Expression and secretion of rabbit plasma cholesteryl ester transfer protein by *Pichia pastoris*

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Abstract The rabbit cholesteryl ester transfer protein (CETP) was expressed in the methylotrophic yeast *Pichia pastoris* by introducing the CETP cDNA under the control of the methanol-inducible alcohol oxidase promoter. The cDNA was cloned from in vitro amplified cDNA of rabbit liver mRNA. The nucleotide sequence of the cloned cDNA differed slightly from the previously published sequence that changed the amino acid sequence in six residues. Interestingly, five of these replacements are identical to the corresponding residues in human CETP. In addition, the encoded mature N-terminal sequence was changed from Cys- to Arg-Glu-Phe- to link the CETP sequence to the yeast acid phosphatase signal peptide. The culture medium of the transformed cells induced with 1% methanol contained both cholesteryl ester and triglyceride transfer activity comparable to that of rabbit plasma. Like rabbit plasma, the lipid transfer activity in the medium could be inhibited by monoclonal antibodies that block CE/TG transfer or TG transfer alone. Immunoblot analysis of the medium detected a major immunoreactive species of M_r = 80 K and minor species of M_r = 60–100 K. In spite of these differences, the specific transfer activity of the recombinant CETP was indistinguishable from that of rabbit plasma CETP of M_r = 74 K. N-Glycosidase F treatment converted both the recombinant and plasma CETP to a single species of M_r = 55 K. Both the plasma and recombinant CETP lost their activity after removal of N-linked carbohydrate and sialic acid. A single 55 K component was found in the cell-lysates. The intracellular form of the recombinant CETP was not modified by N-glycosidase F treatment. In conclusion, the recombinant CETP is synthesized as an inactive polypeptide that is processed and secreted as a functional glycoprotein. In addition, the N-terminal Cys residue of the plasma CETP is not required for its activity.—**Kotake, H., Q. Li, T. Ohnishi, K. W. S. Ko, L. B. Agellon, and S. Yokoyama.** Expression and secretion of rabbit plasma cholesteryl ester transfer protein by *Pichia pastoris*. *J. Lipid Res.* 1996. **37:** 599–605.

Supplementary key words lipid transfer • cholesteryl ester • CETP cDNA • nucleotide sequence

Cholesteryl ester transfer protein (CETP) is a plasma glycoprotein that functions to transfer neutral lipids, such as cholesteryl ester (CE) and triglyceride (TG), among lipoprotein particles (1, 2). The majority of CE in plasma is synthesized in high density lipoprotein (HDL) by lecithin:cholesterol acyltransferase (3) and distributed to low density lipoprotein (LDL) and very low density lipoprotein by the action of CETP, mainly in exchange with TG at least in bulk plasma (4, 5). This transfer of CE can facilitate the efficient clearance of CE from circulation by receptor-mediated uptake of LDL by the liver. Therefore, CETP may play an important role in the pathway by which peripheral tissue cholesterol is removed by lipoproteins and returned to the liver for catabolism. On the other hand, the action of CETP can also result in lower HDL-cholesterol and higher LDL-cholesterol, a lipoprotein profile considered to be atherogenic and a risk for coronary heart disease (6). Thus, the overall role of CETP in plasma lipoprotein metabolism and development of atherosclerosis is unclear.

Rabbit CETP consists of 496 amino acid residues (7) and has a high degree of similarity to the human CETP of 476 amino acid (8). The major difference is the 19-residue insertion near the COOH-terminal in the rabbit protein. Biochemical analysis also show a difference in substrate specificity and distribution in plasma (4, 7, 9). We have previously purified rabbit CETP and shown that human and rabbit CETP have similar specific activities with respect to CE transfer in vitro, although they differ in physicochemical properties, especially in hydrophobicity (10, 11). We also reported the production of novel inhibitory monoclonal antibodies against rabbit CETP that cross-react with human CETP (5). One of the monoclonal antibodies dissociated radioisotopic TG and CE transfer activities of CETP.

Abbreviations: CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; DTT, dithiothreitol.

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In order to further dissect the reaction mechanism of CETP, a reliable supply as well as a facile system to introduce specific modifications to the protein is required. To meet these requirements, we have constructed a vector that directs the production of recombinant rabbit CETP in the yeast, *Pichia pastoris,* which has previously been shown to efficiently express and secrete various recombinant mammalian proteins (12–14). Once the transfected yeast cell line is established, the culture can be grown rapidly, inexpensively, and without the need for a sophisticated culture facility, an advantage compared to the expression systems utilizing mammalian and insect cells (15–17).

MATERIALS AND METHODS

Cloning of rabbit CETP cDNA

Figure 1 shows the overview of the cloning. Total RNA was extracted from the newborn New Zealand White rabbit liver and poly(A) fraction was separated from total RNA by using oligo(dT) latex (18). Two cDNA fragments (encoding from the N-terminal to amino acid residue 262 of rabbit CETP and from the residue 263 to the C-terminal) were produced by in vitro DNA amplification of cDNA reverse-transcribed from rabbit liver RNA. The region of the rabbit cDNA that encodes the mature polypeptide was obtained by in-frame fusion of the two fragments and cloning in pUC19. The sequence was confirmed by sequencing the cloned amplified products using an Applied Biosystem 373A DNA sequencer. The amplified cDNA sequence differs from the previously published sequence in 8 nucleotides causing six amino acid replacements (Asn → Asp at 110, Asp → Gln at 122, Asn → Lys at 185, Val → Met at 284, Ala → Thr at 434 and Thr → Ala at 435). Interestingly, these replacements are identical to the corresponding amino acid residues in human CETP except for that of the residue 122 (8). The rabbit cDNA was constructed to contain an EcoRI and HindIII restriction sites at the 5′ and 3′ ends, respectively. This allows the convenient cloning of the fragment into pHIL-S1 expression plasmid. In addition, the sequence of the 5′ end of the cDNA encoding the mature N-terminal end was designed to allow the in frame fusion with the *Pichia pastoris* acid phosphatase signal sequence encoded by pHIL-S1. This altered the natural N-terminal residue of the mature CETP from Cys to Arg-Glu-Phe. This modification was necessary to allow the cleavage of the acid phosphatase signal sequence encoded by the pHIL-S1 from the recombinant rabbit CETP.

Expression of rabbit CETP in *Pichia pastoris*

Pichia pastoris GS115 (defective in HIS4), pHIL-S1 expression plasmid and transfection reagents were obtained from Invitrogen (San Diego, CA). Transformation of *Pichia pastoris* GS115 with the rabbit CETP expression vector was carried out essentially according to the instructions provided by Invitrogen based on the previous publications (12–14). A recombination event is designed to occur in vivo between the cassette in pHIL-S1 (includes CETP cDNA and HIS4 between 5′ and 3′ ends of alcohol oxidase gene (AOX1) regulatory sequences) and those in the genome of *Pichia pastoris.* The clones with impaired growth in the media containing methanol as the sole carbon and energy source (in which the AOX1 structural gene was replaced with the rabbit CETP expression cassette) were selected (18). The modified cells were grown at 30°C in 150 mL of Minimal Glycerol Medium as described by Invitrogen (San Diego, CA). The AOX1 promoter is used to drive the expression of the rabbit CETP cDNA by incubating the cells in the medium containing methanol (1%, v/v) at 30°C for 48 h. The conditioned media of the cells was collected for the analysis and partial purification of the recombinant CETP. The cells were washed, resuspended in ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, and 5%

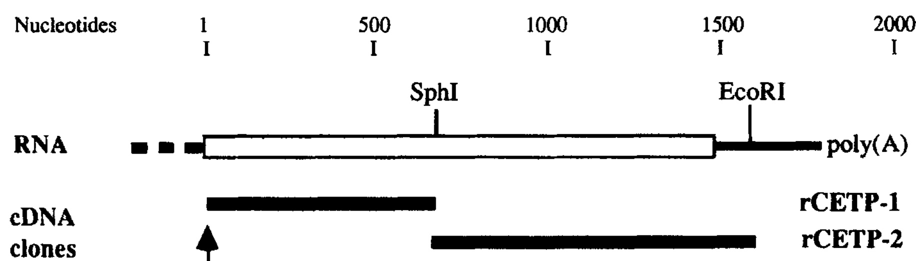

Fig. 1. Cloning of rabbit CETP mRNA obtained from the liver of a newborn rabbit is displayed according to a size scale in nucleotides. Direction of transcription is from left to right. Features of the message are, the undetermined length of 5′-untranslated and leader prepeptide coding region (broken line), mature protein coding region (open box), and 3′-untranslated region ending in poly(A) site (solid line). The positions of restriction sites on the cDNA are indicated by the names of the enzymes. The cDNA clones are displayed as rCETP-1 (5′ fragment) and rCETP-2 (3′ fragment). The arrow indicates the position of mutagenesis from the N-terminal Cys to Arg-Glu-Phe as described the text.

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TABLE 1. Partial purification of recombinant rabbit CETP

	Total Protein	Total Activit	Specific Activit	Purification	Yield
	mg	*nmol/h*	*nmol/h/mg*	*-fold*	*%*
Medium	3180	8999	2.83	1.0	100
Butyl-Toyopearl	38	2435	64.09	22.6	27
(Rabbit plasma)			0.56		

Specific activity was based on the assay of CE transfer from LDL to HDL in the assay mixture (LDL-CE 32 nmol and HDL-CE 30 nmol) described in the text. The values were corrected for the recovery of HDL in the supernatant (10 ± 2%).

glycerol) and vortexed with glass beads. The supernatants of the cell lysate were collected for the analysis.

Lipid transfer activity was quantitated as previously described with slight modifications (5, 19). HDL (d 1.07–1.21 g/mL) and LDL labeled with [^{3}H]cholesteryl oleate (d 1.02–1.063 g/mL) were prepared as described previously (20). The reaction mixture contained the labeled LDL and unlabeled HDL, 32 nmol and 30 nmol of cholesterol moiety, respectively, in a final volume of 237.5 µL in the 10 mM Na-phosphate buffer containing 150 mM NaCl, pH 7.4, in the presence of 5% bovine serum albumin (fatty acid-free, Sigma). The reaction was terminated by adding 12.5 µL of 5% heparin/1 M $MnCl_2$ to precipitate LDL, and the radioactivity in a 100-µl aliquot of the supernatant was counted. The specific lipid transfer activity of CETP is expressed as the CE/TG transfer activity per unit of the immunoreactive mass of CETP as a percentage of that of CETP mass in rabbit plasma (New Zealand White).

The cultured medium of the transformed yeast clones was assayed for lipid transfer activity. One clone that showed both CE and TG transfer activity was selected for detailed analysis (**Fig. 2**). Both lipid transfer activities exhibited a linear dose-dependency, and were inhibited by monoclonal antibody 3-9F, which inhibits both radioisotopic CE and TG transfer activities of rabbit plasma CETP (5). Similarly, monoclonal antibody 14-8H which inhibits only radioisotopic TG transfer activity of rabbit plasma CETP (5) also selectively blocked the TG transfer activity of the recombinant CETP.

Characterization of recombinant rabbit CETP

The recombinant rabbit CETP was partially purified by chromatography on a Butyl-Toyopearl (Supelco Canada) column (2.3 cm diameter × 7 cm) pre-equilibrated with 1 M NaCl solution containing 1 mM EDTA and 1 mM PMSF (equilibration solution) (9, 19). The clarified medium was loaded directly onto the column at 1.0 mL/min. The column was washed with an equal volume of equilibration solution, then with 150 mM NaCl solution containing 1 mM EDTA and 1 mM PMSF until the UV absorbance returned to near base line and finally with 3 column volumes of 1 mM EDTA/1 mM PMSF. Bound proteins (includes lipid transfer activity) were eluted with distilled water. Fractions (2 mL) were collected and aliquots (25 µL) across the protein peak were assayed for lipid transfer activity. The recombinant CETP was purified 22.6-fold from the conditioned medium (**Table 1**).

Immunoblotting was performed after polyacrylamide gel electrophoresis (PAGE) in 0.5% sodium dodecyl sulfate (SDS) and 2.5% of mercaptoethanol (21) as previously described in detail (5, 10). Proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad), reacted with the monoclonal antibody against rabbit CETP (14-8H) (5), and then with horse-

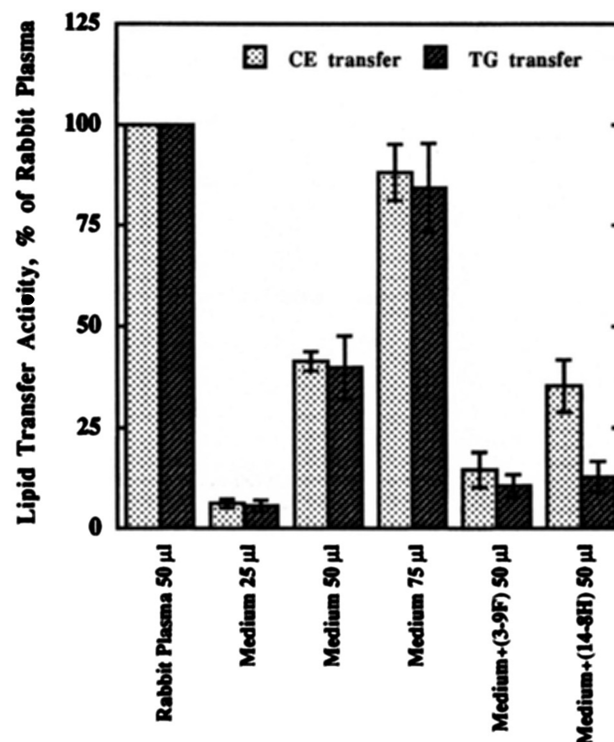

Fig. 2. Relative CE and TG transfer activity in the conditioned medium with 1% methanol of the transformed cells. The assay was carried out as described in the text. The medium was preincubated with the anti-CETP monoclonal antibodies (3-9F, 14-8H) (64 µg IgG/mL) for 1 h to inhibit the activity. Percent of the activity was determined relative to the activity of the rabbit plasma CETP. Data represent the mean ± SE for n = 3.

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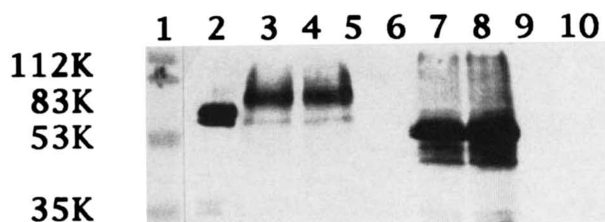

Fig. 3. SDS-PAGE immunoblot of the conditioned medium of the transformed cells and the soluble fraction of the cell-lysate. The medium and the cell-lysate supernatant (20 µl) were subjected to SDS-PAGE, followed by immunostaining of CETP after electrophoretic transfer to a nitrocellulose membrane. For immunostaining, 30 µg of IgG/mL of monoclonal antibody 14-8H was used as the primary antibody to detect CETP by fluorography. Lane 1, prestained SDS-PAGE standards (low molecular weight range); lane 2, rabbit plasma; lanes 3 and 4, the conditioned medium of the transformed cells with lipid transfer activity; lanes 5 and 6, the conditioned medium of transformed cells without lipid transfer activity; lanes 7 and 8, the supernatant of lysate of the transformed cells secreting lipid transfer activity; lanes 9 and 10, the supernatant of lysate of the transformed cells that do not secrete lipid transfer activity.

radish peroxidase-linked sheep anti-mouse immunoglobulin (Amersham). Immunocomplexes were visualized by chemiluminescence. The mass of CETP was determined by densitometric scanning of luminographs.

RESULTS

Immunoblot analysis revealed that the conditioned media of the transformed yeast cells having the lipid transfer activity contained the protein species that reacted to the monoclonal antibody against rabbit CETP (14-8H) while the media without the lipid transfer activity had no protein recognized by the monoclonal antibody (**Fig. 3**). The immunoreactive proteins detected ranged in SDS-PAGE from M_r = 60 K to 100 K but the major form showed the mobility of M_r = 80 K. Thus, the recombinant rabbit CETP differs in size from the rabbit plasma CETP which has a molecular mass of M_r = 74 K. The intracellular form of the recombinant CETP was also examined. The soluble fraction of the transformed cell lysate contained a single immunoreactive protein of M_r = 55 K. This size was equivalent to the peptide molecular weight predicted by the cDNA sequence (54, 674 for 498 amino acid residues). This discrepancy is most likely due to glycosylation, similar to that observed in human CETP (8, 22).

To determine whether rabbit CETP is glycosylated, the rabbit plasma, the conditioned medium, and the soluble fraction of transformed cell-lysate were digested with N-glycosidase F (from *Flavobacterium meningosepticum,* Boehringer Mannheim, Montreal, PQ) to remove N-linked carbohydrate chains. The rabbit plasma, the conditioned media of cells, and the soluble fraction of cell-lysate were dialyzed against 0.1 M sodium phosphate, pH 8.0, containing 10 mM EDTA, 1% Triton X-100, 0.02% SDS, 3 mM dithiothreitol (DTT), and 1 mM PMSF, and then treated with N-glycosidase F at 2.0 unit/mL. The reaction mixture was incubated at 37°C for 18 h under nitrogen. After digestion, the samples were recovered by column chromatography of Butyl-Toyopearl as described above and analyzed by SDS-PAGE.

The glycosidase treatment of the conditioned medium converted all immunoreactive proteins to a single species of 55 kDa in SDS-PAGE (**Fig. 4**). Similarly, the treatment of rabbit plasma reduced the size of CETP to 55 kDa. This is in good agreement with the size of the polypeptide predicted by the rabbit CETP cDNAs as mentioned above. The mobility of the intracellular form of the recombinant protein remained unchanged after the N-glycosidase F treatment. These data indicate that the transformed cells synthesize CETP as a protein of 55 kDa which is subsequently secreted after a varying degree of glycosylation.

The specific lipid transfer activities of partially purified recombinant CETP in N-deglycosylated form were examined. In order to determine the activity of the deglycosylated protein, the N-glycosidase F digestion was carried out at 4.0 unit/mL after dialysis of the samples against the buffer without Triton X-100, SDS, and DTT. The deglycosylation of the CETP produced the same results as those by the digestion in the presence the detergents and the reducing reagent, showing that the N-glycosidase F reaction was completed even without these denaturants (**Fig. 5**). The partially purified

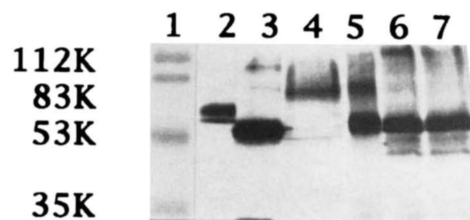

Fig. 4. The treatment of CETP with N-glycosidase F. Rabbit plasma, the conditioned medium of the transformed cells, and the soluble fraction of cell-lysate were incubated with or without 2.0 unit/mL of N-glycosidase F in the presence of the detergents and DTT as described in the text. After the incubation, the samples were subjected to SDS-PAGE and subsequent immunostaining of the CETP after their electrophoretic transfer to nitrocellulose membrane. For immunostaining, 30 µg of IgG/mL of monoclonal antibody 14-8H was used as the primary antibody to detect CETP by fluorography. The following samples are shown: lane 1, prestained SDS-PAGE standards (low molecular weight range); lane 2, rabbit plasma; lane 3, rabbit plasma treated with N-glycosidase F; lane 4, the conditioned medium; lane 5, the conditioned medium treated with N-glycosidase F; lane 6, the supernatant of cell-lysate; lane 7, the supernatant of cell-lysate treated with N-glycosidase F.

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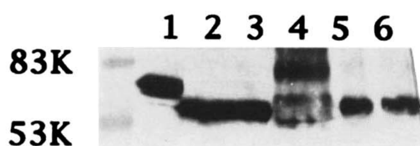

Fig. 5. N-glycosidase F treatment of rabbit plasma and partially purified recombinant CETP with or without Triton X-100, SDS, and DTT. Rabbit plasma and partially purified recombinant CETP were dialyzed against the buffer with or without Triton X-100, SDS, and DTT, and then incubated with 4.0 unit/mL of N-glycosidase F or without enzyme as described in the text. After the incubation, the samples were subjected to SDS-PAGE and immunostaining of the CETP. The following samples are shown: lane 1, rabbit plasma; lane 2, rabbit plasma treated with N-glycosidase F in the presence of Triton X-100, SDS, and DTT; lane 3, rabbit plasma treated with N-glycosidase F in the absence of Triton X-100, SDS, and DTT; lane 4, the conditioned medium; lane 5, the conditioned medium treated with N-glycosidase F in the presence of Triton X-100, SDS, and DTT; lane 6, the conditioned medium treated with N-glycosidase F in the absence of

recombinant proteins showed both CE and TG transfer activities (**Fig. 6**). Specific CE and TG transfer activities were 82% and 112% of that of rabbit plasma, respectively. Removal of N-linked sugar by treatment with N-glycosidase F completely abolished lipid transfer activity of the both recombinant and plasma CETP. Thus, N-linked glycosylation is essential for the lipid transfer activity of rabbit CETP.

The molecular weight of the major form of the secreted recombinant CETP was higher than that of plasma CETP, which may be due to hyperglycosylation as was reported for several heterologous proteins produced in *Saccharomyces cerevisiae* (23, 24). Such attachment of carbohydrate may allow proteins to achieve unique conformations that can influence their biological activities (25, 26). However, the recombinant CETP showed a specific lipid transfer activity similar to that of the plasma protein. Thus, hyperglycosylation does not influence the activity of the recombinant CETP, suggesting that the functional structure is not significantly affected.

DISCUSSION

In our experiment, the lipid transfer activity of the recombinant rabbit CETP and rabbit plasma CETP was completely lost after treatment with N-glycosidase F. This indicates that posttranslative modification by N-linked carbohydrate and sialic acid at Asn residues is essential for the lipid transfer activity of rabbit CETP, though the critical Asn residue responsible for the activity is yet to be determined. Rabbit CETP does not have an Asn residue corresponding to Asn 341 of human CETP (7) but is comparable in activity to human CETP (10). Thus, there are six potential N-linked glycosylation sites in rabbit CETP, at positions 88, 98, 110, 240, and 402, as well as 416 which corresponds to the position 396 of human CETP. Human CETP contains four potential N-linked glycosylation sites at positions 88, 240, 341, and 396 (8), but as suggested above glycosylation at Asn 341 is not essential for the activity. Whereas earlier observations implicated that N-linked carbohydrate and sialic acid are not essential for CETP activity (27), a different conclusion was drawn from recent experiments involving the elimination of Asn residues (22). Mutation of Asn 240 and Asn 396 did not significantly affect the transfer activity, but glycosylation at Asn 88 was essential for the normal lipid transfer activity of human CETP. Thus, our data are consistent with these results, implying that glycosylation at Asn 88 is critical for full lipid transfer activity of CETP.

In conclusion, recombinant rabbit CETP was expressed and efficiently secreted as a glycoprotein in the yeast *Pichia pastoris*. In order to secrete the recombinant CETP, the rabbit CETP sequence was fused to the acid phosphatase signal sequence. This changed the mature N-terminal residue of CETP from Cys to Arg-Glu-Phe.

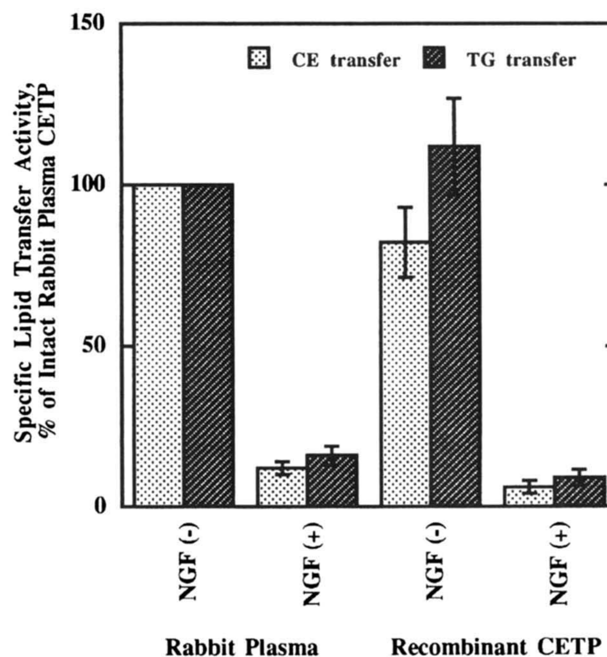

Fig. 6. Specific CE and TG transfer activities of the partially purified recombinant CETP and N-deglycosylated protein. The specific lipid transfer activity for each protein was calculated from CE/TG transfer activity and the immunoreactive mass of CETP protein determined by densitometric scanning of X-ray film. The data for recombinant protein were normalized to the plasma CETP protein values. Data represent the mean ± SE for n = 3.

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Despite this alteration, however, the recombinant CETP was fully active and had specific lipid transfer activities indistinguishable from that of the plasma protein. In addition, the 6 amino acid replacements had no observable effect on activity. These amino acid differences may represent bona fide and normal genetic amino acid heterogeneity of rabbit CETP. N-linked glycosylation was essential for activity and accounted for all carbohydrate modifications of the recombinant and plasma CETP. Thus, in spite of slight structural modification from the wild type, the recombinant rabbit CETP expressed by *Pichia pastoris* represents a good model for structure–function studies and molecular characterization of CETP. These studies include mapping of epitopes of species-specific and substrate-specific monoclonal antibodies (5), investigation of the mechanism for substrate specificity (28), and introduction of site-specific change that causes specific structural and functional alteration.

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REFERENCES

1. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein deficient plasma. *J. Lipid Res.* **23:** 1058–1067.
2. Tall, A. R. 1995. Plasma lipid transfer proteins. *Annu. Rev. Biochem.* **64:** 235–257.
3. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
4. Morton, R. E., and D. B. Zilversmit. 1983. Inter-relationship of lipids transferred by the lipid transfer protein isolated from human lipoprotein-deficient plasma. *J. Biol. Chem.* **258:** 11751–11757.
5. Ko, K. W. S., T. Ohnishi, and S. Yokoyama. 1994. Triglyceride transfer is required for net cholesteryl ester transfer between lipoproteins in plasma by lipid transfer protein. *J. Biol. Chem.* **269:** 28206–28213.
6. Inazu, A., M. L. Brown, C. B. Hesler, L. B. Agellon, J. Koizumi, K. Takata, Y. Maruhama, H. Mabuchi, and A. R. Tall. 1990. Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. *N. Engl. J. Med.* **323:** 1234–1238.
7. Nagashima, M., J. W. McLean, and R. M. Lawn. 1988. Cloning and mRNA tissue distribution of rabbit cholesteryl ester transfer protein. *J. Lipid Res.* **29:** 1643–1649.
8. Drayna, D., A. S. Jarnagin, J. McLean, W. Henzel, W. Kohr, C. Fielding, and R. Lawn. 1987. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. *Nature (London).* **327:** 632–634.
9. Tall, A. R., E. Granot, R. Brocia, I. Tabas, C. Hesler, K. Williams, and M. Denke. 1987. Accelerated transfer of cholesteryl esters in dyslipidemic plasma. Role of cholesteryl ester transfer protein. *J. Clin. Invest.* **79:** 1217–1225.
10. Ko, K. W. S., K. Oikawa, T. Ohnishi, C. M. Kay, and S. Yokoyama. 1993. Purification, characterization, and conformational analysis of rabbit plasma lipid transfer protein. *Biochemistry.* **32:** 6729–6736.
11. Ohnishi, T., L. D. Hicks, K. Oikawa, C. M. Kay, and S. Yokoyama. 1994. Properties of human plasma lipid transfer protein in aqueous solution and at interfaces. *Biochemistry.* **33:** 6093–6099.
12. Ellis, S. B., P. F. Brust, P. J. Koutz, A. F. Waters, M. M. Harpold, and T. R. Gingeras. 1985. Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris. Mol. Cell. Biol.* **5:** 1111–1121.
13. Cregg, J. M., K. J. Barringer, A. Y. Hessler, and K. R. Madden. 1985. *Pichia pastoris* as a host system for transformations. *Mol. Cell. Biol.* **5:** 3376–3385.
14. Cregg, J. M., and K. R. Madden. 1987. Biological Research on Industrial Yeasts. Vol. II. CRC Press, Boca Raton, FL. 1–18.
15. Sarcich, J. L., H. D. Fischer, M. S. Babcock, J. W. Leone, and A. G. Tomasselli. 1995. Expression and purification of recombinant cynomolgus monkey cholesteryl ester transfer protein from Chinese hamster ovary cells. *J. Protein Chem.* **14:** 73–80.
16. Wang, S., L. Deng, M. L. Brown, L. B. Agellon, and A. R. Tall. 1991. Structure–function studies of human cholesteryl ester transfer protein by linker insertion scanning mutagenesis. *Biochemistry.* **30:** 3484–3490.
17. Au-Young, J., and C. J. Fielding. 1992. Synthesis and secretion of wild-type and mutant human plasma cholesteryl ester transfer protein in baculovirus-transfected insect cells: the carboxyl-terminal region is required for both lipoprotein binding and catalysis of transfer. *Proc. Natl. Acad. Sci. USA.* **89:** 4094–4098.
18. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Ohnishi, T., S. Yokoyama, and A. Yamamoto. 1990. Rapid purification of human plasma lipid transfer proteins. *J. Lipid Res.* **31:** 397–406.
20. Nishikawa, O., S. Yokoyama, and A. Yamamoto. 1986. A method for direct incorporation of radiolabeled cholesteryl ester into human plasma low-density-lipoprotein: preparation of tracer substrate for cholesteryl ester transfer reaction between lipoproteins. *J. Biochem.* **99:** 295–301.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
22. Stevenson, S. C., S. Wang, L. Deng, and A. R. Tall. 1993. Human plasma cholesteryl ester transfer protein consists of a mixture of two forms reflecting variable glycosylation at asparagine 341. *Biochemistry.* **32:** 5121–5126.
23. Tarentino, A. L., T. H. Plummer, and F. Maley. 1974. The release of intact oligosaccharides from specific glycopro-

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teins by endo-beta-N-acetylglucosaminidase H. *J. Biol. Chem.* **249:** 818–824.

24. Reddy, V. A., R. S. Johnson, K. Biemann, R. S. Wiliams, F. D. Ziegler, R. B. Trimble, and F. Maley. 1988. Characterization of the glycosylation sites in yeast external invertase. I. N-linked oligosaccharide content of the individual sequons. *J. Biol. Chem.* **263:** 6978–6985.
25. Olden, K., B. A. Bernard, M. J. Humphries, T. Yeo, K. Yeo, S. L. White, S. A. Newton, H. C. Bauer, and J. B. Parent. 1985. *Trends Biochem. Sci.* **10:** 78–82.
26. Drickamer, K. 1991. Clearing up glycoprotein hormones. *Cell.* **67:** 1029–1032.
27. Swenson, T., J. Simmons, C. Hesler, C. Bisgaier, and A. R. Tall. 1987. Cholesteryl ester transfer protein is secreted by HepG2 cells and contains asparagine-linked carbohydrate and sialic acid. *J. Biol. Chem.* **262:f45 16271–16274.**
28. Ohnishi, T., C. Tan, and S. Yokoyama. 1994. Selective transfer of cholesteryl ester over triglyceride by human plasma lipid transfer protein between apolipoprotein-activated lipid microemulsions. *Biochemistry.* **33:** 4533–4542.

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