Effects of Site-Directed Mutagenesis on the N-Glycosylation Sites of Human Lecithin:Cholesterol Acyltransferase†

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ABSTRACT: There are four potential N-glycosylation sites (Asn-X-Ser/Thr) in human lecithin:cholesterol acyltransferase (LCAT, residues 20, 84, 272, and 384). To study the role of the N-linked sugars, the codon for Asn at these positions was replaced with one for Thr (AAC to ACC). The wild-type and mutant LCAT cDNAs were used to transfect COS-6 cells from which RNA was isolated; cDNAs were synthesized by reverse transcription and subjected to the polymerase chain reaction, which showed that all transfectants synthesized LCAT-specific mRNA. No intracellular or secreted LCAT was detected with the Asn^{272}→Thr transfectants, indicating that this residue is essential for intracellular processing. All other single-point transfectants were secretion-competent. Although there was detectable LCAT protein inside the cells and in the media of the transfectant, Asn^{84}→Thr, its specific activity and secreted amount were only 26% and 58% of the wild type, respectively. This implies that Asn^{84} is critical for full activity but not for intracellular processing. The amount secreted, specific activity, and V_{max} of LCAT (Asn^{20}→Thr) were similar to those of the wild-type LCAT. LCAT (Asn^{384}→Thr) differed from the wild-type LCAT only by a lower K_m. These results suggest that glycosylation at residues 20 and 384 is not essential for intracellular processing, secretion, or activity.

Lecithin:cholesterol acyltransferase (LCAT,[1] EC 2.3.1.43) is a key enzyme in the series of steps that transfers cholesterol from peripheral tissue to the liver (Fielding, 1990; Jonas, 1991). LCAT is important in this process because it converts cholesterol, which transfers freely between lipid surfaces, to its esterified form, which transfers only via its carrier, cholesteryl ester transfer protein (CETP). The major substrates for LCAT are the high-density lipoproteins (HDL), which contain the enzyme activator apolipoprotein A-I (apoA-I). Under some conditions, LCAT associates with low-density lipoproteins and catalyzes cholesteryl ester formation or the transesterification of fatty acids between phosphatidylcholine and lysophosphatidylcholine (Subbaiah et al., 1980).

Analysis of the primary structure of LCAT determined from protein and cDNA sequencing revealed regions of high hydrophobicity adjacent to the active-site serine at residue 181 that may be part of the interfacial recognition site that binds to lipid surfaces (McLean et al., 1986; Yang et al., 1987). LCAT is a glycoprotein in which approximately 25% of the molecule is composed of carbohydrate (Chung et al., 1979; Chong et al., 1983; Collet & Fielding, 1991). Upon SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) of plasma LCAT, a broad band corresponding to a range of molecular weights is observed, suggesting the presence of a mixture of LCAT species differing in their carbohydrate content. Treatment of native human plasma LCAT with various endoglycosidases converts the broad band found by SDS–PAGE to a single narrow band corresponding to the molecular weight determined by sequencing of the protein or cDNA.

LCAT contains four potential N-glycosylation sites (Asn-X-Ser/Thr) located at residues 20, 84, 272, and 384. Removal of most of the carbohydrate by neuraminidase is associated with a 50% increase in specific activity (Doi & Nishida, 1983). A series of glucosidase inhibitors had little effect on the quantity of LCAT secreted from stably transfected Chinese hamster ovary cells. Moreover, inhibitors of glucosidases that catalyze the trimming of high-mannose chains were without effect on the specific activity of the secreted LCAT. However, the LCAT specific activities were reduced in those cells that were incubated with tunicamycin, which inhibits N-glycosylation or glucosidases that hydrolyze the glucose caps from the high-mannose chains (Collet & Fielding, 1991). Results using inhibitors of glycosylation could be misinterpreted because they may affect the synthesis and secretion of other proteins that are involved in the processing of LCAT. As an alternative approach, we have tested the effects of replacing the four N-glycosylation sites by site-directed mutagenesis on the synthesis, secretion, and specific activity of human LCAT expressed *in vitro*.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Promega. The Sequenase Version 2.0 DNA sequencing kit used was from United States Biochemical. [α-^{35}S]dATP (1000 Ci/mmol), [α-^{32}P]ATP(5000 Ci/mmol), and $Na^{125}I$ were obtained from Amersham. Rabbit anti-goat IgG was from BioRad, [^{3}H]cholesterol was from Dupont, and the phospholipids were from Avanti Polar Lipids. A full-length cDNA clone of human LCAT (pUCLCAT.10), covering the entire coding region, cloned into the *Eco*RI/*Bam*HI sites of

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[1] Abbreviations: CETP, cholesterol ester transfer protein; DEAE, diethylaminoethyl; DNA, deoxyribonucleic acid; cDNA, complementary DNA; mRNA, messenger ribonucleic acid; ssDNA, single-stranded DNA; HDL, high-density lipoprotein(s); R-HDL, model-reassembled HDL; apoA-I, apolipoprotein A-I; LCAT, lecithin:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-glycerophosphocholine; DAPC, 1,2-diarachidonoyl-*sn*-3-glycerophosphocholine.

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pUC19 was kindly provided by Richard Lawn of Genentech Inc. (McLean et al., 1986). The insert contained 102 nucleotides in the 5′-untranslated region, the entire coding region, and 68 nucleotides in the 3′-untranslated region. Both ends were bounded by multiple linkers.

Construction of the Mutant Clones. The oligonucleotides used as the primers for mutagenesis (Table I) were synthesized on a Cyclone Plus DNA synthesizer and purified by an Oligo-pak oligonucleotide purification column (MilliGen/BioSearch). Before use, the oligonucleotides were 5′-phosphorylated by T_4 polynucleotide kinase. After digestion with *Eco*RI and *Bam*HI, the pUCLCAT.10 was subcloned into M13mp18 and M13mp19. Uracil-containing single-stranded DNA (ssDNA) was prepared by using *Escherichia coli* host strain CJ236 (ung$^-$) to culture the M13mp18 or M13mp19 LCAT clones (Kunkel et al., 1985). These ssDNA were used as a template and hybridized to the oligonucleotides which contained the mismatched nucleotides that coded for the desired mutant amino acid. After hybridization, the second strand was synthesized using T_7 DNA polymerase and T_4 ligase. The double-stranded DNA was transformed into an ung$^+$ *E. coli* strain, DH-5α'. The plaques of mutant cDNA were identified by the dideoxynucleotide method (Sanger et al., 1977). After digestion with *Eco*RI and *Bam*HI, the wild-type and mutant cDNAs were subcloned into a eukaryotic expression vector, pSG5 (Green et al., 1988), and transfected into *E. coli* AG1. The positive clones were checked by restriction enzyme mapping and direct sequencing. The selected mutant cDNAs were prepared using a kit (Qiagen Midi) in larger quantities for expression in mammalian cells.

In Vitro Expression of LCAT. A modified DEAE-dextran method (Selden et al., 1986) was used to transfect wild-type and mutant cDNAs of LCAT into COS-6 cells. Cells were grown in high-glucose DMEM with 10% heat-inactivated fetal bovine serum and 1× antibiotic–antimycotic liquid (Gibco BRL) in 75 cm^2 flasks. After >90% confluence was reached, the cells were subcultured at a 1:4 to 1:6 ratio and incubated for 24–48 h. The cells were transfected with 20 μg of plasmid per flask, incubated with the DNA–DEAE-medium stock and chloroquine for 3 h, and shocked with 10% dimethyl sulfoxide for 2 min. In each experiment, two flasks containing only COS-6 cells and COS-6 cells plus pSG5 plasmids were used as controls. After 72 h of incubation, the medium was collected, centrifuged, and immediately tested for LCAT activity using model-reassembled HDL (R-HDL) as the substrate.

RNA Preparation, cDNA Synthesis, and Polymerase Chain Reaction (PCR). After the medium for LCAT assays was collected, the transfected COS-6 cells were used to prepare total RNA. Extraction of RNA with guanidinium thiocyanate (Chirgwin et al., 1979) was followed by centrifugation in cesium trifluoroacetate (Okayama, 1987) and precipitation in ethanol. The polymerase chain reaction was performed on RNA using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus). For each sample, 1 μg of total RNA was used. PCR was carried out as follows: 2 min at 94 °C; 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C for 30 cycles; 10 min at 72 °C; overnight soak at 4 °C.

Substrate Preparation and LCAT Assays. R-HDL were prepared by a detergent removal technique (Matz & Jonas, 1982). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) or diarachidonoylphosphatidylcholine (DAPC), 2 mol % cholesterol, and a trace of [^{3}H]cholesterol (7500 dpm/pmol) were mixed, and the organic solvent was removed under a stream of N_2. The lipids were suspended in Tris-buffered saline, pH 7.4, by vortexing and combined with human apoA-I at a lipid to protein ratio to 100 to 1 (M/M). Sodium cholate (10%) was added until the turbidity of the solution disappeared and then exhaustively dialyzed against the same buffer. The LCAT assays were performed as previously described (Pownall et al., 1982). Assays for enzyme kinetics were performed similarly, except that the substrate concentration was varied as needed between 0 and 200 mM phosphatidylcholine.

Table I: Synthetic Oligonucleotides Used for LCAT Mutants and PCR Primers[a]

(I) Oligonucleotides for Site-Directed Mutagenesis	
Asn^{20}→Thr	5′-CCG-TGT-GTG-GGT-ACT-GAG-CTC-3′
Asn^{84}→Thr	5′-AGA-GCT-CCG-GGT-GTA-GAC-AAC-3′
Asn^{272}→Thr	5′-GCC-TGT-GTA-GGT-GAA-GCT-GGG-3′
Asn^{384}→Thr	5′-CAG-GGT-CAG-GGT-GCT-GAA-GAC-3′
(II) PCR Primers	
downstream primer	5′-TTC-TGG-CTC-CTC-AAT-GTG-CTC-3′
upstream primer	5′-CAA-GTG-TAG-ACC-GCC-GAG-GTC-3′

[a] The mutagenesis primers are antisense strand; every codon GTT was changed to GGT; the single base shifted is underlined.

Quantification of LCAT and Western Blot Analysis. A solid-phase immunoassay was used to determine the mass of LCAT secreted in the media using purified human plasma LCAT as the standard. The polyclonal antibody to human LCAT used was raised by injection of purified plasma enzyme into a goat. Isolated human LCAT and media from transfected COS-6 cells and the controls (COS-6 cells untransfected or transfected with only the expression vector pSG5) were concentrated 10-fold with a Centriprep-30 (Amicon) and applied to a nitrocellulose membrane in a dot-blot apparatus. The nitrocellulose was then incubated with goat antiserum to human LCAT, and then with rabbit anti-goat [^{125}I]IgG. After extensive washing, autoradiography was performed to ensure the existence of a proper signal/background ratio. The dots were cut, and the radioactivity was measured by γ counting. Electrophoresis was conducted using a 12% SDS–polyacrylamide gel (Laemmli, 1970). The proteins were transferred to a nitrocellulose membrane (Towin et al., 1979) and reacted with goat antiserum to human LCAT. The bands were visualized using rabbit anti-goat IgG(H+L) horseradish peroxidase-conjugated IgG (BioRad).

RESULTS

Site-Directed Mutagenesis and Construction of the Expression Vector. In LCAT, there are four potential N-glycosylation sites, Asn-X-Ser/Thr (McLean et al., 1986; Yang et al., 1987). They are Asn^{20}, Asn^{84}, Asn^{272}, and Asn^{384}. Four mutants of LCAT were prepared in which each of these sites was replaced by a threonine. A fifth tetrapoint mutant, Asn^{Tet}, in which all four asparagine residues were replaced by threonine was also prepared. These mutants are designated as LCAT (Asn^n→Thr) where n represents the location of the mutation within the primary structure. The oligonucleotide primers used to direct the mutations are listed in Table I. The LCAT cDNA sequences of the wild type and the mutants are shown in Figure 1. Before expression, the cDNAs were subcloned into the mammalian expression vector pSG5 (Figure 2).

Polymerase Chain Reaction Analysis. Total RNA isolated from COS-6 cells transfected by pSG5-containing wild-type or mutant LCAT cDNA was used for cDNA synthesis. The primers used for the PCR of these cDNA are listed in Table I. If the LCAT-specific mRNA was present, a fragment of 561 bp should appear in the electrophoresis of the PCR

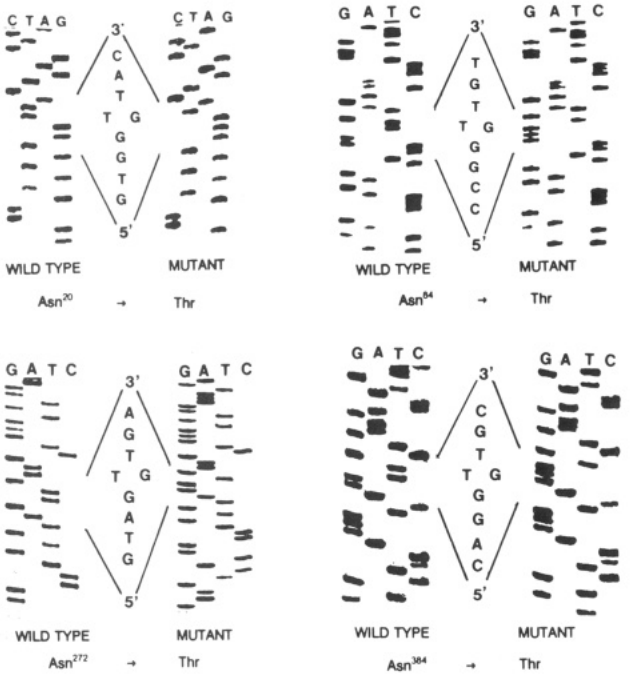

FIGURE 1: Comparison of cDNA sequences of LCAT from wild-type and Asn mutants. Each pair of autoradiograms contains the sequence of interest of both wild-type LCAT and the site-directed mutant. All the sequences are anti-sense strand.

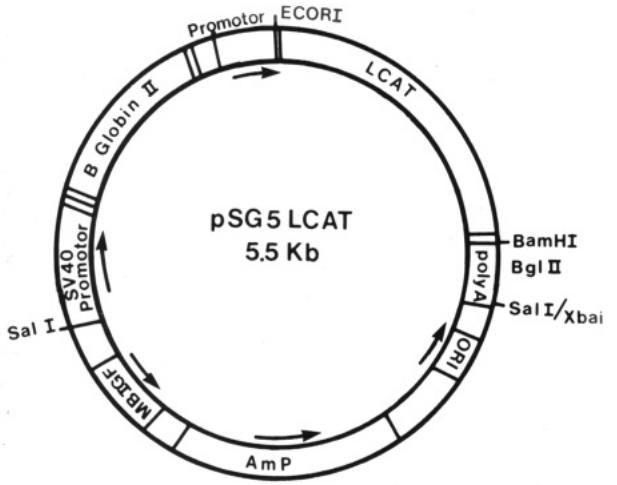

FIGURE 2: Structure of the expression vector for wild-type and mutant cDNAs of LCAT. Plasmid pSG5LCAT and its mutants were constructed as described under Materials and Methods. The components of the plasmid are shown.

products. As shown in Figure 3, no specific band was found for the cells transfected by the pSG5 vector control. In contrast, the electrophoretic patterns of the wild-type and all-Asn mutants contained a band corresponding to the 561 bp fragment, indicating the occurrence of LCAT-specific mRNA in all six transfectants.

Quantification of LCAT and Western Blot Analysis. The results of the determination of LCAT in the media of the transfected cells are shown in Table II. The immunologically determined amounts of LCAT in the media of the transfectants Asn^{20}→Thr, Asn^{84}→Thr, and Asn^{384}→Thr, respectively, were 92%, 58%, and 75% of that of the wild type. In contrast, no LCAT was detected when 20 or 40 μL of media from the Asn^{272}→Thr transfectant was used for the quantitative solid immunoassay. A trace amount of this LCAT mutant could be detected only by applying 80 μL of media to the nitrocellulose membrane. No LCAT was secreted by cells transfected with the tetrapoint mutant.

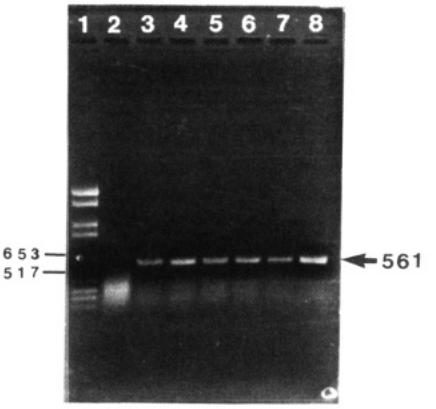

FIGURE 3: Polymerase chain reaction amplification of cDNA from total RNA. The total RNA samples from the expressed COS cells were subjected to PCR. Electrophoresis was performed on a 1.2% agarose gel. From the 100 μL of resulting PCR mixture, 5 μL was applied to each lane. Lanes: 1, standards; 2, cells transfected by pSG5; 3, wild-type LCAT; 4, Asn^{20}→Thr; 5, Asn^{84}→Thr; 6, Asn^{272}→Thr; 7, Asn^{384}→Thr; 8, $Asn^{20,84,272,384}$→Thr.

Table II: LCAT Activity, Specific Activity, and Mass in Media from COS-6 Cells Transfected by Wild-Type and Mutant LCAT cDNA[a]

mutants	act. [nM cholesteryl ester (mL of media^{-1} h^{-1})]	concn (μg/mL media)	sp act. (nM cholesteryl ester/μg of LCAT)
wild type	6.6 ± 0.3	1.2 ± 0.1	5.4 ± 0.3
Asn^{20} to Thr	4.3 ± 0.6	1.1 ± 0.2	3.9 ± 0.5
Asn^{84} to Thr	1.0 ± 0.2	0.7 ± 0.2	1.4 ± 0.3
Asn^{272} to Thr	0.0	trace[b]	0.0
Asn^{384} to Thr	4.3 ± 0.3	0.90 ± 0.1	4.8 ± 0.3
Asn^{Tet} to Thr	0.0	0.0	0.0

[a] Data represent the results from one set of experiments in which for each plasmid triplicate transfections were performed in 75 cm^2 flasks. After 72-h incubation, the media were centrifuged at 1200 rpm for 10 min. Ten microliters was removed from the media of each set of transfected cells to estimate the LCAT activity as described under Materials and Methods. Mass quantification was performed by a dot-blot method. Volumes of 10, 20, 40, 80, and 100 μL of standard LCAT (4 μg/mL) or 2 times concentrated media (20, 40, and 80 μL) were applied. [b] No detectable mass with 20- and 40-μL dots; a trace was visualized with 80 μL.

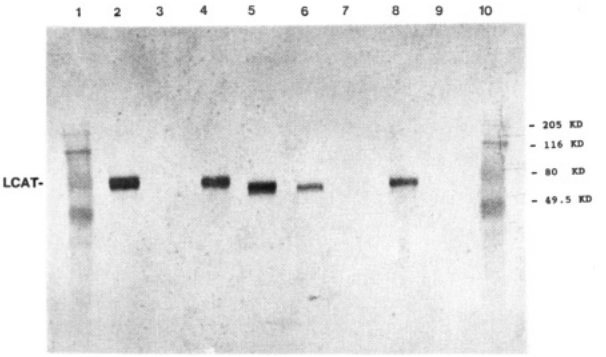

FIGURE 4: Western blot analysis of isolated human LCAT, and wild-type or mutant-transfected COS-6 cell culture media. The medium from COS-6 cells was concentrated 10-fold, and 5 μL was applied to each lane. Lanes: 1 and 10, protein molecular mass standards; 2, human plasma LCAT; 3, cells transfected by pSG5 COS-6 cells; 4, wild type; 5, Asn^{20}→Thr; 6, Asn^{84}→Thr; 7, Asn^{272}→Thr; 8, Asn^{384}→Thr; 9, $Asn^{20,84,272,384}$→Thr.

Results of Western blot analyses of media and cell lysates of LCAT-transfected cells are shown in Figures 4 and 5. LCAT protein was identified in LCAT-transfected cells (Asn^{20}→Thr), LCAT (Asn^{84}→Thr), and LCAT (Asn^{384}→Thr). The LCAT migration distance corresponded, however, to a lower mo-

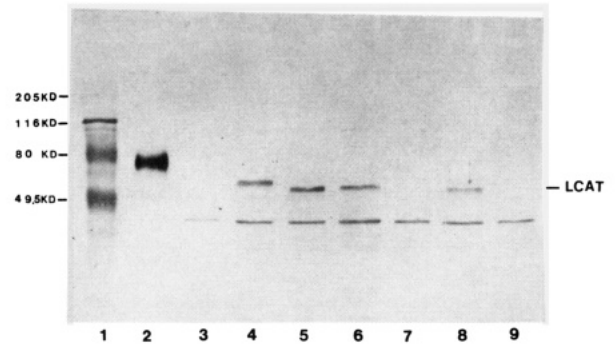

FIGURE 5: Western blot analysis of transfected COS-6 cell lysates from control, wild type, and mutants. After incubation for 72 h, cells were washed with PBS and trypsinized. Cells from each 75 cm^2 flask were homogenized using 1 mL of solubilizing buffer (1.6% Triton X-100, 0.3 mM leupeptin, 5.0 M urea, and 1.5 mM phenylmethanesulfonyl fluoride). The supernatants were concentrated 10-fold, and 10 μL was used for Western blot analysis. Lanes: 1, protein molecular mass standards; 2, human plasma LCAT; 3, COS-6 cells transfected by pSG5; 4, wild type; 5, $Asn^{20}\rightarrow Thr$; 6, $Asn^{84}\rightarrow Thr$; 7, $Asn^{272}\rightarrow Thr$; 8, $Asn^{384}\rightarrow Thr$; 9, $Asn^{20,84,272,384}\rightarrow Thr$.

Table III: Results of Substrate Saturation Kinetics of Wild-Type LCAT and Asn Mutants

	POPC		DAPC		
	V_{max}[a]	$K_m \times 10^6$ [b]	V_{max}	$K_m \times 10^5$	R[c]
wild type	9.0	26.0	1.0	13.0	9.0
$Asn^{20}\rightarrow Thr$	7.0	23.0	1.0	13.0	7.0
$Asn^{84}\rightarrow Thr$	2.0	28.0	0.2	16.0	10.0
$Asn^{384}\rightarrow Thr$	11.0	11.0	1.0	3.0	11.0

[a] V_{max} is expressed in nanomoles of cholesteryl ester formed per milliliter per hour. [b] K_m is expressed in moles of phosphatidylcholine per liter. [c] R is the ratio of V_{max} using POPC vs V_{max} using DAPC.

lecular weight with respect to the wild type, very likely due to the loss of N-linked carbohydrates. No LCAT-specific band was found in the media or in the cell lysates of the $Asn^{272}\rightarrow Thr$ and tetrapoint Asn→Thr transfectants.

Activities of Wild-Type and Mutant LCAT. No measurable LCAT activity was found in the media collected from the COS-6 cells that were not transfected nor from COS-6 cells containing the pSG5 plasmid lacking the LCAT insert. The media from the cells transfected with the wild-type DNA exhibited the highest transacylase activity (100%, Table II). The activities of LCAT ($Asn^{20}\rightarrow Thr$), LCAT ($Asn^{84}\rightarrow Thr$), and LCAT ($Asn^{384}\rightarrow Thr$) were 65%, 15%, and 65%, respectively. No activity was detected in the media of the LCAT ($Asn^{272}\rightarrow Thr$), or the tetrasite mutant. The specific activities of LCAT ($Asn^{20}\rightarrow Thr$), LCAT ($Asn^{84}\rightarrow Thr$), and LCAT ($Asn^{384}\rightarrow Thr$) were 72%, 26%, and 89% of the wild type (Table II).

Substrate Specificity of Mutant and Wild-Type LCAT. The kinetic parameters for binding and catalysis observed with the active mutants ($Asn^{20}\rightarrow Thr$, $Asn^{84}\rightarrow Thr$, and $Asn^{384}\rightarrow Thr$) and wild-type LCAT with substrates of POPC and DAPC were compared. As previously observed (Pownall et al., 1985), the activity of wild-type LCAT with POPC is greater than that observed with DAPC as the substrate. This can be expressed as a ratio, R, of the V_{max} obtained with POPC over that with DAPC. With wild-type LCAT and all of the Asn→Thr mutants with active LCAT, R is between 7 and 10 (Table III). Although the specific activities were always higher with POPC than with DAPC, with a given substrate the values of V_{max} were similar. The one exception was the $Asn^{84}\rightarrow Thr$ mutation for which V_{max} was about 80% lower than that of the wild type when measured with either POPC or DAPC. The only LCAT mutant exhibiting an apparent K_m that was different from that of the wild type was $Asn^{384}\rightarrow Thr$; its lower apparent K_m points to an affinity for both POPC and DAPC substrates that was higher than that of the other mutants and of wild-type LCAT.

DISCUSSION

There are two major steps for the glycosylation of proteins. Glycoproteins acquire their core sugars from dolichol donors in the rough endoplasmic reticulum (Abeijon et al., 1992). Then the glycoproteins migrate from the endoplasmic reticulum to the Golgi complex for further glycosylation. Subsequently they move to other destinations (lysosomes, secretory granules, or plasma membrane) according to signals encoded by their three-dimensional structure (Pfeffer et al., 1987). Proteins that are incorrectly folded or assembled and incompletely oligomerized proteins are generally retained in the endoplasmic reticulum and eventually degraded (Braakman et al., 1992). The inhibition of the sugar attachment to asparagine residues in different glycoproteins can be followed in many cases by intracellular degradation, inhibition of secretion, or loss of activity (Matzuck & Boime, 1988).

The decrease in the molecular weights of the LCAT mutants that was seen in Western blots of cells transfected with LCAT mutant cDNA provides direct evidence that all potential sites of N-glycosylation for the wild-type protein contain carbohydrate. The substitution of threonine for asparagine has the main effect of removing the site of sequence-specific glycosylation. Other differences between asparagine and threonine are small. Like asparagine, threonine is nonpolar and has a similar molecular volume. There can be little doubt that the changes observed in the cells transfected with mutant cDNA are due to the absence of individual N-linked carbohydrate chains rather than to changes in the amino acid itself.

According to PCR analysis of total RNA, all transfected cells contained LCAT-specific mRNA (Figure 3). Therefore, it is likely that differences among the transfectants were produced by posttranslational deficits. The amounts of LCAT found within cells transfected with wild-type or mutant LCAT cDNA, corresponding to the mutations $Asn^{20}\rightarrow Thr$, $Asn^{84}\rightarrow Thr$, or $Asn^{384}\rightarrow Thr$, were similar (Figure 5). Moreover, among these three transfectants there was little difference in the amount of LCAT found in the media (Figure 4, Table II). Thus, glycosylation at Asn^{20}, Asn^{84}, or Asn^{384} is not required for effective secretion of LCAT.

In contrast, LCAT ($Asn^{272}\rightarrow Thr$) was not found in the media or the cell lysates (Figures 4 and 5, Table II). This suggests that the carbohydrate linkage at Asn^{272} is indispensable for secretion-competent LCAT. The absence of measurable amounts of this LCAT mutant product may be due to its retention and subsequent degradation within the rough endoplasmic reticulum. One possible model is that without the glycosylation of Asn^{272} the protein is not properly folded and is consequently degraded. Thus, glycosylation of Asn^{272} may be one of several structural signals that permits LCAT to proceed through the secretory pathway. However, if removal of carbohydrate by neuraminidase increases rather than decreases activity (Doi & Nishida, 1983), this indicates that the essential native structure required for activity has been retained and that sialylation should not affect protein folding within the endoplasmic reticulum. No intracellular or secreted LCAT was associated with cells transfected with the tetrapoint mutation. Since the tetrapoint mutant also contains the $Asn^{272}\rightarrow Thr$ mutation, which is associated with

little or no intracellular or secreted enzyme, it is very likely that the absence of detectable amounts in cell lysates is due to this single mutation without contributions by the other three mutant sites.

Although the amounts of LCAT found in the media of the secretion-competent transfectants of LCAT were similar, there were differences in their activities and kinetic parameters (Table II). None of the glycosylation sites of LCAT are near the active site in the primary structure. Therefore, any effect on kinetic parameters would presumably occur because some of the sites are within or near the interfacial binding and catalytic sites of the folded protein. With the exception of LCAT (Asn^{84}→Thr), V_{max} and molecular specificities, as determined by the relative rates of utilization of POPC and DAPC, were also similar (Table III). These data suggest that carbohydrates at residues 20 and 384 have little or no effect on the activity and specificity of human LCAT. Although the ratio, R, is similar to those of the other LCAT mutants, the specific activity at V_{max} for the Asn^{84}→Thr mutation is lower against substrates containing POPC or DAPC. Since there is little effect on the apparent K_m, the major effect is on the catalytic step. The exact mechanism is not clear but may involve a steric effect if Asn^{84} is close to the active site in the three-dimensional structure. Clearly though, Asn^{84} is essential for the full activity but not for intracellular processing and secretion.

There is one other major difference among the kinetic parameters of the LCAT mutants. The substrate affinity of the LCAT (Asn^{384}→Thr), as determined from its lower apparent K_m, is greater than that of the wild type and the other Asn mutants. The meaning of K_m in connection with soluble enzyme association with insoluble substrates is not clear, and two alternative models have been proposed (Verger & de Hass, 1976). These are that the K_m represents the affinity of the enzyme for (1) the individual molecules of phospholipid or (2) the substrate surface that is composed of many lipid molecules. According to the first model, removal of the carbohydrate may lower the free energy of transfer of the phosphatidylcholine molecules into the active-site region. Alternatively, loss of the carbohydrate, which is very hydrophilic, could increase the hydrophobic association of an interfacial binding site to the substrate surface.

The intracellular processing and activity of LCAT are modulated by glycosylation. Although this study has provided important insight into the functional importance of individual N-glycosylation sites, the mechanisms by which the removal of glycosylation sites affects intracellular processing and enzyme activity are not known. Moreover, it is not known whether or not our findings would be duplicated in other cell types that secrete LCAT *in vivo*. Further studies with other cell types or transgenic species may further complement and validate the physiological importance of glycosylation in human LCAT.

ADDED IN PROOF

Recently, similar results were reported using Chinese hamster ovary cells (Francone et al., 1993).

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