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# Lysosomal Acid Lipase as a Preproprotein

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**Lysosomal acid lipase (LAL; EC 3.1.1.13) hydrolyzes intracellular triglycerides and cholesterol esters taken up by various cell-types. Previously, LAL purified from human liver tissue was described as a preproprotein with a 27 amino acid signal peptide and a 49 amino acid propeptide. Three mutants of the putative proregion of LAL were produced and expressed in *Spodoptera frugiperda* insect cells. Pulse-chase experiments demonstrated that LAL undergoes proteolytical processing. The deletion of the 49 amino acids led to a complete loss of the LAL activity. The two other mutants were produced at the C-terminus of the pro-region, at positions 49 and 50, by site-directed mutagenesis. Mutant K49R showed wild-type LAL activity, but mutant G50A showed significantly reduced enzyme activity compared to wild-type LAL and a greater reduction in culture medium than in detergent cell extracts. Kinetic data suggest that mutant G50A is less stable than wild-type LAL and mutant K49R. In contrast to K49, the highly conserved amino acid residue G50 seems to be in a very important position and its mutation influences both secretion and enzyme activity of LAL. A three-dimensional model of LAL shows that K49 and G50 are localized in the loop-region between two beta-sheets, highly accessible for proteolytic enzymes. These data together indicate that LAL is indeed a preproprotein, in which the pro-region is essential for its folding and stability, secretion, and enzyme activity.**

**Key words: baculovirus expression, lysosomal acid lipase, propeptide, site-directed mutagenesis.**

Abbreviations: AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus; LAL, human lysosomal acid lipase.

Lysosomal acid lipase (LAL, EC 3.1.1.13) is a lipolytic hydrolase involved in the intracellular metabolism of cholesteryl esters and triacylglycerols derived from plasma lipoproteins (*1*). LAL is synthesized in virtually all cells and tissues of the human body, including hepatocytes, fibroblasts, macrophages, and lymphocytes (*2*). After synthesis, the enzyme is targeted to the lysosome (*3*). Lysosomes, lytic organelles in cells, contain many hydrolytic enzymes including phosphatase, glycolytic enzymes such as β-hexosaminidase and β-galactosidase, and proteolytic enzymes such as cathepsins B, D and L (*4*). Lysosomal enzymes are transported as proteolytically inactive precursors that, after uncoupling of receptor-ligand complexes, become matured by proteolytic processing within late endosomes or lysosomes.

Formerly, LAL purified from human liver was sequenced at the N-terminus. From the results, it was concluded that LAL exists in two forms, a 56-kDa and a 41-kDa form (*5*). The thesis is that the 56-kDa precursor protein is subject to post-translational processing. Cleavage of the precursor lipase carboxyl to K49 would then remove 49 N-terminal amino acids and yield a mature liver LAL of 323 amino acids. Propeptides are generally located N-terminally to the mature enzyme. Propeptides vary from a few (*e.g.*, trypsin) to more than 200 amino acid residues (*e.g.*, cathepsin C). In most cases, propeptides are important for the correct folding of enzymes (*6*), are involved in targeting to specific organelles (*7*), and can affect post-translational modifications such as glycosylation (*8*). Propeptides can be cleaved by proteases or by intra- or intermolecular autocatalysis. Lowering of pH, which occurs when the protein enters the lysosome, is most commonly responsible for triggering the activation of proproteins, and it can increase the affinity for protonated groups close to the cleavage site, which often are localized in an easily accesible loop region, or cause a conformational change in the propeptide or enzyme (*9*).

Expression of LAL in insect cells using a baculovirus vector resulted in a purified enzyme with a molecular mass of 51 kDa (*10*). In general, signal peptides, also existing in LAL, are cleaved correctly from diverse membrane-bound or secreted proteins expressed in baculovirus-infected insect cells (*11*). However, there is evidence that some types of post-translational proteolytic and protein modifying processes are lacking or different in insect cells (*12*). We were previously able to show that recombinant LAL is highly N-glycosylated, which is important for both its activity as well as the building of a stabile three-dimensional structure (O. Zschenker *et al.*, unpublished data). The reason for the reduced molecular weight of the recombinant LAL is not cleavage but N-glycosylation, which is not as bulky in insect cells as in mammalian cells (*13*). The lack of cleavage of the potential propeptide at position K49/G50 of recombinant LAL

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compared to purified LAL from human liver allowed the function and importance of the peptide sequence to be determined by mutagenesis in the insect cell system.

In the present study, we investigated three LAL mutants expressed in the baculovirus system. We constructed one LAL enzyme (Δ1–49) with a complete deletion of the potential propeptide sequence, and two mutants at the cleavage site by site-directed mutagenesis, K49R and G50A, respectively.

## MATERIALS AND METHODS

*Vector and Site-Directed Mutagenesis of K49 and G50 of LAL USED for Expression in Insect Cells*—Synthesis of LAL cDNA and insertion into the pBakPak8 baculovirus expression vector (Invitrogen), named pAPLALBacHis, have been described (*10*). For expression, wild-type enzyme was modified by replacing the LAL signal peptide sequence with the alkaline phosphatase signal peptide sequence (*14*). Codons for six histidine residues were added to the C-terminus of the enzyme to allow purification by affinity chromatography. Primer-mediated site-directed mutagenesis was performed according to the supplier (Stratagene). PCR was carried out for K49R mutant with primers K49R-fwd (5′-AGGAAGAACCATTCTGACCGGGGTCCCAAACCAGTTGTC-3′) and K49R-rev (5′-GACAACTGGTTTGGGACCCCGGTCAGAATGGTTCTTCCT-3′) and for G50A mutant with primers G50A-fwd (5′-AGGAAGAACCATTCTGACAAAGCCCCCAAACCAGTTGTC-3′) and G50A-rev (5′-GACAACTGGTTTGGGGGGCTTTGTCAGAATGGTTCTTCCT-3′). At position 49 of LAL cDNA, lysine was converted to an arginine residue; and at position 50, glycine was converted to an alanine residue at the potential C-terminal propeptide cleavage site. Standard PCR reactions were performed with *Pfu*-DNA polymerase, with 16 cycles of 30 sec at 95°C, 1 min at 55°C and 14 min at 68°C. Mutagenesis was verified by sequencing the region surrounding the respective mutation.

*Construction of Baculovirus Expression Vector of Deletion Mutant Δ(1–49) of LAL*—The expression vector of the N-terminal deletion mutant of the potential propeptide in LAL cDNA was constructed by restriction of pAPLALBacHis with *Nco*I and *Not*I. After gel-extraction performed according to the supplier (Qiagen), the product was dephosphorylyzed with alkaline phosphatase. For deletion of the 49 amino acid residues of the potential propetide of LAL, cDNA of LAL was employed as a template and amplified by PCR with primers LAL-D49-*Nco*I-fwd (5′-ACTGCCATGGGGTCCCAAACCAGTTGTCTTCCTG-3′) and LAL-His-*Not*I-rev (5′-ACGTGCGGCCGCTCAGTGATGGTGATGGTGATGCTGATATTTCCTCATTAGATT-3′). The PCR product was purified with the PCR purification kit (Qiagen). Vector-DNA (*Nco*I-pBakPak8-*Not*I) and insert-PCR-product (*Nco*I-APΔ (1–49)LALHis-*Not* I) were ligated and transformed in DH5α-competent cells (Invitrogen) using standard protocols (*15*). Clones were isolated, cultivated and sequenced after DNA-preparation (Qiagen).

*In Vitro Expression, Purification and RT-PCR of Wild-Type and Mutant LAL*—To express wild-type and mutant LAL protein, *S. frugiperda* (*Sf* 9) cells (Invitrogen) were propagated as monolayer cultures in TC-100 medium (Life Technologies, Inc.) and supplemented with 10% heat-inactivated fetal bovine serum, 10,000 IU/ml penicillin and 10 mg/ml streptomycin. At a density of $10^6$ cells per 25-$cm^2$ tissue culture flask, *Sf* 9 cells were co-transfected by lipofection with 0.5 μg of wild-type or mutant plasmids and *Bsu*36I-digested BacPAK6 DNA. Recombinant viruses were plaque-purified to ensure homogeneous virus stocks. PCR amplification of viral DNA using LAL- and BacPAK8-specific primers was used to verify recombination of transfer plasmid with wild-type viral DNA. For the production of a high-titer virus, stocks were amplified three times.

Media used for LAL purification was harvested after 4 d of viral infection, and supplemented with ethylene glycol and Triton X-100 at concentrations of 25% and 0.1%, respectively. LAL wild-type and mutants were purified by $Ni^{2+}$ chelate affinity chromatography on a Ni-NTA matrix (Qiagen) utilizing the carboxyterminal polyhistidine tag of recombinant LAL as previously described (*10*). Aliquots of purified recombinant LAL were separated on SDS-polyacrylamide gels (Biorad) under denaturing conditions, using 4% stacking gels and 12.5% separating gels (*16*). Proteins were visualized by staining with silver nitrate (*17*).

To obtain total RNA for RT-PCR, *Sf* 9 cells were collected after 4 d of infection with wild-type or mutant baculoviruses. After sedimentation of cells, total cellular RNA was extracted according to the protocol of the supplier of the RNA extraction kit (Qiagen). To eliminate residual genomic DNA, 5 μg of total RNA was treated with *DNase* I. After inactivation of *DNase* I, first strand-synthesis was performed by using SUPERSCRIPT™ reverse transcriptase (Invitrogen) with random hexamers. The first-strand cDNA was amplified directly. For PCR reactions, 2 μl of cDNA from first-strand reaction, 5 U/μl *Taq* DNA polymerase (Invitrogen), 10 μM oligonucleotide FS 2/2 (5′-GCTTCATTCTTGCTGATGCTGG-3′) and 10 μM oligonucleotide RS5/2 (5′-AAAGTTTCAAGCCTTTGACTGGGG-3′) were incubated. A 620-bp fragment of LAL was btained. PCR-products were analyzed by 1% agarose gel electrophoresis.

*Immunoblot Analysis*—A semi-dry blot system (Biometra) was used for analysis of wild-type LAL and mutants. In brief, after centrifugation for 5 min at 4°C and 2,000 rpm, cells were suspended in 50 mM Tris-HCl (pH 7.4) 150 mM NaCl and 1 mM EDTA, and again collected by centrifugation. Cells were dissolved in 200 μl containing 10 mM sodium acetate (pH 5.0) 0.1 mM DTT, 1% Triton X-100, and sonified on ice with a Branson device. After electrophoretic separation, samples were transferred to a PVDF membrane for 2 h at 1 mA/$cm^2$. After transfer, the membrane was blocked with PBS/3% BSA for 1 h, washed first with PBS/100mM EDTA, then washed with TBS-Tween, and incubated overnight at 4°C with an anti-$His_6$ antibody (*18*) (Dianova) (1:200 in TBS). Membranes were incubated with a secondary anti-horseradish peroxidase antibody (1:1,000 in TBS) for 1 h at room temperature. The ECL system (Amersham) was used for detection.

*Enzymatic Activities*—Enzymatic activities were determined in cell culture medium and in detergent extracts as described (*5*). In brief, hydrolytic activity of LAL was determined using two substrates: tri-(9,10(*n*)-$^3$H)-ole-

oylglycerol (Amersham), also called $^{3}$H-triolein, and cholesteryl (1-$^{14}$C)oleate (Amersham). Purified protein and protein from cell extracts were measured in buffers containing 1% Triton X-100 and 25% ethylene glycol for stabilization of LAL, and incubated for 30 min at 37°C in buffers with the radioactive-labeled substrates. Reaction products were isolated by organic extraction with specific buffers, and the aqueous phases were separated and quantitated in a scintillation spectrometer. The results represent the means of three independent LAL expressions, with all assays conducted in duplicate.

Protein concentrations were determined by the bicinchonicic acid (BCA) method (Pierce) (*19*) using bovine serum albumin as a reference. The BCA protein assay was insensitive to Triton X-100 or ethylene glycol.

*Enzymatic Activities and Kinetic Determinations*—Enzymatic activities were determined in cell culture medium in three separate experiments, as described. To obtain kinetic data of LAL mutants G50A and K49R, purified LAL preparations were diluted to a concentration of 50 μg/ml. Triolein concentrations were chosen between 100 μM and 1.5 M. To the reactions, 0.109 M sodium acetate (pH 5.0) was added to a total volume of 200 μl. Samples were incubated at 37°C for 30 min, and lipolytic activities were determined as described above. To obtain $K_m$ values, triolein hydrolysis was plotted against substrate concentration using Graph Pad Prism 3® software.

*Pulse Chase*—Pulse chase experiments were performed as described in (*20*). Briefly, $10^6$ *Sf* 9 cells were incubated with 20 moi of baculovirus of wild-type LAL, K49R, G50A and Δ(1–49) mutants. One hour before each time point, medium was removed and replaced with the same volume of methionine-deficient medium (Expression Systems LLC). Cells were incubated for 1 h at 27°C. Methionine-deficient medium was removed, samples were centrifuged for 5 min at 1,000 rpm and resuspended in 0.5 ml of methionine-deficient medium containing 250 μCi $^{35}$S-labeled methionine (Amersham). Cells were incubated for 10 min at 27°C with gentle rocking. At the end of the labeling period, cells were immediately resuspended in warm (27°C) chase medium. Cells were pelleted and resuspended in 1 ml of chase medium for each time period to be analyzed. Cells were washed three times with ice-cold PBS containing protease inhibitor cocktail (Roche) and analyzed by immunoprecipitation as described in next section.

*Immunoprecipitation*—His-tagged proteins were immunoprecipitated using QIAexpress Detection Systems (Qiagen) according to the suppliers protocol. After SDS-PAGE, proteins were visualized by exposure onto Biomax MR-1 (Kodak) after signal amplification with Amplify fluorographic reagent (Amersham Pharmacia Biotech).

*Computer Analysis of LAL*—The crystal structure of LAL is unknown, only a model based on the crystal structure of the very homologous human gastric lipase (*21*). We tried to perform crystallization of LAL by site-directed mutagenesis of glycosylation-sites using a modified baculovirus expressed enzyme, but it failed. The enzyme structure was therefore determined by using computer analysis available on the world wide web. If LAL is cleaved at position K49/G50 as suggested by our group, the possibility of cutting by enzymes exists beside the possibility of autocatalytical cleavage. The former was investigated using the PeptideCutter (http://us.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl). The importance of a possible propeptide for the correct building of the three-dimensional structure of a protein can be determined by interpretation of Kyte-Doolittle hydropathy plots (http://bioinformatics.weizmann.ac.il/hyd-bin/protein_to_res.pl). In addition, a full model analysis of LAL and LAL mutants was performed with Geno3D (Pole Bio-Informatique Lyonnais) (*22*) and Molscript (*23*).

## RESULTS AND DISCUSSION

*Differences in LAL Expressed in Mammalian and Insect Cells*—After purification and N-terminal sequencing of LAL from human liver tissue, it was concluded that LAL exists in two forms, a 56-kDa and a 41-kDa form (*5*). The putative N-terminus of the 41-kDa form was 50 amino acids C-terminally to the regular N-terminus. It is also likely that an N-glycosylation site is present in the propeptide sequence at position N9 (O. Zschenker, unpublished data). N-terminal post-translational processing is described for quite a number of lysosomal enzymes. For example, cathepsin D (*24*, *25*), α-fucosidase (*26*), β-glucoronidase (*27*) and lamb pregastric lipase (*28*) showed evidence of cleavage of specific propeptides in a prelysosmal compartment, which is important for correct folding and enzymatic activity of the enzymes. Propeptides are generally located N-terminally to the mature enzyme. However, after expression of an extracellular endoprotease of *Aeromonas caviae* T-64 in *E. coli*, the enzyme was also shown to have a C-terminal propeptide, one which was not essential for correct folding and enzymatic activity (*29*).

In the baculovirus expression system, no cleavage of the potential propeptide of LAL was observed. In general, propeptides can be cleaved by proteases or by intra- or intermolecular autocatalysis. There are only two proteases which are possible enzymes for cleavage of LAL at the putative cleavage site, namely, lysine C-proteinase and trypsin. Lysine C-proteinase as an extracellular enzyme is out of the question, but trypsin is theoretically possible, because it is an ubiquitous enzyme, mostly localized in microsomes. No trypsin-like endopeptidase activity was detected in *Sf* 9-cells (*11*), besides which, the secretion of proforms of lysosomal enzymes has been observed in physiological and pathological conditions in various cell-types. A defect in M6P-based trafficking is the cause of I-cell disease in which lysosomal enzymes are secreted as proforms from patients' fibroblasts (for review see (*30*)). In insect cells, phosphorylated high-mannose oligosaccharides, which are important for targeting of lysosomal enzymes, are not observed (*31*), supporting the observation that LAL is determined only as a proform in the insect cell system. Another possibility, which is a point for further research, is that LAL is cleaved, but the propeptide remains associated with the mature enzyme, as was observed for cathepsin C (*32*).

Lowering of pH, which occurs when the protein enters the lysosome, is most commonly responsible for triggering the activation and separation of proproteins, and can increase the affinity for protonated groups close to the cleavage site, which is often localized in an easily accesi-

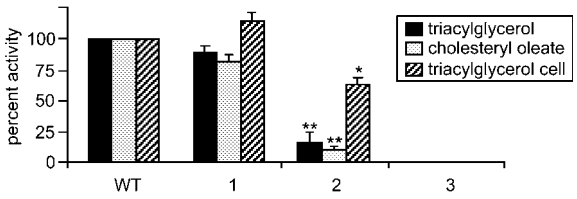


Fig. 1. **Enzymatic activities of LAL propeptide mutants, Δ (1–49) deletion mutant, and K49R and G50A mutants.** LAL wild-type and mutants were expressed in *S. frugiperda* cells, purified from culture supernatant, and their activities were determined ($n$ = 3) with $^{3}$H-labeled trioleoylglycerol (black columns) and $^{14}$C-labeled cholesteryl oleate (spotted columns). Mutant K49R (panel 1) showed wild-type activity. G50A (panel 2) showed significantly reduced activity compared to the wild-type enzyme, and the deletion mutant (Δ1–49; panel 3) displayed no activity compared with wild-type lipase (WT), indicating that the propeptide sequence is essential for LAL activity. Enzymatic activities of LAL with $^{3}$H-labeled trioleoylglycerol were also measured in cell extracts (hatched column). Interestingly, in cell extracts G50A showed significantly more enzymatic activity as purified from cell culture supernatant, but still significantly reduced activity compared to the wild-type enzyme. $^{*}p < 0.05$; $^{**}p < 0.01$, as compared to wild-type LAL.

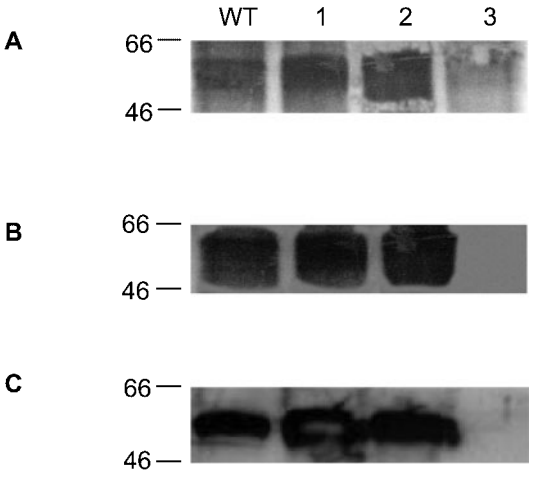


Fig. 2. **SDS-PAGE and immunoblot analysis of propeptide mutants.** Panel A: Mutants and wild-type enzyme purified from supernatant of *S. frugiperda* cells were separated on a 12.5% SDS-PAGE and stained with silver nitrate. Panel B: LAL protein was detected in culture supernatant with an anti-$His_6$ antibody (1:200 in TBS) after transfer of proteins to a PVDF membrane. Panel C: Proteins from cell extracts of *S. frugiperda* cells expressing LAL mutants were separated, blotted and LAL was detected as described above. A horseradish peroxidase conjugated secondary antibody (1:1,000 in TBS) was used with the ECL system. Mutant Δ(1–49) was neither detectable on SDS-PAGE analysis (lane 3 in panel A) nor by antibody detection in culture supernatant or cell extracts (lane 3 in panelsB and C, respectively). WT: wild-type LAL; lane 1: K49R; lane 2: G50A; lane 3: Δ(1–49).

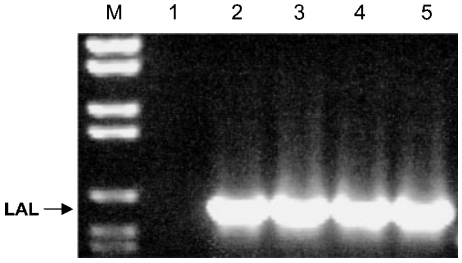


Fig. 3. **Agarose gel electrophoresis of RT-PCR products from LAL wild-type and propeptide mutants.** Total RNA (5 μg) from *S. frugiperda* cells was reverse-transcribed after DNase I digestion and PCR amplified with LAL-specific oligonucleotide primers. Lane 1, no RT-PCR product of cells without baculoviral infection was visible. Lane2, RT-PCR product of cells infected with LAL wild-type baculoviral vector, lane 3, mutant K49R, lane 4, G50A and lane5, RT-PCR product of the propeptide deletion mutant (Δ1–49) of LAL. The size of the amplified LAL product is 620 bp (arrow). The DNA marker used was marker VI (Roche).

ble loop region or cause a conformational change in the propeptide or enzyme (*9*, *33*). This also does not occur in LAL expressed in the insect cell system.

*Expression and Enzymatic Activity of Recombinant LAL and Propeptide Mutants*—Based on the fact that LAL expressed by a baculovirus is not cleaved at position K49/G50, in the same way that LAL purified from human liver tissue is not cleaved, it is possible to determine the function of the possible propetide for LAL enzyme in insect cells. A deletion mutant Δ(1–49) was cloned, and two mutants at the putative LAL cleavage site at the C-terminus of LAL propeptide sequence, K49R and G50A, respectively, were produced by site-directed mutagenesis, and expressed by the baculovirus system in *Sf* 9 cells. Enzymatic activities were determined with $^{3}$H-labeled triacylglycerol and $^{14}$C-labeled cholesteryl oleate as substrates. Mutant K49R showed lipolytic activities comparable to wild-type LAL (Fig. 1). Mutant G50A only showed residual enzymatic activity in cell culture medium (lower than 20%). Interestingly, in detergent extracts of cultured insect cells, G50A showed more than 60% activity compared to wild-type LAL, though it was significantly reduced compared to wild-type LAL (Fig. 1). The Michaelis-Menten value $K_M$ of mutant G50A (1,374 ± 89 μM) purified from insect cell culture medium was significantly increased compared to wild-type lipase (899 ± 451 μM) and mutant K49R (803 ± 397 μM). These results suggest that the enzyme substrate complex of G50A is less stable and G50A mutant has less affinity to triacylglycerol than wild-type LAL and mutant K50R. The deletion mutant Δ(1–49) showed no enzymatic activity, either in culture medium or in detergent cell extracts (Fig. 1). On SDS-PAGE analysis of LAL mutants expressed in *S. frugiperda* cells, Δ(1–49) was the only mutant not detectable (Fig. 2A, lane 3), indicating that Δ(1–49) was not produced. On immunoblot analysis with a monoclonal antibody recognizing the polyhistidine tag of recombinant lipase mutants, Δ(1–49) was the only mutant also not detectable in culture medium (Fig. 2B, lane 3) and in detergent extracts of cultured cells (Fig. 2C, lane 3).

To determine whether LAL-specific RNA was not synthesized or synthesized and degraded in the deletion mutant Δ(1–49), RT-PCR of total RNA of cultured *S. frugiperda* cells was performed to specifically amplify LAL sequences. DNA agarose gel electrophoresis showed that LAL RNA was synthesized in the deletion mutant as in the two others (Fig. 3).

K49R showed no significant difference in expression and activity compared to wild-type LAL, suggesting that

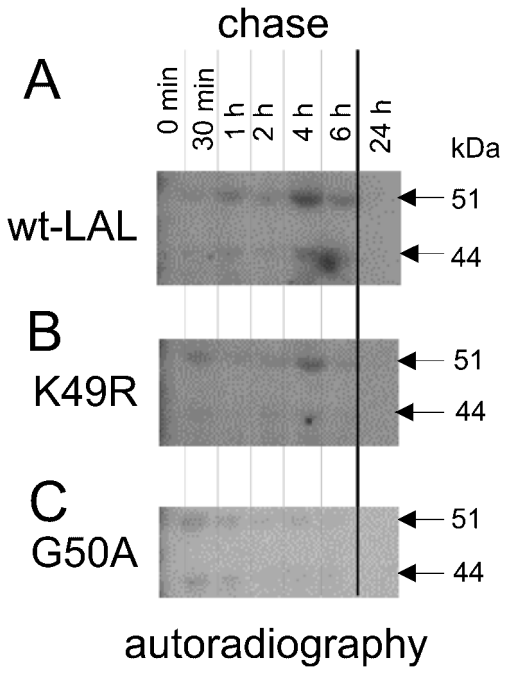


Fig. 4. **Proteolytical processing and stability of wild-type and mutant (K49R; G50A) LAL enzymes expressed in *Sf* 9 cells.** *Sf* 9 cells were infected with recombinant baculoviruses, labeled for 10 min with $^{35}$S-Met and chased with an excess of cold Met for the indicated periods. Cells were lyzed and immunoprecipitated using an anti-His-tag-antibody. The immunoprecipitated enzymes were fractionated by SDS-PAGE. The dried gels were exposed at –80°C for 5–7 d. The arrows indicate two LAL fractions. Wild-type LAL (panel A) and mutant K49R (panel B) showed two fractions, as mutant G50A also does (panel C). However, the two bands of mutant G50A are only visible for the first 1 h of the chase time period, whereas wildtype-LAL and K49R were visible up to 6 h with optimum amounts 4 h after beginning of the chase period. The lower level of expression of mutant G50A was apparently due to the peculiarly low stability of this enzyme in *Sf* 9 cells.

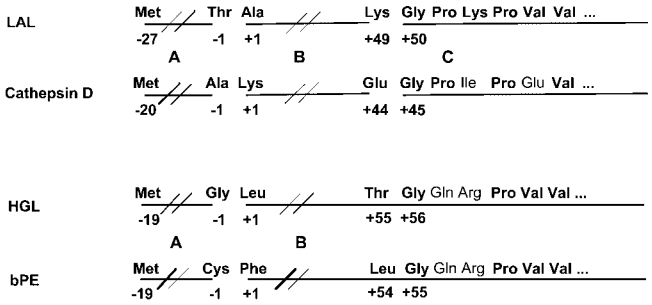


Fig. 5. **Post-translational processing of LAL (hypothesis).** Proline following glycine may be an important amino-acid sequence at the possible cleavage-site of a propetide of an enzyme. As examples of highly homologous enzymes compared to LAL, cathepsin D (*25*) human gastric lipase (HGL) (*21*), and bovine pregastric lipase (bPE) (*47*) are shown. For all named enzymes, a signal peptide (A) is described, although not proven for HGL. Cathepsin D is an enzyme with a propeptide (B). (C): mature protein if there is a propeptide sequence.

this position is not important for processing of LAL. In contrast, Δ(1–49) showed no enzymatic activity and was not detectable in silver-stained SDS-PAGE and immunoblot analysis, indicating that the putative propeptide is important for three-dimensional building up and activity of the LAL enzyme. Many examples of this phenomenon are given in the literature. Protein expression and analysis of an aminopeptidase from *Vibrio proteolyticus* expressed in *E. coli* demonstrated that the N-terminal propeptide was essential to the formation of the active enzyme (*34*). The deletion of the proregion of mouse cathepsin L showed that the propeptide plays an essential role in proper folding of cathepsin L, ER retention and decreased stability (*35*). Because RNA of LAL Δ(1–49) was detectable, it is assumed that this mutant is synthesized but rapidly degraded in the ER. It was shown for cathepsin L that the full-length propeptide is essential for proper folding of the active tertiary structure and for recombinant expression in *E. coli* (*36*, *37*). The propeptide of prouroguanylin, an endogenous ligand of guanylyl cyclase C, regulates the disulfide-coupled folding of the mature region as an intramolecular chaperone (*38*), which may also play a major role in LAL, because its three-dimensional structure is built up and stabilized by a disulfide-bridge (*39*). Obviously, a propeptide seems to be important for recombinant human cathepsin D expressed in insect cells, but processing is also important for generation of an active mature enzyme (*40*), which is not the case in recombinant wild-type LAL.

Proteolytical processing and stability of the expressed enzymes were tested in pulse and chase experiments (Fig. 4). Wild-type LAL and mutant K49R (Fig. 4A and B) were detectable up to 6 h, with an increased amount 4 h after labeling the *Sf* 9 cells with $^{35}$S-Met and chasing them with cold Met. Autoradiographies showed two bands at 51 and 44 kDa, indicating proteolytic processing of wild-type and LAL mutants. Mutant G50A (Fig. 4C) also showed two bands, but only trace amounts were detectable, and moreover, they only were detectable for 1 h of the chase period. These data demonstrate a peculiar low stability of mutant G50A in *Sf* 9 cells.

The calculated molecular mass of the propeptide of LAL predicted from the cDNA is about 5,000 Da. The difference of about 2,000 Da can be explained by the possibility of post-translational modifications like phosphorylation or glycosylation. All references indicated that N-terminal propeptides are important for protein function, but they are not sufficient to direct a secretory protein to the lysosome in fibroblasts or epithelial cells (*41*). There is data indicating that N-glycans in the pro-regions are important for sorting and secretion of proteins (*8*, *42*). But the role of N-glycosylation in propeptide regions is not clear yet, because other examples given in the literature indicate that glycan chains in the pro-regions are not essential for proteins (*43*, *44*). One of putative *N*-glycosylation sites of LAL is located in the propeptide sequence at position 9. Previously, we were able to show that this *N*-glycosylation site was important for secretion of LAL, but not essential for enzyme activity (data not shown).

*Computer Analysis of Structure–Function Relationship of LAL*—Mutant G50A of LAL showed residual enzyme activity in culture medium (Fig. 1) and was secreted in lower amounts than wild-type LAL or mutant K49R (data not shown). Interestingly, G50A showed significantly more enzymatic activity in cell extracts (more

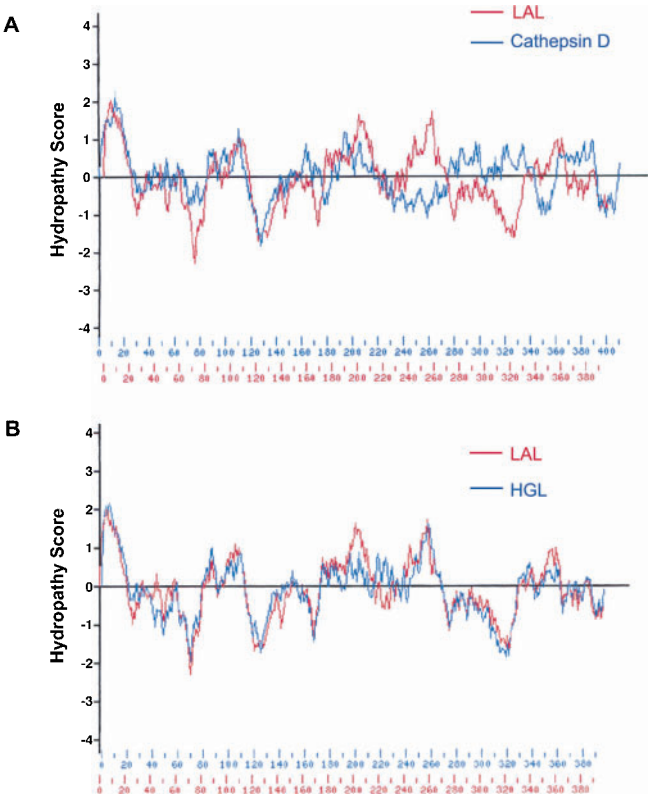


Fig. 6. **Kyte-Doolittle hydropathy plots of (A) LAL compared to cathepsin D and (B) LAL compared to human gastric lipase (HGL).** Plots were designed by using the web-site (http://bioinformatics.weizmann.ac.il/hydroph/hydroph.html). (A) Cathepsin D is a preproprotein, but with only poor homology to LAL, and shows big differences in hydrophilicity upwards of aminoacid position 240. Differences are negligible in the prepropeptide sequences, except for the C-terminus of propeptide sequence. There, the propeptide of LAL is, in contrast to cathepsin D, at the surface of the molecule, which makes it more accessible to proteolytic enzymes. (B) From amino-acid 100 upwards, there is almost no difference between LAL and HGL, but there are quite a few differences in hydrophilicity between the N-terminus of LAL and HGL, which is possibly the reason why LAL has a propeptide and HGL does not.

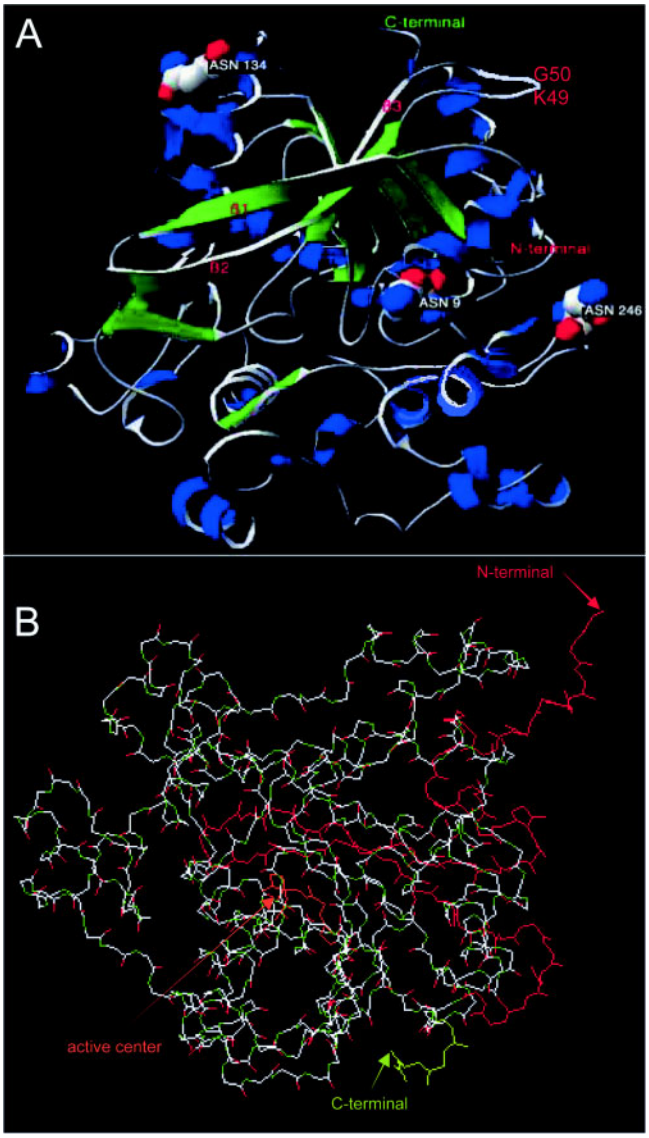


Fig. 7. **Stereo ribbon representation (A) and tertiary skewer configuration (B) of LAL designed by Geno3D, an automatic comparative molecular modeling of proteins available internet (*22*), visualized with SwissPdb Protein Viewer program and Molscript (*23*).** (A) N-terminus without signal peptide sequence, and C-terminus are marked, also the glycosylation sites (*13*) and the three β-structures in the propeptide sequence, showing that K49/G50 is between two β-sheets outside the molecule. (B) The N- and C-terminus and the active center of LAL are marked by arrows. The N-terminus including signal peptide sequence is visualized in red, the C-terminus in green and the enzyme active center in orange.

than 60%); however, it was significantly reduced compared to wild-type LAL. Mutant G50A was detected in silver-stained SDS-PAGE and immunoblot analysis (Fig. 2, A–C, lane 2). Obviously, position 50 (position 1 of mature enzyme) plays an important role in secretion, enzymatic activity, and stability (Fig. 4) of LAL. Cathepsin D is a lysosomal enzyme with a propeptide which has been proven (*45*). The first amino acid residue in the mature cathepsin D is glycine (Fig. 5). Glycine is highly conserved in lipases and esterases, *e.g.*, in human gastric lipase, which has a high homology and identity with LAL (*46*), and bovine pregastric esterase (*47*). However, these two enzymes are not processed at the putative propeptide cleavage site. Fig. 5 shows that the amino acid residue following glycine in the preproproteins Cathepsin D and LAL is proline and not glutamine. Possibly, there have to be some specific amino acid residues at a cleavage site in order that it can be cleaved by enzymes or autoprotolytic processes. We did some Kyte-Doolittle plots to determine whether the propeptide hydrophobicity between LAL and cathepsin D and LAL and HGL are different (Fig. 6) (*48*). The differences in hydrophobicity in the propeptide regions between LAL and HGL are more evident than those between LAL and cathepsin D, suggesting that LAL has a pro-region and HGL has not. But this difference is negligible and does not prove the existence of a propeptide in LAL. More evidence is given by a model of the three-dimensional structure of LAL, based on the crystal structure of the highly homolgous HGL (*21*, *49*)

(Fig. 7). The putative cleavage site (K49/G50) is localized in the "core-domain I," exactly between two beta-sheets (β2 and β3) in a loop-region, which provides good accessbility for protelytic enzymes. The figure also shows that the propeptide is mainly localized on the outside of the enzyme, as is proven by Kyte-Doolittle plots, and covers the active center of LAL. Modelling of a three-dimensional structure of LAL without a propeptide sequence resulted in an enzyme which could not be as stable.

Further investigations are needed to obtain more evidence. We have to do more site-directed mutagenesis in the N- and C-terminal of the putative propeptide sequence of LAL, because, as was shown for ribonuclease A, for examples a single mutation can result in protein stabilization to proteolytic degradation (*50*). We will also try to clone a new baculovirus expressing LAL by a CMV promoter to investigate proteolytic processing also in mammalian cells.

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