Human Plasma Cholesteryl Ester Transfer Protein Consists of a Mixture of Two Forms Reflecting Variable Glycosylation at Asparagine 341

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ABSTRACT: Plasma cholesteryl ester transfer protein (CETP) mediates the transfer of neutral lipids and phospholipids between the plasma lipoproteins. The deduced M_r of the CETP polypeptide from the cDNA is 53 000, but in sodium dodecyl sulfate (SDS) gels plasma CETP appears as a broad band containing two different moelcular forms of M_r 65 000–71 000. The purpose of this study was to see if variable N-linked glycosylation could explain the microheterogeneity of CETP. Recombinant CETP (rCETP), derived from stable expression of the CETP cDNA in Chinese hamster ovary (CHO) cells, appeared as a protein doublet comparable to plasma CETP. Digestion of plasma or rCETP with N-glycosidase F (glyco F, to remove N-linked carbohydrates) resulted in the formation of a lower M_r doublet in which the bottom band approximated the M_r of the CETP polypeptide. Metabolic labeling of the rCETP with [^{3}H]mannose and [^{3}H]glucosamine, followed by digestion with glyco F, suggested that the top band of the doublet contained residual N-linked carbohydrates resistant to glyco F digestion. To explore this hypothesis further, each of the four potential N-linked glycosylation sites of CETP (at amino acid positions 88, 240, 341, and 396) was eliminated by mutagenesis of asparagine to glutamine. The wild-type (WT) and mutant CETP cDNAs were transiently expressed in COS-7 cells. Each mutant CETP showed a lower M_r than WT, indicating that all four sites were occupied by N-linked carbohydrate. Each mutant and WT protein appeared as a doublet except for the 341N → Q mutant, which gave rise to a single protein band coincident with the lower band of the doublet of WT CETP. Furthermore, digestion of the 341N → Q protein with glyco F gave rise to a single band of $M_r \sim$ 53 000. The 88N → Q and 396N → Q mutants were poorly secreted, but the 341N → Q protein was well secreted and displayed moderately increased cholesteryl ester transfer activity compared to WT. The results suggest that plasma or rCETP consists of a mixture of two forms in which amino acid 341 is or is not occupied by N-linked carbohydrate. Although a preliminary survey indicated only slight variations in normolipidemic subjects, variable ratios of the two forms in altered metabolic states could give rise to differences in the specific activity of plasma CETP.

Plasma cholesteryl ester transfer protein (CETP)[1] mediates the transfer of cholesteryl esters (CE) from high- and low-density lipoproteins (HDL and LDL) to triglyceride-rich lipoproteins as well as the reciprocal transfer of triglyceride from very low density lipoproteins (VLDL) to LDL and HDL (Tall, 1986, 1990). CETP, acting in conjunction with lecithin–cholesteryl acyltransferase and hepatic triglyceride lipase, remodels plasma HDL, influencing HDL particle size and composition. Several studies have shown an inverse relationship between CETP activity and HDL cholesterol levels (Agellon et al., 1991; Brown et al., 1989; Inazu et al., 1990; Whitlock et al., 1989), indicating an important role for CETP in the catabolism of HDL CEs. Human genetic CETP deficiency is one striking example of this relationship (Brown et al., 1989; Inazu et al., 1990). Markedly increased HDL cholesterol and HDL2/HDL3 ratios as well as reduced CE in VLDL and LDL were found in homozygotes with this condition, indicating a potential antiatherogenic phenotype (Brown et al., 1989; Inazu et al., 1990). Thus, CETP may be a possible target for drug intervention as the inhibition of CETP would lead to increased HDL cholesterol levels.

CETP is a hydrophobic glycoprotein with a reported M_r of 66 000–74 000 (Hesler et al., 1987; Drayna et al., 1987; Swenson et al., 1987; Ohnishi et al., 1990). The sequence of the human CETP cDNA and the organization of the CETP gene have been elucidated (Drayna et al., 1987; Agellon et al., 1990). The cDNA predicts a polypeptide of M_r 53 000 (Drayna et al., 1987) but plasma CETP appears as a broad band of M_r 65 000–71 000, from which two major forms (M_r 66 000 and 69 000) have been purified (Ohnishi et al., 1990). The molecular weight difference between the CETP polypeptide and plasma CETP is most likely due to posttranslational modifications such as N- or O-linked glycosylation. Four potential N-linked glycosylation sites are found within the CETP sequence (Drayna et al., 1987). Previous studies using tunicamycin, an inhibitor of N-linked glycosylation, have shown that N-linked carbohydrates were necessary for CETP secretion from HepG2 cells (Swenson et al., 1987). However, glycohydrolase treatment of purified plasma CETP resulted in the formation of heterogeneous products which were not as small as the CETP polypeptide (Swenson et al., 1987). The basis of this molecular weight anomaly is not understood. In addition to posttranslational modification, heterogeneity of CETP might reflect alternative splicing of the CETP gene transcript (Inazu et al., 1992).

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[1] Abbreviations: (r)CETP, (recombinant) cholesteryl ester transfer protein; VLDL, very low density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; bp, base pairs; M_r, apparent molecular weight; glyco F, N-glycosidase F; CE, cholesteryl ester; CHO, Chinese hamster ovary; DEAE, diethylaminoethyl; DME medium, Dulbecco's modified Eable's medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WT, wild type.

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Table I: Mutant CETP cDNA Constructs

construct	amino acid change	position	DNA codon change
WT	native		
88N → Q	Asn to Gln	88	AAT to CAG
240N → Q	Asn to Gln	240	AAT to CAG
341N → Q	Asn to Gln	341	AAT to CAG
396N → Q	Asn to Gln	396	AAT to CAG
240/341N → Q	Asn to Gln	240/341	AAT to CAG

The purpose of this study was to investigate posttranslational modifications of CETP which might explain its microheterogeneity as well as the M_r anomaly. After initial results suggested the presence of an occult N-linked sugar, we focused on the role of N-linked sugars in CETP structure and function. We employed site-directed mutagenesis and expression approaches to study the effects of eliminating each N-linked carbohydrate of CETP.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's modified Eagle's (DME) medium, trypsin, penicillin/streptomycin, bovine serum albumin, chloroquine, lysozyme, and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose, acrylamide, bis(acrylamide), ammonium persulfate, SDS, and nitrocellulose filter membranes (0.45 μm) were from Bio-Rad (Richmond, CA). Restriction enzymes were from appropriate commercial sources. *Escherichia coli* DH5aF'IQ were from Gibco–Bethesda Research Laboratories (Rockville, MD). KS⁻ Bluescript expression plasmid and mammalian transfection reagents were obtained from Stratagene (La Jolla, CA). ^{35}S-dATP, [2-^{3}H]mannose, and [6-^{3}H]glucosamine were purchased from New England Nuclear (Boston, MA). HDL, HDL_3 (d = 1.11–1.21 g/mL) containing [^{3}H]CE, and LDL (d = 1.02–1.063 g/mL) were prepared as described previously (Sammett & Tall, 1985).

Oligonucleotide-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was used to alter each of the four potential N-linked glycosylation sites within the human CETP amino acid sequence. This was achieved by changing each of the asparagines to glutamine (N → Q; see Table I). Oligonucleotides were designed to change the codon AAT (N) to CAG (Q). Oligonucleotides were prepared by Oligo Etc's (Gilford, CT) or were prepared on an Applied Biosystems DNA synthesizer and had the following sequences: 88N → Q, 5′-GACCACAGACACCTGCTGAATGGAGAC-3′; 240N → Q, 5′-AGGTCCTCTGAGACCTGCTTGTAGATGAAATGACCCTTGTGATGGGACTCGAGGTAGGAGGCTGT-3′; 341N → Q, 5′-CATCACTGAAGACTGGACCACGACTCC-3′; and 396N → Q, 5′-GCTCTCAGTCAACTGGGAAACAGTCTTTGG-3′. The CETP cDNA was cloned into the KS⁻ Bluescript expression vector for production of single-stranded DNA template. Mutagenesis was performed using the Amersham in vitro mutagenesis kit. Approximately 5 μg of single-stranded DNA template was annealed to 4 pmol of 5′-phosphorylated oligonucleotide primer. Competent *E. coli* DH5aF'IQ cells were transformed with 20 μL of the above reaction, and then the individual resultant colonies were screened. Dideoxynucleotide sequencing was used for screening and to confirm the presence of the desired mutation. In order to eliminate the possibility of secondary mutations, a sequenced fragment containing the mutation was subcloned into the WT cDNA and transferred into the pCMV4 expression vector kindly supplied by Dr. David W. Russell (University of Texas Southwestern Medical Center) (Andersson et al., 1989).

Transfection of COS-7 Cells. Transient expression of WT and mutant constructs was achieved by transfection of the SV-40 transformed monkey kidney cell line COS-7 (American Type Culture Collection, Rockville, MD, CRL 1651). COS-7 cells were grown in DME medium with 10% fetal calf serum (Hyclone, Logan, UT), and the cells were split 1:5 approximately 16 h prior to transfection. Transient expressions were performed by the DEAE-dextran method using a transient expression transfection kit (Stratagene, La Jolla, CA) exactly as described by Wang et al. (1991).

Characterization of Mutant Proteins. The activity, mass, and apparent molecular weight were determined for each mutant and wild-type CETP protein secreted into the culture medium. The CE transfer activity was quantitated using [^{3}H]-CE HDL as the transfer donor and human LDL as acceptor. A 200-μL aliquot of Opti-MEM I medium was incubated for 16 h at 37 °C with labeled HDL (10 000 cpm) and LDL (100 μg of protein) in a total 300-μL incubation volume. LDL was precipitated by the addition of bovine serum albumin, heparin, and $MnCl_2$ to achieve final concentrations of 4%, 75 mM, and 140 mM, respectively (Sammett & Tall, 1985). All assays were performed in the linear range such that the transfer of radiolabeled CE was less than ~20%.

The mass of CETP recovered in the medium was quantitated by slot-blot analysis. A 200–500-μL aliquot of Opti-MEM I medium was applied in duplicate to a nitrocellulose membrane with a slot-blot apparatus (Schleicher and Schuell, Inc.). A CETP standard curve was constructed ranging from 0.5 to 10 ng with rCETP of known concentration as determined by radioimmunoassay (Marcel et al., 1990). The nonspecific sites on the nitrocellulose membrane were blocked by incubation in a 4% milk solution containing 0.1 M Tris, pH 7.6. CETP was detected with a monoclonal antibody, TP-2 (Hesler et al., 1988; Wang et al., 1992), and was amplified with a secondary antibody, antimouse IgG horseradish peroxidase (HRPO). The reaction was developed using the enhanced chemiluminescence (ECL) detection kit from Amersham (Arlington Heights, IL). The relative intensity of each spot was determined by densitometric scanning, and a standard curve was then constructed for determination of CETP in the culture medium. The specific activity (counts per minute per nanogram) for each mutant and wild-type protein was calculated from the CE transfer activity (counts per minute per milliliter) and the immunoreactive mass of CETP protein recovered in the culture medium (nanograms per milliliter). The data for each transfection were normalized to the wild-type values for comparison.

SDS–PAGE. The glycosylation mutants, WT CETP, or plasma CETP immunoprecipitates were subjected to SDS–6% polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were then electrophoretically transferred to 0.45-μm nitrocellulose membranes and probed with CETP mAb (TP-2) as described above or with ^{125}I-TP-2. Molecular weights were determined using 3–4 proteins (Pharmacia, Piscataway, NJ) to construct a standard curve. The following proteins were used: phosphorylase b, M_r 94 000; albumin, M_r 67 000; ovalbumin, M_r 43 000; and carbonic anhydrase, M_r 30 000.

Metabolic Labeling of CHO Cells. In order to investigate the glycosylation of CETP, metabolic labeling studies were carried out. Chinese hamster ovary cells (CHO) were stably transfected with the human CETP cDNA (Inazu et al., 1991) and were grown to 90% confluency in 60-mm dishes in Ham's F12 medium supplemented with L-glutamine, penicillin, and streptomycin. The medium was removed from the cells by

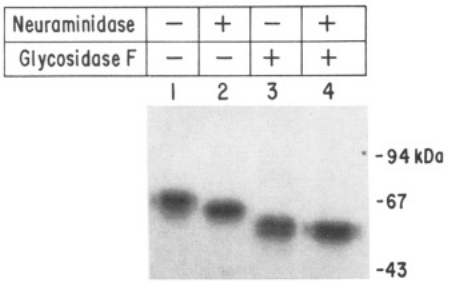

FIGURE 1: Glycohydrolase treatment of immunoprecipitated human plasma CETP. Fresh plasma (150 μL) was immunoprecipitated with TP2-Sepharose, and then the immunoprecipitates were incubated with 0.75 unit of glyco F (+) or neuraminidase (+) or without enzyme (−) in the same buffer solution. In the sequential digestion (+/+) the immunoprecipitate was treated with glyco F, washed, and then incubated with 350 milliunits of neuraminidase. Following the incubations, the immunoprecipitates were electrophoresed on an SDS–6% polyacrylamide minigel and detected immunochemically by Western blotting using ^{125}I-TP-2. The following samples are shown: lane 1, plasma CETP control without glycohydrolase treatment; lane 2, plasma CETP treated with neuraminidase; lane 3, plasma CETP treated with glyco F; and lane 4, plasma CETP treated with both glyco F and neuraminidase. The positions of molecular weight standards are indicated.

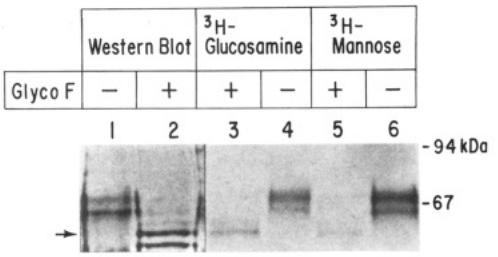

FIGURE 2: Incorporation of [^{3}H]mannose and [^{3}H]glucosamine into human CETP. CHO cells, stably transfected with human CETP cDNA, were grown to 90% confluency and were then radiolabeled with 77 μCi/mL [^{3}H]mannose or [^{3}H]glucosamine or incubated without isotope in glucose-free DME medium for 16 h. CETP was immunoprecipitated from 1.5 mL of the culture medium. The immunoprecipitates were then incubated with glyco F (+) or without enzyme (−) in the same buffer solution as described under Experimental Procedures. The immunoprecipitates were analyzed by SDS–6% polyacrylamide minigel electrophoresis and Western blotting and detected with CETP mAb (lanes 1 and 2) or by fluorography (lanes 3–6). The arrow indicates the position of the M_r 57 000 band of glyco F-treated CETP showing persistant metabolic labeling with [^{3}H]glucosamine and [^{3}H]mannose.

aspiration and the cells were washed with PBS. Glucose-free DME medium (6 mL) was added to the cells, and [^{3}H]mannose or [^{3}H]glucosamine was then added dropwise to the culture medium to achieve final concentrations of 77 μCi/mL. After 16 h of labeling, the medium was collected. Sodium azide, PMSF, aprotinin, leupeptin, and EDTA were added to the culture medium at final concentrations of 0.02%, 1 mM, 1 μg/mL, 1 μg/mL, and 2 mM, respectively. CETP was immunoprecipitated from the cell culture medium using a monoclonal antibody, TP-2, attached to Sepharose beads (Inazu et al., 1991). Immunoprecipitation was carried out overnight at 4 °C. The TP-2-Sepharose–CETP complexes were isolated by centrifugation at 15000*g* for 30 s, washed with 0.10 M ammonium bicarbonate, and then resuspended in buffer for glyco F (from *Flavobacterium meningosepticum*, Boehringer Mannheim Biochemicals, Indianapolis, IN) digestions or were prepared directly for SDS–PAGE and immunoblot analysis or for SDS–PAGE and fluorography.

Glycohydrolase Digestions. For glyco F digestions the immunoprecipitates were resuspended in 0.10 M sodium phosphate, pH 8.0, containing 10 mM EDTA, 1% Triton X-100, 0.02% SDS, 3 mM DTT, and 1 mM PMSF and were then treated with 0.75 unit of glyco F. Glyco F digestions were incubated under nitrogen at 37 °C for 18 h. In some experiments CETP was immunoprecipitated from plasma (150 μL) and was then treated with neuraminidase (*Clostridium perfingens*, Boehringer Mannheim Biochemicals, Indianapolis, IN). The immunoprecipitates were resuspended in 0.05 M sodium acetate, pH 5.0, 0.15 M NaCl, 1% Triton X-100, 0.01% SDS, 3 mM DTT, and 1 mM PMSF and was then incubated under nitrogen with 350 milliunits of neuraminidase at 37 °C for 18 h. After digestion, samples were prepared for SDS–PAGE.

RESULTS

In SDS–6% polyacrylamide gels, human plasma CETP migrates as a broad band with an average M_r of 68 000 ± 3000 (Figure 1, lane 1). Neuraminidase treatment of plasma CETP results in a decrease in M_r of approximately 4500, reflecting the removal of approximately 16 terminal sialic acid residues (Figure 1, lane 2). Treatment of plasma CETP with glyco F, a glycohydrolase which removes N-linked carbohydrate chains at the asparagine–*N*-acetylglucosamine bond (Swenson et al., 1987), results in the formation of a doublet with bands of M_r 57 000 and 54 000 (Figure 1, lane 3). The CETP doublet showed no further change in M_r even with digestion up to 90 h and addition of fresh glyco F. Treatment of the CETP doublet with neuraminidase resulted in a slight decrease in M_r of the top band of the doublet, indicated by a closing of the space between the two bands, while there was no apparent change in M_r of the bottom band (Figure 1, lane 4). These results indicate that plasma CETP consists of a mixture of two major forms, which cannot be accounted for simply by heterogeneous sialation (Hesler et al., 1987; Ohnishi et al., 1990). The persistence of a higher M_r form after glyco F digestion could be due to alternative splicing of a small exon of the CETP gene (Inazu et al., 1992), the persistence of occult N-linked carbohydrate resistant to glyco F treatment, or another form of posttranslational modification (such as O-linked glycosylation).

In order to distinguish among these possibilities, we investigated the glycosylation of recombinant CETP (rCETP) secreted by CHO cells. The rCETP immunoprecipitated from CHO cell media appeared as a distinct protein doublet, M_r 69 000 and 65 000 (Figure 2, lane 1). Treatment of rCETP with glyco F gave rise to a doublet of M_r 57 000 and 54 000 (Figure 2, lane 2) identical in M_r to that derived from plasma CETP (Figure 1, lane 3). Since the rCETP was derived by transfection of a single species of cDNA, the protein doublet is most likely due to heterogenous posttranslational modification.

To assess the possibility that the doublet is due to variable glycosylation, we performed metabolic labeling of rCETP secreted by CHO cells, using [^{3}H]mannose and [^{3}H]glucosamine. [^{3}H]Mannose- or [^{3}H]glucosamine-treated CHO cells synthesized radiolabeled rCETP, indicating incorporation into the complex carbohydrate chains of CETP (Varki, 1991). Both bands of the CETP doublet incorporated both labeled sugars, although the upper band was more intensely labeled, suggesting a higher content of carbohydrate (Figure 2, lanes 4 and 6). Treatment of the radiolabeled rCETP with glyco F resulted in the formation of a single band of M_r 57 000 (Figure 2, lanes 3 and 5), in contrast to the CETP doublet seen by protein detection on the same samples (Figure 2, lane 2). The radiolabeled band had identical migration to the top band (M_r 57 000) of the rCETP doublet resulting from glyco

Table II: Relative Secretion and Specific Activity of Mutant CETP Constructs[a]

construct	transfections (n)	secretion (% wt CETP)	specific activity (% wt CETP)
WT	5	100.0	100.0
88N → Q	4	8.9 ± 1.5[b]	u.d.[c]
240N → Q	3	121.7 ± 15.2	117.0 ± 15.3
341N → Q	5	108.7 ± 15.4	120.8 ± 8.2[b]
396N → Q	4	22.1 ± 10.1[b]	98.0 ± 13.0
240/341N → Q	3	138.8 ± 58.3	93.4 ± 31.5

[a] All values are the mean ± sem. Each transfection consisted of the average of 3 plates of COS-7 cells for each construct. The average secretion and specific activity of wt CETP were 8.1 ± 2.1 ng/mL and 478 ± 208 cpm/ng (mean ± sem, $n = 5$), respectively. The secretion and specific activity of each construct were expressed relative to those of WT CETP. [b] Significantly different from WT CETP using an unpaired t-test analysis, $p < 0.05$. [c] u.d., unable to detect; cpm transferred were too low to be determined.

F treatment (see arrow, Figure 2). There was no labeling of carbohydrate in the position of the lower band (Figure 2, cf. lanes 2, 3, and 5) indicating that the M_r 54 000 species was devoid of carbohydrate. Thus, the upper band of the protein doublet contained residual carbohydrate which could not be removed by glyco F digestion. [^{3}H]Mannose will radiolabel only N-linked carbohydrate, but [^{3}H]glucosamine will label both N- and O-linked carbohydrates (Varki, 1991). Since the M_r 57 000 band was radiolabeled with both [^{3}H]mannose and [^{3}H]glucosamine, it contains residual N-linked sugars. Thus, the data shown in Figures 1 and 2 suggest that plasma and rCETP consist of a mixture of two forms which, following glyco F digestion, do or do not contain residual N-linked sugar. This could arise if one of the N-linked carbohydrate sites of CETP was variably utilized and inaccessible to glyco F.

In order to evaluate this hypothesis further and to study the impact of N-linked sugars on the secretion and function of CETP, we mutagenized each of the four potential sites for N-linked glycosylation. These sites are defined by the three amino acid consensus sequence Asn-X-Ser/Thr and occur at amino acid positions 88, 240, 341, and 396 in the CETP sequence (Drayna et al., 1987). Oligonucleotide-directed mutagenesis was employed to eliminate each potential N-linked glycosylation site by substituting a glutamine for an asparagine (Table I). Following transfection into COS-7 cells, the secretion, CE transfer activity, and M_r of each mutant protein were determined. In addition to the single mutants, a double mutant including amino acids 240 and 341 was prepared, since the single mutants at sites 240 and 341 were found to be well secreted.

The mass of secreted protein and its activity relative to WT are shown in Table II. The values are normalized to those of WT in order to control for differences in transfection efficiency and for the different preparations of substrate lipoproteins used in different experiments. The 88N → Q protein was poorly secreted by the COS-7 cells and did not display detectable CE transfer activity in the medium. The 240N → Q protein displayed similar secretion and activity compared to WT. The 341N → Q protein was also well secreted and displayed significantly increased CE transfer specific activity compared to WT ($p < 0.05$). The 396N → Q protein was not efficiently secreted but the CETP protein that was recovered in the medium has CE transfer specific activity similar to that of WT CETP. The double mutant 240/341N → Q protein was also secreted as well as WT CETP and displayed CE transfer specific activity similar to that of WT CETP. These data suggest that N-linked glycosylation at asparagines 88 and 396 is critical for the efficient secretion

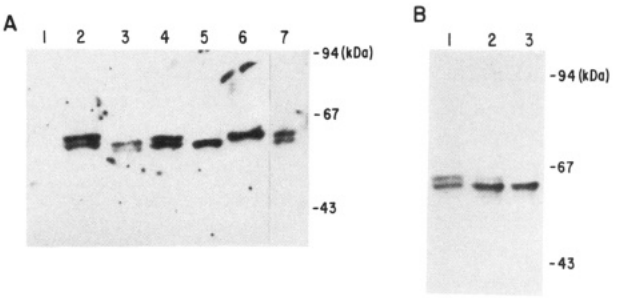

FIGURE 3: Western blots of WT and mutant CETP proteins from COS-7 cell lysates. (A) COS-7 cells were transiently transfected with WT or mutant CETP cDNAs, and the cells were harvested after 72 h of further growth. Transfected COS-7 cells were gently scraped from the plate and washed with PBS buffer. The cells were sedimented by centrifugation and resuspended in 400 μL of lysis buffer containing 0.5 M Tris-HCl, pH 6.8, 2 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM PMSF. The cells were lysed by sonication and then 10 μL of the cell lysates were subjected to high-resolution (12 cm) SDS–6% PAGE. The positions of molecular weight markers are indicated. Lane 1, control (no DNA); lane 2, WT CETP; lane 3, 88N → Q; lane 4, 240N → Q; lane 5, 240/341N → Q; lane 6, 341N → Q; lane 7, 396N → Q. (B) COS-7 cell lysates from WT CETP and 341N → Q were mixed in equal proportions and subjected to high-resolution SDS–6% PAGE and Western blotting. Lane 1, WT CETP; lane 2, WT CETP (50% of the mass in lane 1) + 341N → Q (50% of the mass in lane 3); lane 3, 341N → Q.

of CETP. Single or double mutants involving the other two sites (asparagine 240 and 341) were well secreted and had normal or increased activity.

The molecular weights of the CETP glycosylation mutants were analyzed in COS-7 cell lysates by SDS–PAGE and Western blotting. WT CETP migrates as a doublet with M_r of 62 000 and 60 000 (Figure 3A, lane 2). The lower M_r of CETP secreted by COS-7 cells has been noted previously and is thought to reflect defective sialation by these cells (Wang et al., 1991). The 88N → Q, 240N → Q, and 396N → Q proteins also were found to migrate as discrete doublets with M_rs of about 60 000 and 58 000 (Figure 3A, lanes 3, 4, and 7, respectively); each mutant CETP protein was lower in molecular weight compared to WT CETP by approximately 2000. In contrast, the 341N → Q protein was found to migrate as a single band on SDS–PAGE analysis with an M_r of 60 000 (Figure 3A, lane 6), in this experiment as well as in five other transfections. The 240/341N → Q protein was also found to migrate as a single band with an M_r of 58 000 (Figure 3A, lane 5). The pattern of doublet formation by CETP proteins recovered in the medium was identical to that of the respective protein in cell lysates (data not shown). These results suggest that the doublet present in WT CETP is due to variable glycosylation at amino acid 341.

Consistent with this hypothesis, the mobility of the 341N → Q protein was found to be identical with that of the lower band of the doublet of WT CETP, as shown in the mixing experiment in Figure 3B (lane 1, WT; lane 2, WT + 341N → Q; lane 3, 341N → Q). Furthermore, digestion of the 341N → Q protein with glyco F gave rise to a single band of $M_r \sim 53\,000$, approximating the deduced M_r of the CETP polypeptide, whereas mutants at the other three sites gave rise to doublets when digested with glyco F (data not shown). These results suggest that the N-linked glycosylation sites at amino acid positions 88, 241, and 396 are all utilized and each contribute at least 2000 to the overall M_r of WT CETP. This is a minimal estimate of the contribution of each site, owing to the poor sialation of CETP by COS-7 cells (Wang et al., 1991). In addition, the glycosylation site at asparagine 341

Table III: Distribution of the Plasma CETP Glycoforms in Normocholesterolemic Subjects[a]

	(n)	% top band	% bottom band
females[b]	6	49.8 ± 1.1	50.3 ± 1.1
males[b]	5	49.0 ± 1.5	51.0 ± 1.5

[a] All values shown are the mean ±sd. CETP was immunoprecipitated from plasma (30 μL), digested with glyco F, and then subjected to SDS–PAGE and immunoblotting as described under Experimental Procedures. The relative intensity of each band of the CETP doublet was determined by laser densitometry and the percent distribution of each band was then calculated. [b] No statistically significant difference was found between the percent top versus the percent bottom band using an unpaired t-test analysis.

appears to be variably utilized, and when this site is occupied it contributes approximately 2000 to the M_r of WT CETP. From the ratio of the intensity of each of the bands in the doublet of WT and mutant CETP proteins, it appears that asparagine 341 is glycosylated on ~50% of the CETP molecules synthesized.

The ratio of variably glycosylated forms was investigated in plasma CETP by treating immunoprecipitated plasma CETP with glyco F followed by SDS gel analysis. In a survey of a small group of normocholesterolemic males and females (average total plasma cholesterol concentration 200 ± 33, mean mg/dL ± sd, $n = 7$), only slight variations in the ratios of the two glycoforms were found, as shown in Table III.

DISCUSSION

We have used oligonucleotide-directed mutagenesis to eliminate each potential site of N-linked glycosylation within human CETP. These studies show that every potential site in CETP is glycosylated and that individual sites of N-linked glycosylation influence the biosynthesis, secretion, and biological activity of CETP. Interestingly, the N-linked site at asparagine 341 was found to be variably utilized and accounts for the presence of the two major forms of differing M_r found in plasma and rCETP. Since the two forms have different CE transfer activity, different proportions of the two glycoforms of CETP in different metabolic states could give rise to differences in the specific activity of plasma CETP, and this change in CE transfer could potentially influence HDL cholesterol levels. In addition, the CETP 341N → Q protein is a potential candidate for future crystallization studies since it is more homogeneous than plasma or WT recombinant CETP (Figure 3).

Attachment of carbohydrate may allow proteins to achieve unique conformations which can influence their activities (Olden et al., 1985; Drickamer et al., 1991). When individual sites of N-linked glycosylation were abolished by mutagenesis, the functional activity of CETP was affected (Table II). A significant change in specific activity was not found when asparagine 240 or 396 was changed to glutamine via mutagenesis. However, when asparagine 341 was changed to a glutamine by mutagenesis, a 20% greater specific activity was found compared to WT CETP. Wild-type CETP consists of an equal mixture of the fully and partially glycosylated CETP glycoforms (Figure 3). Thus, the form of CETP without carbohydrate at asparagine 341 has about 40% higher activity than the form with carbohydrate at this site. Although we cannot exclude the possibility that the higher activity resulted from the N → Q mutation per se, inspection of the data in Ohnishi et al. (1990) suggests that the lower M_r form of CETP isolated from plasma probably also had higher activity, suggesting that the absence of carbohydrate at asparagine 341 has a direct effect on activity. This increase in CE transfer activity due to the removal of N-linked sugars could be a result of charge alteration or a decrease in steric hindrance in proximity to the active site (Olden et al., 1985; Drickamer, 1991). Although attachment of carbohydrate frequently influences the folding and secretion of proteins, effects on catalytic activity, as indicated here for CETP, are relatively rare (Drickamer, 1991). An additional result of variable N-linked glycosylation could be different rates of clearance by the hepatic asialoglycoprotein receptor (Drickamer, 1991).

Previous studies have shown that treatment of HepG2 cells with tunicamycin, an inhibitor of N-linked glycosylation, resulted in greater than 90% inhibition of CETP secretion (Swenson et al., 1987). We have shown that asparagine 88 and 396 appear to be the sites required for efficient secretion of CETP, as the mutation of either site resulted in a significant decrease in CETP secretion (Table II). Carbohydrate attachment to newly synthesized proteins is thought to modify the protein's physiochemical properties such as its ability to fold properly, its solubility, and its net charge (Drickamer, 1991). Carbohydrate depletion of CETP as through mutagenesis may modify the folding, causing impaired transit through the secretory pathway. This effect appeared to be greater when the N-linked site at asparagine 88 was deleted compared to asparagine 396. Thus, attachment of carbohydrate at asparagine 88, which is near the N-terminus of the 476 amino acid CETP molecule, may influence the early steps in folding during translocation across the ER.

Plasma CETP migrates as a broad band of M_r 66 000–74 000 from which two major forms (M_r 66 000 and 69 000) have been purified (Ohnishi et al., 1990). Recombinant CETP, derived from cDNA expression in CHO cells, was also found to comprise two major forms of M_r 65 000 and 69 000; and plasma and rCETP formed an indentical doublet after glyco F digestion. We have presented evidence demonstrating that the variable glycosylation of asparagine 341 accounts for the doublet found in rCETP and by analogy in plasma CETP. Metabolic labeling studies of the CETP carbohydrates with [^{3}H]mannose and [^{3}H]glucosamine (Figure 2) indicated that the higher M_r band of the doublet was more heavily glycosylated than the lower band and showed that the presence of residual N-linked sugar in ~50% of the CETP molecules was responsible for the rCETP doublet found after glyco F digestion. These findings suggested that variable usage of one of the N-linked glycosylation sites of CETP, buried with the CETP molecule and therefore inaccessible to glyco F and of limited access to the protein oligosaccharyltransferase during CETP folding, might account for the two forms of CETP. This hypothesis was confirmed by showing that the 341N → Q mutant protein gave rise to a single molecular species, comigrating with the lower band of the CETP doublet (Figure 3B) and degraded by glyco F to a single band of M_r identical that of the CETP polypeptide. By contrast, mutants at each of the other N-linked sites gave rise to doublets both before and after glyco F treatment. Further proof that CETP was variably glycosylated at asparagine 341 would require the isolation of the individual glycoforms and subsequent biochemical analysis for the presence or absence of sugars at this site. However, metabolic labeling and glycohydrolase digestion studies have become accepted as an alternative approach to this analysis for nonabundant proteins (Varki, 1991). Although variable glycosylation of asparagine 341 explains the presence of two major forms of plasma and rCETP, our results do not exclude the possibility of other minor post-translational modifications of CETP. The greater blurring of the bands of plasma CETP which tended to obscure the

doublet (Figure 1) could arise from more heterogeneous glycosylation at each N-linked site or from another modification not seen on rCETP.

The variable glycosylation of asparagine 341 may be a potential mechanism to influence the CE transfer activity in plasma. Preliminary studies in a small set of normocholesterolemic males and females (Table III) did not show a significant alteration in the ratio of the CETP glycoforms. This is consistent with the high correlation between CETP activity and mass found in normolipidemic subjects ($r = 0.86$). However, the correlation of CETP mass and activity is lower in hyperlipidemic subjects ($r = 0.72$) (McPherson et al., 1991). Also, a recent study of alcoholic subjects, who may have defects in protein glycosylation (Stibler et al., 1986), has indicated a moderate increase in specific activity of plasma CETP, compared to nonalcoholic subjects (Hannuksela et al., 1992). Differences in the ratio of the two glycoforms of CETP could be one potential source for variation in plasma CETP specific activity.

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