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Characterization of a naturally occurring new version of the cholesterol ester transfer protein (CETP) from small intestine

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Abstract

The cholesterol ester transfer protein (CETP) is found in plasma mediating the transfer of cholesterol esters and triacylglycerides between lipoproteins. The last 26 amino acids of its carboxy-end correspond to an amphipathic α-helix whose hydrophobic side has been directly involved in the transfer of lipids [1–3]. Alterations in this region lead to the reduction or loss of lipid transfer activity [3–5]. To date, the only variant of the CETP messenger that has been reported lacks exon 9 [6–8], which translates into an inactive isoform regarding neutral lipid transfer [6–8]. In this study, we describe a new version of the messenger RNA of rabbit CETP identified exclusively in the small intestine of wild type (WT) rabbits. This isoform includes several of the intron bases prior to exon 16. The presence of a stop codon within this sequence prevents translation of exon 16, substituting the original carboxy-end sequence and therefore generating a random structure that does not contain the region responsible for neutral lipid transfer. Antibodies generated against a peptide within the carboxy-end sequence of the new isoform show the presence of this new protein in human plasma. (Mol Cell Biochem **245**: 173–182, 2003)

Key words: alpha-helix, CETP, cholesterol, isoform, small intestine

Introduction

The cholesterol ester transfer protein (CETP) mediates cholesterol esters and triacylglycerides transfer among high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very-low-density lipoproteins (VLDL). In humans, although the molecular weight of CETP obtained from its cDNA is 53 kDa, employing denaturing gels CETP isolated from plasma shows two different molecular forms (66 and 74 kDa). This phenomenon may be due to several degrees of glycosilation present in residue 341 [9]. CETP is a highly conserved protein in several mammalian species. CETP in the rabbit presents 496 amino acids, with a sequence homology of 81% against the 476 amino acids of human CETP, in which two thirds of these substitutions are conservative [10]. These data implicate an important structural similarity between human and rabbit CETP [11,12]. The last 26 amino acids of the carboxy-end for CETP present a well-defined amphipathic α-helix structure, whose hydrophobic side has been directly involved in cholesterol ester transfer [1, 2]. Modifications on the region contained between amino acids 470–475 of human CETP and 490–495 in rabbit lead to the reduction or loss of lipid transfer activity without affecting their capacity to associate with lipoproteins [4, 13].

Since rabbit CETP mRNA presents an homology of 85% with its human form and contains a large sequence of 230 pb, it is estimated to have 2.2 kb in length [7]. The CETP messenger is composed of 16 exons containing 8% of the gene sequence [14]. To date, various mutations in the CETP messenger have been reported, some of which derive in alternative splicing that correlates with alterations of HDL and LDL

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levels in plasma. Only one isoform of CETP described in the human has been reported so far. This isoform that lacks exon 9 (CETPΔ9) is poorly secreted at normal plasma lipid levels and has shown to be inactive in the transfer of neutral lipids. Mice bearing the Δ9 CETP transgene have also shown to be able to secrete this isoform into plasma [8]. This isoform seems to be retained in the endoplasmic reticulum and forms intracellular heterodimers with normal CETP. This mechanism has been considered as a regulatory process in the secretion of the complete form of CETP [6,7]. On the other hand, although the highest levels for the CETP messenger have been found in the liver, it has been also reported in a great variety of peripheral tissues, including the small intestine [4, 10]. These results suggest a local induction in the synthesis of CETP, mechanism that might aid in the recycling of cholesterol deposited in peripheral tissues during lipoprotein lipolysis [4, 10, 15, 16].

[1]The present study reports the presence and initial characterization of an alternative form of CETP not described to date. During our investigation, the mRNA of this new isoform called CETPI was only found associated to the small intestine in the rabbit. The CETPI messenger includes at least part of the intron prior to exon 16. Due to the presence of a stop codon within this intron, the translation of exon 16 does not take place, leading to the substitution of the last 24 amino acids of CETP at its carboxy-end. This change in its primary structure implicates the substitution of the original amphipathic α-helix structure [13, 17] by a hydrophilic random structure rich in prolines. The presence of CETPI has been demonstrated in this study to be present in human and rabbit plasma.

Materials and methods

[2]*mRNA Isolation, reverse transcription coupled to the polymerase chain reaction (RT-PCR), and sequence analysis*

Total RNA extractions from adipose tissue, brain cortex, endothelium, heart, kidney, liver, lung, muscle, pancreas, small intestine, spleen, ovary and uterus from female white New Zealand WT rabbits presenting normal plasma lipid levels, were performed using the method described by Sumikawa *et al*. [18]. The fraction of poly(A^+) RNA from all tissues was isolated by column chromatography using oligo(dt)-cellulose. cDNAs were synthesized employing a commercial system for RT-PCR (Perkin-Elmer, Branchburg, NJ, USA) using 100 ng of liver mRNA and 400 ng of mRNA from other tissues. Additionally, cDNAs were synthesized from 3 μg of total liver RNA and 7 μg from small intestine RNA. Primers employed in polymerase chain reaction (PCR) amplifications and sequence reactions were designed with the MacVector software (Genetic Computer Group, Madison, WI, USA), based on the cDNA sequence for the rabbit CETP published by Nagashima *et al*. [10] (Table 1). Primers were also designed for control reactions, based on the rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA sequence reported by Appletquis *et al*. [19] (Table 1). PCR amplifications were carried out in 35 cycles and 55°C with primer pairs RAB5-RAB6 and GA3PF-GA3PR. For the sequence reactions, the RAB4, RAB5, RAB6, GA3PF, and GA3PR primers were used employing the following commercial systems: Secuenase Version 2-0 (Amersham, Cleveland, OH, USA); AmpliTaq DNA polymerase (AmpiCycle Sequencing Kit, Perkin Elmer, Branchburg, NJ, USA) and dsDNA Cycle Sequencing System (Gibco BRL, Gaithersburg, MD, USA). The nucleotide sequences were determined from both DNA strands. The sequences of all PCR products were aligned with the analysis performed using the following data banks: GeneBank, EMBL, DDBJ, and protein data bank (PDB).

[2]*cDNA Probes for Northern and Southern-blot analysis*

The identities of PCR products amplified with the RAB5-RAB6 primers were verified with Southern-blot hybridations, using the RAB4 primer as an internal probe. PCR products were subjected to electrophoresis in 1% agarose gels and transferred to NYLON membranes (HYBOND-N+, Amersham, UK). Membranes were hybridized with the RAB4 probe (19 nt) at 37°C and washed at 58°C with NaCl-Trisodium Citrate Buffer pH 7.0. They were later exposed on X-OMAT autoradiographic plates (Kodak, Rochester, NY, USA). Radioactive labeling of the RAB4 primer was carried out with δ-ATP-[^{32}P] using the Pharmacia Biothec Oligolabelling Kit (Uppsala, Sweden).

For Northern-blot hybridations, the integrity of total RNA and mRNA samples were verified by electrophoresis in 1% denaturing agarose gels. Using denatured twin gels, 100 mg of total liver and small intestine RNA were electrophoresed and transferred to NYLON membranes (HYBOND-N, Amersham, UK). The amplified PCR product with the pair of primers RAB5-RAB6 was used as a probe against CETP, and GA3PF-GA3PR as a probe against GAPDH, both obtained from liver mRNA. Labeling of the probe was carried out with [α-^{32}P] deoxycytidine 5′-triphosphate using the Random Primed DNA Labeling Kit (Boehringer, Mannheim, Ger-

[1]The original nucleotide sequence reported in this paper has been deposited in the DDBJ database under the accession no. 20011025040619.79609

[2]Several protocols and materials described in this paper have been registered according to the International Patent Cooperation Treaty, with International Publication Number WO 02/32935. This application has been registered at the European Patent Office (WIPO) with a priority date of 1999.

Table 1. Primers used in PCR and sequencing reactions

CDNA	Primers	Position	Sequence
CETP	RAB4 Antisense	1462–1443	5' GTGCTTGGGAAAACCGAAG 3'
CETP	RAB5 Sense	1391–1414	5' TCATCAACCCCGAGATTATCACTC 3'
CETP	RAB6 Antisense	1852–1828	5' TCGTTTACTTGAGAGGCAGAGAGAG 3'
GAPDH	GA3PF Sense	79–104	5' TGAAGGTCGGAGTCAACGGATTTGG 3'
GAPDH	GA3PR Antisense	1061–1040	5' GGTGGACCTCATGGCCCACATG 3'

many). The membranes were simultaneously hybridized with the RAB5-RAB6 and GA3PF-GA3PR probes, and later analyzed while exposed on a Phosphorimager screen (Storm System, Pharmacia Molecular Dynamics, Uppsala, Sweden).

[2]*Cloning of CETP and CETPI, cDNAs and recombinant proteins*

PCR products generated with RAB5-RAB6 primers were cloned in the pMos*Blue* T vector (Amersham, UK). They were later subcloned in the pGex-2T expression vector (Pharmacia LKB Biothec, Uppsala, Sweden), generating one control and two recombinant plasmids called pGex-2T, pGex-2T/ID1 and pGex-2T/ID2 respectively. These plasmids were transformed in *Escherichia coli* DH5α strain for the obtention of recombinant proteins fused to glutathione-S-transferase (GST). The transformed bacteria were cultured during 8 h at 37°C in 500 ml of Super Luria Broth with 50 µg/ml of ampicillin. The partially purified recombinant proteins CETP 3′/GST, CETPI 3′/GST and control GST were obtained following the next protocol. Culture induction was performed with 0.4 mM of IPTG during 3 h. The transformed bacteria were suspended in phosphate-buffered saline (PBS), ethylenediaminetetraacetate (EDTA) 50 mM, aprotinin 1%, phenylmethylsulfonyl fluoride (PMSF) 1 mM, benzamidine 5 mM, leupeptine 10 µg/ml and later sonicated in cycles for 10 sec and 30 sec on ice. The lysed products were applied to agarose glutathione columns (Sigma, St. Louis, MO, USA).

[2]*Epitope analysis, peptide antigen design, and antibody production*

The sequences of the different PCR products amplified with the pair of primers RAB5-RAB6 from small intestine and liver mRNA, were translated into amino acid sequences and analyzed with the help of the DNAstar software (Lasergene, Madison, WI, USA). Predictions for the Kyte-Doolittle hydrophobicity index and secondary structure were achieved employing the Chou-Fasman and Garnier-Robson algorithms. Results of these analyses were confirmed by PHD (Profile Network Prediction, Heidelberg). Based on this information, two synthetic peptides with the CETP and CETPI carboxy-end sequences were designed. These peptides were synthesized by PeptidoGenic Research and Co. (Livermore, CA, USA). They included an additional cysteine residue in the amino-end in order to allow direct coupling with KLH (Keyhole Limpet Hemocyanin). The peptides coupled to KLH were used for the production of anti-CETP IgY and anti-CETPI IgY antibodies in white Leghorn chickens, utilizing a standard protocol of 63 days carried out by ADI (Alpha Diagnostic International, San Antonio, TX, USA). Antibody titers in plasma were determined by enzyme-linked immunosorbent (ELISA). IgYs were isolated from 12 eggs. Anti-BSA IgY antibodies (bovine serum albumin antibodies) were also obtained from white Leghorn chickens inoculated subcutaneously once a week in the presence of complete Freund's adjuvant (Sigma Immuno Chemicals, St. Louis, MO, USA) on first application, and with incomplete Freund´s adjuvant (Sigma Immuno Chemicals, St. Louis, MO, USA) in three subsequent applications. Prior to each application, blood was withdrawn, plasma separated and antibody presence titered by ELISA.

The specificity of the antibodies used studied by ELISA and Western-blot analysis, was determined using cross-reaction tests with three antibodies (anti-CETP IgY, anti-CETPI IgY and anti-BSA IgY). Each antibody was tested against both free synthetic peptides CETP and CETPI, BSA, synthetic peptides coupled to BSA, and recombinants fused to GST (control GST, CETP 3′/GST and CETPI 3′/GST). Each test included preimmune chicken antibodies as a control (data not shown).

[2]*Electrophoresis and immunoblotting*

Western-blot tests were performed against crude and lipid-depleted extracts of liver and small intestine in addition to human and rabbit plasmas. Organs employed in these tests were previously perfused with Krebs-Ringer buffer (pH 7.4). Preparation of crude extracts was performed with 0.5 g of tissue homogenized in 5 ml suspension buffer (100 mM NaCl, 10 mM Tris-HCL pH 7.6, 1 mM EDTA-NaOH pH 8.0, 1 µg/ml aprotinin, and 100 µg/ml PMSF). Crude extracts were lipid-depleted employing 60% acetone extractions. Protein quantification in the lipid-depleted extracts was performed employing the BCA Protein Assay Reagent method (PIERCE,

Rockford, IL, USA) and in total plasma samples using the Folin-Lowry method. Crude liver (20 µg protein) and small intestine (30 µg protein) extracts, as well as plasma samples (40 µg protein) were subjected to electrophoresis in 12.5% sodium dodecyl sulfate-acrylamide gels. Three µg of BSA were used for the anti-BSA IgY positive control and 40 µg of BSA as negative control for anti-CETP IgY and anti-CETPI IgY. This material was electrotransferred to 0.45 µm nitrocellulose membranes (Trans-Blot Transfer Medium, BIO-RAD, Hercules, CA, USA). In tissue samples, anti-CETP IgY was used in 1:5,000 dilutions and anti-CETPI IgY in 1:30,000. In plasma samples and negative controls with BSA, anti-CETP IgY 1:5,000 and anti-CETPI IgY 1:15,000 were used. For positive control with BSA, anti-BSA IgY at a dilution of 1:25,000 was employed. In all cases, a peroxidase-conjugated IgG anti-chicken secondary antibody (PIERCE, Rockford, IL, USA) was used in a dilution 1:10,000. All incubations with the different antibodies were carried out using a suspension of 2.5% powdered skimmed milk in tris-buffered saline – Tween 0.1% (TTBS), 37°C for 1 h. Visualization was carried out using SuperSignal Substrate (Pierce, Rockford, IL, USA) on X-OMAT autoradiographic plates (Kodak, Rochester, NY, USA).

Results and discussion

A new version of the CETP messenger expressed in the small intestine

The product we call ID1 was obtained by means of RT-PCR amplifications from mRNA extracted from representative tissues such as liver, heart, small intestine, and adipose tissue. No amplification products were obtained from spleen and uterus mRNA, perhaps due to the fact that mRNA levels of CETP in these tissues are below the detection capacity of the methodology used. The ID1 product had the expected size of 461 pb for amplifications utilizing the RAB5-RAB6 pair of primers, according to the rabbit CETP cDNA sequence reported by Nagashima *et al.* [10] (Fig. 1A). RT-PCR from small intestine mRNA additionally generates a second product of 569 pb that we call ID2 (Fig. 1A). Employing Southern-blot analysis it is confirmed that although very close to each other, both amplified products correspond to CETP cDNA (Fig. 1B). Figure 1C shows the control assay using liver and small intestine without the presence of reverse trancriptase in the mixture reaction. We can conclude that the amplification shown by ID1 and ID2 was obtained from mRNA and not form contaminant DNA.

Sequence analysis of ID1 and ID2 showed that both products were amplified from CETP messengers. The nucleotide sequence of the ID1 fragment comprises from base 1391 located at the 3′ end of exon 15 to base 1852 found in the non-encoding region of exon 16. The ID1 product sequence presents an identity of 100% with the encoding region sequence of rabbit CETP cDNA [10], and of 87% with the reported human CETP sequence [17]. The ID2 product sequence also has an identity of 100% with the reported CETP cDNA sequence; however, it includes a section of 108 pb between exons 15 and 16 that we call Sequence I (Fig. 2).

This is situated between nucleotides 1419 and 1420 at the limit between exons 15 and 16. Figure 2B shows part of the ID2 product sequence, i.e. from nucleotide 1391 in exon 15 to nucleotide 1449 in exon 16 (Sequence I is shown in upper-case letters). Figure 2A, shows the stop codon in position 1491 reported for the messenger of rabbit CETP [10]. Two more stop codons are found within Sequence I, one in position 55 and the other at the 3′ end at position 106 (Fig. 2). According to the translation of nucleotide sequences to amino acid sequences, ID1 corresponds to the previously reported version of rabbit CETP and can be translated into the active protein that is liberated in plasma. On the other hand, the mRNA of ID2 corresponds to a version of the CETP

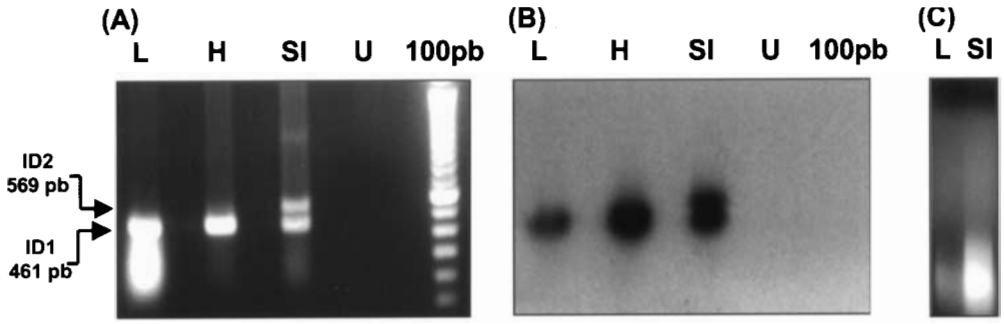

Fig. 1. Southern-blot analysis of RT-PCR products showing ID1 and ID2 amplifications. (A) Agarose gel products amplified by RT-PCR with RAB5-RAB6 primers from mRNA of liver (L), heart (H), small intestine (SI), and uterus (U). (B) Southern-blot radiography employing the RAB4 probe. The small intestine expresses a variant of CETP mRNA represented by the ID2 amplification product. (C) Control assay for liver and small intestine performed without reverse transcriptase.

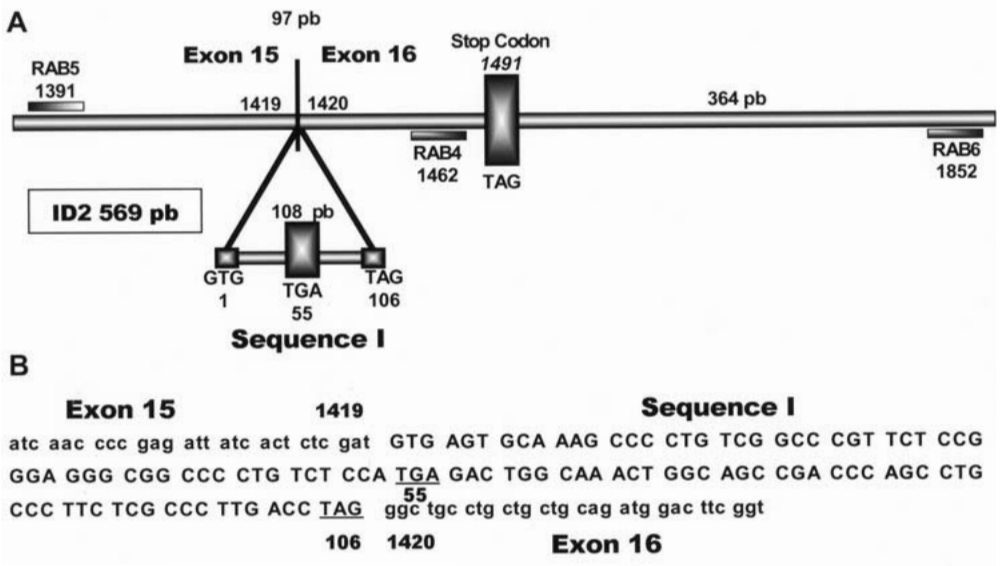

Fig. 2. Schematic representation of ID1 and ID2 showing the localization of Sequence I and stop codons. A) ID1 and ID2 present an identity of 100% when compared to the rabbit CETP sequence. ID2 includes Sequence I containing 108 pb between CETP exons 15 and 16 and nucleotides 1419 and 1420. (B) Within Sequence I (upper-case letters), there are two stop codons (underlined), one in position 55 (TGA) and the other in its 3′ end at position 106 (TAG). Translation stops at codon 55 of Sequence I preventing the entire exon 16 from being formed.

messenger not reported to date, which is expressed in the small intestine of the rabbit and translates into the new isoform CETPI.

Considering that Sequence I is an intron, the ID2 product could have been amplified from non-edited mRNA contaminant in the small-intestine sample. In order to discard this possibility, RT-PCR was performed using an excess of total RNA, of both liver and small intestine. These results showed that the ID1 product is obtained from the samples in both tissues; nonetheless, the ID2 product could only be obtained from the sample of small intestine, similar to the results obtained with mRNA (Fig. 1). Therefore, we conclude that the small intestine expresses a variant of CETP mRNA, represented by the ID2 amplification product.

In order to attempt to establish the approximate molecular weight for this variant of the CETP messenger, Northern-blots were carried out. Figure 3A shows the result of hybridization with the GA3PF-GA3PR control probe. Lanes 1 and 2 (total liver and small intestine RNA respectively) correspond to a band of approximately 1.3 kb that matches the size of rabbit GAPDH mRNA. Figure 3B demonstrates the result of hybridization with the RAB5-RAB6 probe. In lane 3 (total liver RNA), a single band with 2.2 kb can be observed corresponding to the approximate size of the rabbit CETP messenger. The agarose-formaldehyde gel of 18s (1.7 kb) rRNA and 28s (4.7 kb) rRNA shown in Fig. 3A can be used as a reference for relative mobility. In lane 4 (total RNA of small intestine) the same band is observed; however, a second band with lower mobility that could correspond to the transcript of ID2 could not be clearly found. Since the expected size for CETPI mRNA is 2.3 kb compared to 2.2 kb for CETP, such a small difference in size is difficult to resolve in 1% agarose gel.

CETPI lacks a cholesterol esters binding domain

Figure 4A shows a comparison between the amino acid sequence of the carboxy-ends of CETP and CETPI translated from the nucleotide sequences for the RT-PCR ID1 and ID2 products. Translation of the CETP mRNA ends at the stop codon in position 1491, while the CETPI messenger stops at

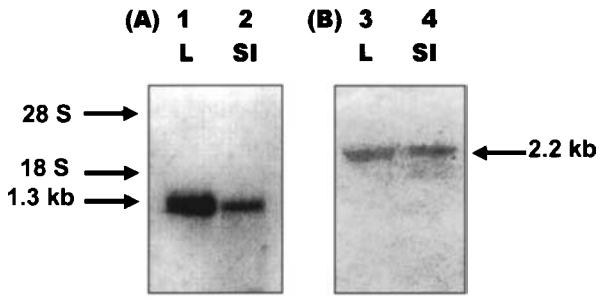

Fig. 3. Northern-blot hybridization of total RNA isolated from different tissues employing the Rab5-Rab6 probe. (A) Hybridization with the GA3PF-GA3PR control probe with total liver RNA (L) (line 1) and total small intestine RNA (SI) (line 2). A band of approximately 1.3 kb was observed, corresponding to the size of rabbit GAPDH mRNA. (B) Hybridization with the RAB5-RAB6 probe against CETP; a band around 2.2 kb was observed in lanes 3 and 4.

A

Isoform	Carboxy-end Sequences	Structure (Prediction)	M.W. (kDa)	I.P.	Peptide
CETP 33 AA	INPEIITLDGCLLLQMD*F*GFPK<u>HL *L*VD*F*LQS*L*S</u> 472	Alpha Helix	3731.10	3.99	Soluble at pH 9.5
CETPI 27 AA	INPEIITLDVSA KPLS<u>A RSPGGRPLSP</u> 472	Random	2785.60	9.00	Soluble at pH 7.0

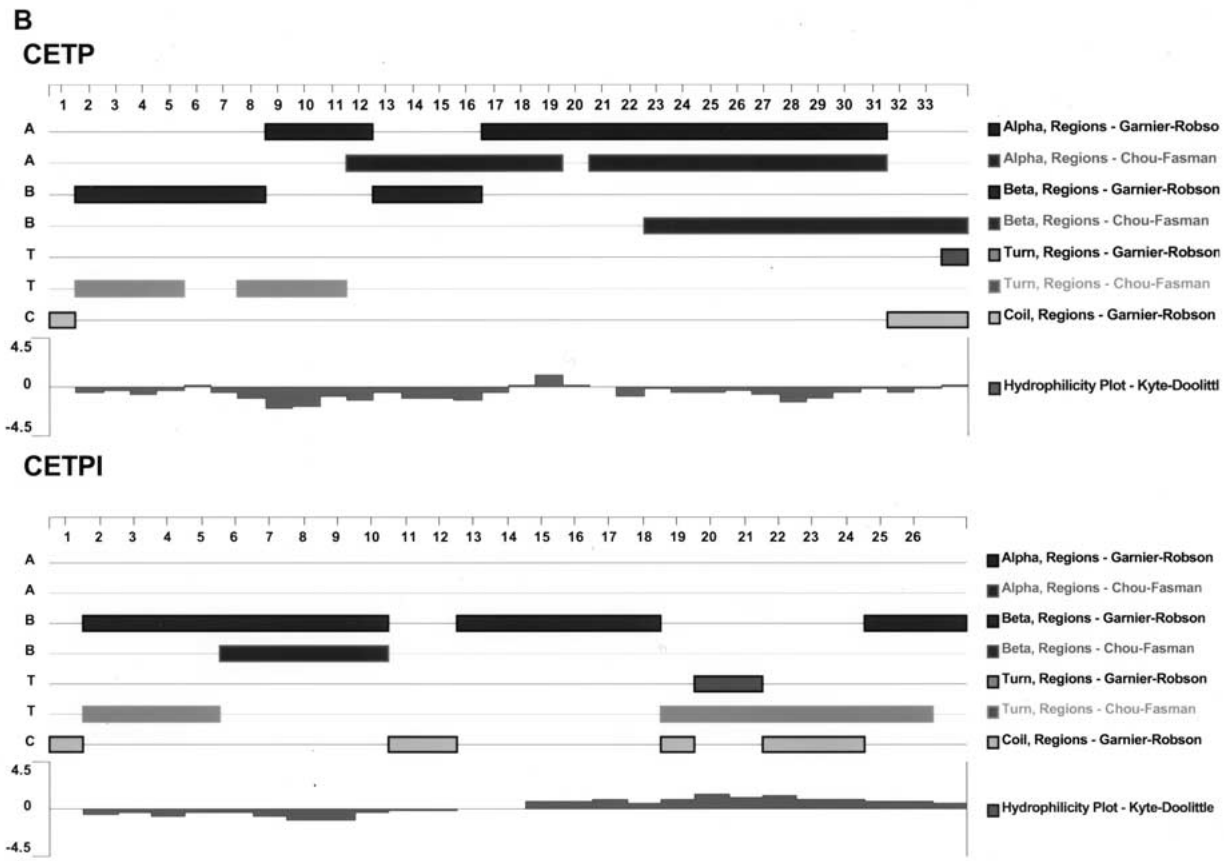

Fig. 4. Comparison between the amino acid sequences of the carboxy-ends of CETP and CETPI. (A) Amino acid sequence translated from ID1 and ID2 RT-PCR product sequences. The underlined regions represent the synthetic peptides designed for antibody production. (B) According to the analysis of the amino acid sequences using the Garnier-Robson, Chou-Fasman, and PHD algorithms, CETPI lacks the C-terminal amphipathic α-helix structure. In its place, the CETPI carboxy-end presents a hydrophilic random structure.

codon 55 of Sequence I, preventing the entire exon 16 from being translated. Therefore, both sequences are identical up to residue D472, the last amino acid encoded by exon 15. The remainder of the primary structure of the carboxy-end of CETPI lacks any homology with the carboxy-end of CETP. These differences in primary structure might be related to their well known properties, such as isoelectric point, molecular weight, hydrophobicity, and probably secondary structure (Fig. 4) [13].

Contained in the sequence of the CETP carboxy-end, residues F481, L488, F491, and L495 are shown in italics in Fig. 4A. These residues have been shown via directed mutagenesis to be important in maintaining the transfer activity of cholesterol esters [4]. As observed in the carboxy-end of

CETPI, the last 24 amino acids of the original form of CETP, including the previously mentioned residues, are substituted with 18 amino acids encoded by Sequence I. According to our calculations the last 26 amino acids of the carboxy-end of CETP have a high content of amphipathic α-helix structure in agreement with other autors [1–7]. Our prediction concerning its secondary structure employing the Garnier-Robson, Chou-Fasman, and PHD algorithms, shows that CETPI does not preserve such an amphipathic α-helix (Fig. 4B). Instead, the secondary structure of the carboxy-end of CETPI acquires a random structure that is hydrophilic and rich in prolines. This information suggests that CETPI most probably lacks a binding site for cholesterol esters, as previously analyzed by us [13].

CETP and CETPI translate in small intestine

Based on the amino acid sequences shown in Fig. 4A, we designed two synthetic peptides corresponding to the carboxy-ends of CETP and CETPI. The sequences for both peptides are shown underlined in Fig. 4A. These synthetic peptides were used in the production of polyclonal antibodies anti-CETP IgY and anti-CETPI IgY. In Western-blot tests employing the anti-CETP IgY antibody against crude extracts of liver and small intestine, we observed that CETP shows a band around 68 kDa, which is expressed in both tissues (Figs 5A and 5B). The use of the anti-BSA IgY antibody against liver, small intestine and BSA itself, give a signal around 66 kDa (shown as reference in Fig. 5E). Figure 5C show that the anti-CETPI IgY antibody only recognizes a single band around 53 kDa, 15 kDa less than CETP that is only detected in extracts from small intestine. Figure 5D shows the result using the mixture of both, the anti-CETP IgY and the anti-CETPI IgY antibodies. The difference in 15 kDa between CETP and CETPI seen in our Western-blot analysis is larger than the change in size expected for a difference of only six amino acid residues. Therefore, we might conclude that additional differences must exist between the two proteins in order to explain this change in mobility. The differences in sequence and predicted secondary structure between CETP and CETPI might be responsible for several of their physicochemical properties (Fig. 4). The isoelectric point predicted for CETP (with 496 aminoacids) is 6.41 compared to the isoelectric point predicted for CETPI (with 490 aminoacids) of 7.09. This change could also affect the relative mobility of CETPI in polyacrylamide gels. Employing lipid-depleted samples, the difference in mobility remained constant; therefore, discarding the possibility that the presence of lipid bound to the proteins might affect their migration in the gels. The possibility of postranslational modifications that might affect the relative mobility of the CETPI is at the moment under consideration in our laboratory.

The presence of CETPI in plasma

To establish whether CETPI is secreted into plasma in a manner similar to the case of CETP, we studied plasma samples from both; normolipidemic human subjects, and normal rabbits. Plasma samples shown in Fig. 6 were studied by Western-blot analysis. Figure 6A shows plasma samples from both species, human and rabbit. Using the anti-CETP IgY antibody, we established that CETP is present in both human and rabbit plasma with an approximate molecular weight around 66 and 68 kDa respectively (Fig. 6B). Employing the anti-CETPI IgY antibody we demonstrated that CETPI is also present in the plasma of both species (Fig. 6C). On other hand

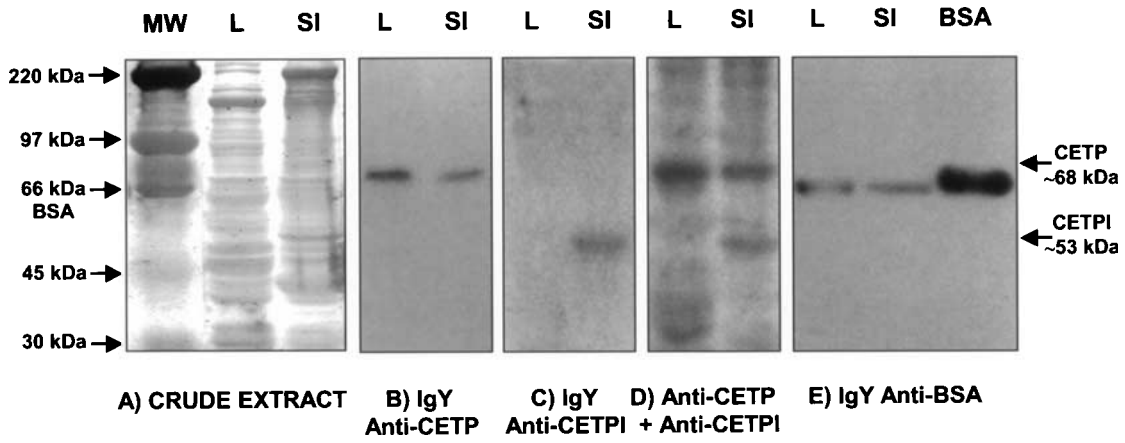

Fig. 5. Identification of CETP and CETPI in tissue extracts by immunoblot analysis. Molecular weight marker (MW) and crude extracts from liver (L) and small intestine (SI) (panel A), incubated with anti-CETP IgY (panel B), anti-CETPI IgY (panel C) and the combinations of both (panel D). Control anti-BSA IgY (panel E).

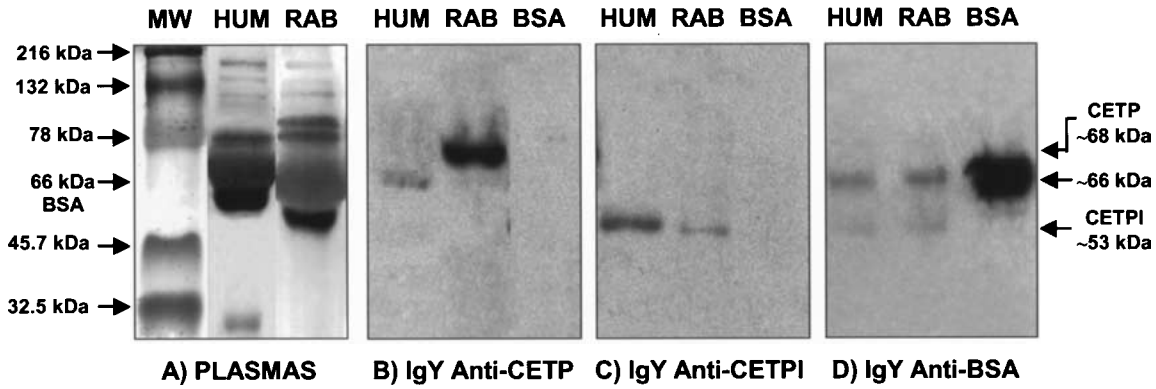

Fig. 6. Identification of CETP and CETPI in human and rabbit plasmas by immunoblot analysis. Molecular weight marker (MW), Human plasma (HUM) and rabbit plasma (RAB) (panel A). Human plasma (HUM) and rabbit plasma (RAB) using the anti-CETP IgY antibody (panel B). Human plasma (HUM) and rabbit plasma (RAB) using the anti-CETPI IgY antibody (panel C). Both plasmas and a BSA control were also incubated with the anti-BSA IgY (panel D).

the antibody anti-CETPI IgY showed that CETPI presents a higher concentration in human than rabbit plasma (Fig. 6C). Taking into consideration that albumin is one of the most abundant proteins in plasma, and CETP presents a very similar molecular weight, we also used the anti-CETP IgY and the anti-CETPI IgY antibodies against BSA in order to discard the possibility of false positives (Fig. 6B and 6C). Reference can be taken from Fig. 6D, in which the anti-BSA IgY antibody was utilized against plasma obtained from the two species.

At this point it has to be considered human intron 'O' located between exons 15 and 16 [14]. If this intron is translated in the original reading frame it could generate a sequence 60 amino acids larger than CETP. Therefore, the minimum calculated molecular weight (non-glycosylated form) for human CETPI would be 59.4 kDa, 6.4 kDa larger than the molecular weight observed in rabbit CETPI. Based on this possibility, we considered that the human CETPI messenger could be related to a spliced product. Although cryptic splice sites are found, these would generate proteins with a molecular weight much larger than 56 kDa. Table 2 shows the comparison between the carboxy-end sequence of human (ID1) and rabbit CETP (ID1), as well as the carboxy-end sequence of rabbit (ID2) and a putative version of human CETPI (ID2). The latest form of human CETPI corresponds to one possibility amongst several others originated by alternative splicing, lacking a segment from base 6 to base 96 of intron 'O'. The stop codon in this product is suggested to be localized in base 1573 of exon 16. This variant would generate a protein 17 amino acids larger than CETP, with a minimum molecular weight of 56 kDa. Although, between the human and rabbit CETP there is an homology that corresponds to 91.7%, the sequence homology for CETPI in both species corresponds to 50% from residue A6 to P12 of Sequence I, the region recognized by the antibody anti-CETPI IgY. It is interesting to note that CETPI either from rabbit or human sources seems to present a random configuration rich in prolines as well as a hydrophilic character. Taking into account that it seems we are dealing with a new variant of CETP with a main deference of six aminoacids that produces a clear effect of its main properties, it remains to be established if posttranscriptional or posttranslational modifications are associated to CETPI.

At present, several mutations in the CETP messenger have been reported, some of which reflect several alterations in the levels of HDL, LDL and VLDL in plasma. A change of a guanine for an alanine in position +1 of intron 14 in the CETP mRNA, does not allow the synthesis of CETP to take place. This simple change causes hypoalphalipoproteinemia promoted by a reduction of 50% in the level of CETP [20,21]. On other hand, Gotoda *et al.* [22] describe a change of a cysteine for a timine in exon 10 of the CETP gene of patients showing hypoalphalipoproteinemia. This substitution results in the replacement of codon 309 CAA(Q) for a premature ter-

Table 2. Sequence comparison of CETP and CETPI carboxy-ends from human and rabbit

Isoform	Carboxy-end Sequences	Structure (prediction)	M.W. (kDa) (prediction)	I.P.
ID1 human 476 AA	GFLLLQMD**F**GFPE HL**L**VD**F**LQS**L**S	Alpha Helix	53.10	5.80
ID1 rabbit 497 AA	GCLLLQMD**F**GFPK HL**L**VD**F**LQS**L**S	Alpha Helix	54.52	6.41
ID2 human 483 AA	VKGPLPAWLPPGGLGVSPARPSLLLPLPSGLPAAADGLWLP	Random	56.06	6.28
ID2 rabbit 491 AA	VSAKPLSARSPGGRPLSP	Random	53.57	7.09

minal codon TAA. In this case the CETP synthesized lacks 168 aminoacids at the carboxy-end region of the protein [21]. Sakai *et al.* [23], reported a cutting defect in intron 10 that excludes exon 10 in the cDNA and promotes the insertion of a 31 pd sequence in intron 13 [23].

Takahashi *et al.* [24], also reported a nucleotide substitution that decreases CETP activity. Only one isoform of human CETP has been described lacking exon 9 (CETP Δ9). This isoform seems to be retained at the level of the endoplasmic reticulum forming intracellular heterodimeric complexes with the normal form of CETP [6,7]. This association has been thought to be related to a regulatory mechanism in the steps previous to the secretion of CETP.

The CETPI messenger presents an approximate size of 2.2 kb and with the exception of Sequence I shows 100% identity with the reported 3′ end of rabbit CETP. Since we have identified the only source for the CETPI transcript to be the small intestine, CETPI found in plasma most probably is originated in this tissue. Based on our results, the CETPI messenger identified as ID2 is translated into an isoform of CETP that is also liberated in the plasma of rabbit and humans, at normal lipid levels. The messenger of CETPI includes 108 bases of the intron previous to exon 16. This change substitutes the last 24 aminoacids in the carboxy-end of CETP for 18 new ones with the subsequent change in the secondary structure of the isoform.

Although CETPI most probably lacks the capacity to bind cholesterol esters, it is possible that the capacity of CETPI to bind phospholipids and in general to associate to lipoproteins might not be altered [2, 12]. Moreover, since CETP is responsible for approximately 30% of the phospholipid transport in plasma [23, 24] and that the phosphate groups constitute the primary site for the interaction of CETP with lipoproteins [25–27], this characteristic might be conserved in CETPI.

For the first time we describe the presence of CETPI in human plasma, a novel protein that places new boundaries in the study of lipid metabolism, both at the plasma level as well as the local level of the small intestine.

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