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Modification of the N-terminal cysteine of plasma cholesteryl ester transfer protein selectively inhibits triglyceride transfer activity

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Abstract

An invariant cysteine residue is found at the N-terminus of cholesteryl ester transfer protein (CETP) isolated from plasma of humans, rabbits and cynomolgus monkeys. We previously reported the expression of recombinant rabbit cholesteryl ester transfer protein in yeast (Kotake et al., J. Lipid Res. 1996; 37: 599–605). The recombinant CETP secreted into the medium contains an altered N-terminal sequence but was fully capable of facilitating both cholesteryl ester (CE) and triglyceride (TG) transfer between lipoproteins. We investigated the importance of the conserved N-terminal cysteine of plasma CETP in the lipid transfer activity by chemical modification of the free sulfhydryl groups of the recombinant CETP and CETP from human and rabbit plasma. The unmodified forms of these CETPs had similar specific activities of CE and TG transfer. Neither 5,5′-dithiobis-(2-nitrobenzoate) nor N-ethyl maleimide altered the lipid transfer activity. In contrast, p-chloromercuriphenyl sulfonate selectively inhibited the TG transfer activity of both human and rabbit plasma CETP. The TG and CE transfer activities of the recombinant CETP, which lacks the N-terminal cysteine residue, was not affected. These results demonstrate that the N-terminal cysteine residue of both human and rabbit plasma CETP is free and is likely to be involved in the construction of a critical part of the active site of CETP that can determine the selectivity of the lipid molecule for the transfer reaction. © 1997 Elsevier Science B.V.

Keywords: Cholesteryl ester transfer protein; Activity; Recombinant protein; Specificity

Abbreviations: CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); NEM, N-ethylmaleimide; pCMPS, p-chloromercuriphenyl sulfonat.

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1. Introduction

Cholesteryl ester transfer protein (CETP) is a plasma glycoprotein that catalyzes the redistribution of neutral lipids, such as cholesteryl esters (CE) and triglycerides (TG), among lipoprotein particles [1–3]. The CETP reaction significantly contributes to plasma lipoprotein metabolism, particularly the level of high density lipoprotein (HDL) [4]. However, the role of the CETP reaction in atherogenesis remains a subject of debate. CETP may be antiatherogenic by facilitating the net transfer of cholesteryl ester from HDL to apolipoprotein B-containing lipoproteins, a step involved in the transport of cholesterol from the peripheral tissues to the liver [5,6]. On the other hand, the CETP reaction may be atherogenic since it results in the unfavorable decrease of HDL cholesterol and the increase of low density lipoprotein (LDL) cholesterol [7].

CETP facilitates the equimolar exchange of neutral lipids between lipoproteins [8]. Using lipid microemulsion activated by surface-bound apolipoprotein as substrates, CETP shows high selectivity for CE over TG in the transfer reaction [9]. This selectivity is decreased by the integration of apolipoproteins into the lipid–protein complex [9,10]. Consequently, the preference of CETP for CE over TG for lipid transfer between native lipoproteins is only moderate. Hence hetero-exchange of CE and TG between CE-rich and TG-rich lipoproteins takes place and leads to a progressive depletion of CE and enrichment in TG of HDL [9,11]. Indeed, selective inhibition of TG transfer resulted in the inhibition of net CE transfer between lipoproteins even when the CE exchange reaction was allowed to occur [12]. Thus, selectivity of lipid of the CETP reaction is one of the key factors that regulate plasma HDL cholesterol level.

The nature of the CETP lipid binding site(s) is not yet fully characterized. Unlike phospholipids, CE and TG compete for the transfer reaction by CETP, whereas neither compete against phospholipids. This indicates the existence of a common binding sites for CE and TG [8,9,13] and likely a unique site for phospholipids. At least 3 monoclonal antibodies against CETP have been shown to inhibit TG transfer but not CE transfer [12,14,15], and one of these antibodies also inhibited the transfer of pyrene-acylester of cholesterol [12]. These results suggested that lipids with large molecular size has weaker affinity for the common lipid interaction site of CETP, and the antibodies can interfere with this weak interaction. Several reports have also shown that cysteine-modifying reagents, particularly mercurial-type reagents such as p-chloromercuriphenyl sulfonate (pCMPS) inhibit TG transfer by CETP leaving CE transfer relatively unaffected [8,14,16]. Interestingly, an organomercurial cholesterol derivative known as U-617, which can potentially react with an unpaired cysteine residues, has recently been reported to inhibit CE transfer with minimal inhibition of TG transfer [15,17]. These observations suggest the involvement of unpaired cysteine residue(s) in the lipid transfer process.

Deletion and mutagenesis studies of CETP [13,18] and epitope mapping of the inhibitory monoclonal antibodies against CETP [19,20] have clearly demonstrated the importance of the domain(s) near the C-terminal of the protein for the transfer activity. The C-terminal region of human CETP (residues 470-475) has been proposed to construct a lipid binding site [18] (see Fig. 1). Rabbit CETP shows a high degree of structural similarity to the human CETP [22] and the proposed lipid binding site for human CETP corresponds to a conserved region (residues 490-495) in the C-terminal end of rabbit CETP (see Fig. 1).

Rabbit CETP contains 9 cysteines, 7 of which are located in the same positions as all 7 cysteine residues of human CETP (Fig. 1). The two additional cysteine residues in rabbit CETP are located near the C-terminus but not in the proposed lipid binding site (Fig. 1). The most interesting of the cysteine residues

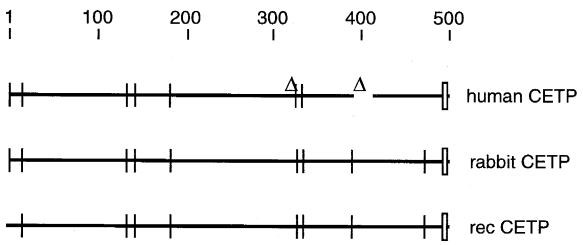

Fig. 1. The linear structure of human plasma CETP (top), rabbit plasma CETP (middle) and recombinant (rec) rabbit CETP (bottom). The perpendicular lines represent the location of cysteine residues. The gaps (△) in the human CETP represent missing sequences that are found in the rabbit CETP (residue 318 and residues 395-413). The putative lipid binding site near the C-terminal region is indicated as an open box.

is the one that makes up the N-terminal cysteine residue of the mature human, rabbit and cynomolgus monkey CETP [23]. Previously, we reported the production of recombinant rabbit CETP in the yeast, *Pichia pastoris* [24]. The recombinant CETP was fully capable of CE and TG transfer even though the N-terminal sequence of the mature protein had been altered. Using this recombinant mutant protein and CETP purified from human and rabbit plasma, we studied the importance of the N-terminal cysteine residue in the lipid transfer activity.

2. Materials and methods

2.1. Isolation of CETP

Methylotrophic *Pichia pastoris* strains expressing recombinant rabbit CETP were established previously [24]. In order for the recombinant CETP to be secreted, the sequence corresponding to the mature portion of the rabbit CETP was fused to a yeast acid phosphatase signal sequence. Recombinant CETP was partially purified from the culture medium of transformed yeast clones by chromatography on Butyl-Toyopearl (Supelco Canada) as described previously [24]. Human CETP was isolated from human plasma as previously described [25]. The bottom fraction of the density 1.23 g/ml (adjusted with NaBr) of fresh plasma from New Zealand White rabbits was dialyzed against the 10 mM Na-phosphate buffer (pH 7.4) containing 150 mM NaCl, and used as a source of rabbit CETP.

2.2. CE and TG transfer assay for CETP activity

Lipid transfer activity of CETP and LDL containing [^{3}H]cholesteryl oleate was prepared according to previously described methods [25,26]. The reaction mixture was composed of labeled LDL and unlabeled HDL (32 nmol and 30 nmol of cholesterol moiety, respectively) and one of the CETP preparations in the 10 mM Na-phosphate buffer (pH 7.4), containing 150 mM NaCl and 5% bovine serum albumin (fatty acid-free, Sigma) in a final volume of 237.5 μl. The reaction was carried out by incubating the mixture at 37°C for 2 h and terminated by adding 12.5 μl of 5% heparin/1 M $MnCl_2$ to precipitate LDL. Radioactivity in a 100 μl aliquot of the supernatant was counted. The HDL recovery in the supernatant has been 85 $\pm$ 3% regardless of the assay mixture. The specific lipid transfer activity of CETP was calculated as the CE or TG transfer activity per unit of immunoreactive CETP mass and expressed as a percentage of that measured in rabbit plasma (New Zealand White). Sulfhydryl modifying reagents, p-chloromercuriphenyl sulfonate (pCMPS), N-ethyl maleimide (NEM), and 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) were added to this assay mixture to make the final concentration described in the legend for each experiment. Each of these reactions was compared to control reactions without the sulfhydryl modifying agents.

3. Results and discussion

3.1. Specific CE and TG transfer activities of the partially purified recombinant rabbit CETP

The expression of recombinant CETP in yeast necessitated the replacement of the mature N-terminal cysteine residue with the tri-peptide Arg-Glu-Phe [24]. The remaining cysteine residues were conserved (Fig. 1). The internal amino acid sequence of the recombinant CETP differs from the sequence described by Nagashima et al. [21] by 6 residues. Interestingly, except for that of the residue 122 the amino acid replacements predicted by the rabbit CETP cDNA cloned by us are identical to the corresponding residues in human CETP [22]. The recombinant rabbit CETP was purified 22.6-fold from the conditioned medium of the transformed yeast clones after chromatography on a Butyl-Toyopearl column. Despite the alteration of the N-terminal sequence, the partially purified recombinant CETP possessed both CE and TG transfer activities comparable to that of rabbit plasma CETP (Fig. 2) [24]. Rabbit CETP and human CETP were previously shown to have the same specific lipid transfer activity [26].

3.2. Effect of sulfhydryl-modifying reagents on CETP activity

The effect of sulfhydryl-modifying reagents on CETP-mediated transfer of CE or TG from LDL to HDL was determined (Fig. 3). As previously noted

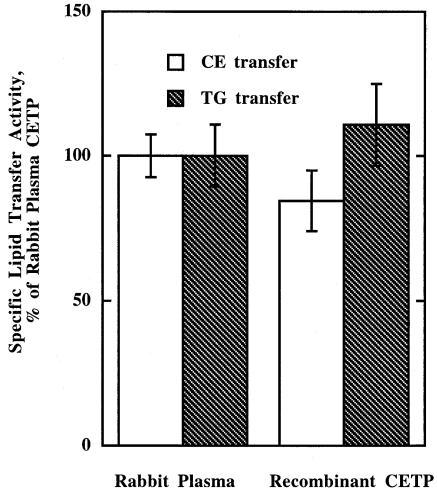

Fig. 2. Specific transfer activity of the partially purified recombinant CETP. The data was adopted from the Ref. [24]. The lipid transfer specific activities for each protein was calculated from CE or TG transfer activity per unit immunoreactive mass of CETP protein determined by densitometric scanning of immunoblots [24]. The CE transfer (open bars) and the TG transfer (hatched bars) activities (mean ± S.E.M. of triplicate assays) are shown normalized to the respective activities of rabbit plasma CETP.

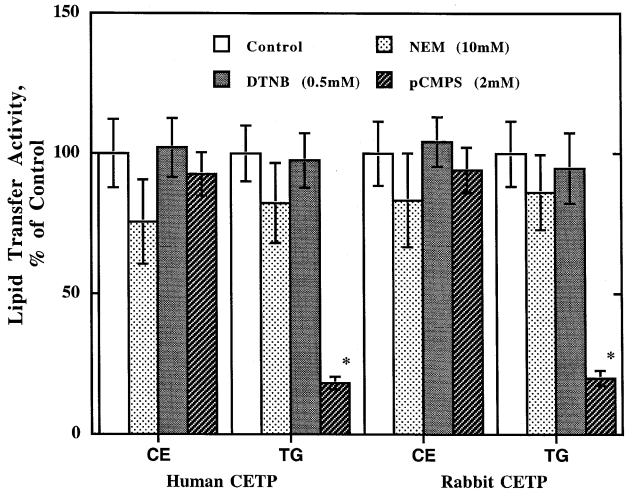

Fig. 3. Effect of sulfhydryl modifying reagents on human and rabbit plasma CETP. The CE and TG transfer activities of human and rabbit plasma CETP was assayed in the presence of each compound indicated. The compounds were added to assay mixture immediately before the reaction. The results are expressed relatively to the control reactions without the reagents. Data represent the mean ± S.E.M. of triplicate assays. Asterisks indicate significant difference from controls ($P < 0.001$, Student's *t*-test).

[8,14,16], TG transfer mediated by human plasma CETP was almost completely abolished by the mercury compound pCMPS, whereas the CE transfer was only slightly decreased. The same effect was also demonstrated with rabbit plasma CETP. The irreversible sulfhydryl alkylating reagent, NEM, insignificantly decreased CE and TG transfer activity of both human and rabbit CETP. However, DTNB inhibited neither TG nor CE transfer mediated by either human or rabbit CETP. The effect of these sulfhydryl modifying reagents on the lipid transfer activity of the recombinant CETP was also examined (Fig. 4). Neither NEM nor DTNB inhibited the lipid transfer activity of the recombinant CETP. In contrast, pCMPS which inhibited the TG transfer of the plasma CETPs had no inhibitory effect on TG transfer mediated by the recombinant CETP. This discrepancy was confirmed by the dose-dependent inhibition of the TG transfer activity of plasma CETP by pCMPS (Fig. 5).

Treatment of plasma CETP with pCMPS resulted in almost complete inhibition of TG transfer, whereas CE transfer was relatively unaffected. This result indicates that unpaired cysteine residue(s) may reside near or in a presumed hydrophobic pocket of CETP that is proposed to interact with the neutral lipid substrate. The recombinant CETP displayed the same

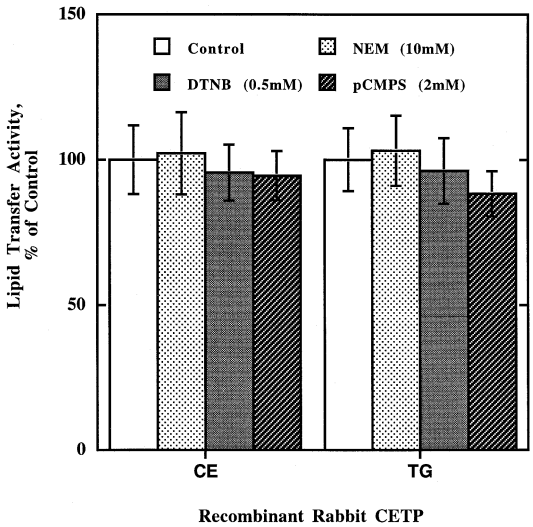

Fig. 4. Effect of sulfhydryl modifying reagents on the activity of the recombinant rabbit CETP. The CE and TG transfer activities of the recombinant CETP were determined in the presence of the sulfhydryl modifying reagents. The values (mean ± S.E.M. of triplicate assays) shown are expressed relative to the control reactions without the reagents.

specific transfer activity of CE and TG despite the alteration of the N-terminal sequence by substitution of cysteine with the tri-peptide, and was not affected by pCMPS treatment. This indicated that the N-terminal cysteine residue of plasma CETP is not involved in a disulfide bond and is responsible for the inhibitory effect of pCMPS.

The experiments using domain deletion and site-specific mutagenesis of human CETP [13,18], as well as those employing a monoclonal antibody [19], have indicated that the region near the C-terminal is responsible for lipid binding and transfer activity. The data from the present study suggest that the N-terminus of CETP may also be involved in, or reside close to, the proposed active site of CETP in its native conformation. Interestingly, N-terminal modification resulted in selective modulation of lipid transfer reaction as only the TG transfer activity was dose-dependently suppressed by pCMPS. The modification of an N-terminal cysteine residue by pCMPS may partially interfere with the accessibility of binding site of lipid to such an extent that allows access to the CE molecule but not TG molecule due to its larger molecular volume [27].

Most of the previous work on the effect of sulfhydryl modifying reagents on CETP activity are consistent with the data and hypothesis presented in this paper [8,14,16]. However, a more recent paper reported that pCMPS and 4,4′-dithiodipyridine blocked both CE and TG transfer activities of human CETP to equal extents with much lower concentration [28]. This finding may indicate an alternative mechanism of inhibition in different experimental conditions such as modification of (an) other cysteine residue(s) due to different conformation of CETP or requirement of an additional non-specific effect of pCMPS that could be implicated by higher concentration for selective inhibition of TG transfer in our data.

The organomercurial derivative of cholesterol known as U-617 has been shown to preferentially inhibit the CE transfer over the TG transfer activity of the recombinant cynomolgus monkey CETP [15,17]. These studies are the first to document the selective inhibition of the CE transfer. Previous studies employing sulfhydryl modifying reagents [8,14,16], monoclonal antibodies [12,14] or a lipid microemulsion system [9] have shown the preference of CETP for CE over TG as the transfer substrate. The mechanism responsible for this observed inhibition is unknown. The cholesterol moiety of CE may interact with the hydrophobic lipid binding pocket of CETP with high affinity. It is conceivable that the U617 compound, which contains a sulfhydryl-modifying group in place of the hydrophobic acyl chain of CE, may covalently modify a cysteine residue that is critical for the transfer of CE molecules. The effect of this modification on TG transfer is minimal since

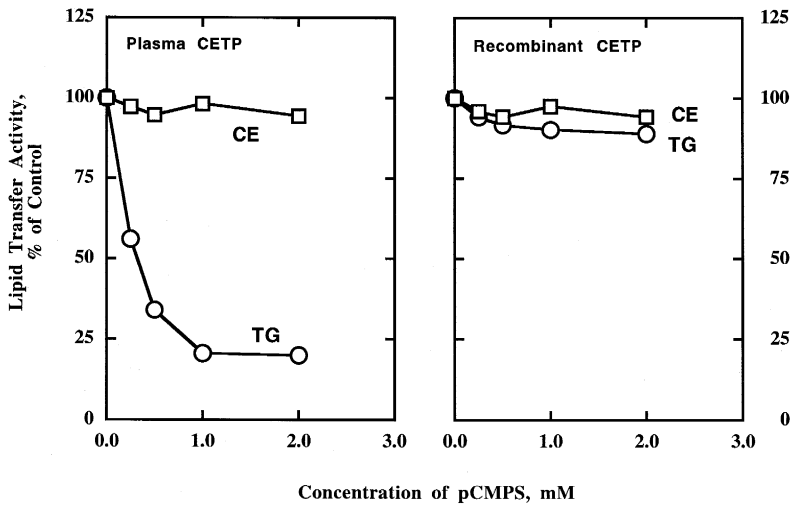

Fig. 5. The dose-dependent effect of pCMPS on the plasma and recombinant rabbit CETP. The CE and TG transfer activities were assayed in the presence of pCMPS at the indicated concentrations. The values are expressed relatively to the control without pCMPS.

the binding of TG to CETP may not be influenced by such a small molecular weight compound.

The results of the present study suggests that the N-terminus of CETP is oriented close to, or may participate with the lipid binding site of CETP. The N-terminus of the mature CETP isolated from the plasma of rabbits and humans (and perhaps cynomolgus monkeys as well) is a free cysteine residue. Chemical modification of this residue resulted in the modulation of lipid selectivity for the transfer reaction. CETP is an important regulator of plasma lipoprotein profile in human plasma, and selectivity of CE and TG transfer is one of the direct determinants of plasma HDL and LDL levels [12]. Alteration of the N-terminus of CETP can result in the selective inhibition of TG transfer, and still cause a decrease in net CE transfer from HDL to other lipoproteins leading ultimately to an increase in HDL cholesterol level.

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