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Structure–function relationships of human cholesteryl ester transfer protein: analysis using monoclonal antibodies

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Abstract Cholesteryl ester transfer protein (CETP), a 476 amino acid glycoprotein, mediates cholesteryl ester (CE), triglyceride, and phospholipid transfer among plasma lipoproteins. A monoclonal antibody (mAb), TP2, specific for an epitope within the last 26 amino acids of CETP has been shown to block all CETP-mediated lipid transfer, apparently by limiting access to lipid-binding sites in the carboxy terminal of CETP. A new panel of 16 anti-human CETP mAbs has now been used to further probe the structure–function relationships of CETP. Of the new mAbs, 9 partially inhibit CETP-mediated CE transfer (24–43%) from HDL to LDL. The corresponding epitopes were mapped within the CETP primary structure by the reactivity of the mAbs with CETP variants having deletions or amino acid substitutions. Of the 9 new, neutralizing mAbs, 6 are specific for epitopes situated between residues 410–450 and two others for epitopes between residues 184–260 and 332–366, respectively. The epitope of one neutralizing mAb could not be mapped. Therefore, binding of mAbs to epitopes situated in four non-overlapping regions within CETP primary structure that are separated by as many as 280 residues can neutralize CETP-mediated CE transfer. Epitopes of mAbs that do not influence CE transfer activity map to the regions 184–260, 261–331, and 367–409, respectively. When pairs of mAbs were tested for their abilities to mutually compete for binding to immobilized CETP, competition was observed for mAbs specific for epitopes that are distant in CETP primary structure. The cross-competition patterns demonstrate that the carboxy terminal 60% of CETP adopts a compact structure. Together with previous mutagenesis studies, the data suggest that a carboxy terminal neutral lipid binding domain may be in close proximity to a lipoprotein binding region within native CETP.—**Roy, P., R. MacKenzie, T. Hirama, X-C. Jiang, P. Kussie, A. Tall, E. Rassart, and R. Milne.** Structure–function relationships of human cholesteryl ester transfer protein: analysis using monoclonal antibodies. *J. Lipid Res.* 1996. **37:** 22–34.

Supplementary key words epitope mapping • antibody competition • lipid transfer

Cholesteryl ester transfer protein (CETP), a 476 amino acid glycoprotein that mediates lipid exchange between plasma lipoproteins, has been shown to be responsible for all of the cholesteryl ester (CE) and triglyceride (TG) transfer activity in human plasma and for about one-half of the plasma phospholipid transfer activity when measured in an in vitro assay (1, 2). Under most circumstances, CETP mediates an equimolar exchange of neutral lipid among lipoproteins (3). As a consequence of this reciprocal transfer, neutral lipid exchange between CE-rich lipoproteins, such as high density lipoproteins (HDL), and TG-rich lipoproteins, such as very low density lipoproteins, will lead to a progressive depletion of CE and enrichment in TG of the HDL. The identification of CETP-deficient humans and the generation of mice, in which a CETP transgene is over-expressed, have helped to define the in vivo consequences of CETP activity on lipoprotein metabolism (reviewed in ref. 4). Nevertheless, the conditions that determine whether CETP promotes or protects against atherosclerosis have yet to be fully elucidated.

Two models of CETP-mediated lipid transfer have been proposed. In one model, CETP would serve as a shuttle protein that binds and carries lipid molecules between lipoprotein particles (5), while in the second,

Abbreviations: CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; TG, triglyceride; HDL, high density lipoprotein; mAb, monoclonal antibody; SPA, surface plasmon resonance; HBS, HEPES-buffered saline; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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CETP would act as a bridge in the formation of a ternary complex that includes donor and acceptor lipoproteins (6). Whether CETP functions as a shuttle protein or facilitates formation of a ternary complex, it is probable that CETP has a number of regions or sites that are important for its function. It is thought that the carboxy-terminus constitutes, or forms part of, the CETP neutral lipid binding site(s) (4) as a CETP variant lacking amino acid residues 470–475 cannot bind CE or TG (7) and binding of neutral lipid to wildtype CETP is blocked by the neutralizing, carboxy-terminal specific, anti-CETP monoclonal antibody (mAb), TP2 (8). Moreover, as TG (2, 9, 10) and CE (11) transfer can be selectively inhibited, there may be discrete binding sites within the carboxy terminal region of CETP for TG and CE, respectively. It is probable that CETP also possesses a separate phospholipid binding site as the transfer of neutral lipid and that of phospholipid appear to be distinct processes (12, 13). Finally, the regions of the CETP molecule that are responsible for binding to lipoproteins are clearly different from the site(s) responsible for binding neutral lipid (8). In order to identify such functionally important regions of CETP and to obtain information concerning CETP tertiary structure, we have generated a new panel of 16 murine anti-human CETP mAbs. A number of the new mAbs can partially inhibit CETP-mediated lipid transfer activity in an in vitro assay. We further demonstrate that the new anti-CETP mAbs that are capable of suppressing lipid transfer are specific for epitopes that are distant in the CETP primary structure from that of the previously described, neutralizing TP2 mAb.

MATERIALS AND METHODS

Production, purification, and radiolabeling of mAbs

The production and characterization of anti-human CETP mAbs TP1, TP2, TP3, and TP4 have been described (2, 8). New hybridomas that secrete anti-human CETP mAbs were obtained by polyethylene glycol-mediated fusion of spleen cells from BALB/c mice immunized with purified plasma CETP (14) with SP2-0 murine plasmacytoma cells (15). The hybridoma supernatants were screened by a solid phase radioimmunometric assay using purified plasma CETP adsorbed to Immunlon II Removawells (Dynatech) as antigen and affinity purified ^{125}I-labeled rabbit anti-mouse IgG to detect bound antibody. Positive hybridomas were twice recloned by limiting dilution at 1 and 0.5 cells/well, respectively. To obtain large amounts of the mAbs, the hybridoma cells were injected into the peritoneal cavity of BALB/c mice and ascites was harvested about 2 weeks later (15). IgG was purified from ascites by affinity chromatography on protein A Sepharose (Pharmacia) (16). Immunoglobulins were labeled as previously described (15) and the isotypes of the mAbs were determined using a Mouse Monoclonal Antibody Isotyping Kit (Gibco BRL).

Surface plasmon resonance

The kinetics of binding of anti-CETP mAbs to purified, immobilized CETP were determined by surface plasmon resonance (SPR) using a BIAcore biosensor system (Pharmacia Biosensor). This technology allows for interactions to be continuously monitored in real time and generates binding profiles, or sensorgrams, from which rate constants can be derived (17). Primary amine groups of CETP were covalently coupled to research grade CM5 sensor chips (Pharmacia Biosensor) using the amine coupling kit supplied by the manufacturer. The proteins were diluted to 20 μg/ml in 10 mM sodium acetate, pH 4.5, and 40-μl aliquots were injected over the activated chip surface. Unreacted moieties were blocked with ethanolamine. All measurements were carried out in HEPES-buffered saline (HBS) which contained: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P-20 (Pharmacia Biosensor). Analyses were performed at 25°C and the binding of each antibody was tested at five concentrations. Immobilizations and binding assays were carried out at a flow rate of 3 μl/min. Sensor chip surfaces were regenerated with 100 mM HCl and surface integrity was periodically checked with control antibody (TP2).

Association and dissociation rate constants were calculated by nonlinear fitting of the sensorgram data (18) using the BIAevaluation 2.0 software (Pharmacia Biosensor). The dissociation rate constant is derived using the equation:

$$R_t = T_{to}\, e^{-k_{off}(t-to)}$$

where R_t is the response at time t, R_{to} is the amplitude of the response and k_{off} is the dissociation rate constant. The association rate constant can be derived using the equation:

$$R_t = \frac{k_{on} C R_{max}\left(1 - e^{-(k_{on}C + k_{off})t}\right)}{k_{on}C + k_{off}}$$

where R_t is the response at time t, R_{max} is the maximum response, C is the concentration of ligate in solution, k_{on} is the association rate constant, and k_{off} is the dissociation rate constant.

Construction of β-galactosidase fusion proteins

The CETP cDNA (1) was excised from a CDM8PI vector construct (19) with restriction enzymes BanI and

HindIII. BanI cuts 15 nucleotides downstream from the CETP signal peptide sequence, while HindIII cuts within the sequence encoding the last CETP amino acid. The excised fragment was end-filled with the Klenow fragment of DNA polymerase I before Gene-clean purification (Bio 101) and ligation into the SmaI site of the vector pEX-1 (20). The ligation mixture (pEX1-CETP) was used to transform POP2136 competent cells (Boehringer Mannheim). Clone pEX1-CETPΔ410-476 was obtained by cutting the parent clone (pEX1-CETP) with PstI, which cuts within the CETP sequence and in the pEX1 polylinker downstream from the CETP sequence and religated before transfection. Clones pEX1-CETPΔ261-476 and pEX1-CETPΔ184-331 were obtained using the same strategy by deletion of the Sph1-Pst1 and BglII-BglII fragments, respectively. Clone pEX1-CETPΔ42-366 was obtained by cutting the parent vector pEX1-CETP with restriction enzyme EcoRV. EcoRV cuts twice within the CETP cDNA sequence and once in the pEX1 vector. The resulting fragments were religated together before transfection in POP2136 cells. Colonies were screened for deletion of the CETP internal fragment. Finally, clone pEX3-CETPΔ5-260 was obtained by ligating the end-filled Sph1-HindIII fragment of CETP in the SmaI site of vector pEX3. The cDNAs encoding variant forms of CETP having short deletions (12) or amino acid substitutions (13) were cloned into pEX1 as end-filled, BanI/HindIII fragments. The sequence of nucleotides spanning the junction between the Lac Z gene and the 5′ end of the CETP insert was determined for each of the constructs to ensure that an open reading frame was maintained.

Expression of β-galactosidase-CETP fusion proteins

Bacteria containing plasmids with appropriate CETP inserts were grown at 30°C until the OD (600 nm) reached 0.3 to 0.6. Transient expression of the fusion proteins was induced by shifting the culture to 42°C for 3 h. Cells (10 ml) were centrifuged at 30°C for 8 min at 3500 rpm and resuspended in 0.5 ml of 50 mM Tris-HCl, pH 7.5, containing 75 mM NaCl and 1 mM EDTA. Cells were then incubated for 15 min at room temperature after the addition of 12 μl of lysozyme (10 mg/ml). The mixtures were then subjected to two cycles of freezing (-40°C), thawing, and vortexing followed by the addition of 6 μl of 1 M $MgCl_2$ and 50 μg of DNaseI before a 20-min incubation at 37°C. The lysate was centrifuged for 30 min at 4°C and the precipitate was resuspended in 0.5 ml of 10% SDS and heated at 60°C for 10 min. The β-galactosidase fusion proteins were found in the precipitate.

Expression of CETP variants in COS7 cells

The preparation of the mammalian expression vectors pCMV4 and CDM8PI containing inserts encoding

TABLE 1. Kinetics and affinities of antibody binding to immobilized CETP

mAb	Isotype	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (M^{-1})
TP1	γ1, κ	5.7×10^3 (± 5.4)[a]	1.4×10^{-4} (± 5.1)	2.4×10^{-8}
TP2	γ1, κ	1.4×10^4 (± 2.8)	2.6×10^{-4} (± 8.6)	1.9×10^{-8}
TP4	γ1, κ	5.8×10^3 (± 6.0)	1.3×10^{-4} (± 3.9)	2.2×10^{-8}
TP5	γ1, κ	1.2×10^3 (± 6.6)	6.6×10^{-5} (± 12.1)	5.4×10^{-8}
TP7	γ2a, κ	1.8×10^3 (± 9.7)	2.4×10^{-5} (± 25)	1.3×10^{-8}
TP8	γ2b, κ	2.0×10^3 (± 9.0)	4.6×10^{-4} (± 2.8)	2.3×10^{-7}
TP9	γ2a, κ	2.2×10^2 (± 12.3)	8.6×10^{-5} (± 2.3)	3.9×10^{-7}
TP10	γ1, κ	3.5×10^3 (± 6.6)	1.9×10^{-4} (± 7.9)	5.5×10^{-8}
TP11	γ1, κ	1.3×10^4 (± 6.5)	1.6×10^{-4} (± 5.6)	1.2×10^{-8}
TP12	γ1, κ	8.6×10^2 (± 5.9)	4.5×10^{-4} (± 5.3)	5.3×10^{-7}
TP13	γ2b, κ	1.3×10^3 (± 8.3)	8.3×10^{-5} (± 3.6)	6.2×10^{-8}
TP14	γ2a, κ	1.3×10^5 (± 5.3)	6.4×10^{-4} (± 2.3)	4.8×10^{-9}
TP15	γ2a, κ	1.4×10^5 (± 6.7)	1.1×10^{-3} (± 6.4)	7.9×10^{-9}
TP16	γ1, κ	7.0×10^2 (± 11.1)	3.4×10^{-4} (± 1.2)	4.8×10^{-7}
TP17	γ2a, κ	3.4×10^3 (± 4.0)	1.7×10^{-4} (± 3.6)	4.9×10^{-8}
TP18	γ2a, κ	6.6×10^4 (± 4.1)	6.4×10^{-4} (± 2.0)	9.7×10^{-9}
TP19	γ2a, κ	1.1×10^4 (± 10.3)	4.4×10^{-5} (± 27.2)	4.1×10^{-9}
TP20	γ1, κ	2.2×10^5 (± 9.1)	3.6×10^{-4} (± 28.35)	1.6×10^{-9}

The association (k_{on}) and dissociation (k_{off}) rate constants ± the standard error of anti-CETP mAbs with CETP were determined by surface plasmon resonance. The values k_{on} and k_{off} represent the means calculated from determinations at five different antibody concentrations.

[a]SEM given as percent.

CETP variants having short deletions (12) or amino acid substitutions (13) and the methods used for transient expression of the CETP variants in COS7 cells and for the preparation of cell extracts (12) have been described previously.

Polyacrylamide gel electrophoresis and immunodetection

Bacterial or eucaryotic cell extracts were subjected to SDS electrophoresis on an 8% polyacrylamide gel. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes and treated with antibodies as described (15). Purified CETP and extracts of either bacteria carrying a pEX plasmid lacking a CETP insertion or of non-transfected COS-7 cells were also run as positive and negative controls, where appropriate.

Measurement of CETP-mediated CE transfer and inhibition by anti-CETP mAbs

Wells of 96-well Multi-screen Filtration Plates having nitrocellulose membrane bottoms (Millipore) were coated with 80 µl of a 2.8 µg/ml solution of purified recombinant CETP in phosphate-buffered saline, pH 7.2 (PBS) for 3 h at 37°C. The wells were washed twice and saturated with 3% polyvinylpyrrolidone (mol wt 10000) in PBS for 1 h at room temperature. The CETP-coated wells were washed twice with 1% BSA in PBS (PBS-BSA) before incubation for 3 h at 37°C with 100 µl of either PBS-BSA or purified antibody, appropriately diluted in PBS/BSA. The wells were washed twice with a solution of 50 mM HEPES, 0.15 M NaCl, 0.1% BSA, 0.05% NaN_3, pH 7.4 (assay buffer). Measurement of [^{3}H]CE from HDL to LDL was determined using the CETP (^{3}H) Scintillation Proximity Assay (SPA) Kit (Amersham). Transfer was initiated by addition of a solution composed of 20 µl of [^{3}H]cholesteryl ester-labeled HDL, 20 µl of biotinylated LDL, and 60 µl of assay buffer to the CETP-coated wells and was incubated, with stirring, for 18 h at 37°C. The reaction mixture was transferred to scintillation vials before addition of 400 µl of stop reagent that includes SPA-streptavidin beads and, after a 1-h equilibration, the samples were counted in a scintillation counter. The liquid phase assay for CETP activity was carried out using the CETP (^{3}H) SPA

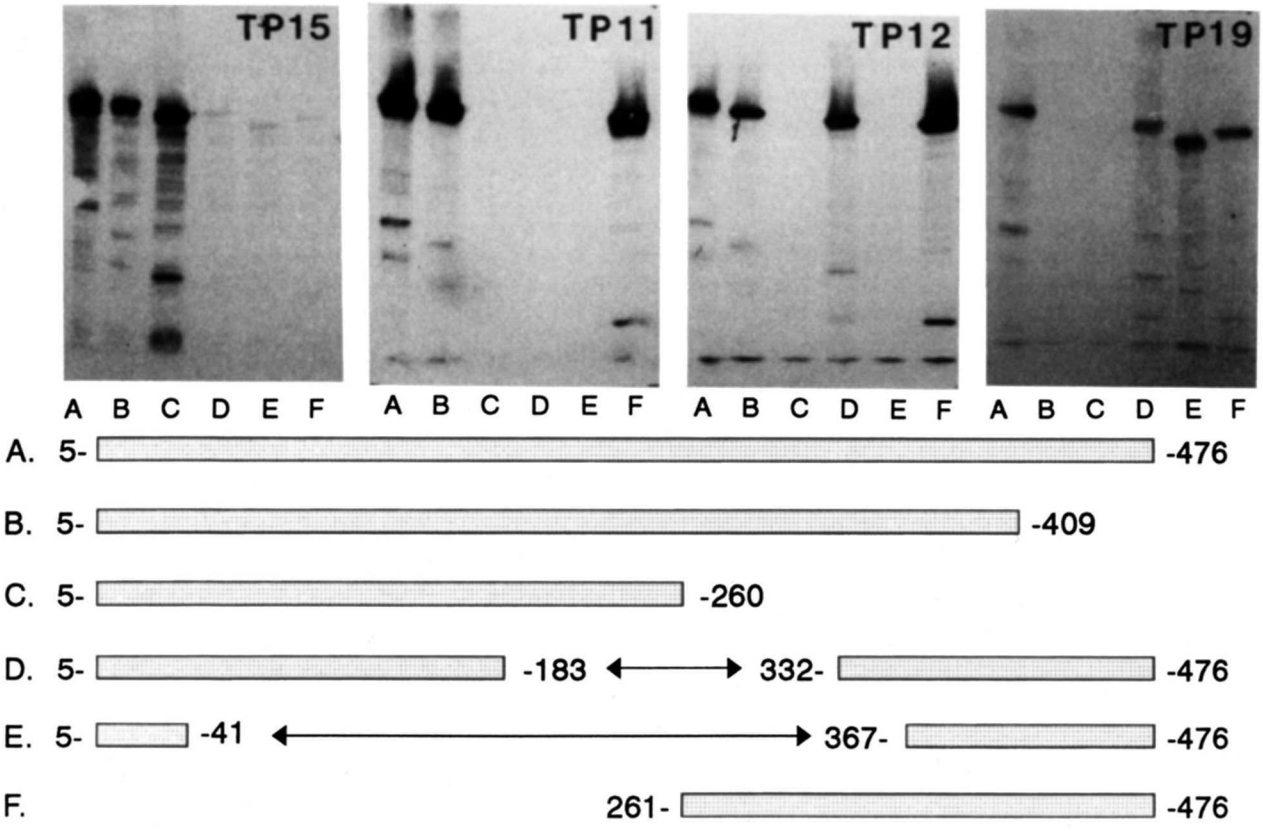

Fig. 1. Reactivity of mAbs TP15, TP11, TP12, and TP19 with fragments of CETP expressed as β-galactosidase fusion proteins. The amino- and carboxy-terminal truncated variants of CETP and the CETP variants having internal deletions that were expressed in *E. coli* in the form of β-galactosidase fusion proteins are schematically represented. Bacterial cell extracts containing the fusion proteins were subjected to SDS polyacrylamide electrophoresis and electrotransfer to nitrocellulose membranes and were tested for reactivity with the mAbs.

kit according to the protocol supplied by the manufacturer.

To estimate the amount of CETP that fixed to the nitrocellulose membranes of the filtration plates in the solid phase assay, ^{125}I-labeled CETP was mixed with unlabeled CETP to give a specific activity of 24 cpm/ng. Serial dilutions of the trace-labeled CETP in PBS were prepared and 80-μl aliquots were added to the wells of the Multiscreen filtration plates. The fixation, washing, and saturation steps were identical to those of the solid phase assay. After the final wash, the filter-associated radioactivity was determined and the mass of bound CETP was calculated.

Antibody competition studies

The ability of unlabeled mAbs to compete with radiolabeled mAbs for binding to CETP adsorbed to Immulon II Removawells was performed as previously described (21).

RESULTS

Monoclonal antibodies and epitope mapping

Sixteen hybridomas that secrete IgG anti-CETP mAbs were identified by a solid phase radioimmunometric assay from a fusion using spleen cells from a mouse that had been immunized with purified human CETP. The cells were twice recloned by limiting dilution and the isolated subclones were used to raise ascites in BALB/c mice. The kinetics of binding of the mAbs to immobilized CETP were determined by SPR and the association and dissociation rate constants were used to calculate the binding affinities. As can be seen in **Table 1,** most of the new mAbs have affinities similar to those of the original panel of anti-CETP mAbs (TP1-TP4).

Mapping of the epitopes for the anti-CETP monoclonal antibodies within the CETP primary structure was carried out by determining the reactivity of the mAbs with CETP deletion mutants expressed in *E. coli*

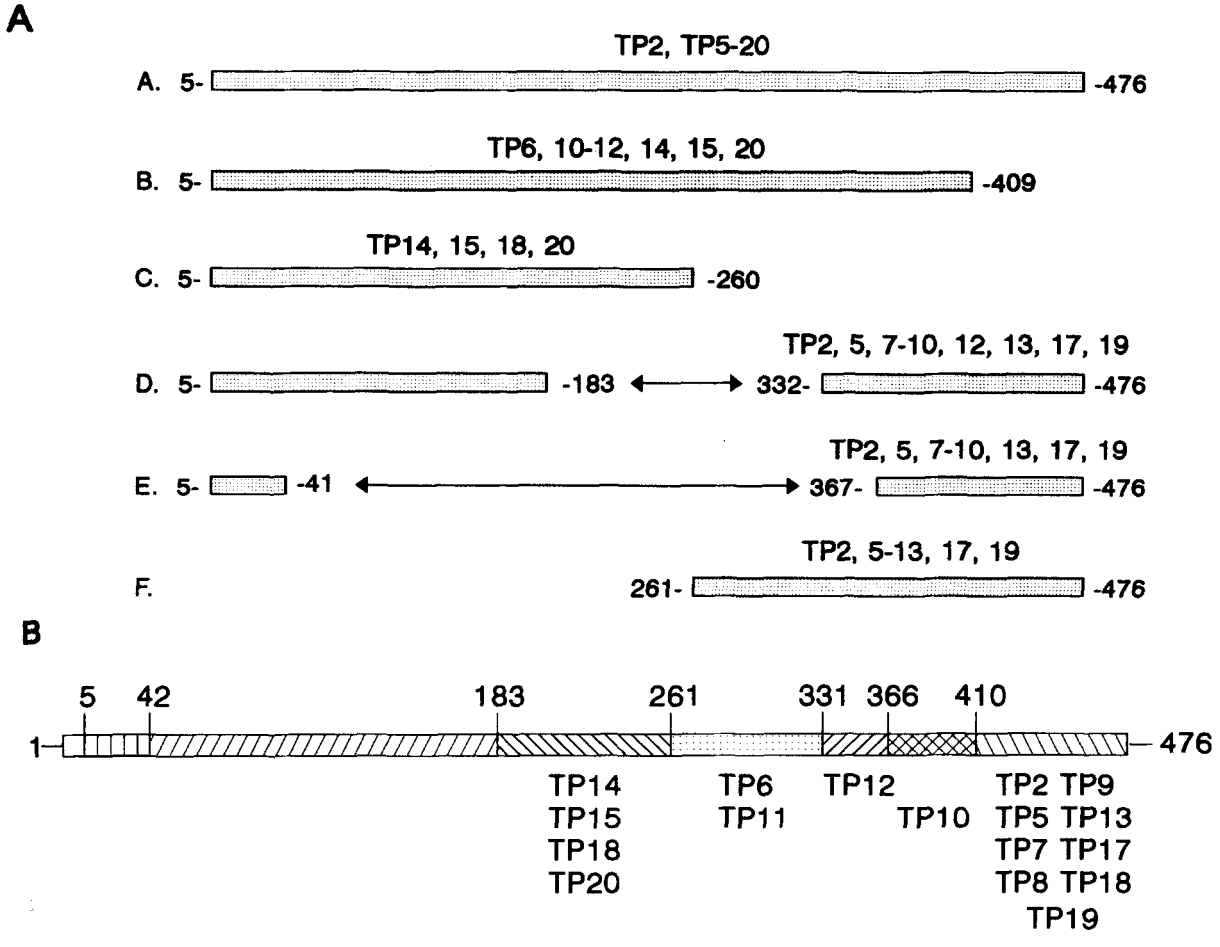

Fig. 2. A summary of the reactivities of the anti-CETP mAbs with CETP/β-galactosidase fusion proteins and the proposed CETP epitope map. A) The anti-CETP mAbs were tested for their reactivities with the β-galactosidase/CETP fusion proteins as described in the legend to Fig. 1. The reactivities of the mAbs with individual fusion proteins are indicated. B) An epitope map of CETP was formulated based on the pattern of reactivities of the mAbs with the expressed CETP fragments as shown in Fig. 2A.

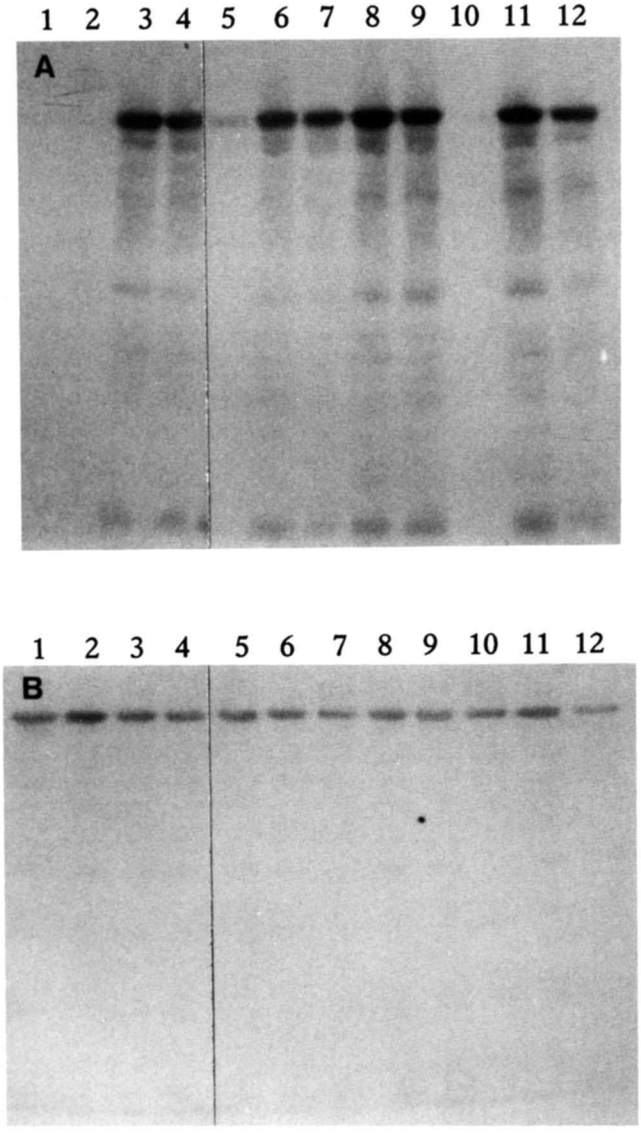

Fig. 3. Reactivity of TP2 and TP19 with CETP variants having short deletions or amino acid substitutions that were expressed as β-galactosidase fusion proteins. Bacterial cell extracts containing the fusion proteins were subjected to SDS polyacrylamide electrophoresis and electrotransfer to nitrocellulose membranes and were tested for reactivity with mAbs TP2 (A) and TP19 (B). Lanes 1–4 are CETP deletion mutants Δ470–475, Δ463–469, Δ457–462, and Δ451–456, respectively. Lanes 5–12 are CETP variants having single amino acid substitutions. Lane 5: His466 → Gln, lane 6: Ser474 → Cys, lane 7: Leu456 → Arg, lane 8: Phe461 → Leu, lane 9: Leu455 → Val, lane 10: Asp460 → Ala, lane 11: Phe461 → Tyr, and lane 12: Asp460 → Gly.

in the form of β-galactosidase fusion proteins. In all cases, the CETP cDNA was cloned into the pEX vectors in frame with the *Lac* Z gene and all constructs were verified by restriction analysis and partial DNA sequencing. As shown in **Fig. 1,** the six CETP variants that were expressed are characterized by internal deletions or amino- or carboxy-terminal truncations. The mAbs were tested for reactivity with CETP fusion proteins after SDS polyacrylamide electrophoresis and electrophoretic transfer to nitrocellulose membranes. The reactivities of four of the mAbs are shown in Fig. 1 and results for all of the mAbs are summarized in **Fig. 2A.** The pattern of reactivity of individual mAbs with the series of CETP fusion proteins allowed us to construct the epitope map shown in Fig. 2B. With the exception of mAb TP16, the epitopes for all of the mAbs could be assigned without apparent ambiguity. The epitopes for mAbs TP2, TP5, TP7, TP8, TP9, TP13, TP17, and TP19 were mapped to the carboxy-terminal 67 amino acids of CETP based on the absence of reactivity with fusion protein B which lacks these residues. TP10 recognized fusion proteins B, E, and F that include, respectively, residues 5–409, 367–476, and 261–476, a pattern that is consistent with its epitope being situated between residues 367 and 409. Assignment of the epitope TP12 was based on its reactivity with fusion proteins D and F, in which CETP residues 184–331 and 1–260 were, respectively, deleted and its failure to recognize fusion protein E that lacked residues 42–366. The epitopes for mAbs TP6 and TP11 were mapped to residues 261–331 by their reactivity with fusion protein F and their lack of reactivity with fusion protein D. Finally, the epitopes for mAbs TP14, TP15, TP18, and TP20 were localized to residues 184–260 as a function of their recognition of fusion protein C and their lack of recognition of fusion protein D. The epitope of TP16 could not be identified as the mAb reacted, presumably nonspecifically, with all of the expressed fusion proteins. No antibodies were obtained that reacted with epitopes in the amino terminal 183 residues of CETP.

To obtain a more precise mapping of CETP epitopes, the anti-CETP mAbs were also tested for their reactivity with CETP variants having short deletions or amino acid substitutions. The variants were expressed in *E. coli* as β-galactosidase fusion proteins and/or as non-fused, glycosylated proteins in COS-7 cells. The expressed proteins were subjected to SDS polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose paper. Results with the mAb TP19, specific for an epitope situated between CETP residues 410 and 476 are shown in **Fig. 3B** and can be compared with the reactivity of TP2 (Fig. 3A). Whereas TP19 reacts with all of the variants, TP2, as previously shown (12), fails to react with variants having deletions of residues 470–475 and 463–469 or amino acid substitutions at residues 460 and 466 (13). Results for all of the mAbs are summarized in **Table 2.** TP1 and TP4 showed a pattern of reactivity identical to that of TP2. In contrast, the other mAbs whose epitope(s) were situated between residues 410 and 476 (TP5, TP7, TP8, TP9, TP13, TP17, and TP19) reacted with the four deletion mutants as well as with all of the variants having amino acid substitutions. It is

therefore probable that this latter group of antibodies reacts with epitopes situated upstream of the most amino terminal of the deletion mutants, Δ451–456. The epitopes for these mAbs could therefore be situated between residues 410 and 450 or could span a region that includes residue 410. The mAbs TP6 and TP11, whose epitopes were mapped to residues 261–331 of CETP, recognized variants having substitutions at residues 271 and 275 (H271A and K275A). TP14, TP15, TP18, and TP20, specific for epitopes between CETP residues 184–260, showed normal reactivity with variants having substitutions at residues 231 (H231A), 233 (K233A), 239 (K239A), and 259 (R259D) as well as with a double mutant having substitutions at residues 233 and 239 (K233D, K239D).

Inhibition of CETP cholesteryl ester transfer activity

We have observed in solid phase radioimmunometric assays that native CETP in solution competed poorly with CETP adsorbed to polystyrene for binding to most of the new mAbs described in this study (results not shown). This poor reactivity with liquid phase CETP was independent of whether the CETP was free or bound to HDL. Of the 16 new mAbs, only TP20 efficiently recognized liquid phase CETP. This poor reactivity with liquid phase CETP was not seen with the original panel of mAbs composed of TP1–TP4 which reacted well with CETP in solution (2). This inability of the new panel of anti-CETP mAbs to recognize CETP in solution prevented us from testing the capacity of the mAbs to neutralize CETP activity using a standard assay format for the determination of CETP-mediated lipid transfer. As a consequence, we have developed an assay of CETP activity in which CETP is immobilized on a solid phase in a conformation that can both mediate lipid transfer and be recognized by the mAbs. In initial experiments, CETP was adsorbed to polystyrene microtitre wells under the same conditions that were used in the solid phase radioimmunometric assay for screening the hybridoma supernatants. While we found that the adsorbed CETP could mediate CE transfer from HDL to LDL, we observed that there was some desorption of the CETP during the incubations and that the desorbed

TABLE 2. Reactivity of anti-CETP mAbs with CETP variants

	Antibody																	
Mutant	TP1	TP2	TP4	TP5	TP6	TP7	TP8	TP9	TP10	TP11	TP12	TP13	TP14	TP15	TP17	TP18	TP19	TP20
K233D/K239D[a]	NT	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+	+	NT	+	NT	+
K233A[a]	NT	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+	+	NT	+	NT	+
K239A[a]	NT	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+	NT	NT	+	NT	+
H231A[a]	NT	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+	NT	NT	+	NT	+
R259D[a]	NT	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	+	NT	NT	+	NT	+
H271A[a]	NT	+	NT	NT	+	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
K275A[a]	NT	+	NT	NT	+	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	NT	NT
D442G[A]	NT	+	NT	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
L455V[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L456R[b]	+	+	+	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
D460A[b]	-	-	-	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
D460G[b]	+	+	+	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
F461L[b]	+	+	+	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
F461Y[b]	+	+	+	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
H466Q[b]	-	-	-	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
S474C[b]	+	+	+	+	NT	+	+	+	NT	NT	NT	+	NT	NT	+	NT	+	+
Δ451-456[ab]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Δ457-462[ab]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Δ463-469[ab]	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Δ470-475[ab]	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
W.T.[ab]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Extracts of Cos7 cells that had been transfected with vectors encoding CETP variants or extracts of bacteria transformed with plasmids that direct the expression of CETP variants in the form of β-galactosidase fusion proteins were subjected to SDS electrophoresis, transferred to nitrocellulose membranes, and reacted with anti-CETP mAbs; *w.t.*, *wild type*; *NT*, *not tested*.

[a]Expressed in Cos7 cells.

[b]Expressed as β-galactosidase fusion protein.

CETP contributed significantly to the transfer activity that was measured (not shown). Immobilization of CETP was, however, shown to be stable when nitrocellulose membranes, in the form of 96-well MultiScreen Filtration plates, rather than polystyrene were used as the solid phase. In this format CETP retained lipid transfer activity and was recognized by the new panel of mAbs as well as by TP2. In initial experiments, increasing amounts of CETP were fixed onto nitrocellulose filters to assess the dose response of CE transfer. The results are shown in **Fig. 4B** and can be compared to a dose response using a liquid phase assay which is shown in Fig. 4A. In both cases CE transfer increased with increasing concentrations of CETP. In Fig. 4B the abscissa represents the ng CETP added to the wells of which only a fraction would have bound to the nitrocellulose membrane whereas, in Fig. 4A, the abscissa represents the amount of CETP present during the incubation with donor and acceptor lipoproteins. In all subsequent experiments a concentration of 224 ng of CETP in 80 µl was used for the adsorption step. From experiments using radiolabeled CETP, we have estimated that, at this concentration, about 40% of the added CETP is bound (results not shown).

Inhibition of CE transfer was assessed by incubating the bound CETP with increasing concentrations of the mAbs (varying from 0.25 to 10 µg/ml) prior to incubation with a mixture composed of labeled [^{3}H]CE HDL and LDL. As shown in **Fig. 5,** mAbs TP5, TP7, TP9, TP12, TP13, TP16, TP17, TP18, and TP19 partially inhibit CE transfer. These mAbs inhibited the transfer by 24% to 43% under conditions in which TP2 reduced activity by 60%. No inhibition was observed with control mAb C7 that is specific for the LDL receptor (22). Most inhibitory mAbs showed a dose-dependent increase in inhibition up to the maximum inhibition that is represented in Fig. 5. Higher concentrations of mAb did not lead to increased inhibition. The dose response for inhibition of CETP-mediated CE transfer paralleled that of mAb binding to the immobilized CETP (results not shown). Contrary to the majority of the inhibitory mAbs, TP12 and TP18 showed maximal inhibition at a

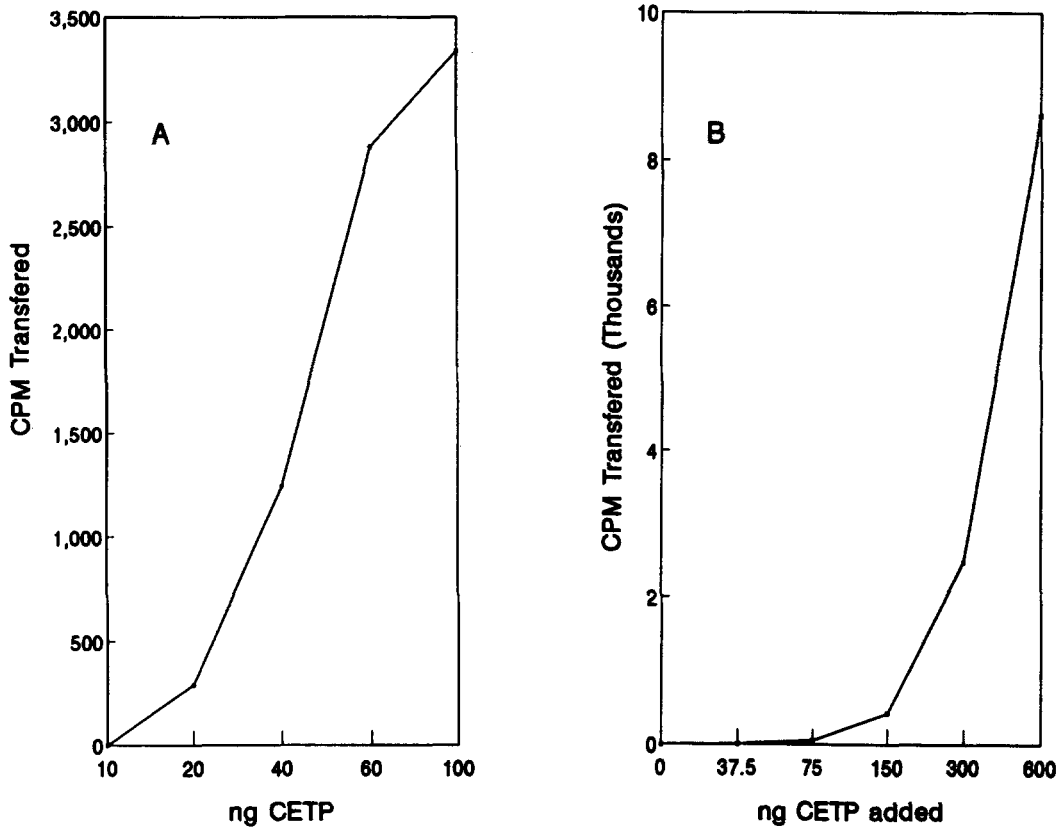

Fig. 4. Comparison of liquid and solid phase assays of CETP-mediated CE transfer. The effect of increasing concentrations of CETP on the transfer of [^{3}H]CE from HDL to LDL using liquid phase (A) and solid phase (B) assays. In the liquid phase assay, the abcissa represents the amount of CETP present during the incubation with the donor and acceptor lipoproteins. In the case of the solid phase assay, the concentration of CETP represented on the abcissa indicates the amount of CETP that was added to the wells during the adsorption step of which only a portion was fixed to the nitrocellulose membrane.

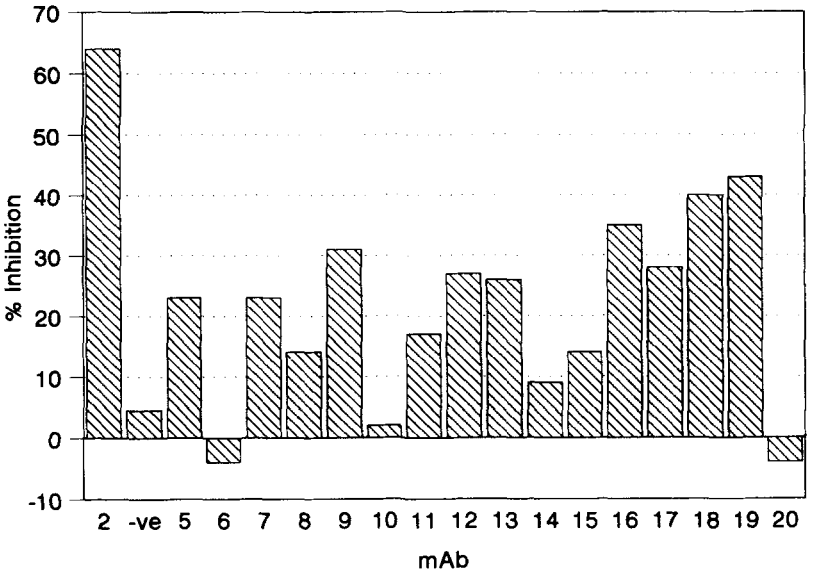

Fig. 5. Inhibition of CETP-mediated CE transfer by mAbs. Purified recombinant CETP adsorbed to nitrocellulose membranes was preincubated for 3 h at room temperature with concentrations of IgG ranging from 0.25 to 10 μg/ml. The CETP-mediated [^{3}H]CE transfer from HDL to LDL was then determined during an 18-h incubation at 37°C. The maximal inhibition observed with each mAb is represented. The anti-LDL receptor mAb, C7, was used as the negative control (-ve).

concentration of 500 ng/ml with higher concentrations leading to decreased inhibitory activity. As with the other mAbs, the dose–response curve for inhibition did parallel the ability of the mAbs to bind to the solid phase CETP (results not shown). The reasons for the atypical binding of TP12 and TP18 to the CETP are not apparent.

Most of the inhibitory mAbs are specific for epitopes that have been mapped to the last 67 amino acids of the CETP polypeptide (Fig. 2B). This could reflect the proximity of their epitopes to that of the previously described neutralizing mAbs TP1–TP3 (8). The other two inhibitory antibodies, TP12 and TP18, recognize epitopes that were mapped to regions encompassing amino acids 332–366 and 184–260, respectively.

Monoclonal antibody competition studies

Ascites from anti-CETP hybridoma-bearing mice were diluted 1/100, 1/200, and 1/400 and tested for their respective capacities to compete with ^{125}I-labeled anti-CETP mAbs for binding to immobilized CETP. Results obtained with ascites diluted 1/100 are shown in **Table 3.** The order of the mAbs in Table 3 reflects the relative positions of their respective epitopes in the CETP primary structure. The original anti-CETP mAbs, TP1–TP4, mutually compete for binding to CETP. In addition, these antibodies could partially compete with radiolabeled TP5, TP7, TP8, TP9, TP13, TP17, and TP19, whose epitope(s) are also situated between CETP residues 410 and 476, and with TP10 whose epitope is located between residues 367 and 409. The competition was not reciprocal as these latter antibodies had little ability to compete with ^{125}I-labeled TP2. The binding of ^{125}I-labeled TP10 and ^{125}I-labeled TP12 (residues 332–366) was inhibited by many of the antibodies and, in some cases, the competition was reciprocal. We could not test ^{125}I-labeled TP6 (residues 261–331); however, unlabeled TP6 totally blocked binding of ^{125}I-labeled TP11, specific for an epitope on the same region of CETP, and partially competed with several other ^{125}I-labeled mAbs. ^{125}I-labeled TP11 binding was primarily inhibited by mAbs specific for epitopes that were mapped to the same or adjacent CETP fragments (TP6, TP12, TP14, TP15, TP18, TP20). The mAbs specific for epitopes between residues 184 and 260 (TP14, TP15, TP18, and TP20) fall into three separate competition groups. TP14, TP18, and TP20 mutually compete but they show only partial competition with TP15. TP14, TP15, and TP18, but not TP20, competed with radiolabeled C-terminal mAbs, TP5, TP7, TP8, TP13, TP17, and TP19, and all four mAbs competed with ^{125}I-labeled TP10. TP16 which appeared to react with all of the CETP β-galactosidase fusion proteins was only moderately efficient in competition with itself and partially competed with ^{125}I-labeled TP13 and ^{125}I-labeled TP10. In contrast, binding of ^{125}I-labeled TP16 was inhibited by all other mAbs, in some cases more efficiently than by TP16 itself.

The ability of antibodies specific for epitopes situated between residues 184 and 260 (TP14, 15, and 18) to compete with antibodies specific for epitopes located in the carboxy terminal 110 residues (TP5, 7, 8, 9, 10, 13, 17, and 19) was unexpected. We have, therefore, done more extensive competition studies using purified IgG from selected clones. It can be seen in **Fig. 6** that TP14

TABLE 3. Competition of antibodies for binding to solid phase CETP

^{125}I-Ab	Competing antibody																			
	TP14	TP15	TP18	TP20	TP6	TP11	TP12	TP10	TP5	TP7	TP8	TP9	TP13	TP17	TP19	TP1	TP2	TP3	TP4	TP16
TP14	3	47	4	21	60	89	96	57	85	90	97	96	93	89	98	94	94	NT	91	97
TP15	72	4	76	81	74	92	100	78	84	85	96	97	99	97	99	89	92	NT	87	97
TP18	14	36	12	35	72	83	86	75	74	84	88	86	70	89	89	76	84	NT	84	85
TP20	10	81	11	9	112	109	109	97	96	114	102	109	96	87	105	92	96	NT	110	111
TP11	53	26	57	58	2	2	68	81	144	109	77	135	118	102	83	89	78	NT	101	122
TP12	48	33	49	59	47	49	15	71	99	87	65	91	89	97	84	67	69	NT	80	119
TP10	60	20	56	46	47	86	86	53	63	70	91	87	86	96	85	47	61	NT	76	71
TP5	54	43	67	102	62	80	99	64	13	9	14	20	8	5	11	52	51	NT	52	114
TP7	40	33	53	87	33	69	72	60	12	3	8	15	6	3	8	37	35	NT	65	97
TP8	52	34	42	108	48	98	81	99	9	5	8	17	6	2	7	70	53	NT	77	96
TP9	45	29	36	106	67	90	83	83	24	42	46	37	24	28	30	49	65	NT	75	112
TP13	32	37	29	88	29	107	84	50	12	5	9	20	5	3	9	38	60	NT	83	53
TP17	48	32	53	99	49	79	68	67	16	7	17	29	12	3	16	47	45	NT	69	96
TP19	56	41	47	77	57	104	90	97	16	6	11	19	8	4	11	69	57	NT	44	100
TP1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8	5	8	4	NT
TP2	85	76	90	96	75	88	95	98	88	82	88	95	95	94	100	22	16	21	21	103
TP4	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	15	2	18	2	NT
TP16	50	32	39	68	61	46	52	62	36	25	59	51	49	43	56	38	56	NT	45	50

Antibodies were radiolabeled with ^{125}I and tested for binding to solid phase CETP in the presence of competing unlabeled antibodies. The results show the binding in the presence of competing antibodies (ascites diluted 1/100) as a percentage of the binding in the absence of competitor; NT, not tested.

and TP15 can compete with ^{125}I-labeled TP10 for binding to immobilized CETP in a dose-dependent manner. The two mAbs are, however, less efficient than TP10 itself.

DISCUSSION

We have prepared 16 new anti-human CETP mAbs. Their respective patterns of reactivity with a series of CETP fragments expressed as β-galactosidase fusion proteins allowed us to formulate the epitope map shown in Fig. 2B. Four mAbs, TP14, TP15, TP18, and TP20 react with epitopes situated between residues 184 and 260. The ability of these four mAbs to mutually compete for binding to CETP with each other and with mAbs specific for epitopes situated elsewhere in the CETP primary structure demonstrates that TP15 and TP20 react with distinct epitopes that are, in turn, different from the epitope(s) recognized by TP14 and TP18. The epitopes for TP6 and TP11, both situated between residues 261 and 331, could not be distinguished or mapped more precisely by competition studies or by the reactivity of the two mAbs with CETP variants having amino acid substitutions in this region. TP12 and TP10 are specific for epitopes that are situated between residues 332 and 366 and between 367 and 409, respectively. The epitopes for seven of the new mAbs, TP5, TP7, TP8, TP9, TP13, TP17, and TP19, co-localize to a region spanning residues 410 through 450 and are thus clearly different from those recognized by the original panel of mAbs TP1–TP4 that are situated in the carboxy-terminal 26 amino acids of CETP.

One unusual characteristic that is shared by all of the members of this second panel of anti-CETP mAbs is that, while they react well with CETP when it is bound to a solid surface, they react poorly with CETP in solution. We have previously produced mAbs against human lecithin:cholesterol acyltransferase (23) and against human apolipoprotein B (21) that also react preferentially with their respective antigens when these latter are immobilized on a solid surface. While the mechanism responsible for the phenomenon is not clear, it could involve the generation of neoepitopes upon adsorption of CETP to the solid phase. The latter case would imply that CETP undergoes a conformational change upon adsorption to the solid phase and that the mAbs would be specific for a non-native conformation of CETP. This altered conformation would nevertheless still be compatible with lipid transfer activity and the expression of the TP2 epitope. Immobilization of CETP could also lead to immunochemical heterogeneity. Differential expression of epitopes on CETP could be one reason why none of the anti-CETP mAbs, including TP2, completely neutralized lipid transfer

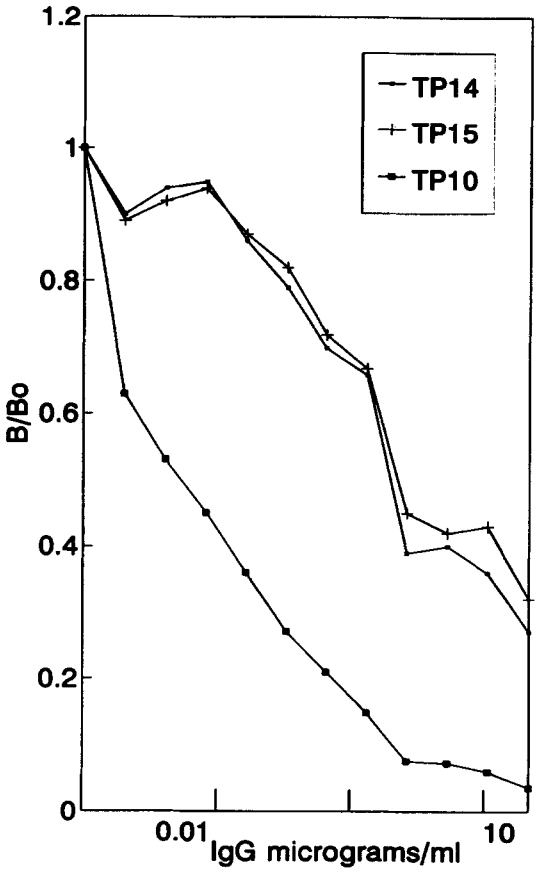

Fig. 6. The abilities of mAbs TP14 and TP15 to compete with ^{125}I-labeled TP10 for binding to immobilized CETP. Increasing concentrations of TP10, TP14, and TP15 IgG were mixed with ^{125}I-labeled TP10 IgG and added to Immulon II microtiter wells to which CETP had been adsorbed. The bound radioactivity in the presence of unlabeled competitor as a percentage of bound radioactivity in the absence of cold competitor is indicated.

activity in the solid phase assay and might also have contributed to the partial or non-reciprocal competition that was observed between certain pairs of mAbs in the antibody competition studies. In addition to the effects of putative conformational changes in CETP upon immobilization, incomplete inhibition of CETP activity by the mAbs could also result from the inaccessibility of epitopes in a subpopulation of adsorbed CETP due to steric hinderance that results from protein–protein interactions or from the orientation of the CETP with respect to the solid phase.

We demonstrate that immobilized CETP can mediate CE transfer and that certain of the mAbs are capable of markedly suppressing this activity. The ability of these mAbs to neutralize CETP activity may reflect the proximity of their corresponding epitopes to a functionally important site on CETP. This would appear to be the mechanism by which TP2 blocks CETP-mediated lipid transfer (7, 8, 12). The carboxy-terminus of CETP, that includes the TP2 epitope, is predicted to form an amphipathic α-helix that appears to include a part of the neutral lipid binding site of CETP. Amino acid substitutions in this region of the molecule that reduce TP2 binding are largely confined to the hydrophilic face of the putative α-helix, whereas substitutions that reduce lipid transfer activity tend to involve the hydrophobic face. Binding of TP2 may therefore limit access to a lipid-binding site partly formed by the hydrophobic surface of a carboxy-terminal helix (4). Antibodies TP5, TP7, TP9, TP13, TP17, and TP19 whose epitopes are situated between CETP residues 410 and 450 inhibited lipid transfer activity. As these mAbs are specific for epitopes relatively close to the carboxy-terminus, they may limit access to the lipid binding site as has been proposed for TP2. Other mAbs that can partially neutralize CETP activity are TP18 and TP12, specific for epitopes that are situated in the regions 184–260 and 332–366, respectively. As these epitopes are distant from those of the carboxy-terminal-specific neutralizing mAbs in the CETP primary structure, TP18 and TP12 may be interfering with mechanisms involved in CETP-mediated lipid transfer that are different from that of

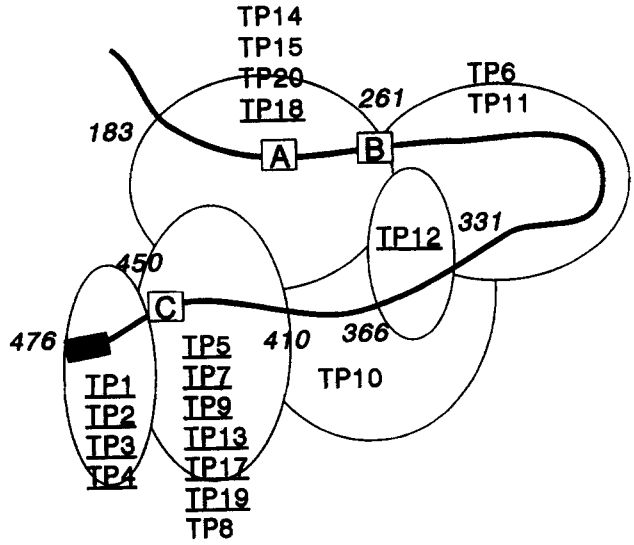

Fig. 7. A model of the carboxy-terminal 60% of CETP based on the results of epitope mapping and antibody competition experiments. The abilities of mAbs to compete for binding to CETP are indicated by the overlapping ellipses. The cross-hatched box represents the predicted carboxy-terminal amphipathic α-helix that is thought to form part of the CETP neutral lipid binding site. The open boxes A and B represent lysines 233 and 259, respectively, that are proposed to be implicated in the binding of CETP to lipoproteins and box C represents Asp442 whose replacement by Gly is associated with CETP deficiency. Antibodies that are capable of greater than 20% inhibition of CETP-mediated CE transfer are underlined. The competition that was observed between mAbs specific for epitopes situated between residues 183 and 261 and mAbs specific for epitopes situated in the regions 410–451 and 366–410 could indicate that the middle of the CETP polypeptide is spatially close to the carboxy terminal in CETP tertiary structure.

neutral lipid binding. Recently, it has been shown that, when Lys233 or Arg259 are replaced by neutral or acidic amino acids, the resulting CETP variants have markedly reduced lipid transfer activity (24). The binding of CETP(Lys233 → Ala) to HDL was shown to be defective and it has been proposed that both Lys233 and Arg259 may be implicated in the association of CETP with the lipoprotein surface. While the neutralizing mAb, TP18, whose epitope is situated between residues 184 and 260, recognizes these CETP variants normally, due to the proximity of its epitope to Lys233 and Arg259 in the CETP primary structure, it may inhibit activity by interfering with the binding of CETP to lipoprotein surfaces. Alternatively, TP18 could sterically encumber another potentially important site centered on residue 165 that was identified by insertional mutagenesis (25).

A second mechanism by which the anti-CETP mAbs could potentially inhibit lipid transfer activity is by either inducing a conformational change in CETP or by altering the equilibrium between an active and an inactive CETP configuration. Certain mAbs specific for tryptophan synthase have been shown to neutralize enzyme activity by trapping a transient, spontaneous, unfolding intermediate that is catalytically inactive (26, 27). Interestingly, these mAbs resemble certain of the neutralizing anti-CETP mAbs (e.g., TP5, TP7, and TP9) in having preferential reactivity for immobilized antigen and reaction kinetics that are characterized by slow k_{on} and k_{off} rate constants (Table 1).

The extensive cross-competition amongst the mAbs for binding to solid phase CETP (Table 3) that we observed indicates a compact tertiary structure for the carboxy terminal 60% of the CETP molecule consistent with previous biochemical experiments (28). Notably, we have shown that TP14, TP15, and neutralizing mAb TP18 that are specific for epitopes between 184–260 efficiently compete with mAbs TP5, TP7, TP8, TP9, TP13, TP17, and TP19 that are specific for epitopes near the carboxy terminus (Table 3). Thus, epitopes such as those of TP14, 15, and 18 that are distant from the carboxy terminus in terms of primary structure may be relatively close in terms of tertiary structure. The results of the antibody competition experiments have been consolidated into the model of CETP structure shown in **Fig. 7.** In the tertiary structure of CETP, the epitopes for all of the neutralizing mAbs may be sufficiently close together that each of the mAbs may potentially interfere with the same related functional sites on CETP. However, whether neutralization of CE transfer activity by different mAbs, specific for epitopes that are separated in CETP primary structure, reflects the proximity of individual epitopes to distinct functional sites on CETP or a compact tertiary CETP structure, the antibody competition experiments clearly demonstrate that, in CETP tertiary structure, residues that have been implicated in the binding of CETP to lipoproteins (24) are relatively close to residues that constitute the putative CETP neutral lipid-binding site(s) (8). It has been proposed that occupation of the CETP neutral lipid-binding site(s) may increase the affinity of CETP for lipoproteins (8), a coordination that may require the propinquity of the two sites. Moreover, as both CETP-mediated phospholipid and neutral lipid activities are neutralized by mAb TP2 (2), it would appear that the residues which constitute the CETP phospholipid and neutral lipid binding sites and those responsible for mediating the binding of CETP to lipoprotein surfaces may be brought together in the native CETP molecule. This spatial proximity of residues directly implicated in lipid transfer may be essential for CETP activity.

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