Role of N-linked glycosylation in the secretion and activity of endothelial lipase

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Abstract **Human endothelial lipase (EL), a member of the triglyceride lipase gene family, has five potential N-linked glycosylation sites, two of which are conserved in both lipoprotein lipase and hepatic lipase. Reduction in molecular mass of EL after treatment with glycosidases and after treatment of EL-expressing cells with the glycosylation inhibitor tunicamycin demonstrated that EL is a glycosylated protein. Each putative glycosylation site was examined by site-directed mutagenesis of the asparagine (Asn). Mutation of Asn-60 markedly reduced secretion and slightly increased specific activity. Mutation of Asn-116 did not influence secretion but increased specific activity. In both cases, this resulted from decreased apparent K_m and increased apparent V_{max}. Mutation of Asn-373 did not influence secretion but significantly reduced specific activity, as a result of a decrease in apparent V_{max}. Mutation of Asn-471 resulted in no reduction in secretion or specific activity. Mutation of Asn-449 resulted in no change in secretion, activity, or molecular mass, indicating that the site is not utilized. The ability of mutants secreted at normal levels to mediate bridging between LDL and cell surfaces was examined. The Asn-373 mutant demonstrated a 3-fold decrease in bridging compared with wild-type EL, whereas Asn-116 and Asn-471 were similar to wild-type EL.—**Miller, G. C., C. J. Long, E. D. Bojilova, D. Marchadier, K. O. Badellino, N. Blanchard, I. V. Fuki, J. M. Glick, and D. J. Rader. **Role of N-linked glycosylation in the secretion and activity of endothelial lipase. *J. Lipid Res.* 2004.** 45: **2080–2087.**

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Endothelial lipase (EL) has been identified as a member of the triglyceride lipase gene family that includes pancreatic lipase, LPL, and HL (1–4). Similar to LPL and HL, EL functions in the plasma compartment, and our initial report showed that overexpression of EL in mice profoundly alters plasma lipoprotein levels (5). More recent studies have indicated that HDL is the preferred substrate of EL (6–8). In contrast to LPL and HL, EL is synthesized in endothelial cells. Initial studies indicated that EL has significant phospholipase activity (5, 9); more recent studies have shown that it also has triglyceride lipase activity (6). Many features of the gene family are conserved in EL, including the three catalytic residues, the cysteine residues that form intramolecular disulfide bonds (10), the lid covering the catalytic pocket (11), and the heparin binding and lipid binding regions (12). Conservation of structure also exists among the N-linked glycosylation sites, with two of the five potential glycosylation sites in human EL conserved in both human LPL and human HL (**Fig. 1**) (13, 14). HL has two additional glycosylation sites that are not present in LPL; EL has three additional potential glycosylation sites that are not present in LPL and are different from those in HL.

Prevention of N-linked glycosylation has a direct effect on the secretion and activity of both LPL and HL. Tunicamycin treatment of LPL- and HL-expressing cells resulted in intracellular accumulation of an inactive protein of reduced size (15–17). Site-directed mutagenesis was used to generate mutant constructs of each putative site in LPL and HL, which were analyzed for effects on secretion and activity. In both LPL and HL, mutation of the conserved glycosylation sites had varying effects on the enzymes. Mutation of the conserved N-terminal site in LPL, Asn-43, completely abolished secretion and activity (16, 18, 19). Mutation of the corresponding site in HL, Asn-56, resulted in a substantial decrease in secretion (19–21) and a reduced specific activity of the enzyme that was secreted (19, 20). Mutation of the C-terminal conserved site in LPL, Asn-359, had no effect on secretion or activity (19), whereas mutation of the homologous site in HL, Asn-375, resulted

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Abbreviations: A/A, antibiotic/antimycotic; EL, endothelial lipase; Endo F, endoglycosidase F; HSPG, heparan sulfate proteoglycan; PBST, PBS plus 0.02% Tween 20.

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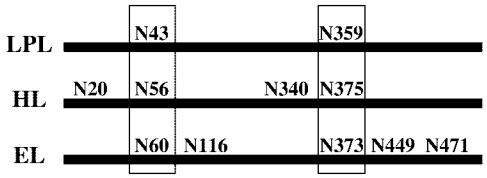

Fig. 1. Potential N-linked glycosylation sites in LPL, HL, and endothelial lipase (EL). Sites conserved among all three enzymes are shown in boxes. Numbering relates to the secreted proteins after cleavage of the signal peptide.

in a substantial decrease in secretion but had no effect on the specific activity of the secreted enzyme (19, 20). Mutation of the other two sites in HL, Asn-20 and Asn-340, had no effect on secretion or specific activity (19).

In this study, we demonstrate that human EL is a glycosylated protein, that four of the five potential N-linked glycosylation sites are used, and that lack of glycosylation at the four N-linked glycosylation sites has different effects on the secretion and specific activity of the enzyme. In addition, because both LPL and HL have been shown to have the ability to promote the binding of lipoproteins to cell surface proteoglycans through a bridging effect (3, 22–24), we tested the role of N-linked glycosylation on the ability of human EL to mediate lipoprotein bridging.

EXPERIMENTAL PROCEDURES

Cell culture

Both COS and 293 cells were grown in DMEM with 10% FBS and 1% antibiotic/antimycotic (A/A) at 37°C and 5% CO_2. COS cells were used for infection with adenovirus encoding EL, whereas 293 cells were used for transient transfection of wild-type and mutant EL cDNAs. Before infection with recombinant adenoviruses (5), COS cells were brought to 90% confluence on 60 mm plates. Cells were then incubated with adenovirus in 1 ml of serum-free medium at a multiplicity of infection of 3,000 particles/cell. Two hours later, 2 ml of serum-free medium containing 10 U/ml heparin was added. Medium was harvested at 24 or 48 h after infection, immediately divided into aliquots, and frozen at −80°C. 293 cells were brought to 90% confluence on 60 mm plates and were transfected using Lipofectamine™ according to the manufacturer's recommendations. At 20 h after transfection, medium on each plate was removed and replaced with 2 ml of serum-free medium containing 1% A/A and 10 U/ml heparin. At 47.5 h after transfection, an additional 10 U/ml heparin was added to the medium on each plate. After a 30 min incubation, harvested medium was divided into aliquots and frozen at −80°C.

Western blotting

Western blotting was done according to standard methods. Samples were separated on Nupage™ 10% Bis-Tris gels and detected as previously described (5) using a rabbit polyclonal antibody directed to a peptide in the N terminus of the EL protein as a primary antibody, an HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) as a secondary antibody, and ECL reagents (Amersham Biosciences).

Digestion of EL with glycosidases

Three glycosidases, endoglycosidase F (Endo F), Endo H, and neuraminidase, were used to digest EL in conditioned media collected from COS cells infected with adenovirus encoding EL. Incubations were for 4.5 h at 37°C, and reactions were stopped by adding 4× sample buffer to each tube and heating at 95°C for 5 min. After cooling on ice, the samples were analyzed by Western blotting.

Treatment of cells expressing EL with tunicamycin

COS cells were infected with adenovirus as described above with the following exception. After the 2 h with adenovirus, the medium was replaced with 2 ml of serum-free medium containing 10 U/ml heparin with and without 0.5 μg/ml tunicamycin. Plates were incubated at 37°C overnight, and both cells and media were collected 24 h after infection. The cells were collected in 0.75 ml of lysis buffer (0.25 M sucrose, 25 mM Tris-HCl, pH 8, and 1 mM EDTA), sonicated, and spun at 13,000 rpm for 30 min at 4°C. Supernatant containing intracellular protein and pellet containing membranes were separated and stored at −80°C. Samples from the medium, lysate, and pellet were analyzed by Western blotting. For measurement of cellular lipase activity, plates were washed three times with PBS and then frozen at −80°C until the day of assay. Cells were collected in 1.75 ml of 50 mM Tris-HCl, pH 8, sonicated, and immediately used for triglyceride lipase and phospholipase activity assays. Cell lysate protein was determined using a Pierce™ Micro BCA protein assay reagent kit.

Generation of site-specific glycosylation mutant plasmids

The Stratagene Quikchange™ site-directed mutagenesis kit was used to mutate the asparagine (Asn) residue at the five putative glycosylation sites (Asn-X-Ser/Thr) identified in EL to either an alanine (Ala) or a glutamine (Gln) residue. For each of the five mutants, PCRs containing 10, 20, and 50 ng of wild-type EL cDNA in the pcDNA3 plasmid vector were combined with 125 ng of the complementary sense and antisense oligonucleotides. PCR parameters were 1 cycle at 95°C for 30 s and 12 cycles at 95°C for 30 s, 55°C for 1 min, and 68°C for 14 min (2 min/kb of plasmid length). The oligonucleotides used for mutagenesis of each construct are as follows (sense sequence): N60A, 5′-CAGCCCTTAGAAGACTGCAGTTTCGCCATGACAGCTAAAACC-3′; N60Q, 5′-GCCCTTAGAAGACTGCAGTTTCCAAATGACAGCTAAAACC-3′; N116A, 5′-CTTTACACGGATGCGGTCGCCAATACCAGGGTGGTG-3′; N116Q, 5′-CTTTACACGGATGCGGTCCAAAATACCAGGGTGGTG-3′; N373A, 5′-GAGCGGATCGAGCAGGCCGCCACCAACACC-3′; N373Q, 5′-GTGGAGCGGATCGAGCAGCAAGCCACCAACACC-3′; N449A, 5′-GTACAGAAGACCCTGAGGCCACCAGCATATCCCCAG-3′; N449Q, 5′-GTACAGAAGACCCTGAGCAGACCAGCATATCCCCAGC-3′; N471A, 5′-GGCTGGAGGATGAAAGCCGAAACCAGTCCCACTG-3′; and N471Q, 5′-GGCTGGAGGATGAAACAGGAAACCAGTCCCACTGTGGG-3′. Mutant sequences were then verified by DNA sequencing of each construct.

Lipase assays

Triglyceride lipase and phospholipase assays were performed in triplicate using glycerol-stabilized substrates as previously described (6). Statistical comparisons were made using an unpaired *t*-test with GraphPad Prism software. A modification of the triglyceride lipase assay was used to determine apparent K_m and V_{max} values for the various mutant enzymes. The following triolein concentrations were used: 1.7, 1.2, 0.8, 0.64, 0.425, 0.215, and 0.21 mM. To ensure the measurement of initial velocities and to keep the utilization of substrate below 10%, samples were incubated for 15 min. Conditioned media (150 μl) prepared by transfection of the wild-type and mutant constructs in 293 cells were used in these assays. Assay blanks containing DMEM were run for each substrate concentration. Because of the large number of assays and the day-to-day variability in the preparation of

the substrate emulsion, separate experiments were performed, each comparing one or two of the mutant enzymes with the same preparation of wild-type EL. K_m and V_{max} values ± SEM were obtained using GraphPad Prism's nonlinear regression analysis, and statistical comparisons of the regression curves were made using a paired *t*-test, also with GraphPad Prism. As shown in **Table 1**, the apparent K_m and V_{max} for the wild-type enzyme varied somewhat between experiments but the ratio of V_{max} to K_m was quite similar.

ELISA for human EL

Rabbit antiserum against human EL (anti-AdhEL) as described elsewhere (25) was used in the ELISA applications. There was no cross-reactivity with either human LPL or human HL. The IgG fraction was purified using a HiTrap protein G column (Amersham Biosciences, Uppsala, Sweden) and biotinylated using sulfo-NHS-LC-biotin (Pierce, Rockford, IL). Free biotin was removed using a desalting column.

For the ELISA assay, unlabeled anti-AdhEL was diluted to 10 μg/ml in carbonate-bicarbonate buffer (16.85 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.2) and then applied (100 μl per well) to a 96-well EIA/RIA plate (Corning, Inc., Corning, NY). The plate was placed at 4°C overnight. Remaining binding sites in those wells, as well as an equal number of empty wells, were then blocked with 3% BSA (Sigma-Aldrich Co., St. Louis, MO) in PBS for 1 h at 37°C. The wells were washed twice with PBS plus 0.02% Tween 20 (PBST) and once with PBS alone. Because EL has not been purified to date, a standard preparation of medium containing human EL from stably transfected 293 cells was used in each assay to generate a standard curve. Masses of EL in the various media are expressed as arbitrary units relative to this EL preparation used as the standard.

All media were diluted in 1% BSA in PBS and added in triplicate to the wells. The plate was incubated at 37°C for 2 h. The wells were washed twice with PBST and once with PBS. Biotin-labeled anti-AdhEL, diluted to 4.0 μg/ml in 1% BSA in PBS, was applied, 100 μl/well. The plate was incubated for 2 h at 37°C. After again washing three times as described, 100 μl of a 1μg/ml streptavidin-horseradish peroxidase conjugate, diluted in 1% BSA in PBS, was applied to each well. The plate was incubated for 1 h 37°C. The plate was then washed five times with PBST and once with PBS. Five milligrams of *o*-phenylenediamine were dissolved in 12 ml of substrate buffer (27 mM citric acid, 51.5 mM Na_2HPO_4-$7H_2O$, pH 5.0), and 12 μl of 30% H_2O_2 was added. The substrate solution was added to each well, 100 μl/well, and color was allowed to develop for 10 min. The reaction was stopped with 100 μl/well of 2.5 M H_2SO_4, and the plate was read at 490 nm on an EL340 Biokinetics Reader (Biotek Instruments, Inc., Winooski, VT).

TABLE 1. Apparent kinetic constants for wild-type and mutant forms of EL

Sample	K_m	V_{max}	V_{max}/K_m
	mM triolein	*nmol/arbitrary unit/h*	
Experiment 1			
Wild-type EL	0.350 ± 0.048	12.4 ± 0.6	35.4
ELN60A	0.177 ± 0.040	13.4 ± 0.8	75.7
ELN116A	0.085 ± 0.011	19.5 ± 0.4	229.4
Experiment 2			
Wild-type EL	0.308 ± 0.024	9.8 ± 0.2	31.8
ELN373A	0.151 ± 0.044	3.3 ± 0.2	21.9
ELN449A	0.275 ± 0.025	9.4 ± 0.3	34.2
Experiment 3			
Wild-type EL	0.273 ± 0.032	8.5 ± 0.3	31.1
ELN471A	0.180 ± 0.024	6.9 ± 0.3	38.3

Values for K_m and V_{max} are ±SEM. EL, endothelial lipase.

Bridging of ^{125}I-LDL and cell surface proteoglycans by wild-type and mutant EL

A cell binding assay was used to examine the abilities of EL and the N-linked glycosylation mutants to mediate bridging between the cell surface heparan sulfate proteoglycan (HSPG) and plasma lipoproteins in vitro. 293 cells cultured on 12-well plates were transfected with vector encoding wild-type EL or glycosylation mutants using Lipofectamine™ reagent. For binding experiments, at 48 h after transfection, media were changed to 0.5 ml of serum-free medium supplemented with 0.2% BSA and ^{125}I-labeled LDL (5 μg/ml) in the absence or presence of heparin (100 U/ml). Cells were incubated at 37°C for 1 h, and then cell-associated ligands were measured. The heparin-sensitive (i.e., HSPG-dependent) component of lipase-mediated binding of ligands was calculated by subtracting the values obtained in the presence of heparin (HSPG-independent) from those obtained in the absence of heparin (total binding). During binding experiments, parallel sets of cells were incubated at 37°C for 1 h in DMEM containing heparin to determine protein mass and phospholipase activity secreted by the cells.

RESULTS AND DISCUSSION

EL is glycosylated

Media were collected from COS cells infected with adenovirus encoding the EL protein and subjected to glycosidase digestion as described in Experimental Procedures. The effect of glycosidase digestion of human EL was assessed by Western blotting (**Fig. 2**). Endo F, Endo H, and neuraminidase digestion each resulted in a reduction in size of the full-length wild-type EL (wild-type full-length EL is ~68 kDa), with the greatest effect by Endo F, an intermediate effect by neuraminidase, and the smallest effect by Endo H. Expression of the human EL cDNA resulted in a 40 kDa band that was probably the result of proteolytic cleavage and was detected with the antibody directed to an N-terminal peptide. A reduction in size of the 40 kDa band was evident in the Endo F digest and to a small degree in the neuraminidase digest, but no shift was observed in the Endo H digest. Digestion with Endo F cleaved N-linked glycans at the N-acetylglucosamine adjacent to the asparagine residue to which they are attached and therefore would be expected to remove all N-linked

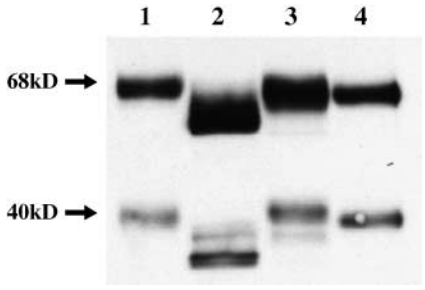

Fig. 2. Effects of glycosidase treatment of wild-type EL. Media samples collected from EL-expressing cells were digested with glycosidases as described in Experimental Procedures and were subjected to Western blotting. Lane 1, untreated EL; lane 2, endoglycosidase F (Endo F)-digested EL; lane 3, Endo H-digested EL; lane 4, neuraminidase-digested EL.

glycans. Consistent with this, Endo F reduced the full-length 68 kDa form to ~55 kDa, which is the predicted size of unglycosylated EL. Digestion with neuraminidase, which trims terminal sialic acid residues, resulted in a more modest decrease in size that was observed for both the 68 and 40 kDa forms. Endo H, which has specificity for N-acetylglucosamine residues of the trimannosyl cores of N-linked glycans, resulted in a modest decrease in the 68 kDa form but no decrease in the 40 kDa form. The lack of effect of Endo H on the 40 kDa form suggests that the glycans at the N-terminal glycosylation sites contained within the 40 kDa form (Asn-60 and Asn-116) lack the Endo H-sensitive sequence.

We used tunicamycin treatment to determine the effects of inhibition of N-linked glycosylation on EL secretion and activity. Media were collected from COS cells infected with adenovirus encoding EL in the presence and absence of tunicamycin using adenovirus encoding green fluorescent protein (GFP) as a negative control. Cellular lysates, cell membranes, and conditioned media were analyzed by Western blot (**Fig. 3**). For cells expressing EL, a protein of reduced mass (~55 kDa) was present in the cell lysate and membrane fraction of the tunicamycin-treated cells compared with untreated cells. A protein of 55 kDa is consistent in size with the unglycosylated EL protein predicted by the amino acid sequence. Secreted EL protein was absent in media of tunicamycin-treated cells but present in the media of untreated cells. Media were then analyzed for both triglyceride lipase (**Fig. 4A**) and phospholipase (Fig. 4B) activity. As expected from the lack of secretion, the data indicate that both triglyceride lipase and phospholipase activities were absent in media from tunicamycin-treated cells. Cellular lysates were also analyzed for both triglyceride lipase and phospholipase activity. The activity in both cases was abolished in the tunicamycin-treated cells compared with untreated cells expressing wild-type EL (data not shown). These studies suggest that loss of N-linked glycosylation as a result of tunicamycin treatment blocks the secretion of EL and leads to the intracellular retention of an inactive protein of reduced size. Previous studies of tunicamycin treatment of LPL- and HL-expressing cells reported similar findings (19, 26, 27). Our results establish that EL, like LPL and HL, requires N-linked glycosylation for its activity and secretion.

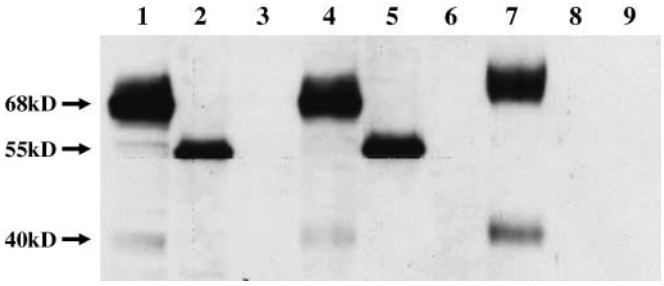

Fig. 3. Effect of tunicamycin treatment of cells expressing EL. COS cells infected with adenovirus encoding EL were treated with tunicamycin as described in Experimental Procedures. Cells and media samples were collected and subjected to Western blotting. Lanes 1–3 are cellular lysates: lane 1, untreated cells (uEL); lane 2, tunicamycin-treated cells (tEL); lane 3, cells expressing green fluorescent protein (GFP). Lanes 4–6 are membrane-bound protein: lane 4, uEL; lane 5, tEL; lane 6, GFP. Lanes 7–9 are media samples: lane 7, uEL; lane 8, tEL; lane 9, GFP.

Four of five predicted N-linked glycosylation sites of EL are utilized

Individual mutant cDNA constructs were generated at each putative N-linked glycosylation site to further characterize glycosylation of the EL protein. These mutants allow for precision in determining whether an individual site is utilized and for studying the impact of loss of individual N-linked glycan chains. The asparagine (N) residue of the Asn-X-Ser/Thr sequence was changed to either alanine (A) or glutamine (Q) via site-directed mutagenesis and transiently transfected in 293 cells in the presence of heparin. Conditioned media were collected and subjected to analysis by Western blot (**Fig. 5**), which indicated that 8 of the 10 mutant constructs resulted in a reduction in molecular mass from the full-length 68 kDa band compared with wild-type EL, the exceptions being the N449A and N449Q mutant constructs. The reduction in molecular mass is consistent with the loss of N-linked glycosylation at the other four sites, indicating that four of the five potential N-linked glycosylation sites in EL are utilized. Mutation of two of the sites, Asn-60 and Asn-116, also resulted in a reduction in molecular mass of the 40 kDa band, whereas mutation of the two other sites, Asn-373 and Asn-471, did not affect the size of the 40 kDa band. This suggests that the site of cleavage in EL lies between amino acids 116 and 373.

Effect of mutations on EL secretion

Because N-linked glycosylation occurs cotranslationally and can have effects on the protein-folding process and secretion (28), it was of interest to compare the amounts of the various mutant ELs that were secreted into the medium. The Western blot (Fig. 5) of the conditioned media suggested that mutation of Asn-60 (to either Ala or Gln) resulted in an observable decrease in secretion compared with wild-type EL. This N-terminal N-linked glycosylation site is conserved among human LPL, HL, and EL (Fig. 1). Mutation of LPL Asn-43 completely abolished secretion of the enzyme (16, 18, 19), and mutation of HL Asn-56 resulted in a substantial reduction in secretion (19, 20). Thus, our results with the mutation of EL at this site are consistent with previous findings for LPL and HL, but more similar to HL, in that secretion was not completely abolished as it was for LPL.

The remaining EL mutations at Asn-116, Asn-373, Asn-449, and Asn-471 had no observable effects on secretion compared with wild-type EL. Of these, only Asn-373 is conserved in LPL and HL (Fig. 1), and as with EL, mutation of the homologous LPL Asn-359 also had no effect on secretion (18–20). Mutation of the homologous HL Asn-375, however, resulted in a 60% decrease in secretion (19, 20). Thus, efficient secretion of HL, but not LPL or EL, is dependent on N-linked glycosylation at this site.

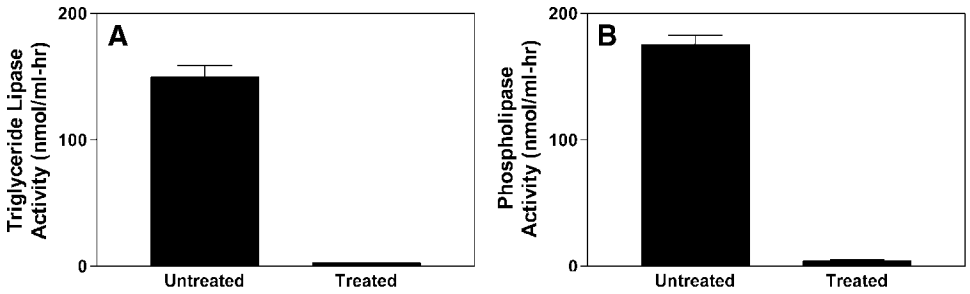

Fig. 4. Effect of tunicamycin treatment on triglyceride and phospholipase activity of cells expressing EL. COS cells infected with adenovirus encoding EL were treated with tunicamycin as described in Experimental Procedures. Media samples were collected and aliquots of medium from the same plate were assayed in triplicate for triglyceride lipase (A) and phospholipase (B) activity using radiolabeled substrates as described in Experimental Procedures. Untreated EL-expressing cells were present as a positive control. Data shown are means ± SD.

Effect of mutations on lipolytic activity

N-linked glycosylation can also influence protein structure and stability (28), both of which might have impact on enzymatic activity. In the same experiment that examined both the asparagine and glutamine mutants, conditioned media samples were assayed for triglyceride lipase and phospholipase activities, assayed for EL mass by ELISA, and specific activities were determined. This experiment revealed differences in specific activity among the mutants (data not shown); however, because the experiment was based on duplicate plates, statistical significance could not be assessed. As there was no evidence that the Ala mutants substantially differed from the Gln mutants, another experiment was performed in which all five of the Ala mutants and wild-type EL were transiently transfected on triplicate plates. Conditioned media from this new triplicate experiment were collected and assayed for triglyceride lipase and phospholipase activities as well as for EL mass by ELISA, and the specific activities were determined (**Fig. 6**).

Although the Asn-60 mutant displayed reduced secretion (17% of wild type; $P = 0.01$), there was no reduction in lipase specific activity (Fig. 6). The trend toward increased specific activity was not statistically significant in this experiment (triglyceride, $P = 0.09$; phospholipase, $P = 0.053$), although in the kinetic analyses described below this mutant had increased activity. Therefore, although its secretion was reduced, the Asn-60 mutant enzyme that was secreted had lipase specific activity that was similar to or slightly higher that wild-type EL. This is in contrast to the homologous mutation in HL at Asn-56, which resulted in a substantial reduction in the specific activity of the secreted enzyme to ~20% of wild-type HL (19–21).

The Asn-373 mutant, although secreted normally (80% of wild type; $P = 0.18$), displayed significantly reduced triglyceride and phospholipase specific activity (triglyceride, $P = 0.0001$; phospholipase, $P = 0.002$) compared with wild-type EL (Fig. 6). This N-linked glycosylation site is also conserved in LPL and HL (Fig. 1). In contrast to EL, mutation of neither LPL Asn-359 (16, 17) nor HL Asn-375 (19, 20) resulted in a reduction in specific activity. Thus, N-linked glycosylation at this site is required for normal specific activity of EL but not for LPL or HL.

Secretion was not affected in the remaining three potential N-linked glycosylation sites in EL, Asn-116 (118% of wild type; $P = 0.32$), Asn-449 (106% of wild type; $P = 0.58$), and Asn-471 (115% of wild type; $P = 0.32$). These sites are unique to EL and are not present in LPL or HL (Fig. 1). Mutation of Asn-449 did not reduce lipase specific activity (triglyceride, $P = 0.15$; phospholipase, $P = 0.36$) (Fig. 6); this is not surprising, as this site does not appear to be utilized as an N-linked glycosylation site, as noted above. Although Asn-471 is clearly glycosylated, mutation of this site did reduce triglyceride lipase specific activity ($P = 0.001$) but did not significantly reduce phospholipase specific activity ($P = 0.07$) (Fig. 6). Finally, these experiments demonstrated that mutation of Asn-116 may lead to increased lipase specific activity; the phospholipase specific activity was significantly higher than that in wild-type EL ($P = 0.003$), although, for the triglyceride lipase activity, it was not statistically significantly different ($P = 0.06$). In

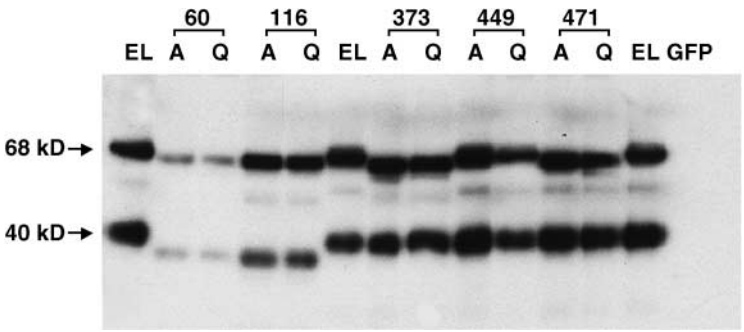

Fig. 5. Effect of site-specific N-linked glycosylation mutants on glycosylation and secretion of EL. Duplicate plates of 293 cells were transiently transfected with wild-type and each of the site-specific mutant EL cDNAs as described in Experimental Procedures. Media samples were collected and subjected to Western blotting. The Western blot shows wild-type EL and GFP present as controls and both the alanine (A) and glutamine (Q) mutant constructs.

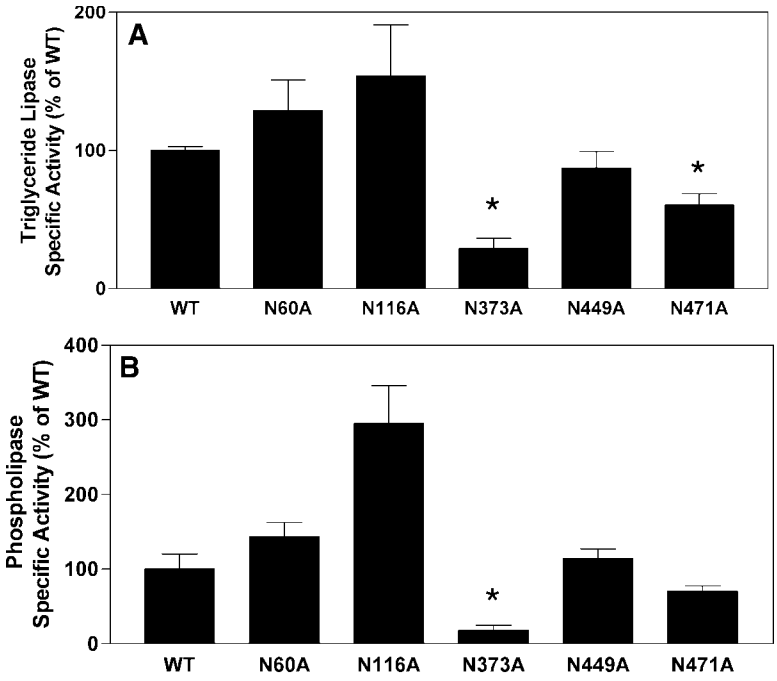

Fig. 6. Comparison of triglyceride lipase and phospholipase specific activities of alanine site-specific glycosylation mutants with wild-type (WT) EL. Triplicate plates of 293 cells were transiently transfected with wild-type and alanine site-specific mutant EL cDNAs as described in Experimental Procedures. Conditioned media was assayed for EL mass in triplicate by ELISA and for triglyceride lipase and phospholipase activities in triplicate using radiolabeled substrates as described in Experimental Procedures. A and B show triglyceride lipase and phospholipase specific activities, respectively, expressed as percentage of that for wild-type EL. The specific activities were calculated using arbitrary ELISA units. * $P < 0.05$ vs. wild type. Data shown are means ± SD for triplicate plates.

seven of eight other experiments that directly compared wild-type EL with the N116A mutant EL, the increased activity of the mutant form was statistically significant. Of note, results from site-directed mutagenesis of the two unique, nonconserved N-linked glycosylation sites in HL (Fig. 1) indicated that, although both sites are utilized for glycosylation, at neither site does the loss of the carbohydrate have an effect on lipase specific activity (19, 20).

Kinetic characterization of mutant EL enzymatic activities

To characterize more rigorously the enzymatic activities of the various mutant enzymes, kinetic analyses were performed as described in Experimental Procedures. Primary data from three separate experiments are presented in **Fig. 7**. In each experiment, separate aliquots of the same preparation of wild-type EL were analyzed to control for variations in preparation of the substrate emulsion. Table 1 shows the kinetic parameters calculated for these experiments. The K_m and V_{max} values presented here must be considered "apparent" values. The rates of hydrolysis are affected by the interfacial binding of the enzyme to the surface, the binding of the active site to a triglyceride molecule in the surface, and hydrolysis of an ester linkage (29). The surface triglyceride undoubtedly represents only a small part of the bulk triglyceride present in the core of the emulsion particle, yet the K_m values represent the total bulk lipid. A useful transformation of the kinetic data, the V_{max}/K_m ratio (30), is the most reliable comparator between experiments. A comparison of the kinetic constants determined for wild-type EL in the three separate experiments (Table 1) demonstrates the variability that is obtained among different substrate preparations. Because of the variability among substrates, comparisons of K_m and V_{max} must be made within a single experiment. For wild-type EL in these experiments, the higher K_m values are associated with higher V_{max} values, and the ratios of V_{max} to K_m are quite similar. Thus, the V_{max}/K_m ratio is the best indicator of meaningful differences in the kinetic parameters and can be used to compare values between experiments.

In experiment 1 (Fig. 7A, Table 1), the curve for ELN60A was significantly different from that for wild-type EL ($P = 0.0016$). Compared with wild-type EL, ELN60A had a lower K_m and similar V_{max}, and the ratio of V_{max} to K_m was nearly doubled. Similarly for ELN116A, the K_m was even lower, the V_{max} was substantially increased, and the V_{max}/K_m ratio increased 6-fold. These data indicate that the loss of glycosylation at position 60 or 116 increased the activity of the enzyme. In experiment 2 (Fig. 7B, Table 1), wild-type EL and ELN449A, the mutant form of the site that appears not to be glycosylated, gave highly similar kinetic constants and V_{max}/K_m ratios, as would be expected, and the curves were not significantly different. However, the curves for wild-type EL and ELN373A were significantly different ($P = 0.0131$). ELN373A had a markedly lower V_{max}, a slightly lower K_m, and a lower V_{max}/K_m ratio compared with wild-type EL. In experiment 3 (Fig. 7C, Table 1), ELN471A showed modest changes in K_m, V_{max}, and V_{max}/K_m relative to wild-type EL. However, a comparison of the curves revealed that they were not significantly different.

In contrast to LPL and HL, in which mutating the N-linked glycosylation sites either produced no change in activity or decreased activity, mutating the N116 site in EL led to a large increase in activity, contributed to by an increased affinity of the enzyme for substrate and an increase in the V_{max}. The N60A mutation had a more subtle but nevertheless positive effect on activity, yielding a 2-fold increase in V_{max}/K_m. Interestingly, an outcome of similar magnitude was reported in a study on lecithin:cholesterol acyltrans-

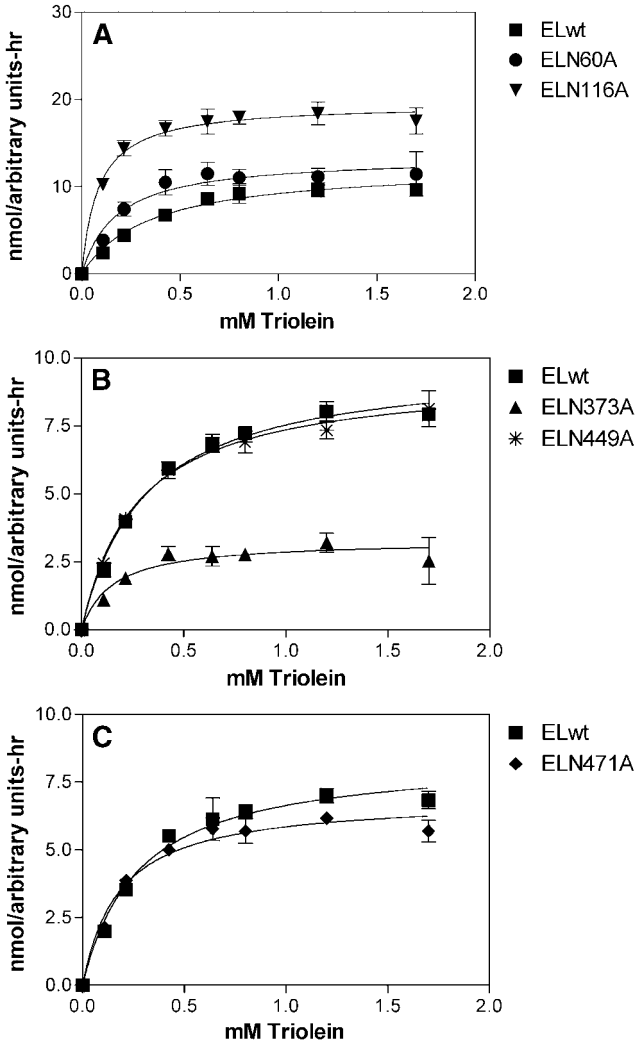

Fig. 7. Kinetic analysis of wild-type and mutant forms of EL. Conditioned media from 293 cells transiently transfected with wild-type (ELwt) and the five mutant EL constructs were subjected to kinetic analysis as described in Experimental Procedures. Initial velocities were normalized to arbitrary units of EL mass as determined by ELISA. Assays were done in triplicate, and data shown are means ± SD.

ferase (30), an enzyme whose natural substrate is also HDL. In that case, mutation of Asn-384 to Gln resulted in a 2-fold increase in V_{max}/K_m, with the increase in the ratio primarily attributable to an increase in V_{max}.

Effects of EL mutants on bridging of lipoproteins and proteoglycans

Although LPL and HL have been shown to have an effect on bridging of lipoproteins with proteoglycans, the role of N-linked glycosylation in the bridging properties of these lipases has not been addressed. To determine the effects of the lack of site-specific N-linked glycosylation on the ability of EL to bridge between lipoproteins and proteoglycans, 293 cells were transiently transfected with wild-type EL and the three alanine mutant EL cDNA constructs that influenced glycosylation and were normally secreted (ELN116A, ELN373A, and ELN471A). Western blotting confirmed expression of the EL proteins at similar concentrations. Binding of ^{125}I-LDL was quantitated in both the absence and presence of a low concentration of heparin (100 μg/ml), a concentration that specifically prevents the interaction of ligands with HSPGs but not with the LDL receptor. Studies were performed in triplicate, and HSPG-specific binding was normalized to cell protein (**Fig. 8**). Of note, the 293 cells that were not expressing EL (i.e., cells transfected with the control GFP plasmid) had a heparin-sensitive ^{125}I-LDL binding of only 2.3 ng/mg, indicating that there was almost no heparin-sensitive (HSPG-mediated) binding in control cells in the absence of expressed EL. The ELN116A and ELN471A mutants were similar to wild-type EL in their ability to mediate HSPG-dependent binding of ^{125}I-LDL. However, the ELN373A mutant had a 3-fold decrease in HSPG-dependent binding of ^{125}I-LDL by the cells compared with wild-type EL. It is interesting that of these three mutants, only the ELN373A mutant has reduced lipase specific activity. Lack of glycosylation at this site may result in an enzyme that is impaired in its ability to bind either proteoglycans or lipoproteins, and this may result in reduced specific activity. Further experiments will be required to address the relationship between specific activity and bridging activity associated with N-linked glycosylation.

In summary, we demonstrate that human EL is a glycosylated protein and that efficient secretion of the enzyme is dependent upon the presence of N-linked carbohydrate. Site-directed mutagenesis of individual N-linked glycosylation sites in EL demonstrated that four of the five putative sites are utilized. Mutation of the conserved N-terminal glycosylation site Asn-60 yielded results similar to mutation of the corresponding sites in LPL and HL in that secretion was reduced. However, unlike LPL and HL, the activity of the enzyme was increased. Mutation of Asn-116, a unique glycosylation site in EL, had no effect on secretion but markedly increased activity. Mutation of the conserved C-terminal glycosylation site Asn-373 was markedly different compared with mutation of the homologous sites in LPL and HL, in that it significantly reduced the lipase spe-

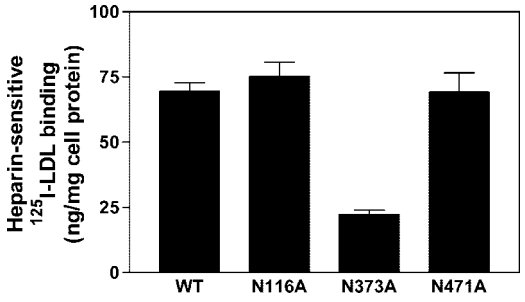

Fig. 8. Effects of site-specific N-linked glycosylation mutants on ^{125}I-LDL bridging. 293 cells were transiently transfected in triplicate with wild-type (WT) and site-specific mutant EL cDNAs as described in Experimental Procedures. At 48 h, cells were incubated with ^{125}I-LDL for 1 h either in the absence or presence of 100 μg/ml heparin, after which heparin-sensitive binding was quantitated as described in Experimental Procedures. Heparin-sensitive binding of ^{125}I-LDL in control-transfected cells was negligible. Data shown are means ± SEM.

cific activity. Mutation of Asn-373 was the only glycosylation mutant to have an effect on bridging of EL to lipoproteins and proteoglycans, indicating a potential role for N-linked glycosylation in this process.

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