Functional role of N-linked glycosylation in human hepatic lipase: asparagine-56 is important for both enzyme activity and secretion

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Abstract Hepatic lipase (HL) and lipoprotein lipase (LPL) are evolutionarily related enzymes that are essential for normal lipoprotein metabolism. While much has been published on the structure-function relationship of LPL, little is known concerning the structural basis of HL action and secretion. Human HL is a glycoprotein and its predicted amino acid sequence contains four putative N-linked glycosylation sites at Asn residues 20, 56, 340, and 375. We studied the role of these residues in the secretion and catalytic activity of hHL by analysis of hHL expressed in stable CHO cell lines. Using site-specific mutagenesis, the wild-type human HL and substitution mutants of each of the four Asn residues were expressed in vitro. The relative sizes of these site-specific mutants indicate that all four putative sites are utilized for glycosylation in CHO cells. Abolition of N-linked glycosylation of three (residues 20, 340, and 375) of the four sites did not affect enzyme secretion or activity. Mutations of Asn-56 to either Gln or Ala resulted in the production of a totally inactive HL which accumulated intracellularly but was not secreted into the culture medium. Therefore, Asn-56 is required for both HL enzyme activity and secretion. The fact that the homologous N-linked glycosylation site (Asn-43) is required for both enzyme activity and secretion for human LPL (Semenkovich et al. 1990. *J. Biol. Chem.* **265:** 5429–5433) indicates that carbohydrate chains at this site are essential for the active conformation and correct folding for secretion of these evolutionarily related lipases. Our observations provide insight into the structural basis of lipase action and secretion.—**Wölle, J., H. Jansen, L. C. Smith, and L. Chan.** Functional role of N-linked glycosylation in human hepatic lipase: asparagine-56 is important for both enzyme activity and secretion. *J. Lipid Res.* 1993. **34:** 2169–2176.

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Hepatic lipase (HL) catalyzes the hydrolysis of mono-, di-, and triacylglycerol, acyl-CoA thioesters and phospholipids (1–3). It is produced mainly, if not exclusively, in the liver (4–6), although HL activity has also been detected in the adrenal glands. The enzyme has been postulated to be involved in the hydrolysis of IDL triacylglycerol to produce LDL, and that of HDL_2 triacylglycerol and phospholipid to produce HDL_3 (7, 8). HL may be essential for the hepatic uptake of HDL triacylglycerol and cholesteryl esters by the liver (9–11). Therefore, it plays a major role in HDL metabolism, and HL activity is inversely correlated with plasma HDL levels (12).

The primary structures of HL in humans (13–15), rat (16), mouse (17, 18), and rabbit (19) have been deduced from their corresponding cDNA sequences. HL shows high homology to lipoprotein lipase (LPL) and pancreatic lipase, and the three lipolytic enzymes are part of a lipase superfamily of genes (14, 20, 21).

Both HL and LPL are glycoproteins. Two putative N-linked glycosylation sites are conserved in these two lipases in mammalian species (**Fig. 1**). Carbohydrate seems to be required for the production of functional HL and LPL (22–24). Tunicamycin treatment markedly reduces the production of active HL from rat hepatocytes (25) and LPL from rat preadipocytes (26), ob/ob adipocytes (27), or 3T3-L1 adipocytes (28). Using in vitro expression of site-specific human LPL mutants in COS cells, Semenkovich et al. (29) found that the N-terminally situated glycosylation site (Asn-43) is essential for both enzyme activity and secretion. In contrast, using site-specific mutants involving the two N-linked glycosylation sites in rat HL expressed in *Xenopus laevis* oocytes, Stahnke et al. (30) found that a mutant lacking the homologous N-linked glycosylation site (Asn-57) was 2.7-times more active than wild-type although it was poorly secreted into the medium. Their experiments suggest a fundamental difference in the structural role of carbohydrates between LPL and HL, a somewhat surprising finding in view of

Abbreviations: HL, hepatic lipase; LPL, lipoprotein lipase; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

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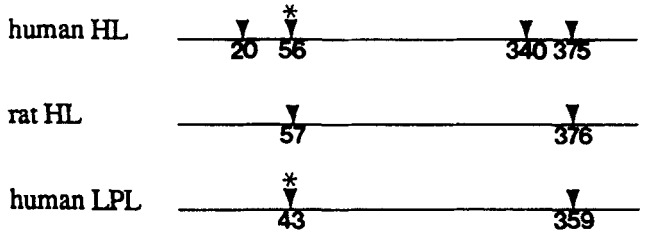

Fig. 1. Alignment of human and rat hepatic lipase and human lipoprotein lipase. A schematic representation of the asparagine-linked glycosylation sites of the three lipases reveals two conserved sites. Asn residues found to be essential for the expression of human HL and LPL are denoted by asterisks.

the high degree of structural and functional similarities between the two enzymes. In this communication, we have examined this apparent difference between LPL and HL by expressing site-specific human HL mutants in stable transformants of CHO cells. Unlike rat HL, human HL contains four N-linked glycosylation sites including the two that are also conserved in rat HL and other vertebrate LPLs. We found that for the human enzyme expressed in mammalian cells, abolition of N-linked glycosylation of three of the four sites did not affect enzyme secretion or activity. Mutations of Asn-56 (which is homologous to Asn-43 in LPL), however, led to the production of a totally inactive enzyme that is not secreted into the medium. Our findings strongly support the essential role of this conserved N-linked glycosylation site in both HL and LPL production and function, and have important implications for the structure-function relationships of the two vascular lipases.

MATERIALS AND METHODS

Human HL cDNA expression vector

A full-length human HL cDNA clone of 1553 nucleotides plus poly(A) tail (15) was constructed from two overlapping cDNA fragments (14), ligated into the *Eco*RI site of M13mp19 and used as a template for site-specific mutagenesis.

Oligonucleotides synthesized on an Applied Biosystems, Inc. 380 DNA Synthesizer were 5'-phosphorylated and annealed with uracil-containing single-stranded template. Mutagenesis was carried out as described by Kunkel (31), using a dut$^-$ ung$^-$ host (*Escherichia coli* CJ 236). After transfection of *E. coli* TG1 cells with the mutant and wild-type hHL cDNA end products, positive clones were identified by direct sequencing (32). For eukaryotic expression, replicative-form DNAs were isolated, digested with *Eco*RI, and inserted into the *Eco*RI site of pEE14 (33, 34). After transformation of *E. coli* JM 109 cells, positive clones were isolated, and orientation of inserts was determined by restriction mapping. All constructs were sequenced again after subcloning into the expression vector pEE14.

In vitro expression and HL enzyme assay

The expression constructs (15–25 μg) were transfected into CHO-K1 cells (1.0 × 10^6/10-cm dish) by the calcium phosphate precipitation method with glycerol shock using a transfection kit from BRL. For each transfection experiment, parallel dishes were subjected to the transfection protocol without plasmid DNA (CHO cells-only condition) and with the vector DNA only. CHO-K1 cells were grown in 10 ml of 10% dialyzed FBS and the glutamine-free medium GMEM-S (Gibco Laboratories, Grand Island, NY) (35, 36). Transfected cells were then selected at 25 μM methionine sulfoximine (Sigma Chemical Co., St. Louis, MO). Transformants from each transfection were picked after about 14 days, seeded in 24-well cell culture plates (Costar Corporation, Cambridge, MA), and grown to confluency. One day prior to the HL enzyme assay, the media were changed to fresh GMEM-S containing 10% FBS, 25 μM methionine sulfoximine, and 40 μg/ml sodium heparin (Sigma). Twenty to 50 clones were usually screened for each expression construct by first assaying HL activity in the media. Lipolytic activity was determined in a similar way as described by Ikeda, Takagi, and Yamamoto (37) using a tri [9,10-^{3}H]olein–gum arabic emulsion as a substrate. Briefly, the final reaction mixture contained 0.2 M Tris-HCl (pH 8.5), 2.5% BSA, 0.75% gum arabic, 0.25 μM of tri [9,10-^{3}H]olein (sp act 1 μCi/μmol), 1 M NaCl, and the sample solution; the final volume was 0.4 ml. The reaction was started by the addition of 100 μl of supernatant or cell extract and then the mixture was incubated for 60 min at 37°C. The released [9,10-^{3}H]oleate was determined by the liquid-liquid partitioning system (38). HL activity is expressed in milliunits (1 milliunit = 1 nmol of fatty acid released/min). For most of the expression constructs, positive clones with the highest HL activity in the media were selected and subsequently transferred from the 24-well plates to T175 tissue culture flasks. Constructs that consistently had no activity in the media were assayed for intracellular activity and mass.

Western blot analysis of HL produced in vitro

Western blot analysis was performed after partial purification through heparin–Sepharose columns (1 ml HiTrap affinity columns from Pharmacia). Different amounts of protein were loaded and separated on a 10% SDS polyacrylamide gel. After transfer of proteins onto nitrocellulose (Schleicher & Schuell), hHL was detected by two polyclonal anti-hHL antibodies. Control experiments showed that these antibodies gave identical signals for the same amount of hHL loaded on the gel. Specificity for hHL was also confirmed by competition analysis. For detection of extracellular protein, a rabbit anti-hHL anti-

body raised against a 13-amino acid long peptide in the N-terminal region of hHL was used in a 1:100 ratio. For detection of intracellular protein, a goat anti-hHL antibody was used in a 1:3000 ratio. This goat antibody gave a lower background for detection of the intracellular hHL than the rabbit anti-peptide antibody. A second rabbit anti-goat antibody (1:3000 ratio) from Pierce was used before the immunoblot alkaline phosphatase assay system (Bio-Rad Laboratories, Inc.) was applied according to the manufacturer's manual. The goat anti-hHL antibody was also used for competition studies of in vitro produced HL. Cold hHL partially purified from human postheparin plasma was added in 200-fold excess to a 1:3000 dilution of goat anti-hHL antibody in a final volume of 4 ml. After 1 h incubation shaking at 4°C, immunoblots were performed as described before.

Developed membranes were photographed immediately and intensity of bands was analyzed by a Model 620 Video Densitometer (Bio-Rad). Specific activities for each construct were calculated by dividing HL enzyme activities by protein mass expressed as arbitrary densitometer units.

RESULTS AND DISCUSSION

To study the structure–function relationship of hHL, we have expressed the wild-type and mutant enzyme in a mammalian expression system. Initially, we used the same strategy that was successful for LPL (29) and expressed hHL in COS cells using the vector p91023(B). Unfortunately, the level of expression was relatively low and the system was not suitable for routine assessment of HL activity (data not shown). We have therefore resorted to a stable expression system in Chinese hamster ovary (CHO) cells. The vector used is pEE14 which is under the control of hCMV 5' sequences and SV40 3' sequences, and contains the glutamine synthetase selectable marker gene (33, 34) (**Fig. 2**). We accomplished high level expression of wild-type and mutant hHL in this system.

The major question being addressed in this study is the role of N-linked glycosylation in HL enzyme secretion and activity. We have therefore expressed the wild-type enzyme as well as site-specific mutants of the four putative N-linked glycosylation sites (Fig. 1), Asn-20, 56, 340, and 375. We assayed for enzyme activity both in the culture medium (secreted) as well as in cellular extracts (intracellular). The enzyme activities of the wild-type and active hHL mutants differed somewhat from experiment to experiment (compare Experiments I and II, **Fig. 3**). However, there is consistently high HL activity in the media and easily detectable activity in the cellular extracts in the stable CHO cell lines expressing wild-type hHL and substitution mutants that individually involve three of the four putative N-linked glycosylation sites. Two

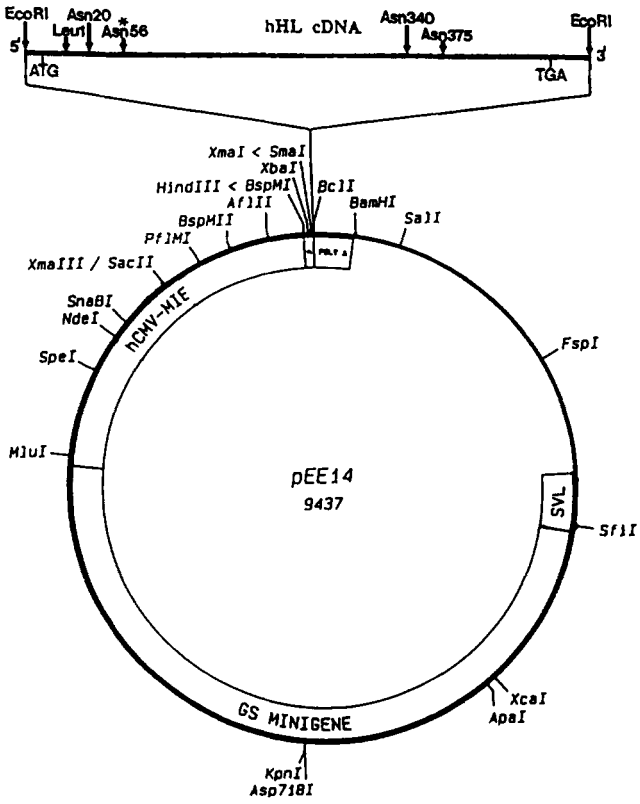

Fig. 2. Structure of human HL expression vector. A full length human HL cDNA clone was inserted at the unique *Eco*RI site in pEE14 and is drawn to scale. Asn residues selected for oligonucleotide-directed site-specific mutagenesis are shown above the HL cDNA. The oligonucleotides used for mutagenesis are: Asn-20/Gln, GCTGTTGAAACACAAAAAACGCTGCAT; Asn-56/Ala, GAGTGCGGCTTCGCCTTCTCCCTGCCT; Asn-56/Gln, GAGTGCGGCTTCCAATTCTCCCTGCCT; Asn-340/Gln, ATCCAGTTCATCCAACAAACTGAGACG; Asn-375/Gln, GGAATTGCTAGTCAAAAAAACGTATTCC. Asn-56, found to be important for both activity and secretion of hHL in this study, is denoted by an asterisk. hCMV-MIE, human cytomegalovirus major immediate early promotor; GS Minigene, glutamine synthetase minigene.

different substitution mutants involving Asn-56 were both devoid of HL activity whether the assay was performed on the media or cellular extracts (Fig. 3). By Northern blot analysis, hHL mRNA was detected in these Asn-56 mutants (data not shown). Therefore, the absence of HL activity in the Asn-56 mutants could be caused by the failure of production of the mutant enzymes at the translational level or by the production of catalytically inactive enzymes by these cell lines.

Because of the variability in the total hHL activities in different experiments, we assayed for the amount of immunoreactive hHL produced by each cell line by Western blot analysis. We used this method because we could study simultaneously the amount and relative size of the hHL produced in vitro. The loading of increasing amounts of intracellular or extracellular hHL produced by the CHO cells gave rise to a linear plot by densitometer quantitation (data not shown). **Fig. 4** is a representative

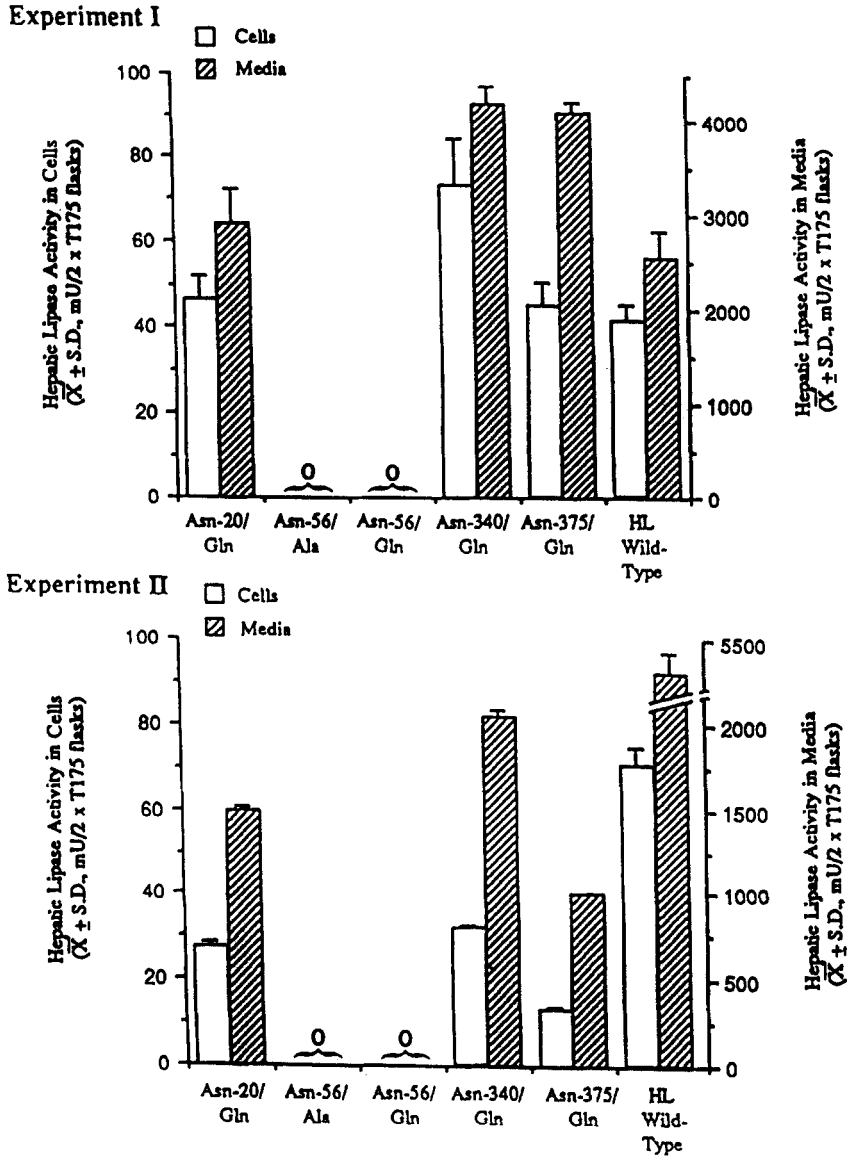

Fig. 3. Total HL activities in CHO cell lines stably transfected with wild-type and mutant hHL constructs. CHO-K1 cells were transfected with wild-type and mutant hHL expression vectors. After selection of positive clones in 24-well culture plates, cells were seeded in two T175 flasks. One day prior to the HL assay, cells were fed fresh media containing 40 μg/ml heparin. Total activities from wild-type and mutant HL CHO cells of two independent experiments were obtained after combining media and cells from two confluent T175 flasks. Values are presented as means ± SD.

Western blot on wild-type and mutant hHLs secreted into the media by the CHO cells. Two interesting features of this blot are evident: *i)* the three mutant hHL proteins that were secreted into the culture medium (Asn-20→Gln, Asn-340→Gln, and Asn-375→Gln) were all smaller than the wild-type hHL protein, suggesting that these sites were actually glycosylated in the wild-type enzyme, and *ii)* cell lines transfected with the two Asn-56 substitution mutants (lane 4, mutated to Gln, and lane 5, mutated to Ala) produced no detectable immunoreactive hHL in the medium.

To ensure whether immunoreactive hHL proteins were produced intracellularly in these stable cell lines, we performed a Western blot analysis on the cellular extracts from each line (**Fig. 5**). Immunoreactive hHL was clearly present in the wild-type cell line as well as in all four N-linked glycosylation site mutants including the Asn-56→Gln (lane 4) and Asn-56→Ala (lane 5) mutants which failed to secrete immunoreactive hHL protein in the media.

Using the relative hHL protein concentrations deduced from the Western blot analysis and the enzyme activity data, we calculated the specific activities of wild-type and

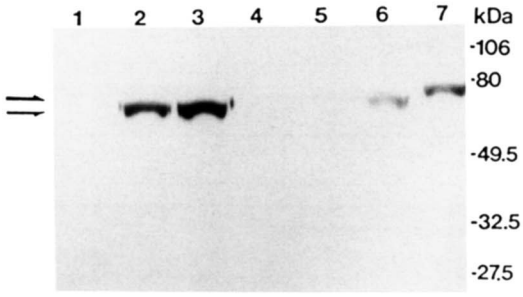

Fig. 4. Western blot analysis of hepatic lipase secreted into tissue culture media. Stable transfected CHO cells from two T175 flasks were grown to confluency. One day before HL purification, cells were fed fresh media containing 40 μg/ml heparin. After partially purifying hepatic lipase from wild-type and mutant transfected CHO cells, Western blot analysis was performed as described under Materials and Methods. Lane 1, CHO cells transfected with pEE14 only (no hHL DNA insert); lane 2, CHO cells transfected with hHL construct containing Asn-375/Gln mutation; lane 3, Asn-340/Gln mutation; lane 4, Asn-56/Gln mutation; lane 5, Asn-56/Ala mutation; lane 6, Asn-20/Gln mutation; lane 7, wild-type hHL expression vector. This blot has been repeated with wild-type hHL run in other lanes indicating that the apparent difference in molecular weight was real and not an "edge artifact."

mutant hHLs produced by the CHO cells (**Table 1**). Although there was substantial difference in the amount of hHL produced by different cell lines in Experiments I and II, the specific activities were very similar. The specific activities of wild-type hHL and the three mutant hHLs that were secreted into the media differed by less than 25%. The specific activities of the intracellular enzymes were low, being 1.5–2.5% that of the secreted enzymes except for the Asn-56 mutants which were totally inactive. Furthermore, the specific activity of the intracellular form of Asn-375→Gln mutant was approximately 55–75% that of the other active intracellular forms in both experiments. In every case (except for Asn-56 mutants), the secreted form of hHL was consistently much more active than the intracellular form. These results are consistent with the interpretation that maturation of the carbohydrate chains is required for maximal HL enzyme activity (23).

The role of N-linked glycosylation in HL secretion was also assessed by the quantitation of hHL mass in the intracellular and extracellular compartments. In the wild-type enzyme and all the active Asn mutants, ~60% of the enzyme was found extracellularly and ~40% intracellularly, indicating that proper glycosylation of Asn-20, Asn-340, or Asn-375 was not required for efficient enzyme secretion. As both the Asn-56→Ala and Asn-56→Gln mutants were detected intracellularly but were totally undetectable in the media, we conclude that glycosylation of Asn-56 is required for hHL secretion.

Our observation that Asn-56 is essential for both enzyme activity and secretion is at variance with that of Stahnke et al. (30) who concluded that in rat HL, Asn-57 (which is the homolog of Asn-56 in hHL) is not required for HL activity. In fact, in their hands, the Asn-57→Gln mutant was superactive with a "specific activity" 274% that of wild-type. The reason for this discrepancy is unclear. Although possible, it is unlikely that the rat and human HLs differ drastically in their structure–function relationship since the two enzymes show high homology. The totally different techniques used in the two studies could explain, at least in part, the divergent results. In the previous study, rat HL was expressed in *Xenopus* oocytes (30). Very small amounts of HL activity were expressed in the oocytes, about 4000-fold less than that in our study (for the wild-type HL, 0.56 mU/20 oocytes in the previous study vs. 2548 ± 292 mU/2 T175 flasks in the current study). Furthermore, the total immunoreactive rat HL mass was not measured in the previous study. Instead, the amount of [^{35}S]methionine incorporated into immunoprecipitable HL, rather than total mass, was used to calculate relative specific activity. As the immunoprecipitable radioactivity does not bear a direct relationship to HL mass, the calculated "specific activity" is not a true specific activity in the usual sense. The use of a different method in the estimation of enzyme activity and the extremely small amount of HL activity produced by the *Xenopus* oocyte may account for the apparent 3-fold higher activity of the Asn-57→Gln rat HL mutant (compared to wild-type) in the previous study.

In contrast to the previous results obtained for rat HL, our findings are consistent with the observations for hu-

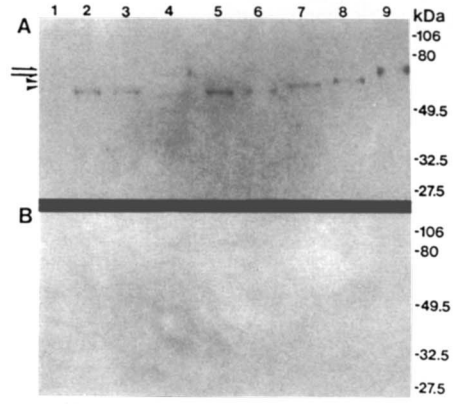

Fig. 5. Western blot analysis of intracellular HL produced in CHO cells. A: After processing of tissue culture supernatants as described in the legend of Fig. 4, transfected CHO cells from two T175 flasks were subjected to the partial HL purification procedure and Western blot analysis as described under Materials and Methods. Lane 1, CHO cells transfected with pEE14 only (no hHL cDNA insert); lane 2, construct containing Asn-375/Gln mutation; lane 3, Asn-340/Gln mutation; lane 4, Asn-56/Gln mutation; lane 5, Asn-56/Ala mutation; lane 6, Asn-20/Gln mutation; lane 7, wild-type hHL expression vector; lane 8, extracellular purified protein from CHO cells transfected with the Asn-20/Gln mutation; lane 9, extracellular purified protein from wild-type transfected CHO cells. This blot has been repeated with wild-type hHL run in other lanes indicating that the apparent difference in molecular weight was real and not an "edge artifact." B: The Western blot was performed in the presence of excess purified human HL.

TABLE 1. Hepatic lipase (HL) enzyme specific activities and distribution of immunoreactive HL mass

Expression Construct	Specific Activity				Distribution of HL Mass (%)			
	Experiment I		Experiment II		Experiment I		Experiment II	
	Cell	Media	Cell	Media	Cell	Media	Cell	Media
Asn-20/Gln	62 ± 8.2	2465 ± 150[a]	65 ± 8.1[b]	2721 ± 106[a]	39.0	61.0	43.5	56.5
Asn-56/Ala	0	0	0	0	100	0	100	0
Asn-56/Gln	0	0	0	0	100	0	100	0
Asn-340/Gln	69 ± 7.2[b]	2645 ± 82[a]	67 ± 4.1[a]	2921 ± 182[a]	40.1	59.9	40.2	59.8
Asn-375/Gln	37 ± 6.3[b]	2634 ± 155[a]	38 ± 4.6[a]	2418 ± 104[b]	44.0	56.0	45.5	54.5
HL wild-type	53 ± 6.3	2008 ± 115	51 ± 1.4	2223 ± 70	38.0	62.0	36.9	63.1

CHO cells were stable transfected with 20 μg of DNA/dish as described under Materials and Methods. HL protein was partially purified for each construct from two T175 flasks by chromatography on a heparin-Sepharose column. Specific activities and distribution of HL mass of two independent experiments are presented. Values are given as means ± SD.
[a] $P < 0.01$, [b] $P < 0.05$, compared to wild-type construct.

man LPL. In the latter enzyme, the homologous N-linked glycosylation site (Asn-43) is required for both enzyme secretion and activity (29). The high degree of sequence homology between HL and LPL and the fact that this particular N-linked glycosylation site is conserved in both proteins across species suggest that carbohydrate chains attached to this site may be important for the active conformation of both HL and LPL.

The crystal structure of pancreatic lipase was reported by Winkler, D'Arcy, and Hunziker (39). There is substantial evidence from site-specific mutagenesis experiments that LPL and possibly HL (because of its high homology to LPL) have three-dimensional structures similar to pancreatic lipase (40–42). We have examined the locations of the N-linked glycosylation sites of hHL projected on the schematic ribbon diagram of pancreatic lipase (**Fig. 6**). All sites appear on the exterior polar surface of the crystal structure and would be accessible to the aqueous solvent. The three sites that can be changed without loss of catalytic activity of HL occur in nonconserved regions and away from the mixed α/β structure containing the catalytic triad. In contrast, the essential glycosylation site of HL occurs in a β-turn on the exterior polar surface of the enzyme. This structural feature separates the random coil region from the highly conserved β-sheet region. The β-sheet observed in the crystal structure of PL is predicted, by the Chou-Fassman algorithm (43), to be conserved in HL and LPL. It is not obvious how the absence of a car-

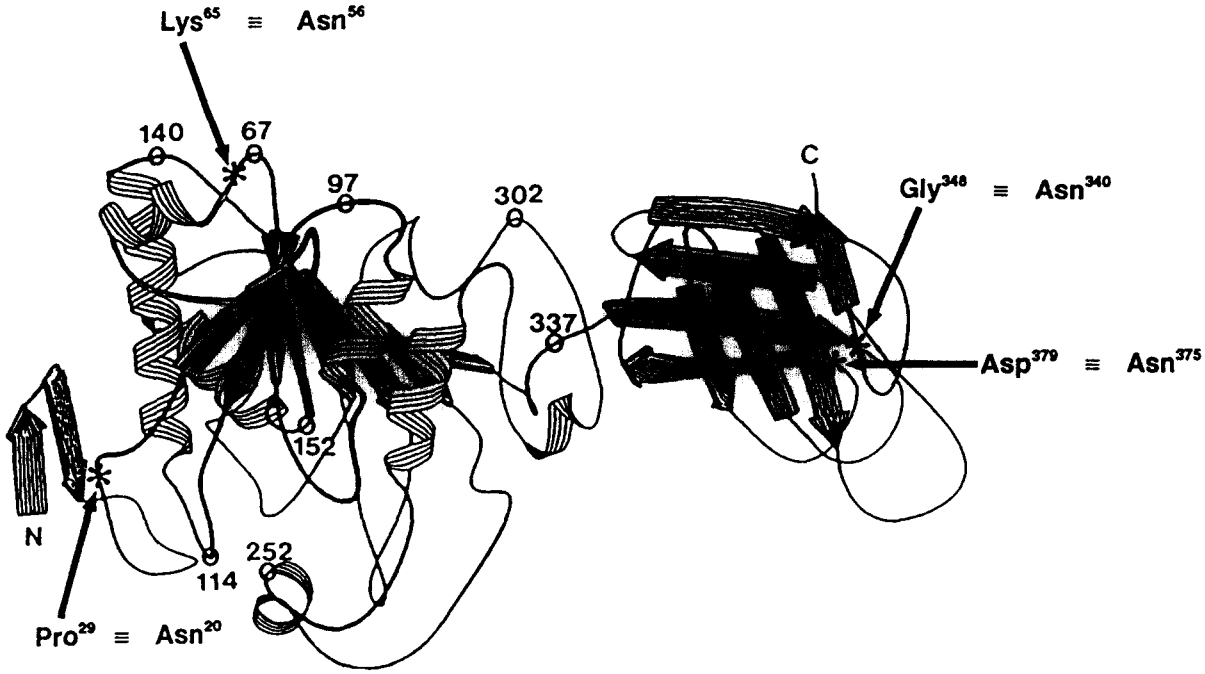

Fig. 6. Schematic ribbon representation of human pancreatic lipase adapted from Gubernator et al. (44). The residues in pancreatic lipase that correspond to the Asn residue of the N-linked glycosylation sites in hHL by sequence alignment are shown.

bohydrate moiety at Asn-56 in HL produces an inactive protein. We speculate that cotranslational modification of the glycosylation site is critical for the interaction of the highly conserved β-sheet with other regions of the enzyme to achieve the appropriate alignment of the residues comprising the catalytically active triad (39). In summary, N-linked glycosylation of HL and LPL is required for both enzyme activity and secretion for the two evolutionarily related lipases.

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