Structure-Function Studies of Human Cholesteryl Ester Transfer Protein by Linker Insertion Scanning Mutagenesis†

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ABSTRACT: Human plasma cholesteryl ester transfer protein (CETP) enhances transfer and exchange of cholesteryl ester (CE) and triglyceride (TG) between high-density lipoprotein and other lipoproteins. To define regions responsible for the neutral lipid transfer activities at the molecular level, a total of 27 linker insertion mutants at 18 different sites along the CETP molecule were prepared and transiently expressed in a mammalian cell line (COS). The inserted linkers were small (usually 6 bp) and did not interrupt the translational reading frame of the CETP cDNA. Although secretion of each mutant protein was less than that of wild-type CETP, the majority of the mutants had normal cholesteryl ester transfer activity (transfer activity per nanogram of CETP in media). However, insertional alterations in three regions severely impaired CE transfer activity: (1) in the region of amino acids 48–53; (2) at amino acid 165; and (3) in the region of amino acids 373–379. Although the impaired activities could also be a result of globally incorrect folding of these CETP mutants, hydrophobicity analysis and secondary structure predictions tended to exclude this possibility for most of the insertion sites at which insertions resulted in inactivation. The insertion at amino acid 379 occurs immediately after a triplet of lysine residues, suggesting that this region might be involved in an essential step in the mechanism of CE and TG transfer, such as the binding of CETP to phosphatidylcholine molecules in the lipoprotein surface. Effects on TG transfer activity were generally similar to those on CE transfer activity, suggesting a similar structural requirement for both neutral lipid transfer activities.

Cholesteryl ester transfer protein (CETP)[1] catalyzes the exchange and transfer of neutral lipids [cholesteryl ester (CE) and triglycerides (TG)] between the plasma lipoproteins (Glomset, 1968; Morton & Zilversmit, 1979). The human plasma CETP is a hydrophobic glycoprotein with an apparent molecular weight of 74000 as determined by SDS-PAGE (Hesler et al., 1987). The human CETP cDNA (Drayna et al., 1987) and the sequence and organization of the CETP gene (Agellon et al., 1990) have also been recently described. The human CETP cDNA encodes a polypeptide of M_r 53000 (476 amino acids). Glycosylation is thought to account in part for the higher apparent molecular weight of plasma CETP (Swenson et al., 1987). The importance of CETP in lipoprotein metabolism is illustrated by human genetic CETP deficiency in which there is markedly increased HDL and reduced LDL (Brown et al., 1989). Since HDL levels are inversely correlated with atherosclerosis in humans, CETP activity could be an important factor influencing this disease.

A limited amount of information is available concerning the structure-function relationships of CETP. The CETP has binding sites for CE, TG, and phosphatidylcholine (PC). CETP binds approximately 1 mol of CE and 11 mol of PC at its maximal capacity (Swenson et al., 1988). The CE bound to CETP can readily equilibrate with lipoprotein lipids. A monoclonal antibody that binds to the carboxy-terminal region of CETP neutralizes the CE and TG transfer activities (Hesler et al., 1988). Limited proteolysis of human CETP showed that the CETP activity is tolerant of partial digestion by trypsin and chymotrypsin. However, an active proteolytic fragment could not be separated from intact CETP, and the results suggest that catalytically active CETP possesses a distinct and highly stable tertiary structure (Hesler et al., 1989).

Very little is known about the primary sequence determinants that endow CETP with the ability to transfer neutral lipids. The present study aims to explore the structure-function relationship of CETP by asking whether one or more functional regions that are responsible for the CE and TG transfer activities of the CETP molecule can be defined. The human CETP cDNA was cloned into a eukaryotic expression vector, mutagenized through linker insertions, and expressed in a transient expression system. The transfer activities for CE and TG of the mutants were then assayed, and correlated with the physical locations of the insertions in the CETP polypeptide.

Materials and Methods

Materials. DME, FCS, trypsin, penicillin/streptomycin, and Opti medium were from Gibco, BRL. Agarose, acrylamide, bis(acrylamide), ammonium persulfate, SDS, and nitrocellulose filter membranes (0.45 μm) were obtained from Bio-Rad. Restriction enzymes were from appropriate commercial sources. TEMED and β-mercaptoethanol were from Kodak. *Escherichia coli* DH5αF'IQ was from Bethesda Research Laboratories. The expression plasmid pCMV4 was kindly provided by Dr. David Russell (University of Texas Southwestern Medical Center). [^{35}S]dATP was from Am-

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[1] Abbreviations: CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DME, Dulbecco's modified Eagle's medium; Tris, tris(hydroxymethyl)aminomethene; RIA, radioimmunoassay; EDTA, ethylenediaminetetraacetic acid; TE, Tris-EDTA buffer; TSE, Tris-saline-EDTA; PEG, poly(ethylene glycol); FCS, fetal calf serum; EtdBr, ethidium bromide; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAB, monoclonal antibody; wt, wild type.

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ersham Corp. Bovine serum albumin, chloroquine, lysozyme, and heparin were from Sigma. HDL, HDL_3 (d = 1.11–1.21 g/mL) containing [^{3}H]cholesteryl ester, and LDL (d = 1.02–1.063 g/mL) were prepared and characterized as described previously (Sammett & Tall, 1985). HDL_3 containing [^{3}H]cholesteryl ester and [^{14}C]triolein was prepared according to the method of Morton and Zilversmit (1979). Transfection reagents were from Stratagene.

Construction of Linker Insertion Mutants. All the linker insertion mutants were derived from a parental plasmid, pCMV4-CETP. This plasmid contained a full-length CETP cDNA including the signal sequence. The eukaryotic expression vector pCMV4 was selected for the mutagenesis (Andersson et al., 1989). This 4.9-kb plasmid contained a bacteriophage f1 origin of replication, an ampicillin-resistance gene, an SV40 replication origin, a promoter of human cytomegalovirus major immediate early gene (Thomsen et al., 1984), a human growth hormone fragment containing transcription termination and polyadenylation signals, a translational enhancer, and a polylinker region. The CETP cDNA fragment was cloned into the *Bgl*II and *Sma*I sites in the multiple cloning site. This construct (~6.4 kb) was used as the parental plasmid for mutagenesis. Defined localized alterations within the CETP were created by introducing 6 bp linkers at various position along the CETP cDNA cloning region. The linkers with a length of 6 bp add two amino acids into CETP for most mutants.

pCMV4-CETP was linearized by partial digestion of the parental plasmid with *Alu*I or *Hae*III. The digestion reaction contained 20 μg of plasmid, 40 μg/mL EtdBr, appropriate restriction enzyme buffer, and 5–10 units of *Alu*I or *Hae*III in a total volume of 200 μL. The reaction proceeded up to 2 h and was stopped at 15-min intervals. The extent of the digestion was monitored by running small aliquots of the reaction mixture on a 0.7% low melting point agarose gel while the reaction was standing by at 0 °C. When the digestion showed linearization of the majority of the plasmid but the digestion was not too extensive, the total reaction mixture was separated on a 0.7% preparative low melting point agarose gel containing 0.5 μg/mL EtdBr in both the gel and the buffer. The band containing the linearized plasmid was then excised; the DNA was extracted and dissolved in TE buffer. This procedure resulted in mostly single-cut, blunt-ended plasmid by *Alu*I and *Hae*III.

Three linker inserts that were to be ligated into the linearized parental plasmid consisted of six nucleotides whose sequences contained the recognition site of *Cla*I, *Hin*dIII, and Asp718. These polynucleotides were self-complementary and could form small blunt-ended double-stranded fragments. They were first phosphorylated with T4 DNA kinase and then ligated into the linearized plasmid with T4 polynucleotide ligase. *Escherichia coli* strain DH5αF'IQ was transformed with the ligation mixture. DNA was isolated from the clones resistant to ampicillin and screened for the presence of a newly created unique site for *Cla*I, *Hin*dIII, or Asp718, indicating the presence of the linker. This procedure usually provided a satisfactory mutagenic rate. If initial screening showed a low rate of mutagenesis, the plasmids were further enriched for the mutagenized fraction of the plasmid pool by digesting the mixed plasmids isolated from a pool of colonies with one of the three restriction enzymes, isolating the cut plasmids in a low melting point agarose gel, religating the linearized plasmids, and transforming the *E. coli* strain followed by screening. The positions of the insertions in the CETP cDNA sequence were determined by restriction digestion and in each case were confirmed by DNA sequencing using the dideoxy method.

In a few cases, the mutants contained a tandem arrangement of the linkers at the insertion positions, resulting in insertion of six or eight amino acids. This was due to the fact that mutants containing only one or two pieces of the linker 5'ATCGAT were not initially selected by *Cla*I digestion because of the methylation of the linker sequence by the *E. coli* strain DH5αF'IQ (*dam*$^+$). The methylase recognizes GATC, so the linker sequence would be methylated if one or two of the linkers was inserted at the *Alu*I or *Hae*III site (AG/CT or GG/CC, respectively). Insertions of three or more linkers at one site were detected by *Cla*I digestion because not all the linkers were methylated in this case. Subsequently, mutants containing 6 nt at this site were obtained by selecting linkers whose sequences were not methylated by the *dam*$^+$ strain.

Transfection of COS7 Cells. Expression of wild-type CETP and mutant CETP molecules was achieved by transfection of the SV40-transformed monkey kidney cell line, COS7 (ATCC CRL 1651). The transfection was performed according to Stratagene's protocol with modifications. The cells were grown in 100-mm plates in DME supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 100 units/mL penicillin/streptomycin. Cells at full confluency were split 1 to 5 in 100-mm plates and allowed to grow for 16–20 h before transfection. DNA used for the transfection was isolated from 500 mL of culture in LB medium by either CsCl gradient centrifugation or PEG precipitation (Sambrook et al., 1989). Both methods yielded similar CETP expression levels. DNA (2–4 μg/plate for CsCl and 10–15 μg/plate for PEG preparations) was diluted in 170 μL of PBS and then mixed with another solution containing 85 μL of PBS and 85 μL of DEAE-dextran (2 mg/mL) and 0.9% (w/v) NaCl to form transfection solution. Medium on the cells was then aspirated, and cells were washed with PBS. The transfection solution was then added dropwise onto the cells. The cells were bathed in the transfection solution for 1 h at 37 °C with swirling at 15-min intervals. The transfection solution was aspirated; cells were rinsed gently with PBS and were further incubated in 3 mL of DME medium containing 10% FCS, 1% Pen/Strep, 1% glutamine, and 100 μM chloroquine for 3 h at 37 °C. This solution was then replaced by Opti-medium (Gibco, BRL). Approximately 72 h after the transfection, the medium was collected for CETP activity and protein analyses. For every set of transfections, multiple plates of COS7 cells were always transfected for each mutant, and control plates transfected with no DNA or wt pCMV4-CETP were also included.

Characterization of Mutant Proteins. To assay the CE transfer activity of CETP, [^{3}H]CE–HDL was used as transfer donor and human LDL as acceptor. [^{3}H]CE–HDL (15 μL) containing approximately 10000 cpm was mixed with 50 μL of LDL [2 mg/mL of 50 mM Tris, 150 mM NaCl, and 2 mM EDTA, pH 7.5 (TSE)]. Samples of 50–200 μL were incubated with the mix in the presence of TSE. Variation in activity measurements on different plates of the same mutants was generally small (<15%). The assays were always conducted in the linear range of the activity assay by varying sample volumes in the incubations. The incubation proceeded for approximately 16 h at 37 °C in a shaking water bath. The volume of the incubation was increased to 1 mL by adding TSE followed by addition of 0.35 mL of LDL precipitation solution composed of 4 parts of 20% BSA (w/v), 1 part of 2 M $MnCl_2$, and 1 part of 10000 units/mL heparin. LDL was precipitated by centrifugation in a microfuge for 15 min at 4 °C. One milliliter of the supernatant was mixed with 2 mL of Hydrofluor scintillation fluid and counted. The counts

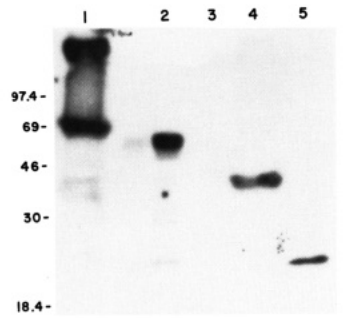

FIGURE 1: Western blots of wt CETP and deletion mutant proteins in media. COS7 cells were transiently transfected, and the media were collected after 3 days of post transfection growth. The proteins accumulated in the media were purified by immunoaffinity chromatography. All the samples were run on a 10% SDS–PAGE, and the proteins were blotted onto a nitrocellulose filter, stained with ^{125}I-TP2, and autoradiographed. Lane 1, CM52 fraction of CETP preparation; the band on the top of the lane represents CETP aggregate retained in the well due to overloading. Lane 2, expressed wt CETP; lane 3, *Xho*I deletion mutant protein; lane 4, *Bgl*II deletion mutant protein; lane 5, *Eco*RV deletion mutant.

represented the amount of CE remaining in the HDL. The concentrations of the LDL and HDL were determined by the method of Bradford et al. (1979). In selected experiments, HDL labeled with both [^{3}H]CE and [^{14}C]TG was used as substrate in the incubation in order to measure CE and TG transfer activities. The CE transfer activities measured with both single- and double-isotope substrates were found to be similar. For CETP mass determination, two or three plates of the transfection media were combined, dialyzed with 3 × 4 L of 2 mM Tris–HCl, pH 7.4, for 2 days, and lyophilized. The CETP mass in the lyophilisate was determined by solid-phase competition radioimmunoassay (Brown et al., 1989).

Results

Expression of CETP Mutant Proteins. To assess the expression of wt CETP or its mutants, aliquots of media of transfected COS7 cells were assayed for CE transfer activity and analyzed by RIA to determine the mass of CETP. The apparent molecular weight of the expressed wt protein was slightly smaller than the molecular weight of plasma CETP as judged by SDS–PAGE and Western blotting of ^{125}I-TP2 (a monoclonal antibody that binds to the C-terminus of human CETP) (Figure 1, lane 1 vs lane 2). This probably resulted from decreased sialation of CETP produced in COS cells, compared to the native CETP purified from plasma. However, the specific activity (cpm transferred/ng of protein) of wild-type CETP from transfected COS7 cell medium was found to be identical with CETP purified from human plasma (Hesler et al., 1987), indicating that CETP obtained through the mammalian expression system had similar activity to authentic human CETP. Initially, we attempted to map the active regions of CETP by deleting portions of the CETP cDNA employing native restriction sites in the cDNA. Two in-frame deletion mutants, one lacking residues 43–366 (*Eco*RV deletion mutant, Figure 1, lane 5) and other lacking residues 183–330 (*Bgl*II deletion mutant, Figure 1, lane 4), were found to be secreted at reduced levels and were completely inactive. Because large fractions of the CETP have been eliminated, the resultant proteins may be folded incorrectly so that an active conformation could not be formed. Another deletion mutant, created by removing a small fragment between two *Xho*I sites in the CETP coding region (nucleotides 1454–1530), was also inactive. This deletion mutant altered the translational reading frame and did not react with the C-terminal antibody (Figure 1, lane 3).

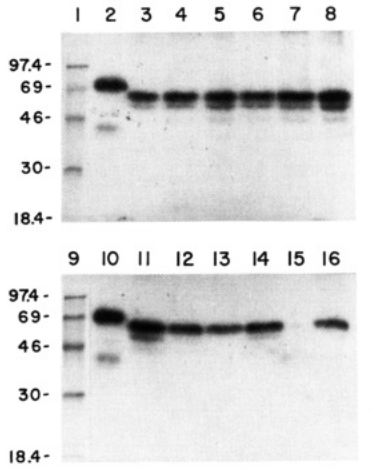

FIGURE 2: Western blots of wt CETP and insertion mutant proteins inside COS cells. COS7 cells were transiently transfected, and the cells were harvested after 3 days of posttransfection growth. Transfected COS7 cells were gently scraped from the plates and washed with PBS buffer. The cells were sedimented by centrifugation and resuspended in 200 μL of lysis buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 μg/mL leupeptin, and 50 μg/mL aprotinin. The cells were disrupted by pumping the cells vigorously through a 26-gauge needle with a 1-mL syringe. The cell lysate (120 μL) was dried under vacuum and dissolved in 30 μL of SDS–PAGE loading buffer. Lanes 1 and 9, ^{14}C-labeled protein molecular weight standards; lanes 2 and 10, purified human plasma CETP; lane 3, 39S:RSF; 4, 277A:EAS; 5, wt CETP; 6, 401S:RSF; 7, 48A:DR(YR)$_2$S; 8, 195A:GVP; 11, 75E:EKL; 12, 165Q:QGT; 13, 177K:KGT; 14, 407S:RSF; 15, mock transfection (no DNA added); 16, 53G:G(SI)$_3$.

As a more subtle approach, we decided to analyze the structure–function relationships of CETP by linker insertion mutagenesis. With this approach, the insertion of only two amino acids introduces local alterations but does not typically induce global changes of conformation of the protein (Barany, 1985; Lobel & Goff, 1984; Lyman & Rohrschneider, 1987; Freimuth & Ginsberg, 1986). A length of 6 bp is also long enough to create a new unique restriction site when the linker is inserted, which greatly facilitates the screening process. A total of 27 different mutants covering 18 restriction sites along the CETP cDNA-coding region were obtained and mapped and confirmed by sequencing. Multiple mutants were prepared at most restriction sites in order to examine the effects of insertion of different amino acids at the same site. Table I summarizes the amino acid alterations generated by each insertion. Most of the mutants contained an insertion of two amino acids. However, a few mutants contained an insertion of six amino acids, and one mutant contained an insertion of eight amino acids (see Materials and Methods).

The expression level for wt CETP was 251 ± 99 ng/plate (mean ± SD, n = 14). However, the mass of each mutant CETP accumulating in media was less than that of wt CETP (varying from 6 to 66% of the simultaneously transfected wt) (Table II). For most of the mutants, SDS gels of cell lysates showed a similar level of CETP to the wt cDNA, suggesting that the lower accumulation in media reflected diminished secretion rather than decreased synthesis (Figure 2). The mass of CETP in media appeared to be a function of the insertion position in the CETP molecule. The mutants having insertions in the N-terminal half tended to show lower levels of secretion than those having insertions in the C-terminal half (Table II). This trend is more clearly seen when mutants containing multiple linkers are excluded from the analysis. The mean secretion of mutants 3′ of an apparent transition region (between 195A:GVP and 274A:GYP) showed significantly greater secretion (24 ± 13 ng/plate, mean ± SD, n = 10,

Table I: Structure of Insertion Mutants in CETP

mutant[a]	restriction site[b]	insertion size (bp)	amino acid changes[a] at	from	to	predicted secondary structure[c] wt	mutant
39S:RSF	296	6	39	S	RSF	E-T-C	
48A:DR(YR)$_2$S	322	18	48	A	DRYRYRS	9H	4E-3T-8E
48A:EAS	322	6	48	A	EAS	9H	11H
53G:G(SI)$_3$	338	18	53	G	GSISISI	6H-2E	6H-8E
53G:GVP	338	6	53	G	GVP	6H-2E	6H-4E
75E:EKL	405	6	75	E	EKL	H	
75E:EGT	405	6	75	E	EGT	H	
149K:KKL	625	6	149	K	KKL	H	
165Q:QGT	675	6	165	Q	QGT	1T-7E	2C-8E
165Q:QKL	675	6	165	Q	QKL	1T-7E	1T-9E
177K:KGT	708	6	176	K	KGT	E-H	
195A:GVP	763	6	195	A	GVP	E-H	
274A:GYP	1000	6	274	A	GYP	H	
277A:EAS	1009	6	277	A	EAS	H	
281G:GVP	1022	6	281	G	GVP	H-E	
281G:GSL	1022	6	281	G	GSL	H-E	H
281G:G(SI)$_3$	1022	18	281	G	GSISISI	H-E	
281G:G(SI)$_4$	1022	24	281	G	GSISISISI	H-E	
319A:GYP	1135	6	319	A	GYP	C-E	
373A:D(RY)$_2$RS	1297	18	373	A	DRYRYRS	9H[d]	2H-8E-5T
379K:KGT	1317	6	379	K	KGT	4H[d]-4E	4T-6E
379K:KKL	1317	6	379	K	KKL	4H[d]-4E	6H[d]-2E-2H
401S:RSF	1382	6	401	S	RSF	E-C-H	
407S:RSF	1400	6	407	S	RSF	H-E	
474S:RSF	1601	6	474	S	RSF	H-C	
475S:RSF	1607	6	476	S	RSF	H-C	
476S:R(SI)$_3$	1607	18	476	S	RSISISI	H-C	

[a] The abbreviations for amino acids are as follows: A, Ala; R, Arg; N, Asn; Q, Gln; E, Glu; G, Gly; I, Ile; L, Leu; K, Lys; F, Phe; P, Pro; S, Ser; T, Thr; Y, Tyr; V, Val. [b] The map position was based on the nucleotide sequence assigned by Drayna et al. (1987). The CETP coding sequence was therefore from 182 to 1612. [c] Analyses based on the method of Garnier et al. (1978). Abbreviations: H, α-helix; E, extended β-sheet; T, β-turn; C, coil. The numbers in front of the predicted secondary structures indicate the numbers of amino acid residues forming these structures. The predicted structures include four flanking amino acids on the left and right sides of the insertion site (wt) or of the insertion (mutant). Thus, the predicted structures for wt contain eight or nine amino acids; for mutants, two, six, or eight additional amino acids were included for the analyses. [d] The same helix.

excluding mutants with multiple linkers) ($P < 0.024$) than that of mutants 5′ of this region (53 ± 38 ng/plate, mean ± SD, $n = 11$, excluding mutants with multiple linkers), perhaps suggesting less constraints in folding in the C-terminal region.

CE Transfer Activity of the Insertion Mutant Proteins. In order to correlate the structural alterations with function, the variants were tested for their ability to transfer CE between lipoproteins. The mean specific activities [relative to wt (=1)] for CE transfer are shown in Figure 3 and also in Table II (where mean ± SDs or means of duplicate experiments are shown). Since the estimation of specific activity for mutants with very low secretion involved some error, the mutants were classified as having low, intermediate, and normal activity to avoid overinterpretation of small differences in activity. Among all the CETP mutants, only five of them possessed low activity (defined as <40% of wild-type activity, which was 1.00 ± 0.20, $n = 6$, mean ± SD). For the rest of the 22 mutants, 15 had activities which were indistinguishable from that of wild-type CETP. The other seven mutants showed intermediate activity levels (between 40% and 75% of wild-type activity). The mutants with low activity had insertions clustered in three different regions: (1) near the N-terminus (at amino acids 48 and 53); (2) at the region around amino acid 165; and (3) in the region of amino acids 373 and 379. However, in each case (except at amino acid 373), there were additional mutants at the same site which displayed normal or intermediate activity levels, indicating that the specific amino acids inserted were just as important as the position in the CETP sequence in the determination of CE transfer activity.

Among the mutants with intermediate activity, 281G:G(SI)$_3$ exhibited the lowest activity (specific activity 0.42). Considering that the other three mutants with insertions at the same

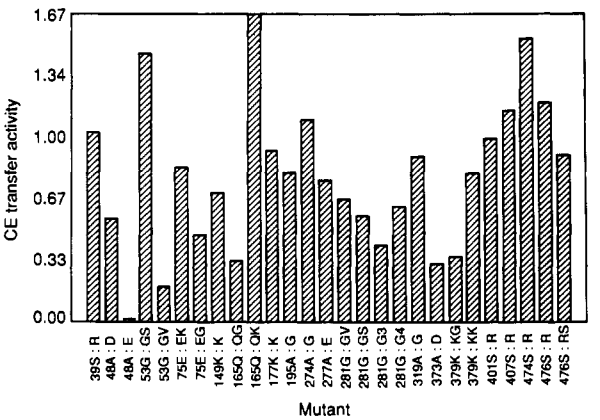

FIGURE 3: CE transfer activities of the insertion mutant proteins in media. The CE transfer activities are the mean specific activities (cpm transfered/ng of CETP) of the mutants relative to that of wt (wt = 1).

location were also only partially active, the region around amino acid 281G may also have an important influence on CE transfer activity.

TG Transfer Activity of the Insertion Mutants. To test the TG transfer activity, double-labeled HDL_3 ([^{3}H]CE/[^{14}C]TG HDL_3) was used as substrate for the mutants in selected experiments, and TG and CE transfer activities were determined simultaneously. The relationship between the TG and CE transfer activities was calculated as a ratio of the two (Table II). The results showed that all sites with low CE transfer activity (i.e., <40% of wt) also had low TG transfer activity and that the ratio of TG/CE was similar to wt (i.e., 1.00 ± 0.12, mean ± SD, $n = 6$) for most mutants. However,

Table II: Secretion, CE Transfer Activity, and TG/CE Ratio of CETP Mutants

mutant	secretion[a] (ng/plate)	CE transfer specific activity[b]	TG/CE[c]
wt	251 ± 119 (14)	1.00 ± 0.20 (6)	1.00 ± 0.12 (6)
39S:RSF	14 (13, 15)	1.03 (0.92, 1.13)	2.10 ± 1.13 (3)
48A:DR-$(YR)_2$S	29 (33, 25)	0.56 (0.28, 0.84)	0.69 ± 0.12 (3)
48A:EAS	32 (24, 40)	0.01 (0.06, −0.05)	1.04 (0.91, 1.17)
53G:D$(SI)_3$	25 ± 12 (3)	1.46 ± 0.37 (3)	0.90 (0.96, 0.83)
53G:GVP	24 (25, 23)	0.19 (0.38, 0.00)	
75E:EKL	36 ± 17 (3)	0.84 ± 0.19 (3)	
75E:EGT	21 (20, 22)	0.47 (0.73, 0.20)	
149K:KKL	52 (38, 66)	0.70 (0.45, 0.83)	0.59 ± 0.12 (4)
165Q:QGT	19 ± 4 (3)	0.33 ± 0.18 (3)	0.61
165Q:QKL	11 (11, 11)	2.16 (3.04, 1.28)	0.91
177K:KGT	14 ± 3 (3)	0.93 ± 0.52 (3)	0.65 (0.57, 0.72)
195A:GVP	18 ± 2 (4)	0.81 ± 0.28 (3)	0.47
274A:GYP	45 (29, 61)	1.10 (1.80, 0.40)	1.07 ± 0.07 (3)
277A:EAS	39 ± 19 (5)	0.77 ± 0.10 (5)	1.27 ± 0.32 (3)
281G:GVP	17 (13, 22)	0.67 (0.61, 0.72)	
281G:GSL	48 ± 11 (3)	0.58 ± 0.12 (3)	0.30
281G:G$(SI)_3$	65 ± 31 (3)	0.42 ± 0.09 (3)	1.22 (1.13, 1.31)
281G:G$(SI)_4$	12	0.63	1.18 (1.26, 1.09)
319A:GYP	44 (54, 34)	0.90 (0.61, 1.18)	0.98 ± 0.11 (3)
373A:D-$(RY)_2$RS	25 (16, 34)	0.32 (0.37, 0.32)	1.51 (1.13, 1.88)
379K:KGT	62 ± 11 (3)	0.36 ± 0.05 (3)	1.22 ± 0.22 (3)
379K:KKL	40 ± 21 (3)	0.81 ± 0.33 (3)	0.79 (0.75, 0.83)
401S:RSF	58 ± 30 (5)	1.00 ± 0.25 (5)	1.33
407S:RSF	34 ± 16 (3)	1.15 ± 0.29 (3)	1.30 (0.94, 1.66)
474S:RSF	31 ± 13 (4)	1.54 ± 0.63 (4)	0.82 (0.80, 0.83)
476S:RSF	161 ± 80 (3)	1.19 ± 0.18 (3)	1.62 (1.67, 1.57)
476S:R$(SI)_3$	11	0.91	

[a] The values are the mean ± SD for $n \geq 3$ or the mean with the individual values in parentheses for $n = 2$. [b] The specific activity of wt CETP was set at 1 in a set of transfections to obtain the relative specific activities for the mutant proteins, and the variation of wt CETP was estimated to be 1.00 ± 0.20 (494 ± 99 cpm/ng, mean ± SD) from six sets of transfections. The number of independent transfection experiments for each mutant is represented as n. A single activity value in the table represents an average activity value assayed on the media from multiple plates of one mutant in one independent transfection experiment. [c] Values represent TG/CE ratios of the mutant proteins relative to wt CETP [therefore, $(TG/CE)_{wt} = 1$] and were given as mean ± SD for $n \geq 3$.

mutants with insertions at Lys149, Gly281, and Ala195 sites had normal, or near-normal, CE transfer activity (Figure 3) but had somewhat reduced (<60%) TG/CE activity (Table II). In two mutants [373A:D$(RY)_2$RS and 476S:RSF], the TG/CE activity ratio appeared to be increased. Overall, the results suggest a large degree of overlap between the sites influencing CE and TG transfer with some moderate differences in transfer activities revealed at selected sites.

Discussion

Linker insertion mutagenesis has been used to identify sites necessary for normal lipid transfer activity in the primary sequence of CETP. The majority of the mutant proteins with amino acid insertions at various locations in the polypeptide had relatively normal cholesteryl ester transfer activity, indicating considerable tolerance to local structural perturbations. This is consistent with an earlier study where CETP remained fully functional despite cleavage at several sites in the polypeptide backbone by trypsin or chymotrypsin (Hesler et al., 1989). Thus, interruption of the primary sequence by proteolytic cleavage or by linker insertion in many cases results in active protein. Selected insertions in three regions were found to impair CE transfer activity. Insertional locations that impaired TG transfer activity were generally parallel to those of CE transfer activity, although moderate differences in the TG/CE activity ratio were also found at several sites. Hydrophobicity and secondary structure predictions suggest that most of the mutations affecting CE transfer activity probably did not result in globally malfolded proteins. Our analysis suggests that local perturbations in secondary structure as a result of specific amino acid insertions may be the major reason for reduced activity.

The reduced secretion of all mutants compared to wt shows that secretion was more sensitive to insertional alterations than activity. One explanation for these finding would be that all mutants in media were globally malfolded but that neutral lipid transfer activity does not require a correctly folded protein. However, this explanation is considered unlikely since CETP or its fragments expressed in *E. coli*, as well as synthetic peptides of CETP (including the epitope of the neutralizing mAB), are totally inactive in neutral lipid transfer (Hesler et al., 1989). A second explanation would be that the mutants were malfolded in varying degrees and that the levels of secretion and activity were both indicative of the extensiveness of the malfolding. However, a regression analysis of 86 CE transfer specific activity values with their secreted protein mass in media showed no correlation between the two parameters ($r = 0.088$, $P = 0.40$), indicating independence of the activity on the secretion. Thus, this explanation is implausible as a general description of the behavior of mutants.

A third explanation for these findings is that the presence of additional amino acids increased the chance of forming incorrectly folded proteins inside the cell and that the cell possesses a highly selective mechanism to abort the secretion of the incorrectly folded population of each mutant protein. Thus, smaller amounts of the mutant proteins would be correctly, or nearly correctly, folded and secreted. This stochastic explanation most readily explains the striking observation that the majority of the mutant proteins remained active but in each case were less well secreted than wt (Figures 1 and 3 and Table II). The extreme sensitivity of CETP secretion to the effects of mutagenesis could be related to its unusual content of hydrophobic amino acids (Hesler et al., 1987), increasing the probability of exposure of hydrophobic regions in malfolded proteins. Although this theory seems to provide the most cogent, general explanation of the activity–secretion behavior of the mutant proteins, it must still be considered that individual mutants with low activity due to global malfolding may be secreted into medium.

With this explanation of the secretion–activity behavior in mind, the dependence of activity on the specific amino acids introduced at each site could thus indicate (1) local alterations in secondary structure which interfere with function without causing global malfolding, or (2) disruption of side chain interactions which are necessary for catalytic function, or (3) changes in the thermodynamic stability leading to global malfolding, e.g., as a result of introduction of a charged amino acid residue into a core region.

The five mutants with markedly impaired CE transfer activity were located in three regions: (1) amino acids 48–53 (48A:EAS and 53G:GVP); (2) amino acid 165 (165Q:QGT); and (3) amino acids 373–379 [373A:D$(RY)_2$RS and 379K:KGT]. The hydrophobicities of the mutants in these three regions were analyzed by the method of Kyte and Doolittle (1982), and their secondary structures were predicted by the method of Garnier et al. (1978) (Table I). Both sites 48A and 53G are probably located within a single hydrophobic α-helix with the some amphipathic character at its N-terminal end. Charged amino acids were inserted in mutants 48A:DR$(YR)_2$S and 48A:EAS, which caused partial inactivation of 48A:D$(YR)_2$S and complete inactivation of 48A:EAS. The

data could reflect a disruption of overall conformation as a result of replacement of the hydrophobic amino acid alanine with a charged amino acid, or removal of a hydrophobic amino acid necessary for catalytic activity (e.g., as part of a lipid binding site). However, it seems more likely that alteration of the helix by changing its local amphipathic nature (48A:EAS) or by abolition of the secondary structure [48A:DR$(YR)_2$S] results in loss of activity. Mutants 53G:G$(SI)_3$ and 53G:GVP were at the same site, but the former was active and the latter was not (Table II and Figure 3). This would be due to the presence of amino acids with flexible hydrophobic side chains at the end of the α-helix in both wt and 53G:G$(SI)_3$ while 53G:GVP has a proline at the equivalent position. This result further suggests that the predicted helix encompassing 48A and 53G may be important for activity.

The insertional sites of mutants 165Q:QGT and 165Q:QKL are neutral in hydrophobicity and could be either located on the surface or buried inside. Since 165Q:QKL introduced a net charge (K) (Table I) into the sequence, inactivation of CE transfer would probably have been observed for this mutant if this site was in the hydrophobic core sequence. Thus, a surface location is probable for mutants 165Q:QKL and 165Q:QGT. However, mutant 165Q:QKL was active in CE transfer while mutant 165Q:QGT was not. At site 165Q, a turn structure may be needed for activity, as in wt and 165Q:QKL, while loss of this structure (165Q:QGT) might inactivate CE transfer (Table I).

The most interesting mutants were those in the region of amino acids 373 and 379. Both sites are located in a hydrophilic region, probably within the same α-helix at the surface of the CETP molecule (Table I). Mutant 379K:KGT had low activity, whereas mutant 379K;KKL, at the same site, had activity indistinguishable from wt. These two mutants with contrasting activity were predicted to have distinct effects on secondary structure (Table I). Whereas the active mutant did not change the helix, the insertion of GT in the inactive mutant resulted in replacement of the helix by a turn at this site (Table I). The same helix is predicted to be disrupted in the low-activity mutant, 373A:D$(RY)_2$RS (Table I). Comparing the predicted secondary structures of wt, 379K:KKL, 379K:KGT, and 373A:D$(RY)_2$RS in Table I suggests that the KKK cluster may have to form a helical structure to be active (wt and 379K:KKL) while changing KKK into a turn inactivates the protein [373A:D$(RY)_2$RS and 379K:KGT].

The putative hydrophilic helix containing the KKK could help to mediate the binding of CETP to the lipoprotein surface. Earlier studies (Pattnaik & Zilversmit, 1979; Sammett & Tall, 1985) have shown that the interaction between CETP and HDL probably involves ionic interaction. An increase in divalent cation concentrations (Ca^{2+} and Mn^{2+}) disrupted the interaction between CETP and HDL. Also, digestion of HDL with phospholipase C which removes choline phosphate, but not phospholipase A_2, abolished its ability to bind CETP, suggesting involvement of the phospholipid head groups. These earlier studies predicted that some basic amino acid residues on CETP interact with negative charges on the surface of the lipoprotein which initiate or stabilize the binding of CETP to HDL.

Hesler et al. (1989) identified this same cluster of Lys residues (i.e., amino acids 377–379) as one of the two sites of preferred tryptic digestion; trypsin-treated CETP was catalytically active but showed decreased binding to a lipid emulsion. It was speculated that these amino acids might exist in a surface region of CETP which interacts with a negatively charged region of the lipoprotein surface to stabilize the binding of CETP. The properties of the mutants in this region are consistent with this concept and suggest that the cluster of lysines may have to form a helical structure to be active.

Monoclonal antibody TP2 binds to the C-terminus of CETP, and it can inhibit the lipid transfer activity of CETP (Swenson et al., 1989). The epitope of this monoclonal antibody was mapped to the last 26 amino acids of the CETP sequence. The exact amino acids involved in the epitope are not known. There were two insertion–mutation sites in this region, Ser474 and Ser476. The insertion mutants at these two sites exhibited normal CE and TG transfer activities. TP2 appeared to bind to the mutants equally well as it bound to the other mutants with no insertions in the epitope region, as revealed on Western blot. The displacement of native CETP by these mutant proteins in the RIA was parallel in different dilutions of the RIA, suggesting a similar affinity for the mAb. The results suggest that these two residues do not have primary importance in the binding sites of the neutralizing antibody. Thus, the epitope of the neutralizing mAb may be located in the inner portion of the C-terminal 26 amino acids.

Insertion mutagenesis utilized in this study proved to be an effective tool for determination of potential active sites in the primary sequence of CETP. It is likely that many important amino acid residues near but not at the insertion sites have not yet been revealed and that a single insertion, even next to an amino acid residue responsible for some function, might not be sufficient to eliminate an activity. Site-directed mutagenesis will be necessary to further define amino acid residues that are of importance for the various lipid transfer activities of CETP.

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Histone Hyperacetylation Does Not Alter the Positioning or Stability of Phased Nucleosomes on the Mouse Mammary Tumor Virus Long Terminal Repeat

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ABSTRACT: Activation of mouse mammary tumor virus transcription by the hormone-bound glucocorticoid receptor results in disruption of a nucleosome that is specifically positioned on the promoter. Limited treatment of cells with the histone deacetylase inhibitor sodium butyrate prevents receptor-dependent promoter activation and nucleosome disruption [Bresnick, E. H., John, S., Berard, D. S., LeFebvre, P., & Hager, G. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3977-3981]. On the basis of this observation, we undertook a series of experiments to compare the structure of normal and hyperacetylated mouse mammary tumor virus chromatin. Although butyrate prevents hormone-induced restriction enzyme cutting specifically in the B nucleosome region, chromatin containing hyperacetylated histones does not differ from normal chromatin in general sensitivity to restriction enzymes. Indirect end-labeling analysis of micrococcal nuclease digested chromatin reveals that nucleosomes are identically phased on the mouse mammary tumor virus long terminal repeat in normal and hyperacetylated chromatin. A synthetic DNA fragment spanning the B nucleosome region was reconstituted into a monosome by using core particles containing normal or hyperacetylated histones. Analysis of the structure of reconstituted monosomes by nondenaturing polyacrylamide gel electrophoresis, salt stability, thermal stability, restriction enzyme accessibility, and exonuclease III or DNase I footprinting reveals no effect of histone hyperacetylation on monosome structure. These observations suggest that histone hyperacetylation does not induce a major change in the structure of mouse mammary tumor virus chromatin, such as nucleosome unfolding. We propose that inhibition of receptor-dependent nucleosome disruption by butyrate is not dependent upon such a general change in chromatin structure and subtle chromatin modifications may modulate the interaction of glucocorticoid receptor with chromatin-associated recognition sites.

The hormone-bound glucocorticoid receptor (GR)[1] regulates MMTV transcription by binding to specific DNA regulatory elements (GREs) on the MMTV promoter. A complete understanding of this transactivation mechanism will require a definition of how the GR interacts with soluble transcription factors to form competent transcription complexes and how the organization of MMTV regulatory sequences into chromatin impacts transcription initiation and elongation.

The MMTV LTR exists as a highly reproducible chromatin structure containing six specifically positioned nucleosomes (Richard-Foy & Hager, 1987). Although initial nucleosome mapping studies were performed with LTR-reporter chimeras in BPV-based episomes (Richard-Foy & Hager, 1987), recent studies have shown that identical nucleosome positions exist on single copy integrated MMTV proviruses (H. Richard-Foy and G. L. Hager, unpublished data). We are using the BPV-based episomal system (Ostrowski et al., 1983) to address two issues: (i) what are the differences between transcriptionally inactive and active MMTV chromatin and (ii) does the specific nucleosomal organization have an active regulatory role in hormone-dependent transcription?

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[1] Abbreviations: BPV, bovine papillomavirus; DNase I, deoxyribonuclease I; DTT, dithiothreitol; Exo III, exonuclease III; GR, glucocorticoid receptor; GRE, glucocorticoid regulatory element; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; NF1, nuclear factor 1; nuc-B, nucleosome B; PBS, phosphate-buffered saline; TA (triamcinolone acetonide), 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; TFIID, transcription factor IID; 30S, rat retroviral-associated cellular DNA sequences.

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