Point Mutagenesis of Positively Charged Amino Acids of Cholesteryl Ester Transfer Protein: Conserved Residues within the Lipid Transfer/Lipopolysaccharide Binding Protein Gene Family Essential for Function[†,‡]

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ABSTRACT: The cholesteryl ester transfer protein (CETP) binds to plasma lipoproteins and transfers neutral lipids between them. Previous studies showed that lipoprotein binding involves ionic interactions between CETP and lipoproteins, with increased binding of CETP to lipoproteins carrying increased negative charge. In order to understand the molecular determinants of lipoprotein binding in CETP, site-directed mutagenesis was carried out on positively charged amino acids within and outside regions of conserved sequence in the putative family of lipid transfer/lipopolysaccharide (LPS) binding proteins (LT/LBP). Within the conserved regions, two mutant proteins, K233A and R259D, were well secreted by the transfected cells but showed markedly reduced cholesteryl ester transfer activity. Separating the bound from free CETP by gel filtration after incubation with HDL, HDL binding by K233A was found to be impaired, suggesting that the binding deficiency of the mutant may be responsible for decreased transfer activity. Kinetic analysis showed a marked increase in the apparent K_m but no change in V_{max}, consistent with a lipoprotein binding defect. Thus, within CETP, K233 and R259 play an essential role in cholesteryl ester transfer activity probably by mediating binding of CETP to lipoproteins. Sequence alignment of CETP, phospholipid transfer protein, LPS binding protein, and bactericidal permeability-inducing protein showed that K223 and R259 were strictly conserved as positively charged amino acids, suggesting a common function within the LT/LBP gene family.

The plasma cholesteryl ester transfer protein (CETP,[1] M_r 74 000, 476 amino acids) plays an important role in lipoprotein metabolism by exchanging cholesteryl esters (CE) and triglycerides (TG) between high-density lipoproteins (HDL) and very low density lipoproteins (VLDL) (Tall, 1993). In human plasma, most CETP is bound to HDL due to the high molar concentration of HDL and higher affinity for HDL than other lipoproteins (Morton, 1985). By epitope mapping and mutagenesis, the hydrophobic face of a putative carboxy-terminal amphipathic helix (amino acids 465−476) of CETP has been determined to be necessary for CE and TG transfer, while amino acids on the polar face of this helix were found to be required for the normal binding of the neutralizing monoclonal antibody TP2 (Swenson et al., 1989; Wang et al., 1992). However, the HDL binding appears to involve other regions, since TP2 does not inhibit HDL binding (Swenson et al., 1989) and the Δ470−475 deletion mutant that is impaired in CE/TG transfer has nearly normal HDL binding activity (Wang et al., 1992).

The binding of CETP to lipoproteins involves hydrophobic and ionic interactions (Nishida et al., 1993). The binding of CETP to lipoproteins is increased when lipoprotein negative charge is increased. For example, lipolysis increases the binding of CETP to VLDL as a result of enrichment of VLDL with negatively charged fatty acids (Sammett & Tall, 1985). Also, increasing the negative charge of LDL by chemical modification or addition of anionic detergents enhances the binding of CETP to these particles and improves their ability to serve as substrates (Nishida et al., 1993), while removal of phospholipid phosphate groups from the surface of HDL by phospholipase C treatment abolishes binding of CETP (Pattnaik & Zilversmit, 1979). These and other findings suggest that the phospholipid phosphate groups are the primary sites for the interaction of CETP with lipoproteins and that the stability of the complexes increases with the negative charge of the lipoproteins. Thus, there may be an ionic interaction between negatively charged groups on lipoproteins and positively charged amino acids on CETP.

There are 36 lysines and arginines scattered almost evenly in the sequence of CETP (Drayna et al., 1987). The human CETP sequence is similar to human lipopolysaccharide binding protein (LBP), bactericidal/permeability-increasing protein (BPI), and phospholipid transfer protein (PLTP), with 24%, 26%, and 20% identity, respectively (Day et al., 1994). Since these proteins all bind lipids, we reasoned that the

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[‡] The sequences of CETP, BPI, LBP, and PLTP have been submitted to GenBank under Accession Numbers M30185, J04739, M35533, and L26232, respectively.

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[1] Abbreviations: BPI, bactericidal/permeability increasing protein; CE, cholesteryl ester; C-terminal, carboxy terminal; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein(s); LBP, lipopolysaccharide binding protein; LDL, low-density lipoprotein(s); LPS, lipopolysaccharide; PLTP, phospholipid transfer protein; TG, triglyceride(s); VLDL, very low density lipoprotein(s).

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regions of conserved sequence among these proteins should contain structural elements that enable lipid binding. In particular, one or more positively charged amino acids located at homologous positions in these proteins may be responsible for the binding of CETP to lipoproteins.

EXPERIMENTAL PROCEDURES

Mutagenesis. CETP mutants were generated according to the method of Eckstein and colleagues (Taylor et al., 1985; Sayer et al., 1988) using the Amersham mutagenesis system. Single-stranded DNA was prepared from *Escherichia coli* DH5aF'IQ (Gibco/BRL) transformed with the pBlueScript KS- vector (Stratagene) containing the CETP cDNA, using helper phage VCS-M13 (Stratagene), and served as the template for the mutagenesis. Oligonucleotides (approximately 40 bases long) containing appropriate changes for silent mutations that created or deleted a restriction site were used. Mutant clones were screened by restriction digestion and confirmed by DNA sequencing using the dideoxy sequencing method (Sanger et al., 1977). The *XbaI*−*Hin*dIII fragment (for R14A), *Eco*RV fragment (for K47E&K56E, K176E, K233D&K239D, K233A, K239A, H232A, R259D, and H271A) and *Bfr*I−*Hin*dIII fragment (for K377A&K378A&K379A), containing the mutagenized site and sequenced in its entirety, were transferred from the KS$^-$-CETP cDNA to pCMV4-CETP(wt) expression vector (Andersson et al., 1989) to replace the corresponding fragment in the wt cDNA.

Transfection of COS7 Cells. Transient expression was performed as described (Wang et al., 1992). CETP was quantitated in media by immunoassay (Wang et al., 1992), using the CETP mAb TP2 with a C-terminal epitope (Swenson et al., 1989) or the mAb TP9 and TP18.[2] CE transfer activity in media was measured as described (Morton & Zilversmit, 1979).

HDL Binding Assay. Wild-type CETP or the K233A mutant was secreted by transiently transfected COS7 cells. HDL (100 μg of protein) was incubated with wild-type or mutant CETP (2 μg, estimated from slot-blotting) at 37 °C for 1 h in a buffer containing 50 mM Tris-CH, pH 7.4, 150 mM NaCl, 1 mM EDTA (TSE) containing 0.25 mM DTT in a final volume of 300 μL. The mixture was then loaded on a Sephadex G-150 column (0.9 × 50 cm) which was equilibrated with TSE. To locate the position of HDL and CETP on the column, HDL (100 μg of protein) and CETP (5 μg) were also run on the column separately. To localize fractions containing CETP, 80 μL of each 500 μL fraction was analyzed by SDS−reducing polyacrylamide gradient gel (4−20%) electrophoresis, followed by immunoblotting and detection with TP2 as described above.

Kinetic Analysis. HDL containing [^{3}H]cholesteryl ester and LDL were prepared as described previously (Morton & Zilversmit, 1979). CE transfer rates were measured by incubating 5 ng of CETP with radiolabeled CE-containing HDL and unlabeled LDL for 1 h, in 30 μL of 50 mM Tris, 150 mM NaCl, and 2 mM EDTA, pH 7.5, then precipitating the LDL by the heparin/Mn method (Swenson et al., 1989), and counting an aliquot of the supernatant. The transfer rates were corrected for the decrease in the specific activity of the CE during transfer by the first-order rate expression (Barter & Jones, 1980).

Sequence Analysis Methods. The sequences of CETP, BPI, LBP, and PLTP (Genbank Accession Numbers M30185, J04739, M35533, and L26232, respectively) were aligned using the MACAW program, that allows the location, analysis, and assessment of statistical significance of regions of local similarity among multiple protein sequences (Schuler et al., 1991).

RESULTS

We used the multiple sequence alignment program MACAW to find regions of local similarity among members of the LT/LBP family. Sequence alignment of CETP, LBP, BPI, and PLTP revealed significant homology ($p < 10^{-19}$) in five sequence blocks within the more N-terminal region (Figure 1). Outside these blocks there were regions that showed significant three-way similarity between LBP, BPI, and PLTP ($p < 10^{-12}$), and two-way similarity between LBP and BPI ($p < 10^{-2}$), but no similarity involving CETP. Thus, the homology of CETP to other LT/LBP gene family members was confined to the more N-terminal region.

In order to identify positively charged residues in CETP that might be involved in ionic interactions with lipoproteins, we carried out mutagenesis studies on lysine or arginine residues within the blocks of conserved sequences. Initially, four mutants, K47E&K56E, K176E, K233D&K239D, and R259D, were constructed with the purpose of creating large charge modifications at the indicated residues. CETP secreted into the medium by the transfected COS cells was assayed for CE transfer activity and CETP mass (Table 1). The K233D&K239D double mutant and the R259D mutant were found to have markedly decreased specific activity. Subsequently, in order to assess whether K233 or R239 was responsible for the decrease in transfer activity in the double mutant, we constructed two more mutants with milder charge modification, converting each of these residues separately to alanines. K233 but not K239 was found to be the critical amino acid (Table 1). An attempt to obtain an R259A mutant was unsuccessful. Further mutants outside of the conserved sequences were also constructed. In summary, 12 single or double point mutants affecting 9 lysines, 2 arginines, and 2 histidines were prepared and characterized. Of these mutants, seven were located within the sequence blocks that showed very significant similarity ($p < 10^{-19}$) among human CETP, LBP, BPI, and PLTP (Figure 1). Residues whose mutation led to decreased specific activity (K233 and R259) were in the conserved blocks and were strictly maintained as positive charges. All mutants except H232A, K377A&K378A&K379A, and K377A were relatively well secreted.

To verify that the conformations of K233A and R259D were not altered in a more subtle way that would not have interfered with secretion, we tested the binding of three anti-CETP monoclonal antibodies with different specificities to them. The mAbs TP2, TP9, and TP18 bound K233A, R259D, and wild-type CETP with similar affinities (Table 2), suggesting that the structure of the mutants was not altered significantly.

Because K233A has a milder change (from lysine to alanine) compared to that of R259D (from arginine to aspartic acid), it was chosen for a more detailed characterization.

[2] R. Milne, A. Tall, and X.-C. Jiang, unpublished results.

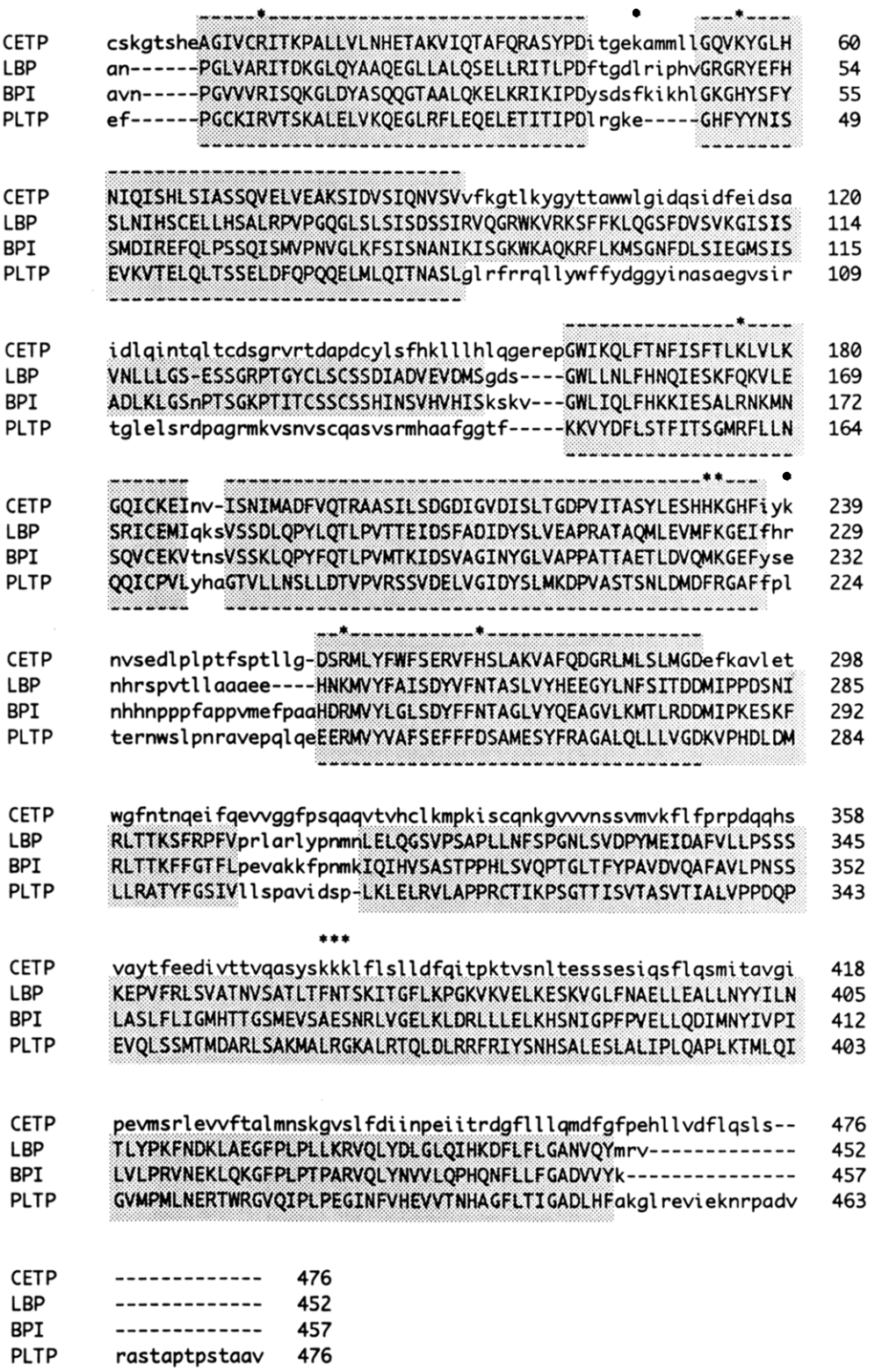

FIGURE 1: Multiple sequence alignment of CETP, BPI, LBP, and PLTP. The amino acids that were mutagenized in this study are indicated by stars above the CETP sequence. Where four sequences show significant similarity, lines are drawn above and below the alignment. Regions where two or more sequences show significant similarity to each other are shaded.

Separating the HDL-bound CETP from free CETP by gel filtration, we found that only about 40% of the K233A mutant was bound to HDL (Figure 2A), compared to 95% of wild-type CETP (Figure 2B), indicating a binding defect of the mutant. In separate experiments, mixtures of HDL and either WT or K233A mutant were analyzed by native gradient gel electrophoresis followed by Western blotting to determine the distribution of CETP in the HDL-bound and free form. Wild-type CETP migrated with HDL while the mutant formed a smear, suggesting dissociation from HDL during the gel run (data not shown).

The K233 mutant was characterized further in a kinetic analysis of CE transfer from HDL to LDL. When the HDL concentration was varied while keeping the LDL concentration constant, the mutant's defect in CE transfer was overcome by increasing HDL concentrations (Figure 3). An

Table 1: Characterization of Mutant CETP Proteins[a]

mutant	CE transfer (%)	protein mass (%)	specific activity (%)
wild type	100	100	100
R14A	92 (89, 95)	91 (90, 92)	100 (99, 101)
K47E&K56E	70 (65, 75)	82 (78, 86)	89 (90, 88)
K176E	75 (81, 69)	50 (56, 44)	110 (121, 99)
K233D&K239D	2 (1, 3)	25 (21, 29)	6 (4, 8)
K233A	16 ± 9	72 ± 12	22 ± 3, $n = 6$
K239A	82 ± 11	92 ± 14	90 ± 19, $n = 4$
H232A	4 ± 2	9 ± 3	45 ± 12, $n = 3$
R259D	10 ± 7	41 ± 14	21 ± 12, $n = 5$
H271A	81 ± 21	83 ± 15	97 ± 22, $n = 4$
K377A	0 (0, 0)	0 (0, 0)	NA[b]
K378A	16 ± 8	23 ± 9	73 ± 16, $n = 4$
K379A	26 ± 9	28 ± 15	91 ± 12, $n = 4$
K377A&K378A&K379A	3 (2, 4)	3 (2, 4)	NA

[a] The values for the mutants are given relative to that of wild-type CETP in one transfection (15). Each value is the average from 2−6 independent transfections, and for each transfection, multiple assays (3−4) were conducted. [b] Not applicable, because of the low value for CETP mass in the culture medium.

Table 2: Relative Binding Strength of Monoclonal Antibodies TP2, TP9, and TP18 to K233A, R259D, and Wild-Type CETP[a]

antibody	epitope[c]	K233A	R259D	wild type
TP2	450−476	++[b]	+++	+++
TP9	410−450	+++	++	+++
TP18	183−261	+++	++	++

[a] Medium from cells expressing wild-type or mutant CETP was assayed for CETP mass by slot-blotting on nitrocellulose with the indicated antibodies (see Experimental Procedures). [b] A relative binding strength of "++" is 50−75% of that designated "+++". [c] Position of epitope within the 476 amino acid CETP sequence.

experiment done at constant [HDL] with variable LDL concentrations gave a similar result (not shown). The apparent K_m and V_{max} values calculated from these two experiment (Table 3) indicate that the K_m of K233A is significantly higher for both HDL (about 6-fold) and LDL (about 4-fold) while its V_{max} is essentially unaltered.

DISCUSSION

Using homology within the LT/LBP gene family as a guide, we have identified two residues in CETP that are essential for normal CE transfer activity, i.e., K233 and R259. The cause of the decrease in activity appears to be a defect in lipoprotein binding, suggesting that these positively charged residues are involved in binding CETP to the lipoprotein surface. An alternative explanation is that the residues are involved in maintaining a tertiary structure of CETP that is involved in lipoprotein binding.

These positively charged residues in CETP are strong candidates for amino acids involved in ionic interactions with the lipoprotein phospholipid head groups (Pattnaik & Zilversmit, 1979). A single ionic interaction is likely to contribute about 1.4 kcal/mol to the binding energy, equivalent to a 10-fold change in K_d (Kim et al., 1991). Thus, it is plausible that the 4−6-fold increase in the K_d of the K233A mutant results from the loss of an ionic bond stabilizing lipoprotein binding (Table 3). The loss of activity seen in the K233A and R259 mutants is unlikely to be due to a structural alteration in the protein since they were relatively well secreted by transfected cells. Prior experiments have shown that cellular secretion of CETP appears to be highly

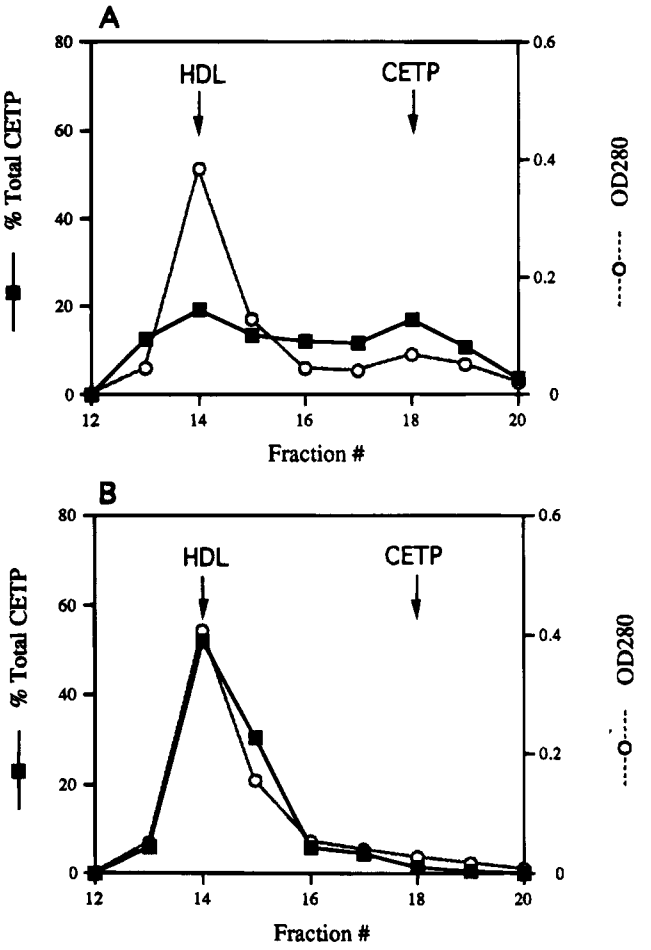

FIGURE 2: HDL binding capacity of wild-type and mutant (K233A) CETP. HDL binding assay for wild-type (A) and the K233A mutant CETP (B) was performed as detailed under Experimental Procedures. The data are representative of two similar experiments.

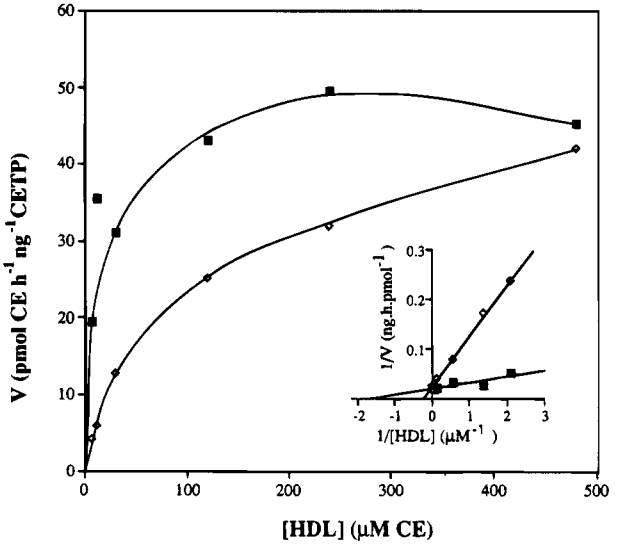

FIGURE 3: Transfer of cholesteryl ester from HDL to LDL by wild-type and K233A mutant CETP. Transfer assays were done at varying [HDL] and constant [LDL] (36 μM LDL-CE). The inset shows the double-reciprocal plot of the data.

sensitive to changes in structure (Wang et al., 1991). In the case of the K233A mutation, the substitution is likely to produce mild perturbation of protein structure. Positively charged amino acids are typically found on the protein surface (Bowie et al., 1990), and substitutions of alanine are

Table 3: Kinetic Parameters of CE Transfer from HDL to LDL by Wild Type and the K233 Mutant of CETP[a]

CETP	K_m (μM CE)	V_{max} (pmol of CE·h^{-1}·ng^{-1})	K_m (μM CE)	V_{max} (pmol of CE·h^{-1}·ng^{-1})
wild type	12 (14, 10)	52 (48, 56)	17 (16, 18)	87 (83, 91)
K233A	80 (84, 76)	48 (42, 53)	64 (59, 69)	67 (67, 67)

[a] Assays were done as detailed under Experimental Procedures by varying the concentration of the indicated lipoprotein and keeping the other one constant at 36 μM CE. The apparent K_m and V_{max} values were determined from double-reciprocal plots of the data. The numbers are the means of results (in parentheses) from two experiments.

considered to be relatively nonperturbing (Cunningham & Wells, 1989). Also, the defect of the K233A mutant was overcome by increasing substrate concentration (Figure 3), making a global folding defect unlikely. Furthermore, the interaction of the K233A and R259D mutants with a panel of CETP mAbs showed the same reaction as WT CETP, suggesting an identical folding pattern. In contrast to these findings, the mutants with markedly impaired secretion (H232A and K277A) probably had major alterations in their secondary structure or folding responsible for low activity and secretion. This may explain the low activity of a linker insertion mutant involving the lysine triplet (KKK 377−379) (Wang et al., 1991). Although this was suggested to be a candidate region for an interfacial recognition site, this was not confirmed herein.

CETP is a surface-active molecule that forms protein monolayers at air−water interfaces (Ohnishi et al., 1994), inserts into lipid monolayers (Weinberg et al., 1994), and binds to lipoproteins, emulsions (Hesler et al., 1987), and vesicles (Ohnishi et al., 1994). However, CETP is highly stable in solution (Hesler et al., 1989), and the binding of CETP to lipoproteins probably does not involve extensive unfolding as it does for apolipoproteins since the increase in surface pressure upon binding to a lipid monolayer is less than for apolipoproteins (Weinberg et al., 1994). It is likely that the surface of the CETP molecule interacting with HDL contains several ionic and hydrophobic residues (Nishida et al., 1993). Amino acids involved in the hydrophobic interactions mediating lipoprotein binding may also be near K233 and R259 within the folded structure of CETP. One candidate region for HDL binding is the C-terminus, since large deletions in this region (451−476) result in decreased HDL binding (Au-Young & Fielding, 1992) and the binding of the mAb TP2 to the polar face of a putative C-terminal amphipathic helix enhances binding of CETP to lipoproteins possibly as a result of exposure of a hydrophobic region (Swenson et al., 1989). Monoclonal antibody epitope mapping studies suggest that K233 and R259 are in the vicinity of the C-terminal domain in the folded structure.[2] Thus, the putative lipoprotein binding surface could involve both the region around K233 and R259 as well as the C-terminal region. Hydrophobic residues in the C-terminus are also necessary for neutral lipid binding by CETP (Wang et al., 1995). Binding of CETP to HDL could be followed by entry of CE into a contiguous lipid binding site.

An alternative interpretation of the results is that K233 and R259, rather than directly binding HDL, are acting in an indirect manner, by joining two domains of CETP that are involved in HDL binding. Such a situation exists with apolipoprotein E isoforms in which ionic interactions between two domains in the protein influence the isoform specificity of lipoprotein binding (Dong et al., 1994). Since K233 and R259 are conserved in the gene family, it would be expected that if they are each part of a salt bridge, the negatively charged residues they would bind to would also be conserved. One such conserved residue, E426, is close to the putative HDL binding site in the C-terminus, and may be a potential partner for one of the two critical positively charged residues we identified.

The low-activity mutants of Lys233 and Arg259 were found within regions of conserved sequence and were absolutely conserved as positively charged residues within the lipid transfer/LPS binding protein family, indicating conservation of structure and function in these regions within the gene family. The conserved function that results from conserved structural elements within the N-terminal region of these proteins may be either LPS binding, phospholipid binding, or membrane binding. Conversely, distinctive functions such as the ability to transfer neutral lipids would be expected to map to the less conserved regions, particularly in the carboxy terminus. Although LBP, BPI, and CETP bind LPS with high affinity (Tobias et al., 1986; Marra et al., 1990), this function appears to be contained in the N-terminal 25 kDa of LBP and BPI (Theofan et al., 1994; Gazzano-Santoro et al., 1992), a fragment that corresponds to the first 200 amino acids of CETP. Therefore, K233 and R259 are probably not involved in LPS binding. On the other hand, these four proteins all have some affinity for lipoproteins or phospholipid membranes. CETP and PLTP are well-known as HDL-associated proteins (Morton, 1985; Tall et al., 1982), and BPI is found associated with the membranes of azurophile granules of myeloid cells (Egesten et al., 1994). Although initially described as a component of lipoprotein-free plasma, LBP was recently shown also to be HDL-associated by apoA-I immunoaffinity chromatography (Wurfel et al., 1994). The lipoprotein binding defect of the K233A mutant suggests that lipoprotein or membrane binding is the conserved function that is implied by the conservation of these two positively charged residues.

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