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Systematic Mutagenesis of Potential Glycosylation Sites of Lysosomal Acid Lipase

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Lysosomal acid lipase (LAL; EC 3.1.1.13) is a key enzyme in the intracellular lipid metabolism. It hydrolyzes exogenous triglycerides and cholesterol esters taken up by various cell types. LAL has six potential *N*-glycosylation sites and one potential *O*-glycosylation site. Elimination of each of the six Asn-(X)-Ser/Thr sites by site-directed mutagenesis and expression in baculovirus-infected *Spodoptera frugiperda* cells resulted in two single-mutant enzymes without lipolytic activities (N134Q and N246Q) and four mutants with preserved activities. The two inactive mutants were not detectable on immunoblot analysis, indicating that they were not secreted. Six double mutants in all possible combinations except for the two inactive single mutants were produced and expressed. Double mutants in combination with the N9 glycosylation site showed reduced activities as compared to the other mutants or the wild-type enzyme. Kinetic data of LAL glycosylation mutants indicate that substrate affinity of N9Q was not changed, but k_{cat} of N9 mutants was reduced distinctly compared to the wild-type enzyme. Peanut agglutinin lectin did not recognize LAL, demonstrating that the protein has no core1 structure (Galβ 1-3 GalNAc) of *O*-glycosylation. These data indicate that at least two of the six *N*-glycosylation sites are used in native lipase. N134 and N246 were found to be essential for LAL activity. We conclude that glycosylation plays an important role in the formation of functional LAL.

Key words: baculovirus expression, lysosomal acid lipase, *N*-glycosylation, 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 hydrolase essential for the intracellular degradation of neutral lipids 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*), where it hydrolyzes triacylglycerol and cholesteryl ester to glycerol, fatty acids and cholesterol, with the latter contributing to cellular sterol homeostasis (*4*).

Genetic defects of LAL lead to two inborn errors of lipid metabolism, Wolman disease (WD, Ref. *5*) and cholesteryl ester storage disease (CESD, Refs. *6* and *7*). In WD, affected infants succumb to hepatic and adrenal failure due to massive lysosomal accumulation of triacylglycerols and cholesteryl esters within the first year of life (*8*). CESD follows a more benign clinical course. However, hypercholesterolemia is often observed in CESD patients and premature atherosclerotic vascular disease may be severe (*8*). Various LAL mutations have been detected in CESD or WD patients (*9–12*). Based on the accumulating data it has been suggested that CESD has a more homogeneous genetic basis than WD (*13*). Individuals with compound heterozygosity for WD and CESD mutations have also been identified (*14*). A mouse model of LAL deficiency generated by targeted disruption of the mouse LAL gene mimicked the CESD phenotype, although histopathological features resembled those encountered in WD patients (*15*).

Expression of LAL in insect cells using a baculovirus vector resulted in a purified enzyme with a molecular mass of 51 kDa (*16*), while the calculated molecular mass predicted from the cDNA of LAL is 42.5 kDa (*17*). This difference is probably attributable to the glycosylation of the enzyme. According to the presence of the glycosylation consensus sequence, Asn-X-Ser/Thr (*18*), LAL has six potential *N*-glycosylation sites. A potential *O*-glycosylation site (*19*) is also present. In order to study the function of carbohydrate residues in LAL, the enzyme was expressed in the *Spodoptera frugiperda* cell system infected with a baculovirus vector. This system was chosen to generate recombinant lipase, since insect cells produce high amounts of LAL for biochemical analysis. In the present study, the six potential *N*-glycosylation sites were investigated by generating Asn-to-Gln mutants, thus preventing glycosylation at the site in question. This allowed the role of glycan moieties in the enzymatic properties of LAL to be determined.

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Table 1. **Amino acid numbering refers to the Asn residue of the putative glycosylation site and is according to human liver LAL (*17*).**

N9Q-fwd	5′-GCTGTGGATCCTGAAACAAACATGCAGGTGAGTGAAATTATCTCT-3′
N9Q-rev	5′-AGAGATAATTTCACTCACCTGCATGTTTGTTTCAGGATCCACAGC-3′
N45Q-fwd	5′-ATTCCTCATGGGAGGAAGCAACATTCTGACAAAGGTCCC-3′
N45Q-rev	5′-GGGACCTTTGTCAGAATGTTGCTTCCTCCCATGAGGAAT-3′
N74Q-fwd	5′-TGGGTCACAAACCTTGCCCAAAGCAGCCTGGGCTTCATT-3′
N74Q-rev	5′-AATGAAGCCCAGGCTGCTTTGGGCAAGGTTTGTGACCCA-3′
N134Q-fwd	5′-TCCATTAACTTCATTCTGCAGAAAACTGGCCAAGAACAAGTG-3′
N134Q-rev	5′-CACTTGTTCTTGGCCAGTTTTCTGCAGAATGAAGTTAATGGA-3′
N246Q-fwd	5′-GGATTTAATGAGAGAAATTTACAGAGTTCTAGAGTGGATGTA-3′
N246Q-rev	5′-TACATCCACTCTAGACATCTGTAAATTTCTCTCATTAAATCC-3′
N294Q-fwd	5′-GCCAAGAATTATTTTCATTACCAGCAGAGTTATCCTCCCACA-3′
N294Q-rev	5′-TGTGGGAGGATAACTCTGCTGGTAATGAAAATAATTCTTGGC-3′

"Fwd" and "rev" denote forward and reverse strand orientation of the oligonucleotide, respectively. The codons imparting the mutations are underlined, converting the Asn residue to a Gln residue, respectively.

MATERIALS AND METHODS

Vector and Site-Directed Mutagenesis Used for Expression of LAL in Insect Cells—Synthesis of LAL cDNA and insertion into the pBakPak8 baculovirus expression vector (Invitrogen) have been described (*16*). For expression, wild-type enzyme was modified by replacing the LAL signal peptide sequence with the alkaline phosphatase signal peptide sequence (*20*). Codons for six histidine residues were added to the C-terminus of the enzyme to allow purification by affinity chromatography. Oligonucleotide-mediated site-directed mutagenesis was performed according to the supplier (Stratagene). The twelve oligonucleotides used for mutagenesis are listed in Table 1. Asn residues of the six potential *N*-glycosylation sites (Asn-X-Ser/Thr) were converted to glutamine residues. Standard PCR reactions were performed with *Pfu*-DNA polymerase, with 16 cycles of 30 s at 95°C, 1 min at 55°C and 14 min at 68°C. Mutagenesis was verified by sequencing the region surrounding the mutation.

In Vitro Expression, Purification and RT-PCR of Wild-Type and Mutant LAL—To express wild-type and mutant LAL protein, *S. frugiperda* cells 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, *S. frugiperda* 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 four times.

Media used for LAL purification were harvested after 96 h of viral infection and supplemented with ethylene glycol and Triton X-100 at concentrations of 25% and 0.1%, respectively. Wild-type and mutant LAL were purified by Ni^{2+} chelate affinity chromatography on a Ni-NTA matrix (Qiagen) utilizing the carboxyterminal polyhistidine tag of recombinant LAL. Briefly, supernatants were incubated in a batch procedure for 1 h at 4°C with Ni-NTA matrix equilibrated with 50 mM sodium phosphate (pH 7.0), 300 mM NaCl, 20 mM imidazole, 25% ethylene glycol, and 0.1% Triton X-100. After sedimentation of the Ni-NTA matrix in a column, the matrix was washed with 50 mM sodium phosphate (pH 6.5), 300 mM NaCl, 25% ethylene glycol, and 0.1% Triton X-100. Recombinant LAL was eluted in two fractions of 2 ml each with 50 mM sodium phosphate (pH 4.5), 300 mM NaCl, 20 mM imidazole, 25% ethylene glycol, and 0.1% Triton X-100. Aliquots of 500 ng of purified recombinant LAL were separated on SDS–polyacrylamide gels under denaturing conditions, using 4% stacking gels and 12.5% separating gels (*21*). Proteins were visualized by immunoblotting and detection with an anti-histidine antibody (see below).

To obtain total RNA for RT-PCR, *S. frugiperda* cells were collected after 96 h of infection with wild-type or mutant baculoviruses. After sedimentation of cells, total cellular RNA was extracted according to the supplier (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 of oligonucleotide FS 2/2 (GCTTCATTCTTGCTGATGCTGG) and 10 μM of oligonucleotide RS5/2 (AAAGTTTCAAGCCTTTGACTGGGG) were incubated. A 620 bp fragment of LAL was obtained. 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 mutant. For purified protein from supernatant, protein amounts were adjusted to 500 ng per sample. For samples from cell extracts, total protein was loaded. Therefore, 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 3 h at 1 mA/cm^2. After transfer, the

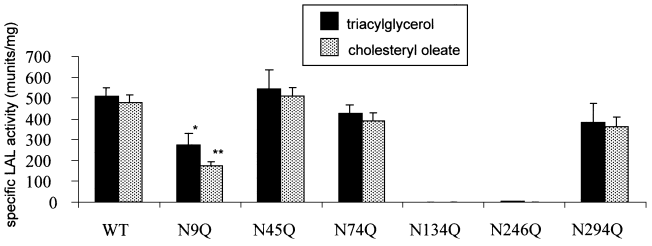

Fig. 1. **Enzymatic activities of Asn-to-Gln single glycosylation site mutants.** Enzymatic activities of LAL expressed in *S. frugiperda* cells were determined for purified LAL from culture supernatant using ^{3}H-labeled trioleoylglycerol (black columns), and ^{14}C-labeled cholesteryl oleate (hatched columns). Three separate expression experiments were performed. Mutant N9Q showed a reduced specific activity in culture supernatants. Mutants N134Q and N246Q displayed no specific activity. $*p < 0.05$; $**p < 0.01$, as compared to wild-type LAL.

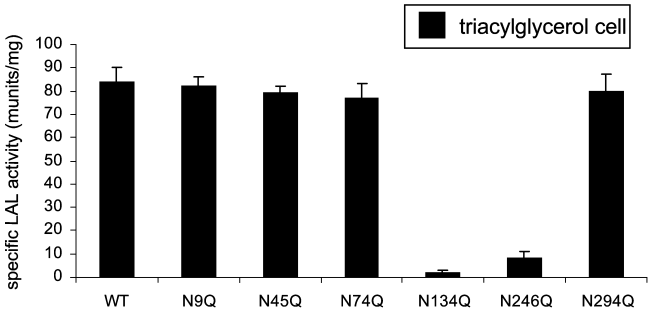

Fig. 2. **Enzymatic activities of Asn-to-Gln single glycosylation site mutants.** Enzymatic activities of LAL expressed in *S. frugiperda* cells were determined for cell extracts using ^{3}H-labeled trioleoylglycerol (black columns). Three separate expression experiments were performed. Interestingly, mutant N9Q showed no reduced specific activity compared to wild-type LAL. Mutant N134Q showed no specific activity, and N246Q showed only residual activity in cell extracts.

membrane was blocked with PBS/3% BSA for 1 h, washed with TBS-Tween, and incubated overnight at 4°C with an anti-His_6 antibody (*22*) (Dianova) (1:200 in TBS). Membranes to which purified LAL had been transferred were blocked for another 30 min with PBS, 2 mg/ml BSA, 100 mM EDTA to bind free nickel ions. Membranes were then 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.

Binding of Peanut Agglutinin to Recombinant Wild-Type LAL—Peanut agglutinin (PNA) recognizing the core disaccharide galactose β(*1,3*) *N*-acetyl galactosamine was used to identify putative *O*-linked carbohydrate side chains. Purified LAL was separated by a 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with the specific lectin conjugated to the steroid hapten digoxigenin (Roche). The bound lectin was detected using an anti–digoxygenin Fab antibody fragment conjugated to alkaline phosphatase. A colorimetric reaction in 50 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris-HCl (pH 9), containing 400 μg/ml 4-nitro blue tetrazolium chloride and 200 μg/ml 5-bromo-4-chloro-3-indolyl phosphate was used.

Deglycosylation and Inhibition of Glycosylation of LAL—To study the effect of deglycosylation on LAL structure and function, 2 μg of the purified lipase was incubated with 9 U of *N*-glycosidase F (PNGase F) overnight at 37°C. Enzyme was denatured by adding 1% SDS and 0.5% Nonidet P-40 and heating for 20 min at 100°C. Purified and denatured LAL was separated by SDS-PAGE and visualized by staining with silver nitrate (*23*).

To determine the effects of inhibiting glycosylation during LAL expression, tunicamycin was added to the tissue culture medium at a concentration of 6 μg/ml. As a control, LAL was expressed in the absence of tunicamycin. Media and cells were separated, and cell extracts were analyzed in an immunoblot assay using an anti-His_6 antibody as described above.

Enzymatic Activities and Kinetic Determinations—Enzymatic activities were determined in cell culture medium and in detergent extracts in three separate experiments, as described (*17*). To obtain kinetic data of LAL mutants, 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_{cat} and K_M values, triolein hydrolysis was plotted against substrate concentration to determine K_M values using Graph Pad Prism 3® software.

RESULTS

We have previously determined the extent of glycosylation for purified human liver LAL and demonstrated that *N*-linked oligosaccharides can be cleaved by endoglucosaminidase H, releasing high mannose-type oligosaccharides with a molecular mass of 4,000 to 6,000 Da (*17*). However, digestion with endoglycosaminidase H is known to be incomplete, *i.e.*, an *N*-acetylglucosamine residue remains attached to the Asn residue of the *N*-glycosylation recognition sequence. Based on data from the cloned cDNA, LAL contains six Asn-X-Ser/Thr consensus sequences for posttranslational glycosylation (*17–24*). To determine which of the six potential *N*-glycosylation sites are utilized in the mature lipase, Asn residues of the glycosylation recognition sites were systematically mutagenized to prevent glycosylation. Six single and six double mutants were generated by site-directed mutagenesis using a PCR overlap strategy to obtain Asn-to-Gln mutants. Glutamine was chosen because of its chemical and structural similarity to asparagine. Therefore, mutation *per se* should not lead to huge alterations in structure and function of LAL enzyme. Enzymatic activities were determined with ^{3}H-labeled triacylglycerol and ^{14}C-labeled cholesteryl oleate as substrates. Mutants N45Q, N74Q, and N294Q had lipolytic activities comparable to wild-type LAL (Fig. 1). Mutants N134Q and N246Q showed a complete loss of enzymatic activities. These two mutants showed enzymatic activities neither in culture medium nor in detergent extracts of cultured *S. frugiperda* cells (Figs. 1 and 2). Mutant N9Q showed significantly reduced enzymatic activities with both substrates. Interestingly, N9Q showed a preserved lipolytic activity in cell extracts (Fig. 2), indicating that mutation at amino

Enzyme	WT	WT	N9Q	N9Q
N-glycosidase F	-	+	-	+
Lane	1	2	3	4

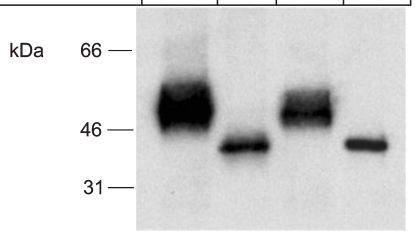

Fig. 3. **Effects of *N*-glycosidase F on LAL expressed in *S. frugiperda* cells.** Wild-type LAL (0.2 μg) was incubated without (lane 1) or with (lane 2) *N*-glycosidase F after denaturation by boiling for 20 min in buffer containing 1% SDS and 0.5% Nonidet P-40. Mutant N9Q was also incubated without (lane 3) and with *N*-glycosidase F (lane 4). Proteins were resolved on 12.5% SDS-PAGE, transferred to a PVDF membrane, and probed with a monoclonal anti-His6 antibody.

	WT	WT	N9Q	N9Q	WT	WT
	cell	cell	cell	cell	sup	sup
Tunicamycin	-	+	-	+	-	+
Lane	1	2	3	4	5	6

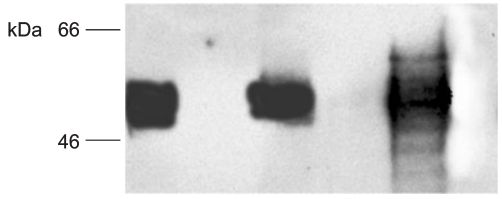

Fig. 4. **Effects of tunicamycin on LAL expression in *S. frugiperda* cells.** Cells were infected with wild-type or N9Q mutant and incubated without (lanes1, 3, 5) or with tunicamycin (lanes 2, 4, 6), and were analyzed after immunoblotting as described for Fig. 1. Wild-type LAL and N9Q mutant proteins were detectable in cell extracts after incubation without tunicamycin (lanes 1 and 3, respectively). Wild-type LAL and N9Q mutant proteins were undetectable (lanes 2 and 4, respectively). Wild-type LAL was undetectable in culture supernatants after tunicamycin treatment (lane 6), in contrast to untreated Sf 9 cells (lane 5). "Cell" denotes cell extract; "sup" denotes cell culture supernatant. Lanes 1 through 4, total cell extract; lanes 5 and 6, purified wild-type LAL.

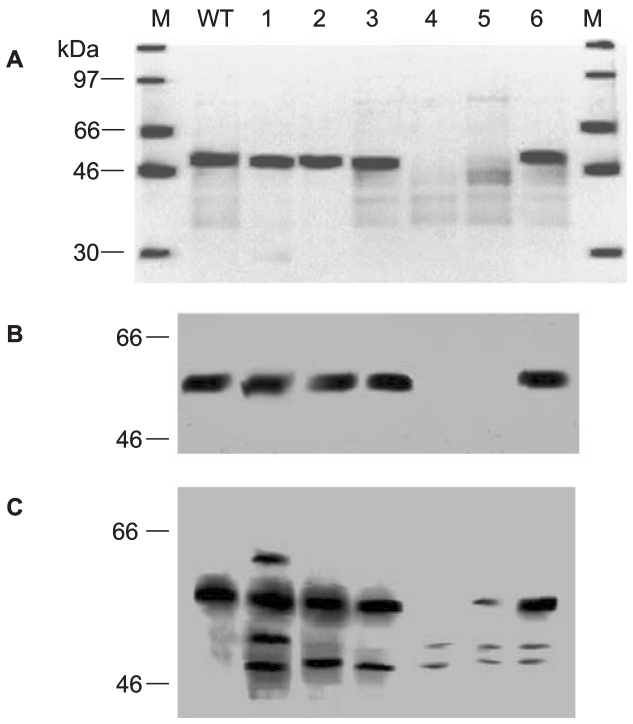

Fig. 5. **SDS-PAGE and immunoblot analysis of single glycosylation mutants.** Panel A, mutants and wild-type enzyme purified from supernatant of *S. frugiperda* cells were separated on 12.5% SDS-PAGE and stained with silver nitrate. Panel B, LAL protein was detected in culture supernatant with an anti-His_6 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. Mutants N134Q and N246Q were undetectable by SDS-PAGE analysis or antibody detection in culture supernatant or cell extracts (lanes 4 and 5 in panels B and C, respectively). WT, wild-type; lane 1, N9Q; lane 2, N45Q; lane 3, N74Q; lane 4, N134Q; lane 5, N246Q; lane 6, N294Q.

acid position 9 of LAL may interfere with secretion of the enzyme.

To determine whether LAL can be more completely deglycosylated than indicated in our initial experiments (*17*), *N*-glycosidase F (*25*) was utilized. A reduction in molecular mass of 6,000 to 7,000 Da was observed after *N*-glycosidase F incubation as compared to untreated LAL (Fig. 3, lanes 1 and 2), indicating a more complete deglycosylation with *N*-glycosidase F. Mutant N9Q showed no reduction in molecular mass, indicating that LAL is not glycosylated at this position. However, the signal on the immunoblot analysis was more homogeneous (Fig. 3, lane 3). Lectin blot analysis using peanut agglutinin revealed that purified recombinant LAL is not glycosylated with core 1 structures (Galβ1-3GalNAc) of *O*-glycosylation (data not shown). To determine whether glycosylation is required for the biosynthesis of an active lipase, *S. frugiperda* cultures were incubated with tunicamycin, a competitive inhibitor of glycosylation. In detergent-solubilized extracts of infected cells incubated in the presence of tunicamycin for four days, LAL protein was absent (Fig. 4, lanes 1 and 2). Lipolytic activities were undetectable both in cell extracts and in culture supernatants (data not shown). When grown in the presence of tunicamycin, LAL protein was also undetectable in the culture supernatant of *S. frugiperda* cells (Fig. 4, lane 6). Also, mutant N9Q was undetectable in cell extracts of *S. frugiperda* cells treated with tunicamycin (Fig. 3, lane 4). These data suggest that glycosylation is required for the synthesis of LAL in *S. frugiperda* cells.

To find out whether the mutants without lipolytic activity, N134Q and N246Q, were deficient in synthesis or secretion, SDS-PAGE analysis and immunoblotting of LAL mutants expressed in *S. frugiperda* cells were performed. On SDS-PAGE stained with silver nitrate, the two inactive mutants were undetectable (Fig. 5 A, lanes 4 and 5). On immunoblot analysis of recombinant lipase

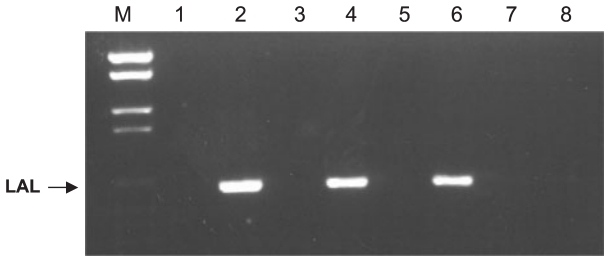

Fig. 6. **Agarose gel electrophoresis of RT-PCR products from LAL wild-type and glycosylation mutants N134Q and N246Q.** Total RNA (5 μg) from *S. frugiperda* cells was reverse-transcribed after DNase I digestion and PCR amplified with LAL-specific oligonucleotide primers. Lane 1, RT-PCR product of cells without baculoviral infection. Lane 2, RT-PCR product of cells infected with LAL wild-type baculoviral vector. Lane 3, RT-PCR product of cells infected wild-type LAL virus without reverse transcriptase. Lane 4, mutant N134Q. Lane 5, mutant N134Q without reverse transcriptase. Lane 6, mutant N246Q. Lane 7, mutant N246Q without reverse transcriptase. Lane 8, control without RNA. The size of the amplified LAL product is 620 bp (arrow). The DNA marker used was marker VI (Roche).

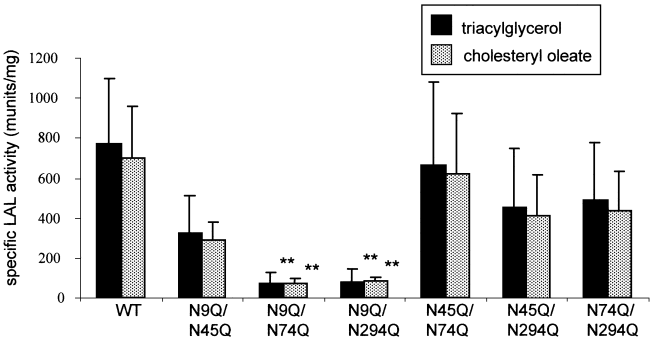

Fig. 7. **Enzymatic activities of double glycosylation site mutants.** Double mutants in all possible combinations except for positions N134 and N246 were constructed and expressed in *S. frugiperda* cells. Enzymatic activities of mutants purified from cell culture supernatants were determined in three separate experiments, using ^{3}H-labeled triacylglycerol (black columns) or ^{14}C-labeled cholesteryl oleate (hatched columns). Double mutants in combination with N9 showed reduced lipolytic activities compared to other mutants, indicating that N9 is glycosylated but not essential for enzymatic activity. $^{**}p < 0.001$, as compared to wild-type LAL.

mutants secreted into the culture media using a monoclonal antibody recognizing the polyhistidine tag, the two inactive mutants N134Q and N246Q were also undetectable (Fig. 5B, lanes 4 and 5). In detergent extracts of *S. frugiperda* cells, protein species with a lower molecular mass were detected (Fig. 5C, lanes 4 and 5), most likely representing degradation products of LAL. Mutant N9Q, which had significantly reduced lipolytic activities, was found on SDS-PAGE and immunoblot analysis in amounts comparable to wild-type LAL (Fig. 5, lane 1 in panels A to C).

To determine whether LAL-specific mRNA was synthesized in the two inactive mutants N134Q and N246Q or mRNA degradation occurred, RT-PCR of total RNA of cultured *S. frugiperda* cells was performed to specifically amplify LAL sequences (Fig. 6). DNA agarose gel electrophoresis showed that LAL mRNA was synthesized in

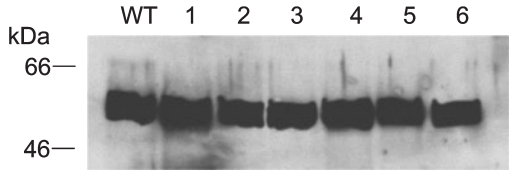

Fig. 8. **Immunoblot analysis of double glycosylation site mutants.** Wild-type LAL and six double mutants were purified from culture supernatant, separated by SDS-PAGE and transferred to a PVDF membrane. An anti-His_6 antibody was used for detection and developed with a horseradish peroxidase–coupled secondary antibody and the ECL system. All double mutants were detectable. WT: wild-type LAL; lane1, N9Q/N45Q; lane 2, N9Q/N74Q; lane 3, N9Q/N294Q; lane 4, N45Q/N74Q; lane 5, N45Q/N294Q; lane 6, N74Q/N294Q.

both mutants (Fig. 6, lanes 4 and 6, respectively). It is thus assumed that both mutant proteins are synthesized but rapidly degraded.

For further characterization of individual Asn residues, glycosylation double mutants were generated. Double mutants in combination with N9Q displayed reduced lipolytic activities (Fig. 7). Cholesteryl oleate hydrolysis was found reduced in mutants N45Q/N74Q, N45Q/N294Q, and N74Q/N294Q. Mutant proteins were detectable on immunoblot analysis of LAL secreted into culture media for all analyzed mutants (Fig. 8). Mutants were also detectable in detergent extracts of *S. frugiperda* cells (data not shown).

To obtain kinetic data of purified wild-type LAL and glycosylation site mutants, enzymatic triacylglycerol hydrolysis was determined with constant amounts of protein (1 μg) and various triacylglycerol concentrations. All mutants had similar K_M values to wild-type LAL. Mutants N9Q, N9Q/N74Q, and N9Q/N294Q showed significantly reduced k_{cat} values. k_{cat}/K_M values were significantly reduced for mutants N9Q/N74Q and N9Q/N294Q (Table 2). These results indicate that mutations leading to preserved or reduced hydrolytic activities may not result in changes in enzyme–substrate interaction.

Taken together, these data indicate that Asn residues 134 and 246 are glycosylated in native LAL. Glycosylation at these positions seems to be essential for the biosynthesis of active lipase. Mutation of Asn residue 9 leads to reduction in enzyme activity, but this residue is not essential for LAL. Enzymatic activities toward cholesteryl oleate as substrate were lower in all analyzed mutants than those toward triolein.

DISCUSSION

Glycosylation is a major and complex covalent modification that modulates the structure and function of membrane and secretory proteins in eukaryotes. Carbohydrate groups can control conformation, folding, or stability of proteins. Protection from proteolytic degradation, modulation of enzyme activity, cell recognition, and targeting to intra- and extracellular destinations have also been ascribed to sugar moeities (*26*, *27*). Oligosaccharides are attached to proteins by either an *N*-linked asparagine residue, an *O*-linked serine or threonine residue, or glycosylphosphoinositol located at the C-terminus

Table 2. **Kinetic data were determined at 37°C. and pH 4.5, using tri-[9,10(*n*)-^{3}H]oleoylglycerol as substrate.**

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
Wild-type LAL	0.413 ± 0.107	924 ± 473.2	447 ± 226
N9Q	0.178 ± 0.032*	768 ± 299.7	232 ± 90
N45Q	0.386 ± 0.085	1,145 ± 463.5	337 ± 182
N74Q	0.355 ± 0.074	1,013 ± 149.0	350 ± 96
N294Q	0.324 ± 0.112	590 ± 115.6	549 ± 168
N9Q/N45Q	0.250 ± 0.028*	578 ± 155.4	432 ± 180
N9Q/N74Q	0.125 ± 0.040*	1,012 ± 63.1	124 ± 63*
N9Q/N294Q	0.071 ± 0.032*	1,139 ± 515.0	62 ± 28*
N45Q/N74Q	0.323 ± 0.025	820 ± 80.4	394 ± 31
N45Q/N294Q	0.329 ± 0.014	726 ± 297.3	453 ± 46
N74Q/N294Q	0.308 ± 0.140	663 ± 452.2	464 ± 310

Activities are shown as mean values ± standard deviation. Three separate determinations were performed. *Significant at $p < 0.05$.

of membrane proteins. Interestingly, in some proteins, sugar residues can be removed by glycosidases without immediate structural consequences for the protein (*28*).

N-linked oligosaccharide side chains are found on all soluble lysosomal enzymes. Oligosaccharide residues are of particular relevance for these enzymes, since phosphorylation of mannose residues mediates the targeting of these hydrolases to the lysosome via mannose-6-phosphate receptors (*29*). The possible function of oligosaccharides in the synthesis and stabilization of active LAL has been a matter of discussion. From studies on human fibroblast LAL (*30*) and our previous data on human liver LAL (*17*) it is known that 4,000 to 6,000 Da can be removed by endoglycosidase H treatment. A small proportion of oligosaccharides was retained on the lipase, however. In the present study we therefore used endoglycosidase F, an enzyme hydrolyzing all classes of *N*-linked oligosaccharides, which may allow complete deglycosylation of LAL. Data from endoglycosidase F incubations indicate that LAL contains 6,000 to 7,000 Da of oligosaccharide residues. Assuming an average molecular mass of 3,000 Da per residue, this suggests that two of the possible six *N*-glycosylation sites are used. To examine the requirement for *N*-linked glycosylation in LAL synthesis and secretion of *S. frugiperda* cells, we utilized the glycosylation inhibitor tunicamycin. Consistent with previous studies of others (*31*), tunicamycin could completely suppress synthesis and secretion of active lipase to the media of cultured cells. In cell extracts, LAL was also undetectable by immunoblotting. We conclude from these studies that *N*-linked glycosylation of LAL is essential for the biosynthesis and secretion of enzymatically active lipase.

To precisely determine which of the six putative *N*-linked glycosylation sites of LAL are used in the active lipase, we mutated the LAL cDNA such that the six potential *N*-glycosylation sites were individually removed. We replaced the amino acid Asn with Gln at all potential glycosylation sites by site-directed mutagenesis, because Gln is chemically and structurally very similar to Asn. For this reason, we assume that the substitution itself did not lead to structural and functional differences between the mutants and wild-type LAL enzyme, but that any such differences had to be based on loss of glycosylation. A total of twelve single and double mutants were generated, transiently expressed in *S. frugiperda* cells and chromatographically purified. Enzymatic activities toward the two natural substrates of LAL, triacylglycerol and cholesteryl oleate, were determined in cell extracts and culture supernatants. In two LAL mutants, N134Q and N246Q, synthesis was virtually abolished. Only small amounts of degraded lipase were found on immunoblotting of extracts of *S. frugiperda* cells infected with these mutants. The lack of synthesis of the two mutants may have been due to a reduced or absent mRNA synthesis or a premature degradation of mRNA. RT-PCR experiments detected LAL-specific mRNA, however, making this an unlikely explanation.

The function of oligosaccharide side chains has been investigated in a number of lipolytic enzymes. Gastric lipase, an enzyme with high homology and identity to LAL, comprises four potential *N*-linked glycosylation sites (*32*). Site-directed mutagenesis of each of these sites did not significantly reduce the enzymatic activity of gastric lipase. Only complete prevention of *N*-linked glycosylation resulted in markedly reduced lipolytic activites. Glycosylation appeared to contribute to enzyme stability in that it prevented proteolytic degradation, rather than to enzyme biosynthesis (*32*). PNA lectin did not recognize either normal or recombinant gastric lipase, suggesting that these proteins are not *O*-glycosylated (*32*). This result is consistent with the fact that we have not found *O*-glycochains using peanut agglutinin in the highly homologous LAL enzyme. In human acid ceramidase, a glycosylated lysosomal enzyme, five of six potential *N*-glycosylation sites were found to be used in the native enzyme. Site-directed mutagenesis of each of these sites generated three mutants with complete loss of enzymatic activity due to an inhibited heterodimer formation. These mutants were degraded early after biosynthesis (*33*). In lipoprotein lipase, a neutral lipolytic enzyme localized to the endothelial surface of skeletal muscle and adipose tissue, a glycosylation site mutant resulted in the retention of lipase in the ER. In that mutant, N43A, premature degradation of the non-glycosylated enzyme was observed (*34*).

LAL belongs to a family of esterases containing a highly conserved serine residue as part of a catalytic Asp-

His-Ser triad (*24*, *35*). Based on the tertiary structure of human gastric lipase (*36*), a model structure was established for LAL (*37*). According to this model, the catalytic serine is covered by a stretch of 30 amino acid residues forming a lid structure. In the presence of lipid substrate the lid amino acids are displaced, allowing access of the substrate to the serine residue. The inactive LAL mutant N134Q may have an altered structure in the vicinity of this lid. The absence of the oligosaccharide residue on Asn134 may then impede the substrate interaction with the catalytic serine residue and thus abolish enzymatic activity of the mutant. Furthermore, glycochains at positions N134 and N246 are important for the stability of the enzyme, because mutants lacking glycosylation at these positions were not detectable.

Determination of K_M values of N9 single and double mutants showed that substrate affinities of mutant enzymes were unchanged. However, k_{cat} values of N9 mutants were significantly reduced. Recently, we demonstrated that LAL enzyme is a preproprotein. Asn9 is located in the pro-region of LAL (see Ref. *38* for details). Since the pro-region is removed by proteolysis during the course of secretion, it is unlikely that the sugar chain attached to the N9 site interacts with the mature LAL directly. Perhaps, the mutation of N9 may influence the folding of nascent LAL polypeptide and its tertiary structure. Interestingly, Dustin et al. suggested that a phosphorylated glycopeptide is bound at a propiece peptide of LAL enzyme (*39*). This is consistent with our finding that mutant N9Q showed reduced lipolytic activity after purification from supernatant. Unfortunately, we were not able to show a reduced mass in SDS-PAGE analysis of this mutant compared to wild-type LAL. Based on these findings, we assume that LAL is perhaps *N*-glycosylated at position 9, but this has to be investigated in more detail in mammals.

Glycosylation of recombinant proteins depends on the cell system used for protein expression. In these studies, *S. frugiperda* cells infected with a baculovirus vector were employed. In contrast to the highly heterogeneous glycosylation observed in mammalian cells, *S. frugiperda* cells were found to trim *N*-glycans to trimannosyl core structures modified with fucose residues, resulting in short oligosaccharide chains with considerably less heterogeneity (*40*). However, phosphorylated high-mannose oligosaccharides are not observed in insect cells (*41*). Although this is definitely relevant to proteins, such as LAL, that are subject to intracellular sorting *via* the mannose-6-phosphate receptor pathway (*42*), it has been shown that expression of posttranslational modifications of proteins in insect cells is most similar to mammalian cells compared to other expression systems (*43*).

In conclusion, this simple insect cell expression system is an adequate source for the analysis of glycosylation of low-expressed LAL. Data indicate that at least two of the six *N*-glycosylation sites are used in LAL and play an important role in the formation of functional LAL. N134 and N246 were found to be essential, and N9 was also assumed to be glycosylated, but further experiments have to be done to validate this assumption.

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