methods

Quantitation of the active and low-active forms of human plasma phospholipid transfer protein by ELISA

Sarah Siggins,[1] Minna Kärkkäinen,[1] Jukka Tenhunen, Jari Metso, Esa Tahvanainen, Vesa M. Olkkonen, Matti Jauhiainen, and Christian Ehnholm[2]

Department of Molecular Medicine, National Public Health Institute, Biomedicum, FIN-00251 Helsinki, Finland

Abstract **Human plasma contains two forms of phospholipid transfer protein (PLTP), one catalytically active [high-activity PLTP (HA-PLTP)] and the other a low-activity (LA-PLTP) form. We present here a PLTP ELISA that allows not only for accurate measurement of PLTP concentration in plasma but also of the distribution of both LA- and HA-PLTP. To achieve similar immunoreactivity of the two PLTP forms, a denaturing sample pretreatment with 0.5% SDS was required. Distribution of LA- and HA-PLTP in plasma was assessed using size-exclusion chromatography, Heparin-Sepharose chromatography, anti-PLTP immunoaffinity chromatography, and dextran sulfate-$CaCl_2$ precipitation. All four methods demonstrated that ~60% of plasma PLTP represents LA-PLTP and 40% represents HA-PLTP. According to the modified ELISA, the total serum PLTP concentration in a random Finnish population sample (n = 80) was 5.81 ± 1.33 mg/l (mean ± SD) (range, 2.78–10.06 mg/l) and the mean activity was 5.84 ± 1.39 μmol/ml/h (range, 3.21–11.15 μmol/ml/h).** **To quantitate both forms of PLTP in sera from this sample, we combined dextran sulfate-$CaCl_2$ precipitation with the modified PLTP ELISA. The HA-PLTP mass (mean, 1.87 ± 0.85 mg/l) correlated significantly with serum PLTP activity, whereas that of LA-PLTP (mean, 3.94 ± 1.4 mg/l) showed no correlation with phospholipid transfer activity.**— Siggins, S., M. Kärkkäinen, J. Tenhunen, J. Metso, E. Tahvanainen, V. M. Olkkonen, M. Jauhiainen, and C. Ehnholm. **Quantitation of the active and low-active forms of human plasma phospholipid transfer protein by ELISA.** ***J. Lipid Res.*** **2004.** 45: **387–395.**

Supplementary key words lipid transfer protein • enzyme-linked immunosorbent assay • high-activity form of phospholipid transfer protein • low-activity form of phospholipid transfer protein

In vitro studies have identified a number of functions for phospholipid transfer protein (PLTP) in lipoprotein metabolism (1, 2). These include the transfer of phospholipids (PLs), diacylglycerol, free cholesterol, vitamin E, and lipopolysaccharide between lipoproteins and cells. Other in vitro functions demonstrated are proteolysis of apolipoprotein A-I (apoA-I) (3), HDL interconversion (4, 5), and the stimulation of LCAT and cholesteryl ester transfer protein activity (6).

Studies in genetically manipulated mice with altered PLTP expression have advanced our understanding of the metabolic functions of PLTP (7–16). PLTP has three major functions in HDL metabolism: it transfers surface remnants from VLDL to HDL during lipolysis (7), it increases the catabolism of HDL and the generation of pre-β-HDL, and it stimulates cellular cholesterol/PL efflux (9–12). PLTP has also been shown to play a role in the secretion of apoB-containing lipoproteins (15, 17, 18).

Although the in vitro role of PLTP in gene-targeted animal models has been studied intensively, the physiological role of PLTP in human lipid metabolism is far from resolved. To obtain information about the role of PLTP in human metabolism, one approach has been to assess PLTP activity under different physiological conditions (19–22). Moreover, to gain a more complete understanding of the metabolic role of PLTP, its mass in plasma should also be recorded (23–26). To date, three different methods to assay plasma PLTP mass have been reported, with conflicting results (23–25). A possible reason for the discrepancies may be differences in the immunoreactivity of the antibodies used, as reported by Murdoch et al. (27), and the presence of two forms of PLTP in the circulation (28, 29). The two forms, one catalytically active [high-activity PLTP (HA-PLTP)] and the other low-activity (LA-PLTP), are associated with distinct macromolecular complexes of different size and eventually display different immunochemical reactivities. The observation that PLTP mass and activity in human plasma do not correlate (24, 25) further suggests differences in the reactivity of antibodies to HA-

Manuscript received 1 July 2003 and in revised form 1 October 2003.

Published, JLR Papers in Press, November 16, 2003.
DOI 10.1194/jlr.D300023-JLR200

Abbreviations: HA-PLTP, high-activity PLTP; LA-PLTP, low-activity PLTP; MAb, monoclonal antibody; PLTP, phospholipid transfer protein.

[1] S. Siggins and M. Kärkkäinen contributed equally to this work.

[2] To whom correspondence should be addressed.
e-mail: christian.ehnholm@ktl.fi

PLTP versus LA-PLTP. Only in one study was a significant correlation between PLTP mass and activity observed (23).

To resolve these discrepancies, we undertook to reassess our ELISA method. We now report that by including a denaturing pretreatment of the samples with SDS, it is possible to reliably measure the mass of both forms of plasma PLTP.

MATERIALS AND METHODS

Collection of human plasma for PLTP purification

Human plasma was obtained by plasmapheresis. The protease inhibitors, Trasylol (50 U/ml), dichloroisocoumarin (DCIC, 0.4 mM), E-64 (1 mM), 3-amidinophenylmethanesulfonyl fluoride (APMSF, 1 mM), and leupeptin (10 μM) were immediately added to the plasma.

Subjects

For PLTP analysis, serum samples from 80 subjects (age range, 25–75 years) were randomly chosen as a subsample of the Health 2000 Health Examination Survey study (30) carried out in Finland. Serum samples were stored at −70°C before PLTP analysis.

Treatment of human plasma with detergents

The detergents used were cationic cetyltrimethylammonium bromide [CTAB; critical micellar concentration (CMC) 0.03%; Merck], anionic SDS (CMC 0.3%; BDH), zwitterionic CHAPS (CMC 0.5%; Sigma), nonionic *n*-octyl-β-D-glucopyranoside (OG; CMC 0.1%; Sigma), and polyoxyethylene sorbitan monolaurate (Tween 20; CMC 0.007%; Fluka). The effect of the detergents on the immunoreactivity of PLTP was determined by the ELISA method as follows. Normolipidemic human plasma (50 μl) was mixed with an appropriate amount of the detergent. All detergents were tested at three concentrations: at their CMC, at 0.1% (v/v), and at 0.5% (v/v). Plasma-detergent mixtures were incubated for 30 min at 22°C and then diluted 1:50 with PBS. The dilutions (200 μl) were added in duplicate to microtiter plate wells coated with the anti-human PLTP monoclonal antibody (MAb) JH66, and all subsequent steps were performed according to the PLTP ELISA method described previously (24). Control wells with detergents but without plasma were incubated to assess background values. The resulting absorbances with different detergents were compared with the control plasma sample without any detergent, the immunoreactivity of which was set at 100%.

Purification of the HA and LA forms of human plasma PLTP

The two forms of PLTP, HA-PLTP and LA-PLTP, were isolated as described (29).

Production and isolation of recombinant PLTP

A hexahistidine tag was engineered into the C-terminal end of full-length human PLTP cDNA using PCR. The cDNA was transferred into the baculovirus expression vector pVL1393 (Pharmingen), and virus stocks and recombinant PLTP (r-PLTP) were produced as described previously (31, 32).

Recombinant PLTP (r-PLTP) was purified using two chromatographic steps: hydroxylapatite chromatography (Bio-Gel HTP; Bio-Rad) and nickel-nitrilotriacetic acid (Ni-NTA) agarose Superflow Agarose chromatography (Qiagen). Briefly, 100 ml of the r-PLTP-containing Sf9 cell growth medium was applied onto a hydroxylapatite column (20 ml) equilibrated with 1 mM sodium phosphate buffer, pH 6.8, and 150 mM NaCl, at a flow rate of 1 ml/min. After extensive washing with the same buffer, the bound material was eluted with 75 mM sodium phosphate buffer, pH 6.8, containing 150 mM NaCl, and 2 ml fractions were collected. The fractions containing PLTP activity were combined and, after addition of NaCl to a final concentration of 0.3 M, applied to Ni-NTA Superflow Agarose. After this, the column was washed with 50 mM sodium phosphate buffer, pH 8.0, containing 0.3 M NaCl, 1 mM imidazole, and 0.5% Tween 20. A second wash was performed with the same buffer without imidazole. Finally, the bound protein was eluted with 50 mM sodium phosphate buffer, pH 8.0, containing 250 mM imidazole, 0.3 M NaCl, and 0.1% Tween 20. The eluted material was analyzed for PLTP activity and mass and was immediately used as a primary calibrator to standardize the ELISA method. The purified r-PLTP was stored in small aliquots at −70°C.

Calibration of the ELISA and determination of PLTP concentration in human plasma

Calibration of the ELISA and measurement of human plasma PLTP concentration were performed essentially as described earlier (24), but with modifications in sample treatment and using another MAb, JH66. For the primary standardization, r-PLTP, as well as HA-PLTP purified from human plasma (24), were incubated with 0.5% SDS for 30 min at 22°C. After the SDS pretreatment, the mixtures were diluted in sample buffer (0.1% Tween 20 in PBS) and appropriate amounts of the calibrators were added in duplicate wells in a total volume of 200 μl (the final SDS concentration being 0.02%). The amounts used were 0, 25, 50, 75, and 100 ng/ml. To create a secondary standard calibration curve, human plasma was incubated with 0.5% SDS for 30 min and thereafter diluted 1:20, 1:40, 1:80, and 1:160 in sample buffer, and 200 μl of the dilutions was added to duplicate wells. All calibration assays were repeated six times, and the PLTP concentration in the secondary standard was determined as an average of these repetitions. The other ELISA steps were performed as described (24).

Preparation of antibodies

The MAb and polyclonal antibodies against PLTP were produced and isolated as previously described (24). As judged from a molecular model (33), the epitope region of the MAb JH66 is located on the surface of PLTP and is well exposed (amino acids 225–235) (34). The specificity of the antibodies was confirmed by SDS-PAGE and Western blotting.

Assay of PLTP activity

PLTP activity was measured using the radiometric assay described by Damen, Regts, and Scherphof (35) with minor modifications (4).

Size-exclusion chromatography of human plasma

Fresh human plasma (1 ml) was subjected to a fast-performance liquid chromatography system containing two Superose 6HR 10/30 size-exclusion chromatography columns (Amersham Pharmacia Biotech) connected in tandem. The columns were equilibrated with Tris-buffered saline (10 mM Tris-HCl and 154 mM NaCl, pH 7.4). Chromatography was performed at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected and used for PLTP activity and mass analyses.

Heparin-Sepharose chromatography of human plasma

Fresh human plasma sample (1 ml) was applied onto a 1 ml Hi-Trap Heparin-Sepharose column (Amersham Pharmacia Biotech) at a flow rate of 1 ml/min at room temperature. The column was previously equilibrated with 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. The column was washed with the equilibrating buffer, after which bound proteins were eluted with 1 M NaCl. The fractions were analyzed for PLTP activity and mass.

Immunoaffinity chromatography

Anti-PLTP MAb JH66 was coupled to cyanogen bromide-activated (CNBr) Sepharose 4B as described (29). The anti-PLTP column (1 × 3 cm) was equilibrated with PBS and then with PBS containing 0.2% Tween 20. A plasma sample (1 ml) was applied to the column at a flow rate of 0.5 ml/min, and the nonbound material was eluted. The protein that had bound to the antibody column was eluted with 0.1 M glycine and 0.2% Tween 20, pH 2.5, and fractions (1 ml) were collected into tubes containing 1 M Tris-HCl, pH 8.5, for neutralization. The fractions were analyzed for PLTP activity and mass.

Precipitation of plasma with dextran sulfate-$CaCl_2$

Plasma samples were treated with dextran sulfate-$CaCl_2$ using the method of Kato et al. (36) with minor modifications. Briefly, 200 μl of plasma was mixed with 300 μl of water and 200 μl of 1% dextran sulfate sodium salt (dextran molecular weight ~500,000; Amersham Pharmacia Biotech, code No. 17-0340-02; dialyzed against water before use). The mixture was incubated on ice for 20 min with intermittent mixing. After this, $CaCl_2$ was added to a final concentration of 0.1 M. The mixture was then centrifuged (16,000 *g*) for 5 min at room temperature, and the clear supernatants were collected and assayed for PLTP activity and mass. NaCl was added to the supernatants to a final concentration of 1 M before the PLTP activity assays.

General procedures

SDS-PAGE was performed by the method of Laemmli (37), followed by Coomassie staining or Western blotting (38) and enhanced chemiluminescence detection. Gels were scanned and the images quantitated using a Kodak scanner. Protein concentration was determined by the method of Lowry et al. (39) or by using the CBQCA Protein Quantitation Kit (Molecular Probes) with BSA as a standard.

Statistical analysis

Statistical testing was performed using the Statistical Package for Social Sciences (SPSS) version 11.5 (SPSS, Chicago, IL). The correlations between PLTP activity and HA and/or LA mass were performed with the Pearson correlation test using unadjusted values.

RESULTS

Effect of detergents on the immunoreactivity of PLTP

The two forms of PLTP, LA-PLTP and HA-PLTP, were isolated from human plasma (29). They were then quantitated using the conventional ELISA assay (24). To study whether the two forms have different reactivity in the ELISA assay, similar amounts (40 ng as determined by ELISA of HA-PLTP and LA-PLTP) were subjected to SDS-PAGE, transferred to a nitrocellulose filter, and immunoblotted (**Fig. 1A**). Semiquantitative scanning of the gels revealed that the intensity of HA-PLTP was ~3-fold stronger (162,110 pixels) than that of LA-PLTP (54,030 pixels). Assuming that the Western blot gives an accurate measure of PLTP mass, this result suggests that the immunoreactivity of HA-PLTP in the ELISA is underestimated compared with that of LA-PLTP and suggests that a denaturation step before antibody binding may improve the detectability of the protein, especially that of the active form. Therefore, we decided to evaluate if pretreatment with a denaturant could improve the reactivity of human PLTP also in the ELISA method. The effects of five detergents on the immunoreactivity of human plasma PLTP were evaluated. They included anionic (SDS), cationic (CTAB), nonionic (OG and Tween 20), and zwitterionic (CHAPS) species. All detergents were assayed at CMC, 0.1%, and 0.5% concentrations. The evaluation was carried out using the previously described ELISA method (24).

The results (**Table 1**) indicate that, of the detergents tested, the negatively charged SDS caused the largest increase in the immunoreactivity of plasma PLTP. When plasma was treated with 0.5% SDS before the assay, the immunoreactivity increased 4-fold compared with plasma assayed without any detergent pretreatment. Higher SDS concentrations did not result in higher reactivity (data not shown). The other detergents tested had a deleterious effect (CTAB), no effect at all (OG and CHAPS), or caused only a slight increase of immunoreactivity (Tween 20). Based on these observations, we decided to repeat the calibration of our ELISA method by incorporating a pretreatment of the samples with 0.5% SDS.

Development of a denaturing ELISA for determination of PLTP mass

For calibration of the ELISA, r-PLTP produced in the baculovirus protein expression system was used (32). The purified r-PLTP, when subjected to SDS-PAGE and visualized by silver staining, showed one major 76 kDa protein band, which represented more than 95% of total protein in the preparation as determined by scanning of the gel. The purified PLTP preparations typically contained 80–100 mg/l r-PLTP protein.

To obtain a standard curve for the PLTP ELISA, the primary standards, r-PLTP and PLTP purified from plasma, were treated with 0.5% SDS for 30 min at 22°C and were thereafter used to create standard curves in the range of 25–100 ng PLTP/ml. As shown in **Fig. 2**, the ELISA is linear over the entire range used and is suitable for the quantification of PLTP levels as low as 0.0125 mg/l. Between runs (n = 3), each primary standard value differed on average 4.3% from the mean value. When the primary standard r-PLTP protein was compared with PLTP protein purified from human plasma or with the secondary calibrator plasma, similar calibration curves were obtained (Fig. 2).

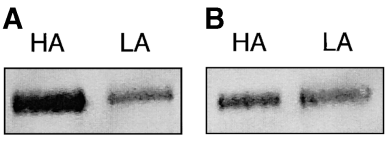

Fig. 1. Western blot analysis illustrating the difference in the reactivity of antibodies to the high-activity (HA) versus low-activity (LA) phospholipid transfer protein (PLTP). A: Equal amounts of HA-PLTP and LA-PLTP (40 ng of each) as determined by ELISA (24) were subjected to SDS-PAGE on 12.5% gels under reducing conditions, transferred to a nitrocellulose filter, and immunoblotted. Western blotting was performed using the monoclonal antibody (MAb) JH66. B: Equal amounts of HA-PLTP and LA-PLTP (40 ng of each) as measured using the new ELISA method, which involves pretreatment of the samples with 0.5% SDS.

TABLE 1. Effect of different detergents on the immunoreactivity of phospholipid transfer protein in human plasma

Detergent	Immunoreactivity (%)		
	Critical Micellar Concentration	0.1% Concentration	0.5% Concentration
		v/v	
None[a]	100	100	100
CTAB	120	64	30
SDS	275	135	430
CHAPS	110	95	110
Tween 20	92	102	203
OG	95	81	100

[a] The immunoreactivity of the control plasma sample without detergent.

To study whether serum interferes with the measurement of either HA-PLTP or LA-PLTP, we added increasing amounts of each PLTP form to serum before ELISA measurement. The mean recoveries of added PLTP were 109% for LA-PLTP and 90% for HA-PLTP, indicating that serum does not interfere with the measurement of either one.

Having calibrated the ELISA, we established a secondary standard using a normolipidemic plasma sample from a healthy male volunteer. When analyzed after SDS treatment using different plasma dilutions (1:20, 1:40, and 1:80), the mean PLTP mass in our secondary standard was 5.9 mg/l. To obtain a standard curve, the secondary standard was pretreated with 0.5% SDS and diluted to cover the PLTP mass range from 25 to 200 ng PLTP protein/ml. The slope of the secondary standard curve did not differ significantly from the slope of the curve obtained with the primary standard (**Fig. 3**). To avoid the potential nonlinearity caused by very low or high absorbance, PLTP concentrations in plasma samples were measured using two dilutions (1:40 and 1:80). The intra- and inter-assay variations were 8% and 13%, respectively.

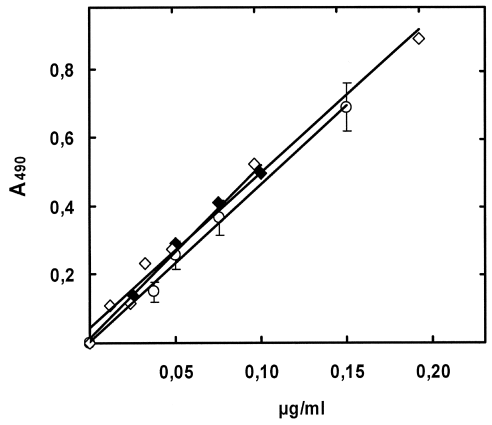

Fig. 2. Calibration of the ELISA for determination of PLTP mass. The preparations [recombinant-PLTP (open diamonds), purified plasma PLTP (closed diamonds), and secondary plasma calibrator (open circles)] used for calibration of the ELISA were incubated with 0.5% SDS for 30 min at 22°C. Dilutions were performed (0.1% Tween 20 in PBS) to obtain the standard curve in the range of 25–100 ng PLTP protein/ml. The ELISA assays were performed as described in Materials and Methods. MAb JH66 was used as the capture antibody, and polyclonal antibody R176 was used for detection. The curves are representative of three independent measurements. The ±SD of the secondary calibrator, plasma, is displayed. A_{490}, absorbance at 490 nm.

Use of the ELISA to determine total PLTP concentration in serum

Having established the ELISA method, PLTP concentrations were determined in human serum samples obtained from 80 randomly selected individuals. The mean PLTP mass in the subjects was 5.81 ± 1.33 mg/l (mean ± SD) (range, 2.78–10.06 mg/l), and the mean activity was 5.84 ± 1.39 μmol/ml/h (range, 3.21–11.15 μmol/ml/h). In contrast to the previously reported ELISA method (24), the present assay revealed a weak, yet significant, correlation ($r = 0.345$, $P < 0.01$) between total serum PLTP activity and mass.

Measurement of the HA and LA forms of plasma PLTP

To be able to study the reactivity of the two forms of plasma PLTP using the new ELISA assay and their proportions in plasma, separation of LA-PLTP and HA-PLTP was necessary. We first applied size-exclusion chromatography (28) to separate the two forms of PLTP (**Fig. 4**). The differences in the reactivity of antibodies to HA- versus LA-PLTP using this chromatographic method have been dem-

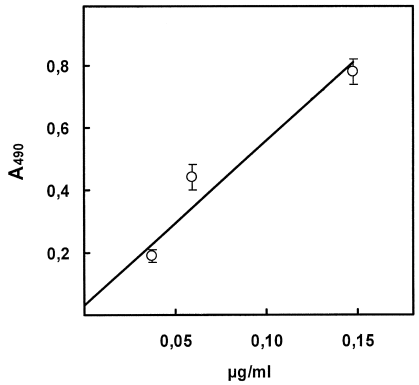

Fig. 3. Reproducibility of the PLTP ELISA. To study the reproducibility of the assay, plasma was treated with 0.5% SDS and thereafter subjected to ELISA as described in Materials and Methods. Between runs (n = 8), each standard point differed by an average of 8.25% from the mean value.

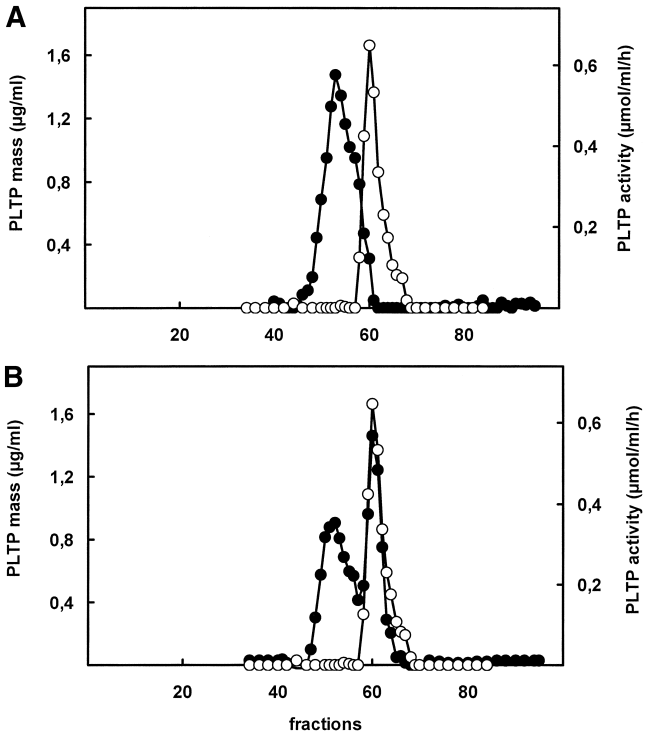

Fig. 4. Separation of the HA and LA forms of plasma PLTP by size-exclusion chromatography. Human plasma (1 ml) was subjected to size-exclusion chromatography. PLTP activity (open circles) and mass (closed circles) in the eluted fractions were analyzed. The mass assay was carried out either without (A) or with (B) SDS pretreatment of the fractions before ELISA, as described in detail in Materials and Methods.

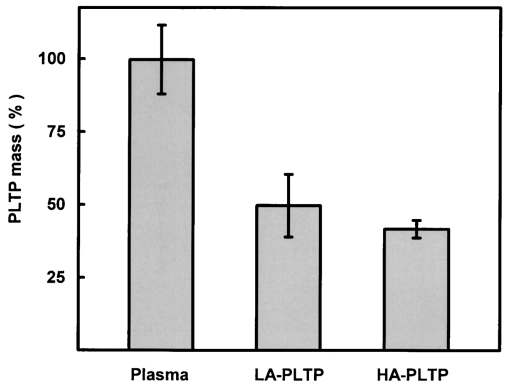

Fig. 5. Distribution of HA- and LA-PLTP mass after Heparin-Sepharose affinity chromatography of human plasma. Heparin-Sepharose affinity chromatography of six plasma samples was performed as described in Materials and Methods. The distribution of PLTP mass (mean ± SD) between the unbound (LA-PLTP) and bound (HA-PLTP) fractions was analyzed. All of the PLTP activity applied to the column was retained (activity recovery >95%) and could be eluted with 1 M NaCl.

onstrated (27). As previously described (29), PLTP activity eluted in fractions corresponding to the elution position of 160 kDa. When the previous nondenaturing ELISA was used, practically all of the detectable PLTP mass eluted in the position of LA-PLTP. However, after pretreatment of the fractions with SDS, it was evident that PLTP mass could also be reliably measured in the fractions displaying PLTP activity (35% HA-PLTP, 65% LA-PLTP). The data clearly support the recent suggestion (27) that different reactivities of anti-PLTP antibodies to HA- and LA-PLTP occur when they are in their native conformation.

In addition to size-exclusion chromatography, LA- and HA-PLTP forms can be separated by Heparin-Sepharose affinity chromatography (29). To verify that our new modified ELISA accurately measures both forms of PLTP in plasma, we performed repeated plasma runs on Heparin-Sepharose affinity chromatography and measured PLTP mass from the unbound and bound fractions (**Fig. 5**). It is evident that, of the PLTP mass applied on the Heparin-Sepharose column, ~50% is not retained by the matrix, representing LA-PLTP, and ~40% of the mass is bound. As more than 95% of the PLTP activity applied is bound (data not shown), the bound fractions represent the HA form of PLTP. The separation of the two forms of PLTP by Heparin-Sepharose affinity chromatography was reproducible as evaluated by repeated plasma runs. When the mass of LA-PLTP and HA-PLTP, separated by Heparin-Sepharose affinity chromatography, was assayed using the new modified ELISA and a calculated 40 ng of each was subjected to SDS-PAGE and Western blot analysis, scanning of the blots revealed that the intensities of LA-PLTP (28,760 pixels) and HA-PLTP (35,500 pixels) were similar (Fig. 1B). This experiment suggests that our modified ELISA measures both forms of plasma PLTP equally.

To further verify the new ELISA method and to study the immunoreactivity of the MAb JH66 antibody to the two forms of plasma PLTP, we performed immunoaffinity chromatography of plasma on Sepharose containing covalently linked MAb JH66. It was revealed that HA-PLTP does not bind to the antibody matrix, whereas LA-PLTP bound and could be eluted by a low-pH buffer (**Fig. 6**). Of the PLTP activity applied to the immunoaffinity column, none was retained as all could be recovered in the unbound fractions. Of the total plasma PLTP mass, 35% was not retained. After elution with a low-pH buffer (0.1 M glycine, pH 2.5), no activity but 67% of the PLTP mass could be recovered in fractions 17–19. These mass recoveries were obtained using the denaturing SDS modification of our ELISA. If no denaturing SDS pretreatment of the samples was performed, almost no PLTP mass was measurable in the nonretained fractions. These results support the conclusion that our new ELISA measures both forms of plasma PLTP.

SDS-polyacrylamide gel electrophoresis of the two PLTP forms followed by Western blotting with the MAb JH66 revealed that under denaturing conditions, both forms of PLTP could be visualized (data not shown). These experiments indicate that MAb JH66 recognizes only the LA-PLTP form in human plasma, but after SDS denaturation, both forms are reactive with the antibody.

In addition, we treated plasma with 0.5% SDS for 30 min and thereafter diluted the plasma sample to 0.02% SDS concentration and applied it to a MAb JH66 immu-

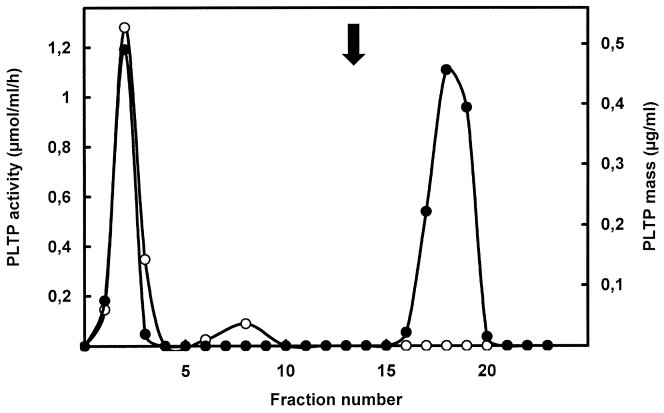

Fig. 6. Anti-PLTP immunoaffinity chromatography of human plasma. Immunoaffinity chromatography of plasma (1 ml) on an anti-PLTP antibody (MAb JH66) column was performed as described in Materials and Methods. Fractions were analyzed for PLTP activity (open circles) and mass (closed circles). The arrow indicates the start of low pH elution.

noaffinity chromatography column. All of the PLTP protein was retained by the column, demonstrating again that after SDS pretreatment of plasma, all PLTP protein reacts with our MAb JH66.

Although size-exclusion, Heparin-Sepharose, and immunoaffinity chromatography methods facilitate the separation and quantitation of the two forms of PLTP, they are too cumbersome to be used in measurements of clinical samples. To overcome this problem, we have introduced a new method, dextran sulfate ($DxSO_4$)-$CaCl_2$ precipitation, which has been used as a purification step for PLTP (5). Using our previous ELISA, it was evident that only trace amounts of PLTP protein could be measured in the $DxSO_4$-$CaCl_2$ supernatant, which contained almost all of the PLTP activity, suggesting that most of the LA-PLTP protein was in the precipitate. This was also confirmed by Western blotting (data not shown). Therefore, we subjected plasma samples (n = 8) to $DxSO_4$-$CaCl_2$ precipitation and analyzed the supernatant for PLTP activity and mass with the new ELISA to determine whether this method could be used to separate HA-PLTP and LA-PLTP in plasma samples. After $DxSO_4$-$CaCl_2$ precipitation of normolipidemic plasma, 84% of the PLTP activity and 37% of PLTP protein mass could be recovered in the supernatant fraction. This suggests that the 63% of PLTP mass precipitated by $DxSO_4$-$CaCl_2$ represents LA-PLTP and that HA-PLTP is almost quantitatively recovered in the supernatant. The mass distribution obtained with this method is similar to that obtained by the three chromatographic methods, suggesting that $DxSO_4$-$CaCl_2$ precipitation can be used as a rapid method to separate LA- and HA-PLTP when assaying a larger number of samples.

By subtracting the HA-PLTP mass measured in the $DxSO_4$-$CaCl_2$ supernatant from total plasma PLTP mass, a good estimate of the mass of LA-PLTP is obtained. Having established the assay method, we analyzed the mass of HA- and LA-PLTP from the serum of 80 randomly selected Finnish subjects, a subsample of the Health 2000 Health Examination Survey. The mean concentration of HA-PLTP protein was 1.87 ± 0.85 mg/l (mean ± SD), and the LA-PLTP in the precipitate represented 3.94 ± 1.40 mg/l. Therefore, the mass distribution of the two forms of PLTP in serum was 32% HA-PLTP and 68% LA-PLTP. The average PLTP activity recovered in the supernatant after $DxSO_4$-$CaCl_2$ precipitation represented 88% of the activity measured in serum before precipitation. The concentration of HA-PLTP from the $DxSO_4$-$CaCl_2$ supernatant showed a stronger positive correlation with PLTP activity in serum ($r = 0.536$, $P < 0.01$) than did total PLTP mass ($r = 0.345$, $P < 0.01$), whereas the LA-PLTP mass did not correlate with PLTP activity (**Fig. 7**).

DISCUSSION

In the present study, we report a novel, modified ELISA assay for PLTP mass determination. As suggested by Murdoch et al. (27), the previously described assay preferentially detects LA-PLTP from human plasma and reacts poorly with HA-PLTP. In the present assay, a pretreatment of the primary calibrator, the secondary standard used, and the plasma specimens to be analyzed with SDS detergent partially denatures PLTP. This apparently increases the accessibility of epitopes on HA-PLTP, resulting in similar immunoreactivity of LA-PLTP and HA-PLTP in the ELISA assay. This development enables quantitation of the distribution of human PLTP protein between the LA and HA forms and thus provides a tool for detecting possible changes in distribution in clinical serum samples.

The present assay is based on calibration with human r-PLTP produced using a baculovirus/insect cell system and then purified by a simple protocol involving two affinity chromatography steps. The calibrator is easy to produce and purify under readily controllable conditions and therefore provides a highly reproducible calibration tool. This makes the assay applicable for wider use in laboratories investigating lipoprotein metabolism. The total PLTP concentration in serum samples obtained with the present assay (5.81 ± 1.33 mg/l) is considerably lower than those reported previously with similar assays [15.6 ± 5.1 mg/ml (24) and 12.0 ± 3.0 mg/l (25)] but close to that reported using a different, competitive immunoassay (23). The reason for the difference in PLTP mass values measured is obviously the fact that the calibrator that was previously used was from purified active plasma PLTP (24) or r-PLTP (25). Therefore, in the absence of a denaturing treatment, these calibrators react poorly with the antibodies used in the assays. This results in low absorbance values obtained with the calibrators and, correspondingly, too high values are obtained for serum samples that contain abundant LA-PLTP. In the present assay, the denaturing treatment equalizes the immunoreactivity of the calibrator and the PLTP in serum samples, thus yielding more reliable PLTP mass values.

Why do the two forms of PLTP in plasma show different immunoreactivity? We have previously demonstrated by size-exclusion chromatography that LA-PLTP and HA-

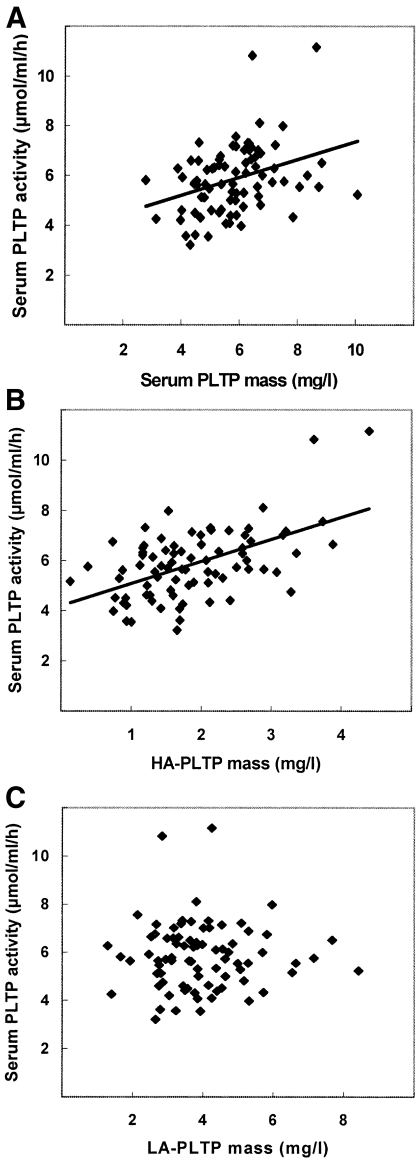

Fig. 7. Correlation between PLTP activity and LA- and HA-PLTP mass. PLTP activity and mass (using the denaturing ELISA) were determined in sera collected from 80 randomly chosen Finns. Sera were precipitated with $DxSO_4$-$CaCl_2$ as described in Materials and Methods. PLTP activity and mass in the supernatant were determined. The mass of LA-PLTP was calculated by subtracting the mass of HA-PLTP in the supernatant from total serum PLTP mass. A: Correlation between serum PLTP mass and activity ($r = 0.345$, $P < 0.01$). B: Correlation between HA-PLTP mass and serum PLTP activity ($r = 0.536$, $P < 0.01$). C: Correlation between LA-PLTP mass and serum PLTP activity ($r = 0.006$, $P =$ NS).

PLTP are associated with complexes of different size, LA-PLTP eluting at a position corresponding to 520 kDa and HA-PLTP at 160 kDa (28). Furthermore, LA-PLTP is found associated with apoA-I, whereas HA-PLTP is not (29), and active PLTP secreted by HepG2 hepatoma cells (resembling HA-PLTP in plasma) is found associated with apoE (40). As suggested previously (27), it is possible that PLTP adopts a different conformation when associated with particles of different size and surface curvature, which may lead to differential exposure of epitopes. On the other hand, interactions with other proteins and possibly lipids may result in masking of epitopes on the surface of PLTP. According to molecular modeling (33), the epitope for MAb JH66 should be exposed on the surface of PLTP. However, the model leaves open the possibility that the epitope may be hidden upon a change of conformation or upon interaction with a binding partner in the HA-PLTP complex. Partial denaturation by SDS most likely either disrupts interactions with protein or lipid partners, masking the epitope, and/or causes a conformational change, exposing the epitope.

As the present assay detects both forms of plasma PLTP, the only way to determine the mass of the individual LA- and HA-PLTP fractions is by separating the two PLTP populations before mass determination. In the present study, we used four methods to accomplish this: size-exclusion chromatography, Heparin-Sepharose affinity chromatography, immunoaffinity chromatography using the monoclonal PLTP antibody JH66, and $DxSO_4$-$CaCl_2$ precipitation. The results given by these four independent methods to separate the LA- and HA-PLTP are remarkably similar, suggesting that ~60% of plasma PLTP mass resides in the LA-PLTP fraction and 40% in the HA-PLTP fraction. Furthermore, when we applied the active fractions, as separated by the three chromatographic methods, to the non-SDS ELISA, we detected only negligible PLTP mass.

For the analysis of a large number of serum samples, $DxSO_4$-$CaCl_2$ precipitation is the most suitable approach and is therefore the method of choice for clinical use. PLTP mass can be analyzed from the total serum sample and from the supernatant obtained after precipitation. The mass value obtained from the supernatant is a measure of HA-PLTP, which can then be subtracted from the total serum PLTP mass, yielding LA-PLTP mass. We fractionated 80 serum samples from randomly selected Finnish individuals by $DxSO_4$-$CaCl_2$ precipitation and analyzed the concentrations of the LA and HA forms. The HA-PLTP mass measured from the $DxSO_4$-$CaCl_2$ supernatant correlated more strongly with PLTP activity than the total PLTP mass, thus demonstrating successful separation of HA-PLTP from the LA form. The concentration of LA-PLTP as calculated from the $DxSO_4$-$CaCl_2$ method did not correlate with serum PLTP activity, an observation that further illustrates the specificity of this method in the isolation of the two forms of PLTP. In addition, the mean specific activity of total PLTP from the 80 serum samples is 1.0 μmol/μg/h. Assuming that our $DxSO_4$-$CaCl_2$ precipitation results in complete separation of HA- and LA-PLTP, the anticipated specific activity recovered in the supernatant would be ~3.1 μmol/μg/h. Our observation that the PLTP present in the supernatant yields a specific activity of 2.6 μmol/μg/h, similar to that obtained for active PLTP secreted by HepG2 hepatoma cells (40), suggests that the separation we achieved is close to optimal.

In conclusion, the development of a PLTP ELISA mass assay presented in this study allows not only for the accurate measurement of total PLTP protein concentration in plasma and/or serum samples but also for the determina-

tion of the distribution of PLTP between LA-PLTP and HA-PLTP. In view of that, this methodology will be extremely valuable for future analyses of both PLTP forms and subsequently for establishing the relationship that PLTP mass and activity have with associated lipoproteins, plasma factors, and lipid metabolism overall.

The authors thank Sari Nuutinen, Ritva Nurmi, and Seija Puomilahti for their expert technical assistance. The authors would also like to thank Prof. Arpo Aromaa, the principal investigator of Health 2000 Health Examination Survey for providing serum samples. This work was supported by the International HDL Research Awards Program (to C.E., M.J., and V.M.O.), the Finnish Foundation for Cardiovascular Research (to M.K. and M.J.), the Sigrid Juselius Foundation (to V.M.O. and M.J.), Finska Läkaresällskapet (to S.S.), Magnus Ehrnrooths Stiftelse (to S.S.), and the Wihuri Research Foundation (to M.K.).

REFERENCES

1. Huuskonen, J., V. M. Olkkonen, M. Jauhiainen, and C. Ehnholm. 2001. The impact of phospholipid transfer protein (PLTP) on HDL metabolism. *Atherosclerosis.* **155:** 269–281.
2. Tall, A. 1995. Plasma lipid transfer proteins. *Annu. Rev. Biochem.* **64:** 235–257.
3. Jauhiainen, M., J. Huuskonen, M. Baumann, J. Metso, T. Oka, T. Egashira, H. Hattori, V. M. Olkkonen, and C. Ehnholm. 1999. Phospholipid transfer protein (PLTP) causes proteolytic cleavage of apolipoprotein A-I. *J. Lipid Res.* **40:** 654–664.
4. Jauhiainen, M., J. Metso, R. Pahlman, S. Blomqvist, A. van Tol, and C. Ehnholm. 1993. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem.* **268:** 4032–4036.
5. Tu, A-Y., H. Nishida, and T. Nishida. 1993. High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. *J. Biol. Chem.* **268:** 23098–23105.
6. Lagrost, L., A. Athias, P. Gambert, and C. Lallemant. 1994. Comparative study of phospholipid transfer activities mediated by cholesteryl ester transfer protein and phospholipid transfer protein. *J. Lipid Res.* **35:** 825–835.
7. Jiang, X. C., C. Bruce, J. Mar, M. Lin, Y. Ji, O. Francone, and A. R. Tall. 1999. Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. *J. Clin. Invest.* **103:** 907–914.
8. Kawano, K., S. C. Qin, M. Lin, A. R. Tall, and X. C. Jiang. 2000. Cholesteryl ester transfer protein and phospholipid transfer protein have nonoverlapping functions in vivo. *J. Biol. Chem.* **275:** 29477–29481.
9. Foger, B., S. Santamarina-Fojo, R. D. Shamburek, C. L. Parrot, G. D. Talley, and H. B. Brewer, Jr. 1997. Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. *J. Biol. Chem.* **272:** 27393–27400.
10. Jiang, X. C., O. L. Francone, C. Bruce, R. Milne, J. Mar, A. Walsh, J. L. Breslow, and A. R. Tall. 1996. Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. *J. Clin. Invest.* **98:** 2373–2380.
11. Ehnholm, S., K. W. van Dijk, B. van 't Hof, A. van der Zee, V. M. Olkkonen, M. Jauhiainen, M. Hofker, L. Havekes, and C. Ehnholm. 1998. Adenovirus-mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. *J. Lipid Res.* **39:** 1248–1253.
12. van Haperen, R., A. van Tol, P. Vermeulen, M. Jauhiainen, T. van Gent, P. van den Berg, S. Ehnholm, F. Grosveld, A. van der Kamp, and R. de Crom. 2000. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1082–1088.
13. Qin, S., K. Kawano, C. Bruce, M. Lin, C. Bisgaier, A. R. Tall, and X. C. Jiang. 2000. Phospholipid transfer protein gene knock-out mice have low high density lipoprotein levels, due to hypercatabolism, and accumulate apoA-IV-rich lamellar lipoproteins. *J. Lipid Res.* **41:** 269–276.
14. Bouly, M., D. Masson, B. Gross, X. C. Jiang, C. Fievet, G. Castro, A. R. Tall, J. C. Fruchart, B. Staels, L. Lagrost, and G. Luc. 2001. Induction of the phospholipid transfer protein gene accounts for the high density lipoprotein enlargement in mice treated with fenofibrate. *J. Biol. Chem.* **276:** 25841–25847.
15. Jiang, X. C., S. Qin, C. Qiao, K. Kawano, M. Lin, A. Skold, X. Xiao, and A. R. Tall. 2001. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat. Med.* **7:** 847–852.
16. Kawano, K., S. Quin, M. Lin, A. R. Tall, and X. C. Jiang. 1999. A synergistic increase in FC and CE in VLDL/LDL in mice with combined HL/PLTP deficiency suggests a joint role in selective uptake from VLDL. *Circ. Suppl.* **100:** I.
17. Lie, J., R. de Crom, T. van Gent, R. van Haperen, L. Scheek, I. Lankhuizen, and A. van Tol. 2002. Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. *J. Lipid Res.* **43:** 1875–1880.
18. Van Haperen, R., A. van Tol, T. van Gent, L. Scheek, P. Visser, A. van Der Kamp, F. Grosveld, and R. de Crom. 2002. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J. Biol. Chem.* **277:** 48938–48943.
19. Riemens, S. C., A. van Tol, W. J. Sluiter, and R. P. Dullaart. 1998. Plasma phospholipid transfer protein activity is related to insulin resistance: impaired acute lowering by insulin in obese type II diabetic patients. *Diabetologia.* **41:** 929–934.
20. Riemens, S. C., A. van Tol, W. J. Sluiter, and R. P. Dullaart. 1999. Plasma phospholipid transfer protein activity is lowered by 24-h insulin and acipimox administration: blunted response to insulin in type 2 diabetic patients. *Diabetes.* **48:** 1631–1637.
21. Kaser, S., A. Sandhofer, B. Foger, C. F. Ebenbichler, B. Igelseder, L. Malaimare, B. Paulweber, and J. R. Patsch. 2001. Influence of obesity and insulin sensitivity on phospholipid transfer protein activity. *Diabetologia.* **44:** 1111–1117.
22. Cheung, M. C., G. Wolfbauer, H. Kennedy, B. G. Brown, and J. J. Albers. 2001. Plasma phospholipid transfer protein activity in patients with low HDL and cardiovascular disease treated with simvastatin and niacin. *Biochim. Biophys. Acta.* **1537:** 117–124.
23. Desrumaux, C., A. Athias, G. Bessede, B. Verges, M. Farnier, L. Persegol, P. Gambert, and L. Lagrost. 1999. Mass concentration of plasma phospholipid transfer protein in normolipidemic, type IIa hyperlipidemic, type IIb hyperlipidemic, and non-insulin-dependent diabetic subjects as measured by a specific ELISA. *Arterioscler. Thromb. Vasc. Biol.* **19:** 266–275.
24. Huuskonen, J., M. Ekström, E. Tahvanainen, A. Vainio, J. Metso, P. Pussinen, C. Ehnholm, V. M. Olkkonen, and M. Jauhiainen. 2000. Quantification of human plasma phospholipid transfer protein (PLTP): relationship between PLTP mass and phospholipid transfer activity. *Atherosclerosis.* **151:** 451–461.
25. Oka, T., T. Kujiraoka, M. Ito, M. Nagano, M. Ishihara, T. Iwasaki, T. Egashira, N. E. Miller, and H. Hattori. 2000. Measurement of human plasma phospholipid transfer protein by sandwich ELISA. *Clin. Chem.* **46:** 1357–1364.
26. Oka, T., S. Yamashita, T. Kujiraoka, M. Ito, M. Nagano, Y. Sagehashi, T. Egashira, M. N. Nanjee, K. Hirano, N. E. Miller, Y. Matsuzawa, and H. Hattori. 2002. Distribution of human plasma PLTP mass and activity in hypo- and hyperalphalipoproteinemia. *J. Lipid Res.* **43:** 1236–1243.
27. Murdoch, S. J., G. Wolfbauer, H. Kennedy, S. M. Marcovina, M. C. Carr, and J. J. Albers. 2002. Differences in reactivity of antibodies to active versus inactive PLTP significantly impacts PLTP measurement. *J. Lipid Res.* **43:** 281–289.
28. Oka, T., T. Kujiraoka, M. Ito, T. Egashira, S. Takahashi, M. N. Nanjee, N. E. Miller, J. Metso, V. M. Olkkonen, C. Ehnholm, M. Jauhiainen, and H. Hattori. 2000. Distribution of phospholipid transfer protein in human plasma: presence of two forms of phospholipid transfer protein, one catalytically active and the other inactive. *J. Lipid Res.* **41:** 1651–1657.
29. Kärkkäinen, M., T. Oka, V. M. Olkkonen, J. Metso, H. Hattori, M. Jauhiainen, and C. Ehnholm. 2002. Isolation and partial characterization of the inactive and active forms of human plasma

phospholipid transfer protein (PLTP). *J. Biol. Chem.* **277:** 15413–15418.
30. Aromaa, A., and S. Koskinen, editors. 2002. Health and Functional Capacity in Finland: Baseline Results of the Health 2000 Health Examination Survey. National Public Health Institute, Helsinki, Finland.
31. Huuskonen, J., V. M. Olkkonen, M. Jauhiainen, T. Sareneva, P. Somerharju, and C. Ehnholm. 1998. Oxidative modification of HDL_3 in vitro and its effect on PLTP-mediated phospholipid transfer. *Biochim. Biophys. Acta.* **1391:** 181–192.
32. Huuskonen, J., V. M. Olkkonen, C. Ehnholm, J. Metso, I. Julkunen, and M. Jauhiainen. 2000. Phospholipid transfer is a prerequisite for PLTP-mediated HDL conversion. *Biochemistry.* **39:** 16092–16098.
33. Huuskonen, J., G. Wohlfahrt, M. Jauhiainen, C. Ehnholm, O. Teleman, and V. M. Olkkonen. 1999. Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis. *J. Lipid Res.* **40:** 1123–1130.
34. Settasatian, N., M. Duong, L. K. Curtiss, C. Ehnholm, M. Jauhiainen, J. Huuskonen, and K-A. Rye. 2001. The mechanism of the remodelling of high density lipoproteins by phospholipid transfer protein. *J. Biol. Chem.* **276:** 26898–26905.
35. Damen, J., J. Regts, and G. Scherphof. 1982. Transfer of [^{14}C]phosphatidylcholine between liposomes and human plasma high-density lipoprotein. Partial purification of a transfer-stimulating plasma factor using a rapid transfer assay. *Biochim. Biophys. Acta.* **712:** 444–452.
36. Kato, H., T. Nakanishi, T. Arai, H. I. Nishida, and T. Nishida. 1988. Purification, microheterogeneity, and stability of human lipid transfer protein. *J. Biol. Chem.* **264:** 4082–4087.
37. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
38. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76:** 4350–4354.
39. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
40. Siggins, S., M. Jauhiainen, V. M. Olkkonen, J. Tenhunen, and C. Ehnholm. 2003. Phospholipid transfer protein (PLTP) secreted by HepG2 cells resembles the high-activity PLTP form in human plasma. *J. Lipid Res.* **44:** 1453–1461.