Immunochemical evidence that cholesteryl ester transfer protein and bactericidal/permeability-increasing protein share a similar tertiary structure

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(RECEIVED June 29, 1999; ACCEPTED August 9, 1999)

Abstract

Cholesteryl ester transfer protein (CETP) plays an important role in plasma lipoprotein metabolism through its ability to transfer cholesteryl ester, triglyceride, and phospholipid between lipoproteins. CETP is a member of a gene family that also includes bactericidal/permeability-increasing protein (BPI). The crystal structure of BPI shows it to be composed of two domains that share a similar structural fold that includes an apolar ligand-binding pocket. As structurally important residues are conserved between BPI and CETP, it is thought that CETP and BPI may have a similar overall conformation. We have previously proposed a model of CETP structure based on the binding characteristics of anti-CETP monoclonal antibodies (mAbs). We now present a refined epitope map of CETP that has been adapted to a structural model of CETP that uses the atomic coordinates of BPI. Four epitopes composed of CETP residues 215–219, 219–223, 223–227, and 444–450, respectively, are predicted to be situated on the external surface of the central β -sheet and a fifth epitope (residues 225–258) on an extended linker that connects the two domains of the molecule. Three other epitopes, residues 317–331, 360–366, and 393–410, would form part of the putative carboxy-terminal β -barrel. The ability of the corresponding mAbs to compete for binding to CETP is consistent with the proximity of the respective epitopes in the model. These results thus provide experimental evidence that is consistent with CETP and BPI having similar surface topologies.

Keywords: bactericidal/permeability-increasing protein; cholesteryl ester transfer protein; epitope; molecular models; monoclonal antibodies

Human plasma contains two lipid transfer proteins: cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). CETP is responsible for all of the cholesteryl ester and triglyceride transfer activity and for about 50% of the protein-mediated phospholipid transfer activity in human plasma (Tall et al., 1983; Hesler et al., 1988). The non-CETP-mediated, facilitated phospholipid transfer activity in plasma has been attributed to PLTP (Tall et al., 1983; Tollefson et al., 1988). Because the cDNA encoding human CETP was cloned and characterized in 1987 (Drayna et al., 1987), there has been a major effort to elu-

cidate the structure, biology, and clinical relevance of CETP (Bruce et al., 1998b). By the use of animal models and through the characterization of individuals with defects in the CETP gene, it has been shown that CETP activity has a major influence on plasma lipoprotein profiles. Although CETP is thought to participate in reverse cholesterol transport, a process considered to be anti-atherogenic, the conditions that determine whether CETP promotes or protects against atherosclerosis have yet to be fully elucidated (Marotti et al., 1993; Zhong et al., 1996; Kuivenhoven et al., 1998).

CETP has an apparent molecular weight of between 66 and 74 kDa. It is composed of 476 amino acids with four potential N-glycosylation sites and numerous hydrophobic regions spread throughout the primary structure (Drayna et al., 1987). It is a member of a gene family that also includes PLTP, lipopolysaccharide binding protein (LBP), and bactericidal/permeability-increasing protein (BPI) (Day et al., 1994). LBP and BPI can both bind bacterial endotoxins and can modulate the host response to Gram-negative bacterial infections. Whereas BPI is bactericidal and can

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Abbreviations: β gal, β -galactosidase; BPI, bactericidal/permeability-increasing protein; CETP, cholesteryl ester transfer protein; LBP, lipopolysaccharide-binding protein; mAb, monoclonal antibody; PLTP, phospholipid transfer protein.

prevent the neutrophil response to endotoxin (Marra et al., 1990), LBP promotes lipopolysaccharide-induced cell activation, probably through an interaction with cell surface CD14 (Ulevitch & Tobias, 1995). Both CETP (Jiang & Bruce, 1995) and PLTP (Hailman et al., 1996) can bind and, in the case of PLTP, neutralize lipopolysaccharide. For humans, CETP amino acid homology is higher with LBP and BPI (26%) than with PLTP (20%), and is highest in the amino terminal half of the molecule (Jiang et al., 1995). In addition to the sequence homologies between members of the family, the genes are also similar in their respective intron/exon organization (Tu et al., 1995). The human PLTP, LBP, and BPI genes are on the q arm of chromosome 20, whereas the CETP gene is on chromosome 16 (Whitmore et al., 1995). The crystal structure of human BPI has recently been solved (Beamer et al., 1997). It is a boomerang-shaped molecule composed of two domains that have a very similar fold. Each domain includes a hydrophobic pocket that opens onto the concave surface of the boomerang. In the BPI crystal structure, each hydrophobic pocket contained a phospholipid molecule that was bound through an interaction that involved primarily the acyl chains.

Both immunochemistry and site-directed mutagenesis have been used to assess the structure–function relationships of CETP. A monoclonal antibody (mAb), TP2, that is specific for an epitope that has been mapped to the carboxy-terminal 26 residues of CETP primary structure can inhibit CETP-mediated cholesteryl ester, triglyceride, and phospholipid transfer (Hesler et al., 1988; Swenson et al., 1989). This mAb does not prevent binding of CETP to the lipoprotein surface, but appears to neutralize cholesteryl ester and triglyceride transfer by preventing access to a neutral lipid-binding site on CETP (Swenson et al., 1989). The mechanism responsible for TP2 inhibition of CETP-mediated phospholipid transfer has yet to be determined. A CETP variant that lacks residues 470–475 is unable to bind or to mediate the transfer of neutral lipids but does retain its ability to transfer phospholipid (Wang et al., 1992, 1995). As an increased negative surface charge of lipoproteins is associated with a higher binding affinity for CETP and an increased rate of CETP-mediated lipid efflux, electrostatic interactions are thought to be important in the association of CETP with the lipoprotein surface (Nishida et al., 1993). Replacement of Lys233 or Arg259 of CETP by neutral residues decreases binding of CETP to lipoproteins, although it is unclear whether or not these basic residues are directly implicated in the CETP-lipoprotein interaction (Jiang et al., 1995; Bruce et al., 1998a). Based on both biochemical (Hesler et al., 1989) and immunochemical (Roy et al., 1996) evidence, we have predicted that CETP adopts a compact tertiary structure. Here, we present a refined epitope map for CETP and show that the immunochemical data support a model of CETP tertiary structure that is based on the crystal structure of BPI.

Results

We have previously described a panel of 20 mAbs specific for human CETP (Hesler et al., 1988; Swenson et al., 1989; Roy et al., 1996). A number of the mAbs can block the cholesteryl ester transfer activity of CETP. The neutralizing mAb, TP2, is specific for an epitope in the carboxy terminal 26 residues of CETP and appears to block neutral lipid transfer activity by preventing access to a neutral lipid binding site on CETP. In addition to TP2, mAbs specific for epitopes situated between residues 331–365 and 410–450, respectively, as well as one of four mAbs whose epitopes are located between residues 183–260 also neutralize CETP-mediated

cholesteryl ester transfer activity. In contrast, mAbs specific for epitopes between CETP residues 261–330 and 366–409, respectively, do not influence CETP cholesteryl ester transfer activity (Roy et al., 1996).

The mAbs that are specific for epitopes between CETP residues 183 and 260 are of particular interest. The replacement of two basic residues within this region, Lys233 and Arg259, by neutral amino residues results in CETP variants that are defective in binding to lipoproteins and in mediating lipid transfer (Jiang et al., 1995). Of the four mAbs that are specific for epitopes in this region (TP14, TP15, TP18, and TP20), only TP18 can block CETP-mediated cholesteryl ester transfer activity (Roy et al., 1996). Thus, TP18 could potentially inhibit cholesteryl ester transfer by interfering with the binding of CETP to lipoproteins. In addition, however, TP14, TP15, and TP18 can compete with a group of neutralizing mAbs whose epitope(s) are located between CETP residues 410 and 450. In terms of CETP tertiary structure, the TP18 epitope could be close to the carboxy-terminus of the molecule, a region that modulates or is responsible for neutral lipid binding. Therefore, this group of mAbs should be useful as probes of CETP tertiary structure and for defining the functional domains of CETP.

We have previously localized the epitopes within CETP primary structure based on the reactivities of the corresponding mAbs with fragments of CETP that had been expressed as β -galactosidase fusion proteins in *Escherichia coli*. The four mAbs react with a β gal-CETP fusion protein that included CETP residues 5–260, but not with a β gal-CETP fusion protein in which CETP residues 183–332 were deleted. To more precisely map the epitopes of mAbs TP14, TP15, TP18, and TP20, we have tested the mAbs for their respective abilities to recognize a series of carboxy-terminally truncated variants of a β gal-CETP5–260 fusion protein that was generated by unidirectional exonuclease III digestion of the pEX-CETP5–260 plasmid (Fig. 1). The time of digestion was varied from 0.5 to 2 min to yield variants with progressively larger truncations. After trimming of the single stranded overhangs and ligation, the plasmids from each time point were used to transform *E. coli*. Expression of the β gal-CETP fusion proteins was induced in the liquid cultures prepared from individual colonies. Clones were characterized by DNA sequencing and the corresponding fusion proteins, after separation by SDS-PAGE, were analyzed for their immunoreactivities with TP14, TP15, TP18, and TP20. The β gal-CETP fusion proteins that were most informative for mapping the epitopes are expressed by bacterial clones that had been transformed with plasmids that had been digested for 1.5 min with Exonuclease III. A selection of immunoreactions is shown in Figure 2, and all of the results are summarized in Table 1.

Each of the mAbs shows a characteristic pattern of reactivity with the series of β gal-CETP fusion proteins. TP20 reacts with all fusion proteins that contain CETP sequences that extend beyond residue 217. TP14 recognizes a fusion protein that terminates at CETP residue 223, but fails to react with a fusion protein that ends at CETP residue 221. Likewise, the TP18 epitope is detected on β gal-CETP5–225 but not on β gal-CETP5–223. Finally, TP15 reacts with β gal-CETP5–258 but not with β gal-CETP5–225. It is quite remarkable that the TP18, TP14, and TP20 epitopes can be distinguished by the reactivity of the corresponding mAbs with four fusion proteins that differ by sequential carboxy-terminal truncations of two, two, and four residues, respectively. Although the three epitopes can be differentiated, given their apparent proximity within CETP primary structure, it is probable that they overlap.

Fig. 1. The strategy for production of truncated β -gal-CETP fusion proteins. The pEX-CETP5-260 and pEX-CETP261-476 plasmids were cut with restriction endonucleases and subjected to unidirectional exonuclease III/mung bean nuclease digestion (horizontal arrows) as described in Materials and methods. Restriction sites within the CETP cDNA are indicated by the codon number and refer to the sequence encoding mature CETP protein.

Not surprisingly, therefore, we have previously shown that TP20, TP14, and TP18 mutually compete for binding to CETP (Roy et al., 1996). These antibody competition studies also indicated that this cluster of epitopes is spatially separated from the TP15 epitope. Although TP14 and TP18 but not TP20 could partially compete with TP15 for binding to CETP, TP15 was ineffective as a competitor for the binding of TP14, TP18, or TP20.

A revised epitope map based on the results of Table 1 is presented in Figure 3. For the TP20, TP18, and TP14 epitopes, we have assumed that the carboxy terminal residue of the longest β -gal-CETP fusion protein that fails to react with the corresponding mAb represents the center of the epitope in terms of CETP primary structure. We further assume that the minimal epitope would include several flanking residues. The individual epitopes may extend farther toward the amino terminus and, in native CETP, noncontiguous residues from other regions of the molecule could contribute to assembled, conformational epitopes. We have assigned the TP15 epitope to a region of CETP primary structure circumscribed by residues 225 and 258. Although we cannot formally exclude that the TP15 epitope is centered on residue 225, we consider that this is unlikely, in view of the antibody competition studies.

Fig. 2. Reactivity of anti-CETP mAbs with truncated variants of the β -gal-CETP5-260 fusion protein. Complementary DNA that encodes human CETP residues 5-260 in the pEX1 plasmid was subjected to exonuclease III digestion, and the resultant carboxy-terminally truncated variants were expressed in *E. coli* as β -gal-CETP fusion proteins. After separation on SDS-PAGE, the migrated proteins were transferred to nitrocellulose membranes and tested for immunoreactivity with anti-CETP mAbs TP20, TP14, TP18, and TP15, and with an anti- β -gal mAb. As a control for nonspecific reactivity of the anti-CETP mAbs, all mAbs were tested for reactivity with an extract of bacteria that had been transformed with the empty pEX1 plasmid (β -gal).

A similar strategy of deletion mutagenesis has been used to define more precisely the epitope(s) of a second group of mAbs, TP5, TP7, TP8, TP13, and TP19 that can neutralize CETP-mediated cholesteryl ester transfer activity. Our previous studies have indicated that the epitope(s) is situated between CETP residues 411 and 450, and that its accessibility can be limited by the binding of mAbs TP14, TP18, and TP15 (Roy et al., 1996). As the present study suggests that the five mAbs recognize the same epitope, only the results of TP13 and TP19 are shown. A pEX clone into which the cDNA encoding CETP residues 261-476 had been inserted in-frame with the β gal coding sequence (Roy et al., 1996) was subjected to unidirectional exonuclease III digestion as described in Materials and methods. The pool of modified plasmids was used to transform *E. coli*, and bacterial clones were identified that contained plasmids of an appropriate size. In contrast to what we had observed with the pEX- β -gal-CETP5-260 plasmid, in the case of the pEX- β -gal-CETP261-476 plasmid, there was a poor correlation between the time and the extent of exonuclease III digestion. The immunoreactivities of the mAbs with the expressed β -gal-CETP fusion proteins are summarized in Table 1. TP13 and TP19 reacted with β -gal-CETP261-451, as well as with longer fusion proteins, but not with β -gal-CETP261-444. TP10, whose epitope had been previously mapped to a region between CETP residues 366 and 410 (Roy et al., 1996), reacted with all of the truncated fusion proteins. We propose, therefore, that mAbs TP13 and TP19 are specific for an epitope between residues 444 and 450 (Fig. 3). This is consistent with our previous observations that these mAbs show normal reactivity with CETP variants in which there are deletions of residues 451-456, 457-462, 463-469, and 470-475, respectively, and with a variant that has an aspartic acid $\rightarrow$ glycine substitution at residue 442 (Roy et al., 1996).

Exonuclease III/mung bean nuclease digestion of pEX-CETP261-476 also permitted a more accurate mapping of the TP10, TP11, and TP12 epitopes. Our previous studies indicated that TP11, TP12, and TP10 recognize epitopes between CETP residues 261-330, 331-365, and 366-410, respectively (Roy et al., 1996). Based on the results shown in Table 1, we now propose that the TP11, TP12, and TP10 epitopes are situated between CETP residues 317-331, 360-366, and 393-410, respectively (Fig. 3). We have previously shown that TP12, but neither TP10 nor TP11, can partially block CETP-mediated cholesteryl ester transfer.

Discussion

The crystal structure of BPI shows that it is a two-domain protein in which the two domains share an almost identical structural fold

Table 1. Immunodetection of β gal-CETP fusion proteins with anti-CETP mAbs^a

Starting clone	Restriction fragment	CETP residues	Antibodies									
			TP14	TP15	TP18	TP20	TP11	TP12	TP10	TP13	TP19	Anti- β gal
pEX1-CETP5-260	BamHI/PstI	5-260	+	+	+	+	NT ^b	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-259	+	+	+	+	NT	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-258	+	+	+	+	NT	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-225	+	—	+	+	NT	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-223	+	—	—	+	NT	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-221	—	—	—	+	NT	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-217	—	—	—	—	NT	NT	NT	NT	NT	+
pEX1-CETP5-260	BamHI/PstI	5-217	—	—	—	—	NT	NT	NT	NT	NT	+
pEX3-CETP261-476	AflII/PstI	261-363	NT	NT	NT	NT	+	—	NT	NT	NT	+
pEX3-CETP261-476	AflII/PstI	261-347	NT	NT	NT	NT	+	—	NT	NT	NT	+
pEX3-CETP261-476	AflII/PstI	261-335	NT	NT	NT	NT	+	—	NT	NT	NT	+
pEX3-CETP261-476	AflII/PstI	261-317	NT	NT	NT	NT	—	—	NT	NT	NT	+
pEX3-CETP261-476	PstI/XbaI	261-393	NT	NT	NT	NT	NT	+	—	NT	NT	+
pEX3-CETP261-476	PstI/XbaI	261-390	NT	NT	NT	NT	NT	+	—	NT	NT	+
pEX3-CETP261-476	PstI/XbaI	261-387	NT	NT	NT	NT	NT	+	—	NT	NT	+
pEX3-CETP261-476	PstI/XbaI	261-360	NT	NT	NT	NT	NT	—	—	NT	NT	+
pEX3-CETP261-476	BamHI/XbaI	261-476	NT	NT	NT	NT	NT	+	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-474	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-471	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-469	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-467	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-460	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-456	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-451	NT	NT	NT	NT	NT	NT	+	+	+	+
pEX3-CETP261-476	BamHI/XbaI	261-444	NT	NT	NT	NT	NT	NT	+	—	—	+
pEX	—	β gal	—	—	—	—	—	—	—	—	—	+

^aThe pEX-CETP plasmids were cut with the indicated restriction enzymes, subjected to exonuclease III digestion as described in Materials and methods, religated and used to transform DH5 α bacteria. The CETP residues that were encoded in the exonuclease III-digested plasmids were deduced by cDNA sequencing. Expression of the CETP- β gal fusion proteins was induced, and the bacterial extracts were tested for their reactivity with anti-CETP mAbs.

^bNT, not tested.

that gives the molecule a pseudo-twofold axis of symmetry (Beamer et al., 1997). Each domain consists of a five-stranded antiparallel β -sheet and two α -helices that, together, adopt a β -barrel structure. A central seven-stranded β -sheet provides the interface between the two domains. In each domain, the two α -helices form the entrance to a large apolar ligand-binding pocket. When the amino acid sequences of CETP, PLTP, and LBP are aligned with that of BPI, it is observed that residues that are important in determining the higher order structure of BPI are conserved in the four proteins (Beamer et al., 1997). The cysteine residues that form the only

disulfide bond in BPI are conserved in CETP, PLTP, and LBP, and the pattern of hydrophilic and hydrophobic residues that characterizes the β -strands of BPI is also apparent in the other members of the family. As a consequence, it has been proposed that CETP, PLTP, and LBP have tertiary structures that are similar to that of BPI (Beamer et al., 1997).

We have attempted to determine if our immunochemical data are consistent with CETP having a tertiary structure similar to that of BPI. We have superimposed the linear CETP epitope map (Fig. 3) on to the crystal structure of BPI (accession number 1bp1) (Beamer

Fig. 3. A linear epitope map of CETP based on the reactivity of anti-CETP mAbs with β gal-CETP fusion proteins. An epitope map was derived from the results presented in Table 1 based on the criteria described in the text.

et al., 1997). The alignment of the BPI and CETP sequences was that proposed by Beamer et al. (1997) and was based on a comparison, using CLUSTAL (Higgins & Sharp, 1989), of the sequences of 11 members of the lipopolysaccharide-binding/lipid transport protein family. Figures 4A and 4B represent, respectively, views of the convex and concave surfaces of the "boomerang"-shaped CETP model in a space-filling display. It is evident that all of the epitopes are largely confined to the convex surface of the molecule. The same view of the convex surface that is shown in Figure 4A is presented in a ribbon display in Figure 5. In this model, the residues that constitute the minimal epitopes recognized by the mAbs TP20 (blue), TP14 (green), and TP18 (yellow) would contribute to the loops and β -strands of the central β -sheet that forms the interface between the two domains. As the antibodies would not have access to the hydrophobic side chains of residues of the β -strands that form the putative hydrophobic binding pocket of CETP (Fig. 4), the epitopes of these mAbs would be composed of side chains that extend out from the concave surface of the molecule. The residues that compose the TP13 and TP19 epitope(s) (cyan, residues 444–450) would also be included in this central β -sheet. The TP15 epitope has been mapped to CETP residues 225–258 (red), a region that would be predicted to include the loop that links the amino- and carboxy-terminal domains of the molecule. The TP11 (pink, residues 317–331) and TP12 (orange, residues 360–366) epitopes would be situated in regions that participate in the β -strands and loops of the carboxy-terminal β -barrel and the TP10 epitope (purple, residues 393–410) would include residues from the distal end of the long helix of this domain.

As can be seen in Figure 6, the model of CETP tertiary structure would be consistent with the results of the mAb competition studies. It is notable that all pairs of mAbs that compete for binding to CETP are specific for epitopes that are on the convex surface of the molecule. Residues that contribute to the TP14 and TP18 epitopes are predicted to be within 15 Å of the residues that participate in the TP13/TP19 epitope(s). As the footprint of an antibody

Fig. 4. A three-dimensional epitope map of CETP based on the atomic coordinates of BPI is shown in a space-filling representation. Views of the (A) convex and (B) concave surfaces of the CETP model represent a rotation of 180° about the X-axis. The sequences to which the epitopes have been mapped are indicated according to the color code: TP20 (residues 215–219, blue), TP14 (residues 220–223, green), TP18 (residues 224–227, yellow), TP15 (residues 228–258, red), TP11 (residues 317–331, pink), TP12 (residues 360–366, orange), TP10 (residues 393–410, purple), and TP13/19 (residues 444–451, cyan). The model uses the atomic coordinates of BPI (Beamer et al., 1997) (accession number 1bp1), and the images were produced using MolMol (Koradi et al., 1996).

on a protein antigen surface in an antibody–antigen complex represents about 750 Å² (Amit et al., 1986), binding of TP14 or TP18 to CETP could preclude the binding of TP13 and TP19 through steric hindrance. Likewise, the predicted proximity to the TP14, TP18, TP20, and TP13/TP19 epitopes of CETP residues 226–228 that would include the TP15 epitope would also be consistent with the antibody competition results. Other pairs of antibodies that compete for binding to CETP (e.g., TP15 and TP10) are specific for epitopes that are separated by less than 30 Å.

A model of CETP based on the crystal structure of BPI gives some clues to the mechanism by which the anti-CETP mAbs might block CETP-mediated lipid transfer activity. The neutralizing TP2 mAb is specific for an epitope in the carboxy-terminal 26 residues of CETP and is thought to limit access to a neutral lipid binding site. CETP residues 454–461 and 466–476 have been predicted to form amphipathic α -helices that are joined by a short flexible linker (Tall, 1993). Site-specific mutagenesis of CETP has shown that residues required for TP2 epitope expression are localized on the hydrophilic face of the putative amphipathic helix composed of residues 466–476, whereas residues that are necessary for lipid transfer activity are on the hydrophobic face (Wang et al., 1993). It has been proposed that the hydrophobic surfaces could form part of a flexible, neutral lipid-binding pocket at the carboxy terminus of CETP. Furthermore, it was suggested that flexibility of the carboxy-terminus of the molecule may be intrinsic to the mechanism of CETP-mediated neutral lipid transfer and that the capacity of TP2 to block CETP activity may reside in its ability to limit the mobility of this region (Tall, 1993). Although the sequence of BPI residues 446–553 is very similar to CETP residues 454–461, BPI terminates at residue 456, and thus lacks the putative amphipathic helix represented by CETP residues 466–476. Although not present in the model, one would predict that residues 466–476 are situated close to the entrance of the amino terminal apolar pocket in CETP (Bruce et al., 1998a). Accessibility to one or both of the apolar binding pockets could be restricted in TP2-CETP immune complexes. It is notable that only the TP2 epitope would be exclusively situated on the concave surface of the molecule and TP2 fails to compete with any of the other antibodies for binding to CETP.

TP12, TP18, and TP13/TP19 also neutralize CETP-mediated CE transfer. Based on the predicted topology of the CETP molecule, it would be improbable that the binding of these mAbs would directly impair access to either of the putative ligand-binding pockets of CETP or directly prevent association of CETP with the lipoprotein surface. More likely, the mAbs have an indirect effect on the ability of CETP to bind neutral lipid or interact with lipoprotein surfaces. Binding of TP12, TP18, or TP13/TP19 could induce a change in conformation of the ligand-binding pockets of CETP, alter an equilibrium between an active and an inactive conformation of CETP or, as has been suggested for TP2, limit the mobility of the carboxy-terminus of CETP. Recently it has been proposed that CETP undergoes a conformational change following association with, and preceding dissociation from, the lipoprotein surface that is obligatory for both neutral lipid and phospholipid transfer activity (Bruce et al., 1998a). The neutralizing mAbs may lock CETP in one or the other of these two conformational states.

Based on sequence analysis, it has been proposed that CETP and BPI share similar tertiary structures. If this were proven to be the case it would have a major influence on our concepts concerning the structure–function relationships of CETP. Here we present immunochemical evidence that is consistent with such a postulate. In a structural model of CETP based on the BPI crystal structure,

Fig. 5. A three-dimensional epitope map of CETP based on the atomic coordinates of BPI is shown in a ribbon representation. The view of the convex surface of the CETP model is identical to that in Figure 4A, and the color code of the epitopes is that described in the caption to Figure 4. Images were produced using MolMol (Koradi et al., 1996).

epitopes of mAbs that can compete for binding to CETP are predicted to be close together in CETP tertiary structure, although widely separated within the primary structure. Furthermore, by adapting our linear epitope map to a three-dimensional model of CETP topology, we have gained new insights into possible mechanisms by which anti-CETP mAbs can neutralize CETP-mediated cholesteryl ester transfer.

Materials and methods

Monoclonal antibodies

The production and characterization of antihuman CETP mAbs have been described previously (Hesler et al., 1988; Swenson et al., 1989;

Roy et al., 1996). Monoclonal anti- β -galactosidase (β gal) IgG was purchased from Gibco BRL (Gaithersburg, Maryland).

Bacterial expression of CETP fragments as β -galactosidase fusion proteins

The construction of pEX plasmids for the expression of CETP residues 5–260 (pEX-CETP5–260) and 261–476 (pEX-CETP261–476), respectively, as β -galactosidase fusion proteins has been described in detail (Roy et al., 1996). Four exonuclease III digestions of the pEX-CETP plasmids were undertaken (Fig. 1). The pEX1–CETP5–260 was digested with Bam HI and Pst I, which cut at sites that are 3 and 19 base pairs, respectively, downstream of the 3' end of the CETP insert. After ethanol precipitation, 25 μ g of the plasmid were digested with 500 units of exonuclease III (Stratagene, La Jolla, California) at 20 °C according to the manufacturer's recommendations. At 30 s intervals after the addition of the enzyme, aliquots were removed and extracted with phenol/chloroform. The single-stranded overhangs were removed by digestion with mung bean nuclease (Stratagene), again following the recommendations of the manufacturer. A similar protocol was used for exonuclease III digestion of pEX3–CETP261–476 that had been cut with Afl II that cleaves within codon 381 of mature CETP cDNA and with Pst I that cleaves within codon 410 of mature CETP cDNA and 19 base pairs downstream of the CETP insert. The protocol was modified for other digestions of the pEX3–CETP261–476 due to the Pst I site within the CETP coding sequence. The pEX3–CETP261–476 plasmid was digested with Xba I that cuts at a site, 57 base pairs downstream of the 3' end of the CETP insert. The 5' restriction overhangs were filled in using the Klenow fragment of DNA polymerase I and α -thio-deoxynucleotide triphosphates. The plasmid was then digested with Bam HI and, after ethanol precipitation, with exonuclease III and mung bean nuclease as described above. Finally, pEX3–CETP261–476 was cut with Xba I and end-filled with α -thio-deoxynucleotide triphosphates. The plasmid was then cleaved with Pst I, and the resulting 3' overhangs were removed with mung bean nuclease before sequential digestion with exonuclease III and mung bean nuclease. In each case, the digested plasmid was precipitated with ethanol, redissolved in water, and one-half of each sample was subjected to agarose gel electropho-

Fig. 6. A view of the convex face of the central β -sheet and carboxy-terminal β -barrel that demonstrates the proximity of epitopes of anti-CETP mAbs that compete for binding to immobilized CETP. The view of the convex surface of the CETP model is identical to that in Figure 4A, and the color code of the epitopes is that described in the caption to Figure 4. The arrows summarize mAb competition studies that have been reported previously (Roy et al., 1996). Solid and broken lines indicate strong and weak competition, respectively. Single-headed arrows represent unidirectional competition. The color of the arrow is the same as that of the epitope of the antibody that is capable of competition. White double-headed arrows represent bi-directional competition.

resis to monitor the efficiency of the exonuclease III/mung bean nuclease digestion. The other one-half of each sample was incubated with T4 DNA ligase (Gibco BRL) overnight at 16 °C and was used to transform competent POP2136 *E. coli*. Conditions for expression of the CETP fragments as β -galactosidase fusion proteins and the protocols for extraction of the fusion proteins from the bacteria and their detection after SDS-PAGE have been described previously (Roy et al., 1996). The 3' ends of the truncated CETP cDNA inserts were sequenced by the dideoxy chain termination method (Sanger et al., 1977).

Acknowledgments

This work was supported by the Heart and Stroke Foundation of Ontario through operating grant B-3452 and a John D. Schultz Science Student Scholarship to V.T. We thank Drs. Benoit Gauthier, Roger McLeod, Yves Marcel, Joshua Schultz, and Zemin Yao for suggestions and for stimulating discussions.

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