Interfacial and Lipid Transfer Properties of Human Phospholipid Transfer Protein: Implications for the Transfer Mechanism of Phospholipids†

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ABSTRACT: In circulation the phospholipid transfer protein (PLTP) facilitates the transfer of phospholipid-rich surface components from postlipolytic chylomicrons and very low density lipoproteins (VLDL) to HDL and thereby regulates plasma HDL levels. To study the molecular mechanisms involved in PLTP-mediated lipid transfer, we studied the interfacial properties of PLTP using Langmuir phospholipid monolayers and asymmetrical flow field−flow fractionation (AsFlFFF) to follow the transfer of ^{14}C-labeled phospholipids and [^{35}S]PLTP between lipid vesicles and HDL particles. The AsFlFFF method was also used to determine the sizes of spherical and discoidal HDL particles and small unilamellar lipid vesicles. In Langmuir monolayer studies high-activity (HA) and low-activity (LA) forms of PLTP associated with fluid phosphatidylcholine monolayers spread at the air/buffer interphase. Both forms also mediated desorption of [^{14}C]dipalmitoylphosphatidylcholine (DPPC) from the phospholipid monolayer into the buffer phase, even when it contained no physiological acceptor such as HDL. After the addition of HDL_3 to the buffer, HA-PLTP caused enhanced lipid transfer to them. The particle diameter of HA-PLTP was ∼6 nm and that of HDL_3 ∼8 nm as determined by AsFlFFF analysis. Using this method, it could be demonstrated that in the presence of HA-PLTP, but not LA-PLTP, [^{14}C]DPPC was transferred from small unilamellar vesicles (SUV) to acceptor HDL_3 molecules. Concomitantly, [^{35}S]-HA-PLTP was transferred from the donor to acceptor, and this transfer was not observed for its low-activity counterpart. These observations suggest that HA-PLTP is capable of transferring lipids by a shuttle mechanism and that formation of a ternary complex between PLTP, acceptor, and donor particles is not necessary for phospholipid transfer.

The phospholipid transfer protein (PLTP)[1] is a member of the lipid transfer/lipopolysaccharide binding protein family that also includes the cholesterol ester transfer protein (CETP), the lipopolysaccharide (LPS) binding protein (LBP), and bactericidal/permeability increasing protein (BPI) (*1*). The BPI protein has been crystallized and the X-ray structure determined (*2*). On the basis of these data and the sequence for PLTP from several species a molecular model of PLTP has been constructed (*3*). The model predicts a two-domain architecture with conserved N- and C-terminal lipid-binding pockets consisting of hydrophobic residues in each domain. In BPI these pockets bind phospholipid molecules whereas LBP can bind both LPS and phospholipids.

Human PLTP contains 467 amino acids and is highly glycosylated (*1*, *4*). In humans, PLTP expression is highest in the placenta, pancreas, adipose tissue, and lung with lower levels found in liver, kidney, and heart (*1*, *5*). PLTP is also found in human tear fluid where it may play a role in the maintenance of the exterior lipid film (*6*, *7*). In vitro studies have identified a number of functions for PLTP in lipoprotein metabolism (*8*). These include the transfer of phospholipids (PL), diacylglycerol (*9*), free cholesterol (*10*), α-tocopherol (vitamin E) (*11*), and lipopolysaccaride between lipoproteins and cells (*12*, *13*). A role for PLTP in atherogenesis has been suggested (*14*). Functions of PLTP which may influence the formation of atherosclerotic lesions include the generation of acceptors for lipid efflux from cells, regulation of plasma high-density lipoprotein (HDL) levels, protection of lipoproteins from oxidation, and regulation of production of atherogenic lipoproteins (*14*, *15*). In human plasma two distinct forms of PLTP are present, one with high activity (HA-PLTP) and another with low activity (LA-PLTP) (*16*). These two forms are associated with different apolipoproteins during their purification (*17*). Intriguingly, serum apolipo-

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[1] Abbreviations: apoA-I, apolipoprotein A-I; AsFlFFF, asymmetrical flow field−flow fractionation; DPPC, dipalmitoylphosphatidylcholine; HDL, high-density lipoprotein; PL, phospholipid; PLTP, phospholipid transfer protein; LA-PLTP, low activity PLTP; HA-PLTP, high activity PLTP; POPC, palmitoyloleoylphosphatidylcholine; SUV, small unilamellar vesicle; π, surface pressure.

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proteins do not seem to be a requisite for PLTP activity as we recently observed that human tear fluid contains high concentrations of highly active PLTP although it contains no apolipoproteins (*6*). The physiological significance of this finding is yet to be revealed as well as the function and possible interacting protein partners present in tear fluid.

Although the role of PLTP in lipoprotein metabolism and atherogenesis has been intensively studied in gene-targeted mouse models and using in vitro experiments, the physiological role of PLTP in human metabolism is far from resolved. Another unsolved question is by what mechanisms do PLTP as well as CETP mediate lipid transfer between the lipid donors and acceptors? On the basis of kinetic evidence it has been suggested that CETP facilitates lipid transport by a *shuttle* mechanism rather than by forming a *ternary complex* between CETP, HDL, and the acceptor lipoprotein particles like VLDL and LDL (*18*−*20*). In favor for this model of lipid transport it was later demonstrated that CETP can absorb lipids from phospholipid monolayers residing at the air−water interface (*21*). However, a matter of controversy whether CETP also can facilitate transport of lipids via formation of a ternary complex still remains.

To elucidate the mechanism(s) by which PLTP transfers its principal substrate, phospholipids, we studied its interactions with phospholipid monolayers and demonstrate that PLTP is surface active. We suggest a model where PLTP first binds to fluid phospholipid monolayers and from them facilitates desorption of phospholipid molecules which it transfers to acceptor particles and finally releases the lipid from the protein. Monolayer studies were further complemented by using asymmetrical flow field−flow fractionation (AsFlFFF), which is an analytical separation technique capable of separating polymers and particles ranging in size from a few nanometers (approximately 1 nm) to micrometers (approximately 50 μm). Multimodal distributions differing by 10% in diameter can easily be determined. Compared to other FFF methods, AsFlFFF is more universal and efficient with a broader application range. It has been used for the separation and characterization of a large number of different analytes, including proteins, lipoproteins, liposomes, chromosomes, DNA, viruses, blood cells, emulsions, drug carriers, starch, polysaccharides, diagnostic particles, and latex dispersions. The instrumental setup of an AsFlFFF system is comparable to a size exclusion chromatography; however, fractionation of samples takes place in a separation channel instead of a separation column. Since separation in AsFlFFF takes place in an empty spacer, interactions between analytes and the stationary phase, which are often encountered in size exclusion chromatography, can be minimized. Taken together, the data suggest that PLTP transfers phospholipids by a shuttle mechanism, a conclusion compatible with that demonstrated for other lipid transfer proteins.

EXPERIMENTAL PROCEDURES

Isolation of Lipoproteins and PLTP. HDL_3 was isolated from human plasma by sequential ultracentrifugation. PLTP was purified from human plasma, and the two forms of PLTP, HA-PLTP and LA-PLTP were isolated as described earlier (*17*, *22*). Reconstituted apolipoprotein A-I (apoA-I) particles were prepared by the cholate dialysis method. In brief, to a glass vial were added 7.7 mg of egg yolk phosphatidylcholine [egg PC, Sigma, 10 mg/mL stock in chloroform−methanol, 9:1 (v/v)] and 0.193 mg of cholesterol (Sigma, 1 mg/mL stock in benzene), and the organic solvents were evaporated under nitrogen at room temperature. After this the mixture was lyophilized for 30 min to remove all traces of solvents. After lyophilization 3 mL of TBS buffer (10 mM Tris-HCl, 1 mM EDTA, and 140 mM NaCl, pH 7.4) was added, the mixture was vigorously vortexed, and finally 1 mg of apoA-I was added. Sodium cholate (stock solution: 0.725 M in TBS) was then added (55 mM final concentration), and the mixture was gently mixed on vortex (4 × 15 s) avoiding foaming. The mixture was next incubated for 20 min at 24 °C in a shaking water bath. Finally, the mixture was dialyzed against TBS for about 72 h at 4 °C using 3500 cutoff dialysis tubing. After dialysis the final volume was adjusted to 4.0 mL. The procedure was exactly the same for preparing apoA-I−egg PC discoidal particles. The molar ratios for the discoidal particles were as follows: apoA-I:PC:chol, 1:137:12.5 (mol/mol), and apoA-I:PC, 1:137 (mol/mol).

Analysis of PLTP Activity and Mass. PLTP activity was measured using the radiometric assay described by Damen et al. (*23*) with minor modifications (*22*). PLTP mass was determined with the ELISA assay (*24*).

Preparation of Vesicles. POPC (palmitoyloleoylphosphatidylcholine) was purchased from Avanti (Birmingham, AL). [^{14}C]DPPC (dipalmitoylphosphatidylcholine; specific activity 2.22 GBq/mmol, purity ∼99%) was obtained from Amersham Biosciences and used without further purification. Phospholipid stock solutions were made in chloroform. [^{14}C]-DPPC SUVs were prepared as previously described (*22*). In brief, 10 μmol of egg PC (Sigma), 20 μL (corresponding to 1 μCi) of [^{14}C]DPPC, and 100 nmol of butylhydroxytoluene (BHT) antioxidant (stock: 1 nmol/μL in chloroform) were pipetted into glass tubes on ice. Organic solvent was evaporated under nitrogen until dry and residual organic solvent was removed by lyophilization for 30 min. Onto the dry lipid film was added 1 mL of PLTP buffer (10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.4), and the mixture was vortexed vigorously to solubilize the lipid. The suspension was then probe sonicated for 3 × 5 min, carefully keeping the tube in the ice bath. After each 5 min sonication, the tubes were left to stand on ice for 1 min, and then sonication was continued, avoiding foaming. After this protocol, the mixture became slightly opaque. The sonicated material was subsequently transferred into Eppendorf tubes (1 mL) and centrifuged for 10 min at 15000 rpm at room temperature to pellet particulate material and titanium residual released from the sonicator probe. The opalescent PC vesicles were transferred to a new 1 mL Eppendorf tube, and the tube was filled with nitrogen and kept at 4 °C wrapped in aluminum foil. Using this procedure, radioactivity counting of 15 μL of this substrate yielded ∼15000 cpm (16300 dpm, counting efficiency 92%).

Radiolabeling of PLTP. Purified human PLTP was radiolabeled with the [^{35}S]sulfur labeling reagent (Amersham Biosciences) using the method described earlier (*25*). Shortly, purified HA-PLTP (24 μg) and LA-PLTP (300 μg) were dialyzed against 0.1 M sodium borate buffer, pH 8.5, in the volume of 1 mL. The dialyzed samples were then mixed with [^{35}S]sulfur labeling reagent (5 μCi/mg of protein), and

the mixture was kept on ice for 45 min. The reaction was stopped by adding 50 μL of 1 M glycine in 0.1 M sodium borate buffer, pH 8.5, and kept on ice for 5 min. Unbound radioactivity was removed on a PD-10 gel filtration column (Pharmacia, Uppsala, Sweden). PLTP-facilitated PL transfer activity was 10900 nmol mL^{-1} h^{-1}, indicating a very good recovery of 98% when compared to the starting material. Specific radioactivity of the HA-PLTP after labeling was 4000 dpm/μg and that for LA-PLTP 24800 dpm/μg.

Interaction of PLTP with Lipid Monolayers. Lipid monolayers residing on an air−buffer interface provide a convenient means to assess membrane partitioning of PLTP by monitoring the increase in surface pressure caused by insertion of the protein into the film. Penetration of PLTP into zwitterionic POPC/DPPC (80:20 molar ratio) or [^{14}C]-DPPC films was measured using circular Teflon-coated wells (subphase volume 1200 μL). Surface pressure (π) was monitored with a Wilhelmy wire attached to a microbalance (μTroughS; Kibron Inc., Helsinki, Finland) and connected to a Pentium personal computer. The monolayer was spread on an air−buffer (25 mM sodium phosphate buffer and 150 mM NaCl, pH 6.8) interface by dropwise addition of phospholipid−chloroform (1 mg/mL) spreading solution from a Hamilton syringe to different initial surface pressures (π_0). The resulting monolayers were allowed to equilibrate for 15 min before addition of HA-PLTP (10 μL; stock solution of 160 μg of HA-PLTP/mL in sodium phosphate buffer and 150 mM NaCl, pH 6.8) or LA-PLTP (10 μL; stock solution of 150 μg of HA-PLTP/mL in the above buffer) into the subphase. The addition of an equal volume of buffer did not change π. The increment in π from the initial surface pressures (π_0) after the addition of PLTP was complete in <60 min, and the difference between π_0 and the value observed after binding of PLTP into the film (π_f) was taken as $\Delta\pi$. All measurements were performed at ambient temperature (~24 °C). The data are represented as $\Delta\pi$ versus π_0.

Absorption of DPPC from the Air−Buffer Interface. A monolayer of [^{14}C]DPPC was spread on an air−buffer (25 mM sodium phosphate and 150 mM NaCl, pH 6.8) interface to an initial surface pressure of ~7 mN/m. The surface pressure was controlled as described above. Monolayer was allowed to equilibrate for 15 min before the addition of 10 μL of either HA-PLTP (stock solution: 160 μg/mL) or LA-PLTP (stock solution: 150 μg/mL), HDL_3 (6.3 mg/mL), or a mixture of either LA- or HA-PLTP combined with HDL_3 into the subphase. On the basis of earlier reaction kinetics of apolipoproteins and CETP, the lipid/buffer system was incubated for 3 h (*21*), and one-fourth (300 μL) of the subphase volume was collected for liquid scintillation counting (Wallac Winspectral 1414 liquid scintillation counter, Turku, Finland). The results (dpm) are expressed as a mean (±standard deviation) from triplicate wells.

Asymmetrical Flow Field−Flow Fractionation. Miniaturized and conventional asymmetrical flow field−flow fractionation (mAsFlFFF and AsFlFFF, respectively) channels constructed in-house were utilized. Detection was performed with a UV/vis detector (HP1050 model 79853C, Tokyo, Japan). The geometrical area of the accumulation wall and volume were 6 cm^2 and 0.25 mL for the miniaturized scale and 76 cm^2 and 3.7 mL for the conventional scale. Dimensions for mAsFlFFF were 11 cm, 0.7 and 0.35 cm at inlet and outlet, with a spacer thickness of 500 μm. Dimensions for the AsFlFFF were 38 cm × 2 cm × 500 μm.

For both systems, a regenerated cellulose acetate ultrafiltration membrane with a molar mass cutoff of 10 kDa (DSS-RC70PP, Nakskov, Denmark) was laid on top of the porous frit. An HPLC pump (model PU-980, Jasco International Co., Ltd., Tokyo, Japan) was used to deliver carrier solution during the injection−relaxation−focusing and running periods. During the injection−relaxation−focusing period the carrier liquid was delivered from both the front and back side of the channel. The outlet flow from the channel was monitored at 254 or 280 nm.

The carrier liquid used in AsFlFFF was 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, and 0.02% NaN_3 at pH 7.4. The temperature was 22 ± 2 °C. The actual channel thickness was calculated from the retention time of bovine serum albumin in 10 mM phosphate−150 mM NaCl solution, at 20 °C, having a diffusion coefficient of 6.21×10^{-7} cm^2/s.

Relaxation−focusing conditions using mAsFlFFF for HA-PLTP, HDL_3, and apoAI were as follows: frontal flow rate, 0.1 mL min^{-1}; flow inward from outlet, 1.4 mL min^{-1}; injection, 0.5 mL min^{-1} for 1−2 min; relaxation time, 20 min. Flow rates during elution were 0.3 and 0.5 mL min^{-1} for the main flow rate at the outlet and cross-flow rate, respectively.

Relaxation−focusing conditions using AsFlFFF for SUV, purified HA-PLTP + HDL_3 + SUV, LA-PLTP + HDL_3 + SUV, HA-PLTP + phospholipid−cholesterol discoidal particles + SUV, and LA-PLTP + phospholipid−cholesterol discoidal particles + SUV were as follows: frontal flow rate, 0.1 mL min^{-1}; flow inward from outlet, 2.6 mL min^{-1}; injection, 1 mL min^{-1} for 5 min; relaxation time, 30 min. Flow rates during elution were 0.9 and 2.2 mL min^{-1} for the main flow rate at the outlet and cross-flow rate, respectively.

Lipid Transfer Assays and Asymmetrical Flow Field−Flow Fractionation. A volume of 15 μL of the donor (DPPC small unilamellar vesicles, 150 nmol calculated as phosphatidylcholine) and 250 μg of acceptor (HDL_3, apoA-I−phospholipid or apoA-I−phospholipid−cholesterol discoidal particles, calculated as protein) were placed in polypropylene microsample tubes (Eppendorf), and the mixture was diluted to 400 μL with the phosphate buffer solution (10 mM phosphate, 150 mM NaCl, and 1 mM EDTA, pH 7.4). The microsample tubes were placed in a horizontal shaking bath at a speed of 100 cycles/min for 45 min at 37 °C. After incubation was completed, the sample was diluted to 10 mL with the same buffer and then injected to AsFlFFF at 1.0 mL/min for 10 min.

To elucidate the ability of PLTP to transfer lipids from donor SUVs to acceptor particles, either LA (c = ~32 μg/mL) or HA (c = ~10−15 μg/mL) PLTP was added separately to the mixture before incubation. To demonstrate the actual transfer of the lipid and movement of the PLTP protein itself, [^{14}C]DPPC-labeled SUV and ^{35}S-labeled PLTP were used. After AsFlFFF the radioactivity of collected fractions was measured with liquid scintillation counting.

RESULTS

Interaction of PLTP with Phospholipid Monolayers. To assess the partitioning of PLTP between the phospholipid

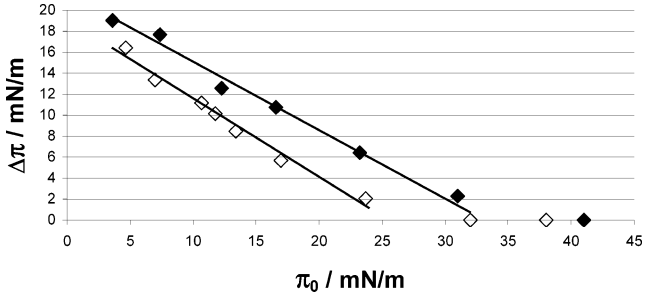

FIGURE 1: Interaction of PLTP with lipid monolayers. HA- (open squares) and LA- (solid squares) PLTP was injected into the subphase beneath POPC/DPPC monolayers spread at increasing initial surface pressures (π_0). The surface pressure was monitored until no changes were seen.

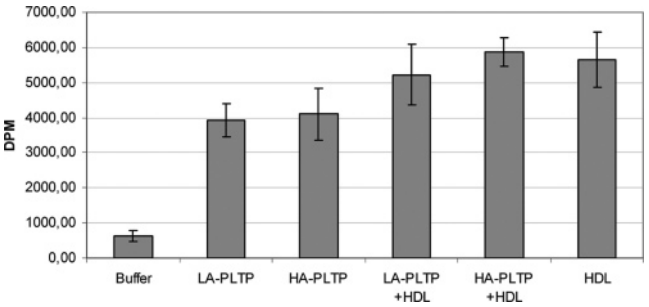

FIGURE 2: PLTP-mediated desorption of phospholipids from a phospholipid monolayer. LA- or HA-PLTP and HDL_3 molecules were injected into the subphase beneath a [^{14}C]DPPC monolayer at an initial surface pressure of ~7 mN/m. The radioactivity (dpm) of the subphase collections was measured after a 3 h incubation.

monolayer and the aqueous subphase as a function of monolayer surface pressure, we prepared POPC/DPPC (80:20 molar ratio) phospholipid monolayers in a Teflon-coated well. The initital surface pressures ranged from 4 to 41 mN/m. To these monolayers was injected 1.6 μg of HA-PLTP or 1.5 μg of LA-PLTP into the subphase, and the adsorption of PLTP to the interface was monitored by the change in surface pressure ($\Delta\pi$) (Figure 1). The binding of HA-PLTP to the lipid−buffer interface decreased linearly with increasing π_0 (Figure 1); linear extrapolation of the $\pi_0-\Delta\pi$ curve indicated that HA-PLTP could not penetrate the PL monolayer surface at $\pi_0 \geq 25$ mN/m. At higher π_0 values no changes in surface pressure were detected. Similar results were obtained when using LA-PLTP, except that the critical surface penetration pressure was estimated to be 33 mN/m (Figure 1). Also isolated lipid-free apoA-I was incorporated into the PL monolayer, albeit the interaction was weaker (data not shown).

Transfer of Phospholipid from the Monolayer to the Aqueous Phase. To study whether PLTP can cause desorption of PL from the fluid monolayer and transfer it to the aqueous subphase, we prepared [^{14}C]DPPC monolayers. The initial surface pressure for these monolayers was 7 ± 0.5 mN/m. The desorption of PL from the monolayer and transfer to the subphase following the addition of PLTP, HDL_3, or a combination of them are displayed in Figure 2. After a 3 h incubation following the addition of either LA-PLTP or HA-PLTP, the radioactivity recovered in the subphase was increased almost 7-fold as compared to that observed for the control monolayers.

We analyzed PLTP mass in the subphase by using the ELISA method. From the added total HA-PLTP 10.3% (134 ng) was in the subphase; i.e., 89.7% of the HA-PLTP was associated with the lipid monolayer. Analogously 11.0% (167 ng) of LA-PLTP was found in the subphase, and therefore 89.0% is bound on the monolayer. From the added HDL (119 μg) equal amounts were recovered in the lipid monolayer and in the subphase. We then analyzed the amount of total desorbed DPPC-associated radioactivity in the subphase. Without any added proteins 4.2% of the total radioactivity could be accounted in the subphase. After 3 h incubation with LA-PLTP 26.4% of the total radioactivity was encountered in the subphase, whereas in the presence of HA-PLTP and HDL_3 27.6% and 38%, respectively, were found in the subphase. In the presence of both LA-PLTP or HA-PLTP and HDL_3, 35.2% and 39.5%, respectively, were found in the subphase.

The measured radioactivities (dpm) of collected subphases clearly demonstrated the protein/lipoprotein enhanced transfer of DPPC from the PL monolayer (Figure 2). The measured transfer properties for PLTP and HDL_3 were similar, while the mixture of them showed a small increase in lipid transfer to the subphase. Estimation of the stoichiometry and the specific activity of DPPC/PLTP binding from the subphase indicated that on average 5.5 mol of DPPC/mol of PLTP was desorbed to the subphase. Accordingly, in addition to the two defined lipid-binding pockets more lipid molecules are attached to the surface of PLTP in accordance with previous results (*10*).

Studies on PLTP-Mediated Phospholipid Transfer Using Asymmetrical Flow Field−Flow Fractionation. Flow field−flow fractionation has previously been shown to be a useful method to separate lipoprotein particles (*26*−*28*). In this work AsFlFFF was used for the separation and fractionation of HA-PLTP, HDL_3, SUV, and their mixtures. The components were efficiently separated, and the diameter of HA-PLTP was ~6 nm, that of HDL_3 ~8 nm, and that of SUVs ~25 nm (Figure 3). Discoidal reconstituted apoA-I−phospholipid−cholesterol particles were resolved in three peaks due to the formation of three discoidal subgroups which have also previously been detected by native gradient gel electrophoresis (*29*, *30*).

To clarify the mechanism whereby PLTP facilitates lipid transfer, we incubated radioactively labeled ([^{14}C]DPPC) egg PC−SUVs and HDL_3 in the presence of HA-PLTP (activity 10 μmol mL^{-1} h^{-1}, Figure 4A) or LA-PLTP (activity 10 nmol mL^{-1} h^{-1}, Figure 4B). After incubation the mixture was subjected to AsFlFFF analysis. Figure 4 reveals that the radioactive phospholipid at time 0 was almost exclusively associated with the SUVs. During the 45 min incubation, however, approximately 30% of the radioactivity was transferred to a position corresponding to HDL_3 particles. This transfer only occurs in the presence of HA-PLTP while the inactive form, LA-PLTP, does not mediate transfer of phospholipids (Figure 4B). Neither did incubation of SUVs and HDL_3 in the absence of LA- or HA-PLTP cause transfer of SUV-associated radioactivity (data not shown). The size of the different particles remained unaltered (Figure 4).

In order to record the distribution of PLTP among the different particles (either SUVs or HDL_3) during the phospholipid transfer process, we used ^{35}S-labeled HA- and LA-PLTP. At the start (time point, 0 min) both forms of [^{35}S]PLTP were associated with the same fractions as the SUVs (Figure 4C,D). However, after a 45 min incubation a

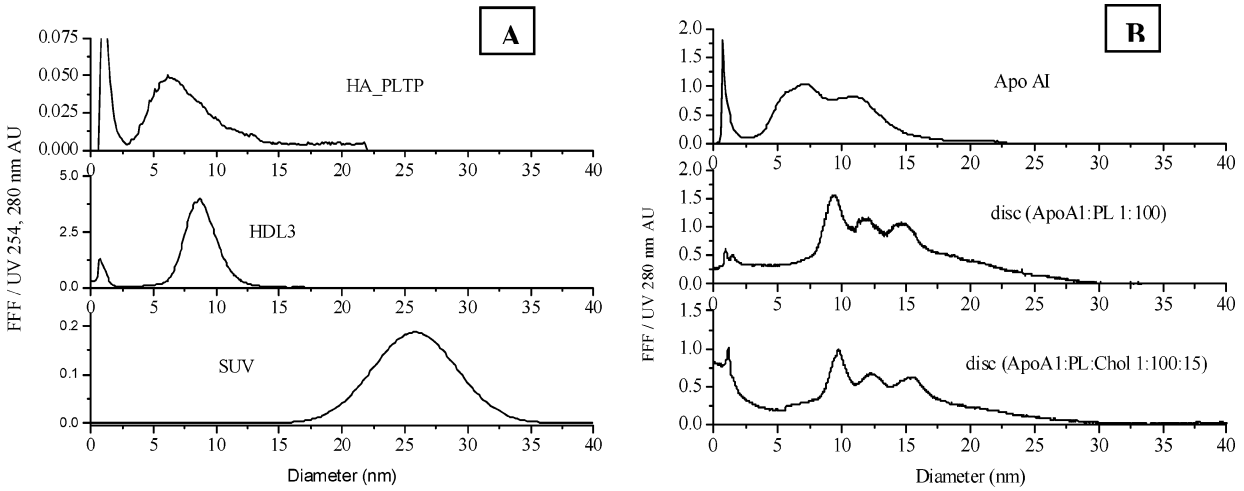

FIGURE 3: Lipoprotein particle diameters measured by AsFlFFF. Panel A shows the particle diameters of HA-PLTP (~6 nm), HDL_3 (~8 nm), and SUV (~26 nm) and panel B those of apoA-I and apoA-I−phospholipid−cholesterol discoidal particles. Carrier solution: 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4. See Experimental Procedures for details.

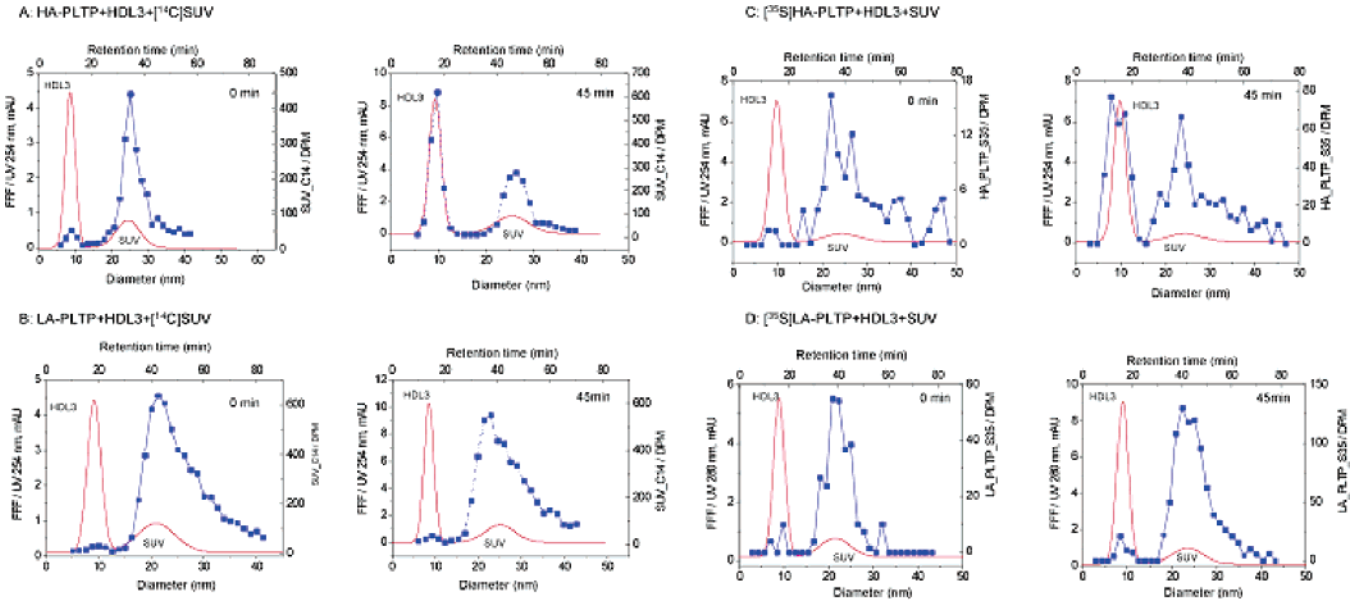

FIGURE 4: AsFlFFF analysis of lipid transfer from SUVs to HDL_3 by PLTP. [^{14}C]DPPC-labeled SUVs (~25 nm, measured by AsFlFFF) and HDL_3 molecules (~8 nm) were incubated in the presence of HA-PLTP (panel A) and LA-PLTP (panel B), and the radioactivity of radioactive DPPC was monitored at 0 and 45 min. In another set of experiments ^{35}S-labeled HA-PLTP (panel C) and [^{35}S]-LA-PLTP (panel D) were incubated with HDL_3 and nonlabeled SUVs after which radioactivity was measured from the AsFlFFF fractions. The solid lines are particle size or retention time fractograms obtained by AsFlFFF. Carrier solution: 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4. See Experimental Procedures for details.

marked transfer (approximately 26%) of the radioactively labeled [^{35}S]-HA-PLTP to the HDL_3 position was evident (Figure 4C). No such transfer of radioactively labeled [^{35}S]-LA-PLTP was observed (Figure 4D). These results demonstrate that while HA-PLTP has the ability to attach and detach from lipid environments and transfer lipids to HDL_3, LA-PLTP cannot perform these functions.

To determine the ability of discoidal apoA-I−phospholipid and apoA-I−phospholipid−cholesterol particles to receive lipids from PLTP molecules, these particles were incubated with [^{14}C]DPPC containing SUV donors. After incubation for 45 min and following AsFlFFF, no transfer of radioactivity was detected in any of our experiments (Figure 5). The data indicate that these disk-shaped apoA-I particles do not act as acceptors in phospholipid transfer processes as mediated by active PLTP.

DISCUSSION

In human plasma, lipids can be exchanged and transferred between different lipoprotein classes by spontaneous, temperature-dependent transfer or by protein-mediated transfer (*31*). Protein-mediated transfer and exchange of lipids as facilitated by the two known lipid transfer proteins in plasma, CETP and PLTP, have not been fully investigated. PLTP in human plasma exists in two different forms, one with high activity (HA) and the other with low activity (LA) (*16*). Only one gene has been reported to code for both forms of the protein, indicating that PLTP activity regulation occurs at the posttranslational level. The two PLTP forms in human plasma have been shown to associate with distinct macromolecular populations. In size exclusion chromatography, PLTP activity has been demonstrated to elute with an average mass of 160 kDa, whereas the inactive form of PLTP has

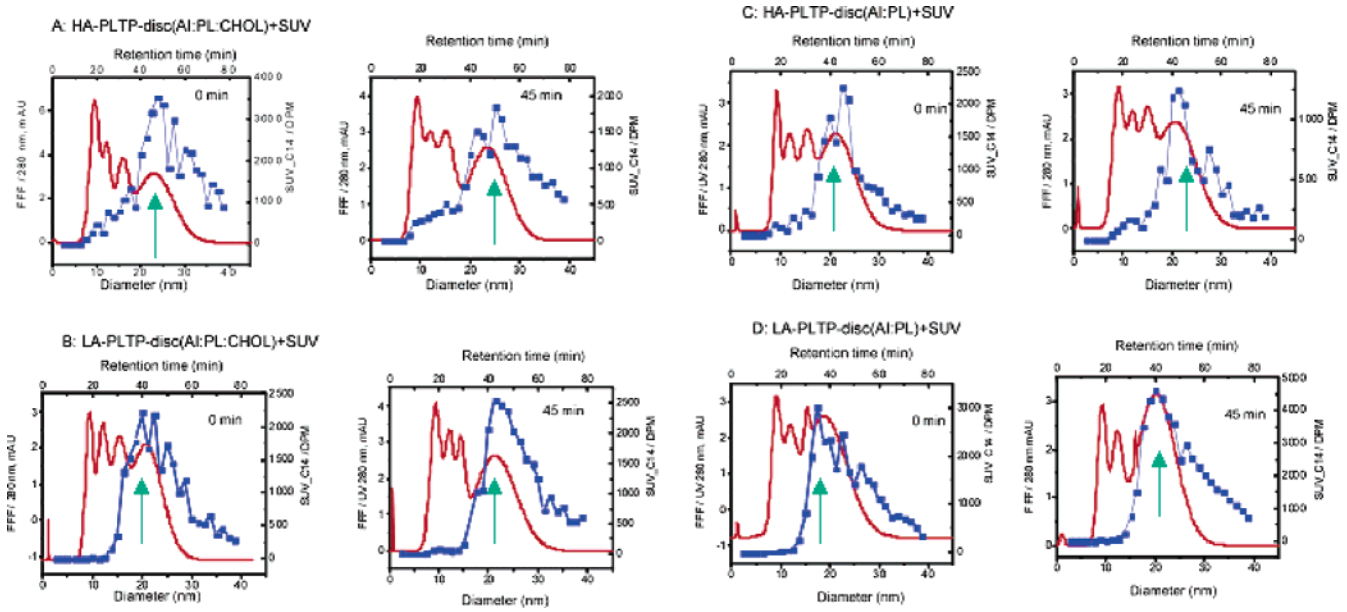

FIGURE 5: PLTP-facilitated lipid transfer from SUVs to apoA-I−phospholipid−cholesterol discoidal particles. HA- or LA-PLTP was incubated with SUVs and discoidal apoA-I particles, and the radioactivity was monitored from the collected fractions essentially as described in Figure 4. Carrier solution: 10 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4. See Experimental Procedures for details. The arrow indicates the particle size position for SUVs. Solid lines represent particle size or retention time fractograms obtained be AsFlFFF. Squares represent radioactivity in the fractions.

been shown to elute in a position corresponding to a molecular size of 520 kDa (*16*). In plasma of mice expressing human PLTP, two populations of PLTP have also been observed. One population has been shown to have low specific activity, whereas the other population displayed high specific activity (*32*). Furthermore, PLTP secreted by the human hepatoma cell line, HepG2, has been shown to resemble the high-activity form of PLTP found in human plasma. We have recently reported that human tear fluid contains only the high-activity form of PLTP which can be isolated by heparin−Sepharose affinity chromatography and elutes at the position of 160 kDa in gel filtration corresponding to its active counterpart in plasma (*6*). In addition, active PLTP has been demonstrated to coelute with apoE but not with apoA-I or apoB-100, and the activity could be inhibited only with antibodies against PLTP or apoE (*33*), suggesting in vivo selectivity in its interaction between apolipoproteins. In order to investigate the molecular mechanism behind the PLTP-facilitated lipid transport from donor vesicles to HDL acceptors, we utilized two methodological approaches, lipid monomolecular films and AsFlFFF. On the basis of current views two models have been proposed: (i) *a shuttle model* in which PLTP physically transports lipids between lipoprotein particles and (ii) *a ternary complex model* in which PLTP forms a bridge between two lipoprotein particles, enabling them to exchange lipids by diffusion (*31*, *34*). We first reasoned that nevertheless the mechanism PLTP has to be surface active, as was verified here experimentally (Figure 1). Then we reasoned that if active PLTP would act as a shuttle, it should be able to absorb lipid molecules from the lipid interface as demonstrated (Figure 2). This activity per se is not needed in the ternary complex model. We then proceeded to use AsFlFFF as a novel technique to study lipid transport. The validity of this method in the separation of lipoproteins and lipid-containing particles has been documented (*26*−*28*). To begin with, we confirmed by using this method that radiolabeled lipids were transferred from SUVs to HDL_3 following addition of HA-PLTP, but not LA-PLTP. The particle size of HDL_3 was not altered in either experiment, suggesting that no ternary complexes were formed. Although a priori one might think that the particle size of HDL_3 should increase, this does not actually happen because the amount of transferred lipids (compared to the amount of lipids in the HDL_3 particle) is low and the time of incubation is short. These results strongly suggest that the shuttle mechanism is the preferred mechanism of PLTP-induced lipid transfer. To show that the active PLTP itself was transferred (with the lipids) from SUVs to HDL_3, we radiolabeled HA-PLTP (and also LA-PLTP) and demonstrated that the majority of the [^{35}S]-HA-PLTP but not the [^{35}S]-LA-PLTP at the end of the incubation was found in HDL_3, further strengthening our hypothesis. These data are also in line with our previous findings where PLTP was demonstrated to bind to both apoA-I and apoA-II and that the PLTP binding domain of apoA-I resided in the N-terminal region (*35*). Furthermore, in another set of experiments using PLTP mutants, we showed that the C-terminal lipid-binding pocket may also be involved in the association of PLTP with HDL (*3*). Finally, formation of a ternary complex has some inherent problems associated with the model: the formation and disassembly of the ternary complex has to be somehow regulated in order that this mechanism would be efficient enough. Yet these factors have not been found. It has been shown that in plasma HA-PLTP is associated with apoE, and therefore this apolipoprotein might function as regulating the formation of the ternary complex. Yet, we have shown (*6*) that in human tear fluid PLTP exists only in the HA form, but tear fluid totally lacks apolipoproteins based on recent proteosome mapping (*7*) and our previous immunoblotting (*6*).

Our data establish that HA- and LA-PLTP are surface-active proteins. Intriguingly, HA-PLTP binds to monolayers with a critical surface penetration pressure somewhat lower compared to other plasma proteins/apolipoproteins: CETP,

31 mN/m (*21*); lecithin−cholesterol acyltransferase (LCAT), 29 mN/m (*36*); apolipoproteins apoA-I, 33 mN/m (*37*); apoA-II, 34 mN/m (*38*); apoA-IV, 29 mN/m (*39*); apoC-II, 34 mN/m; and apoC-III, 31 mN/m (*40*); as well as LA-PLTP, 33 mN/m. This may be important for more efficient release of HA-PLTP from the hydrophobic environment compared to other apolipoproteins and also LA-PLTP which may be more strongly docked to the phospholipid monolayer surface of lipoprotein particles. The reason why the surface exclusion pressure of LA-PLTP was so much higher than its active counterpart remains to be studied. The very stable tertiary structure and relatively low surface exclusion pressure of HA-PLTP suggest that, when bound to the interface, HA-PLTP does not unfold like many other apolipoproteins. Furthermore, it may be suggested that HA-PLTP does not penetrate deeply into the fatty acyl chain region of the lipid layer but mainly resides at the headgroup level. This is in accordance with the proposed shuttle mechanism of PLTP in which PLTP efficiently should be able to attach on and detach from the donor and acceptor particles.

The PLTP-enhanced lipid absorption from the monolayer suggested that PLTP acts as a carrier shuttling phospholipids between the air−buffer interface and bulk subphase also in the absence of acceptor molecules. As discussed earlier, in human plasma PLTP is seen in two different forms, LA- and HA-PLTP. The former of these is copurified preferentially with apoA-I while the latter is associated with apoE. Both LA- and HA-PLTP molecules were able to absorb lipids from the lipid surface residing at the air−water interface (Figure 2). The calculated stoichiometry of 1 PLTP molecule to desorb 5−6 molecules of DPPC is within the same magnitude when compared to earlier estimations of the unspecific phospholipid binding of the PLTP molecule outside the N-terminal pocket (*10*). This unspecific binding explains why LA-PLTP is also able to absorb phospholipids from the phospholipid monolayer. Strangely enough, this absorption of lipids was not seen when bilayers were used as substrates. The reason for this remains unknown at this stage. The increase in absorption was seen when HA-PLTP and HDL_3 molecules were mixed (Figure 2). This may indicate that, after absorbing lipids from the lipid monolayer, PLTP docks to the HDL_3 particle where it can release bound lipids or also HDL_3 can directly absorb lipids from the lipid monolayer. Since LA-PLTP and HDL_3 mixtures also show increased subphase radioactivity, we consider the latter mechanism more likely.

The ability of PLTP to move and transfer lipid molecules to suitable acceptors was assigned by AsFlFFF of different particles during the lipid transfer process. The data showed efficient movement of radioactively labeled HA-PLTP molecules to HDL_3. Our result also confirmed the lack of transfer capability of LA-PLTP. Although in monolayer series both LA- and HA-PLTP showed equal lipid-binding capability, LA-PLTP was unable to deliver lipids to the acceptor particle. Accordingly, AsFlFFF results suggest that although lipids are unspecifically bound to the surface remnants of LA-PLTP, the complex cannot dock on the acceptor. In keeping with this, similar results were seen when the structure of the acceptor particles was changed: both apoA-I−phospholipid discoidal and apoA-I−phospholipid−cholesterol particles were unable to receive lipids from PLTP. In the phospholipid transfer assay where SUVs and HDL_3 are used as substrates for PLTP, the curvature of the SUV and HDL_3 may be an important determinant for the activity of PLTP. We consider that the monolayer displays a very small degree of curvature and thus is not selective between LA- and HA-PLTP. In case of SUV and especially HDL_3 they have high degree of curvature and display surface layer distortion needed for the formation of sufficient gaps that may accommodate the interfacial binding site of HA-PLTP and proper interaction of the active site with the phospholipid to be transferred. We suggest that because LA-PLTP lacks this active folding, proper interactions between SUV and HDL_3 are hindered.

Taken together, our present data support the *shuttle* mechanism for PLTP-induced lipid transfer: (1) PLTP can transfer lipids from surfaces without any detectable acceptor, (2) a subphase mixture of HA-PLTP and HDL_3 increased the desorption of lipids from the monolayer, possibly by direct transfer from PLTP to HDL_3, and (3) AsFlFFF confirmed the movement of radioactively labeled HA-PLTP to acceptor particles. We propose that when HA-PLTP encounters HDL_3, it discharges the lipid into the outer leaflet of HDL_3 and does not dissociate until its lipid-binding site is once again full. Alternatively, HA-PLTP is taken up by some apolipoprotein particles, such as apoA-I, and this binding would change the conformation of HA-PLTP into LA-PLTP. The former mechanism would be compatible with the AsFlFFF results showing that after incubating [^{35}S]-HA-PLTP with SUVs and HDL_3 the radioactivity initially found in SUVs is transferred into HDL_3. In this sense it is interesting that intracellular phospholipid transfer proteins may utilize similar types of mechanisms: after binding of the lipid onto the surface of the protein, the protein diffuses into its acceptor where it discharges its lipids and resides on the membrane until its lipid-binding pocket is filled with another lipid. No net mass transfer of lipids is observed (*41*).

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