# Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis

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**Abstract The plasma phospholipid transfer protein (PLTP) is an important regulator of high density lipoprotein (HDL) metabolism. We have here, based on sequence alignments of the plasma LPS-binding/lipid transfer protein family and the X-ray structure of the bactericidal/permeability increasing protein (BPI), modeled the structure of PLTP. The model predicts a two-domain architecture with conserved lipid-binding pockets consisting of apolar residues in each domain. By site-directed mutagenesis of selected amino acid residues and transient expression of the protein variants in HeLa cells, the pockets are shown to be essential for PLTP-mediated phospholipid transfer. A solid phase ligand binding assay was used to determine the HDL-binding ability of the mutants. The results suggest that the observed decreases in phospholipid transfer activity of the N-terminal pocket mutants cannot be attributed to altered HDL-binding, but the C-terminal lipid-binding pocket may be involved in the association of PLTP with HDL. Further, the essential structural role of a disulfide bridge between cysteine residues 146 and 185 is demonstrated. The structural model and the mutants characterized here provide powerful tools for the detailed analysis of the mechanisms of PLTP function.**—Huuskonen, J., G. Wohlfahrt, M. Jauhiainen, C. Ehnholm, O. Teleman, and V. M. Olkkonen. **Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis.** ***J. Lipid Res.*** **1999.** 40: **1123–1130.**

**Supplementary key words** HDL metabolism • lipid transfer proteins • homology modeling

A wealth of evidence shows that the risk for coronary heart disease is inversely correlated with the concentration of cholesterol in high density lipoproteins (HDL) (1, 2). One of the major hypotheses explaining this antiatherogenic effect is the ability of HDL to remove excess cellular cholesterol from peripheral cells for transport into the liver, a process called reverse cholesterol transport (3). A subpopulation of HDL, preβ-HDL, is thought to act as the initial acceptor of excess cellular cholesterol (4). Recent studies using transgenic mice (5, 6) and adenoviral overexpression of phospholipid transfer protein (PLTP) (7, 8) have clearly demonstrated that this plasma protein has a marked effect on HDL metabolism. Decrease in plasma total HDL, enhanced clearance of HDL phospholipids, cholesteryl esters, and apolipoprotein A-I (apoA-I), and accelerated regeneration of lipid-poor apoA-I (preβ-HDL) have been attributed to overexpressed PLTP. The ability of PLTP to generate preβ in vitro (9, 10) and in vivo (5, 7) has focused the attention on this potentially antiatherogenic plasma factor.

Besides being able to modulate the size and composition of HDL particles, PLTP is able in vitro to transfer phospholipids (11, 12) and other lipid-like substances among lipoprotein particles (13–15). The cloning of the PLTP cDNA (16) made it possible to address in detail the structure–function relationships of the protein. Recently we have characterized the biosynthesis and secretion of PLTP using stably transfected HeLa cells (17). The major secreted forms of PLTP carry complex N-glycans which are crucial for efficient secretion. Furthermore, DTT treatment of the transfected cells caused a reversible arrest of PLTP secretion, suggesting a role of disulfide bonds in the correct folding of the polypeptide. Analysis of C-terminally truncated PLTP variants demonstrated the importance of sequences in the vicinity of the C-terminus for secretion as well as phospholipid transfer activity of the protein (17).

PLTP belongs to the family of plasma LPS-binding/lipid transfer proteins, together with cholesteryl ester

Abbreviations: BPI, bactericidal/permeability increasing protein; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LBP, lipopolysaccharide binding protein; PC, phosphatidylcholine; PLTP, phospholipid transfer protein.

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transfer protein (CETP), lipopolysaccharide binding protein (LBP), and bactericidal/permeability increasing protein (BPI) (16). The 3-D structure of BPI has recently been determined by X-ray crystallography (18). Based on this structure, a molecular model of LBP has been constructed (19). These proteins are suggested to have a boomerang-shaped structure consisting of two domains, both of which contain an apolar lipid-binding pocket. In the present study we have used sequence alignments of the entire protein family and the BPI crystal structure to build a molecular model of PLTP. To study key features of this model we designed a set of point mutations and expressed the protein variants in HeLa cells. The results demonstrate the presence of an essential sulfur bridge between cysteine residues 146 and 185 and the importance of the conserved lipid-binding pockets for the phospholipid transfer activity of PLTP.

## MATERIALS AND METHODS

### Modeling of the human PLTP structure

The 13 published amino acid sequences of mammalian PLTP, BPI, LBP, and CETP were aligned with CLUSTALW 1.7 (20) in order to identify conserved sequence regions. The accession numbers of the sequences in the Swiss Prot database are: PLTP human P55058, mouse P55065, pig L78843; LBP mouse Q61805, rat Q63313, human P18428, rabbit P17454; CETP human P11597, macaque P47896, rabbit P22687; BPI human P17213, bovine P17453, rabbit Q28739. Also, a multiple alignment based on a hidden Markov model (HMM) (21) was made with the HMMER program suite by S. R. Eddy. This showed only minor differences from the CLUSTALW alignment. Based on the HMM alignment and the X-ray structure of human BPI (18), which is the only structure in the Brookhaven Protein Data Bank (PDB) (22) that is homologous to PLTP, a molecular model of PLTP was constructed with the homology module of the InsightII program package (23). The sequence alignment contains one region with a five amino acid deletion and three one amino acid deletions. For the model construction three deletions were shifted manually for one or two positions to move them out of the secondary structure regions in BPI (Fig. 1). The backbone structure for the deletion regions was generated by searching the PDB for parts of proteins whose alpha carbon distances best fit into the model. The C-terminus (residues 465–493) extends beyond the BPI sequence and is therefore not included in the model. The side chains in the BPI structure were changed to the corresponding residues in PLTP and orientations of the new side chains were optimized by the method suggested by Novotny (24). The minor steric clashes and bond strain due to building the new loops were regularized by energy minimization and 10-ps molecular dynamics runs for the loop regions with the AMBER program (25). The resulting structure was refined by energy minimization (with all coordinates free). The all-atom force field calculations were performed with a 10 Å non-bonded cutoff and a distance-dependant dielectric constant. Analysis of the final model using the ProStat tools of InsightII suggested a typical distribution of torsional angles and only W89 has disallowed Ramachandran angles as for the corresponding R96 in BPI. Two phosphatidylcholine molecules in the conformation observed in BPI were fitted to the proposed lipid-binding pockets and the complex structure was energy minimized.

### Site-directed mutagenesis and expression of PLTP

Mutations in the full-length human PLTP cDNA were generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions, and verified by dideoxy sequencing using the Sequenase 2.0 kit (United States Biochemicals). The constructs in pTRE (Clontech) were transfected using the Superfect reagent (Qiagen) into HeLa tet-on cells (Clontech) seeded on 6 cm culture dishes 1 day before transfection. The cells were thereafter incubated for 72 h in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mm l-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) in the presence of 2 μg/ml of doxycyclin (inducing conditions). The culture medium was harvested and the detached cells were removed by centrifugation at 500 *g* for 5 min at 4°C. A stably transfected HeLa-PLTP cell line (17) was used to produce PLTP containing medium for setting up the HDL binding assay.

### Specific phospholipid transfer activity of PLTP mutants

Phospholipid transfer activity in culture supernatants was determined by incubating 10 μl of medium together with $^{14}$C-labeled dipalmitoyl-PC donor vesicles and acceptor $HDL_3$ (26). The relative mass of secreted PLTP mutants was determined by precipitating the total protein in the transfected HeLa medium with acetone, followed by SDS-PAGE and Western blotting using a polyclonal anti-PLTP antibody R180 raised against a peptide comprising amino acid residues 470–493 of the protein. Thus, the epitopes recognized by the antibody do not coincide with any of the amino acid residues mutated in this study. The bound antibodies were detected with $^{35}$S-labeled protein A (Amersham Pharmacia Biotech) and quantitated with the Fujifilm BAS-1500 Imaging system. Mutant F464E was not recognized by the antibody R180 and hence was quantitated using the monoclonal antibody JH59 (17), goat anti-mouse IgG (Bio-Rad), Enhanced ChemiLuminiscence detection system (Amersham), and densitometric scanning with the Millipore Bio Image equipment. The specific activity is expressed relative to that of wild-type PLTP and was obtained by dividing the phospholipid transfer activity by the PLTP mass.

### Binding of PLTP to $HDL_3$

$HDL_3$ was isolated from human plasma by sequential ultracentrifugation (27). A solid-phase ligand-binding assay described in (28) was modified as follows. Nunc-Immuno 96-well plates were coated for 2 h with $HDL_3$ (20 μg HDL protein/well) diluted in PBS. Post-coating was performed with 1% fat-free milk powder (Valio, Finland) in PBS for 1 h. In order to bind sufficient amounts of recombinant PLTP in the wells, five consecutive aliquots of culture medium were applied in the wells, with 30 min incubation between the applications. To reliably determine the binding of the individual PLTP mutants to HDL, we used five different total amounts of each transfection culture medium (200, 400, 600, 800, 1000 μl; diluted with mock-medium to final total volume of 1.0 ml). The bound PLTP was detected with 1 μg/well of monoclonal antibody JH59 and horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). Formation of *o*-phenylenediamine chromogenic product was measured at $A_{492}$ nm. The antibodies were diluted with 0.5% milk/PBS. All incubations were carried out at 37°C and the wells were washed 6 times with PBS between the incubations. To determine the HDL binding relative to the wild-type PLTP, the mean of relative absorbance values from four different applied amounts (within the linear range of the binding curve) was used. The results were normalized for the mass of secreted PLTP.

## RESULTS

### Sequence alignments and the structural model

The structure of PLTP was modeled using sequence alignments of the plasma LPS-binding/lipid transfer protein family and the known crystal structure of human BPI (18). For the alignment, the sequences of PLTP (3 species), LBP (4 species), BPI (3 species), and CETP (3 species) were used. The sequence identity of human BPI and PLTP in the HMM based alignment (**Fig. 1**) is 24% and the overall homology is 51%, using only the groups of the Gonnet Pam250 matrix which score higher than 0.5 (29). The sequence identity of the 35 residues whose side chains are in close contact (4 Å) with the phospholipid in the N-terminal pocket of BPI is 31% (homology 74%) and for the 32 residues in the C-terminal pocket 34% (homology 66%). The higher conservation of the amino acids in the proposed lipid-binding pockets is an indication that they are probably conserved in structural terms.

A schematic representation of the modeled PLTP structure is shown in **Fig. 2**. The model predicts a boomerang-shaped, two-domain structure. At each end of the protein there are barrel-type structural units, and a central β-sheet forms an interface between these units. Each domain contains a predicted lipid-binding pocket. As expected from a model based on homology, larger differences between the backbone of BPI and the PLTP model occur only in the newly introduced loops which represent the regions with

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PLTP     18      EFPGCKIRVTSKALELVKQEGLRFLEQELETITIPDLRGKE-----GHFY
BPI              vnpGVvvriSqkGLdyaSqqgtaalqkeLkrIkIpdysdsfkikhlgkgh
                     bbbbbbaaaaaaaaaaaaaaaaaa        bbbbbbb   bbbb

PLTP     63      YNISEVKVTELQLTSSELDFQPQQELMLQITNASLGLRFRRQLLYWFFYD
BPI              YsFysMdIreFqLpsSqIsMvpnvglkfsIsnAnIkIsgkWkAqkrflkm
                 bbbbbbbbbbbb    bbbbbb    bbbbbbbbbbbbbbbbbbbbb  bbb

PLTP    113      GGYINASAEGVSIRTGLELSRDP-AGRMKVSNVSCQASVSRMHAAFGGT-
BPI              sgnFdLsIegMsIsAdlklgsnptsgkptItCssÇsShInsVhVhIsksk
                 bbbbbbbbbbbbbbbbbbbbb       bbbbbbbbb     bbbbb

PLTP    161      FKKVYDFLSTFITSGMRFLLNQQICPVLYHAGTVLLNSLLDTVPVRSSVD
BPI              VgwLiqlFhkkIesaLrnkMnsqVçekVtnsvssklqpyfqtlpvmtkId
                  aaaaaaaaaa aaaaaaaaaaaaaaaaaaaaaaaaaaa      bbb

PLTP    211      ELVGIDYSLMKDPVASTSNLDMDFRGAFFPLTERNWSLPNRAVEPQLQEE
BPI              svAGInYgLvapPaTtaeTLdvqMkGeFysenhhnpppfaPpvMeFpaah
                   bbbb        bb    bbbbbb  bbbb

PLTP    261      ERMVYVAFSEFFFDSAMESYFRAGALQLLLVGDKVPHDLDMLLRATYFGS
BPI              drmvyLGLSdyFfnTaglvyqeagvlkmtLrddmIpkeskfrLtTkfFgt
                    bbbbbbaaaaaaaaaaaaa     bbbb 333           aaaa

PLTP    311      -IVLLSPAVIDSPLKLELRVLAPPRCTIKPSGTTISVTASVTIALVPPDQ
BPI              fLpeVakkfpnmkIqIhvsastpphlsVqptgLtfypavdVqAfAvlpns
                     aaaa      bbbbbbb     bbbbb  bbbbb bbbbbbbbb

PLTP    360      PEVQLSSMTMDARLSAKMALRGKALRTQLDLRRFRIYSNHSALESLALIP
BPI              alasLFlIgMhTtgsMevsAesnrLvgeLkldrLlLelkhSnIgpfpvel
                  bbbbbbbbbb  bbbbbbbb  bbbbbbbb       bbbb      333

PLTP    410      LQAPLKTMLQIGVMPMLNERTWRGVQIPLPEGINFVHEVVTNHAGFLTIG
BPI              LqdIMnyIvpilvlprvneklqkGfpLPtparvqLynvvlqphqnFLLFG
                 3aaaaaaaaaaaaaaaaaaaaa bb       bbbbbbbbbbb  bbbbb

PLTP    460      ADLHFAKGLREVIEKNRPADVRASTAPTPSTAAV
BPI              Advvyk
                 bbbbb
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**Fig. 1.** The sequence alignment of human PLTP and BPI used for the construction of the homology model (part of the complete HMM alignment with 13 members fo the LPS-binding/lipid transfer protein family; see Materials and Methods). The alignment is annotated with BPI's three-dimensional structural features calculated with the JOY program (39). Key: UPPER CASE in BPI sequence = solvent inaccessible residues; lower case in BPI sequence = solvent accessible residues; aaa = α-helix; bbb = β-strand; 333 = $3_{10}$-helix; Ç = disulfide bond in BPI. The C-terminal tail of PLTP, which is not included in the modeling, is underlined. The numbering refers to PLTP amino acid residues. The residues mutated in this study are indicated by dots above the corresponding PLTP amino acids.

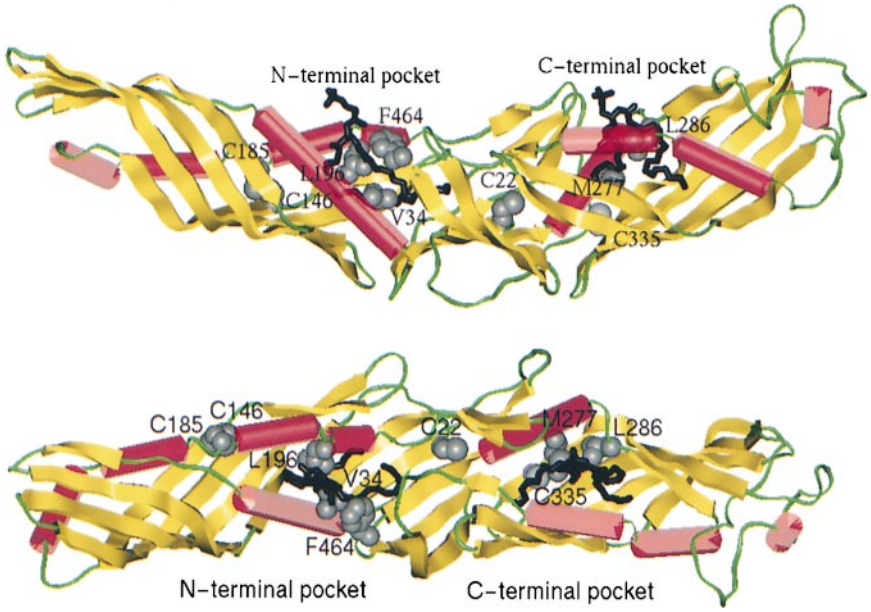


**Fig. 2.** A schematic representation of the structural model of human PLTP. Two projections are shown; rotation angle between the images is 90° around the x-axis. The α helices are shown as transparent red cylinders, the β strands as yellow arrows, and the phosphatidylcholine molecules as black sticks. The side chains of the amino acids mutated here and C22 are shown as grey balls. The illustrations were created with the InsightII program package (23). The coordinates of the atoms in the model are available via electronic mail from Vesa.Olkkonen@ktl.fi upon request.

gaps in the sequence alignment (Fig. 1). They are situated at the left and right ends of the structure shown in Fig. 2 at a distance of about 25 Å from the binding pockets which are investigated in this study. As in BPI, only apolar side chains form the inside of the pockets while polar residues are found near their entrances. A single disulfide bond between C146 and C185, which are totally conserved in the LPS-binding/lipid transfer protein family, is found in an identical position as in the BPI structure. The alpha carbons of C22 and C335, which are only found in PLTP, are separated by a distance of 16 Å in the structural model.

To probe the function of the lipid binding pockets, we planned a series of site-directed mutations which should block the lipid binding without affecting the proper fold of the protein (**Table 1**, Figs. 1 and 2). In the N-terminal pocket we changed V34 and L196 to bulky tryptophans, which can be accommodated by the apolar environment but do not leave enough space for the fatty acyl chains, and F464 to aspartate, which should disturb the interaction with non-polar parts of the lipids. V34 is situated internally in the pocket, while L196 and F464 are at the entrance more exposed to the solvent. In the C-terminal pocket, C335 was mutated to serine and M277 to tryptophan. Both are situated inside the proposed pocket. L286 situated close to the entrance of this pocket was changed to tryptophan. To study the importance of the disulfide bridge, C146 and C185 were mutated to serines. The consequences of these mutations were compared to that of a similar mutation in C335, a residue not involved in disulfide bonding.

TABLE 1. Site-directed mutations generated in PLTP

| Mutation | Predicted Target |
|---|---|
| V34W | N-terminal pocket |
| L196W | N-terminal pocket |
| F464E | N-terminal pocket |
| M277W | C-terminal pocket |
| L286W | C-terminal pocket |
| C335S | C-terminal pocket |
| C146S | disulfide bond |
| C185S | disulfide bond |
| V34W+M277W | |
| V34W+L286W | |
| V34W+C335S | |
| L196W+M277W | |

### Secretion and phospholipid transfer activity of the PLTP mutants

Wild-type PLTP and the mutants were transiently expressed in HeLa cells. All the mutants, except C146S and C185S, were secreted by the cells at levels comparable to that of the wild-type protein (**Fig. 3**). Secretion of the PLTP variants was efficient: 80% of the total PLTP activity (in growth medium + sonicated cell lysate) in each case was recovered in the culture medium (data not shown). This indicates that the point mutations did not severely affect the folding and secretion competence of PLTP. The C146S and C185S mutants were not detectable in the culture medium but were synthesized and remained intracellular as judged from immunofluorescence microscopic observation (data not shown). This suggests an important

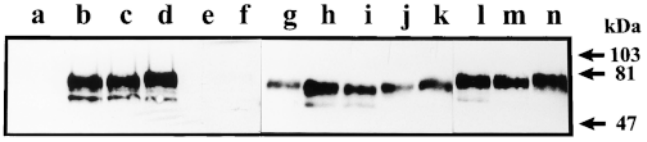


**Fig. 3.** Secretion of PLTP variants into HeLa cell growth medium. HeLa cells were transfected with the PLTP constructs, the growth medium (at 72 h post-transfection) was acetone-precipitated and run on 10% SDS-PAGE. The proteins were Western blotted using a monoclonal anti-PLTP antibody and Enhanced ChemiLuminescence system. The lanes are: (a) medium of mock-transfected cells, (b) wild-type PLTP, (c) V34W, (d) F464E, (e) C146S, (f ) C185S, (g) L196W, (h) M277W, (i) C335S, ( j) L286W, (k) L196W+M277W, (l) V34W+C355S, (m) V34W+M277W, (n) V34W+L286W. Molecular weight markers are indicated on the right.

structural role for the disulfide bridge formed by these cysteine residues as predicted by the model. In contrast, mutating C335 that does not participate in disulphide bond formation, did not affect the secretion process.

The mutations under study had a wide range of effects on the specific phospholipid transfer activity of PLTP (**Fig. 4**). All three mutations affecting the N-terminal lipid binding pocket (V34W, L196W, and F464E) severely impaired the activity, resulting in 65–85% inhibition of phosphatidylcholine transfer. Two of the mutations in the C-terminal lipid binding pocket (M277W and C335S) had a modest effect (20–40% inhibition), whereas mutation L286W almost totally abolished the transfer. These results strongly indicate that both lipid-binding pockets play an important role in PLTP-mediated phospholipid transfer, the N-terminal pocket possibly having a more central role in the process. To elucidate the functional relationship of the two lipid-binding pockets, double mutants with amino acid substitutions in both pockets were constructed (Table 1). In all cases the specific activity of the double mutants was slightly lower than that of the single mutation having a more severe inhibitory effect on phospholipid transfer (Fig. 4). The mean activity values obtained for the four double mutants (as a group) do not significantly differ from those expected for a multiplicative inhibitory effect, i.e., the specific activities of the double mutants are close to the products of the activities displayed by the individual mutants.

### Binding of PLTP mutants to $HDL_3$

In order to determine the relationship of the specific PC transfer activity of the PLTP variants and their binding to HDL, we set up a ligand-binding assay on microtiter plates. Incubation of increasing amounts of PLTP containing HeLa cell medium on HDL-coated wells resulted in a linear, specific increase of absorbance, demonstrating the binding of PLTP to $HDL_3$ (**Fig. 5A**). The three proteins carrying mutations in the N-terminal pocket (V34W, L196W, F464E), which all showed a marked decrease in the transfer activity, behaved differently in the HDL binding assay: V34W displayed a modest reduction in binding compared to wild type PLTP, L196W showed no change in binding, whereas F464E displayed slightly increased HDL binding (Fig. 5B). Of the C-terminal pocket mutations with only a modest defect in PC transfer, M277W showed a mild reduction in HDL binding, while C335S displayed inhibition of approximately 30%. In contrast, the L286W mutant that has a very low PC transfer activity also displayed a drastic defect in binding to HDL. The binding efficiencies of the C-terminal pocket mutants thus displayed a pattern remarkably similar to their specific PC transfer activities.

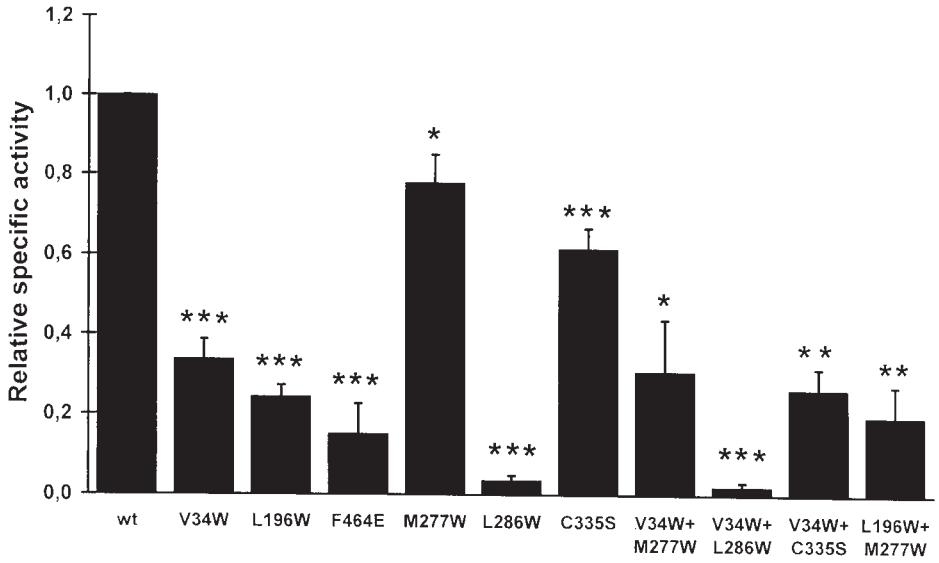


**Fig. 4.** Specific PC-transfer activity of PLTP mutants. HeLa cells were transfected with the PLTP cDNA constructs (indicated at the bottom; wt = wild-type). At 72 h post-transfection, phospholipid transfer activity in the serum-free growth medium was measured, and the relative quantity of the secreted PLTP was determined by Western blotting using anti-PLTP R180, detection with $^{35}$S-protein A, and the Fujifilm BAS-1500 Imaging System. The results are mean values (±SEM) of 5–8 (single mutants) or 3 (double mutants) independent experiments, and are expressed relative to the activity of the wild-type protein (set at 1.0). The asterisks indicate a statistically significant difference (*t*-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$) from the wild-type PLTP.

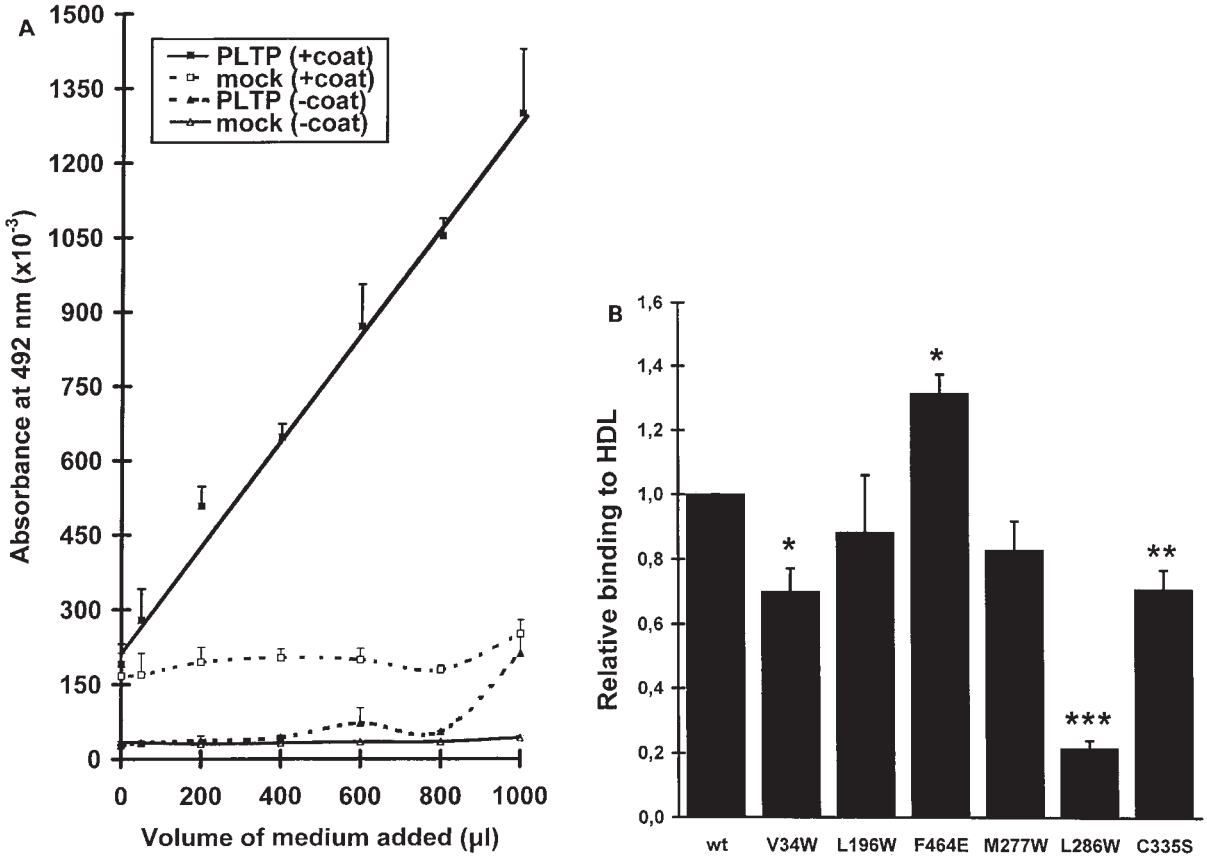


**Fig. 5.** Binding of PLTP to immobilized $HDL_3$. Interaction of PLTP secreted into HeLa cell growth medium with HDL immobilized on Nunc-Immuno 96 plates was determined as specified in Materials and Methods. A: Validation of the binding assay. Growth medium of untransfected HeLa cells (mock) or stably transfected HeLa-PLTP cells (PLTP; (17)) was applied in either non-coated (−coat) or $HDL_3$-coated (+coat) wells. The absorbance values obtained are plotted against the growth medium volume. The results are mean values (±SEM) of three independent experiments. B: Binding of PLTP mutants (identified at bottom; wt = wild-type) to HDL. The results are mean values (±SEM) of 3 independent experiments, and are expressed relative to the binding of the wild-type protein (set at 1.0). The asterisks indicate a statistically significant difference (*t*-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$) from the wild-type PLTP.

## DISCUSSION

In this study we have used sequence alignments of the plasma LPS-binding/lipid transfer protein family and the BPI crystal structure to build a model of human PLTP structure. The central features of the model were studied by site-specific mutagenesis and transient expression of the protein variants in HeLa cells. The model predicts a two-domain structure with hydrophobic lipid-binding pockets in each domain. A structurally important disulfide bridge is predicted to be present between cysteines 146 and 185, which are fully conserved in the entire protein family. Mutating these residues led to a complete secretion arrest of the protein variants, confirming the essential role of the predicted disulfide bridge. We have previously reported a reversible arrest of PLTP secretion upon treatment of cells with the reducing agent DTT (17), which agrees perfectly with the present results. Further, mutagenesis studies on the related BPI have suggested disulfide bonding between the corresponding cysteines 135 and 175 (30, 31).

The C-terminus (residues 465–493) of PLTP extends beyond the sequence of BPI, which forms the basis for the modeling, and was therefore excluded from the model. We have previously shown that deletion of up to 30 amino acids from the PLTP C-terminus (464–493) does not affect secretion of the protein (17). Therefore, we assume that the C-terminal tail does not have an essential role in formation of the general fold of PLTP. However, we have suggested the importance of aa residues 464–468, and especially the conserved F464, for the phospholipid transfer activity of PLTP (17). The present data confirm the essential role of F464 located at the entrance of the N-terminal lipid-binding pocket. Interestingly, the R180 antiserum raised against the amino acids 470–493 failed to recognize the F464E mutant protein on Western blots. This may be due to a structural change in the C-terminal tail of PLTP caused by the mutation, leading to increased protease susceptibility. Therefore, F464 may be essential in maintaining the proper conformation of the C-terminal tail of PLTP.

Site-specific mutagenesis of residues forming the lipid-binding pockets demonstrates the significance of the two pockets for the phospholipid transfer activity of PLTP. Both of the pockets are functionally important, the N-terminal one probably playing a more central role in the transfer

process. Data supporting this interpretation have been reported for the related BPI, the N-terminal domain of which retains the bactericidal, LPS-binding and -neutralizing activities of the intact protein (31–33). In order to decipher the functional relationship between the N- and C-terminal pockets, the specific transfer activity of double mutants with substitutions in both pockets was analyzed. The inhibitory effects of the double mutations were not simply the arithmetic sum of the effects of the corresponding single substitutions, but rather followed a multiplicative pattern, suggesting that the two pockets do not act independently but are functionally interconnected in the phospholipid transfer process.

PLTP is known to associate with the plasma HDL fraction (34, 35). Further, HDL appears to be the most efficient phospholipid donor and acceptor in PLTP-mediated transfer reactions between lipoprotein particles (12). We therefore assessed the relationship between phospholipid transfer activity of the PLTP variants and their binding to acceptor HDL particles. There was no positive correlation between the PC transfer activity and HDL binding of the N-terminal pocket mutants. However, these two parameters showed a remarkably similar pattern in the C-terminal pocket mutants, indicating that this pocket may play an important role in the HDL-PLTP interaction. The L286W mutant, which has a very low PC transfer activity, displayed drastically reduced HDL interaction. Leucine 286 localizes at the entrance of the C-terminal pocket, on the concave surface of the boomerang-shaped molecule. It is unlikely that this mutation would cause a major distortion in the structure of PLTP. The results therefore also indicate that the concave surface where the lipid-binding pockets open is involved in forming the contact surface with HDL.

The exact mechanism of PLTP-mediated phospholipid transfer is currently under debate. A carrier-type transfer mechanism has been suggested both for PLTP (15) and the related CETP (36, 37). On the other hand, the wide substrate specificity spectrum of PLTP (11, 12) and the apparent absence of a stable PLTP–phospholipid intermediate (11) argue against a traditional carrier mechanism. The present data on the role of the lipid-binding pockets does not allow detailed mathematical analysis of the mechanism of PLTP-mediated phospholipid transfer but does provide essential new information on the structure–function relationships of the protein. There are several alternative functional hypotheses that may be compatible with the specific transfer activity data on the single and double mutants. *i*) Both of the lipid-binding pockets could be directly involved in binding the substrate phospholipid to be transferred. It is possible that binding of PL in one pocket modifies the interaction of lipid substrate with the other site. Alternatively, the phospholipid substrate could pass through both pockets sequentially. *ii*) One of the pockets could be involved in the interaction of PLTP with HDL, while the other accommodates the lipid to be transferred. The pockets have different functions and interfering with either function is sufficient to disturb the transfer process. The C-terminal lipid-binding pocket could be envisioned to be important for the interaction of PLTP with HDL, whereas the N-terminal lipid-binding pocket could accommodate the substrate lipid to be transferred. *iii*) The present data do not necessarily prove that the substrate phospholipid transferred is accommodated in the predicted lipid-binding pockets. It is possible that the bound lipids play a merely structural role. Inhibition of lipid insertion into the pockets could cause minor changes in the fold of the protein or its molecular interactions with HDL apolipoproteins (38) or phospholipids (15), causing the observed differences in phospholipid transfer activity. In this model PLTP would facilitate the transfer of phospholipids from the donor to the acceptor by bringing these particles in close contact.

The structural model generated in the present study forms a firm starting point for the elucidation of the structure–function relationships in PLTP, an important regulator of HDL metabolism. The involvement of the lipid-binding pockets in substrate transfer processes is documented here for the first time in the LPS-binding/lipid transfer protein family. The mutants characterized in this study will in the future be subjected to large scale production/purification to be used as tools in deciphering the precise mechanism of PLTP-mediated phospholipid transfer and HDL conversion.

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