in COS cells (LAL). Mutant LALs were all stably expressed in COS cells. Extracts of COS or HeLa cells (10 μg protein/lane) were subjected to 12% polyacrylamide gel electrophoresis and Western blotted. The blots were probed with antiHSV peptide. The estimated molecular masses are indicated ($M_r \times 10^{-3}$).

TABLE 1. Acid lipase activity of mutant LALs

LAL Variants	Triacylglycerol Lipase	Cholesteryl Esterase	Acid Esterase
T267I	2.9 ± 0.8	3.6 ± 0.8	ND
G66V	3.3 ± 1.5	3.9 ± 0.9	ND
Q64R	2.7 ± 1.8	2.7 ± 1.0	ND
P181L	5.2 ± 2.1	3.4 ± 0.8	7.0 ± 1.3
H274Y	3.0 ± 0.9	2.9 ± 0.6	7.7 ± 2.4
L273S	3.1 ± 1.1	1.9 ± 0.5	3.6 ± 0.9
Δ254-277	0	0	0
Y303X	0	0	0

The acid lipase activities of recombinant clones expressing fully glycosylated mutant LALs were determined. Data (mean ± SD) are expressed as percentage of the activity of normal LAL and are the result of three independent determinations using two different clones of each mutant. The specific activities of normal LAL clones toward triolein and cholesteryl oleate averaged 1005 and 296 pmol fatty acid released/h/μg protein, respectively. The acid esterase activity for the same clones averaged 546 pmol of methylumbelliferone released/min per μg protein. Y303X was found in a Wolman case (V.M.). The Δ254–277 protein was associated with both WD and CESD. All the other mutants were associated with CESD; ND, not determined.

LAL exon 8 splicing analysis

As the complete exclusion of exon 8 from LAL mRNA gives rise to the inactive LAL protein Δ254–277, we analyzed the efficiency of splicing of two G→A exon 8 splice site substitutions (in position −1 and +1) that produce this deleted protein. We constructed different LAL hybrid minigenes (**Fig. 3a**). LALwt contains the normal 3′ and 5′ splice sites. In LAL CESD the normal G at position −1 of the 5′ splice site was substituted with A whereas in LAL WD the normal G at position +1 of the 5′ splice site was substituted with A. As a control of splicing efficiency LAL Art contained an A to G mutation at position −2 of the 3′ acceptor splice. These constructs were transfected into COS cells and the splicing pattern of the mRNA derived from the hybrid minigenes was analyzed by RT-PCR using specific primers. The amplifications can produce two different bands of 302 and 230 bp. Direct sequencing of these bands showed that they correspond to the presence or absence of exon 8, respectively.

Transfection of LALwt showed a complete inclusion of exon 8, while complete exon skipping was evident with LAL Art (Fig. 3b). The G to A substitution at the −1 position of the donor splice site (LAL CESD) resulted in an incomplete exon exclusion, with about 5% of processed mRNA retaining the exon. In contrast, the G→A mutation at the +1 position (LAL WD) resulted in complete exon exclusion.

DISCUSSION

The molecular basis responsible for the very different phenotypes of WD and CESD, both of which originate from a deficiency in LAL activity, is not yet completely understood. Some of the mutant alleles of the LAL gene found in these patients produce truncated or deleted proteins due to stop codons or splicing defects (4, 9–11, 13). Alternatively, missense substitutions can produce single
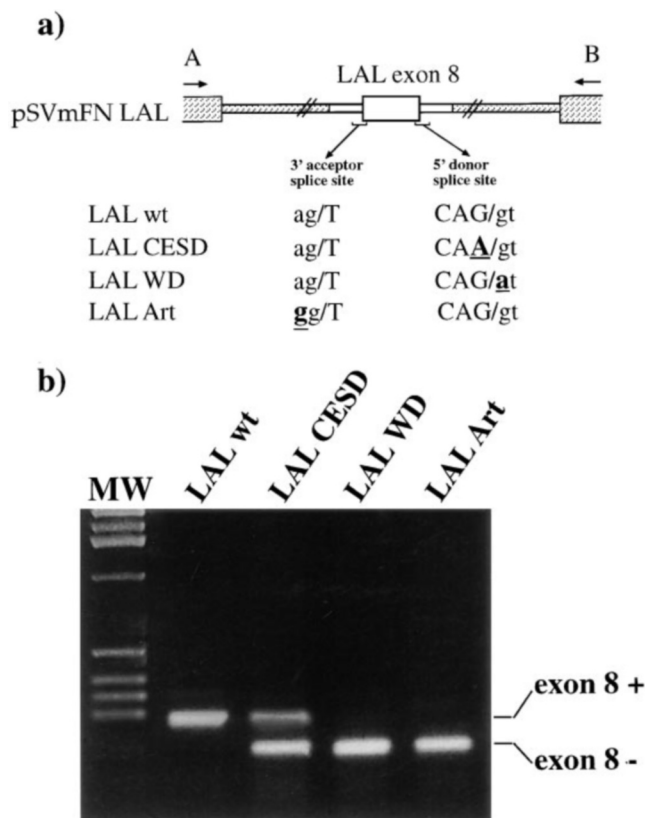

Fig. 3. Expression of the exon 8 LAL minigene variants in COS cells. a) Schematic representation of the hybrid minigenes containing the different splice site variants of LAL exon 8. The genomic portion of LAL encompassing exon 8 (white rectangles) was cloned inside the αglobin–mFN plasmid (barred rectangles). Exons and introns are indicated by wide and narrow rectangles, respectively. The location of the primers used in the RT-PCR assay is shown. The sequence of the intron–exon junctions flanking the exon 8 in the wild type LAL (LAL wt), in the mutant alleles present in CESD (LAL CESD) and Wolman (LAL WD), and in an artificial mutant (LAL Art) are shown. Nucleotides different from those of normal LAL are underlined. b) RT-PCR analysis of mRNA isolated from cells lines transfected with the different LAL constructs, using primers A and B. The bands corresponding to exon 8 inclusion (302 bp) and exclusion (230 bp) products are indicated.

amino acid changes (4, 13). We have previously shown that some of these mutants are directly involved in the pathogenesis of LAL deficiency (4). However, the methodology used to analyze the recombinant LALs (transient expression with the T7 vaccinia system) was not sufficiently accurate to establish a phenotype–genotype correlation. This was due to the fact that fully active, glycosylated LALs were produced together with substantial amounts (about 50%) of partially active, deglycosylated forms (Fig. 2) (4). In the present study we have characterized the LAL gene mutations in three new patients with LAL deficiency and determined the catalytic activity of different mutant LALs stably expressed in mammalian cells. These recombinant LALs become fully glycosylated (5) and can be distinguished from the endogenous LAL background by a HSV epitope tag. We demonstrate that the less severe form of CESD is due to mutants that retain a

significant residual enzymatic activity toward both of the two natural substrates, cholesteryl ester and triglycerides.

The Y303X, T267I, and Q64R mutant alleles identified in the three patients (Fig. 1), along with the previously reported missense substitutions H274Y, L273S, P181L, G66V and the deleted LAL Δ254–277 were expressed in stable clones. All the missense substitutions were associated with CESD. Two of them, H274Y and T267I, have been detected in homozygosity; L273S and P181L were associated with a null allele and with an exon 7 splice site substitution, respectively; and Q64R and G66V were associated with an exon 8 donor splice site substitution (G→A in position −1). The Δ254–277 protein may originate from exon 8 skipping due to either of two different splicing defects at the 5′ splice site: G→A in position −1 of the acceptor splice site, observed in CESD, or G→A in position +1 of the acceptor splice site, observed in WD (10). The G→A substitution at the position −1 is the most frequently found mutation in CESD and it has never been detected in WD (4, 7–9). The Y303X allele that produces a truncated protein is found in homozygosity in a WD case (Fig. 1a).

The results obtained show that all the missense substitutions associated with CESD result in a residual level of enzymatic activity (3–8% of normal) (Table 1). In addition, the relative activity toward each of the two natural substrates cholesteryl ester and triglyceride was very similar. This excludes the possibility of a differential activity toward one of the two natural substrates generating the different phenotypes of WD and CESD.

In contrast to the missense substitutions, the Δ254–277 and the Y303X proteins are completely inactive (Table 1). As the skipping of exon 8 resulting in Δ254–277 can occur in both WD and CESD, we have investigated the efficiency of splicing of exon 8 displayed by the two different splice site mutants found. This analysis was performed by transfection of COS cells with LAL hybrid minigenes containing the CESD allele (G→A in position −1 of the acceptor splice site) or the WD allele (G→A in position +1 of the acceptor splice site) (Fig. 3). These experiments showed that normal splicing is only possible, although with a greatly reduced efficiency, in the case of the splice site mutation of the CESD allele. On the other hand, the WD allele does not produce any normal mRNA, indicating that this is a completely null allele. In addition, our results exclude that other genetic defects located in regions presumed to regulate splicing (i.e., introns) could, in linkage disequilibrium with the G to A CESD substitution, be involved in the aberrant splicing. The hybrid minigene experiments indicate that about 5% of normal mRNA may be produced from the splice site mutant G→A (−1) allele. The patients carrying this allele present a classic (late onset) CESD phenotype, which is less severe than that of the patients with the substitutions H274Y, T267I, and P181L, who develop an early hepatic involvement and adrenal calcifications. This phenotype variability could be due to a higher LAL residual activity in classic CESD as compared with early onset CESD. Different levels of residual activity originating from the combination of the various alleles might be responsible for CESD phenotype variability.

In conclusion, a residual acid lipase activity within the range of 3–8% of that of normal LAL would be sufficient to prevent the excessive accumulation of cholesteryl esters and triglycerides inside lysosomes, leading to the expression of CESD phenotypes of different severity. On the other hand, a complete absence of activity, as in the WD splice site mutation or in Y303X, produces only inactive LAL proteins and generates the severe phenotype of Wolman disease.▋

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