Molecular and Macromolecular Specificity of Human Plasma Phospholipid Transfer Protein†

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ABSTRACT: Phospholipid transfer protein (PLTP), also known as lipid transfer protein 2 (LTP-2), mediates a transfer of phospholipids between high-density lipoproteins (HDL). The molecular and macromolecular specificities of recombinant human PLTP were studied using a fluorometric assay based on the excimer fluorescence of pyrenyl lipids. To determine lipoprotein specificity of PLTP, donor very low density lipoproteins (VLDL), low-density lipoproteins (LDL), and HDL were labeled with 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine (PPyDPC) and incubated with unlabeled acceptor VLDL, LDL, and HDL in every pairwise combination. The highest rate of PPyDPC transfer mediated by PLTP occurred between donor HDL and acceptor HDL. Reassembled HDL (rHDL) consisting of 1-palmitoyl-2-oleoylphosphatidylcholine, apolipoprotein A-I, and pyrene lipids (100:1:4) were used to demonstrate that PLTP transfers diacylglyceride > phosphatidic acid > sphingomyelin > phosphatidylcholine (PC) > phosphatidylglycerol > cerobroside > phosphatidylethanolamine. Thus, PLTP transfers a variety of lipids with two carbon chains and a polar head group. Unsaturation of one PC acyl chain greatly increased transfer rate, whereas increasing chain length and exchanging *sn*-1/*sn*-2 position had only small effects. The rate of PPyDPC transfer by PLTP decreases with increasing free cholesterol content in rHDL and with decreasing HDL size. In contrast to spontaneous transfer, PLTP mediates the accumulation of PC in small rHDL particles. PLTP may be important *in vivo* in the recycling of PC from mature HDL to nascent HDL, the latter of which are the initial acceptors of cholesterol from peripheral tissue for reverse cholesterol transport to the liver.

Numerous studies have shown that plasma lipoprotein levels are important determinants of atherosclerosis and cardiovascular disease. Whereas high plasma levels of low-density lipoprotein (LDL)[1] cholesterol are associated with a high incidence of cardiovascular disease (Levine et al., 1995), high plasma levels of high-density lipoprotein (HDL) cholesterol appear to be cardioprotective (Gordon et al., 1989). It is clear from many studies that LDL is formed from the remodeling of very low density lipoprotein (VLDL) by the activities of lipoprotein lipase and hepatic lipase, which remove the triglyceride core by hydrolysis, and cholesteryl ester transfer protein (CETP), which exchanges the triglycerides of the apolipoprotein B-containing lipoproteins for the cholesteryl esters of HDL (Bruce & Tall, 1995; Fielding & Fielding, 1995; Krauss, 1994). In contrast, the source(s) of HDL and the mechanisms by which HDL are remodeled in plasma remain unclear.

The mature forms of HDL are thought to form through the coordinated activities of lecithin:cholesterol acyltransferase (LCAT), hepatic lipase, CETP, and PLTP (Fielding & Fielding, 1995). Any mechanistic model of how these three proteins modify HDL must include their specificity for various components of lipoproteins. The molecular specificities of LCAT, hepatic lipase, and CETP have been reported by several investigators. All exhibit a broad specificity. In the formation of cholesteryl esters from cholesterol, LCAT utilizes phosphatidylcholines and phosphatidylethanolamines as acyl donors, is selective for lipids having one or more double bonds, and is most efficient against substrate surfaces composed of fluid lipids (Pownall et al., 1985). Hepatic lipase hydrolyzes tri-, di-, and monoacylglycerols and phospholipids, among which the preferred substrate is phosphatidylethanolamine (Jensen et al., 1982; Newkirk & Waite, 1973; Waite & Sisson, 1973). CETP, which is also known as lipid transfer protein 1,

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[1] Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low density lipoproteins; rHDL, reassembled high-density lipoproteins; TBS, Tris-buffered saline; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; LCAT, lecithin:cholesterol acyl transferase; PC-TP, intracellular phosphatidylcholine transfer protein from bovine liver; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; apo A-I, apolipoprotein A-I; pyCE, cholesteryl 12-(1-pyrenyl)dodecanoate; PPyDDG, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]glycerol; PPyDPA, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidic acid; PPyDPC, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine; PPyDPE, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylethanolamine; PPyDPG, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylglycerol; PyDSM, [10-(1-pyrenyl)decanoyl]sphingomyelin; PyDCB, [10-(1-pyrenyl)decanoyl]cerebroside; 16:0,10:0pyPC, 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine; 18:0,10:0pyPC, 1-stearoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine; 20:0,10:0pyPC, 1-arachidoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine; 18:1,10:0pyPC, 1-oleoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine; 18:2,10:0pyPC, 1-linoleoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine; 16:0,6:0pyPC, 1-palmitoyl-2-[6-(1-pyrenyl)hexanoyl]phosphatidylcholine; 6:0py,16:0PC, 1-[6-(1-pyrenyl)hexanoyl]-2-palmitoylphosphatidylcholine; 18:2,9:0pyPC, 1-linoleoyl-2-[9-(1-pyrenyl)nonanoyl]PC; 9:0py,18:2pyPC, 1-[9-(1-pyrenyl)nonanoyl]-2-linoleoylphosphatidylcholine.

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transfers cholesteryl esters, triglycerides, and phosphatidylcholines (Albers et al., 1984; Hesler et al., 1987; Drayna et al., 1987) and exhibits small differences in the rates of transfer of different molecular species of cholesteryl esters (Morton, 1986). PLTP, also called lipid transfer protein 2, is a 476 amino acid protein with 20% sequence homology with CETP that was originally found as a second phospholipid transfer activity during purification of CETP (Tall et al., 1983a; Albers et al., 1984; Tollefson et al., 1988). PLTP does not transfer cholesteryl esters or triglycerides (Albers et al., 1984; Tollefson et al., 1988). Although PLTP is reported to transfer phosphatidylcholine (Albers et al., 1984; Tollefson et al., 1988; Lusa et al., 1996), lipopolysaccharide (Hailman et al., 1996), and α-tocopherol (Kostner et al., 1995), a systematic investigation of its specificity with respect to a broader range of lipids and lipid or lipoprotein surfaces has not been reported. This information is important for (1) the rational design of future studies of the mechanism by which HDL are remodeled in plasma and (2) refinement of current models of the role of reverse cholesterol transport (Fielding & Fielding, 1995) in the cardioprotective effect of HDL. Herein, we report the molecular specificity of PLTP against a wide spectrum of lipids and lipid surfaces.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human PLTP based on the previously published cDNA sequence (Day et al., 1994) was expressed as described previously (Albers et al., 1995). It was stored and used in 50 mM $NaPO_4$/300 mM NaCl, pH 4.0. Lipoproteins were isolated from human plasma from The Methodist Hospital Blood Donor Center, Houston, TX. Bio-Gel A-5m 200−400 mesh molecular sieve was from Bio-Rad Laboratories, Richmond, CA. The buffer used in the fluorometric assay and Superose 6 FPLC was pH 7.4 Tris-buffered saline (TBS) consisting of 10 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1 mM Na azide. The following pyrene-labeled lipids were from Molecular Probes, Inc., Eugene, OR: 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylcholine (PPyDPC), 1-palmitoyl-2-[10-(1-pyrenyl)-decanoyl]phosphatidylglycerol (PPyDPG), 1-palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidylethanolamine (PPyDPE), [10-(1-pyrenyl)decanoyl]sphingomyelin (PyDSM), 12-(1-pyrenyl)dodecanoic acid, 10-(1-pyrenyl)decanoic acid, and 9-(1-pyrenyl)nonanoic acid. 1-Palmitoyl-2-[6-(1-pyrenyl)-hexanoyl]phosphatidylcholine and 1-[6-(1-pyrenyl)hexanoyl]-2-palmitoylphosphatidylcholine were purchased from KSV Chemicals (Helsinki, Finland). The other phosphatidylcholines were synthesized from the appropriate lysophosphatidylcholine and pyrenyl fatty acid as described by Patel et al. (1979a) and Homan and Pownall (1988). 1-Arachidoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, L-α-monostearoyl-lecithin, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids, Birmingham, AL. 1-Linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine was purchased from Doosan Serdary Research Laboratories, Englewood Cliffs, NJ. Apolipoprotein A-I (apo A-I) was purified from human HDL as previously described (Pownall et al. 1978). [10-(1-Pyrenyl)decanoyl]cerobroside (PyDCB) and essentially fatty acid-free human serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Cholesterol was purchased from Calbiochem, La Jolla, CA. Cholesteryl 12-(1-pyrenyl)dodecanoate was synthesized and purified as described in (Patel et al., 1979b).

1-Palmitoyl-2-[10-(1-pyrenyl)decanoyl]phosphatidic acid (PPyDPA) was derived from PPyDPC by digestion with phospholipase D and purified using silica gel chromotography with a methanol/chloroform gradient (0−40% methanol) (Bergelson, 1980). 1-Palmitoyl-2-[10-(1-pyrenyl)decanoyl]-glycerol (PPyDDG) was derived from PPyDPC using phospholipase C. Briefly, 1 mg (1.3 μmol) of PPyDPC was dissolved in 2 mL of diethyl ether and 1 mL of 50 mM KPO_4/30 mM HBO_3, and then shaken for 3 h at room temperature. The PPyDDG was extracted twice with 2 mL of hexane. Purity was tested with silica gel plate thin-layer chromotography using the solvent hexane/ethyl acetate (80:20) and an ultraviolet lamp for visualization.

Methods

Fluorometric Assay of PLTP Activity. Phospholipid was measured using the method of Bartlett (1959). Unless otherwise specified, donor native lipoprotein/rHDL equivalent to 10 μM total phospholipid and acceptor lipoprotein/rHDL equivalent to 50 μM phospholipid were warmed to 37 °C in separate quartz cuvettes in an SLM Instruments Model 8000 (Urbana, IL) photon-counting spectrofluorometer. An emission scan of the donor was recorded with excitation at 342 nm. The emission at 395 nm was used as the monomer value. Recording in the slow kinetics mode (excitation at 342 nm and emission at 475 nm for concentration-dependent pyrene excimer fluorescence), the donor was allowed to stabilize for 200 s, and then the acceptor was added to the donor cuvette. At 600 s, 60 nM PLTP was added. Excimer fluorescence readings were recorded for 1 s every 10 s for a total of 5000 s. Samples were stirred at slow speed throughout preincubations and kinetic measurements. To account for nonhomogeneous labeling and slight variations in final concentration, excimer fluorescence values were divided by the constant monomer value to give an *e*/*m* fluorescence value. In experiments using the same donor (effect of PLTP concentration and acceptor to donor ratio) or similar donors labeled at once (effect of HDL size), the rate (*k*) of transfer is reported. To correct for differences in the amount of pyrene lipids in donors that were made separately (as more pyrene lipid substrate would give a decreased *k* value), initial flux values were calculated as the product of initial *e*/*m* and *k*. Points between the addition of acceptor and addition of PLTP were used to determine spontaneous transfer of the pyrene lipid when spontaneous transfer was not masked by photobleaching. Points after the addition of PLTP were used to determine initial total transfer of pyrene lipid. SAAM II kinetic analysis software (SAAM Institute, University of Washington, Seattle, WA) was used in the compartmental mode with two compartments, bidirectional (intercompartment only) flux to determine total initial flux of pyrene lipid out of the donor compartment. The corresponding numerical mode equation [adapted from Johnson et al. (1986)] is

$$ya_{(t)} = Ak(a_2 + k)^{-1} \exp[-(a_2 + k)t] + Aa_2(a_2 + k)^{-1}$$

where $ya_{(t)}$ is excimer fluorescence intensity, *A* is starting excimer fluorescence intensity, *k* is the partial rate constant

for flux from the excimer (donor) compartment to the nonexcimer (acceptor + PLTP + aqueous solution) compartment, and a_2 is the partial rate constant for flux from the nonexcimer compartment to the excimer compartment. For use in the numerical mode, the data must be corrected for PLTP addition time delay and nonzero $ya_{(\infty)}$. A one-compartment, flux to environment model was used to determine spontaneous flux out of the donor compartment. The corresponding numerical mode equation is $ya_{(t)} = A \exp[-kt]$, where k is the partial rate constant for flux from the excimer (donor) compartment to the nonexcimer (acceptor + aqueous solution) compartment. The difference between total and spontaneous transfer was calculated to be the PLTP-mediated transfer.

Lipoprotein Isolation and Labeling. Lipoproteins were isolated according to Rudel et al. (1974). Human plasma was adjusted to a density of 1.225 g/mL with KBr and ultracentrifuged for 40 h at 48 000 rpm in a Beckman 50.2 Ti rotor. The rotor was stopped with brake off. The floating lipoprotein mixture was collected and a portion was applied to a 1-m × 1.5-cm A-5m column. The lipoproteins were eluted with TBS at 4 °C with a flow rate of 15 mL/hour. Fractions (5 mL) were collected and used as acceptor lipoproteins.

Donor lipoproteins were labeled with PPyDPC before fractionation. PPyDPC (5 mM) in 250 mM sodium cholate was diluted 1:400 in a solution of lipoprotein containing 125 mM phospholipid. Thus approximately 4% of donor lipoprotein phospholipid was PPyDPC. Donor lipoproteins were labeled with cholesteryl 12-(1-pyrenyl)dodecanoate (PyCE) using the CETP activity of lipoprotein-deficient plasma. PyCE was dried onto the inside of a test tube. Total lipoproteins and plasma fraction $d > 1.225$ were added and the mixture was incubated at 37 °C with intermittent stirring for 24 h; the ratio of PyCE to lipoprotein was 8:100. Lipoproteins were separated by sequential ultracentrifugation (Scanu, 1966) and HDL with density between 1.063 and 1.21 g/mL was used.

Reassembled HDL. Donor rHDL composed of POPC, apo A-I, and pyrene-labeled lipid (molar ratio 100:1:4) was made by the cholate removal method (Jonas et al., 1989; Pownall et al., 1982) except cholate was removed during size fractionation. Acceptors were of the same composition except without pyrene-labeled lipid. rHDLwere size-fractionated using Superose 6 column FPLC with 0.5 mL/min flow rate and 4-min fraction collection. With a void time of 30 min, rHDL peaks consistently appeared at 50, 56, and 59 min. Standard human HDL2 and HDL3 (isolated by sequential ultracentrifugation $1.063 < d$(HDL2) < 1.120 g/mL and $1.120 < d$(HDL3) < 1.21 g/mL) had retention times of 56 and 59 min, respectively. Fractions 15 and 16 with retention times 56−64 min were pooled for use. When two donor sizes were used, the large particles had retention times of 51−56 min and the small particles had retention times of 56−60 min.

To investigate the effect of the polar head group on transfer rate, each of the following pyrene-labeled lipids was incorporated into rHDL: PPyDDG, PyDSM, PPyDPA, PPyDPC, PyDCB, PPyDPG, and PPyDPE. To investigate the effect of different acyl chains on the transfer of phosphatidylcholine, the following pyrene-labeled PCs were used in rHDL: PPyDPC (16:0,10:0pyPC), 1-stearoyl-2-[10-(1-pyrenyl)decanoyl]PC (18:0,10:0pyPC), 1-arachidoyl-2-[10-(1-pyrenyl)decanoyl]PC (20:0,10:0pyPC), 1-oleoyl-2-[10-(1-pyrenyl)decanoyl]PC (18:1,10:0pyPC), 1-linoleoyl-2-[10-(1-pyrenyl)decanoyl]PC (18:2,10:0pyPC), 1-palmitoyl-2-[6-(1-pyrenyl)hexanoyl]PC (16:0,6:0pyPC) ,1-[6-(1-pyrenyl)hexanoyl]-2-palmitoylPC (6:0py,16:0PC), 1-linoleoyl-2-[9-(1-pyrenyl)nonanoyl]PC (18:2,9:0pyPC), and 1-[9-(1-pyrenyl)nonanoyl]-2-linoleoylPC (9:0py,18:2PC). To investigate the effect of free cholesterol, rHDL included PPyDPC and increasing amounts of cholesterol. The incorporation of cholesterol was such that starting mixtures of 5%, 20%, and 50% cholesterol/total phosphatidylcholine gave rHDL with 0.4, 2.7, and 5.0% cholesterol/total phosphatidlycholine. Cholesterol content of rHDL was measured using the method of Heider and Boyett (1978).

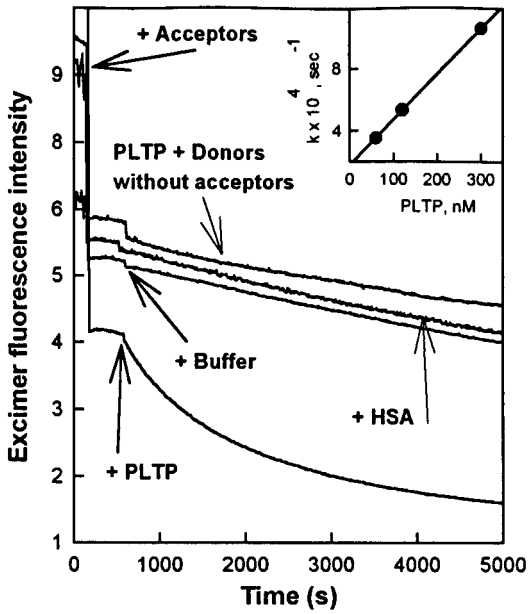

FIGURE 1: Kinetic traces of the phospholipid transfer activity of PLTP compared to controls TBS buffer and human serum albumin. Donor HDL (10 μM total PL, 200 nM HDL) was incubated with a 5-fold excess of acceptor HDL, except for the uppermost trace, which contains no acceptors and shows the change in fluorescence following the addition of TBS alone. PLTP (3 μg/mL), human serum albumin (3 μg/mL), and buffer were added as indicated. A similar curve was obtained when either the phosphate buffer (50 mM $NaPO_4$/300 mM NaCl/pH 4) used to store PLTP or TBS buffer was used. (Inset) Dependence of PPyDPC transfer rate on PLTP concentration. Donor HDL (10 μM total PL, approximately 200 nM HDL) was incubated with a 15-fold excess of acceptor HDL and 60, 120, or 300 nM recombinant PLTP at 37 °C for 80 min. A fluorometer was used to trace decreasing concentration-dependent excimer fluorescence (excitation wavelength = 342 nm, emission wavelength = 475 nm) of donor HDL. To account for nonhomogeneous labeling, rates were calculated from the ratio of excimer fluorescence to monomer fluorescence (emission wavelength = 395 nm). The rate vs PLTP concentration line has $r^2 = 1.000$.

RESULTS

Activity of Recombinant Human PLTP. Preliminary experiments were done to ascertain that the recombinant PLTP used in these studies exhibited the phospholipid transfer activity ascribed to the PLTP protein purified from plasma. Figure 1 shows a typical fluorescence trace beginning with initial donor HDL excimer fluorescence followed by a precipitous drop due to the dilution of donor with the addition of acceptor HDL volume, a slow decrease due to slow donor photobleaching and spontaneous transfer, and finally the characteristic rapid decrease from protein-mediated

PPyDPC transfer upon addition of PLTP. Donor HDL (10 μM total PL, 200 nM HDL) were incubated with a 5-fold excess of acceptor HDL. Equal volumes of 3 μg/mL PLTP, 3 μg/mL human serum albumin in TBS, TBS only, or the PLTP vehicle 50 mM $NaPO_4$/300 mM NaCl, pH 4, were added. There is a slight decrease in fluorescence due to dilution with the mixing of each of the solutions. The phospholipid transfer activity of PLTP is not exhibited by the similar-sized lipid carrier protein human serum albumin or the buffers. Fluorometer traces with human serum albumin and with TBS do not mimic the rapid decrease in excimer fluorescence apparent in the trace with PLTP. An assay was conducted with donor, addition of TBS without acceptor, and TBS instead of PLTP. There was a slow loss of excimer fluorescence due to photobleaching (data not shown). An assay run with donor, no acceptor, and PLTP showed the same slow loss of excimer fluorescence; thus PLTP alone has no effect on donor excimer fluorescence without acceptor. The loss of donor excimer fluorescence upon incubation with acceptors (and no PTLP) is slightly faster than without acceptors (with or without PLTP), showing that spontaneous transfer occurs in the former case only. However, this slight difference is not apparent early in the incubation and photobleaching may at that time mask slow spontaneous transfer. The inset to Figure 1 furthermore shows that the PPyDPC transfer activity of PLTP is dependent on PLTP concentration. Donor HDL (10 μM total PL, approximately 200 nM HDL) were incubated with a 15-fold excess of acceptor HDL and 60, 120, or 300 nM recombinant PLTP at 37 °C for 80 min. The rate vs PLTP concentration line has $r^2 = 1.000$. Taken together, these data indicate that the PPyDPC transfer activity represented by the rapid decrease in excimer fluorescence is in fact an activity mediated by and specific to PLTP.

It is possible that the decrease in concentration-dependent excimer fluorescence results from label dilution from PLTP mediated fusion of donor and acceptor lipoprotein particles rather than from label transfer. To exclude this mechanism, donor HDL were labeled with PyCE instead of PPyDPC. Since PLTP does not transfer cholesteryl esters, a decrease in excimer fluorescence upon addition of acceptor HDL and PLTP would provide evidence for label dilution by particle fusion. Because of less incorporation of PyCE than PPyDPC in the previous experiments, 3.6 μM PyCE donor HDL was used with a 5-fold excess of acceptor HDL at 37 °C. This concentration of HDL also exhibited the characteristic decrease in PPyDPC excimer fluorescence upon addition of PLTP (data not shown). The fluorometer trace in Figure 2 shows no decrease in excimer fluorescence of PyCE upon addition of acceptors and 60 nM PLTP. (No change was seen up to 70 min; data not shown.) To demonstrate that the excimer fluorescence of the amount of PyCE incorporated was not too low to be reduced further, it was reduced by transfer of the label to LDL by CETP, also known as cholesteryl ester transfer protein. (There is an increase in signal intensity due to the contributions of the light scattering by the added LDL.) It is somewhat surprising that CETP apparently did not catalyze an HDL to HDL cholesteryl ester transfer, since Ohnishi et al. (1994) showed that CETP can transfer cholesteryl esters between HDL donors and acceptors. However, although the conditions (particularly donor and acceptor concentrations) of our experiment were sufficient for detecting fusion had it occurred, they were not

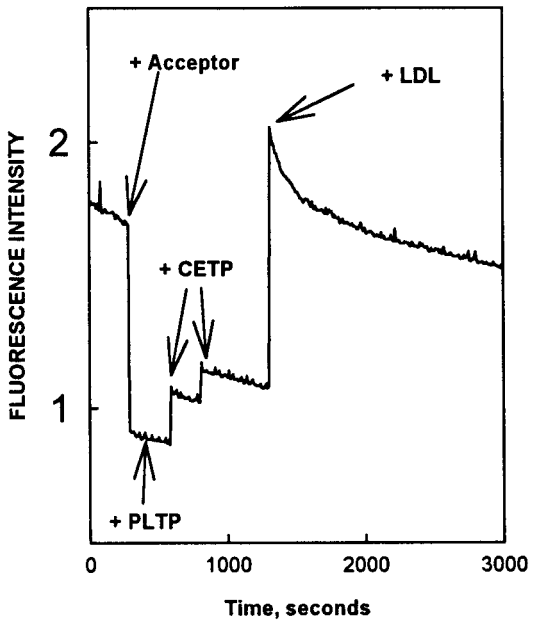

FIGURE 2: Absence of PLTP-mediated HDL fusion in the 1-h time span of kinetic experiments. Cholesteryl pyrene-dodecanoate donor HDL (3.6 μM) and a 5-fold excess of acceptor HDL were incubated at 37 °C. The fluorometer trace showed no decrease in excimer fluorescence upon the addition of 60 nM PLTP. (No change was seen up to 70 min; data not shown.) When an equal amount of CETP was added (split into two aliquots), followed by acceptor LDL, excimer fluorescence decreased.

optimized for cholesteryl ester transfer. We used a better acceptor (LDL) to show such transfer. A recent report by Connolly et al. (1996) indicates that LDL is preferred over HDL as substrate for CETP, with K_m values in the low range of normal physiological concentration for each lipoprotein (LDL K_m = 700 nM, HDL K_m = 2000 nM in particles). We used only 1800 nM HDL acceptor (=K_m); this may have been too low for our assay to show transfer. The 1400 nM LDL ($2K_m$) used showed a small transfer, which was itself enhanced both by the greater affinity of CETP for LDL and by PLTP potentiating cholesteryl ester transfer by CETP (Lagrost et al., 1994). It is possible that a greater incorporation of pyrene-labeled cholesteryl ester would have shown more transfer with either acceptor. In any case, this experiment does exclude fusion.

The absence of particle fusion by PLTP was further demonstrated by size analysis of the PyCE donor/acceptor/PLTP reaction mixture at 0, 1, and 24 h using Superose 6 FPLC. As seen in Figure 3, the decrease in labeled-particle retention time seen at 24 h (panel C), corresponding to a 60% increase in molecular weight as calculated from HDL2 and HDL3 standards, is not yet apparent at 1 h (panel B).

The dependence of PPyDPC transfer by PLTP on acceptor to donor ratio is shown in Figure 4. Donor HDL (10 μM total PL, 200 nM HDL) were incubated with acceptor HDL in 1:1, 1.6:1, 8:1, 16:1, and 25:1 acceptor:donor ratios. PLTP was added to a final concentration of 60 nM. PPyDPC flux increases with increasing acceptor:donor ratio, reaching a plateau by a ratio of 8:1. It must be noted that this reflects not only donor−acceptor collisions but also the fact that in this assay system more acceptor dilutes the transferred PPyDPC to below-excimer, i.e., undetected, concentrations. A 5-fold excess of acceptor with 60 nM PLTP was chosen for subsequent experiments; it supports a high rate without waste of acceptor. Spontaneous transfer flux did not

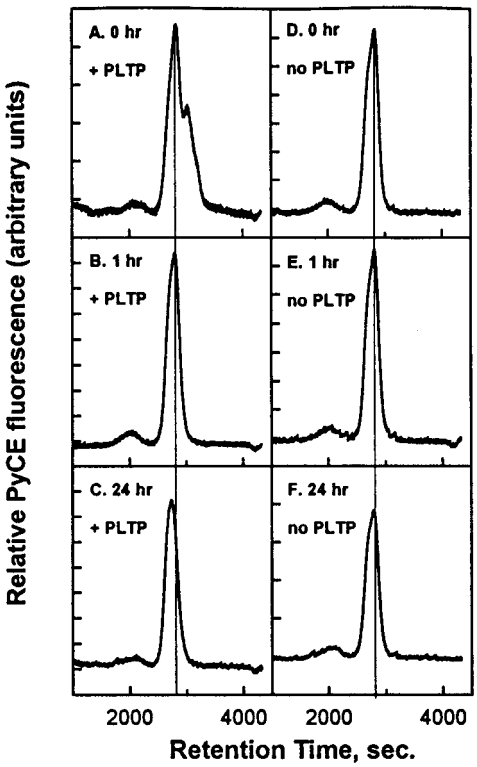

FIGURE 3: Absence of PLTP-mediated HDL size change in 1 h. The same donor HDL and acceptor HDL concentrations were incubated with and without PLTP at 37 °C. Samples were taken at 0, 1, and 24 h and placed on ice. Samples were analyzed with Superose 6 column FPLC. Up to 1 h, PLTP mediated no change in HDL retention time. There was a decrease in HDL retention time with PLTP at 24 h representative of a 60% increase in molecular weight.

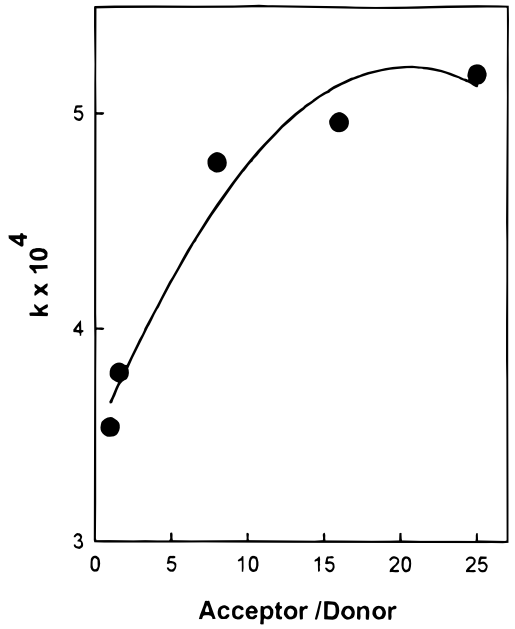

FIGURE 4: Dependence of PPyDPC transfer rate on acceptor to donor ratio. Donor HDL (10 μM total PL, 200 nM HDL) was incubated with acceptor HDL in 1:1, 1.6:1, 8:1, 16:1, and 25:1 acceptor:donor ratios. PLTP was added to a final concentration of 60 nM. Spontaneous transfer flux did not measurably change with acceptor:donor ratio (data not shown).

measurably change with acceptor:donor ratio (data not shown).

Lipoprotein Specificity of PLTP. Donor VLDL, LDL, or HDL (10 μM total PL each; 2.2 μM VLDL, 16 μM LDL, and 200 nM HDL) were incubated with a 5-fold (by phospholipid) excess of acceptor VLDL, LDL, or HDL in every pairwise combination. Figure 5 shows that the PLTP-mediated transfer rate was fastest with acceptor and donor HDL and slowest with acceptor and donor LDL, with acceptor and donor VLDL in between. Combinations of donor and acceptor including HDL gave faster reaction kinetics, and combinations with LDL gave slow kinetics. The HDL to HDL transfer had a significantly low spontaneous flux ($p < 0.05$, ANOVA).

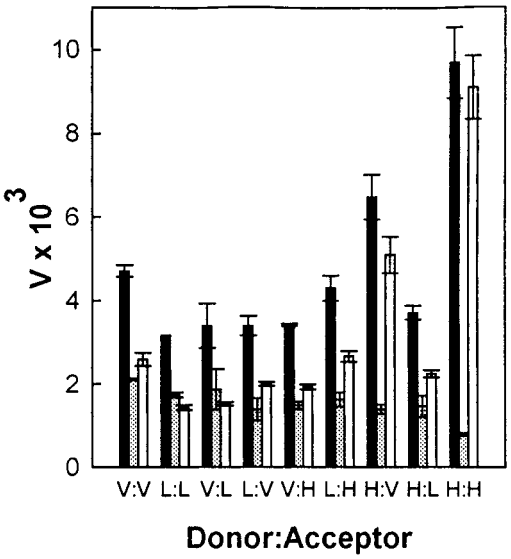

FIGURE 5: Lipoprotein specificity of PLTP. Donor VLDL, LDL, or HDL (10 μM total PL each; 2.2 μM VLDL, 16 μM LDL, and 200 nM HDL) was incubated with a 5-fold excess (by phospholipid) of acceptor VLDL, LDL, or HDL in every pairwise combination. Assays described previously were done in duplicate, except HDL to HDL was done in quadruplicate. Total flux and spontaneous transfer flux were determined from the data, and PLTP-mediated flux was calculated as the difference between the two. Solid bar, total flux; stippled bar, spontaneous flux (lowest value may be photobleaching only); open bar, PLTP-mediated flux.

Molecular Specificity of PLTP. The use of donor rHDL incorporating 4% of various lipids showed that PPyDPC, PPyDDG, PyDSM, PPyDPA, PPyDPG, PyDCB, and PPyDPE were all transferred by PLTP to acceptor rHDL (Figure 6).

Figures 7 and 8 show that PC transfer by PLTP was influenced greatly by unsaturation, by pyrene-labeled acyl chain length, and to a small extent by unlabeled acyl chain length and *sn*-1/*sn*-2 position. Figure 8, panel A, shows that PCs with an acyl chain of 16 or 18 carbons are transferred equally and slightly more rapidly (by 20%) than a PC with a 20-carbon acyl chain ($p < 0.05$, ANOVA). Figure 8, panel B, shows that PCs with an acyl chain with one or more double bonds are transferred equally and more rapidly (170% faster) than a PC with no double bonds ($p < 0.05$, ANOVA). Figure 8 panel B, shows that although total flux of PCs with a shorter pyrene-labeled chain is greater, correction for the higher spontaneous transfer rate gives a decreased PLTP-mediated transfer rate for those compounds ($p < 0.05$, ANOVA). Switching *sn*-1 and *sn*-2 acyl chains makes no significant difference when one is a 16 carbon saturated chain. Figure 8, panel A, shows that PLTP-mediated transfer also decreases with shortened pyrene-labeled chains, but the 18-carbon unsaturated chain gives a slightly (35%) but significantly faster rate in the *sn*-1 position than in the *sn*-2

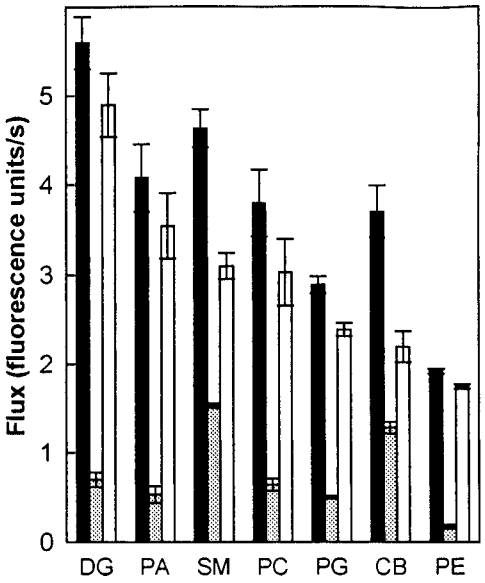

FIGURE 6: Variety of lipids transferred by PLTP. Acceptor rHDL was made with POPC and apo A-I in a molar ratio of 100:1. Donors were made with POPC, apo A-I, and pyrene-labeled lipid in a molar ratio of 100:1:4. Assays were done in triplicate to determine total flux and spontaneous flux; PLTP-mediated flux was calculated as the difference. Solid bar, total flux; stippled bar, spontaneous flux (lowest value may be photobleaching only); open bar, PLTP-mediated flux.

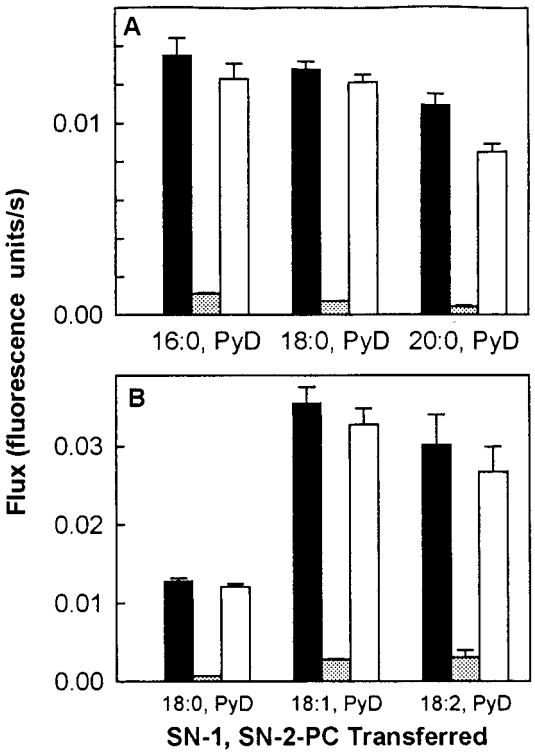

FIGURE 7: Effect of phosphatidylcholine acyl chain length and unsaturation on transfer rate. Panel A shows the transfer of phosphatidylcholines with increasing chain length in the *sn*-1 position. Panel B compares the transfer of phosphatidylcholines with an unsaturated *sn*-1 acyl chain to the transfer of phosphatidylcholine with a saturated *sn*-1 acyl chain. Solid bar, total flux; stippled bar, spontaneous flux (lowest value may be photobleaching only); open bar, PLTP-mediated flux.

position ($p < 0.05$, one-tailed t test). Although not physiologically important, this slight difference between panels A and B may give insight into the structure/mechanism of PLTP activity.

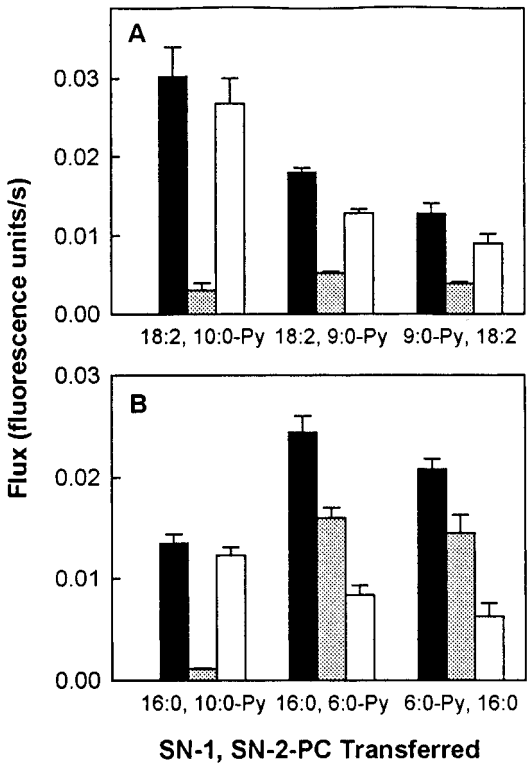

FIGURE 8: Effect of shortened chain length and exchanging *sn*-1/*sn*-2 position on transfer rates of phosphatidylcholines. Panel A compares PPyDPC with phosphatidylcholines with short pyrene-labeled acyl chains in the *sn*-1 and then *sn*-2 positions. The 18-carbon unsaturated chain gives a slightly (35%) but significantly faster rate in the *sn*-1 position than in the *sn*-2 position. Panel B also shows the effect of shortened pyrene-labeled chains. Switching *sn*-1 and *sn*-2 acyl chains makes no significant difference when the other is a 16-carbon saturated chain. Solid bar, total flux; stippled bar, spontaneous flux (lowest value may be photobleaching only); open bar, PLTP-mediated flux.

Lipoprotein Surface Specificity of PLTP. Figure 9 shows that the rate of PPyDPC transfer by PLTP decreases logarithmically with increasing free cholesterol in rHDL. Donor and acceptor rHDL were made with increasing amounts of free cholesterol, the maximum equal to physiological levels of 5% cholesterol/phospholipid (mol/mol). The line of log flux vs % chol/phospholipid has $r^2 = 0.99$.

The rate of PPyDPC transfer between HDL particles by PLTP also decreases with decreasing HDL particle size (Figure 10). A series of fluorometry-based PLTP transfer assays was done using the original A-5M-fractionated, size-matched matched donor and acceptor HDL. Spontaneous flux was unaffected by HDL size (data not shown). The line of log k vs fraction number (decreasing size with increasing fraction number) has $r^2 = 0.996$. This finding suggests that large HDL particles are better donors. Since the fluorometric assay can only be used to definitively analyze the efficiency of donors and not of acceptors, one cannot conclude from these data alone that PLTP will mediate the accumulation of PPyDPC on the less efficient small HDL donors. No such accumulation would occur if small HDL are inefficient acceptors as well. To pursue this, a transfer experiment was done using large and small rHDL donors (no unlabeled acceptors). The reaction mixture was analyzed for correlation of PPyDPC accumulation with particle size using Superose 6 FPLC. Figure 11 shows that PLTP mediates a transfer of PPyDPC from large (i) to small (ii) rHDL particles. Fluorescence, initially equal between

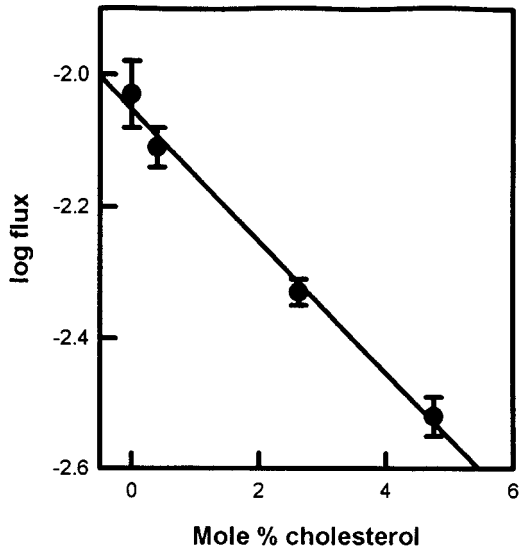

FIGURE 9: Rate of PPyDPC transfer by PLTP decreases logarithmically with increasing free cholesterol in rHDL. Donor and acceptor rHDL were made with increasing amounts of free cholesterol, the maximum equal to physiological levels. All rHDL was size-fractionated using Superose 6 column FPLC and corresponding size fractions were used. Transfer assays were done in triplicate. Spontaneous flux was not measurably affected by free cholesterol content (data not shown). The line of log flux vs mol % cholesterol has $r^2 = 0.99$.

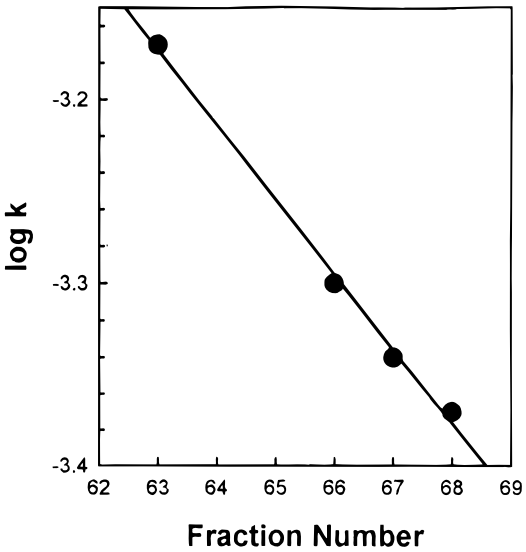

FIGURE 10: Rate of PPyDPC transfer between HDL particles by PLTP decreases with decreasing rHDL size. A series of transfer assays was done using A-5M-fractionated, size-matched matched donor and acceptor HDL. Spontaneous flux was not measurably affected by HDL size (data not shown). The line of log *k* vs fraction number (decreasing size with increasing fraction number) has $r^2 = 0.996$.

the two rHDL sizes, increases at 15 and 60 min in the large particles (i) without PLTP but in the small particles (ii) with PLTP.

DISCUSSION

There have been two barriers hindering the study of PLTP: (1) only small amounts of the protein can be purified from human plasma, where the concentration is estimated to be just 2 mg/L (Tu et al., 1993), and (2) unlike cholesteryl ester and triglycerides, which are essentially insoluble in aqueous solution and therefore have no spontaneous transfer,

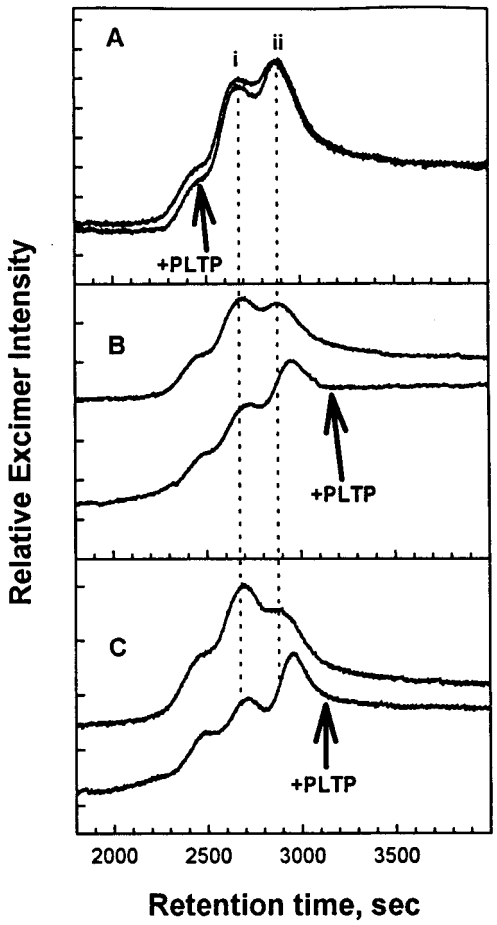

FIGURE 11: PLTP mediates a transfer of PPyDPC from large to small rHDL particles. Total phospholipid equivalents (30 μM each) of large (i) and small (ii) size rHDL, as fractionated by Superose 6 FPLC, were combined and incubated at 37 °C with or without 60 nM PLTP for 1 h. At 0, 15, and 60 min, samples were taken and placed on ice. Samples were run through Superose 6 FPLC. Panel A, 0 min; panel B, 15 min; panel C, 60 min.

phospholipids undergo spontaneous transfer at a rate that makes assays dependent on separation of donor and acceptor unreliable. Both of these problems have been solved in the present study. PLTP was recently cloned and sequenced (Day et al., 1994) and large amounts of highly active and stable recombinant human PLTP were available for this and ongoing studies. Pyrene-labeled lipids provided a means to collect kinetic data in real time (Pownall & Smith, 1989). Not only did this pyrene lipid assay system avoid transfer occurring beyond the intended time, but with otherwise identical donor and acceptor, transfer of components from acceptor to donor did not change the donor surface during the duration of the experiment and there could be no preferential association of PLTP with acceptor to confound results. This assay allowed the investigation of both physiologically relevant lipoprotein mixtures as well as experimentally meaningful artificial donors and acceptors with only one variable parameter. Furthermore, the measurement of transfer as loss of excimer fluorescence from donors rather than the gain of small amounts of monomer fluorescence in acceptors made this assay more accurate than other fluorescence assays. A previously published report shows that this fluorescence assay and an assay using radioactively labeled phospholipids yield similar transfer rates (Massey et al., 1985).

The first set of experiments were done to show that the recombinant human PLTP used has phospholipid transfer activity. The negative result with vehicle alone and increasing rate with increasing PLTP concentration indicated that phospholipid transfer activity resulted from the addition of PLTP. The negative result with human serum albumin indicates that the activity is not shared by all phosphatidylcholine-binding (Jonas, 1975) or simply highly hydrophobic proteins. Several groups have reported that PLTP can mediate conversion of HDL particles into larger and perhaps smaller particles after extended incubations of 3, 6, or 24 h. However, this study confirms the findings of Lusa et al. (1996) that no change in size occurs in 1 h, indicating that the important activity of PLTP in the short time span is lipid transfer and not lipoprotein fusion.

PLTP-mediated transfer differs from spontaneous transfer in several respects. Because the rate-limiting step for spontaneous transfer is desorption of the lipid from the donor surface into the aqueous phase, the identity and concentration of the acceptor have little effect (Massey et al., 1982); however, PLTP-mediated transfer was clearly dependent on acceptor (lipoprotein) identity and concentration. PLTP-mediated transfer probably occurs through protein and lipoprotein bi- or trimolecular collisions. Like spontaneous transfer, PLTP-mediated transfer of phosphatidylcholines increases with acyl chain unsaturation; however, unlike spontaneous transfer, increasing molecular hydrophobicity through changes in the head group or increased acyl chain length does not consistently decrease the rate of PLTP-mediated transfer. The more hydrophobic phosphatidylethanolamine transferred more slowly than phosphatidylcholine by both spontaneous and PLTP-mediated transfer ($p < 0.05$, ANOVA). Yet, the more hydrophobic diacylglyceride was transferred faster than any of the other lipids studied. The cerebroside exhibits a high spontaneous transfer rate, whereas the PLTP-mediated transfer rate was relatively low. There was no consistent correlation between acyl chain length and the rates of PLTP-mediated transfer. Increasing the acyl chain length from 16 to 18 methylenes had no effect on the rate, whereas increasing the chain length to 20 methylenes or decreasing the chain length from 10 to 6 methylenes was associated with slower PLTP-mediated transfer rates.

PLTP shares some characteristics with the intracellular phosphatidylcholine transfer protein (PC-TP) from bovine liver but also differs in some ways since there is no significant sequence homology between the two (Day et al., 1994). Unlike PLTP, PC-TP is specific for PC (Massey et al., 1985). The association of ligand with either transfer protein probably involves hydrophobic interactions. Decreasing acyl chain length decreases transfer rate by PC-TP (Van Loon et al., 1985) and PLTP (Figure 8B). Whereas increased hydrophobicity favors association with the transfer protein, decreased hydrophobicity is necessary for removal from the donor. Child et al. (1985) reported that PC-TP activity increases with degree of phosphatidylcholine unsaturation but warn that the increase may actually in some part result from increased spontaneous transfer. In the present experiments, spontaneous transfer increased with unsaturation but was not significantly different between one or two double bonds. PLTP activity increased far more greatly with unsaturation and was the same for phosphatidylcholines with one or two double bonds. Unsaturation decreases the efficiency of phospholipid packing and probably thereby eases the extraction of the transferring molecule. PC-TP is believed to have distinct binding sites for the *sn*-1 and *sn*-2 acyl chains of phosphatidylcholine (Van Loon et al., 1985). The *sn*-2 position is believed to be more sensitive to chain length, as transfer rate is halved when a 12-carbon chain is switched to the *sn*-2 position and an unsaturated 18-carbon chain is switched to the *sn*-1 position. (Yet no positional preference was evident when the 12-carbon chain was replaced with a 14-carbon chain.) In contrast, switching *sn*-1/*sn*-2 chains had little to no effect on PLTP activity. Switching the position of a saturated chain, in agreement with Huuskonen et al. (1996), had no effect on the rate of PLTP-mediated or spontaneous transfer. The small *sn*-1/*sn*-2 difference only became apparent when one chain was optimized in length and unsaturation. In this case, the 18-carbon unsaturated chain (linoleic acid) was preferred in the physiologically common *sn*-1 position. Since pyrene is equivalent to 10 carbons, an even shorter pyrene-labeled chain may further differentiate *sn*-1 and *sn*-2 positions, but such a short chain would result in an unmanageable spontaneous transfer rate. PLTP seems to differ from PC-TP in distinctness of *sn*-1/*sn*-2 binding sites, if discrete sites do exist.

The most important finding of this study regarding molecular (non)specificity is that PLTP mediates the transfer of diacylglycerides between rHDL particles. Diacylglyceride underwent spontaneous transfer at a rate not significantly different from phosphatidylcholine but underwent PLTP-mediated transfer significantly (62%) faster ($p < 0.05$, ANOVA). Diacylgycerides are released by lipoprotein lipase in capillaries and during lipolysis in adipose tissue and are therefore important in localized lipoprotein metabolism. Vieu et al. (1996) recently reported that HDL particles carry a substantial amount of diacylglycerides, equal to twice the amount of triglycerides. Although Massey et al. (1985) reported that the diacylglyceride was the slowest transferred lipid, even slower than phosphatidylethanolamine, and that the fastest lipid was the cerebroside, their plasma-purified PLTP may not have been quite pure such that experiments might have reflected the combined activities of PLTP, CETP, and other lipid transfer proteins. The lower rate of PLTP-mediated diacylglyceride transfer reported by Massey et al. (1985) is probably a consequence of the inaccessibility of the shorter-chained (14:0,py9:0) pyrene diglyceride used in that study. The study by Vieu et al. (1996) suggests that diacylglycerides with shorter chains (16-carbon vs 18 vs 20) are more likely to be found in the core rather than on the surface of native HDL. PLTP seems to transfer surface lipids faster than core lipids.

The physiological importance of PLTP is substantiated by the macromolecular specifities of PLTP studied. The preference of HDL as both donor and acceptor for PLTP-mediated transfer contrasts with the low spontaneous transfer of phosphatidylcholine between HDL donor and acceptor. Because PLTP transfer is dependent on protein and lipoprotein bimolecular or trimolecular collisions and 89 mol % of lipoproteins in men and 94 mol % of lipoproteins in women (Smith et al., 1983) are HDL, HDL to HDL transfers would by far constitute the bulk of PLTP-mediated transfers. Thus PLTP would be important in HDL remodeling. Decreasing particle size, and hence increasing surface curvature, might be expected to accelerate loss of phospho-

lipid through spontaneous transfer. This does not take effect in PLTP-mediated transfer. Spontaneous transfer caused accumulation of phospholipid in large rHDL whereas PLTP mediated a transfer to small particles. In vivo, small HDL particles are the preferred acceptors of free cellular cholesterol, which is esterified there by LCAT. In this study, PLTP removes phosphatidylcholines less efficiently from rHDL with high free cholesterol content. Free cholesterol, by increasing phospholipid packing efficiency in the fluid rHDL surface, would be expected to hinder penetration of PLTP into the donor to bind and abstract the phospholipid molecule(s). LCAT is more efficient with surfaces composed of fluid lipids (Pownall et al., 1985). A transfer assay could not be done with donors of different free cholesterol content (like with different sizes) because the rapid spontaneous transfer of cholesterol would equalize all rHDL cholesterol content in the mixture; however, the result with identical acceptor and donor still suggests that PLTP may cause accumulation of phosphatidylcholine in HDL with high free cholesterol. The role of PLTP *in vivo* may therefore be to deliver phosphatidylcholine to small, cholesterol-accepting nascent HDL, in opposition to spontaneous transfer. Phosphatidylcholine is the essential cholesterol-solubilizing component of HDL and is the acyl donor for the LCAT esterification of that cholesterol. CETP and LCAT with their respective actions in the transport of peripheral cholesterol to the liver, or reverse cholesterol transport, would be a source of efflux of phosphatidylcholine from those particles. LCAT mediates growth in HDL size as cholesterol ester products augment the HDL core; PC is needed both as a substrate and to cover the accumulating product. LCAT is selective for lipids with one or more double bonds (Pownall et al., 1985), the lipids that are delivered the fastest by PLTP. The role of PLTP in reverse cholesterol transport may be to recycle phosphatidylcholine from large, mature HDL to nascent HDL as a supply of substrate for LCAT and CETP.

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