Biosynthesis and secretion of human plasma phospholipid transfer protein

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Abstract **Plasma phospholipid transfer protein (PLTP) plays a critical role in lipoprotein metabolism and reverse cholesterol transport. We have studied the biosynthesis and secretion of PLTP using a stably transfected inducible HeLa cell line. Pulse-chase analysis revealed that: *i*) the major secreted forms of PLTP carry complex N-glycans; *ii*) N-glycosylation is crucial for PLTP secretion; *iii*) Endo H-resistant forms of PLTP could not be enriched using a 20°C temperature block, indicating that the transport of PLTP from the endoplasmic reticulum to the Golgi apparatus is exceptionally sensitive to low temperatures; and *iv*) treatment of the PLTP-producing cells with the reducing agent dithiothreitol caused a reversible secretion arrest, suggesting a role of disulfide bonds in the correct folding of PLTP. Transient expression of C-terminally truncated PLTP variants in COS cells demonstrated that: *i*) the 30 C-terminal amino acids are dispensable for PLTP secretion, whereas deletion of 35–50 residues results in a complete absence of secretion; and *ii*) the deletion of 30 C-terminal amino acid residues almost completely abolished the phospholipid transfer activity of PLTP. The present study describes for the first time the biosynthesis of phospholipid transfer protein and provides tools for detailed elucidation of the structure–function relationships in the protein.—**Huuskonen, J., M. Jauhiainen, C. Ehnholm, and V. M. Olkkonen. **Biosynthesis and secretion of human plasma phospholipid transfer protein. *J. Lipid Res.* 1998.** 39: **2021–2030.**

Supplementary key words HDL metabolism • lipid transfer • PLTP • protein secretion

The risk for coronary heart disease is inversely correlated with the concentration of cholesterol in plasma high density lipoproteins (HDL) (1, 2). One of the major hypotheses explaining the beneficial role of HDL is its involvement in reverse cholesterol transport (RCT), a process by which excess cholesterol is removed from peripheral cells and transported to the liver for excretion (3). A subpopulation of HDL particles, denoted pre-β-HDL, are thought to act as the primary acceptors of cellular cholesterol in RCT (4). The human plasma phospholipid transfer protein (PLTP) is suggested to play an important role in the generation of these cholesterol acceptor particles (5–7).

In vitro, PLTP facilitates the transfer of several lipids and lipid-like substances between lipoprotein particles (8–12). In addition, PLTP modulates the size of HDL particles. In this process, termed HDL conversion, human HDL is converted to novel populations of larger particles and smaller pre-β-type HDL (5, 13–15). Although PLTP was already isolated from plasma in the 1980s (16, 17), even today little is known of the basic structure–function relationships involved in its activities. The cloning of the cDNA (18) opened new possibilities to study the molecular biology of PLTP. It also revealed that PLTP displays sequence homology to several plasma lipid binding/transfer proteins: cholesteryl ester transfer protein (CETP), lipopolysaccharide binding protein (LBP), and bactericidal/permeability increasing protein (BPI). Most studies on recombinant PLTP have so far concentrated on confirming the activities observed for the purified plasma protein (8, 9, 19, 20), whereas the biosynthesis and the structural characteristics of the protein have not been approached experimentally. The physiological importance of PLTP in the modulation of plasma HDL populations has been highlighted in recent studies using transgenic mice (7, 21) and adenoviral expression of PLTP (22, 23). Overexpression of the protein resulted in a marked decrease in plasma HDL, enhanced the clearance of HDL phospholipids, cholesteryl esters, and apolipoprotein A-I (apoA-I), and accelerated the regeneration of lipid-poor apoA-I, an efficient acceptor of cellular cholesterol. These studies have convincingly demonstrated that PLTP plays a critical role in HDL metabolism and reverse cholesterol transport.

PLTP is expressed in a wide range of tissues and secreted into the circulation (18). Therefore, its biosynthesis and secretion are processes that deserve special atten-

Abbreviations: apoA-I, apolipoprotein A-I; BPI, bactericidal/permeability increasing protein; CETP, cholesteryl ester transfer protein; DTT, dithiothreitol; ECL, enhanced chemiluminiscence; Endo H, endoglycosidase H; ER, endoplasmic reticulum; HDL, high-density lipoprotein; LBP, lipopolysaccharide binding protein; mAb, monoclonal antibody; NEM, N-ethylmaleimide; PC, phosphatidylcholine; PNGase F, peptide: N-glycosidase F; PLTP, phospholipid transfer protein; RCT, reverse cholesterol transport.

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tion when considering the fundamental parameters affecting the PLTP and HDL levels in the plasma. In this study we report the characterization of PLTP biosynthesis and secretion in a stably transfected HeLa cell line that produces the protein in an inducible manner. Pulse-chase analysis is used to analyze the role of N-glycans and putative disulfide bridges in the secretion process. The importance of the PLTP carboxyterminal portion for the secretion and specific activity of the protein is elucidated by transient expression of truncated protein variants in COS cells.

MATERIALS AND METHODS

Cell culture and selection of stable transfectants

HeLa tet-on cells (Clontech) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 μg/ml geneticin (G418; GibcoBRL), 2 mm l-glutamine, streptomycin (100 μg/ml), and penicillin (100 IU/ml). Stable transfectants where generated by co-transfecting the cells with pTRE carrying human PLTP cDNA and the selection plasmid pPUR (Clontech), followed by selection with puromycin (0.2 μg/ml, Sigma). To induce PLTP production, 1.5×10^6 cells were seeded on 6-cm dishes 1 day before induction, which was carried out by adding 2 μg/ml of doxycyclin (Sigma). COS-1 cells were grown in DMEM supplemented with 10% FBS, l-glutamine, streptomycin, and penicillin. Cells (1.2×10^6) were seeded on 6-cm culture dishes the day before transfection. The cells were transfected using the Superfect reagent (Qiagen) according to the manufacturer's protocol, and grown in serum-free medium.

Antibodies

Polyclonal antibody R176 against the carboxyl terminus of PLTP (amino acids 425–493) and antibody R164 against the amino terminus (amino acids 18–144) were produced by immunizing New Zealand White rabbits with glutathione-S-transferase (GST) fusion proteins expressed in *E. coli.* The insoluble proteins were purified by preparative SDS-PAGE, solubilized in 0.1% SDS/PBS and injected to rabbits. Monoclonal antibodies JH59 and JH66 were obtained by injecting Balb/c mice with a full-length recombinant PLTP produced in *E. coli*. Hybridomas were generated essentially as described in (24, 25), cloned twice by limiting dilution, and passaged in Pristane-primed Balb/c mice for ascites production. The immunoglobulins were purified on either protein A (for polyclonal) or protein G (for monoclonal antibodies) Sepharose columns (Pharmacia) according to the manufacturer's instructions. The rabbit polyclonal antibody against influenza virus hemagglutinin was a gift from Dr. Ari Ora (Haartman Institute, University of Helsinki).

Metabolic labeling and immunoprecipitation

The cells cultured on 6-cm dishes were labeled 24 h after the induction (HeLa) or 48 h after transfection (COS-1). The cells were incubated with methionine- and cysteine-free medium for 1 h at 37°C, and labeling was routinely carried out by the addition of 140 μCi of Redivue Pro [^{35}S]methionine/cysteine labeling mix (Amersham) for 30 min. The labeling medium was removed, and chase was carried out by adding fresh medium containing 300 μg/ml of l-methionine and l-cystine. Doxycyclin was present in HeLa cultures throughout the protocol. Before the immunoprecipitations, the culture medium was centrifuged at 500 *g* for 5 min at 4°C in order to remove detached cells, and 1% Triton X-100 (Tx-100) was added. The cells were washed twice with ice-cold PBS and lysed in 10 mm HEPES, pH 7.4, 1 mm $MgCl_2$, 0.1 mm EGTA, 140 mm KCl, and 1% Tx-100 (RIP-buffer). The cellular debris was removed by centrifugation at 15 000 *g* for 10 min. A mixture of protease inhibitors, 5 μg/ml of chymostatin, leupeptin, antipain, and pepstatin A (CLAP) was added to both the medium and the cells to prevent proteolysis. For the immunoprecipitations, 770 μl of growth medium or 700 μl of cell suspension (representing half of the total material from one 6-cm plate) was used. The specimens were incubated overnight at 4°C together with 40 μl of tosyl-activated Dynabeads (Dynal A.S., Norway) carrying 4 μg of coupled polyclonal antibody R176 directed towards the C-terminus of PLTP. After incubation, the immunoprecipitates were washed once with 1 ml of 1% TX-100 in PBS and twice with 500 μl of PBS. Finally, the precipitates were suspended in 30 μl of SDS-sample buffer, boiled for 5 min, and resolved by SDS-PAGE (10%). The gels were fixed for 30 min in 10% acetic acid, incubated in Amplify™ (Amersham) for 30 min, dried, and exposed on X-ray film (Kodak). Quantitation of the results was carried out with the Millipore Bio Image equipment. Immunoprecipitation of PLTP purified from human plasma (15) was carried out as above. Infection of HeLa-PLTP cells with influenza N-virus, metabolic labeling, and immunoprecipitation of the viral hemagglutinin were carried out essentially as described previously (26).

In vitro translation

The full-length human PLTP cDNA in the pGEM3Zf(+) (8) was in vitro transcribed/translated in the presence of [^{35}S] methionine using the TNT T7 coupled transcription/translation kit (Promega) according to the manufacturer's instructions.

Western blotting

For Western blotting, the proteins were resolved by 10% SDS-PAGE and electrotransferred to Hybond-C membranes (Amersham), which were probed with the mAb JH59 or the polyclonal antibody R164. The bound primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse IgG (Nordic Immunology, the Netherlands) or [^{35}S]-Protein A (Amersham).

Glycosidase digestions

Endo H and PNGase F were obtained from New England Biolabs and O-glycosidase and neuraminidase were from Boehringer Mannheim. Digestions of immunoprecipitates were performed according to the manufacturer's instructions with 500 U of EndoH, 1000 U of PNGase F, 2 mU of O-glycosidase or 20 mU of neuraminidase per sample. All digestions were carried out for 20 h at 37°C. After this, the proteins were analyzed by SDS-PAGE as above.

Tunicamycin and DTT treatments

Tunicamycin (5 μg/ml, Sigma) was added to induced HeLa-PLTP cells during the methionine/cysteine depletion step (at 1 h before labeling) and was present throughout the labeling and chase periods. Dithiothreitol (DTT, 2 mm, Sigma) was added to the cells 5 min before the metabolic labeling. In samples chased in the absence of DTT, the agent was washed out carefully with PBS. In experiments for visualization of the abnormal mobility of PLTP synthesized in the presence of DTT, the alkylating agent N-ethylmaleimide (NEM, 20 mm, Sigma) was added to the culture medium and the cells prior to the immunoprecipitation in order to prevent re-formation of disulfide bonds. NEM (100 mm) was also added to the SDS-PAGE samples after the immunoprecipitates were boiled in non-reducing sample buffer and cooled to room temperature before analysis in 7.5% SDS gels.

Immunofluorescence microscopy

Immunofluorescence analysis of HeLa-PLTP cells was carried out as described previously (27).

Carboxyterminal truncations of PLTP

The full-length human PLTP cDNA was cloned into the pSV-poly vector (Pharmacia). A series of carboxyterminal truncations were created by PCR using the Vent polymerase (New England Biolabs) and cloned into the same expression vector. All the constructs were sequenced using the Sequenase 2.0 kit (United States Biochemicals) and were found to be free of additional PCR-derived mutations. The relative amount of secreted PLTP or its truncated variants was determined by precipitating the total protein in the COS-1 cell growth medium with acetone, followed by Western blotting using the polyclonal anti-PLTP antibody R164 directed towards the amino terminus of PLTP and quantitation with the Fujifilm BAS-1500 Imaging system.

Measurement of PLTP activity

PLTP activity in the cell culture medium was measured using the radiometric assay described previously (28).

RESULTS

Characterization of the inducible HeLa cell line expressing PLTP

When the expression of PLTP cDNA in the stably transfected HeLa cells (denoted as HeLa-PLTP) was induced with doxycyclin, a time-dependent increase in phospholipid transfer activity in the culture medium was observed (**Fig. 1A**). After 24 h, the activity was 300 nmol/ml per h, and by the 72-h time-point a plateau at 1400 nmol/ml per h was reached. No activity was secreted by the parental HeLa cell line. In the uninduced state, the HeLa-PLTP cells secreted approximately 10% of the PLTP activity produced by the induced cells, indicating that the regulation of the PLTP cDNA expression in this cell line was not perfectly tight.

Immunoprecipitation protocol was set up to characterize the synthesized forms of PLTP and to monitor the biosynthesis and secretion of the protein. After a 30-min labeling with [^{35}S]methionine/cysteine, imunoprecipitation and SDS-PAGE showed a specific 67-kDa band in the induced HeLa-PLTP cells (Fig. 1B). This band was not present in immunoprecipitates of the parental HeLa cells (denoted as HeLa). During a 2-h chase, the labeled PLTP was secreted into the culture medium, where it was observed as a major group of bands in the size range of 75–80 kDa (denoted as 77 kDa form), and a minor 67 kDa band. Western analysis using the mAb JH59 verified the identity of the immunoprecipitated products.

Secretion time course of PLTP

In order to study the kinetics of PLTP secretion in the HeLa cell system, induced HeLa-PLTP cells were labeled with [^{35}S]methionine/cysteine for 10 min followed by chase time series. A time-dependent disappearance of radioactively labeled intracellular PLTP was evident (**Fig. 2A**), and coincided with the appearance of extracellular forms of the protein in the culture medium (Fig. 2B). Quantitation by densitometric scanning revealed that the half-time of PLTP secretion was approximately 55 min. In order to confirm that the decrease of the intracellular form of PLTP was not due to degradation, we performed a similar experiment in the presence of brefeldin A (5 μg/

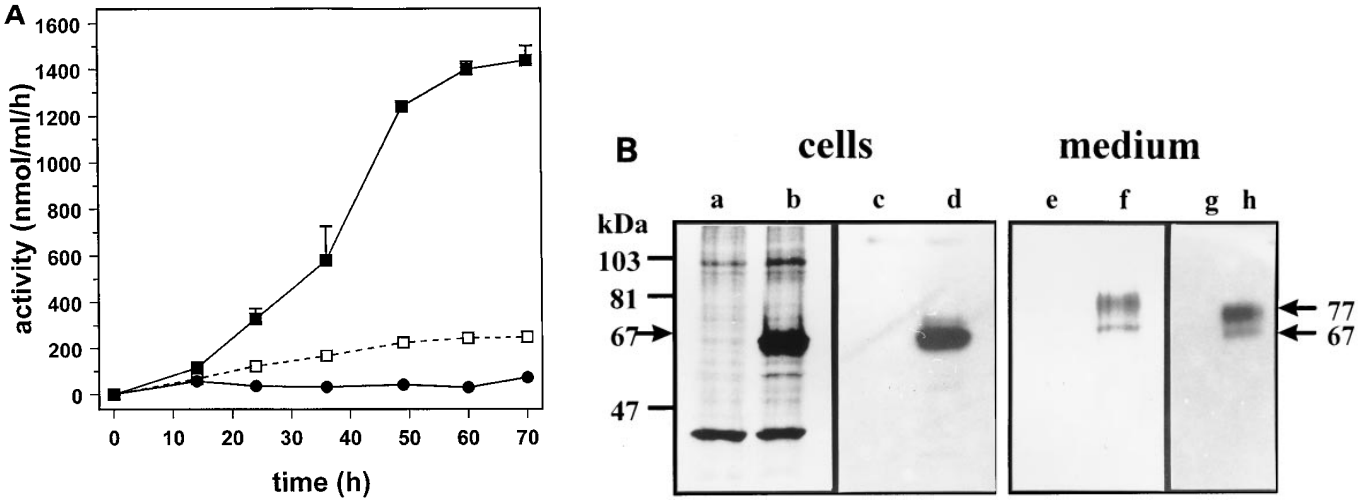


Fig. 1. Synthesis of PLTP by the stably transfected HeLa-PLTP cells. A: Secretion of active PLTP into the growth medium. The PLTP activity in the serum-free growth medium of uninduced (□) or induced (■) HeLa-PLTP cells or induced parental HeLa cells (●) at the indicated time points (t = 0 indicates the addition of the inducing agent, doxycyclin, 2 μg/ml). The results are based on three independent experiments. The error bars in the two bottom graphs have been omitted for the sake of clarity. B: Identification of the induced PLTP protein by immunoprecipitation using the rabbit polyclonal antibody R176. The HeLa or HeLa-PLTP cells were induced for 24 h, labeled for 30 min with [^{35}S]methionine/cysteine, and chased for 2 h. Immunoprecipitates of cell lysates directly after the pulse (lane a, HeLa; lane b, HeLa-PLTP) and growth medium after the chase (lane e, HeLa; lane f, HeLa-PLTP) are shown. Western blot analysis of the immunoprecipitates using the PLTP-specific mAb JH59:cell lysates (lane c, HeLa; lane d, HeLa-PLTP) and growth medium (lane g, HeLa; lane h, HeLa-PLTP). The mobilities of molecular mass markers are indicated on the left and major PLTP forms are identified with arrows.

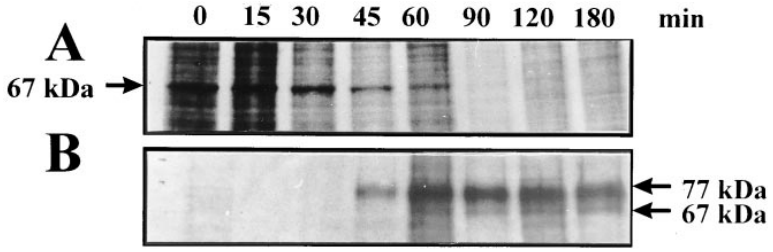


Fig. 2. Time course of PLTP secretion. Induced HeLa-PLTP cells were pulsed for 10 min followed by a chase time series. At the indicated time points, cell lysates (A) and culture medium (B) were immunoprecipitated with a rabbit polyclonal anti-PLTP antibody R176, and analyzed by SDS-PAGE (10%) and fluorography. The apparent molecular masses of PLTP are indicated.

ml), an agent that blocks the transport of proteins in the endoplasmic reticulum (ER). During 3 h of chase, no degradation of the intracellular 67-kDa form of PLTP was observed (data not shown).

Glycosylation of PLTP

The molecular mass of the PLTP polypeptide deduced from the cDNA is 54 kDa. However, all the forms, both intra- and extracellular, that were observed in SDS-PAGE, were considerably larger. The sequence of PLTP contains 6 putative N-glycosylation sites and several potential O-glycosylation sites (18), but the glycosylation pattern of the protein has not been experimentally addressed. We therefore carried out a basic characterization of the putative glycans on PLTP.

Extracellular forms of PLTP. The size of the extracellular PLTP was reduced to two forms (a major 58-kDa and a minor 54-kDa product) upon digestion with PNGase F, a glycosidase removing the entire N-linked oligosaccharide chains of a polypeptide (29); **Fig. 3A**). The major 58-kDa product had clearly slower mobility than the in vitro translated protein. Treatment of the extracellular PLTP with O-glycosidase had no effect on its apparent size in SDS-PAGE. To eliminate the possibility that the bulky N-glycans mask the O-glycans from digestion, the extracellular protein was digested concomitantly with PNGase F and O-glycosidase. The resulting PLTP band remained in the same location as that yielded by PNGase F digestion alone (data not shown). However, PLTP digested simultaneously with PNGase F and neuraminidase migrated midway between the 58-kDa and in vitro translated forms (Fig. 3A), suggesting that the modification responsible for the difference between these two forms is a sialylated glycan.

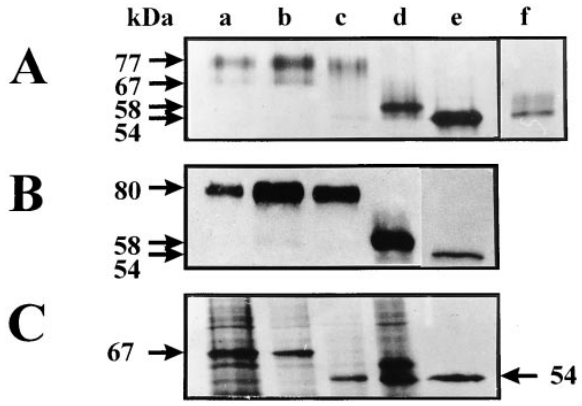


Fig. 3. Analysis of PLTP glycosylation. Extracellular HeLa-PLTP after a 2-h chase (A), purified unlabeled plasma PLTP (B), or intracellular HeLa-PLTP directly after 30-min labeling (C) were immunoprecipitated with anti-PLTP R176 antibody. The precipitates were subjected to glycosidase digestions (see Materials and Methods) followed by SDS-PAGE and fluorography (A,C) or Western blotting with mAb JH59 (B). Lane a, undigested sample; lane b, O-glycosidase; lane c, Endo H; lane d, PNGase F; lane e, in vitro translated PLTP; lane f, concomitant digestion with neuraminidase and PNGase F. The apparent molecular masses of the major PLTP forms are indicated.

The PLTP N-glycans appeared to be mainly of the complex type, as the major extracellular PLTP (77 kDa) was essentially resistant to endoglycosidase H (Endo H), which cleaves the chitobiose core of high mannose and some hybrid oligosaccharides (29). However, a slight shift of the major form towards higher mobility upon Endo H digestion was detectable, indicating that one or two of the glycans on each PLTP molecule may be of high mannose or hybrid type. Interestingly, a minor 67-kDa extracellular form of PLTP was sensitive to Endo H and was reduced to a 58-kDa protein. This suggests that a portion of the molecules is secreted in a high mannose/hybrid glycosylated form.

As there are cell type-specific differences in protein glycosylation, we also treated purified human plasma PLTP with the glycosidases. The apparent molecular weight of the untreated plasma PLTP is slightly higher (80 kDa) than that of HeLa-PLTP (77 kDa), indicating a subtle difference in the extent of glycosylation (Fig. 3B). The pattern obtained upon glycosidase digestion of plasma PLTP was highly similar to that of HeLa-PLTP, the only difference being the absence of the secreted Endo H-sensitive 67-kDa form. This validates the HeLa cell system as a model suited for the study of PLTP glycosylation.

Intracellular forms of PLTP. The major intracellular form of PLTP detected after the pulse and various chase times had the apparent size of 67 kDa (Figs. 1, 2). This form was reduced down to 54 kDa upon treatment with PNGase F (Fig. 3C), matching the mobility of the in vitro translated PLTP. Besides the 54-kDa band, another larger form was observed. In contrast to the major extracellular forms of PLTP, the 67-kDa intracellular form was under all conditions sensitive to Endo H digestion, suggesting the absence of complex glycans. Thus, the major intracellular form detected mainly represents ER and/or *cis*-Golgi forms of PLTP. As also shown for the extracellular PLTP, O-glycosidase treatment had no effect on the mobility of the intracellular form of the protein.

In the medial Golgi compartment, the proteins acquiring

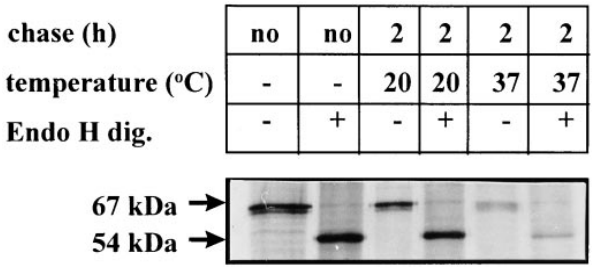


Fig. 4. The effect of reduced temperature on PLTP processing. The induced HeLa-PLTP cells were labeled for 30 min and chased at either 20°C or 37°C, immunoprecipitated with anti-PLTP R176, and digested with Endo H. The samples were subjected to SDS-PAGE analysis and fluorography. The treatments of the individual samples (chase time; "no" refers to analysis directly after pulse–chase temperature–Endo H digestion) are shown on the top. The apparent molecular massses of the PLTP forms detected are indicated.

complex N-glycans become resistant to Endo H treatment (30). In order to follow the maturation of PLTP, we attempted to enrich the nascent PLTP in the *trans*-Golgi network using 20°C temperature block (31). Directly after the pulse, the newly synthesized PLTP was sensitive to Endo H (**Fig. 4A**), and surprisingly, no enrichment of Endo H-resistant forms in the cells was detected during a 2-h chase at 20°C. The temperature block was effective, however, as the labeled PLTP remained intracellular under these conditions. To monitor the intracellular transport of PLTP morphologically, immunofluorescence microscopy of induced HeLa-PLTP cells was carried out (**Fig. 5**). At 24 h post-induction (steady state distribution), PLTP displayed a reticular staining that included the nuclear membrane and concentrated in the perinuclear region, thus mainly representing the ER (Fig. 5A). During a 2-h incubation at 37°C in the presence of cycloheximide, the intracellular staining disappeared almost completely, consistent with efficient secretion of the pre-existing PLTP (Fig. 5B). The brightly stained dotty structures remaining in the cells probably represent aggregates of misfolded PLTP. Upon incubation at 20°C the intracellular staining of PLTP remained identical to the steady state distribution (Fig. 5C), supporting the notion that PLTP was arrested in the ER at the 20°C. Under these conditions, influenza haemagglutinin concentrated in infected HeLa-PLTP cells to the Golgi apparatus (Fig. 5D) (31) and acquired Endo H resistance (data not shown).

Role of N-glycans in PLTP secretion

To study the importance of the N-glycans for PLTP secretion, pulse-chase analysis of induced HeLa-PLTP cells was carried out in the presence of tunicamycin, a drug that inhibits the formation of N-glycans on the newly synthesized polypeptide. After drug treatment, the intracellular form of PLTP was smaller (54 kDa) than in the absence of the drug (67 kDa), demonstrating that N-glycosylation

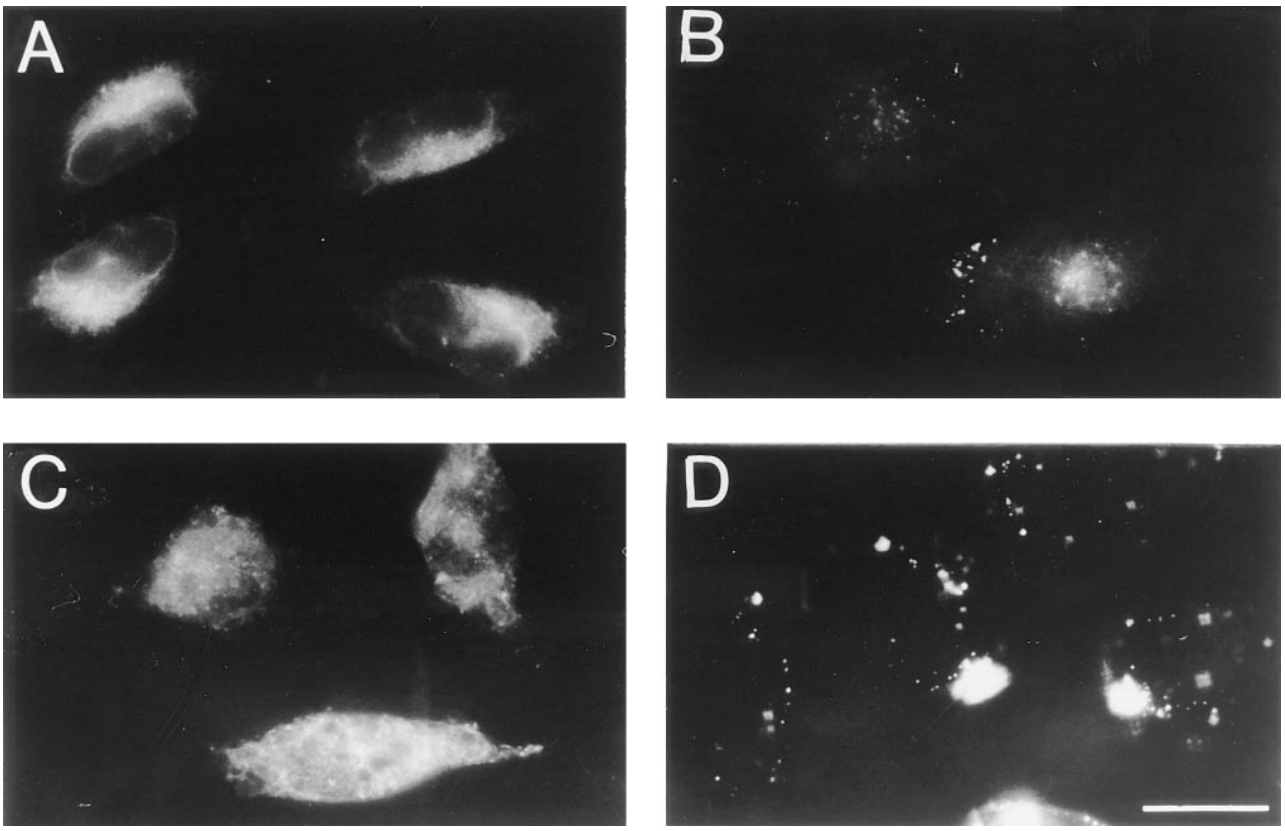


Fig. 5. Visualization of intracellular PLTP by immunofluorescence microscopy. Staining of the induced HeLa-PLTP cells with the anti-PLTP mAb JH66 before (A) or after a 2-h treatment with cycloheximide (100 μg/ml) at 37°C (B) or at 20°C (C). Induced cells infected for 3.5 h with influenza N-virus followed by a 2-h chase at 20°C and stained with anti-haemagglutinin antibody (D). The bar equals 5 μm.

had been inhibited (**Fig. 6**). After a 2-h chase at 37°C, the quantity of intracellular PLTP was clearly reduced. However, only trace amounts of the non-glycosylated protein were secreted into the culture medium, indicating that the protein was degraded within the cells.

Effect of DTT on PLTP biosynthesis and secretion

PLTP has four cysteine residues and is thus potentially capable of forming two intrachain disulfide bonds (18). The formation of disulfide bridges can be prevented by treating cells with a reducing agent, dithiothreitol (DTT) (32). We combined the pulse-chase approach with DTT treatment of the HeLa-PLTP cells in order to monitor the significance of disulfide bond formation for PLTP folding and secretion. The secretion of PLTP was totally blocked when DTT was present during both the pulse and the chase (**Fig. 7**). When DTT was included only in the chase medium, PLTP was secreted into the growth medium, although at reduced efficiency. PLTP synthesized in the presence of DTT was efficiently secreted when the chase was performed in the absence of the reducing agent, demonstrating that the effect of DTT on PLTP folding/secretion is reversible.

The rearrangement of disulfide bonds can in certain cases be observed as a change in the mobility of the protein in non-reducing SDS-PAGE (26, 33). This approach was used to monitor a possible effect of DTT on PLTP structure. After a 30-min pulse, the intracellular PLTP migrated in non-reducing gels as a zone obviously consisting of several closely spaced bands (**Fig. 8**). After 30 min of chase, the PLTP migrated as a focused band in the lower part of the original band pattern. No major shift in the mobility of PLTP was observed when the protein was synthesized in the presence of DTT. However, a subtle but well-reproducible change in the migration of the protein was detectable directly after the pulse. The protein appeared as a single narrow band corresponding to the upper part of the band pattern observed in the absence of DTT. The normal mobility was replenished when chase was carried out in the absence of DTT.

Carboxyterminal truncations of PLTP

Studies on two proteins displaying sequence homology to PLTP, cholesteryl ester transfer protein (CETP) and bactericidal/permeability-increasing protein (BPI), have demonstrated the importance of the C-terminal portion of the molecules for their function (34–37). To elucidate the importance of the C-terminal sequences of PLTP, we generated a series of cDNA deletions (**Fig. 9A**) and expressed the truncated proteins in COS-1 cells. At 48 h post-transfection, the culture media were analyzed by Western blotting to determine the efficiency of secretion of these truncated forms of PLTP (Fig. 9B). Protein variants lacking 5–30 amino acids from the C-terminus (PLTPΔ5–PLTPΔ30) were found in the culture medium at amounts similar to that of the wild-type PLTP. Longer deletions resulted in complete absence of the proteins in the culture medium. Pulse labeling of the transfected cells showed that all the PLTP variants studied were synthesized at approximately equal rates (data not shown). To be able to estimate the relative specific activity of the secreted PLTP variants, the proteins in the medium were quantified and the PLTP activity was determined (**Fig. 10**). The specific activities of PLTPΔ5 and PLTPΔ10 did not significantly differ from the wild-type PLTP. However, the forms lacking 20, 25, or 30 amino acids displayed a significantly reduced specific activity. Interestingly, deletion of 30 amino acids almost completely abolished the

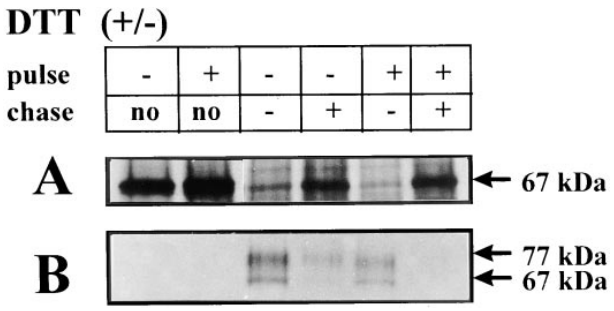


Fig. 7. The effect of DTT treatment on PLTP secretion. Induced HeLa-PLTP cells were pulsed for 30 min and chased for 2 h followed by immunoprecipitation with anti-PLTP R176, SDS-PAGE, and fluorography. The presence or absence of DTT (2 mm) during the pulse and/or chase periods in the individual culture dishes is indicated on the top. "No" refers to samples analyzed directly after pulse. Panel A, cellular fractions; panel B, culture medium. The apparent molecular masses of the PLTP forms detected are indicated.

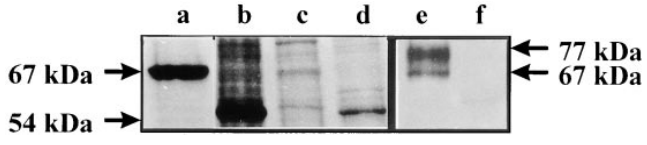


Fig. 6. The role of N-glycosylation in PLTP secretion. Induced HeLa-PLTP cells were labeled for 30 min and chased for 2 h in the absence or presence of tunicamycin (5 μg/ml), followed by immunoprecipitation with anti-PLTP R176, SDS-PAGE analysis, and fluorography. Cells after the pulse: untreated (lane a), tunicamycin-treated (lane b); cells after the chase: untreated (lane c), tunicamycin-treated (lane d); medium after the chase: untreated (lane e), tunicamycin-treated (lane f). The apparent molecular masses of the PLTP forms detected are indicated.

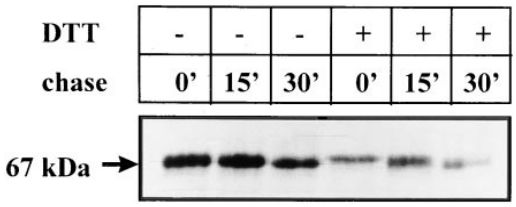
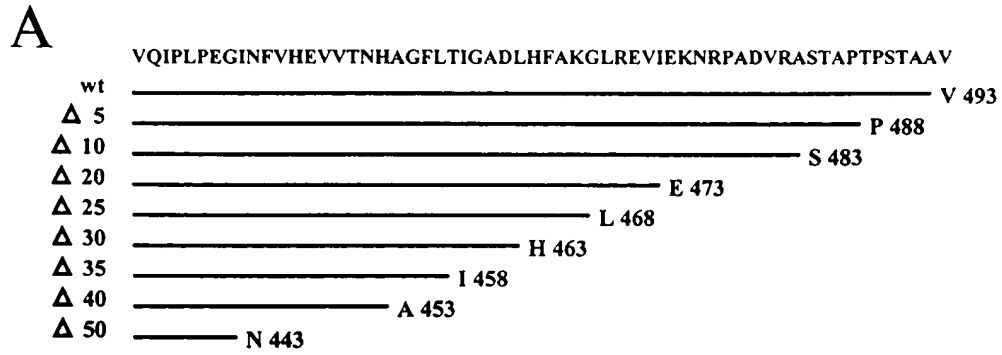


Fig. 8. The effect of DTT on PLTP mobility in non-reducing gels. Induced HeLa-PLTP cells were pulsed for 30 min and chased for 15 or 30 min, immunoprecipitated with rabbit anti-PLTP R176, and the cellular fractions were analyzed by non-reducing SDS-PAGE as described in Materials and Methods. The presence or absence of DTT (2 mm) during the pulse and the chase time (in the absence of DTT) are indicated on the top.



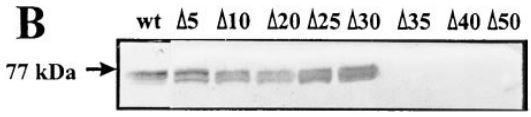


Fig. 9. The secretion of C-terminally truncated PLTP variants. A, Diagram identifying the C-terminally truncated proteins expressed in COS cells. The sequence of the PLTP C-terminus (60 aa) is shown on the top. The last amino acid residue present in each variant and its aa number are indicated. Δ5–Δ50 indicate the number of residues deleted and correspond to the nomenclature used in the text. B, Western blot analysis of the PLTP variants (identified on the top) secreted into the culture medium. The supernatants of COS cells at 48 h post-transfection were analyzed by Western blotting using a rabbit polyclonal antibody R164 against the amino-terminus of PLTP.

PC transfer activity of the protein without markedly affecting its secretion.

DISCUSSION

Interest in the physiological role of phospholipid transfer protein has greatly increased after the recent reports demonstrating the key role of PLTP in HDL metabolism in mouse models (7, 21–23). However, cell biological studies on the biosynthesis of PLTP have not been carried out, largely due to low endogenous expression levels of PLTP in cultured cell lines. Generation of an inducible, stably transfected HeLa cell line in this study allowed detailed analysis of PLTP biosynthesis. Using the HeLa-PLTP cell line we have studied the role of N-glycosylation, intrachain disulfide bonds, and the carboxyterminal portion of PLTP in the biosynthesis and secretion of the protein.

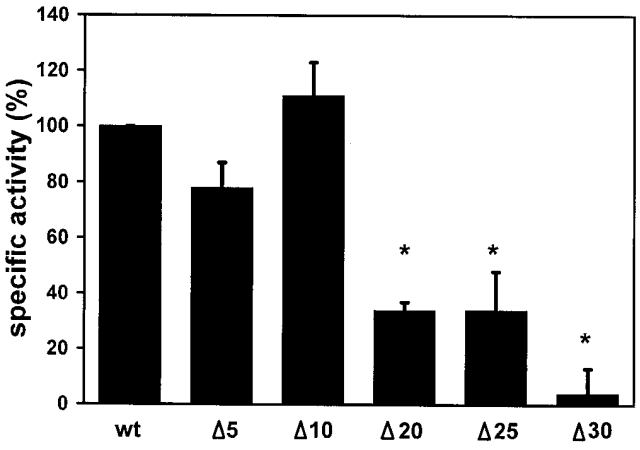


Fig. 10. The phospholipid transfer activity of the secreted PLTP variants. COS cells were transfected with the truncated PLTP cDNA constructs (indicated at the bottom). At 48 h post-transfection, phospholipid transfer activity in the serum-free growth medium was measured, and the relative quantity of the secreted PLTP was determined by Western blotting using anti-PLTP R164, detection with [^{35}S]-protein A, and the Fujifilm BAS-1500 Imaging System. The results are mean values (±SEM) of three independent experiments and are expressed relative to the activity of the wild-type protein (set at 100%). The asterisks indicate a statistically significant difference ($P < 0.01$; *t*-test) from the wild-type PLTP.

In the cell system used, the secretion half-time of PLTP was approximately 55 min. This is well within the range of half-times determined for several plasma proteins (38–40). Furthermore, the glycosylation patterns of HeLa-PLTP and purified plasma PLTP are highly similar. Therefore, the inducible HeLa cell line used in this study probably does not drastically differ from the endogenous sources of PLTP and provides, due to the efficient and controllable protein production, a useful model system for PLTP biosynthesis.

The PLTP secreted by the HeLa-PLTP cells showed size heterogeneity in SDS-PAGE analysis. The major form of secreted PLTP (77 kDa) resembling in mobility the plasma protein was insensitive to Endo H but sensitive to PNGase F, demonstrating the presence of complex N-glycans. However, one of the secreted forms (67 kDa) was Endo H sensitive, thus representing a high mannose or hybrid glycosylated form. This form is not present in plasma, and its secretion by the HeLa-PLTP cells may be due to an insufficient capacity to fully glycosylate the overexpressed protein. Successful production of fully active PLTP in Sf-9 insect cells (41) synthesizing only high-mannose type N-glycans (42) demonstrates that complex N-

glycans are not required for efficient secretion or activity of PLTP. Further characterization of the glycosylation by site-directed mutagenesis and identification of the precise glycan structures on PLTP will reveal whether some of the oligosaccharide moieties or specific sugars therein are essential for the activities of PLTP or whether they merely have a structural role.

The PNGase F-treated immunoprecipitates of the HeLa-PLTP cell growth medium contained PLTP (58 kDa) that was approximately 4 kDa larger than the in vitro translated protein (54 kDa). This indicates that the protein *i*) either contains N-glycans that are uncleavable with PNGase F, or *ii*) carries another type of post-translational modification. Digestion with O-glycosidase failed to demonstrate the presence of O-glycans on the secreted PLTP. However, a simultaneous treatment with PNGase F and neuraminidase yielded a product smaller than 58 kDa, suggesting that the unknown modification is a sialylated glycan. The protein secreted in trace amounts in the presence of tunicamycin displayed the 58 kDa mobility (data not shown), suggesting that the unknown glycan is not N-linked. Therefore, the secreted PLTP most probably carries also O-linked glycans.

We were able to detect only one major intracellular, Endo H-sensitive form of PLTP (67 kDa), which represents ER and/or *cis*-Golgi forms of the protein. Endo H-resistant medial/*trans*-Golgi forms of PLTP could not be accumulated using a 20°C temperature block, indicating that the intracellular transport of PLTP is more sensitive to low temperatures than that of the viral glycoproteins used to monitor events on the secretory pathway (31, 43). This unique behavior may be due to either formation of an unusually stable complex with the ER folding machinery or exceptional sensitivity to changes in membrane lipid fluidity.

Blocking the N-glycosylation with tunicamycin had a dramatic inhibitory effect on PLTP secretion and further, the non-glycosylated form of PLTP appeared to be intracellularly degraded at an increased rate. Blocking the ER exit of normally core-glycosylated PLTP with brefeldin A did not result in a corresponding destabilization. This indicates that the PLTP N-glycans are important for the protein to acquire a secretion-competent conformation. A similar inhibition of secretion in the presence of tunicamycin has been reported for the related CETP (34, 44), reflecting the structural relatedness of these proteins.

PLTP contains four cysteine residues at positions 22, 146, 185, and 335. The role of these residues in the folding of PLTP has not been addressed experimentally. However, a pair of conserved cysteines that form a disulfide bridge in the related BPI is also found in PLTP (37). Treatment of cells with the reducing agent DTT is reported to result in the inhibition of disulfide bond formation, defective folding, and ER arrest of several proteins (45, 46). When present during both pulse and chase, DTT completely inhibited PLTP secretion, indicating a role of disulfide bonds in creating a transport-competent conformation of PLTP. The effect was reversible, suggesting that when PLTP was folded and oxidized posttranslationally, its transport through the secretory pathway resumed. Further, we were able to demonstrate a subtle change in the gel mobility of PLTP synthesized in the presence of DTT. The observed change may reflect a direct effect on the molecular architecture of PLTP via defective disulfide bond formation, or defective trimming of glycan structures resulting from impaired interaction of the protein with the ER/*cis*-Golgi glycosidases.

Mutagenesis studies on the related CETP have demonstrated the crucial role of the sequence in the immediate vicinity of the C-terminus for its neutral lipid transfer activity and secretion (34–36). Further, the crystal structure of the BPI, another member of the protein family, indicates structural importance of C-terminal sequences (37). Deleting up to 30 amino acids from the PLTP C-terminus had little effect on the secretion of the protein. This region can thus be removed without detriment to the protein fold, indicating that it forms a flexible tail or flap on the PLTP molecule. However, PLTPΔ35-PLTPΔ50 were not secreted into the culture medium, suggesting that the proteins were misfolded. These deletions did not destroy any of the putative N-glycosylation sites nor cysteine residues, indicating that the defect was independent of the N-glycans or disulfide bonds. A possible explanation for the secretion defect is found in the crystal structure of BPI. The residues in the homologous C-terminal region participate in β-sheets that form the interface between the BPI major structural domains (37). Thus, deletions extending into this region are likely to cause a major folding defect.

The very C-terminus (10 residues) was found to be dispensable for the PC-transfer activity of PLTP, whereas PLTPΔ20 and PLTPΔ25 displayed mildly decreased specific activity. Interestingly, the PLTPΔ30 variant was efficiently secreted but displayed a very low PC transfer activity. This suggests that the region of amino acids 464–468 contains residues important for the PLTP phospholipid transfer activity. The aromatic residue 464 is conserved throughout the family of PLTP-related proteins (Phe in PLTP and CETP; Tyr in BPI and LBP) and is involved in a phospholipid binding pocket of BPI (37). The extreme C-terminus is essential for the normal neutral lipid transfer activity and lipid substrate specificity of CETP (35, 36). In addition to phospholipids, PLTP has been reported to transfer several lipid-like substances: diacylglycerol, cholesterol, lipopolysaccharide, and α-tocopherol (9–12). Analysis of the substrate specificity of PLTPΔ30 displaying a low PC transfer activity and the other PLTP variants generated here is in progress.

To delineate in detail the residues and subdomains of PLTP responsible for binding and transfer of lipid substrates and interactions with protein components of HDL, careful molecular modelling based on the BPI crystal structure and site-specific mutagenesis are required. The methodology developed during the present study offers tools for the analysis of the biosynthesis and activities of PLTP variants and provides information essential for the future work aimed at elucidation of the structure–function relationships in PLTP.

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