The cholesteryl ester transfer protein (CETP; 476 amino acids) mediates the transfer of neutral lipids and phospholipids between plasma lipoproteins. Previous studies showed that the epitope of a neutralizing monoclonal antibody (mAb) was located within the C-terminal 26 amino acids (aa) of CETP. To determine possible involvement of this region in lipid transfer activities, we generated six deletion mutants between Arg-451 and Leu-475 by in vitro mutagenesis and expressed mutant proteins in mammalian cells. Only deletion mutants between aa Phe-463 and Leu-475 failed to bind TP2; these mutant proteins were well secreted by cells but showed markedly reduced cholesteryl ester transfer activity. One of the deletion mutants (A470-475) showed similar reductions in cholesteryl ester and triglyceride transfer activities but normal or increased phospholipid transfer activity. Limited proteolysis of this mutant protein indicated a similar overall folding pattern to the wild-type protein. Thus, aa between Phe-463 and Leu-475 are necessary for binding TP2. Deletions within this sequence selectively impair neutral lipid transfer activity, suggesting a direct involvement in neutral lipid transfer.

The plasma CETP is a hydrophobic glycoprotein (M, 74,000) which mediates the transfer and exchange of neutral lipids and phospholipids between the plasma lipoproteins (1, 2) and plays an important role in the catabolism of HDL cholesterol esters (CE) (3, 4). Although there is kinetic evidence to support both carrier-mediated (5) and collisional (6) mechanisms of lipid transfer, the detailed mechanisms and the relationship of function to the structure of CETP are poorly understood. An essential step in the lipid transfer mediated by CETP is the binding of CETP to plasma lipoproteins (2, 7). Also, the CETP itself has binding sites for neutral lipids and phospholipids which can exchange with lipids of lipoproteins (8). Earlier studies identified a monoclonal antibody (mAb), TP2, that could neutralize CE transfer activity, and the epitope of the antibody was localized to the C-terminal 26 amino acids (aa) of CETP (9). However, mAbs are large molecules which often act by indirect mechanisms. In order to determine if aa within the C-terminal region are directly involved in lipid transfer activity we have constructed a series of small deletion mutants and tested them for TP2 binding and lipid transfer activity. The results indicate a defined region of aa mediating binding of TP2 and necessary for neutral lipid transfer activity. The sequence implicated by our studies is different from that suggested in a recent report (10).

**MATERIALS AND METHODS**

HDL, HDL₂, (d = 1.11-1.21 g/ml) containing [3H]cholesteryl ester, and LDL (d = 1.02-1.063 g/ml) were prepared and characterized as described previously (11). HDL₂ containing l-a-dipalmitoyl-[2-palmitoyl-9,10-3H]phosphatidylethanolamine ([H]PC) or [cholesteryl-1,2,6,7-3H]cholesteryl linoleate/[carboxyl-14C]triolein ([H]CE/[C]TG) or l-a-dioleoyl-1,1-14C]phosphatidylcholine/[oleate-1-14C]cholesteryl ([H]PC/[C]CE) (Du Pont New England Nuclear) were prepared as described (2).

**Mutagenesis—** CETP mutants were generated according the method of Eckstein and colleagues (12, 13) using the Amersham mutagenesis system. Single-stranded DNA was made from E. coli DH5αF′IQ (Gibco/BRL) transformed with ks (Stratagene) containing CETP cDNA with helper phage VGS-M13 (Stratagene) and served as the template for the mutagenesis. Oligonucleotides (approximately 40 bases) containing appropriate changes for loop-out deletion mutagenesis, in addition to 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 (14). The BfrI-HindIII fragment, containing the mutagenized site and sequenced in its entirety, was transferred from the ks'-CETP cDNA to pCMV4-CETP expression vector (15).

**Transformation of COS7 Cell—** Transient expression was performed as described (16) except that 3 ml of serum-free Opti-MEM medium (Gibco/BRL) was used for the 3-h incubation with chloroquine. To measure CETP mass, different amounts of medium (0.1-0.5 ml) from mutant or wt CETP were blotted onto nitrocellulose filters using a slot blower. The blot was immobilized and detected with an enhanced chemiluminescence detection kit (ECL, Amersham Corp.) using TP2 or TP14 as primary antibody and an anti-mouse g F(ab')₂ linked to horseradish peroxidase (Amersham Corp.) as secondary antibody. Films were quantitated by laser densitometry. Assay for CE transfer activity of CETP from transfected COS7 cells was performed as described (16).
Δ470–475, or pCMV4 alone (30 μg) were mixed with vector pLAY (10 μg) containing a mouse dhfr cDNA under control of a β-globin promoter (17). The mixture was added to a calcium phosphate solution, and this transfection solution was then added dropwise to the medium of the CHO cells. After continuous growth for 24 h, the medium with DNA was removed and cells were washed with phosphate-buffered saline. Selective medium (Ham's F-12 lacking thymidine and hypoxanthine, 10% dialyzed fetal bovine serum) was applied.

The minor band below CETP in Fig. 1 was due to nonspecific binding by a monoclonal antibody with an epitope of sequences between Glu-465 and Leu-475 in CE transfer activity.

The specific activity was the ratio of the CE transfer activity/mg of CETP/mg of medium maintained in selection medium and then adapted to serum-free Opti-MEM medium.

Limited Protease Digestion of CETP—CETP in 3 ml of COS7 cell medium was digested by Staphylococcus aureus V8 protease (Proteins, Inc.) in the presence of 50 mM NH4HCO3, pH 8.5, for 1 h at 37 °C. The reaction was stopped by the addition of 5 μl of 10 mg/ml soybean trypsin inhibitor, and the mixture was dried of reaction was 350 μl. The reaction was stopped with protease inhibitors (0.4 mM phenylmethylsulfonyl fluoride, 1 pg/ml leupeptin, 1.4 pg/ml pepstatin, and 2 mM EDTA-Na2). Salt concentration was raised to 3 M by adding NaCl, and 30 μl of butyl-Toyopearl (M650) beads (Toyosha, Philadelphia, PA) were added. The mix was end-to-end rotated overnight, and proteins adsorbed on the beads were eluted with SDS sample buffer and run on 12% SDS-PAGE (18). Trypsin (Sigma) digestion was performed similarly except wt and Δ470–475 in Opti-MEM medium secreted by CHO cells were used, and the total volume of reaction was 350 μl. The reaction was stopped by the addition of 5 μl of 10 mg/ml soybean trypsin inhibitor, and the mixture was dried under vacuum and dissolved in 60 μl of SDS-PAGE sample buffer. Sample (25 μl) was loaded onto 4–20% gradient SDS-PAGE. After electrophoresis the gels were Western-blotted and detected with TP2 or TP14 using ECL.

RESULTS

Six deletion mutants within the C terminus of CETP were prepared by in vitro mutagenesis and transiently expressed in COS7 cells (Fig. 1). A monoclonal antibody with an epitope outside the C terminus of CETP (TP14) readily detected all the mutant proteins in lysates of COS cells at a similar level to wt CETP (Fig. 2A). However, the same proteins were not uniformly detected by the neutralizing mAb TP2 (Fig. 2B). The four overlapping deletion mutants toward the C terminus (Δ470–475, Δ467–475, Δ465–475, and Δ463–469) showed no significant binding of TP2. However, the two innermost deletion mutants (Δ451–456, Δ457–462) displayed normal TP2 binding. A point mutant of aa 476 showed normal binding of TP2, indicating no involvement of the side chain of aa 476 in anti-TP2 binding (not shown). The results are summarized in Table I and indicate that mAb TP2 binds primarily to aa 463–475 within the C-terminal 26 residues.

CETP secreted into the medium by the transfected COS7 cells was assayed for CE transfer activity (Table I). All deletion mutants had low activity in the media (<25% of that of wt CETP). However, only mutants Δ470–475, Δ467–475, and Δ465–475 were secreted by COS7 cells at a level comparable with that of wt. The specific activities of these mutant proteins were 4–24% of that of wt, suggesting a direct involvement of sequences between Glu-465 and Leu-475 in CE transfer. The other three mutants with deletions between Arg-451 and Val-469 were not secreted at a high enough level for a confident determination of specific activity. The very low levels of secretion suggest that deletion of these sequences results in malfolded CETP molecules.

In contrast, PC, and Val-469 were not secreted at a high enough level for a confident determination of specific activity. The very low levels of secretion suggest that deletion of these sequences results in malfolded CETP molecules.

To carry out more detailed characterization of deletion mutants with low specific activity, the Δ470–475 mutant was stably expressed in CHO cells, and the deletion mutant protein was collected in defined (low protein) medium. The ability to transfer different lipids from HDL to LDL was tested using HDLs containing different radiolabeled lipids (Table II). Using a dual labeled substrate, the deletion mutant protein showed marked decreases in both cholesteryl ester and triglyceride transfer activities (Table II). In contrast, PC transfer activity of the mutant protein was normal or increased using HDLs containing radiolabeled phospholipids with saturated or unsaturated fatty acids, respectively (Table II). Similar results were obtained whether lipoproteins were separated by ultracentrifugation or by precipitation; the ac-

**TABLE I**

| Protein mass in medium | Specific activity | TP2 reactivity |
|------------------------|------------------|---------------|
| %                      | %                | %             |
| wt                     | 100              | 100           | 100 |
| Δ470–475               | 20               | 83            | 24  |
| Δ467–475               | 9                | 40            | 19  |
| Δ465–475               | 2                | 42            | 4   |
| Δ463–469               | 7                | 5             | UD  |
| Δ457–462               | 12               | 13            | UD  |
| Δ451–456               | 12               | 13            | UD  |

a Relative CE transfer by wt and mutants. The activity for wt was 1535 ± 675 cpm/100 μl of medium; n = 8 transfections.

b Relative CETP mass of wt and mutants detected with TP14. The mass of wt in the medium was 77 ± 33 ng/plate (mean ± S.D., n = 8).

c The specific activity was the ratio of the CE transfer activity/CE mass. The specific activity of wt was 1594 ± 701 cpm/ng of CETP (mean ± S.D., n = 8) with a 15-h incubation. UD, unable to determine due to low secretion by COS7 cells.

d Transfected COS7 cell lysates were assayed for TP2 binding activity as described in Fig. 2 and quantitated by densitometry. The values were normalized for mass of CETP determined by TP14.

**FIG. 1.** CETP C-terminal amino acid sequence (20) and deletion mutants. The boxes under the wt sequence represent the missing aa.

**FIG. 2.** Western blots of CETP deletion mutant proteins immunostained by mAb TP14 (A) and TP2 (B). Transfected COS7 cells were collected and resuspended in 1 ml of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA in the presence of protease inhibitors. After brief sonication to break the cells and low speed centrifugation to remove cell debris, 50 μl of the cell lysates were dried and electrophoresed on 12% SDS-PAGE. The gels were blotted to nitrocellulose filter papers and detected by the ECL method with TP14 or TP2. The minor band below CETP in A was due to nonspecific binding by TP14 because it also appeared in the −DNA sample.
TABLE II

| Substrate in HDL | Lipid transfer activity | %tot |
|------------------|-------------------------|------|
| A. "H]CE/HDL     | 26 ± 10 (n = 4)         |      |
| B. "H]CE/["C]TG/HDL | 18 ± 3 (33)          |      |
| C. "H]DOPC/["C]CE/HDL* | 7 ± 1 (4, 10)  |      |
| D. "H]DPPC/HDL*  | 86 ± 14 (n = 4)        |      |

* DOPC, dioleoylphosphatidylcholine.
* DPPC, dipalmitoylphosphatidylcholine.
* Values are averages of two determinations in parentheses.

**FIG. 3. Protease digestion patterns of CETP, wt, and deletion mutant 470–475.** Limited digestions by S. aureus V8 protease (A) and trypsin (B). See “Materials and Methods” for details.

Activity was abolished by incubation at 0 °C. Thus, the mutant protein (Δ470–475) displayed authentic PC transfer activity but had a selective defect in neutral lipid transfer activity.

The decreased specific activity of the deletion mutants (Table I) could have resulted from incorrect folding. To assess this possibility, the inactive mutant Δ470–475 and wt CETP were subject to limited protease digestions by S. aureus V8 protease and trypsin (Fig. 3). The digestion patterns between the mutant and wt CETP were very similar, suggesting a correct folding of the mutant proteins.

**DISCUSSION**

In the present study we have localized a major portion of the epitope of the neutralizing mAb TP2 to a region between aa Phe-463 and Leu-475 of CETP. An antibody may alter the activity of an enzyme by binding directly to an active site or by indirect mechanisms. An antibody molecule could cover the epitope of the neutralizing mAb TP2 to a region between aa 465–475 (Tables I and II) and was used as the blank. Reactions were incubated with the presence of 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA. Medium of CHO cells mock-transfected with pCMV4 and pLAY had no detectable PC, CE, or TG transfer activity and was used as the blank.

Different lipid transfer activities of the CETP deletion mutant protein (aa 470–475)

CHO cell-produced wt CETP or mutant (Δ470–475) protein (12 ng/assay) was mixed with radiolabeled HDL (approximately 10,000 cpm/assay) and LDL (80 μg of protein) in a total volume of 270 μl in the presence of 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA. Medium of CHO cells mock-transfected with pCMV4 and pLAY had no detectable PC, CE, or TG transfer activity and was used as the blank. Reactions were incubated with slow shaking at 37 °C for 3 h. n represents the number of independent determinations, and each determination (±S.D.) is average of 2–4 assays.

A specific involvement of the epitope sequences in neutral lipid transfer is further indicated by normal or increased phospholipid transfer activity of the deletion mutant protein (Δ470–475) (Table II). Even though the mAb TP2 partly inhibits PC transfer activity of CETP (9) this is presumably by an indirect mechanism. The normal phospholipid transfer activity indicates that distinct regions of CETP are involved in neutral lipid and phospholipid transfer, and tends to exclude a mechanism of CETP activity that involves a general mixing of lipoprotein components, such as lipoprotein fusion. The results also suggest that the mechanism of impaired neutral lipid transfer activity is not due to a general defect, such as malfolding or loss of a lipoprotein binding site (10). The similar effects of deletions on CE and TG transfer activities suggest a common mechanism of transfer, consistent with the results of an earlier mutagenesis study (16).

Other evidence also indicated that the reduced neutral lipid transfer activity resulting from C-terminal deletions was not the result of global malfolding. Limited proteolysis with trypsin and S. aureus V8 protease showed very similar digestion patterns for wt and deletion mutants, suggesting a similar distribution of accessible charged residues. Also, deletion mutants between aa 465–475 were well secreted by COS cells. In an earlier study we showed that secretion of CETP by COS 9 cells was highly sensitive to small changes in structure, such as insertions of two aa (16). By contrast, the surprisingly good secretion of the C-terminal deletion mutants indicates that this region can be removed without detriment to the protein globule, suggesting that it forms a flexible tail or flap on the CETP molecule.

Recently, it was suggested that aa 454–457 of CETP are involved in the catalysis of neutral lipid transfer by mediating the normal binding of CETP to lipoproteins (10). This result is to be contrasted to the findings in the present study, implicating aa 465–475 in neutral lipid transfer activity. The conclusions by Au-Young and Fielding (10) were based on results obtained by expression of three very large deletion mutants (deletions of 66, 48, and 26 aa) in insect cells using a baculovirus expression vector. We had previously reported that several different large deletions in different parts of the CETP sequence resulted in poorly secreted inactive proteins; such large deletions were felt to result in uninformative global folding defects (16). Our results showing very poor secretion of much smaller deletions involving aa 454–457 implicated in the recent work (10) suggest that some of these amino acids play a structural role in the CETP molecule.

It is uncertain how aa 470–475 are involved in the catalysis of neutral lipid transfer. This region is predicted to consist of the normal binding of CETP to lipoproteins (10). This result is to be contrasted to the findings in the present study, implicating aa 465–475 in neutral lipid transfer activity. The conclusions by Au-Young and Fielding (10) were based on results obtained by expression of three very large deletion mutants (deletions of 66, 48, and 26 aa) in insect cells using a baculovirus expression vector. We had previously reported that several different large deletions in different parts of the CETP sequence resulted in poorly secreted inactive proteins; such large deletions were felt to result in uninformative global folding defects (16). Our results showing very poor secretion of much smaller deletions involving aa 454–457 implicated in the recent work (10) suggest that some of these amino acids play a structural role in the CETP molecule.

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