L273S missense substitution in human lysosomal acid lipase creates a new *N*-glycosylation site

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Abstract Human lysosomal acid lipase (LAL), when expressed in HeLa cells using the Vaccinia T7 expression system, showed four major molecular forms ranging from 42 to 54 kDa. Treatment with endoglycosidase H resulted in a 42 kDa protein, indicating that the molecular weight variations were due to *N*-glycosylation. A missense substitution, L273S, previously detected in a patient with cholesteryl ester storage disease (CESD), produced catalytically inactive LAL showing a largest molecular mass form of 56 kDa instead of 54 kDa. Analysis of the amino acid sequence in the close proximity of the mutation (NMS→NML) indicated that the L273S mutation creates an additional *N*-glycosylation consensus (N-X-S/T) in this region. Two site directed mutants disrupting this consensus, QMS and QML, when expressed in HeLa cells, did not show the 56 kDa form but the normal 54 kDa band whereas deglycosylation always resulted in the major 42 kDa form, as observed with normal LAL and the L273S mutant. These data confirmed that an additional *N*-glycosylation at N271 was responsible for the 56 kDa form of the protein produced from the L273S allele. Furthermore, deglycosylation of normal LAL reduced the acid hydrolase activity towards both tri-oleyl glycerol and cholesteryl oleate by 50%, strongly suggesting that *N*-linked carbohydrate residues are important for optimal catalytic activity.

Key words: Lysosomal acid lipase; Cholesteryl ester storage disease; Vaccinia T7 expression system; Glycosylation; Epitope tagging

1. Introduction

Hydrolysis of cholesteryl esters and triglycerides, delivered to the lysosomes through the receptor mediated endocytosis of lipoprotein particles, is exclusively mediated by lysosomal acid lipase (LAL) [1]. The amino acid composition of LAL, derived from the cloned cDNA sequence, predicts a 378 amino acid polypeptide chain with an estimated molecular mass of 42.5 kDa [6]. The primary amino acid sequence of LAL suggests the possibility of a 27 amino acid N-terminal signal peptide and six consensus *N*-glycosylation (N-X-S/T) sites. Three of these consensus sites, at N15, N80 and N252, are conserved among the members of the acid lipase gene family including rat lingual lipase and human gastric lipase [6]. The presence of *N*-glycosylation signals within the LAL primary structure indicates mannose-6-phosphate receptor mediated specific targeting to the lysosomal compartment [1,2].

Acid lipase deficiency is associated with two autosomal recessive, lysosomal storage disorders, cholesteryl ester storage disease (CESD) and Wolman disease [1], both characterized by a massive accumulation of cholesteryl esters and triglycerides in different tissues of the body including liver, spleen and adrenals. The most severe form of the disease, Wolman's disease, is lethal during the first year of life, while CESD follows a more benign clinical course frequently characterized by dislipidemia and hepatomegaly during childhood. A low residual activity of LAL has been suggested to be responsible for the different phenotypic expression of CESD and Wolman disease [1,7].

Different mutations in the LAL gene have been reported in patients with CESD and Wolman disease [8–13]. We have recently described the molecular basis of CESD in three patients with the identification of three different missense substitutions, two splicing defects that do not alter the open reading frame and a null allele [14]. One of these patients was a compound heterozygote for a missense substitution (L273S) and a null allele.

The very low amount of LAL that can be isolated from mammalian cells has limited its extensive characterization. Different molecular sizes have been reported for purified human LAL. A 47 kDa form was reported to be secreted from fibroblasts [2], and two forms of 56 and 41 kDa were purified from human liver [3]. On the other hand, two molecular forms of 46 and 41 kDa have been reported for human LAL expressed in insect cells, a system in which the targeting of lysosomal enzymes is not regulated by the canonical mammalian mannose-6-phosphate receptor [4,5]. The origin of the different molecular mass forms of LAL is not yet clear, while both selective proteolysis and *N*-glycosylation have been suggested to be responsible for their occurrence. Discrepancies in the results might be due to biased purification procedures or the type of expression system employed [2–4], particularly considering that very little is known about the glycosylation pattern of mutant LALs and the effect of glycosylation on their catalytic activity.

In this report, we have evaluated the effect of the missense substitution L273S on the glycosylation pattern of recombinant LAL protein. In addition, we have examined the general effect of glycosylation on the enzymatic activity of LAL.

2. Materials and methods

2.1. PCR amplification and oligonucleotides

The sequences of the oligonucleotides used for mutagenesis are listed on the following page.PCR amplifications were performed according to Saiki et al. [15] using Taq polymerase (Boehringer Mannheim), with 50 pM of each corresponding set of oligonucleotides and

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Abbreviations: LAL, lysosomal acid lipase; CESD, cholesteryl ester storage disease; HSV, herpes simplex virus

PII S0014-5793(96)01134-9

LALF782 TGCACTCATGTCATACTGAA
LALJ1rev ACCTCCGCCAGATCCCTGATATTTCCTCATTAGA
QS1 AACAGATGTCACACTGGAGCCAG
QS2 TGTGACATCTGTTGCACAGAAGT
QL1 AACAGATGTTACACTGGAGCCAG
QL2 TGTAACATCTGTTGCACAGAAGT

5 U of Taq polymerase for 35 cycles (45 s at 93°C, 60 s at 52°C and 90 s at 72°C).

2.2. Construction of HSV tagged LAL mutants by site directed mutagenesis

Normal (pLALtag) and mutant LALs were tagged with a 11 amino acid peptide (QPELAPEDPED) derived from herpes simplex virus glycoprotein D. This peptide was linked to the carboxyl end of LAL by a 5 amino acid connecting segment (GSGGG) using the PCR- based overlap extension method [16]. Two site directed mutants at residues 271–273, QML and QMS, were constructed using the same technique. The central portion of pLALtag, between the *Sac*I and *Acc*I restriction sites, was substituted with the mutagenized amplified fragments. pLALtag was amplified with primer pairs LALF782/QS1 or QL1 and LALJ1rev/QS2 or QL2. The two corresponding fragments were mixed and reamplified with LALF782/LALJ1rev. The product of the second PCR was digested with *Sac*I and *Acc*I restriction endonucleases and recloned in pLALtag. The resulting plasmid DNAs, purified on miniprep Spun Columns (Pharmacia Biotech, Sweden), were sequenced according to the dideoxy chain terminating method using [α-^{35}S]dATP and recombinant T7 DNA polymerase (Pharmacia Biotech, Sweden) [17,18].

2.3. Expression of LAL mutants

Expression of LAL by means of the Vaccinia T7 expression system was performed according to [19]. Recombinant Vaccinia virus vTF7-3, bearing the bacteriophage T7 1 gene, was a generous gift of T. Fuerst and B. Moss [19]. HeLa cells grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum were infected with vTF7-3 at a multiplicity of 30 plaque-forming units per cell, in serum-free medium. The virus was removed after 30 min incubation at 37°C and replaced with serum-free medium. Cells were transfected with 2.8 μg of each LAL plasmid DNA plus 0.2 μg of a CAT expressing plasmid (pULB), complexed with 20 μg of cationic liposome DOTAP (Boehringer Mannheim). The control transfection was performed with pULB plasmid alone. Cells were harvested 16–24 h after infection-transfection. Cell pellets were resuspended with 0.16 ml of 100 mM sodium acetate buffer (pH 4.8) containing the protease inhibitors leupeptin (15 μg/ml), aprotinin (15 μg/ml), pepstatin (5 μg/ml), PMSF (0.25 mM) and EGTA (0.7 mM), at 0°C.

2.4. Endoglycosidase treatment and measurement of acid lipase activity

Cell extracts were prepared by sonication for 10 s at an amplitude of 10 μm (Soniprep150, MSE) with the subsequent addition of Triton X-100 (0.2% w/v final concentration). Cell extracts were incubated at 37°C with or without 50 mU of endoglycosidase H (Boehringer Mannheim), for 12 h. Aliquots of each sample were subjected to immunoblotting and assayed for acid lipase activity.

The reaction for the measurement of acid lipase activity mixtures contained cell extract (30–50 μg of protein), 100 mM sodium acetate buffer (pH 4.8), 0.2% (w/v) Triton X-100 and either cholesteryl-[^{14}C]oleate (0.2 mM, 5 μCi/ml) or tri-[^{14}C]oleyl-glycerol (0.3 mM, 5 μCi/ml), in a total volume of 0.1 ml. After incubation at 37°C for 30 min, the reaction product ([^{14}C]oleate) was separated by extraction of the reaction mixtures with benzene-chloroform-methanol (10:5:12 v/v/v) containing 0.3 mM oleic acid, as described by Pittman et al. [20]. The aqueous upper phases containing the partitioned [^{14}C]oleate were counted for β-radioactivity. Cholesteryl oleate and tri-oleyl-glycerol hydrolase activities are expressed as pmol of [^{14}C]oleate released/μg of protein/h.

2.5. Immunoblotting

SDS-PAGE was performed according to the method of Laemmli [21]. Extracts of HeLa cells (40 μg protein) expressing normal or mutant LALs were resuspended in denaturing sample buffer containing 4% SDS and 3% dithiothreitol. After 5 min at 100°C the samples were loaded onto 12% polyacrylamide gels and subjected to electrophoresis. Western blotting of the proteins to nitrocellulose membranes was performed for 2 h at 200 mA. After transfer, the membranes were stained with Ponceau Red S, washed and blocked for 1 h in TBS containing 5% skimmed milk. Tag antigens were detected using anti-HSVtag monoclonal antibody (Novagen, Madison, WI) followed by an alkaline phosphatase-conjugated goat anti-mouse IgG.

3. Results and discussion

Since LAL is ubiquitously expressed [1], in this study transfected LALs were distinguished from the endogenous one by tagging at the carboxyl terminus with a herpes simplex virus epitope of 11 amino acids (Fig. 1). At the same time, the endogenous acid lipase activity was reduced by the recombinant Vaccinia virus infection used to enhance the expression of transfected LALs. Normal LAL expressed in HeLa cells using this system showed four major molecular forms of 54, 50–51, 43 and 42 kDa (Fig. 2, LAL). The expression of the L273S CESD allele resulted in a different pattern of expression, showing the presence of a larger molecular mass form of 56 kDa (Fig. 2, L273S).

Careful examination of the amino acid sequence in the vicinity of the mutation revealed that the leucine to serine substitution at position 273 creates a new consensus for *N*-glycosylation at asparagine residue 271 (Fig. 1) that could account for the higher molecular weight form of the protein expressed by this mutant. In order to verify this possibility, two site directed mutants were constructed. The asparagine at position 271 was replaced with glutamine keeping leucine or serine at position 273 (QML and QMS, Fig. 1). Expression of these mutants in HeLa cells resulted in the same pattern of M_r forms as the normal LAL (Fig. 2, QMS and QML). The absence of the 56 kDa form in QMS confirms that the serine of L273S per se is not responsible for the 56 kDa protein and strengthens the view that an oligosaccharide chain linked to the asparagine at position 271 is responsible for the higher molecular mass of the L273S mutant protein.

Although it is known that *N*-linked glycosylation is important for lysosomal targeting of LAL via the mannose-6-phosphate receptor, its involvement in the generation of the different molecular forms of LAL expressed in mammalian cells has not been investigated. In order to determine the glycosylation status of these forms, extracts of cells transfected with normal

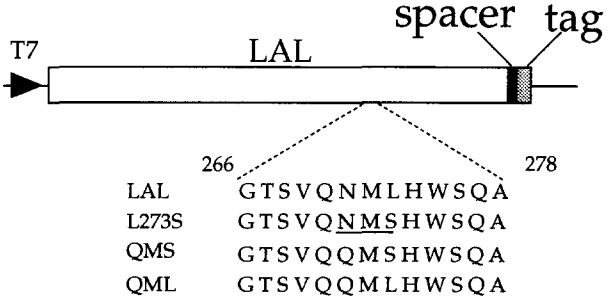

Fig. 1. Schematic representation of the LAL constructs used in the Vaccinia T7 expression system. Normal and mutant LALs, cloned under the T7 promoter, were extended at the 3′ end with a 5 amino acid spacer region (black box) followed by an 11 amino acid stretch from the HSV epitope tag (gray box). The expanded amino acid region between residues 266 and 278 of human lysosomal acid lipase shows the position of the amino acid changes in the CESD allele (L273S) and in the site directed mutants. The leucine to serine substitution in L273S creates a new consensus *N*-glycosylation site (underlined).

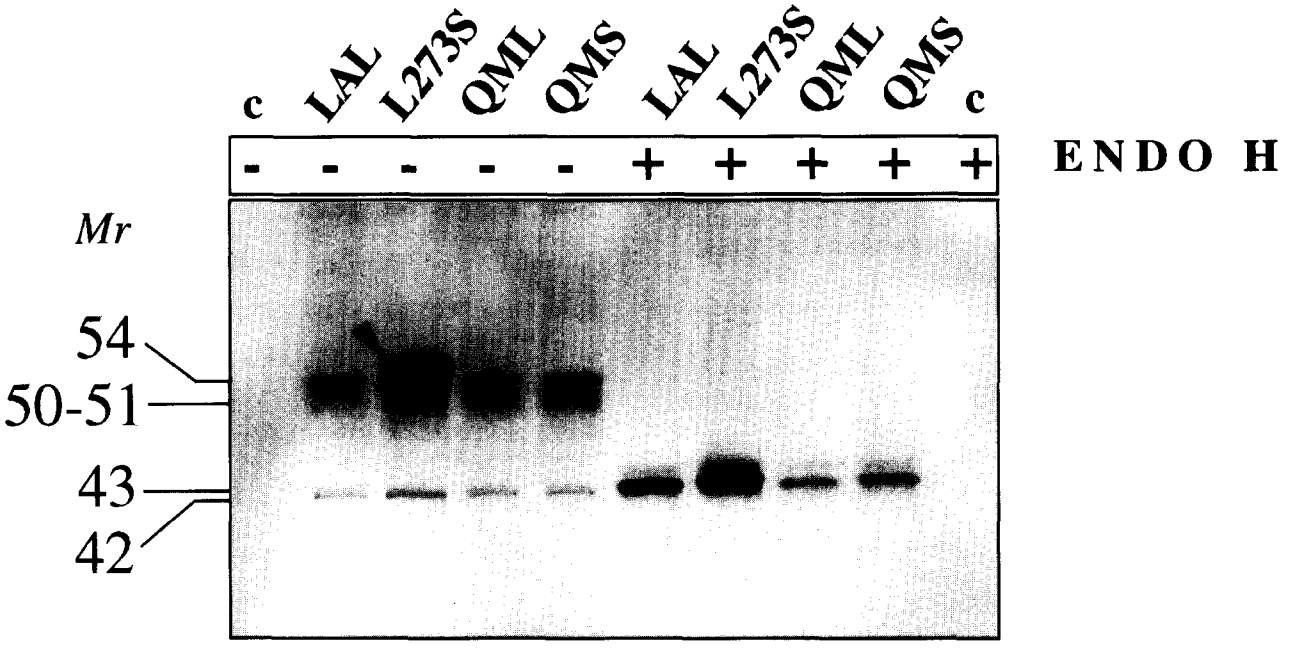

Fig. 2. Immunoblots of normal and mutant LALs expressed in HeLa cells before and after treatment with endoglycosidase H. The 56 kDa form expressed by L273S mutant is indicated by the arrow head. It can be noted that this band is also reduced to 42 kDa form after endoglycosidase H treatment (L273S, Endo H+) just as the 54 kDa form of normal LAL and QMS/QML mutants.

LAL and the different mutants were treated with endoglycosidase H. In the case of normal LAL, a major 42 kDa form was detected after digestion with endoglycosidase H (Fig. 2, LAL endo+). This indicates that the larger molecular forms of 54 and 50–51 kDa represent completely and partially glycosylated forms respectively, whereas the smaller 42 kDa band represents the non-glycosylated form. Similarly, the 42 kDa form was present in all the other mutants after treatment with endoglycosidase H. It should be noted that the 56 kDa form of L273S was also reduced to the smaller 42 kDa form, indicating that the increase from 54 to 56 kDa was due to additional glycosylation. The same results were obtained after deglycosylation with PNgase F (data not shown). In view of these results, it is plausible that all the 6 putative *N*-glycosylation sites of LAL could be occupied by carbohydrate chains contributing an average of 2 kDa to the molecular weight of the protein.

The influence of glycosylation on the catalytic activity of LAL was investigated by measuring the acid cholesteryl esterase and triacylglycerol hydrolase activities in the extracts of HeLa cells infected and transfected with normal and mutated LALs, with and without endoglycosidase H treatment. Transfection with normal LAL increased both activities 8–10 times above the control (90 ± 10 pmol/μg/h for triacylglycerol hydrolase and 30 ± 5 pmol/μg/h for cholesteryl esterase). Endoglycosidase H treatment of the extracts reduced both cholesteryl esterase and triacylglycerol hydrolase activities by approximately 50% (Fig. 3, LAL Endo H +/−) compared to the control extracts incubated in the same way but in the absence of endoglycosidase H. On the contrary, transfection with the L273S CESD allele and the site directed mutants (QMS, QML) showed both activities at background control levels (Fig. 3, QML, QMS Endo H +/−). The absence of activity of QMS and QML indicates that the asparagine residue at position 271 plays an important role concerning the formation of an active conformer, probably independent of glycosylation.

Previous studies have analyzed the involvement of glycosylation in the catalytic activity of LAL, with contradictory results. Expression of human LAL in Sf9 insect cells produced two molecular forms of 41 and 46 kDa and only the higher, glycosylated form was suggested to be active [4]. However, expression of highly glycosylated proteins in this heterologous system does not seem convenient to study the processing of lysosomal enzymes as these cells produce an aberrant pattern of glycosylation and do not follow the typical mammalian mannose-6-phosphate receptor pathway for lysosomal targeting [5]. The relative abundance of inactive, non-glycosylated

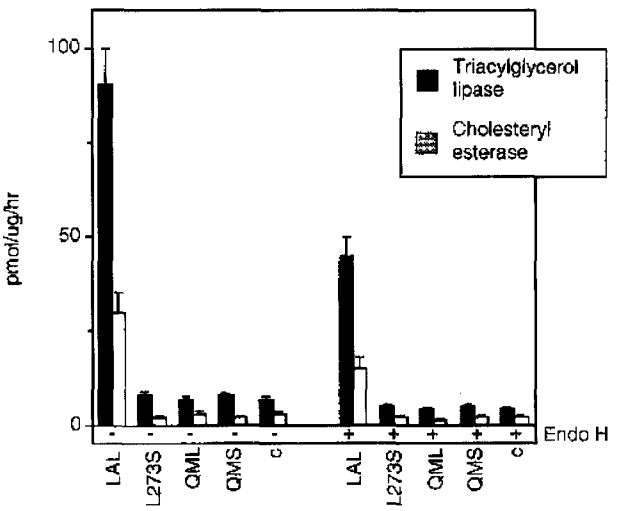

Fig. 3. Acid lipase activity of Vaccinia infected HeLa cells transfected with normal and mutated LALs. The effect of deglycosylation on enzymatic activity is indicated. Each value represents the mean ± S.D. of three independent experiments.

41 kDa form reported in this case may indeed reflect the abnormal processing of LAL in insect cells.

Two major forms of LAL of 56 and 41 kDa, similar to the recombinant proteins we have detected in HeLa cells with the Vaccinia T7 system, have been purified from human liver [3]. Partial amino acid sequencing of these two hepatic forms suggested that the 56 kDa form is a precursor protein from which 49 amino acids are cleaved off from the N-terminus yielding a mature LAL protein. The presence of *N*-linked carbohydrate residues was investigated only in the case of the 41 kDa form, showing a reduction of approximately 5 kDa after deglycosylation [3].

Our experiments indicate that the size heterogeneity of LAL expressed in HeLa cells is completely due to *N*-glycosylation, as endoglycosidase H treatment always resulted in a major 42 kDa form (Fig. 2). Expression of a LAL construct lacking the N-terminal 49 amino acids resulted in a catalytically inactive, non-glycosylated protein of 37 kDa that could be easily differentiated from the 42 kDa form by the electrophoretic mobility (data not shown). We do not have amino acid sequence information about the lower molecular mass forms of LAL observed in HeLa cells. However, our expression studies clearly indicate that proteolytic processing is not involved in the maturation of the N-terminus of LAL or, alternatively, that the N-terminal proteolysis is tissue specific and occurs in hepatocytes but not in HeLa cells. It will be necessary to express LAL in different cell types in order to verify whether such a tissue-specific processing exists.

The 50% reduction in both cholesteryl esterase and triacylglycerol hydrolase activities following deglycosylation suggests that *N*-linked carbohydrate residues, although not essential for LAL activity, may have an important role in the maintenance or stabilization of a fully active conformation. Changes in the tertiary structure of non-glycosylated LAL might interfere with the lipid binding of the enzyme or the structure of the active site, consequently reducing the efficiency of catalysis.

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