

Glycosylation of endothelial lipase at asparagine-116 reduces activity and the hydrolysis of native lipoproteins in vitro and in vivo

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Abstract We previously identified that four of five putative *N*-linked glycosylation sites of human endothelial lipase (EL) are utilized and suggested that the substitution of asparagine-116 (Asn-116) with alanine (Ala) (N116A) increased the hydrolytic activity of EL. The current study demonstrates that mutagenesis of either Asn-116 to threonine (Thr) or Thr-118 to Ala also disrupted the glycosylation of EL and enhanced catalytic activity toward synthetic substrates by 3-fold versus wild-type EL. Furthermore, we assessed the hydrolysis of native lipoprotein lipids by EL-N116A. EL-N116A exhibited a 5-fold increase in LDL hydrolysis and a 1.8-fold increase in HDL₂ hydrolysis. Consistent with these observations, adenovirus-mediated expression of EL-N116A in mice significantly reduced the levels of both LDL and HDL cholesterol beyond the reductions observed by the expression of wild-type EL alone. Finally, we introduced Asn-116 of EL into the analogous positions within LPL and HL, resulting in *N*-linked glycosylation at this site. Glycosylation at this site suppressed the LPL hydrolysis of synthetic substrates, LDL, HDL₂, and HDL₃ but had little effect on HL activity. These data suggest that *N*-linked glycosylation at Asn-116 reduces the ability of EL to hydrolyze lipids in LDL and HDL₂.—Brown, R. J., G. C. Miller, N. Griffon, C. J. Long, and D. J. Rader. Glycosylation of endothelial lipase at asparagine-116 reduces activity and the hydrolysis of native lipoproteins in vitro and in vivo. *J. Lipid Res.* 2007. 48: 1132–1139.

Supplementary key words lipase • lipoprotein hydrolysis • adenovirus • glycosylation • site-directed mutagenesis • heparin • heparan sulfate proteoglycan

Endothelial lipase (EL) belongs to a superfamily of lipases (EC 3.1.1.3) that includes LPL and HL (1–6). These three lipases have both triglyceride (TG) lipase and phospholipase activity, but EL has relatively more phospholipase activity compared with LPL, which has predominantly TG lipase activity (7). Overexpression of EL in mice was shown

to significantly reduce high density lipoprotein cholesterol (HDL-C) (1, 8, 9), whereas loss-of-function studies in mice result in significantly elevated plasma HDL-C (8, 10, 11).

In vitro and in vivo studies using chimeric proteins of LPL and HL have shown that the differences in substrate specificity between these two lipases are governed by a 22 amino acid loop, or “lid domain,” within the N-terminal domain of the respective lipases that covers the catalytic site (12–14). The shorter 19 amino acid lid domain within EL partially contributes to its substrate specificity (15); other elements affecting substrate specificity remain to be elucidated.

Human EL is translated as a 500 amino acid 57 kDa peptide that is processed into a mature 480 amino acid protein with an apparent molecular mass of 68 kDa after the loss of its signal peptide and the addition of *N*-linked glycosylation (1, 2). EL has five putative *N*-linked glycosylation sites [identified by the presence of asparagine-X-serine/threonine (Asn-Xaa-Ser/Thr) motifs]. We previously reported that four of the five sites, specifically Asn-60, Asn-116, Asn-373, and Asn-471, are utilized (16). Abolishment of *N*-linked glycosylation at Asn-116 by mutagenesis of Asn to alanine (Ala) resulted in a surprising increase of catalytic activity, whereas removing *N*-linked glycosylation at other sites produced EL proteins with either decreased or unaffected catalytic activities (16). Asn-116 of EL is not conserved in either LPL or HL. Thus, the *N*-linked glycosylation at Asn-116 of EL may play a unique function that regulates enzyme activity. In this study, we confirmed through additional mutagenesis that the *N*-linked glycosylation at Asn-116 reduces activity against synthetic substrates, and we further explored its role in modulating the ability of EL to hydrolyze native lipoproteins ex vivo and

Abbreviations: A/A, antibiotic/antimycotic; DPPC, dipalmitoyl-phosphatidyl choline; EL, endothelial lipase; FPLC, fast-performance liquid chromatography; HDL-C, high density lipoprotein cholesterol; LDLR, low density lipoprotein receptor; PL, phospholipid; TC, total cholesterol; TG, triglyceride.

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in vivo. Our data support the conclusion that the N-linked glycosylation at Asn-116 of EL affects the substrate specificity of the enzyme by limiting the hydrolysis of substrates in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials

Triolein, dipalmitoylphosphatidyl choline (DPPC), cholesteryl oleate, fatty acid-free BSA, heparin, and FBS were purchased from Sigma. DMEM, antibiotic/antimycotic (A/A), Lipofectamine™, and Nupage™ 10% Bis-Tris gels were purchased from Invitrogen. HRP-conjugated goat anti-rabbit IgG and HRP-conjugated rabbit anti-mouse IgG antibodies were purchased from Jackson ImmunoResearch. [³H]triolein was purchased from Perkin-Elmer. [¹⁴C]DPPC was purchased from American Radio-labeled Chemicals, Inc. A polyclonal anti-human EL antibody was generated as described previously (17). The monoclonal anti-bovine LPL antibody 5D2, with cross-reactivity to human LPL (18), was a gift of Dr. John Brunzell (University of Washington). The monoclonal anti-human HL antibody XHL3-6 (19) was a gift of Dr. André Bensadoun (Cornell University).

Preparation of lipase expression plasmids

The cDNAs for human EL (NM006033), human LPL (NM000237), and human HL (NM000236) were inserted into the pcDNA3 mammalian expression vector (Invitrogen). Mutagenesis of Asn-116 from human EL into Ala (N116A) was described previously (16). Mutagenesis of Asn-116 from EL into Thr (N116T), Asn-117 into Ala (N117A), and Thr-118 into Ala (T118A) was performed using the Quikchange™ mutagenesis kit (Stratagene) with previously described polymerase chain reaction conditions (16). Complementary sense and antisense oligonucleotides (toward nucleotides 647–677) to generate these mutants are as follows (sense sequence): N116T, 5'-CGGATGCGGTCACTAATACACGGTGGTG-GG-3'; N117A, 5'-CGGATGCGGTCAATGCTACCAGGGTGGTGGG-3'; and T118A, 5'-CGGATGCGGTCAATAATGCCAGGGTGGTGGG-3'. Mutagenesis of glycine-99 (Gly-99) from LPL into either Asn (G99N) or Ala (G99A) was performed as described above. The sense sequences of the oligonucleotides (toward nucleotides 538–569) to generate the LPL mutants are as follows: G99N, 5'-CCAGTGTCCGCGAACTACACCAAACCTGGTGGG-3'; and G99A, 5'-CCAGTGTCCGCGGCTACACCAAACCTGGTGG-3'. Mutagenesis of arginine-113 (Arg-113) from HL into either Asn (R113N) or lysine (Lys) (R113K) was performed as described above. The sense sequences of the oligonucleotides (toward nucleotides 441–472) to generate the HL mutants are as follows: R113N, 5'-CCACTACACCATCGCCGTCAACAACACCCGCC-3'; and R113K 5'-CTACACCATCGCCGTCAAGAACACCCGCCCTTGTGG-3'. All mutant sequences were verified by DNA sequencing.

Cell culture

293 cells were cultured in DMEM containing 10% FBS and 1% A/A. Cells were grown to 90% confluency (in 60 mm dishes), and 1 μg of plasmid expressing lipase was transfected using Lipofectamine™ according to the manufacturer's instructions. At 24 h after the transfection, media were removed and replaced with serum-free media containing 1% A/A and 10 U/ml heparin. To promote lipase dissociation from cells, at 47.5 h after the transfection, an additional 10 U/ml heparin was added to the media in each plate. At 48 h after the transfection, media were collected and centrifuged at 1,200 rpm for 10 min to remove any cell debris. The supernatant was divided into aliquots and stored

at –80°C. The total extracellular EL released from transfected cells over 16 h in the absence versus presence of heparin was determined as described previously for HL (20).

Protein analyses

Proteins in conditioned media samples from transfected cells were separated on Nupage™ 10% Bis-Tris gels, and gels were transferred to nitrocellulose membranes. Nitrocellulose membranes were subjected to chemiluminescent immunoblot analyses for EL (using a 1:5,000 dilution of the anti-human EL polyclonal antibody and a 1:5,000 dilution of HRP-conjugated anti-rabbit IgG), LPL (using a 1:3,000 dilution of 5D2 and a 1:5,000 dilution of HRP-conjugated anti-mouse IgG), and HL (using a 1:10,000 dilution of XHL3-6 and a 1:5,000 dilution of HRP-conjugated anti-mouse IgG). The mass of recombinant LPL and HL, expressed as arbitrary units, was determined from standard densitometry curves of immunoblots. Briefly, conditioned media (0–10 μl) of wild-type or mutant lipase were separated on the same Bis-Tris gel and subjected to immunoblot analyses with the appropriate antibodies, as described above. The intensity of detected protein for each dilution was quantified using scanning densitometry of the immunoblot, and standard curves were generated for the lipase pair. The mass of all EL proteins, semiquantified as arbitrary units, was determined using an ELISA (16, 17) in the same assay.

Lipase assays

TG lipase and phospholipase assays using the glycerol-stabilized substrates triolein and DPPC, respectively, were performed as described previously (7). LDL, HDL₂, and HDL₃ were isolated by potassium bromide density gradient ultracentrifugation (21). Assays of lipoprotein lipid hydrolysis by recombinant lipases were performed as described previously (15). The free fatty acids generated by the hydrolysis of lipoproteins were measured using a commercial kit (Waco Pure Chemical Industries) according to the manufacturer's instructions. All activity data were corrected for protein mass (determined as described above) and were normalized to the percentage of wild-type lipase.

In vivo studies

Adenoviruses expressing wild-type human EL (Ad.EL) and control adenoviruses without a cDNA insert (Ad.null) were generated previously (1). Adenoviruses expressing EL-N116A (Ad.EL-N116A) were generated from the mutant cDNA using previously described methods (1). Female low density lipoprotein receptor (LDLR)-null mice in a C57BL6/J background (Jackson Laboratories) were maintained on a normal chow diet and a 12 h light/12 h dark cycle. Mice were injected with 3×10^{10} particles of Ad.EL, Ad.EL-N116A, or Ad.null via the tail vein. Before adenovirus injection and at 7 d after injection, blood was collected from the retro-orbital plexus. Postheparin plasma was collected from the retro-orbital plexus at 5 min after tail vein injection of 500 U/kg heparin. Plasma TG, total cholesterol (TC), HDL-C, and phospholipid (PL) were measured using commercially available kits. For separation of lipoproteins, pooled plasma (100 μl) from control, wild-type EL-, and EL-N116A-expressing mice were loaded onto two in-series Superose-6 columns that had been equilibrated with phosphate-buffered saline and fractionated by fast-performance liquid chromatography (FPLC). Five hundred microliter fractions were collected, and TC from each fraction was measured.

Statistical analyses

Where statistical values are provided, the data were analyzed using the paired *t*-test. Error bars indicate ±SD.

RESULTS

Effect of disruption of *N*-linked glycosylation at Asn-116 of EL on the hydrolysis of native lipoproteins *ex vivo* and *in vivo*

We previously reported that EL-N116A abolished *N*-linked glycosylation at Asn-116 of EL (16). Additional EL mutants were generated to disrupt the Asn-Asn-Thr sequence of this glycosylation site. The EL mutations N116T and T118A also had reduced molecular masses of EL, consistent with a loss of glycosylation (Fig. 1A). In contrast, mutagenesis of Asn-117 to Ala (N117A) did not affect glycosylation, which was expected because Asn-117 is not critical to the *N*-linked glycosylation consensus sequence. Consistent with our previous report on EL-N116A, both EL-N116T and EL-T118A had significantly greater specific activities toward triolein and DPPC, whereas EL-N117A was no different than wild-type EL (Fig. 1B).

We tested the catalytic activity of the EL-N116A mutant against native lipoproteins using LDL, HDL₂, and HDL₃ as

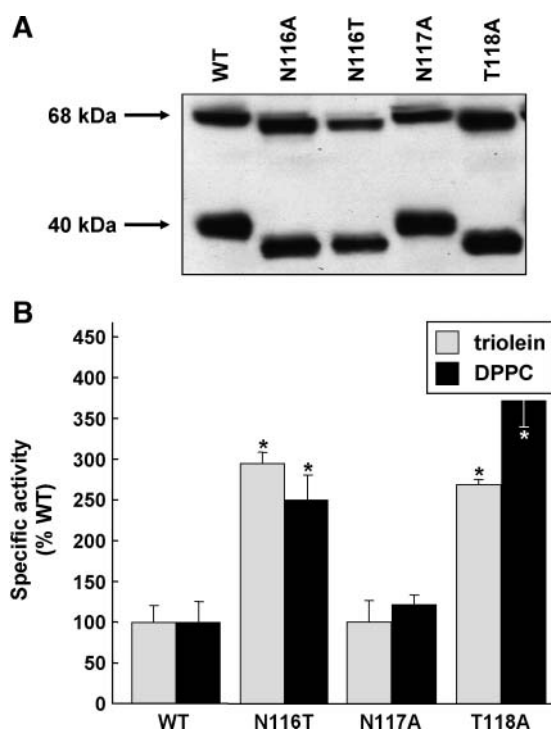


Fig. 1. Transient expression and activities of recombinant endothelial lipase (EL). Wild-type (WT) EL and site-directed mutants of EL affecting the *N*-linked glycosylation site at asparagine-116 (Asn-116) were transiently expressed using 293 cells in the presence of heparin. A: Media samples were collected and EL proteins were visualized by immunoblotting using the anti-human EL antibody. The 68 kDa mature wild-type EL protein and a 40 kDa cleavage product of wild-type EL are indicated. B: Media from cells transiently expressing wild-type EL, EL-N116T, EL-N117A, or EL-T118A in the presence of heparin were collected and assayed for hydrolysis of triolein and dipalmitoylphosphatidyl choline (DPPC). Free fatty acids released were normalized for EL protein and quantified as described in Experimental Procedures. The specific activity data for the EL variants were normalized to 100% of wild-type EL. Assays were performed at least in triplicate. Error bars indicate \pm SD. * $P < 0.001$ versus the wild type.

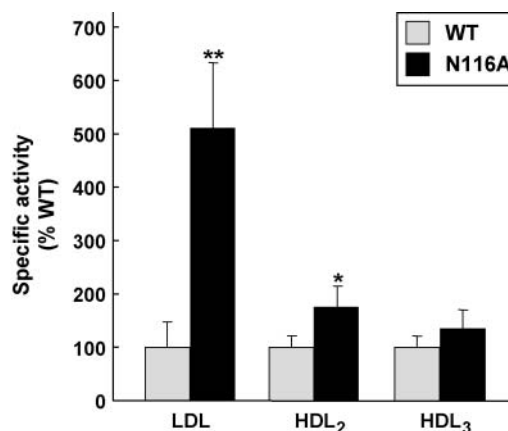


Fig. 2. Activity of recombinant EL toward lipoproteins. Media from cells transiently expressing wild-type (WT) EL or the N116A mutant of EL in the presence of heparin were collected and assayed for lipid hydrolysis using LDL, HDL₂, and HDL₃ as substrates. Free fatty acids were quantified in triplicate from at least triplicate assays, normalized for EL protein, and quantified as described in Experimental Procedures. The specific activity data for the EL variants were normalized to 100% of wild-type EL. Error bars indicate \pm SD. * $P = 0.03$ versus the wild type; ** $P = 0.003$ versus the wild type.

substrates (Fig. 2). The specific hydrolytic activity toward LDL by EL-N116A ($511 \pm 123\%$) was significantly greater versus wild-type EL ($100 \pm 48\%$). A significantly greater increase of specific activity by EL-N116A ($177 \pm 40\%$) versus wild-type EL ($100 \pm 22\%$) was also observed toward HDL₂. However, no significant change of specific activity by EL-N116A ($136 \pm 36\%$) was observed versus wild-type EL ($100 \pm 21\%$) toward HDL₃.

In an attempt to understand the physiological role of *N*-linked glycosylation at Asn-116, we assessed the effects on lipids by wild-type EL and EL-N116A *in vivo* using adenovirus-mediated expression in LDLR-null mice. As shown in Table 1, by day 7 after injection of adenovirus, the TG, PL, TC, HDL-C, and non-HDL-C levels were significantly lower in mice expressing EL-N116A than in mice expressing wild-type EL. The relative mass measured by ELISA of EL-N116A in postheparin plasma (0.92 ± 0.24 arbitrary units) was comparable to that of wild-type EL (1.00 ± 0.42 arbitrary units). No human EL mass was detected in plasma from Ad.null-infected mice. Fractionation of lipoproteins by FPLC from pooled plasma of mice at 7 days after the injection of Ad.EL showed that the decrease of lipid levels in mice expressing wild-type EL was primarily in the HDL fraction (Fig. 3); however, the expression of EL-N116A induced a greater reduction in both the intermediate density lipoprotein/LDL and HDL fractions. Although we were unable to measure changes in the mass of endogenous mouse lipases, our *in vivo* results are consistent with a greater activity by EL-N116A toward lipoprotein lipids *in vitro*.

To ensure that our observation of enhanced activity *in vivo* by EL-N116A was not attributable to altered heparan sulfate proteoglycan association, we tested the cell surface association of wild-type EL and EL-N116A using

TABLE 1. Lipid levels in LDL-receptor-null mice infected with adenoviruses expressing wild-type or N116A EL

Days after infection	Triglyceride (n = 4)	Phospholipid (n = 4)	Total Cholesterol (n = 4)	HDL-C (n = 4)	Non-HDL-C (n = 4)
<i>mg/dl</i>					
Null virus					
Day 0	38.8 ± 4.2	173.8 ± 9.2	166.5 ± 9.7	61.3 ± 4.1	105.3 ± 6.4
Day 7	41.3 ± 5.5	208.0 ± 7.8	193.0 ± 13.3	67.3 ± 2.2	125.8 ± 12.3
Wild-type EL					
Day 0	53.5 ± 14.2	188.8 ± 8.1	177.8 ± 8.9	66.5 ± 2.9	111.3 ± 8.0
Day 7	45.8 ± 13.7	118.0 ± 27.2 ^a	112.0 ± 22.5 ^a	30.3 ± 9.7 ^a	81.8 ± 13.8 ^a
N116A EL					
Day 0	53.8 ± 21.8	205.5 ± 24.4	190.0 ± 27.1	71.3 ± 6.9	118.8 ± 21.2
Day 7	20.8 ± 9.9 ^b	41.8 ± 15.3 ^b	46.0 ± 11.6 ^b	8.3 ± 1.9 ^b	37.8 ± 9.7 ^b

EL, endothelial lipase; HDL-C, high density lipoprotein cholesterol.

^a *P* < 0.005 versus wild-type day 0.

^b *P* < 0.03 versus N116A day 0 and wild-type day 7.

transiently transfected 293 cells (Fig. 4). Immunoblot analyses show that the EL released into media, in the absence versus the presence of heparin during a 16 h incubation period, was comparable for both wild-type EL and EL-N116A (Fig. 4A). The release of uncleaved EL-N116A into heparin-free media was 11% (calculated from densitometry data of immunoblots from triplicate transfections), which was comparable to the 15% release of uncleaved (68 kDa) wild-type EL (Fig. 4B).

Introduction of the Asn-116 *N*-linked glycosylation site in LPL and HL and effect on lipolytic activity

The Asn-116 residue that is glycosylated in EL is not conserved in either LPL or HL. However, when the corresponding residues in LPL (Gly-99) and HL (Arg-113) are

mutagenized into Asn, putative *N*-linked glycosylation sites are generated. We hypothesized that introduction of *N*-linked glycosylation at the corresponding sites in LPL and HL would reduce catalytic activity. To test this hypothesis, we mutated Gly-99 to Asn in LPL (LPL-G99N) and Arg-113 to Asn in HL (HL-R113N). The expression of LPL-G99N in 293 cells resulted in increased molecular mass (Fig. 5A), consistent with newly introduced *N*-linked glycosylation. In contrast, mutagenesis of Gly-99 to Ala (LPL-G99A) did not affect the molecular mass of LPL, as expected. The

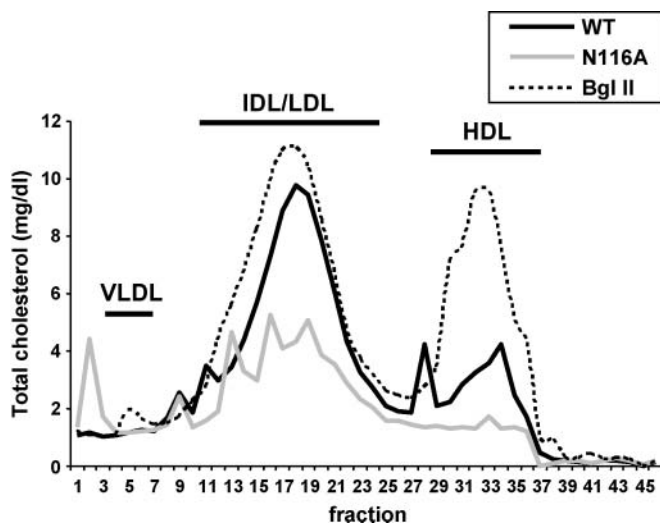


Fig. 3. Plasma lipoprotein profiles from wild-type (WT) EL or EL-N116A-expressing mice. Blood was collected from low density lipoprotein receptor-null mice at 7 days after infection with adenovirus (3×10^{10} particles) encoding wild-type EL, EL-N116A, or null virus (BglII). Plasma samples were pooled from four mice (total of 100 μ l) and fractionated (500 μ l of each fraction) by fast-performance liquid chromatography. Total cholesterol in each fraction was quantified. IDL/LDL, intermediate and low density lipoproteins.

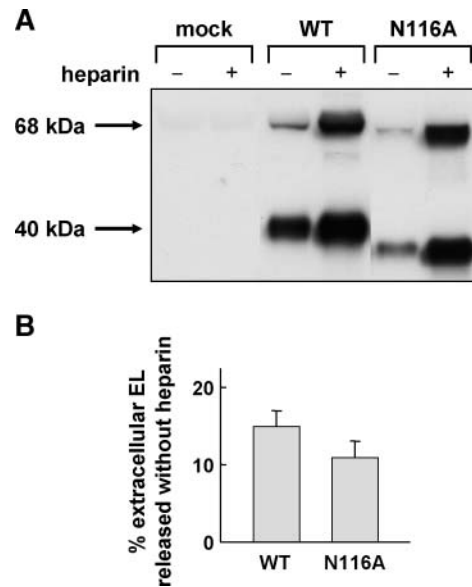


Fig. 4. Analysis of EL secretion in the absence or presence of heparin. Wild-type (WT) EL and EL-N116A were transiently expressed using 293 cells. A: Wild-type EL- and EL-N116A-expressing cells were incubated with or without 100 U/ml heparin for 16 h. Media EL was analyzed by immunoblot analysis using the anti-human EL antibody. Mock, mock-transfected media. B: The intensities of uncleaved media EL protein secreted in the absence or presence of heparin from triplicate transfections were quantified by scanning densitometry of the immunoblots. Data are presented as total extracellular EL released into heparin-free medium as a percentage of that released into medium containing heparin. Error bars indicate \pm SD.

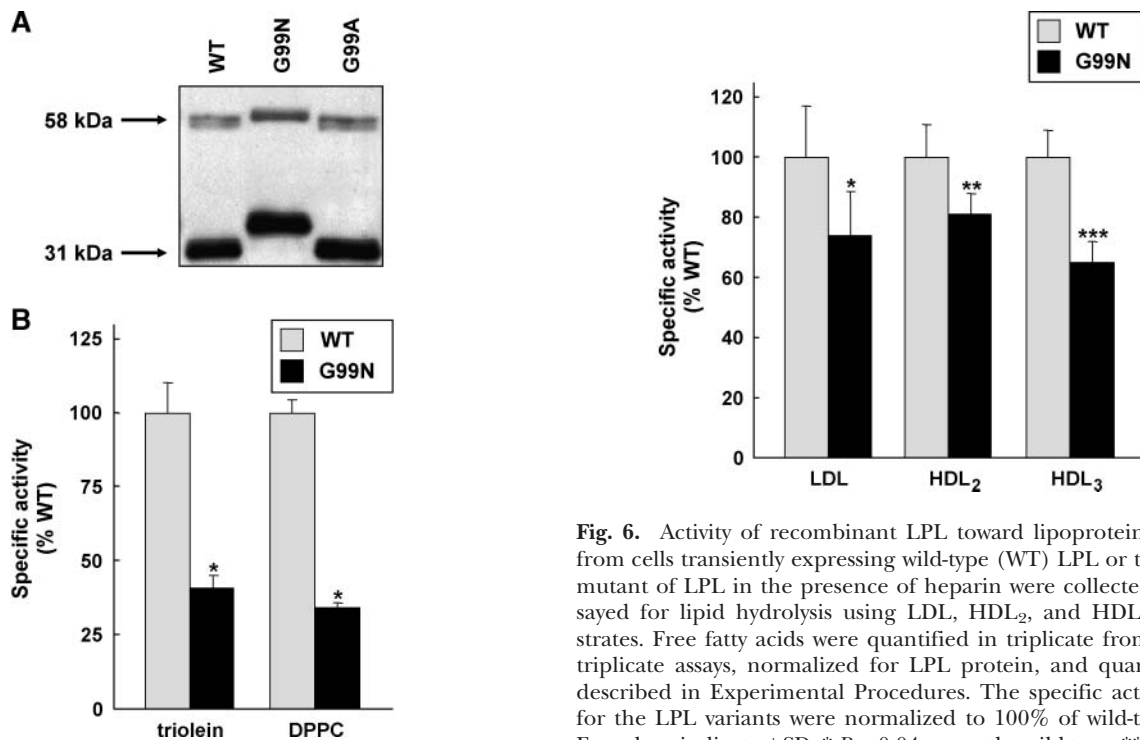


Fig. 5. Transient expression and activities of recombinant LPL. Wild-type (WT) LPL and site-directed mutants of LPL introducing either an *N*-linked glycosylation site at glycine-99 (G99N) or alanine (G99A) were transiently expressed using 293 cells in the presence of heparin. A: Media samples were collected and LPL proteins were visualized by immunoblotting using the 5D2 antibody. The 58 kDa mature wild-type LPL protein and a 31 kDa cleavage product of wild-type LPL are indicated. B: Media from cells transiently expressing wild-type LPL or LPL-G99N in the presence of heparin were collected and assayed for hydrolysis of triolein and DPPC. Free fatty acids released were normalized for LPL protein and quantified as described in Experimental Procedures. The specific activity data for the LPL variants were normalized to 100% of wild-type LPL. Assays were performed at least in triplicate. Error bars indicate \pm SD. * $P < 0.001$ versus the wild type.

specific activity of LPL-G99N toward triolein ($38 \pm 4\%$) and DPPC ($34 \pm 1\%$) was reduced significantly compared with that of wild-type LPL (Fig. 5B). Using LDL, HDL₂, and HDL₃ as substrates, LPL-G99N exhibited significantly decreased hydrolytic activities toward each lipoprotein (Fig. 6). These data show that the addition of *N*-linked glycosylation to amino acid residue 99 of LPL indeed reduced catalytic activity.

The expression of HL-R113N in 293 cells also resulted in increased molecular mass (Fig. 7A), consistent with newly introduced *N*-linked glycosylation. In contrast, mutagenesis of Arg-113 to Lys (HL-R113K) did not affect the molecular mass of HL, as expected. The specific activity of HL-R113N toward triolein ($83 \pm 6\%$) was slightly but significantly reduced compared with that of wild-type HL, but the specific activity of HL-R113N toward DPPC ($111 \pm 4\%$) was not significantly different from that of wild-type HL (Fig. 7B). Using LDL, HDL₂, and HDL₃ as substrates, HL-R113N exhibited a small but significant

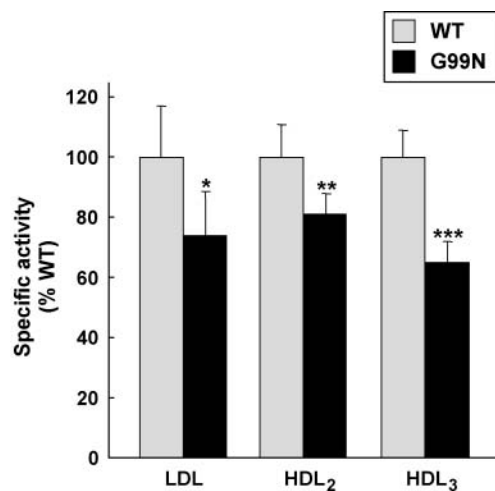


Fig. 6. Activity of recombinant LPL toward lipoproteins. Media from cells transiently expressing wild-type (WT) LPL or the G99N mutant of LPL in the presence of heparin were collected and assayed for lipid hydrolysis using LDL, HDL₂, and HDL₃ as substrates. Free fatty acids were quantified in triplicate from at least triplicate assays, normalized for LPL protein, and quantified as described in Experimental Procedures. The specific activity data for the LPL variants were normalized to 100% of wild-type LPL. Error bars indicate \pm SD. * $P = 0.04$ versus the wild type; ** $P = 0.01$ versus the wild type; *** $P = 0.001$ versus the wild type.

decrease of hydrolytic activity toward LDL, but not toward HDL₂ and HDL₃ (Fig. 8). These data suggest that unlike LPL, the addition of *N*-linked glycosylation to amino acid residue 113 of HL had relatively minor effects on catalytic activity.

DISCUSSION

This study extends our previously reported data suggesting that the abolishment of *N*-linked glycosylation at Asn-116 in human EL by site-directed mutagenesis (generating and expressing the N116A mutant) led to enhanced enzymatic activity (16). These results show that additional mutagenesis to the *N*-linked glycosylation recognition sequence (generating and expressing the N116T and T118A mutants) also abolishes the *N*-linked glycosylation at Asn-116 and results in up to a 3-fold increase in both triolein and DPPC hydrolysis. We also show that the association of wild-type EL and EL-N116A to cell surfaces in the absence or presence of heparin is comparable. These data confirm that it is specifically the loss of glycosylation at Asn-116 (and not some other structural effect of the N116A mutant) that results in the enhanced catalytic activity of EL. We further demonstrate that loss of the carbohydrate at Asn-116 results in increased activity of EL toward LDL and HDL₂ *ex vivo* as well as a greater reduction of LDL and HDL with expression *in vivo*. Finally, we mutated the homologous amino acid residues in LPL and HL (LPL Gly-99 and HL Arg-113) to Asn, introducing a utilized *N*-linked glycosylation site in both enzymes. The addition of this site reduced the hydrolytic

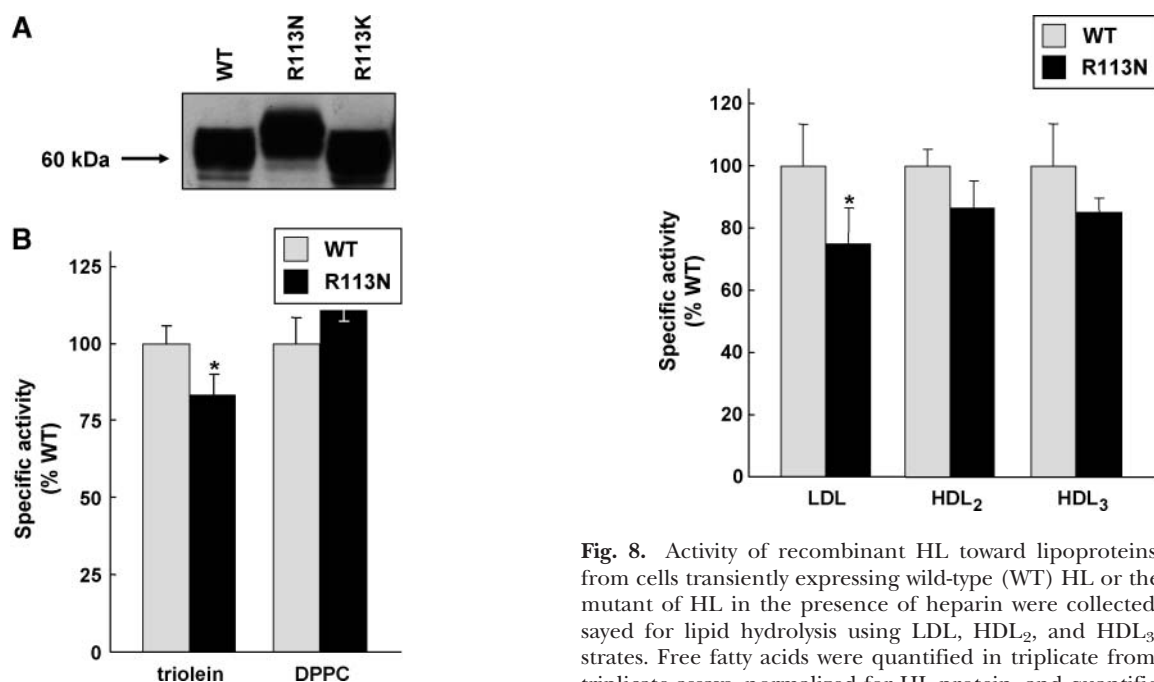


Fig. 7. Transient expression and activities of recombinant HL. Wild-type (WT) HL and site-directed mutants of HL introducing either an *N*-linked glycosylation site at arginine-113 (R113N) or lysine (R113K) were transiently expressed using 293 cells in the presence of heparin. **A:** Media samples were collected and HL proteins were visualized by immunoblotting using the XHL3-6 antibody. The 60 kDa mature wild-type HL protein is indicated. **B:** Media from cells transiently expressing wild-type HL or HL-R113N in the presence of heparin were collected and assayed for hydrolysis of triolein and DPPC. Free fatty acids released were normalized for HL protein and quantified as described in Experimental Procedures. The specific activity data for the HL variants were normalized to 100% of wild-type HL. Assays were performed at least in triplicate. Error bars indicate \pm SD. * $P = 0.04$ versus the wild type.

activity of LPL but had little effect on the catalytic activity of HL.

The suppression of biological activities by *N*-linked glycosylation has been reported for other proteins, including LCAT (22), the human immunodeficiency virus-1 envelope protein gp120 (23), influenza virus hemagglutinin (24), human protein C (25), and human granulocyte-macrophage colony-stimulating factor (26). For example, the abolishment of *N*-linked glycosylation at Asn-384 of LCAT enhanced its catalytic activity by 2-fold, and it was proposed that glycosylation at this site sterically hindered the access of substrate to the catalytic site (22). The enhanced lipolytic activity with the loss of *N*-linked glycosylation at Asn-116 of EL, and the enhanced activity of wild-type LPL versus the G99N mutant of LPL, may be attributable in part to the absence of a carbohydrate chain sterically hindering the access of substrates to the catalytic triad of these lipases.

Unlike for EL and LPL, *N*-linked glycosylation introduced at residue 113 of HL had little effect on hydrolytic activity toward synthetic substrates or lipoproteins. The lack of difference versus wild-type HL suggests that the

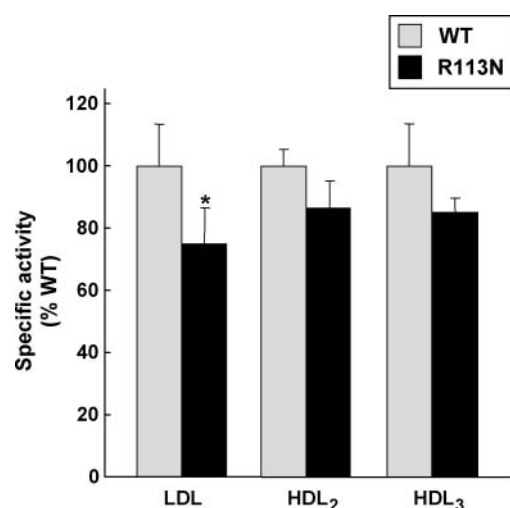


Fig. 8. Activity of recombinant HL toward lipoproteins. Media from cells transiently expressing wild-type (WT) HL or the R113N mutant of HL in the presence of heparin were collected and assayed for lipid hydrolysis using LDL, HDL₂, and HDL₃ as substrates. Free fatty acids were quantified in triplicate from at least triplicate assays, normalized for HL protein, and quantified as described in Experimental Procedures. The specific activity data for the HL variants were normalized to 100% of wild-type HL. Error bars indicate \pm SD. * $P = 0.02$ versus the wild type.

tertiary structure of HL in the region of Arg-113 is different from the analogous regions of both EL and LPL. This would not be a surprise, because phylogenetic analyses of the lipase superfamily suggest that HL has a greater divergence from both EL and LPL than EL and LPL have from each other (27, 28). It is interesting that the carbohydrate at Asn-116 of EL appears to inhibit activity toward LDL and HDL₂ but not toward HDL₃. Thus, the presence of *N*-linked glycosylation at Asn-116 of EL may have evolved in its divergence from LPL, in part as a means to direct greater specificity of EL *in vivo* away from apolipoprotein B-containing lipoproteins and toward HDL. EL activity on HDL has been shown to have several effects, including enhanced adenosine triphosphate binding cassette transporter A1-mediated cholesterol efflux capacity of plasma (29), increased cholesteryl ester selective uptake (30), increased peroxisome proliferator-activated receptor α signaling in endothelial cells (31), and altered macrophage inflammatory responses (32). Of note, HL also acts on HDL but prefers larger TG-rich HDL₂ particles (33). Thus, *N*-linked glycosylation may be another structural feature, along with the sequence of the lid domain (15) and the C-terminus (34), that contributes to the type of lipoproteins these enzymes preferentially hydrolyze.

Roles for the *N*-linked glycosylation sites at Asn-60 and Asn-373 of EL have been established, such that these sites must be glycosylated for proper secretion by cells and for specific activity, respectively (16). These two sites are the only conserved *N*-linked glycosylation sites between LPL (Asn-43 and Asn-359) and HL (Asn-56 and Asn-375). The Asn-43 of LPL and the Asn-56 of HL need to be glycosylated for secretion and also proper specific activity,

whereas mutagenesis of Asn-359 of LPL had no effect on secretion or activity but mutagenesis of Asn-375 of HL caused a substantially reduced secretion from cells (35–39). We propose that the role of *N*-linked glycosylation at Asn-116 of EL is to limit the hydrolysis of lipids preferentially to those in smaller HDL particles; however, we cannot rule out the possibility that the glycan chain possesses other functions. A wide variety of roles are supported by *N*-linked glycosylation. In addition to the regulation of catalytic activity as demonstrated in the current study, other roles include intracellular protein folding and sorting (40), cell signaling (41), protein stability (42), and regulation of protein half-life in vivo (43). Although not addressed in this study, the biochemical and physiological roles of *N*-linked glycosylation sites within EL (Asn-471), LPL (Asn-359), and HL (Asn-20 and Asn-340), where mutations of the respective Asn residues do not substantially impair catalytic activity or secretion from cells, remain to be elucidated. These roles may be identified in extensive future cell biology and in vivo studies.

In summary, we have proven that the removal of *N*-linked glycosylation at Asn-116 in human EL results in a substantial increase in specific activity against both synthetic substrates (triolein and DPPC) and native lipoprotein substrates (LDL and HDL₂) ex vivo and in vivo. This glycosylation site, which is not conserved in LPL or HL, may direct EL activity to a greater extent toward smaller HDL₃ lipoprotein particles. **■**

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