Molecular cloning and functional expression of cDNA encoding the pig plasma phospholipid transfer protein

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Abstract Humans and the pig show marked similarities in lipoprotein metabolism; therefore, the pig has been used as a model in numerous nutritional studies. Pig plasma displays no activity of cholesteryl ester transfer protein (CETP), which is known to be responsible for half of the phospholipid mass transfer in human plasma, the other half being accounted for by the plasma phospholipid transfer protein (PLTP). This makes the pig an ideal subject for the study of PLTP structure and function. Here we report the molecular cloning of pig PLTP and the eukaryotic cell expression of its complementary DNA. Pig PLTP was found to share 93% amino acid sequence identity with human PLTP and 81% with mouse PLTP. Tissue expression of PLTP mRNA was examined by a method based on reverse transcription-polymerase chain reaction (RT-PCR) and solid-phase minisequencing in nine pig tissues. The highest PLTP mRNA levels were found in the pancreas, brain, lung, and liver. Medium from COS-1 cells expressing PLTP possessed phospholipid transfer activity, and the secreted recombinant PLTP was detectable by Western blotting in the culture supernatant. A mutant protein with a substitution of Cys at position 22 by Arg was found to display impaired secretion into growth medium indicating a role for cysteines in the correct folding of PLTP. This study forms the basis for future work on the structure-function relationships in pig PLTP.


Supplementary key words pig lipoproteins

The mechanisms underlying the association between high density lipoprotein (HDL) concentration and coronary heart disease are not well understood, but the anti-atherogenic effect of HDL is usually accounted for by its role in the reverse cholesterol transport. In this pathway HDL mediates the transfer of cholesterol from peripheral tissues to the liver for excretion. It has been demonstrated that the initial acceptors of cell membrane cholesterol belong to a minor HDL subclass called preβ2-HDL (1). HDL subclass distribution is regulated by enzymes, lecithin:cholesterol acyltransferase (LCAT), lipoprotein lipase (LPL), and hepatic lipase (HL), as well as by plasma lipoprotein transfer proteins, the cholesteryl ester transfer protein (CETP), and the phospholipid transfer protein (PLTP) (2). In addition to its phospholipid transfer activity, PLTP facilitates modulation of HDL size and composition (3, 4). Preβ2-HDL particles are generated by this HDL conversion process (5).

The molecular cloning and sequencing of full-length cDNAs encoding human (6) and mouse (7, 8) PLTP has recently been reported. The deduced proteins share 83% overall sequence identity. Human and mouse recombinant PLTP secreted by transfected baby hamster kidney (BHK) cells manifest both phospholipid transfer and HDL conversion activity (8). The structure of the human PLTP gene has been determined (9), and the gene has been mapped to chromosome 20q12–q13.1 (10). The human PLTP gene has a structure remarkably similar to that of the CETP gene (9), and the encoded proteins consist of an identical number of amino acids and show 20% sequence identity. PLTP is also homologous to human lipopolysaccharide-binding protein and neutrophil bactericidal permeability increasing protein (24 and 26% amino acid identity, respectively) (6).

Due to similarities between the human and pig lipoprotein systems, the pig has been considered an attrac-

Abbreviations: bp, base pair; cDNA, complementary DNA; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; mRNA, messenger RNA; nt, nucleotide; ORF, open reading frame; PLTP, phospholipid transfer protein; RT-PCR, reverse transcription-polymerase chain reaction.

1The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number L78843.

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tive animal model in atherosclerosis research. We have earlier described the purification and characterization of pig PLTP (11), which is similar to human PLTP in many respects. The pig and the human proteins have similar molecular masses (81 kD), comparable phospholipid transfer activity, and HDL conversion activity. They also share immunological epitopes. There are, however, a number of crucial differences in lipid metabolism between these two species. Pig HDL consists of a homogeneous population of particles, in which the major protein is apoA-I and no apoA-II can be detected (12). Moreover, pig has no functional CETP (13, 14), suggesting that PLTP alone may be responsible for the phospholipid transfer activity in pig plasma (15). For these reasons we have chosen the pig as a model in our attempts to clarify the physiological role of PLTP. We have, therefore, cloned the cDNA encoding pig PLTP, analyzed its nucleotide and deduced the amino acid sequence, determined the distribution of PLTP mRNA in pig tissues, and expressed the cDNA in COS-1 cells.

MATERIALS AND METHODS

cDNA library screening

A porcine lung cDNA library in the λgt11 vector (Clontech) was screened with the full-length human PLTP cDNA labeled with [32P]dCTP by random priming. The Hybond-C filters (Amersham) with 500,000 lifted plaques were hybridized overnight at 42°C in 40% formamide, 5× Denhardt’s solution, 5× SSC (SSC: 15 mm sodium citrate, pH 7.0, 150 mm NaCl), 1% SDS (16), and washed in 2× SSC, 0.1% SDS, at 37°C. Films were exposed overnight at −70°C, and the four positive plaques detected were collected. A second round of screening was performed to plaque purify the clones and the inserts were amplified by PCR with λgt11 specific primers for sequence verification.

DNA sequencing and sequence analysis

Both strands of the cDNA inserts were sequenced by “primer walking” using a system identical to the Sequenase 2.0 kit (United States Biochemical) with T7 DNA-polymerase (Pharmacia). The sequences were analyzed with the University of Wisconsin GCG programs (17).

RNA isolation, cDNA synthesis, and PCR cloning

RNA was prepared from pig tissues using the guanidinium isothiocyanate method (16). The polyA(+) fraction was enriched with the QIAGEN Oligotex-dT mRNA Mini system. The placenta mRNA was reverse transcribed to first strand cDNA under standard conditions using the Promega AMV reverse transcriptase. The cDNA was used as a template for PCR with oligonucleotide primers corresponding to the start and stop codon regions of the PLTP open reading frame (ORF): 5’-GA GCCCCACTCGGCAATGCCG-3’ (Fig. 1A; 1) and 5’-GAC TGAGGGCTAGACACGTGC-3’ (Fig. 1A; 2). The PCR fragment of 1.6 kb containing the entire coding region of pig PLTP was cloned, using the XbaI restriction sites included in the primers, into pBluescript (Stratagene) and sequenced on both strands by the dideoxy chain termination method. To detect mutations generated during the amplification, the entire sequence was determined from three independent PCR products.

Northern blot analysis

The mRNAs (1.2 µg/specimen) were denatured at 68°C for 10 min in 1.3 × sample loading buffer (50% formamide, 20 mM MOPS, 6% formaldehyde) and electrophoresed through 1% agarose, 20 mM MOPS, 6% formaldehyde gel using 20 mM MOPS, 3% formaldehyde as the running buffer. The mRNAs were transferred to a Hybond-N membrane (Amersham) in 10× SSC, and the blots were blocked for 2 h at 80°C under vacuum. The membrane was probed with the 32P-labeled full-length pig PLTP cDNA (3.5 × 10⁶ cpm/ml) overnight at 42°C in 50% formamide, 5× SSPE (50 mM sodium phosphate, pH 7.4, 750 mM NaCl, 5 mM EDTA), 5× Denhardt’s solution, 1% SDS, and washed in 0.5× SSC, 0.5% SDS, at 60°C. The film was exposed for 10 days at −70°C.

mRNA quantitation

The relative quantity of the PLTP mRNA in different pig tissues was determined by a method based on RT-PCR and solid-phase minissequencing (18). PolyA(+) RNAs (0.6 µg/tissue) were reverse transcribed under standard conditions using the Promega AMV reverse transcriptase and a gene-specific primer, 5’-GTCCTCCA GCTCCTGCTCCAG-3’ (Fig. 1A; 3). Different amounts (0.32, 1.6, 8, or 40 pg) of a plasmid containing a PLTP cDNA with the C22R mutation (substitution of nt 77, T, by C; see Results) were added in the cDNAs as an internal standard, and PCR was subsequently carried out using a biotinylated 5’ primer, 5’-CCTCTTCCTAGT GCTGCTGG-3’ (Fig. 1A; 4) and a 3’ primer, 5’-AGCGC AGCCCGCTTCTTGCTC-3’ (Fig. 1A; 5). The ratio of wild-type PCR product (representing the reverse transcribed cDNA strand) to the mutant one (representing the plasmid standard) was determined by minissequencing using the primer 5’-GAGGTGATGCGGATTT TGC-3’ (Fig. 1A; 6). The ratio represents a direct measure of the mRNA quantity.

Expression of pig PLTP in COS-1 cells

The pig PLTP cDNA was cloned into the XbaI site of the mammalian cell expression vector pSVL.
macia), and transfected into COS-1 cells by the DEAE-dextran method as described previously (19). The phospholipid transfer activity in the culture supernatants and cells broken by sonication was determined at 24 h, 48 h, and 72 h after transfection (20). The HDL conversion activity of the culture supernatant was assessed as described earlier (11).

**Immunofluorescence microscopy**

Transfected COS-1 cells grown on coverslips were fixed for 30 min in 4% paraformaldehyde, 250 mM HEPES, pH 7.4, and permeabilized for 30 min with 0.1% Triton X-100 in PBS. After blocking nonspecific binding of antibodies with 10% fetal calf serum/PBS, incubation was continued for 30 min at 37°C in the presence of primary monoclonal antibody, G11 (11) diluted in 5% serum/PBS. The bound antibody was detected with tetramethylrhodamine-isothiocyanate-conjugated goat anti-mouse F(ab)2 (Immunotech), and the specimens were viewed with a Zeiss Axiophot fluorescence microscope.

**RESULTS**

**Cloning of the pig PLTP cDNA**

Screening of porcine lung cDNA library with the human PLTP probe yielded short truncated clones containing a sequence from the 3'end of the PLTP cDNA. However, one clone contained a rearranged sequence including a short stretch of the PLTP ORF 5'-end. Oligonucleotides corresponding to the ends of the open reading frame were synthesized and used for amplification of the full-length pig PLTP coding region by PCR. The obtained 1500 bp fragment was cloned into pBlue-script for sequencing and further manipulations.

**Sequence analysis**

The cDNA contains an open reading frame of 1488 bp, encoding a deduced 496 amino acid protein (Fig. 1) with a predicted molecular weight of 54,922 Da. SYNthesis of a polypeptide of the expected size from the cDNA was confirmed by in vitro transcription/translation, which yielded a product with the approximated molecular weight of 52 kDa (not shown). The pig protein is 93% identical with the human (6), and 81% with the mouse (7) counterpart. At the nucleotide level the homology is 91% and 81%, respectively. Most of the amino acid differences between the pig, human, and mouse proteins are conservative, and the diverging residues are evenly distributed along the whole sequence (Fig. 1). The pig polypeptide contains more positively charged residues (calculated pI 7.89) than the human (pI 7.00) and mouse (pI 6.72) polypeptides. Structural predictions of the proteins made using the GCG PEPTIDESTRUCTURE program reveal no significant differences. All three proteins are characterized by extensive hydrophobic regions concentrating in the C-terminal half of the proteins. The human and pig proteins contain six and the mouse homologue seven potential N-glycosylation sites. Close to the C-terminus, the pig protein displays insertion of three amino acids (aa 482–484; Fig. 1) absent from both the human and the mouse sequences.

**Tissue distribution of the pig PLTP mRNA**

Expression of PLTP mRNA in adult pig tissues was assessed qualitatively by Northern blotting and quantitatively by RT-PCR/minisequencing analysis. This method based on inclusion of an internal standard is well established and yields an accurately quantifiable result (18, 21, 22). A single mRNA species of 1.6 kb was detected in the tissues studied, the signal being strongest in the pancreas, brain, and lung. The quantitative analysis (Fig. 2) was in agreement with the Northern blot results, revealing highest expression levels in pancreas and brain, intermediate in lung, liver, and heart, and low but detectable levels in aorta, kidney, intestine, and placenta. This distribution is similar to that reported for mouse PLTP, but shows some differences to the human mRNA distribution. A high expression level was detected in human placenta, and a low one in the brain (8). In general, the tissue-specific differences in the pig mRNA level were less pronounced than those reported for human and mouse PLTP mRNAs (8).

**Functional expression of the cDNA in COS-1 cells**

To verify that the cDNA encodes an active phospholipid transfer protein, the cDNA was transiently expressed in COS-1 cells. PLTP activity in the culture supernatants and cellular fractions was determined using an assay that measures PLTP-mediated phospholipid transfer from phosphatidylcholine vesicles to human HDL4 acceptor particles. Active PLTP was secreted in a time-dependent manner into the growth medium of cells transfected with the PLTP cDNA construct (Fig. 3A). The activity was first detected at 24 h post transfection and increased in a linear fashion up to the last (72 h) time point analyzed. At the 72 h time point approximately 60% of the total activity measured was found in the growth medium and 40% in the cellular fraction. The secreted recombinant PLTP was also detectable by Western blotting in the culture supernatant (Fig. 3B). The protein appeared as a multiple band in the molecular weight region of 75–83 kDa. This size is similar to the apparent molecular weight of PLTP purified from pig plasma, 81 kDa (11) which, however, runs as a single band. No significant activity was detected in the me-
Fig. 1.
Fig. 1. Nucleotide sequence of pig PLTP cDNA and the deduced amino acid sequence of the protein. A: The nucleotide sequence of pig PLTP cDNA. The start and stop codons are underlined, and the oligonucleotide primers mentioned in the text are marked in bold, the arrowheads underneath indicating their polarity. The primer 4 was biotinylated. Nucleotide 77 changed in the C22R mutant is boxed, and nucleotides differing between the pig and human sequences are indicated with dots. B: Alignment of the predicted amino acid sequences of pig (pPLTP), human (hPLTP), and mouse (mPLTP) PLTP. The numbers indicate amino acid residue position.
Fig. 2. Relative quantity of PLTP mRNA in various pig tissues. The mean value of three assays and the standard deviations are given.

Fig. 3. Functional expression of the PLTP cDNA in COS-1 cells. A. The pig PLTP cDNA was transfected into COS-1 cells as described. The phospholipid transfer activity in the culture supernatant (medium) and cells broken by sonication (cells) was determined at 24 h, 48 h, and 72 h. pSVL, cells transfected with the plain vector plasmid; mt, cells transfected with the mutant cDNA, C22R; wt, cells transfected with the wild-type cDNA. B. The secreted recombinant PLTP detected by Western blotting with monoclonal anti-PLTP antibody, G11. Equal volumes of the transfected cell culture supernatant were immunobotted from 12.5% SDS-PAGE. a: Cells transfected with the plain vector plasmid, 72 h. b–d: Cells transfected with the mutant cDNA, C22R, 24 h (b), 48 h (c), and 72 h (d). e–g: Cells transfected with the wild-type cDNA, 24 h (e), 48 h (f), and 72 h (g).
Fig. 4. Immunofluorescence microscopy analysis of the transfected COS-1 cells. The expressed PLTP was stained using the anti-pig PLTP MAb G11 and a TRITC-conjugated secondary antibody. A,B,C: cells transfected with the wild-type PLTP cDNA. D,E,F: cells transfected with the mutant type PLTP cDNA, C22R. Expression time: A and D, 24 h; B and E, 48 h; C and F, 72 h.

dium of cells transfected with the plain vector plasmid (mock-transfected cells). Large scale production, purification, and detailed functional characterization of recombinant PLTP are underway in the laboratory.

One of the cDNA clones isolated contained a PCR-derived mutation (nt 77, T → C transition) which changed the cysteine at amino acid position 22 to an arginine (see Fig. 1). To determine whether this cysteine residue is important for the correct folding and/or secretion of the protein, the mutant cDNA was expressed in COS cells along with the wild type one (Fig. 3). The mutant protein, denoted as PLTP C22R, also appeared in the culture medium, and displayed phospholipid transfer activity. However, the activity was significantly reduced throughout the time course when compared to the wild type protein. The lower amount of secreted PLTP C22R was also verified by Western blotting. No accumulation of active PLTP within the cells was observed. However, immunofluorescence microscopic analysis of the COS cells using the anti-pig PLTP MAb G11 (11) revealed that PLTP C22R accumulated within the secretory pathway membrane compartments to an extent clearly higher than that of the wild-type protein (Fig. 4). The staining consisted of reticular-appearing structures apparent already at the 24 h expression time point probably representing the endoplasmic reticulum. At later times large vacuolar-appearing elements with a very strong labeling appeared. By this technique the wild-type protein was rarely detectable in the cells at 24 h, and accumulation within the secretory pathway occurred more slowly.

DISCUSSION

We report here the isolation and characterization of cDNA encoding the pig plasma phospholipid transfer protein (PLTP). The deduced protein is highly similar to the previously identified human (93% identical) and
mouse (81% identical) homologue proteins. The main features distinguishing the pig protein from the human and mouse proteins are an insertion of three amino acids near the C-terminus and relative abundance of positively charged residues. The insertion may have functional relevance as the C-terminus is known to be crucial for the neutral lipid transfer of CETP (23), and phospholipid transfer activity of PLTP (J. Huuskonen, V. Olkkonen, and C. Ehnholm, unpublished data).

The PLTP mRNA was found to be expressed ubiquitously in adult pig tissues. The tissue-specific differences in the expression level were clearly less pronounced than those reported previously for the human and mouse PLTP mRNAs. This is probably due to the fact that mRNA quantitation was here performed by an RT-PCR/minisequencing technique, whereas the previous results are based on densitometric scanning of autoradiograms, which tends to exaggerate differences due to non-linear response of X-ray film to exposure. Interestingly, the highest expression level was detected in the pancreas. Whether PLTP is secreted with pancreatic fluid into the intestinal lumen, where it could be involved in phospholipid absorption, is an interesting subject of future work. High expression levels were also found in brain and lung. Brain tissue displays a high lipid content and rapid turnover of phosphatidylcholine and phosphatidylinositol (24). On the other hand, normal mature lung function depends on a constant supply of a phospholipid, dipalmitoylphosphatidylcholine, which is a constituent of pulmonary surfactant (25). PLTP might play a role in the vigorous phospholipid transport processes occurring in these tissues.

The cDNA was in COS cell expression experiments found to encode an active phospholipid transfer protein secreted to the growth medium. A mutant protein, PLTP C22R, was secreted less efficiently and tended to accumulate in the secretory pathway of the COS cells. This indicates that the cysteine at amino acid position 22 plays a role in the correct folding of the protein, perhaps by forming a disulfide bridge that facilitates the folding process.

PLTP displays two in vitro activities: it mediates phospholipid transfer between lipoproteins, and between liposomes and HDL (20) and, on the other hand, facilitates conversion of HDL particles (3, 4, 11). The kinetics of these two processes induced by PLTP are quite different; the phospholipid transfer can be detected in minutes, whereas the changes in HDL particle size require long incubations, 6–72 h (8, 26). Furthermore, the HDL apolipoprotein A-II/apoA-I molar ratio has a strong effect on the conversion process, while the phospholipid transfer is independent of this ratio (27). This indicates that the two activities of PLTP do not directly correlate. Our preliminary comparisons of the activities of recombinant and plasma PLTP support this notion. Large scale production and purification of recombinant PLTP will, in the future, provide us with the tools for elucidation of the detailed mechanisms of PLTP function.

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