Molecular characterization of rabbit phospholipid transfer protein: choroid plexus and ependyma synthesize high levels of phospholipid transfer protein

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Abstract **Phospholipid transfer protein (PLTP) plays an important role in plasma lipoprotein metabolism. However, PLTP is expressed in a wide range of tissues suggesting additional local functions. To analyze the tissue distribution of PLTP in an animal with high-level expression of the structurally and functionally related CETP, we have cloned the full-length cDNA of rabbit PLTP (1,796 bp). Rabbit PLTP cDNA shows high homology to human, murine, and porcine PLTP cDNA, averaging 86.1%, 80.4%, and 86.1%, respectively. Interestingly, the C-terminus contains a unique seven amino acid insertion not found in previously characterized mammalian PLTPs. In clear contradistinction to human PLTP, rabbit PLTP mRNA was prominent in brain. In situ hybridization studies revealed specific, high-level synthesis of PLTP mRNA in choroid plexus and ependyma, the organs responsible for production of cerebrospinal fluid. Consistent with these findings, PLTP activity in cerebrospinal fluid amounted to 23% ± 3% of that in rabbit plasma. In contrast, neither CETP mRNA nor CETP activity were detectable in rabbit brain. A role of PLTP in the central nervous system could involve some of its actions previously established in vitro, like proteolysis of apolipoproteins, and be physiologically relevant for neurodegenerative disorders such as Alzheimer's disease.**—Gander, R., P. Eller, S. Kaser, I. Theurl, D. Walter, T. Sauper, A. Ritsch, J. R. Patsch, and B. Föger. **Molecular characterization of rabbit phospholipid transfer protein: choroid plexus and ependyma synthesize high levels of phospholipid transfer protein. *J. Lipid Res.* 2002.** 43: **636–645.**

Supplementary key words phospholipid transfer protein • rabbit • cDNA • choroid plexus • ependyma • cerebrospinal fluid

At the outset of the present study, human (1), mouse (2), and pig (3) PLTP cDNA and human (4) and mouse (5) PLTP gene had already been identified. Nonetheless, we felt it would be worthwhile to clone and sequence the rabbit PLTP cDNA for basically three reasons.

First, PLTP is a member of the lipid transfer-lipopolysaccharide binding protein (LT-LBP) family comprised of, in addition to PLTP, CETP, bactericidal permeability increasing protein (BPI), and lipopolysaccharide binding protein (LBP). PLTP is essential for normal secretion (6) and lipolytic degradation of apolipoprotein B (apoB)-containing lipoproteins (7, 8) as well as for adequate lipidation (7, 8) and remodelling (9–11) of HDL. In the same vein, CETP is essential for intravascular remodelling of apoB-containing lipoproteins and HDL (12), leading to the appropriate speciation of LDL (13) and to generation of pre-β HDL (14, 15). Thus, both lipid transfer proteins are structurally homologous (1, 12), are phospholipid transfer proteins (16), are HDL conversion factors (9–11, 17, 18), impact on both forward (12) and reverse (19) cholesterol transport, and have been demonstrated to interact both in vitro (16) and in vivo (20). In addition to PLTP, humans express CETP. Rabbits express even higher levels of CETP (21), whereas mice and pigs lack any appreciable amount of CETP expression. Given the close interrelationship between PLTP and CETP, rabbit data on PLTP regulation may therefore more closely mirror the human situation than data from mice or pigs.

Second, rabbits are well established models for atherosclerosis induced by a high cholesterol diet (22). Importantly, inhibition of CETP activity by specific CETP-antisense oligonucleotides (ODNs) (23), chemical compounds (24), and antibodies (25) leads to a substantial reduction of diet-induced atherosclerosis emphasizing the importance of lipid transfer proteins in atherogenesis. This is of special interest, as CETP overexpression in transgenic mice has proved to be pro-atherogenic in some models (26, 27), but clearly anti-atherogenic in others (28, 29). Thus, opposite conclusions regarding the atherogenic potential of a gene can result from studies in different metabolic contexts or animal models, as also nicely exemplified by models of LCAT overexpression (30). If so, specific

Abbreviations: LTP, lipid transfer protein; ODN, oligonucleotide; PLTP, phospholipid transfer protein; SSC, sodium chloride-sodium citrate.

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inhibition of PLTP by antibodies, chemical inhibitors, or ODNs in the rabbit, which, like humans, expresses both PLTP and CETP, could likely be extrapolated to humans with a higher fidelity than rodent data.

Third, PLTP is expressed in a number of tissues not thought to be substantially involved in plasma lipoprotein transport, like pancreas, placenta, lung, gonads, and brain (2), suggesting a tissue-specific local function. Therapeutic manipulation of plasma lipid transfer proteins may inadvertently lead to side effects by compromising the local function of PLTP or CETP, i.e., by the use of inhibitors unable to discriminate between the LTPs (24). Thus, again, a detailed knowledge of PLTP expression in the rabbit may allow easier monitoring of potential adverse effects of inhibition of lipid transfer proteins in a model closely resembling the human situation.

Therefore, the rabbit model may provide unique insights into PLTP regulation and its effects on atherogenesis and may prove complementary to studies in mice overexpressing (11, 31–33) or lacking (6, 8, 20, 34) PLTP, which have greatly advanced our knowledge on this subject in the past 5 years. The present paper for the first time examines rabbit PLTP on a molecular level and reports the cloning and sequencing of the full-length rabbit PLTP cDNA, its functional expression in COS-7 cells, the tissue distribution of PLTP mRNA in nine different rabbit tissues, analysis of lipid transfer activities in rabbit cerebrospinal fluid and plasma, and a detailed characterization of brain regions synthesizing lipid transfer proteins by in situ hybridization studies.

MATERIALS AND METHODS

Samples

All samples were taken from Chinchilla Bastard rabbits aged between 5 and 15 months. Rabbits were housed at the Central Laboratory Animal Facilities in Innsbruck under protocols approved by the Austrian Animal Care and Use Comittee. Before taking blood, tissue, or cerebrospinal fluid samples, the rabbits were sacrificed by a 3-fold overdose of pentobarbital (Nembutal, Abbott Laboratories, Abbot Park, IL). Tissue samples for RNA isolation were frozen in liquid nitrogen until use. Cerebrospinal fluid was obtained by suboccipital puncture. Protein content of cerebrospinal fluid and plasma was determined according to Lowry et al. (35). Both were stored at −80°C. Rabbit brains were snap-frozen for 30 s in liquid-nitrogen-cooled 2-methylbutane immediately after removal and stored at −80°C until sections were performed.

RNA isolation and reverse transcription

Total RNA from rabbit tissues was prepared using a modified guanidinium isothiocyanate method (RNA-Clean, Dipro, Wiener Neustadt, Austria) and checked by photometric scanning and by agarose gel electrophoresis. Rabbit lung mRNA for rapid amplification of cDNA ends (RACE) was purchased from Clontech (Palo Alto, CA). The first strand cDNA synthesis of the rabbit lung mRNA was performed by using the AMV reverse transcription system from Clontech. The second strand was synthesized by a cocktail of *Escherichia coli* DNA polymerase I, *E. coli* DNA ligase, *E. coli* Rnase H, and T4 DNA Polymerase (Clontech).

cDNA cloning and PCR

Full length cDNA of rabbit PLTP was cloned in two steps. First a 645 bp fragment (175–819) of rabbit PLTP cDNA was amplified by PCR using redundant oligonucleotide primers deduced from the human and murine sequence (forward 645, reverse 645, MWG-Biotech, Ebersberg, Germany), HotStarTaq polymerase (Qiagen, Germantown, MD) and doublestranded cDNA derived from rabbit lung mRNA as described above. After sequencing, new divergently directed primers were designed for RACE. The RACE procedure was carried out using the Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions.

Vector cloning

PCR products for sequencing were cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) by overhanging adenine residues produced by Taq polymerase. Complete rabbit PLTP cDNA was amplified by RT-PCR (forward rP, reverse rP, MWGBiotech, Ebersberg, Germany) using rabbit liver total RNA. Sequence analysis showed that using rabbit liver total RNA as a template for RT-PCR yields the same PLTP cDNA sequence as RACE-PCR from rabbit lung mRNA. In addition, we generated a hybrid cDNA comprising the complete rabbit PLTP cDNA and an additional 39 bp fragment (forward rPE = forward rP, reverse rPE, MWG-Biotech) coding for an E-tag (Amersham Pharmacia Biotech, Uppsala, Sweden) in order to afford convenient immunological detection of the secreted fusion protein by specific antibodies to the E-tag. The E-tag forms the carboxy terminus of the fusion protein. The complete rabbit PLTP cDNA and the hybrid rabbit PLTP-E-tag cDNA were cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) using *Hin*dIII restriction sites at the 5′-side and *Eco*RI and *Not*I at the 3′-side, respectively. The expression constructs contained an upstream CMV promotor and an SV-40 polyadenylation signal downstream of the expression cassette. The control plasmid [pcDNA3.1-green fluorescent protein (GFP)] contained the coding sequence of the GFP (36). All primers were designed with the help of the MacDNAsis V3.6 software (Hitachi Software Engineering, Berks, Great Britain). The expression plasmids were checked by sequencing.

Transient expression of rabbit PLTP and rabbit PLTP-E-tag in COS-7 cells

COS-7 cells (ATCC CRL-1651, Manassas, VA) were transfected by electroporation with 30 μg circular vector DNA (pcDNA3.1-rPLTP, pcDNA3.1-rPLTPE, or pcDNA3.1-GFP, respectively). PLTP and CETP activities were measured as previously described (37, 38) in samples of the cell-culture supernatants after 24 h, 48 h, 72 h, and 96 h and in the cell lysate after 96 h incubation. Transfer activities were corrected for cell-protein content measured by protein quantification (35). Culture supernatants of cells transfected with the rabbit PLTP-E-tag or the GFP construct were analyzed on 7.5% SDS-PAGE and blotted on hydrophobic polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Immunodetection of the recombinant rabbit PLTP-E-tag was carried out by using a monoclonal mouse anti E-tag antibody (Amersham Pharmacia Biotech). After immunodetection with a secondary, horseradish-peroxidase-conjugated goat anti mouse IgG antibody (Dako, Glostrup, Denmark) and chemoluminescent reaction using the electrochemiluminescence kit (ECL) Western blot reagent (Amersham Pharmacia Biotech) the blots were exposed to XAR films (Kodak, Vienna, Austria) and visualized by the Fluor-S-MultiImager (BioRad, Hercules, CA) using the Quantity One V1.1 software (BioRad).

In situ hybridization

Antisense PLTP or CETP oligonucleotides (PLinsitu A–D, CEinsitu A–D), designed to have a GC content of 40% to 60% and a

length of 45 bp, were labeled with α^{32}P dCTP (Code AA0075, Amersham Pharmacia Biotech) by the terminal deoxynucleotidyl transferase of calf thymus (Amersham Pharmacia Biotech) according to the manufacturer's instructions and diluted with hybridization buffer [4 × SSC, 5 × Denhardt's solultion, 200 μg/ml sheared salmon sperm DNA (Sigma Aldrich, St.Louis, MO, USA), 100 μg/ml long chain polyadenylic acid (Sigma Aldrich), 120 μg/ml heparin (Sigma Aldrich), 25 mM sodium phosphate, and 1 mM sodium pyrophosphate] to an activity of 5,000 cpm/μl. Frozen tissue samples were cut into 10 μm sections, fixed in 4% paraformaldehyde, submerged in 96% ethanol, and stored at 4°C until use. Labeled DNA probes were hybridized with the brain sections at 42°C for at least 12 h in a humified atmosphere. Subsequently, excess probe was removed by stringent washing for several times with 1 × SSC at 55°C followed by 50%, 75%, and 96% ethanol. Finally, sections were exposed to XAR films (Kodak) for 10 days. Control experiments were carried out by incubation of the brain sections with radiolabeled sense oligonucleotides. The sequences of the oligonucleotides can be obtained on request.

Statistics

Descriptive data are expressed as mean ± SD. Groups were compared by unpaired *t*-test. Statistical significance was inferred at a two-tailed $P < 0.05$ (SPSS for Windows 8.0).

RESULTS

Cloning of full length rabbit PLTP cDNA

Full-length cDNA of rabbit PLTP was cloned in two steps. First, we amplified a 645 bp-part of rabbit PLTP cDNA by RT-PCR, using redundant primers based on the human and murine PLTP cDNA (1, 5) and subcloned it into the pCR2.1 vector. Subsequently, we designed new divergently-directed primers for the RACE procedure based on the sequence data of the rabbit PLTP cDNA fragment and obtained the complete rabbit PLTP cDNA (1,796 bp).

Analysis of rabbit PLTP cDNA

Our rabbit PLTP cDNA clone comprised 44 bp in 5′-direction of the putative translation start site, the entire coding region, and a 240 bp 3′-noncoding region including a poly(A)-tail (full length cDNA at the GenBank server, Accession Number AY072718). Rabbit PLTP cDNA contains an open reading frame of 1,512 bp predicting a protein of 503 amino acids. The coding cDNA region of rabbit PLTP shares an overall nucleotide identity of 86.1% with the human (1), 80.4% with the murine (2), and 86.1% with the porcine (3) cDNA, respectively. The deduced rabbit PLTP has 84.6% amino acid identity with human (1), 77.3% with murine (2), and 84.5% with porcine PLTP (3) (**Table 1**). As a largely conserved signal peptide of 17 amino acids is cleaved from the N-terminus of PLTP before its secretion (1), we predict the mature rabbit PLTP to comprise 486 amino acids. The calculated molecular mass of the mature rabbit PLTP is thus 53.4 kDa (Compute pI/Mw Tool, http://www.expasy.ch). The cystein residues at amino acid positions 5, 129, 168, and 318 of the secreted protein which are important for synthesis, secretion, and specific activity of PLTP (39, 40), are conserved in human, murine, porcine, and also in rabbit PLTP. Interestingly, the C-terminal region of rabbit PLTP shows a characteristic seven amino acid insertion (amino acids 477–483) which does not share with PLTP of any other species (Table 1). The three amino acid insertion (amino acids 488–490) at the C-terminus described by Pussinen et al. (3), for porcine is also present in rabbit PLTP but not in human or murine PLTP. The calculated isoelectric point (pI) of rabbit PLTP is 6.14. Murine PLTP has a pI of 6.19 while human, and in particular porcine, PLTP have much higher pIs according to the prediction model we used (Compute pI/Mw Tool). However, due to the conservative character of most substitutions, prediction of the secondary structures, *O*- and *N*-linked glycosylation sites (NetOGlyc 2.0, http://www.expasy.ch) revealed no major differences between rabbit PLTP and the PLTPs of other species.

Expression of functional rabbit PLTP in COS-7 cells

Expression constructs containing either just the rabbit PLTP coding region, rabbit PLTP cDNA including an additional carboxy-terminal E-tag sequence, or, as a positive control, the cDNA of GFP designed to allow convenient detection by specific antibodies, were transfected into COS-7 cells. After incubation for 24 h, 48 h, 72 h, and 96 h, samples from cells transfected with either rabbit PLTP cDNA (**Fig. 1A**) or rabbit PLTP cDNA with an added E-tag sequence (Fig. 1B), facilitated radioactive phospholipid transfer, whereas supernatants from cells transfected with GFP cDNA did not (Fig. 1A and B). When compared with the amount of active PLTP present in the supernatants, trace amounts of active PLTP were found in cell lysates, indicating efficient secretion of both native rabbit PLTP and rabbit PLTP carrying an E-tag (**Fig. 2A** and **B**). Transfection of cells with the recombinant rabbit PLTP-E-tag allowed also immunodetection (**Fig. 3**) indicating an approximate molecular mass of 75 kDa. Thus, the calculated mass of rabbit PLTP (53.4 kDa) is substantially increased by glycosylation similar to PLTP isolated from human or porcine plasma.

Tissue specific expression of rabbit PLTP

PLTP mRNA tissue distribution was examined in three individual Chinchilla Bastard rabbits by Northern blots. The PLTP mRNA yielded a single band of 1.8 kb length and was normalized to the respective β-actin mRNA. In all three rabbits, pancreatic tissue consistently expressed the highest amounts of PLTP mRNA followed by adipose tissue and the brain. Moderate levels were found in heart and liver tissues, whereas kidney, lung, and spleen yielded quite low levels. Skeletal muscle showed nearly no PLTP signal (**Fig. 4**). Our results are very similar to those of the pig which was investigated by a different quantitation method based on RT-PCR (3). Previous data of human and murine PLTP mRNA reported somewhat different expression patterns of PLTP (2). Interestingly, human PLTP mRNA in these reports was barely detectable in the brain.

Phospholipid transfer activity in rabbit plasma and cerebrospinal fluid

Protein content of plasma and cerebrospinal fluid averaged 55.5 ± 4 mg/ml and 0.412 ± 0.063 mg/ml, which

TABLE 1. Amino acid sequence alignment of PLTP from four species

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                10         20         30         40         50         60
Rabbit  MALFGAVFLA LLAGAHAEIP GCKIRVTSEA LALVKQEGLR FLEQELEAIT IPDLRGREGH
Pig     MALLGALFLV LLAGAHAELP GCKIRITSKA LELVKQEGLR FLEQELETIT IPDLRGREGH
Mouse   MVLLWALFLA LLAGAHAELP GCKIRVTSAA LDLVKQEGLR FLEQELETIT IPDVYGAKGH
Human   MALFGALFLA LLAGAHAEFP GCKIRVTSKA LELVKQEGLR FLEQELETIT IPDLRGKEGH

                70         80         90        100        110        120
Rabbit  FYYNISDVKV TELQLTGSEL HFQPEQELAL QITNASLGLR FRRQLLYWFF YDGGYINASA
Pig     FYYNISEVKV TELQLTSSDL HFQPEQELML QINNGSLGLR FRRQLLYWFF YDGGYINASA
Mouse   FYYNISDVRV TQLHLISSEL HFQPDQDLLL NISNASLGLH FRRQLLYWFL YDGGYINASA
Human   FYYNISEVKV TELQLTSSEL DFQPQQELML QITNASLGLR FRRQLLYWFF YDGGYINASA

               130        140        150        160        170        180
Rabbit  EGVSIRTALQ LSQGPAGQMR VSNVSCLASV SRMHAAFGGT FRKVYEFLST FITSGMRFLL
Pig     EGVSIHTALQ LSRDPTGRIK VSNVSCQASV SRMHAAFGGT FKKVYEFLST FITSGMRFLL
Mouse   EGVSIRTGLQ LSQDSSGRIK VSNVSCEASV SKMNMAFGGT FRRMYNFFST FITSGMRFLL
Human   EGVSIRTGLE LSRDPAGRMK VSNVSCQASV SRMHAAFGGT FKKVYDFLST FITSGMRFLL

               190        200        210        220        230        240
Rabbit  NLQICPVLYH AGMVLLNSLL DTVPVRSAVD ELVGIDYSLL KDPVVSNSYL DMEFRGAFFP
Pig     NQQICPVLYH AGMVLLNSLL DTVPVRGAVD ELVGIDYSLL KDPVASTSNL DMEFRGAFYP
Mouse   NQQICPVLYH AGTVLLNSLL DTVPVRSSVD DLVGIDYSLL KDPVVSNGNL DMEFRGAFFP
Human   NQQICPVLYH AGTVLLNSLL DTVPVRSSVD ELVGIDYSLM KDPVASTSNL DMDFRGAFFP

               250        260        270        280        290        300
Rabbit  LAEGNWSFLN RAVEPQLQEE ERMVYVAFSE FFFDSAMESY FRAGALKLSL VGDKVPHDLD
Pig     LAEGNWSLPN RRVEPQLQEE ERMVYVAFSE FFFDSAMESY FRAGALKLSL VGDKVPHDLD
Mouse   LKEDNWSLPN RAVEPQLEDD ERMVYVAFSE FFFDSAMESY FQAGALQLTL VGDKVPSDLD
Human   LTERNWSLPN RAVEPQLQEE ERMVYVAFSE FFFDSAMESY FRAGALQLLL VGDKVPHDLD

               310        320        330        340        350        360
Rabbit  ILLRATYFGS IVLLSPAVID SPLKLELQVT APPRCTIKPS GTTISVTATV TIALVPPMQP
Pig     MLLRATYFGS IVLLSPAVID SPLKLELRVM APPRCTIKPS GTTISVTASV TIALVPPGQP
Mouse   MLLRATYFGS IVLLSPTVIN SPLKLKLEAT SPPRCTIKPS GTTISITASV TITLAPPMLP
Human   MLLRATYFGS IVLLSPAVID SPLKLELRVL APPRCTIKPS GTTISVTASV TIALVPPDQP

               370        380        390        400        410        420
Rabbit  EVQLSSMVMD ARFSAKMALR GKALRTQLDL RKFRIYSNQS AIESLALIPL QAPLKTLLQI
Pig     EVQLSSMTMD ARLSAKMALR GKALRTQLDL RRFRIYSNQS ALESLALIPL QAPLKTMLQI
Mouse   EVELSKMIME GRLSAKLTLR GKALRVKLDL RRFQIYSNQS ALESLALIPL QAPLKTLLQI
Human   EVQLSSMTMD ARLSAKMALR GKALRTQLDL RRFRIYSNHS ALESLALIPL QAPLKTMLQI

               430        440        450        460        470        480
Rabbit  GVMPMLNERT WRGVQIPLPE GINFVREVVT NHAGFLTIGA DLHFAKGLRE VIDKNREVID
Pig     GVMPMLNERT WRGVQIPLPE GINFVREVVT NNAGFLTIGA DLHFAKGLRE VIEKNR----
Mouse   GVMPLLNERT WRGVQIPLPE GINFVREVVT NHAGFVTVGA DLHFAKGLRE VIDKNR----
Human   GVMPMLNERT WRGVQIPLPE GINFVHEVVT NHAGFLTIGA DLHFAKGLRE VIEKNR----

               490        500        510
Rabbit  KNPPAATRQP QASEAPLPAP AAD*......
Pig     ---PADTRGP QASSAPPPST AAV*......
Mouse   ---PADV--- AASHVPPPSA AAA*......
Human   ---PADVR-- -ASTAPTPST AAV*......
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PLTP sequences are shown in one-letter-code. Differences are highlighted white, the numbering is according to amino acid sequence.
* Indicates in-frame stop codons.

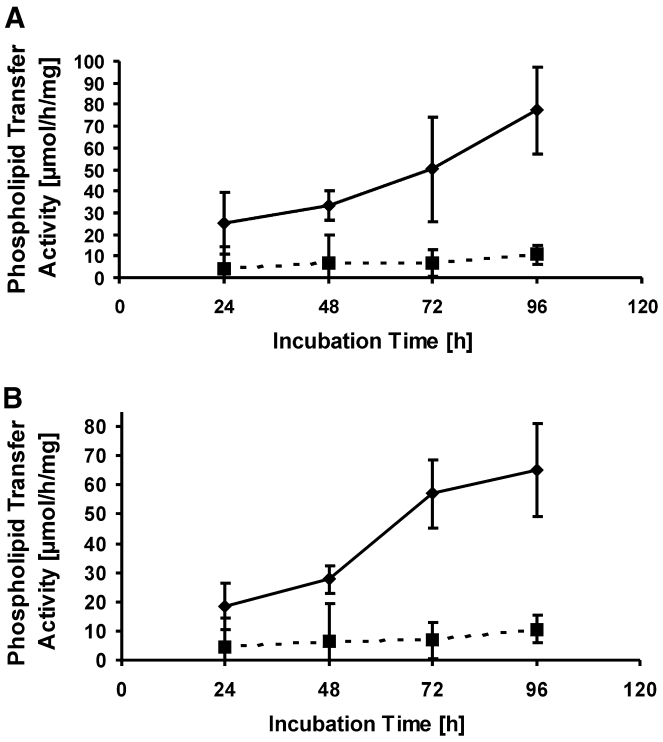

Fig. 1. Transient expression of rabbit phospholipid transfer protein (PLTP) in COS-7 cells. Phospholipid transfer activities after 24 h, 48 h, 72 h, and 96 h incubation. A: Time course of phospholipid transfer activities in cell culture supernatants of rabbit-PLTP-transfected COS-7 cells (closed diamond) and a control experiment with green fluorescent protein (GFP)-transfected COS-7 cells (closed square). B: Phospholipid transfer activity of rabbit-PLTP-E-tag-transfected COS-7 cells (closed diamond) and the GFP-control (closed square). Samples for both experiments were taken 24 h, 48 h, 72 h, and 96 h after transfection. Data are mean ± SD for three independent experiments and were corrected for protein content of cell lysates.

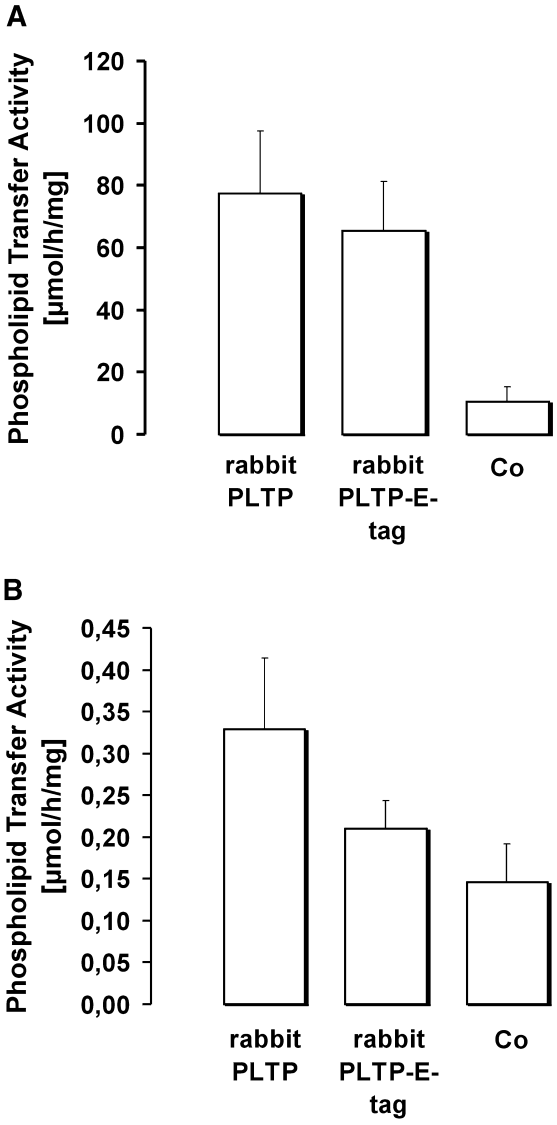

Fig. 2. Transient expression of rabbit PLTP in COS-7 cells. Phospholipid transfer activities in cell culture supernatants (A) and cell lysates (B) after 96 h incubation. Data are mean ± SD for three independent experiments and were corrected for protein content of cell lysates.

falls within the normal range of reported rabbit values (41). Equal volumes of cerebrospinal fluid and plasma from three individual rabbits were pooled for one experiment because of the relatively low amount of cerebrospinal fluid that could be obtained from a single animal. Phospholipid transfer activity in cerebrospinal fluid was linear for sample volumes up to 125 µl (**Fig. 5A**). Phospholipid transfer activities detected in cerebrospinal fluid averaged approximately 23% of plasma values. Thus, normalized for protein content, disproportionately high amounts of active PLTP are present in rabbit cerebrospinal fluid.

Cholesteryl ester transfer activity in rabbit cerebrospinal fluid and plasma

Phospholipid transfer activities in the cerebrospinal fluid could simply be due to plasma PLTP transported into the cerebrospinal fluid via the blood-cerebrospinal-fluid barrier. If so, one would expect to also find another lipid transfer protein, CETP, which is also bound to HDL, in cerebrospinal fluid. CETP activity in plasma was linear up to 15 µl. However, no cholesteryl ester transfer could be detected in cerebrospinal fluid samples up to 50 µl (Fig. 5B).

In situ hybridization of rabbit PLTP and CETP mRNA in brain sections

Paramedian sections of rabbit brain showed pronounced staining in the periventricular area, especially in the choroid plexus and the ependyma of the lateral ventricle (**Fig. 6A** and **B**). After prolonged exposure we observed additional, more subtle differences, in PLTP expression among brain regions. Cortical white matter contained lower amounts of PLTP mRNA than corpus striatum and cortical gray matter (Fig. 6B). Sequential frontal sections of the right brain hemisphere (Fig. 6D, E, G, and H) confirmed that choroid plexus and ependyma of the lateral ventricle were most strongly stained. In addition, corpus striatum showed relatively strong signals in the more cranial sections (Fig. 6E).

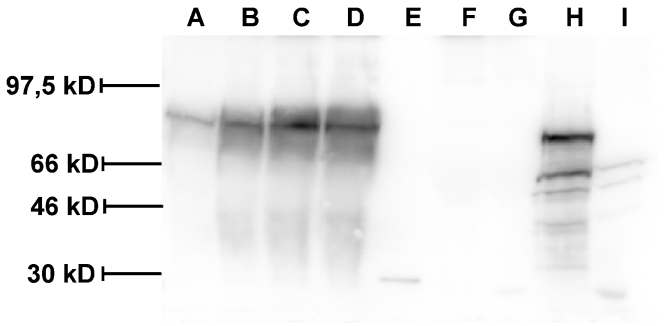

Fig. 3. Western blot analysis of recombinant rabbit PLTP. COS-7 cells were transfected with plasmids containing either recombinant rabbit PLTP-E-tag, native rabbit PLTP, or GFP using a monoclonal antibody against the E-tag contained in recombinant rabbit PLTP. Western blots of cell culture supernatants after 24, 48, 72, and 96 h (A–D) and lysates of COS-7 cells transfected with rabbit PLTP-E-tag after 96 h (lane H) showed a band corresponding to a molecular weight of approximately 75 kDa. Expectedly, no bands were detected in the cell culture supernatant of cells transfected with rabbit PLTP (F) or GFP (G) and the lysate of COS-7 cells transfected with GFP after 96 h incubation (I). Lane E shows a 30 kDa band corresponding to a E-tag positive control protein.

The same set of experiments was also carried out with CETP probes which revealed no signals even after exposure for more than 10 days (data not shown). Negative control experiments revealed no detectable signal (Fig. 6C, F, and I).

DISCUSSION

This paper lays the basis for further studies examining PLTP regulation, its role in atherogenesis, and potential tissue-specific functions of PLTP in an animal with high level expression of the related CETP. Even from the present study, however, interesting findings have emerged both from analysis of rabbit PLTP structure and from a detailed characterization of its expression, especially in rabbit brain.

While rabbit PLTP shares a high overall homology with human (86.1%) (1), murine (5), and porcine (3) PLTP

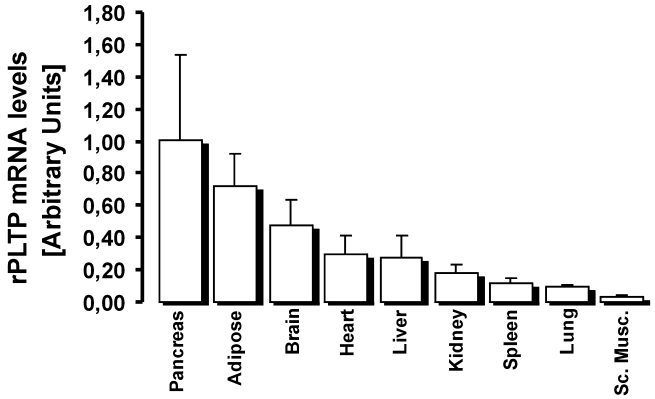

Fig. 4. PLTP multiple tissue Northern blot. PLTP mRNA in nine different rabbit tissues normalized for β-actin mRNA content. Data are mean ± SD for three independent experiments.

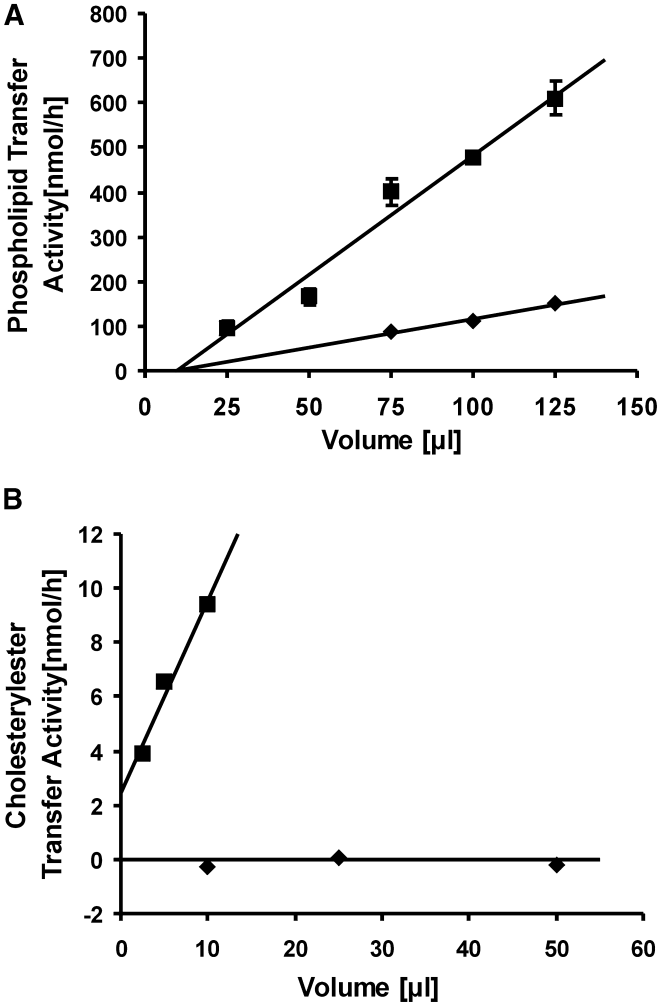

Fig. 5. Phospholipid and cholesteryl ester transfer activities in rabbit cerebrospinal fluid and plasma. A: Phospholipid transfer activities of rabbit plasma (closed square) and rabbit cerebrospinal fluid (closed diamond). B: Cholesteryl ester transfer activities of different volumes of rabbit plasma (closed square) and rabbit cerebrospinal fluid (closed diamond). Data are mean ± SD for three independent experiments.

on a nucleic acid basis, two remarkable differences in amino acid sequence are nevertheless evident. As previously pointed out for porcine PLTP (3), an insertion of three amino acids at positions 488–490 is present in rabbit PLTP, which is not found in human and murine PLTP. A second insertion of seven amino acids at positions 477–483 is actually unique for rabbit PLTP. Whether or not the above or other changes in primary structure alter any of the multiple molecular functions of PLTP in the rabbit remains to be determined. However, studies on site-directed mutagenesis show that C-terminal deletion variants of 20 amino acids have specific activities of only approximately 30% compared with wild-type human PLTP (40). Thus, relatively subtle modification of the amino acid sequence of the C-terminus, as present in rabbit PLTP, may change function and/or substrate binding of PLTP.

PLTP and CETP are synthesized both in tissues with a major role in lipoprotein metabolism (1, 3, 5, 42–46), like

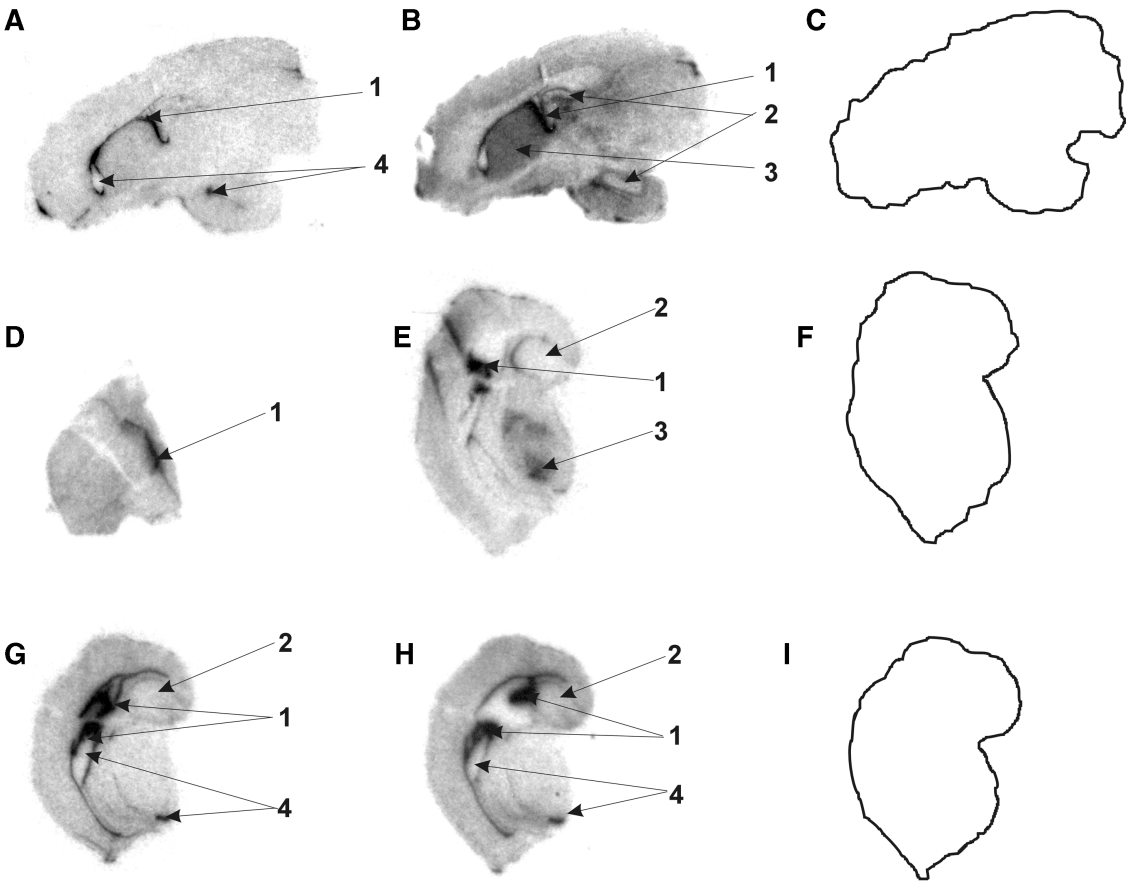

Fig. 6. In situ detection of PLTP mRNA in rabbit brain. A and B show paramedian brain sections after hybridization with radiolabeled antisense PLTP oligonucleotides. The section showed at A has been exposed to a X-ray film for 8 days, the section at B for 11 days. D, E, G, and H show a sequence of frontal sections of the right brain hemisphere from cranial (D) to caudal (H) after hybridization with radiolabeled antisense PLTP oligonucleotides. C, F, and I show representative experiments with radiolabeled PLTP probes of sense polarity. The edges of the sections shown in C, F, and I are indicated by black linings. Arrows indicate specific neuro-anatomic structures: 1, choroid plexus and ependyma; 2, hippocampus; 3, corpus striatum; 4, lateral ventricle.

liver and adipose, and in those without (1, 3, 5, 42–46), like pancreas and spleen. If there were a major overlap in physiological function between CETP and PLTP, one might expect to see a broader tissue pattern of PLTP expression in animals lacking CETP, like mouse and pig, than in the rabbit, which highly expresses both lipid transfer proteins. PLTP expression in the rabbit in this study, however, fails to confirm this expectation and is actually very similar to the tissue pattern of murine (2) and porcine (3) PLTP with high levels of PLTP mRNA in pancreas, placenta, lung, brain, adipose, liver, and heart and lower levels in skeletal muscle, spleen, and kidney. The similar tissue pattern confirms the concept that PLTP and CETP have specialized non-overlapping functions in vivo (20) and extends its validity from plasma lipoprotein metabolism to the cellular level.

Interestingly, brain ranks second in PLTP mRNA content in both mouse (2) and pig (3), and third in the rabbit (Fig. 4). In contrast, PLTP mRNA content in human brain consistently ranks last of all tissues examined (1, 2). Thus, brain is the only tissue with a major species-specific variation in PLTP expression identified so far. Generally, tissue-specific expression of PLTP seems to be more conserved than that of CETP, where some species completely lack CETP (21) and others fail to express CETP in critical organs like the liver (45). Taken together, tight preservation of tissue-specific expression of PLTP and regulation by local factors (47) strongly suggest important local functions of PLTP.

A high content of PLTP mRNA in rabbit brain does not necessarily imply that bioactive PLTP is actually secreted by this tissue. We therefore quantified PLTP-activity in cerebrospinal fluid and found that it amounted to roughly 23% of the plasma activity on a v/v basis (Fig. 5). Given the fact that the protein content of normal rabbit cerebrospinal fluid is approximately two orders of magnitude lower than that of plasma (41) and assuming a normal specific activity of PLTP in cerebrospinal fluid, we estimate the relative PLTP concentration of rabbit cerebrospinal fluid to exceed that of plasma by at least 20-fold. If the cerebrospinal fluid, like plasma (48), were to contain both active and inactive forms of PLTP, extrapolation from activity to PLTP mass data would be further complicated. Even the relatively high PLTP activity in the cere-

brospinal fluid need not be due necessarily to local PLTP synthesis, but could reflect efficient active transport from plasma. In order to further explore the possibility that PLTP activity in cerebrospinal fluid derives from plasma, we determined the activity of CETP in cerebrospinal fluid. As both the literature (42–46) and our own data in the rabbit show that brain lacks CETP mRNA, the only conceivable source of CETP in cerebrospinal fluid would be transport from plasma. CETP shares close structural homology with PLTP (12, 49) and is transported in plasma bound to a similar subset of HDL particles (37) making a selective transport of PLTP, but not CETP, quite unlikely. Expectedly, we found substantial CETP activity in rabbit plasma, but, interestingly, none in rabbit cerebrospinal fluid (Fig. 5), strengthening the notion that PLTP is indeed synthesized locally within the central nervous system (CNS). Even in humans, with clearly lower levels of PLTP mRNA in the CNS, recently PLTP activity and mass was shown to be present in cerebrospinal fluid (50), averaging about 1% of plasma values. This seemingly low level of activity should be seen in the context of cerebrospinal fluid lipid and apoA-I (51) concentrations, which also average about 1% of plasma levels, indicating that PLTP activity, even in humans, clearly has the potential to substantially modulate the metabolism of lipoproteins in cerebrospinal fluid.

Brain comprises various neuroanatomic structures with differing functions. Thus, we were interested if secretion of PLTP was a global property of all brain structures or specific for some regions. In situ hybridization experiments demonstrated choroid plexus and ependyma to be by far the most prominently stained structures (Fig. 6). Choroid plexus and ependyma have evolved to produce and resorb the cerebrospinal fluid filling ventricles, a compartment turning over four to five times each day (52). In hindsight, high level secretion of PLTP by the plexus should not have been a surprise, as PLTP is prominently expressed in endothelial cells and was actually first cloned from a human endothelial cDNA library (1). However, coordinated high-level expression of PLTP both in endothelial cells of the choroid plexus and in epithelial cells of the adjacent ependyma very strongly supports a functional role of PLTP in the cerebrospinal fluid.

In addition to the prominent expression of PLTP in choroid plexus and ependyma, PLTP expression was clearly detectable in corpus striatum and cortical gray matter, and at lower levels also in cortical white matter. On the other hand, the lack of CETP activity in the cerebrospinal fluid and the completely negative results of the in situ hybridization with CETP-antisense probes clearly show the lack of functional overlap between the two transfer proteins in rabbit brain. This observation is of some interest, as CETP activity has been reported to be detectable in human brain (53).

Recently, a growing body of evidence supports an important role of cerebrospinal fluid lipoproteins in the transport of essential fatty acids, lipid-soluble vitamins, cholesterol, and also in the removal of oxysterols formed by degenerative processes (51). A number of components of the lipid transport system in cerebrospinal fluid, such as some apolipoproteins (i.e., apoE, apoD, and apoJ), receptors (i.e., LDL-receptor, LRP, and megalin), and enzymes [i.e., LCAT (50)] are synthesized locally, others are imported from plasma [i.e., apoA-I (51, 54)]. Our data add PLTP to the roster of enzymes secreted by mammalian brain and detail its neuroanatomical localization. Functionally, PLTP might be involved in the proteolysis of apolipoproteins (55), in the remodeling of cerebrospinal fluid HDL, in the stimulation of cholesterol efflux from neuronal cells, or in the equilibration of vitamin E between lipoproteins and cells (56, 57). A specific down-regulation of PLTP in the central nervous system of patients with Down syndrome (58) is in line with a role of cerebrospinal fluid lipoproteins and PLTP in neurodegenerative disease. Which precise physiological role PLTP plays in the CNS remains speculative.

In conclusion, elucidation of the role of PLTP in cholesterol and phospholipid homeostasis in vasculature and brain may provide important clues for the prevention and treatment of atherosclerosis and neurodegenerative diseases, i.e., Alzheimer's disease. We hope that studies of PLTP function and regulation in the rabbit will significantly contribute to this promising area of investigation.

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