Plasma Cholesteryl Ester Transfer Protein Has Binding Sites for Neutral Lipids and Phospholipids*

(Received for publication, August 3, 1987)

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The Journal of Biological Chemistry
Vol. 263, No. 11, Issue of April 15, pp. 5150-5157, 1988
Printed in U.S.A.

The plasma cholesteryl ester-transfer protein (CETP) redistributes lecithin:cholesterol acetyltransferase-derived cholesteryl esters from their sites of synthesis in HDL to the less dense triglyceride-rich lipoproteins (1). The transfer of neutral lipids and phospholipids between the lipoproteins is facilitated by a plasma CETP (2). In addition to its role in redistributing lecithin:cholesterol acetyltransferase-derived CE from HDL to other lipoproteins, the CETP has recently been demonstrated to promote transfer of CE from HDL into cultured HepG2 cells (3). The CETP (M, 74,000) has been purified to homogeneity (4). Amino acid analysis revealed that the CETP has an unusually high content of nonpolar residues compared to other apolipoproteins (4), suggesting the possibility of neutral lipid binding sites in the CETP. Subsequent reports have described a cholesteryl ester transfer protein of identical Mr, pl, and N-terminal sequence and have confirmed the high content of hydrophobic amino acid residues (5, 6).

Kinetic studies of the CETP have generated conflicting results with regard to the mechanism of facilitated lipid transfer between lipoproteins. CETP has been postulated to act as a carrier of lipid between lipoprotein particles (7), or to enhance the exchange of lipids during formation of a ternary collision complex involving donor and acceptor lipoprotein and CETP (8). A major difficulty in ascertaining the mechanism of facilitated lipid transfer has been the inability to isolate and characterize a stable intermediate, i.e. either a ternary complex consisting of a donor and acceptor lipoprotein and CETP or a CETP lipid carrier that has acquired lipid from a donor and is subsequently able to donate that lipid to a new acceptor particle in the absence of the original donor particle. In the present study we have documented the rapid transfer of PC, CE, and triglyceride from vesicles to the CETP. The CETP can then donate these lipids to an acceptor lipoprotein (LDL), suggesting that the CETP can operate by a carrier-mediated mechanism.

**MATERIALS AND METHODS**

RESULTS

Transfer of Cholesteryl Ester from Vesicles to CETP and Subsequent Donation to LDL—Earlier investigations have documented the stable binding of CETP to PC vesicles (13). However, we found that under certain conditions the CETP did not associate with the vesicles (see Miniprint Supplement). Rather, radiolabeled CE was transferred from the vesicles to CETP, and subsequently, the CE could then be donated to LDL (Fig. 1). The elution profiles of unilamellar PC/CE vesicles (Fig. 1A) and partially purified CETP (Fig. 1B) are shown. CETP was incubated for 15 min at 37°C with a small mass of PC/CE vesicles containing a trace of high specific activity cholesteryl ester, and then passed over Sephadex G-200 to separate the vesicles and CETP. A substantial proportion of the [14C]cholesteryl ester and a smaller amount of [3H]phosphatidylcholine were transferred from the vesicles to the peak of CETP activity (Fig. 1C). Ninety-eight percent of the [3H]activity transferred to CETP was shown to be present in CE by thin layer chromatography. The peak of CETP was collected, concentrated, and rechromatographed alone (Fig. 1D) or after incubation with LDL (Fig. 1E). The elution volume of the reisolated [3H]CE CETP was altered

* Portions of this paper (including "Materials and Methods," part of "Results," and Figs. 7-9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Lipid Binding to Transfer Protein

FIG. 1. Transfer of CE and PC from \([^{14}C]PC/[^{3}H]CE\) vesicles to partially purified CETP and subsequent donation to LDL. Partially purified CETP (4.0 mg) was incubated at 37 °C with \([^{14}C]PC/[^{3}H]CE\) vesicles (27 nmol of PC) for 15 min, and then eluted (3.5 ml/h) on a 100-cm Sephadex G-200 column equilibrated with 50 mM Tris-HCl, pH 7.4, 2 mM EDTA at 4 °C. Fractions were analyzed for both CETP activity and radioactivity as described under "Materials and Methods." A, vesicles alone; B, partially purified CETP alone; C, partially purified CETP incubated with vesicles. \([^{3}H]CE\) CETP eluting as free CETP in panel C was re-isolated and divided into two portions, D, one-half of the sample (\([^{3}H]CE\) CETP alone) was re-chromatographed on Sephadex G-200; and E, the other half of the sample (\([^{3}H]CE\) CETP from C) was incubated 15 min, 37 °C, with LDL (0.3 nmol of CE) and re-chromatographed over Sephadex G-200. O, cholesteryl ester transfer activity; \(\bullet\), \([^{3}H]CE\) radioactivity; and \(\bullet\), \([^{14}C]PC\) radioactivity.

slightly but consistently \((n = 10)\), when rechromatographed on Sephadex G-200, eluting with an apparent \(M_r\) of 93,000 ± 3,000 (Fig. 1D) as compared to the major peak of partially purified CETP \((M_r 72,000 ± 2,500)\) which had not previously been incubated with a donor particle (Fig. 1B). When incubated with LDL, the majority (88%) of the \([^{3}H]CE\) was transferred from the CETP to the LDL (which elutes in the void) (Fig. 1E). The PC radioactivity associated with CETP was also transferred to LDL. These experiments suggest that radiolabeled PC and CE can be transferred from vesicles to CETP, and that the CETP-bound lipids can then be donated to LDL. Since these experiments were performed with partially purified CETP, it was possible that other proteins played a role in CE binding and transfer. However, cholesteryl ester binding and transfer were also demonstrated for CETP purified to homogeneity. Although purified CETP had a greater tendency to bind to vesicles, it was still possible to identify a peak of non-vesicle-associated CETP which bound vesicle lipids (Fig. 2A). The secondary peak of free CETP was collected, incubated with LDL, and rechromatographed (Fig. 2B). As demonstrated with the partially purified protein, purified CETP was able to donate most (89%) of its acquired lipid to the LDL. In analogous experiments with PC/triglyceride vesicles, purified CETP was also shown to bind radiolabeled triglyceride. Thus, CETP has binding sites for PC, CE, and triglyceride, and the bound radiolabeled lipids can be donated to an acceptor lipoprotein (LDL).

Specificity of CE Uptake by CETP—The specificity of CE uptake by CETP was evaluated in a variety of ways. Since preparations of partially purified CETP contain many hydrophobic proteins other than CETP (resulting from the binding of relatively apolar proteins to phenyl-Sepharose during the initial step of purification), the ability of specific anti-CETP IgG to precipitate CETP-bound radiolabeled CE from a partially purified preparation was investigated. Immunoprecipitation of partially purified CETP, using nonimmune IgG had no effect on the transfer of PC and CE radioactivity from vesicles to CETP (Fig. 3A). In contrast, immunoprecipitation with anti-CETP IgG removed 100% of CE transfer activity (not shown) and virtually abolished the secondary peak of CE

The later eluting (non-vesicle-associated) CETP is referred to as "free" even though it contains small amounts of lipid.
radioactivity (Fig. 3B). Although most of the secondary peak of PC radioactivity was also removed, there was consistently a residual shoulder on the void eluting vesicles, suggesting the presence of another PC-binding protein (Fig. 3B). Similar results were found with PC/triglyceride vesicles. The results indicate that >90% of the non-vesicle-associated CE and triglyceride radioactivity and >80% of the PC radioactivity is bound to CETP, even though CETP is only a minor constituent (<1%) of the partially purified fraction (4). To evaluate further the specificity of the binding of CE to CETP, PC/triglyceride vesicles were incubated with large amounts of albumin or with apoA-I. Vesicles alone are shown in Fig. 3C and the incubations with albumin and apoA-I are shown in Fig. 3, D and E, respectively. Although the large amount of albumin (1 mg) resulted in slight trailing of CE radioactivity above the base line, there was no definite secondary peak of CE radioactivity (Fig. 3D). There was no binding of CE to apoA-I (50 μg) (Fig. 3E); the multiple protein peaks reflect self-association of apoA-I and binding of apoA-I to vesicles. Similarly, there was no binding of CE to apoA-I when higher masses were used (100 or 200 μg) or to apo-holo-HDL (200 μg), which contains apoA-II, apoE, and apoC's in addition to apoA-I (not shown). Thus, the transfer of CE from vesicles to CETP under these conditions is a highly specific phenomenon not shown to any significant degree by the other hydrophobic proteins co-purifying with CETP, or by albumin or HDL apoproteins.

**Fig. 3. Specificity of CE binding to the CETP.** A and B, partially purified CETP was immunoprecipitated with either non-immune IgG (A) or anti-CETP IgG (B), then incubated with 27 nmol of [3H]CE/[14C]PC unilamellar vesicles, and passed over 20-cm Sephadex G-200 columns equilibrated in 5 mM NH4HCO3, 0.3 mM EDTA, pH 7.5, at 23°C. Column fractions were analyzed for CE transfer activity (O), [3H]CE (●), and [14C]PC (□). The activity measurements for B are not shown since there was no detectable activity. C, D, and E: 27 nmol of 3 mol % [14C]CE/[3H]PC unilamellar vesicles were chromatographed on Sephadex G-200 alone (C), with 1 mg of bovine serum albumin (D), or with 50 μg of apoA-I (E). ApoA-I samples were prepared by dissolving apoA-I in 1 M fresh urea, and diluting to 0.1 M ura when mixed with vesicles. Fractions were analyzed for [14C] CE (●) and protein (△) was determined by the method of Bradford (12).
group blocking reagent, inhibits both triglyceride, and to a lesser extent, CE transfer mediated by CETP (14, 15). Previous experiments have suggested that the inhibitory effect of pCMPS is due to a direct effect of pCMPS on CETP, and not to a modification of the donor or acceptor lipoproteins (15). To see if the inhibitory effect of pCMPS might be mediated by decreased binding of neutral lipid to the CETP, PC/triglyceride vesicles were incubated with CETP and the distribution of CETP was determined by measurement of its CE transfer activity in individual fractions (Fig. 6, open symbols). Although there was inhibition of CETP activity, the distribution of CETP was similar, with or without pCMPS. There

was a pronounced decrease in the binding of triglyceride by the CETP (53%) (Fig. 6, closed symbols). In a series of experiments performed with different concentrations of pCMPS the decrease in binding of triglyceride to the CETP was found to parallel the inhibition of triglyceride transfer activity (Table I). In other experiments, pCMPS was found to inhibit the transfer of CE from PC/CE vesicles to CETP (by 30%) in parallel with a 35% inhibition of CE transfer activity. Since it is possible that the partially purified fraction might contain other proteins which modify triglyceride binding, similar experiments were performed with homogenous, delipidated CETP. In the presence of 5 mM pCMPS the transfer of triglyceride from vesicles to purified CETP was inhibited by approximately 80% (not shown). Under conditions where CETP was predominantly bound to vesicles (longer incubation or higher vesicle/CETP ratio), pCMPS did not inhibit the binding of CETP to the vesicles. These results suggest that the inhibitory effect of pCMPS is mediated by a decrease in the binding of neutral lipid to CETP.
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Fig. 6. pCMPS inhibits the transfer of triglyceride (TG) from PC/triglyceride vesicles to CETP. Partially purified CETP (1.0 mg) was incubated for 4 h, at 37 °C either with or without 5 mM pCMPS, mixed with [3H]PC/PC/triglyceride vesicles (37 mmol of neutral PC) containing 1 mol % [3H]triolein (also adjusted to ± 5 mM pCMPS), and immediately chromatographed on a 20-cm Sephadex G-200 column equilibrated to 5 mM Tris-HCl, pH 7.4, 2 mM EDTA. Fractions were analyzed for [3H]triolein radioactivity, closed symbols; and CE transfer activity, open symbols. ○, ○, without pCMPS; △, △, with 5 mM pCMPS.

Table I

Effect of pCMPS on triglyceride transfer and binding to CETP

<table>
<thead>
<tr>
<th>pCMPS</th>
<th>Binding</th>
<th>Transfer</th>
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<tr>
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<tr>
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<tr>
<td>0.48</td>
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* Values are expressed relative to the sample without pCMPS.

DISCUSSION

We have demonstrated the rapid transfer of small amounts of neutral lipid and phospholipids from unilamellar PC vesicles to CETP. Subsequently, the radiolabeled lipids bound to CETP were readily donated to an acceptor lipoprotein, LDL. The consistency, rapidity, and specificity of lipid binding by CETP, and its inhibition by pCMPS, an inhibitor of neutral lipid transfer, strongly suggest that lipid binding sites on CETP are involved in the facilitation of lipid transfer by CETP. These findings fulfill two of the criteria of a carrier-mediated process: 1) the isolation of the carrier with bound lipid, and 2) the ability of that carrier to donate lipid to a lipoprotein. The CETP can exist in two major states: either associated with vesicles or lipoproteins (13) or as an apparent monomer with bound lipid. The ready exchange of CETP between these two states, and the instability of the binding of CETP to vesicles or lipoproteins (13, 16), are also consistent with a carrier-mediated mechanism of lipid transfer.

Several lines of evidence suggest that the binding of neutral lipid by CETP is intrinsic to the mechanism of facilitated lipid transfer. Although a variety of apolipoproteins can bind to egg phosphatidylcholine vesicles, they do not readily bind neutral lipids, unless co-sonicated with the lipid (17). ApoA-I, apo-HDL, albumin, and a variety of other hydrophobic proteins co-purifying with CETP did not demonstrate binding of neutral lipid or phospholipid when incubated with PC/CE vesicles under conditions similar to those employed for CETP. These observations indicate that this phenomenon is relatively specific for CETP. Furthermore, an inhibitor of CE and triglyceride transfer, pCMPS, was found to decrease the binding of neutral lipid to CETP. Although inhibition was incomplete, the magnitude of the effect of pCMPS on neutral lipid transfer is similar to its effect on neutral lipid binding by CETP: a 20–30% decrease in CE binding, and a 50–80% decrease in triglyceride binding. Also, the decrease in triglyceride binding was found to parallel the inhibition of triglyceride transfer (Table I). These observations suggest that the binding of neutral lipid by CETP is directly related to the transfer process and is not an unrelated epiphenomenon.

To determine the amount of lipid bound to CETP, an increasing mass of PC/CE or PC/triglyceride vesicles was incubated with a constant amount of partially purified CETP. As the vesicles were increased, there was increased binding of PC, CE, and triglyceride to the CETP. By Scatchard analysis, the CE and triglyceride binding data could be reasonably well fitted by linear regression analysis, suggesting a single class of CE or triglyceride lipid binding sites. This analysis also demonstrated that the binding of CE and triglyceride to the CETP reached a limiting value, corresponding to about 0.9 mol of CE/mol of CETP and 0.2 mol of triglyceride/mol of CETP. Given the uncertainties in the estimation of CETP mass by immunoblotting and the scatter in the data (Fig. 5A), the CE binding data is consistent with a single binding site for each CETP molecule. However, the binding of less than 1 mol of triglyceride/mol of CETP requires further explanation. One possibility is that the population of CETP molecules is non-uniform, with some molecules unable to bind triglyceride. However, a comparison of CE and triglyceride transfer activities through the various purification steps used to prepare CETP for these experiments shows no selective loss of triglyceride transfer activity. Another possibility is that the result reflects loss of triglyceride from CETP during column chromatography. Loss of small amounts of triglyceride from CETP could have a large impact on the results of Fig. 5B without an appreciable effect on overall recovery during column chromatography. A more definitive appraisal of the stoichiometry of triglyceride binding by CETP will require an approach which can measure the binding of triglyceride by CETP in equilibrium mixtures with lipoproteins or vesicles.

Although the neutral lipid binding site(s) would readily take up CE or triglyceride from vesicles, the affinity for CE or triglyceride was similar to that of the vesicles, since at all vesicle/CETP ratios the neutral lipid was distributed between the vesicles and CETP. A variable stoichiometry of lipid binding by CETP (e.g. from 0.1 to 0.9 mol of CE/mol of CETP in Fig. 5) could form the basis of a mechanism for the net transfer of lipids. The direction and rate of neutral lipid transfer should be influenced by the ratio of lipoprotein surface neutral lipid to CETP, since this would be expected to affect the amount of lipid bound to CETP (Fig. 5). Although the CETP promotes an equimolar exchange of CE and triglyceride between lipoproteins under many conditions (2, 18, 19), the purified CETP can promote net transfer of CE

* C. B. Hesler and A. R. Tall, unpublished data.
complexes can be isolated by precipitation methods but dis-
teins or certain cells to effect transfer of CE and triglyceride.

The CETP was also found to bind variable amounts of phos-
pholipid, from about 2 to 11 mol/mol of CETP. The variable elution volume of CETP on Sephadex G-200 columns (corresponding to an apparent M, of 75,000-93,000) probably largely results from the uptake of different amounts of phos-
pholipid. The bound phospholipid seems to play an essential
role in activity since extensive delipidation of CETP results
in an inactive preparation, which can be reactivated following
reintroduction of PC, using a PC/cholate dialysis procedure.5

Amino acid analysis of CETP has established that it is an
extremely hydrophobic protein, compared to other plasma
proteins and apolipoproteins (4). The protein amino acid
sequence, derived from the cDNA, shows clusters of hydro-
phobic residues distributed throughout the protein (5). Upon
folding of the protein, the hydrophobic regions may form one
or more pockets to accommodate neutral lipid and phos-
pholipid fatty acyl chains. It is possible that the phospholipids
form part of the neutral lipid binding site(s) of CETP.

The transfer of radiolabeled lipids from vesicles to CETP
was more rapid than the formation of stable complexes be-
tween CETP and LDL or very low density lipoprotein; these
complexes may represent a lipid transfer which act by binding with high affinity to this site.

REFERENCES


Continued on next page.
Lipid Binding to Transfer Protein

METHODS

Preparation of phosphate-activated myelin

Myelin vesicles containing either cholreny alcohol (CC) or triglyceride (TG) were prepared using the following protocol. Lipid was dissolved in CHCl₃ to a concentration of 5 mg/ml and was added to a solution of 10 mM triethanolamine, pH 7.4, and 1 mM MgCl₂. This mixture was then subjected to a temperature of 37°C for 30 minutes. The resulting solution was then filtered through a 0.22-μm filter and used immediately.

Protein binding

Protein binding was measured by incubating 50 μg of protein with 200 μg of myelin vesicles in 0.1 M Tris-HCl, pH 7.4, for 1 hour at 37°C. The reaction was then stopped by the addition of 200 μM NaOH. The resulting mixture was then centrifuged at 100,000 g for 1 hour. The supernatant was then removed and the pellet was resuspended in 0.1 M Tris-HCl, pH 7.4, and 10 mM MgCl₂. The resulting solution was then filtered through a 0.22-μm filter and used immediately.

Results

Table 1. Effect of variable binding on the distribution of CEP and CEP after incubation for 15 min with myelin vesicles is shown in Figure 3.

![Figure 3](image-url)
Lipid Binding to Transfer Protein

at low ionic strength (5 mM Tris-HCl, pH 7.4, 1 mM EDTA, Fig. 6A). CTP was largely associated with vesicles, and 2% of the radiolabeled CE eluted in the CTP peak. At 10 mM NaCl (Figure 6B), 7% of the CTP was associated with the vesicles, and there was a parallel change in the distribution of CE radioactivity. At 150 mM and 500 mM NaCl (Fig. 6C and D), 90% of the CTP was associated with the vesicles. In the absence of NaCl, a small second peak of CE radioactivity was apparent. At higher ionic strength (250 mM NaCl), the majority of the CTP was non-vesicle bound, but the recovery of CTP was much lower (not shown). The results show that the vesicle-associated CTP is largely associated with the vesicles, and that the CTP is also found in the non-vesicle fraction. These results may reflect the presence of free CTP and the association of CTP with the vesicles.

The effect of increasing the vesicle/CTP ratio on the distribution of CTP and CE is shown in Figure 7. The binding of CTP to the vesicles increases as the ratio of vesicles to CTP increases. The distribution of CTP radioactivity followed that of the CTP. Figure 7A shows a range of CE vesicle/CTP ratios that were higher than those employed to assess the cholesterol content of lipid binding to CTP (Fig. 8A).

Figure 7

The effect of vesicle/CTP ratio on the distribution of CTP and CE radioactivity. The CTP-CE vesicles containing CTP were added to partially purified CTP in 1.5 mg and immediately chromatographed on 20 cm octadecyl-DEAE-cellulose equilibrated in 1 mM Tris-HCl, pH 7.4, 1 mM EDTA at some temperatures. Fractions were analyzed for CTP radioactivity (A), and for CE radioactivity (B) and (C). CE vesicle (C) and CTP vesicle (P).

A: 27 mmol vesicles (P); B: 34 mmol vesicles (P); and C: 68 mmol vesicles (P).