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Phospholipid Transfer Protein in Human Plasma Associates with Proteins Linked to Immunity and Inflammation[†]

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ABSTRACT: Phospholipid transfer protein (PLTP), which associates with apolipoprotein A-I (the major HDL protein), plays a key role in lipoprotein remodeling. Because its level in plasma increases during acute inflammation, it may also play previously unsuspected roles in the innate immune system. To gain further insight into its potential physiological functions, we isolated complexes containing PLTP from plasma by immunoaffinity chromatography and determined their composition. Shotgun proteomics revealed that only 6 of the 24 proteins detected in the complexes were apolipoproteins. The most abundant proteins were clusterin (apoJ), PLTP itself, coagulation factors, complement factors, and apoA-I. Remarkably, 20 of the 24 proteins had known protein−protein interactions. Biochemical studies confirmed two previously established interactions and identified five new ones between PLTP and proteins. Moreover, clusterin, apoA-I, and apoE preserved the lipid-transfer activity of recombinant PLTP in the absence of lipid, indicating that these interactions may have functional significance. Unexpectedly, lipids accounted for only 3% of the mass of the PLTP complexes. Collectively, our observations indicate that PLTP in human plasma resides on lipid-poor complexes dominated by clusterin and proteins implicated in host defense and inflammation. They further suggest that protein−protein interactions drive the formation of PLTP complexes in plasma.

Phospholipid transfer protein (PLTP)[1] is an 80-kDa glycoprotein that binds phospholipids and facilitates their transfer between lipoproteins in plasma. It is expressed by macrophages and many other tissues (*1, 2*). PLTP associates with apoA-I and apoE (*3, 4*), as well as with several unidentified proteins (*4*). Two forms of PLTP have been detected in human plasma: an "active" form that transfers phosphatidylcholine from phospholipid vesicles to high-density lipoproteins (HDL) and an "inactive" form that lacks this capability (*4−6*). The apparent molecular mass of the "active" form is similar to that of small HDL particles (∼160 kDa), while the inactive complexes appear to be much larger (apparent MW ∼520 kDa) (*4−6*). However, the plasma fraction showing the greatest PLTP activity has a density of 1.24 g/mL, which is significantly greater than that of HDL (1.063−1.21 g/mL). Thus, PLTP complexes appear to be poorly lipidated (*7*).

PLTP was initially identified by its ability to transport phospholipid between lipoproteins in vitro (*8−10*), and studies using genetically engineered mice confirmed this function in vivo (*11*). PLTP can also bind and transfer free cholesterol (*12*) and vitamin E (*13*) among lipoproteins and between lipoproteins and cells. Like apoA-I, PLTP facilitates cholesterol efflux from cells (*14*) by interacting with the ATP-binding cassette transporter A1 (ABCA1) (*15*). Consequently, it is an important modulator of the level and composition of circulating lipoproteins (*11, 16*). It can also modify the antioxidative potentials of lipoproteins and tissues (*13*).

PLTP is a member of the lipid transfer/lipopolysaccharide binding protein family, which includes cholesteryl ester transfer protein (CETP), bactericidal permeability-increasing protein (BPI), and lipopolysaccharide-binding protein (LBP) (*17*). Both PLTP and CETP transport lipids between lipoprotein classes. However PLTP, like BPI and LBP, binds the lipid A component of lipopolysaccharide (LPS) (*18*), the biochemical hallmark of Gram-negative bacteria, suggesting that PLTP might be involved in host defense mechanisms and inflammation. Indeed, PLTP levels increase during acute inflammation (*19−21*), and PLTP activity associates with inflammatory markers in patients with type 2 diabetes (*22*) and cardiovascular disease (*23*), two disorders linked to systemic inflammation. Moreover, because PLTP binds LPS but does not transfer it to CD14, it neutralizes the polysaccharide's inflammatory effects (*18*). Importantly, PLTP deficiency increases mortality in a mouse model of endotoxemia (*24*).

To test the hypothesis that PLTP interacts with proteins implicated in the inflammatory response, we isolated PLTP complexes from plasma by immunoaffinity chromatography and analyzed their lipid and protein composition (*25, 26*). We found that PLTP resides in lipid-poor complexes rich in proteins implicated in the

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[1]Abbreviations: BPI, bactericidal permeability-increasing protein; HDL, high-density lipoproteins; LBP, lipopolysaccharide-binding protein; LDL, low-density lipoprotein; PC, phosphatidylcholine; PLTP, phospholipid transfer protein; PS, phosphatidylserine; rPLTP, recombinant PLTP. For other proteins and corresponding gene names see Table 1.

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acute phase response and coagulation/complement pathways. Thus, PLTP might participate in innate immunity and inflammation. Importantly, because PLTP complexes are lipid-poor and the majority of the proteins identified have known protein—protein interactions, our data suggest that such interactions are the driving force for the assembly of these complexes.

EXPERIMENTAL PROCEDURES

Human Studies. All studies involving human material were approved by the Human Studies Committee at the University of Washington. Blood was obtained from eight healthy young adults (four male and four female, ages 20−30 years) after an overnight fast using Vacutainer tubes (Becton-Dickinson) containing disodium EDTA. Plasma prepared by low-speed centrifugation for 30 min at 4 °C was promptly supplemented with a protease inhibitor cocktail (Calbiochem) (final concentrations: 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 150 nM aprotinin, 0.5 mM EDTA, 1 μM leupeptin hemisulfate, and 1 μM E-64), and PLTP complexes were immediately isolated.

Antibodies and Immunoadsorbents. Polyclonal antibodies against PLTP were produced by immunizing chickens with purified full-length recombinant PLTP (rPLTP). The rPLTP was produced in BHK-570 cells transfected with human PLTP-His tag cDNA and purified from the serum-free conditioned medium of these cells with Ni-NTA agarose (Qiagen) (*1*). Specific chicken anti-PLTP antibodies were isolated from egg yolk IgY by affinity chromatography using rPLTP covalently coupled to CNBr-activated Sepharose 4B according to the manufacturer's protocol (GE Life Sciences). Affinity-isolated anti-PLTP antibodies reacted with a single band of material in human plasma (∼80 kDa) that comigrated with rPLTP subjected to SDS gel electrophoresis. These antibodies completely inhibited rPLTP's phospholipid transfer activity and >95% of human plasma phospholipid transfer activity. Chicken anti-PLTP immunoadsorbent was prepared by conjugating 12 mg of the antibody to 2 g of CNBr-activated Sepharose 4B. Identical amounts of IgY obtained from the same chickens prior to immunization ("preimmune IgY") were similarly conjugated to 2 g of CNBr-activated Sepharose 4B to create a control immunoadsorbent.

We also prepared Mab4, a monoclonal antibody to PLTP that recognizes a population of plasma PLTP that is unable to transfer phospholipid from liposomes to HDL (*6*). Mab4 was produced by injecting pristine primed Balb/c mice with the Mab4 hybridoma cells, and the antibody was purified from the ascites with fast-flow protein A−Sepharose (GE Life Sciences). Purified Mab4 (27 mg) was conjugated to 2.5 g of CNBr-activated Sepharose 4B to generate the Mab4 immunoadsorbent. All affinity gels were packed into columns and stored in 0.01 M Tris buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, and 0.01% sodium azide (Tris buffer).

Isolation of PLTP-Containing Complexes by Immunoaffinity Chromatography. PLTP complexes were isolated from plasma (45−60 mL) using two immunoaffinity columns in sequence: first the Mab4 column (8 mL) to remove an inactive PLTP population, followed by chicken anti-PLTP column (7 mL). Plasma was applied to the columns at 8 mL/h, and the columns were subsequently washed with Tris buffer, initially at 8 mL/h then at a flow rate up to 90 mL/h, until the 280 nm absorbance of the wash fell to zero. PLTP complexes bound to the chicken anti-PLTP immunoadsorbent were eluted with 0.1 M glycine (pH 2.8) at 90 mL/h. The pH of the eluted material was immediately adjusted to 7 with 1 M Tris (pH 8.6), and assays for PLTP activity were

preformed. All active fractions were combined and concentrated under vacuum, using a membrane filter that excluded molecules with MWs <10000 (Spectrum Laboratories). Contaminating immunoglobulins were removed by exposing concentrated PLTP complexes to protein A and protein G coupled to Sepharose beads (GE Life Sciences). Recovery of PLTP from the columns after concentration and protein A and protein G adsorption was 64 ± 9%.

To assess nonspecific protein binding to the immunoaffinity column, the flow-through plasma of the chicken anti-PLTP immunoadsorbent column was applied to the preimmune IgY immunoadsorbent at 8 mL/h, and the column was washed with Tris buffer until the 280 nm absorbance of the wash fell to zero. Material bound to the preimmune column was eluted with 0.1 M glycine (pH 2.8) and immediately neutralized with 1 M Tris, pH 8.6. Eluted materials were monitored with absorption spectroscopy. All materials with 280 nm absorption >0 were pooled, concentrated, and exposed to protein A and protein G coupled to Sepharose beads as described above for the anti-PLTP column.

Protein Digestion. Isolated complexes (10 μg of protein) were adjusted to a final concentration of 100 μg/mL with digestion buffer (0.1% Rapigest, Waters; 50 mM Tris buffer, pH 8.0). Samples were reduced, alkylated, and digested at 37 °C with two aliquots of trypsin (1:50 (w/w) trypsin:protein; Promega), first for 2 h and then overnight. Proteolysis was stopped by adding HCl (final concentration 50 mM). The samples were incubated for 45 min at 37 °C to hydrolyze the Rapigest detergent and then clarified by centrifugation. The supernatants were dried under vacuum and resuspended in 5% acetonitrile (0.3% acetic acid) for mass spectrometric analysis.

LC-ESI-MS/MS. Tryptic digests (2 μg of protein) were injected onto a trap column (Paradigm Platinum Peptide Nanotrap, 0.15 × 50 mm; Michrom Bioresources, Inc.), desalted for 5 min with 1% acetonitrile/0.1% formic acid (50 μL/min), eluted onto an analytical reverse-phase column (0.15 × 150 mm, Magic C18AQ, 5 μm, 200 A; Michrom Bioresources, Inc.), and separated at a flow rate of 1 μL/min over 180 min, using a linear gradient of 5% to 35% buffer B (90% acetonitrile, 0.1% formic acid) in buffer A (5% acetonitrile, 0.1% formic acid). Mass spectra were acquired in the positive ion mode, using electrospray ionization in a linear ion trap mass spectrometer (LTQ; Thermo Electron Corp., San Jose, CA) with data-dependent acquisition (one MS survey scan followed by MS/MS scans of the eight most abundant peaks in the survey scan). Samples from four subjects from the PLTP affinity column and from three subjects from the control IgY column were analyzed in duplicate.

Protein Identification by LC-ESI-MS/MS. MS/MS spectra were matched against the human International Protein Index (IPI) database v.3.4 (12/1/2006) (*27*) using the SEQUEST (v 2.7) search engine with fixed Cys carbamidomethylation and variable Met oxidation modifications. The mass tolerance for precursor ions was 2.5 Da, and the SEQUEST default tolerance was accepted for product ions (*28*). SEQUEST results were further validated with PeptideProphet and ProteinProphet (*29*, *30*) using an adjusted probability of ≥0.90 for peptides and ≥0.95 for proteins. Each charge state of a peptide was considered a unique identification.

Protein Quantification. For LC-ESI-MS/MS analysis, proteins were quantified by spectral counting (*31−33*). To account for proteins that bound nonspecifically to the preimmune IgY column, spectral counts were normalized to the albumin content of material isolated from the anti-PLTP and preimmune IgY

columns, respectively. The spectral counts for each protein detected in the preimmune material were then subtracted from those detected in the PLTP complexes. Corrected spectral counts were normalized to the molecular weight of each protein (*34*). Total protein was quantified by light absorption at 280 nm. ELISAs were used to quantify PLTP (*35*), apoA-I (AlerCHEK), and clusterin (BioVendor).

Protein Identification by MALDI-TOF/TOF. Proteins separated by SDS−PAGE were digested in the gel with trypsin and analyzed by matrix-assisted laser desorption ionization−time-of-flight−time-of-flight (MALDI-TOF/TOF) MS/MS. The proteins were identified by their MS and MS/MS spectra using the MASCOT search engine (Matrix Science v2.0) (*36*) and Swiss-Prot protein database (v.10/25/2004). Database searches were restricted to human proteins (with fixed Cys carbamidomethylation, variable Met oxidation, 50 ppm precursor, and 0.1 Da fragment ion accuracy). Protein identification required a ≥98% confidence based on the MOWSE score.

High-Resolution Size-Exclusion Chromatography. Isolated PLTP complexes were subjected to size-exclusion chromatography by FPLC on a Superose 6 column that was eluted at 0.5 mL/min with phosphate-buffered saline (PBS). Fractions (0.5 mL) were collected and assayed for PLTP activity and for PLTP and clusterin mass. Reference and calibration proteins were HDL, LDL, blue dextran, thyroglobulin, ferritin, catalase, and aldolase.

PLTP Binding and Functional Studies. Binding of PLTP to proteins identified in the PLTP complexes was quantified by a solid-phase binding assay. Purified protein (100 μL of 5 μg of protein/mL in 100 mM carbonate buffer, pH 9.6) was incubated in 96-well microtiter plates (MaxiSorp, Nunc) at 4 °C overnight. Protein-coated wells were washed three times with PBS buffer containing 0.05% Tween 20 (PBS-T) (Sigma-Aldrich) and blocked with Starting Block (Pierce Biotechnology) at room temperature for 2 h. Wells were washed three times with PBS-T and then incubated at 37 °C with 100 μL of recombinant PLTP (rPLTP; 0.5−5 μg/mL in PBS-T) for 2 h. After three washes with PBS-T, PLTP bound to the wells was detected with affinity-isolated polyclonal anti-PLTP antibodies labeled with horseradish peroxidase, with *o*-phenylenediamine dihydrochloride as substrate. The reaction product was monitored by absorbance at 490 nm. Results were corrected for nonspecific rPLTP binding, using wells lacking protein that were blocked with Starting Block. Standard curves were generated by coating wells with affinity-purified anti-PLTP antibodies and then incubated with 100 μL of rPLTP at concentrations between 25 and 200 ng/mL as described (*35*).

To determine the effect of protein binding on PLTP function, rPLTP stored at −70 °C was thawed and diluted to 20 μg/mL in Tris buffer. PLTP alone, with a test protein (1:2 (mol/mol) PLTP: protein), or with phospholipid liposomes containing phosphatidylcholine (PC) and phosphatidylserine (PS) (4:1 (mol/mol) PC: PS) was incubated for 1 h at 37 °C, and phospholipid transfer activity was determined immediately by measuring the transfer of [^{14}C]phosphatidylcholine from phospholipid liposomes to HDL as described. Briefly, each assay tube contained 50 μL of HDL$_3$ with 150 nmol of phospholipids, 50 μL of ^{14}C-PC-labeled liposomes containing 50 nmol of PC, 5 μL of test samples, and Tris-buffered saline to bring the total assay volume to 400 μL. All samples were incubated at 37 °C for 15 min. Donor and acceptor particles were separated by precipitation with dextran sulfate and magnesium chloride (*37*).

ApoA-I used in these experiments was isolated from delipidated human plasma HDL by ion-exchange chromatography (*38*).

The sources of other proteins were as follows: plasma apoE (rPeptide), recombinant clusterin (BioVendor), plasma vitronectin (R&D Systems), plasma protein S, serum two-chain complement C1r, and plasma transthyretin (Calbiochem), plasma glu-plasminogen and fibrinogen (plasminogen depleted) (Enzyme Research Laboratories), and essentially fatty acid-free serum albumin and serum immunoglobulin G (Sigma-Aldrich).

Immunoblot Analysis. Proteins in PLTP complexes were reduced and separated in SDS on either 4%−20% Tris-HCl polyacrylamide gels (Bio-Rad) or 4%−12% polyacrylamide gels in MES buffer (NuPAGE; Invitrogen), transferred to 0.2 μm nitrocellulose or PVDF membranes, and probed with antibodies. PLTP was detected with affinity-purified chicken anti-PLTP antibodies and horseradish peroxidase- (HRP-) conjugated goat anti-chicken IgY (Kirkegaard and Perry) with a chemiluminescent substrate (Pierce). Other primary and secondary antibodies were as follows: purified mouse anti-human clusterin monoclonal antibody (BD Pharmingen), murine monoclonal anti-human vitronectin (Quidel), affinity-purified goat anti-human protein S antibody (R&D Systems), affinity-purified goat anti-human complement C1r antibody (R&D Systems), HRP-conjugated goat anti-mouse immunoglobulin specific antibody (BD Pharmingen), and rabbit anti-goat IgG, Fc fragment-specific HRP conjugate (Calbiochem).

Quantitation of Lipids. Phosphatidylcholine (PC), sphingomyelin (SM), triacylglycerol (TAG), total cholesterol, and free cholesterol (FC) were quantified in PLTP complexes using a triple-quadrupole mass spectrometer equipped with an electrospray ion source (*39*, *40*). Cholesteryl ester (CE) was calculated as the difference between total and FC. Molecular weights used to convert the mole contents of these lipids to weight were PC and SM (710), TAG (850), cholesterol (387), and CE (650).

Gene Ontology and Protein−Protein Interaction Analysis. Functional annotation of proteins detected in PLTP complexes was obtained from the Gene Ontology database (http://www.geneontology.org; Gene Ontology Consortium) using the DAVID Bioinformatics Resource (NIAID, NIH v.2007) (*41*) and BINGO plugin (*42*) in Cytoscape (*43*). We also searched PubMed for each protein and the following keywords: acute phase response, lipid metabolism, apoptosis, complement, and coagulation.

Protein−protein interaction networks were constructed with Cytoscape, using the MiMI plugin interrogating the Michigan Molecular Interactions database (*44*). Protein identifications (IPIs) were cross-referenced to Uniprot accession numbers.

RESULTS

LC-ESI-MS/MS Reveals That Immunoaffinity-Isolated PLTP Complexes Carry a Diverse Protein Cargo. We used shotgun proteomics to identify the proteins associated with PLTP complexes isolated by immunoaffinity chromatography from the plasma of eight healthy subjects. After digesting the complexes with trypsin, we analyzed the resulting peptide mixture by LC-MS/MS and matched the peptide tandem mass spectra with spectra derived from sequences in a protein database.

We used two complementary criteria to determine whether a protein was associated with PLTP. First, we applied rigorous criteria for protein identification: (a) detection of at least three unique peptides with a peptide probability of >0.90 by PeptideProphet; (b) a protein probability of >0.95 by ProteinProphet; and (c) detection of the protein in ≥4 independent preparations of PLTP complexes. The ProteinProphet estimated protein false

Table 1: Proteins Identified in Immunoaffinity-Isolated PLTP Complexes[a]

IPI	protein	gene	spectral counts[b]	moles per complex[c]	mole fraction[d]
IPI00291262	clusterin	CLU	286.3	4.1	24.4
IPI00298497	fibrinogen β chain	FGB	100.1	1.4	8.5
IPI00021891	fibrinogen γ chain	FGG	94.1	1.4	8.2
IPI00021885	fibrinogen α chain	FGA	93.3	0.8	4.4
IPI00022432	transthyretin	TTR	24.6	1.2	7.1
IPI00022733	PLTP	PLTP	81.4	1.0	6.1
IPI00019580	plasminogen	PLG	88.8	0.7	4.2
IPI00021841	apolipoprotein A-I	APOA1	28.8	0.7	3.9
IPI00294004	vitamin K-dependent protein S	PROS1	52.4	0.5	3.2
IPI00296165	complement C1r subcomponent	C1R	40.4	0.5	2.9
IPI00303482	fibrinogen-like protein 1	FGL1	23.8	0.5	2.7
IPI00218732	serum paraoxonase/arylesterase 1	PON1	19.2	0.4	2.6
IPI00298971	vitronectin	VTN	31.3	0.4	2.6
IPI00022371	histidine-rich glycoprotein	HRG	29.4	0.3	2.0
IPI00006662	apolipoprotein D	APOD	10.4	0.3	2.0
IPI00216882	mannan-binding lectin serine protease 1 isoform 3	MASP1	19.7	0.3	2.0
IPI00299307	mannan-binding lectin serine protease 1 isoform 1	MASP1	6.5	0.1	0.3
IPI00290283	mannan-binding lectin serine protease 1 isoform 2	MASP1	9.6	0.1	0.5
IPI00021854	apolipoprotein A-II	APOA2	10.7	0.3	1.9
IPI00021842	apolipoprotein E	APOE	16.4	0.3	1.8
IPI00029061	selenoprotein P	SEPP1	16.2	0.3	1.5
IPI00017530	ficolin-2	FCN2	12.5	0.3	1.5
IPI00477597	haptoglobin-related protein	HPR	5.2	0.1	0.8
IPI00186903	apolipoprotein-L1	APOL1	7.8	0.1	0.8
IPI00044369	plexin domain-containing protein 2	PLXDC2	9.2	0.1	0.6
IPI00024284	perlecan	HSPG2	65.2	0.1	0.6
IPI00064667	carnosine dipeptidase 1	CNDP1	6.0	0.1	0.5
IPI00021727	C4b-binding protein α chain	C4BPA	5.9	0.1	0.4

[a]PLTP complexes ($N = 8$) and nonspecifically adsorbed material ($N = 3$) were prepared from fresh human plasma using affinity-purified chicken anti-PLTP antibodies and preimmune IgY, respectively. All protein identifications required the detection of at least three unique peptides from at least four individuals. [b]Mean spectral counts detected for each protein in PLTP complexes corrected for spectral counts detected for each protein in nonspecifically adsorbed material. [c]Based on corrected spectral counts normalized to the albumin content of material isolated from the anti-PLTP and preimmune columns and the molecular weight of each protein, using the relationship normalized $SC = f[(SC_P/SC_{Alb})_{PLTP} - (SC_P/SC_{Alb})_{preimmune}]/MW_P$, where SC_P and SC_{Alb} are spectral counts for a given protein and albumin, respectively, in either PLTP complex or nonspecifically adsorbed material, MW_P is the molecular weight of the protein, and f is a scaling factor (10^6). Details are provided in ref *34*. [d]The sum of all proteins detected in the particles was assumed to equal 100%.

discovery rate with these criteria at <5%. Second, we corrected the protein composition of affinity-isolated PLTP complexes for proteins detected in material eluted from a preimmune IgY column. To consider an identified protein as a component of the PLTP complexes, we required that the level of spectral counts in the material isolated from the immune column be significantly greater than that of material from preimmune columns, as assessed by the Mann−Whitney nonparametric U test.

Based on these stringent criteria, our mass spectrometric analysis identified 28 unique proteins (including three forms of MASP1 and three chains of fibrinogen) in immunoaffinity-isolated PLTP complexes (Table 1). Isoform 1 of PLTP (full length) was identified in all samples. In contrast, isoform 2 (a highly homologous truncated form of PLTP with an F/L variant at residue 109 and deletion of residues 110−141) was not detected. However, because only one unique tryptic peptide distinguishes the two isoforms and because detection of individual peptides by MS/MS is probabilistic, the presence of isoform 2 cannot be excluded. Twenty-six of the 28 proteins were detected in PLTP complexes isolated from all eight subjects; carnosine dipeptidase and apoA-II were detected in seven of the eight subjects. We identified all three chains of fibrinogen and three isoforms of mannose-binding lectin-associated serine protease 1 (MASP1). For simplicity, we refer to these as fibrinogen and MASP1, respectively. Thus, we detected 24 proteins in the PLTP complexes (Table 1).

Of these 24 proteins, six (vitamin K-dependent protein S, perlecan, fibrinogen-like protein 1, ficolin-2, plexin domain-

containing protein 2, and mannan-binding lectin serine protease) were not previously known to reside in HDL (*25, 26, 45*).

Clusterin and ApoA-I Are Major Apolipoproteins in the PLTP Complexes. Studies of model systems strongly support the proposal that spectral counting, summing all of the peptide identifications derived from a single protein in an LC-ESI-MS/MS analysis, can quantify proteins in complex mixtures (*31−33*). To assess the relative abundance of proteins in the PLTP complexes, we therefore used the following strategy. First, we optimized chromatographic conditions to provide extensive data-dependent MS/MS sampling, which is critical for obtaining enough spectral counts to estimate relative protein abundance. Second, after correcting the spectral counts of proteins detected in PLTP complexes for proteins detected in material isolated from a preimmune IgY column, we normalized the corrected spectral counts to the molecular weight of each protein. Normalization adjusts for the probability that smaller proteins are less likely to yield proteotypic peptides (*34, 46*).

This approach demonstrated that clusterin (apoJ) was the most abundant protein; it accounted for ∼25% of the mole fraction in PLTP complexes. The mole fractions of PLTP and apoA-I were ∼6% and ∼4% (Table 1), respectively, suggesting that the molar ratio of clusterin, PLTP, and apoA-I in PLTP complexes was ∼4:1:1. The mole fractions of fibrinogen, transthyretin, and plasminogen ranged from 4%−8%, which were similar to that of PLTP and apoA-I. It is important to note that these results are semiquantitative, because many factors affect peptide detection by LC-ESI-MS/MS.

We also used externally calibrated ELISAs to quantify the amounts of clusterin, PLTP, and apoA-I in the PLTP complexes. This independent approach indicated a mean molar ratio of ~5:1:1, which is in excellent agreement with the MS results.

SDS−PAGE and MALDI-TOF/TOF Analysis Confirm That Clusterin, Complement C1r, and Fibrinogen Are Major Components of PLTP Complexes. We used SDS−PAGE and MALDI-TOF/TOF to further probe the protein composition of affinity-isolated PLTP complexes. Imperial Blue (Pierce) staining revealed multiple bands of material when the proteins in the complexes were reduced and separated under denaturing conditions (Figure 1A). We obtained a similar pattern of protein staining with the PLTP complexes isolated from all eight study subjects. Major protein bands were detected in apparent MW regions around 30, 40, and 80 kDa. Several bands with MWs between 50 and 70 kDa were also detected.

To identify the proteins, we excised the stained bands, digested them with trypsin, eluted the peptides, and subjected them to MS/MS analysis by MALDI-TOF/TOF. The major proteins in the 30, 40, and 80 kDa bands were apoA-I, clusterin, and PLTP, respectively. The major proteins within the 50−70 kDa region were identified as different subunits of fibrinogen (FG). The 80 kDa band also contained substantial amounts of the Ig μ-C-chain, presumably derived from immunoglobulins not removed by adsorption to protein A and protein G. Furthermore, a band with an apparent MW of 90 kDa was identified as complement C1r. Each protein migrated with an apparent MW anticipated for the intact protein, indicating that full-length proteins rather than proteolytic fragments associate with PLTP (*47*). Other major proteins identified by shotgun proteomics but not identified by this approach were plasminogen (expected at ~90 kDa) and transthyretin (expected at ~14 kDa). Their apparent absence most likely resulted from the low-resolution of SDS−PAGE (unresolved multiple bands in the 90 kDa region) and the limit of detection of Imperial Blue stain.

Immunoblotting Detects Clusterin, Protein S, Vitronectin, and Complement C1r in PLTP Complexes. Immunoblot analysis of proteins separated by SDS−PAGE confirmed that the clusterin, protein S, vitronectin, and C1r that we identified in the proteomic analysis were indeed associated with PLTP complexes. The apparent MWs of the immunoreactive material were consistent with those expected for the full-length proteins (Figure 1B). Additionally, precipitation of plasma with anti-clusterin yielded a precipitate that contained PLTP by immunoblot (data not shown).

SDS−PAGE and immunoblotting with affinity-isolated anti-PLTP antibody demonstrated that most of the PLTP in the complexes had the expected MW of 80 kDa. In addition, some immunoreactive material was also detected with apparent MWs of 50, 60, and 170 kDa (Figure 1B). The smaller forms likely represented PLTP fragments or truncated isoform 2, while the larger form presumably was a PLTP dimer.

PLTP Complexes Are Heterogeneous in Molecular Size. PLTP complexes isolated from the plasma of three subjects were analyzed by high-resolution size-exclusion chromatography to determine their molecular sizes and the distribution of clusterin and PLTP. PLTP activity and mass were detected in fractions spanning the entire HDL size range as well as in fractions approaching the size of LDL (Figure 2). Peak PLTP activity was located in fractions 16−20, with estimated MWs of 400 to 600 kDa, while peak PLTP mass was detected in fractions 13−16, with estimated MWs of 500 to >670 kDa. Of particular importance
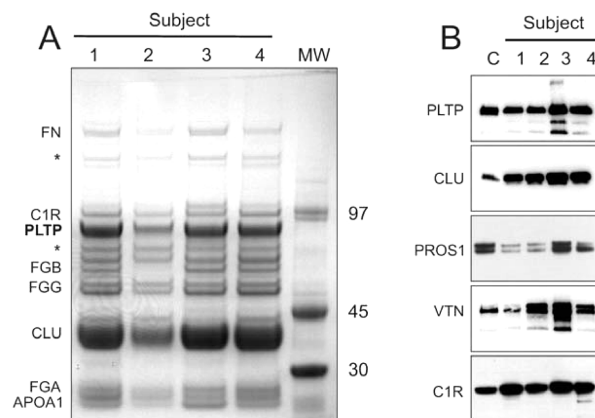
FIGURE 1: Protein composition of PLTP complexes. (A) ApoA-I, clusterin, and PLTP in PLTP complexes were identified by SDS−PAGE and MALDI-TOF/TOF. PLTP complexes (2 μg) isolated from four plasma samples (lanes 1−4) were reduced, separated on 4%−12% SDS gel, and stained with Imperial Blue stain (Pierce). ApoA-I, clusterin, and PLTP were identified in prominent bands around the 30, 40, and 80 kDa positions by in-gel digestion and mass spectrometry. The apparent MWs of the proteins were deduced from the electrophoretic mobilities of a standard protein mixture. Fibronectin (FN) was shown by proteomics to be a nonspecific contaminant (*, various forms of immunoglobulin identified in the bands). (B) Immunoreactive protein S, vitronectin, and complement C1r were identified in PLTP particle. Immunoblot detection of PLTP, clusterin (CLU), protein S (PROS1), vitronectin (VTN), and complement C1r (C1R) in PLTP complexes subjected to SDS−PAGE. Lane C is either purified protein standard (PLTP, CLU, PROS1, VTN) or human plasma (C1R). Lanes 1−4 are PLTP complexes isolated from the plasma of four subjects.

is that clusterin was present in most of the fractions containing PLTP. Thus, PLTP complexes appear to be heterogeneous, both in size and in lipid-transfer activity.

PLTP Complexes Are Lipid-Poor. Pooled PLTP complexes were analyzed for lipid by isotope dilution MS. Lipids represented ~3% of the mass of apoA-I, PLTP, and clusterin in the complexes, as monitored by ELISA. Thus, PLTP complexes are clearly lipid-poor. The approximate molar ratios of PC, SM, FC, CE, and TAG were 28:11:6:12:22, indicating that core lipids (CE, TAG) accounted for ~35% of the lipid moiety and surface lipids accounted for 65%.

Clusterin and ApoA-I Bind to rPLTP and Stabilize Its Activity. We used two complementary approaches to determine whether PLTP binds to any of the proteins we detected in the PLTP complexes and whether such interactions might be functionally significant. First, we determined whether nine of the complexes' abundant proteins can interact with rPLTP in a solid-phase binding assay. For comparison, we included two highly abundant plasma proteins, albumin and immunoglobulin G.

Clusterin, apoA-I, apoE, plasminogen, fibrinogen, transthyretin, and complement C1r all bound to rPLTP in this assay (Figure 3). In contrast, the two control proteins, albumin and immunoglobulin G, failed to interact with rPLTP. We also observed little detectable binding of rPLTP to protein S or vitronectin (data not shown).

To determine if the association of proteins detected in the PLTP complexes might be functionally significant, we took advantage of the fact that rPLTP rapidly loses its lipid-transfer activity unless stabilized with phospholipid liposomes. When rPLTP was incubated without liposomes for 1 h at 37 °C, it lost ~80% of its activity. Importantly, in the absence of liposomes, apoA-I, clusterin, apoE, and, to a lesser extent, C1r were each able to
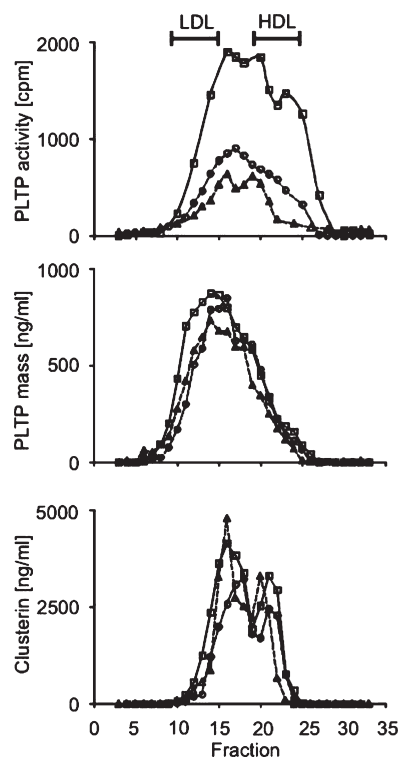
FIGURE 2: PLTP and clusterin coelute on size-exclusion chromatography (SEC). Immunoaffinity isolated PLTP complexes were separated by FPLC using a Superose 6 10/30 column. Column fractions were assayed for PLTP activity, PLTP mass, and clusterin. The panels, from top to bottom, show the distribution of PLTP activity, PLTP mass, and clusterin mass, respectively, of PLTP complexes isolated from three individuals. (Bars at the top indicate where LDL and HDL elute on this SEC column.)
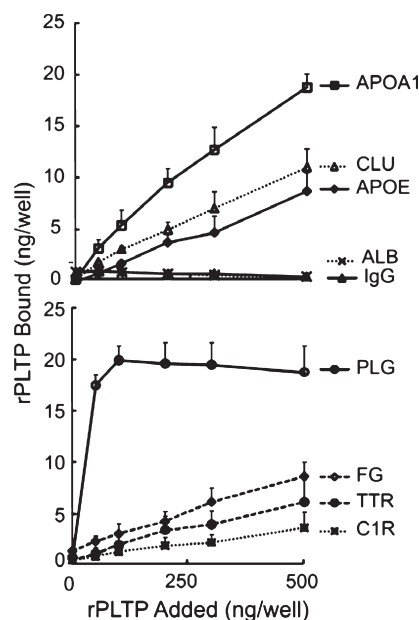

FIGURE 3: PLTP specifically binds to major proteins identified in PLTP complexes. Purified proteins immobilized on microtiter plates were reacted with rPLTP. After extensive washing, PLTP bound to the proteins was detected with anti-PLTP antibodies labeled with horseradish peroxidase; *o*-phenylenediamine dihydrochloride was used as the substrate. Data represent the mean and standard deviation of three experiments for apoA-I (APOA1), apoE (APOE), clusterin (CLU), fibrinogen (FG), plasminogen (PLG), transthyretin (TTR), and complement C1r (C1R) and two experiments for human albumin (ALB) and immunoglobulin G (IgG).

significantly stabilize PLTP's lipid-transfer activity (Figure 4). In contrast, fibrinogen, transthyretin, albumin, or immunoglobulins failed to do so, and PLTP activity was significantly lower ($p < 0.05$) in the presence of plasminogen.

Together with the results of the solid-phase binding studies, these data strongly suggest that clusterin, apoA-I, and apoE can bind directly to PLTP and that this interaction stabilizes PLTP's lipid-transfer activity. In contrast, binding of plasminogen to PLTP may destabilize the protein or inhibit its ability to transfer phospholipids.

DISCUSSION

Proteins and Lipids in PLTP Complexes. PLTP associates with apoA-I and apoE (*3*, *4*), and PLTP in plasma coelutes with HDL on size-exclusion chromatography (*3*, *5*). However, our observations indicate that PLTP isolated from human plasma associates with an array of proteins that are distinct from those of HDL. We also found that lipid accounts for only 3% of the mass of PLTP complexes. The latter observation, though surprising, is consistent with earlier reports that most plasma PLTP activity resides in a lipoprotein-deficient fraction of density >1.21 g/mL (*5*, *7*). Using shotgun proteomics, a global protein identification approach, we identified 24 proteins associated with PLTP, including apoA-I, clusterin, fibrinogen, plasminogen, and C1r. Strikingly, this protein repertoire differed markedly from that of HDL particles isolated by ultracentrifugation and affinity chromatography (*25*, *26*, *45*, *48*). While apoA-I and apoA-II are the dominant proteins in HDL, clusterin was the major protein identified in the PLTP complexes. Moreover, we detected a number of

proteins not known to reside in the HDL fraction, including vitamin K-dependent protein S, perlecan, fibrinogen-like protein 1, ficolin-2, plexin domain-containing protein 2, and mannan-binding lectin serine protease. The observation that 90% of plasma paraoxonase associates with a species of apoA-I particles that also contains clusterin (*49*) is consistent with our identification of clusterin, paraoxonase, and apoA-I in PLTP complexes.

Molecular Size of PLTP Complexes. The apparent MW of affinity-isolated PLTP complexes (as assessed by both mass and activity) ranged from ~160 to 670 kDa, which is consistent with the size distribution of PLTP in whole plasma (*5*). Significantly, the clusterin identified in the affinity-isolated PLTP complexes coeluted with PLTP in size-exclusion chromatography. Together with our finding that PLTP can directly bind clusterin, these observations strongly suggest that PLTP and clusterin directly associate through protein−protein interactions. Both proteomics and biochemical analyses (ELISA) revealed that clusterin, PLTP, and apoA-I accounted for ~25%, 6%, and 4%, respectively, of the mole fraction of proteins in immunoaffinity-isolated PLTP complexes. Previous studies have shown that clusterin and apoA-I associate in the same population of particles, with an estimated molar ratio of ~5:1 (*50*). Interestingly, we observed a similar mole fraction in our affinity-isolated PLTP complexes. The molar ratio of PLTP to apoA-I in the PLTP complexes was ~1:1. Assuming that each PLTP complex carries one PLTP, one apoA-I, and four to five clusterin molecules, the range of apparent MWs would be ~400−600 kDa, which is consistent with the particle masses we estimated from size-exclusion chromatography.

Protein−Protein Interactions in the PLTP Complexes. Lipid−protein interactions are thought to play a major role in the formation of lipoprotein particles that contain >30% (by mass)

of lipid. Because lipids account for only ~3% of the mass of PLTP complexes, it is likely that protein−protein interactions, rather than protein−lipid interactions, are more important in assembling PLTP complexes. Indeed, 20 of the 24 proteins have experimentally documented protein−protein interactions according to protein−protein interaction databases (Figure 5). Our solid-phase binding studies confirmed that PLTP interacts directly with apoA-I and apoE, as well as with five additional major proteins in the complexes, including clusterin, C1r, transthyretin, fibrinogen, and plasminogen (Figure 3). Significantly, when rPLTP was incubated in lipid-free buffer, its lipid-transfer activity was maintained by including apoA-I, clusterin, or apoE (and
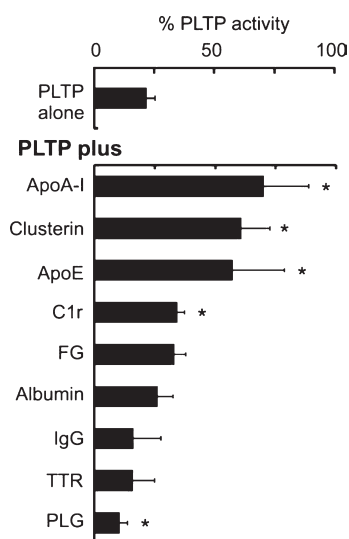

FIGURE 4: ApoA-I, clusterin, and apoE stabilize PLTP's lipid-transfer activity. rPLTP was incubated alone or with apoA-I, clusterin, apoE, complement C1r (C1r), fibrinogen (FG), plasminogen (PLG), transthyretin (TTR), albumin, or immunoglobulin G (IgG) at a molar ratio of 1:2 (PLTP:protein) for 1 h at 37 °C. The PLTP activity of each incubation mixture was immediately measured and compared with that of rPLTP diluted in phospholipid liposomes and incubated under the same conditions. The data represent the mean and standard deviation of three or four experiments and are expressed as the percent of the activity of the PLTP sample incubated in liposomes for 1 h at 37 °C. * denotes significantly different from PLTP incubated alone.

partially by complement C1r) in the buffer, suggesting that such interactions are functionally important. Collectively, these observations indicate that protein−protein interactions are likely to determine which proteins reside in PLTP complexes.

PLTP Complexes Carry Distinct Families of Proteins Related to Inflammation and Immunity. To gain further insight into the possible biological functions of PLTP complexes, we used Gene Ontology analysis and PubMed searches to connect the array of proteins to biological processes. Remarkably, 14 of the 24 proteins were acute-phase-response proteins ($p < 1 \times 10^{-4}$) whose plasma concentrations are altered markedly by both acute and chronic inflammation (Figure 6). Twelve were involved in innate immunity ($p = 2 \times 10^{-5}$), eight in complement activation/regulation ($p = 1 \times 10^{-5}$), and only eight in lipid metabolism ($p = 4 \times 10^{-5}$). Six proteins were also associated with coagulation and thrombosis ($p = 6 \times 10^{-7}$). It is therefore interesting that PLTP, as a member of the lipid transfer/lipopolysaccharide binding protein gene family, is related to LBP and BPI, which play key roles in host responses to Gram-negative bacteria. Studies by Barlage and colleagues (*19*) have shown that in patients with a bacterial infection with or without severe sepsis, PLTP activity is increased, and this increase is paralleled by a redistribution of PLTP into a population of small (120−200 kDa) particles. Our earlier studies have shown that PLTP extracts LPS from bacterial membranes (*51*) and transfers LPS to HDL (*18*) while others have shown that PLTP can mediate the transfer of LPS from HDL to LDL (*52*). Also, Oslakovic et al.(*53*) have recently shown that PLTP mediates the transfer of procoagulant anionic phospholipids to HDL and LDL, thereby neutralizing the effect of the procoagulant liposomes. It is clear that PLTP is an LPS binding protein and PLTP transfers LPS. The increase in PLTP-mediated phospholipid transfer activity in inflammation may represent (1) a protective mechanism against the deleterious effects of LPS in endotoxemia by facilitating the transfer of LPS to HDL and thereby neutralizing its activity (*24*) and (2) the facilitation of the transfer of phospholipid and alpha tocopherol to tissues needed for the protection or regeneration of cellular membranes such as those from vascular endothelial cells, that may be targets of the activated components of the complement and coagulation systems during inflammation (*13, 19, 54*).
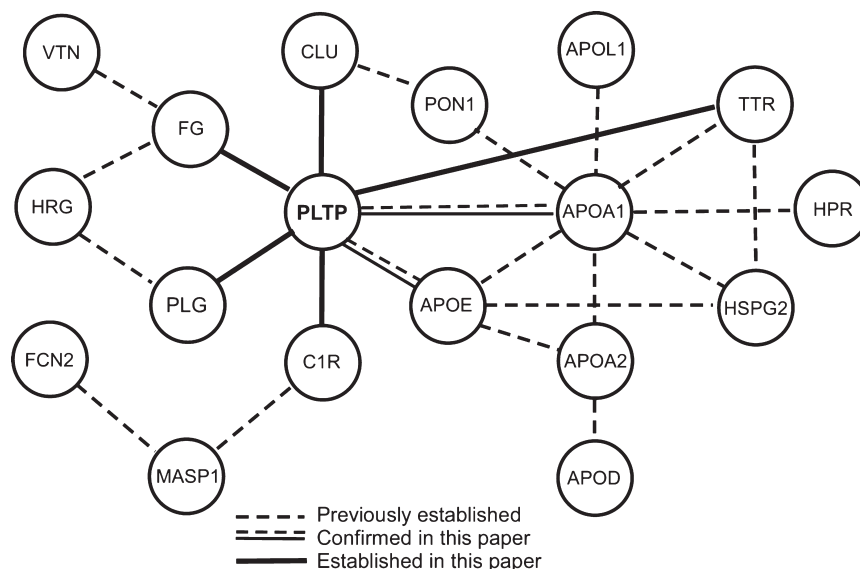

FIGURE 5: Most proteins in PLTP complexes have established protein−protein interactions. A protein interaction network for the proteins detected in PLTP complexes was constructed using the MiMI plugin in Cytoscape and the Michigan Molecular Interactions database.
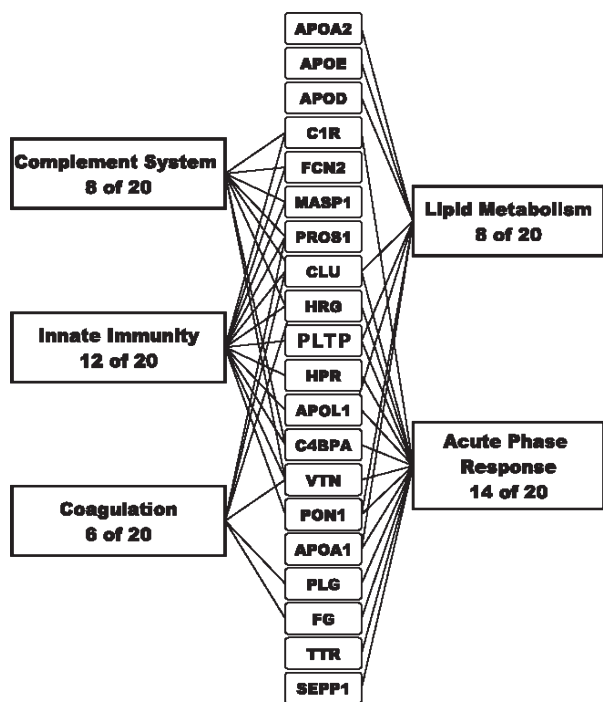
FIGURE 6: The proteome of PLTP complexes associates with components of the immune system. Using Gene Ontology annotation analysis and PubMed searches, we assigned major functional categories to the proteins that associated specifically with PLTP in plasma.

The ability of PLTP to transfer phospholipids appears to depend, in part, on the size of the PLTP complexes as large PLTP complexes (520 ± 120 kDa; 12 to >17 nm) contain an inactive form of PLTP, while complexes associated with an active form of PLTP are smaller (160 ± 40 kDa) (7.6−12 nm) (*5*, *6*). It is not known if the larger inactive PLTP complexes have more lipids than the smaller active PLTP complexes as would be expected if these complexes possess a structure similar to HDL particles. However, as these complexes appear to possess as little as 3% lipid, their structure presumably differs from bulk HDL particles. The fact that these PLTP complexes contain little lipid may actually enhance their ability to shuttle phospholipids to and from cell surfaces and thereby modulate inflammation and the host defense response. Collectively, these observations strongly suggest that PLTP may play previously unsuspected roles in modulating innate immunity and may also regulate the complement and coagulation cascades, other key components of the host response to infection and tissue damage.

Summary. PLTP is expressed in many tissues with various functions (*1*). Significantly, it is also expressed by macrophages (*2*), key cells of the innate immune system, and macrophage PLTP contributes significantly to plasma PLTP levels in mouse models (*55*). The wide array of functionally distinct proteins detected in our affinity-isolated PLTP complexes and the size heterogeneity of those complexes may reflect multiple origins and functions of plasma PLTP. Interestingly, most of the proteins we found associated with PLTP have established functions in inflammation and immunity. Thus besides participating in lipid transport, PLTP may play previously unsuspected roles in immune responses and inflammatory pathways. Furthermore, our results indicate that affinity-isolated PLTP complexes, while lipid-poor, are dominated by clusterin and that most of their proteins are known to interact directly with each other. Therefore, protein−protein interactions, rather than lipid−protein interactions, likely drive the assembly of plasma PLTP complexes.

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