.html

Interaction of phospholipid transfer protein with human tear fluid mucins[s]

Niko L. Setälä,[*,**,§§] **Juha M. Holopainen,*** **Jari Metso,**[**] **Gebrenegus Yohannes,**[†] **Jaakko Hiidenhovi,**[***] **Leif C. Andersson,**[§] **Ove Eriksson,**[††] **Alexandra Robciuc,**[*,***] **and Matti Jauhiainen**[1,**]

Departments of Ophthalmology,* Chemistry,[†] and Pathology,[§] University of Helsinki, Finland; National Institute for Health and Welfare, Public Health Genomics Research Unit and Institute for Molecular Medicine (FIMM),** Biomedicum, Helsinki, Finland; Research Program of Molecular Neurology,[††] Institute of Biomedicine, Biomedicum, Finland; Department of Ophthalmology,[§§] University of Turku, Turku, Finland; and MTT Agrifood Research Finland,*** Biotechnology and Food Research, Jokioinen, Finland

Abstract In addition to circulation, where it transfers phospholipids between lipoprotein particles, phospholipid transfer protein (PLTP) was also identified as a component of normal tear fluid. The purpose of this study was to clarify the secretion route of tear fluid PLTP and elucidate possible interactions between PLTP and other tear fluid proteins. Human lacrimal gland samples were stained with monoclonal antibodies against PLTP. Heparin-Sepharose (H-S) affinity chromatography was used for specific PLTP binding, and coeluted proteins were identified with MALDI-TOF mass spectrometry or Western blot analysis. Immunoprecipitation assay and blotting with specific antibodies helped to identify and characterize PLTP-mucin interaction in tear fluid. Human tear fluid PLTP is secreted from the lacrimal gland. MALDI-TOF analysis of H-S fractions identified several candidate proteins, but protein-protein interaction assays revealed only ocular mucins as PLTP interaction partners.[s] We suggest a dual role for PLTP in human tear fluid: (1) to scavenge lipophilic substances from ocular mucins and (2) to maintain the stability of the anterior tear lipid film. PLTP may also play a role in the development of ocular surface disease.—Setälä, N. L., J. M. Holopainen, J. Metso, G. Yohannes, J. Hiidenhovi, L. C. Andersson, O. Eriksson, A. Robciuc, and M. Jauhiainen. **Interaction of phospholipid transfer protein with human tear fluid mucins.** *J. Lipid Res.* 51: 3126–3134.

Supplementary key words lipid transfer • tear lipid film • lacrimal gland

Dry eye syndrome (DES), the most common ocular disorder that affects around 14% of individuals aged 65 ± 10 years (1), poses a considerable public health problem as well as an economic burden to patients and the community. DES is considered to arise from the interplay of inadequate tear production, increased tear evaporation, and altered composition of the tear film. The underlying biochemical and physiological events in the development of DES and precise composition of the human tear fluid are only partially understood. Until now the model of the tear film has been a three-layered structure (2, 3): the inner mucin-enriched phase and the middle aqueous layer with soluble proteins form a gel-like structure while the outermost layer consists of lipids. On the basis of analysis of meibomian gland secretions, the lipid layer is suggested to be composed of wax esters, sterol esters, and polar lipids (4, 5). It has been suggested, based on the hydrophobic effect, that the charged (polar) phospholipids are disposed adjacent to the aqueous-mucin layer and, externally to this, a layer composed of nonpolar lipids, such as cholesteryl esters and triglycerides, face the tear-air interface (6–8). This type of lipid organization is believed to strongly oppose evaporation. Yet, the ocular and mucin components become vulnerable to lipid contamination, which would lead to dewetting of the corneal epithelium. A mechanism to organize and maintain homeostasis of the lipid layer and to prevent epithelial or mucin contamination is needed. Indeed, lipocalin, one such kind of protein, has been confirmed to efficiently remove lipids from the corneal surface (9). Yet this observation does not ex-

clude the possibility that other tear proteins are capable of performing similar functions.

Earlier we have shown that active form (high active; HA) of the phospholipid transfer protein (PLTP), a glycoprotein with phospholipid transfer activity, is a normal component of the human tear fluid (10). This finding has been recently verified with liquid chromatography (LC) MALDI-TOF mass spectrometry studies by Li et al. (11). PLTP was originally found in plasma, where it has an important role in lipoprotein metabolism (12). In plasma, PLTP transfers phospholipids, but not neutral lipids, between lipoprotein particles (13). The PLTP gene is also highly expressed in alveolar type II cells and is induced during hypoxia and in emphysema (14), indicating surface protective properties. Notably human lung tissue, where PLTP is found in an air-water environment (i.e., similar to tear fluid), displays high PLTP expression levels compared with other tissues.

We have been unable to unambiguously demonstrate the function of PLTP in tear fluid. To shed light on the function of PLTP, we first examined the cellular site for PLTP secretion into the tear film. To elucidate the function of PLTP, we then searched the proteins that are possibly interacting and forming a functional complex with PLTP in the human tear fluid. Here we demonstrate that human tear fluid PLTP is secreted form lacrimal gland, and it interacts with mammalian mucins. Our data suggest that PLTP has an important role in the maintenance of lipid balance of the human tear fluid and could in part attenuate the development of DES.

EXPERIMENTAL PROCEDURES

Protein samples and antibodies

PLTP was purified from human plasma as described (15, 16). In the present study, the purified active PLTP was used in experiments. Mouse monoclonal (MAb59 and MAb66 IgG) and rabbit polyclonal (R290 IgG) antibodies against human PLTP were produced as described earlier (13). A soluble form of ovomucin was purified from egg-white as described earlier (17).

Bovine submaxillary gland mucin (BSM; type I-S) and lactoperoxidase were from Sigma (St. Louis, MO), and lysozyme was from Abcam (Cambridge, UK). Monoclonal antibodies against lipocalin and serum amyloid A were from Abcam, and lactoperoxidase and proline rich-protein 1 monoclonal antibodies were from Sigma. Monoclonal mouse anti-human mucin 5AC (MUC 5AC, clone 2-12M1) was purchased from AbD Serotec (Oxford, UK), and the polyclonal rabbit anti-MUC5B antibody was from Sigma Prestige Antibodies (St. Louis, MO).

Enzyme conjugated secondary antibodies, goat anti-mouse IgG Horseradish peroxidase (HRP), and goat anti-rabbit IgG HRP were purchased from Bio-Rad (Bio-Rad Laboratories, CA).

Lacrimal gland samples

Pieces of main lacrimal gland and the adjacent conjunctiva were obtained from nine subjects with whom the lacrimal gland was excised for diagnostic purposes (one prominent, three suspicious for sarcoidosis, three excised with removal of prolapsed orbital fat, and two lid tumors with no involvement of the lacrimal gland).

Tear fluid samples

Onion vapor-induced tear fluid samples were collected from the lower conjunctival sac of two healthy subjects by means of 25 µL micropipettes (Blaubrand Intramark; Brand GMBH, Wertheim, Germany) causing minimal conjunctival irritation. The samples were immediately cooled to 4°C, pooled, and stored at −70°C until analyzed. Total protein content in tear fluid was 9.6 mg/ml as analyzed by the Lowry method, and of that, the PLTP mass was 9.6 µg/ml. This study was performed according to the principles of the Helsinki Declaration and approved by the Ethics Review Committee of the Department of Ophthalmology, University of Helsinki. Informed consent was obtained from each subject. The permission to use tissue samples was obtained from the Finnish National Authority for Medicolegal Affairs.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissues containing the lacrimal gland and the adjacent conjunctiva were incubated with monoclonal antibodies to PLTP, MAb59 and MAb66. The sections were processed in a microwave oven (2×5 min at 900 W) and treated with a methanol-perhydrol solution for 30 min to block endogenous peroxidase activity. Immunohistochemical staining was performed using a commercial Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA). The sections were blocked with CAS-Block solution (Zymed, San Francisco, CA) for 20 min, followed by incubation overnight at 4°C with monoclonal MAb 59 or MAb66 antibody diluted 1:1000 in Chem Mate (Dako, Glostrup, Denmark). Nonimmunized mouse serum served as a primary antibody source in all control staining. The slides were rinsed three times (15 min each) with phosphate-buffered saline (PBS; pH 7.2) between every staining step, and all incubations were carried out in a moist chamber. The bound peroxidase activity was visualized with a specific substrate, 3-amino-9-ethylcarbazole (Sigma) solution (0.2 mg/ml in 0.05 M acetate buffer containing 0.03% perhydrol; pH 5). The slides were stained with hematoxylin, washed with distilled water, and mounted in Aquamount (BDH, Poole, UK).

Heparin trapping of PLTP

To determine possible PLTP-protein interactions in tear fluid, Heparin-Sepharose affinity chromatography was used. Briefly, pooled tear fluid samples in a total volume of 400 µl were applied to a 1 ml HiTrap Heparin column (GE Healthcare, Buckinghamshire, UK) that was equilibrated with buffer A (25 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA). The column was washed with buffer A (flow rate 0.5 ml/min), and the bound material was eluted with a 0.5 M NaCl in buffer A (flow rate 0.5 ml/min; fraction size 1.0 ml). The active PLTP fractions were combined and dialyzed against buffer A and then applied to same 1 ml HiTrap Heparin column. The bound PLTP was eluted with a linear 0–0.5 M NaCl gradient (flow rate 0.5 ml/min; fraction size 0.5 ml). The fractions were analyzed for PLTP activity (18). The active fractions were pooled and concentrated with nanosep 10K omega system (Pall Life Sciences, UK). Lyophilized material was loaded to 12.5% SDS-PAGE gel and stained with Coomassie R-250. The detected protein bands were cut apart and transferred to mass spectrometry.

Measurements of PLTP activity

PLTP activity was recorded by measuring the transfer of ^{14}C-PC from radiolabeled PC vesicles to unlabeled HDL3 acceptors, with minor modifications (10).

Immunoprecipitation of PLTP-protein complex with monoclonal and polyclonal antibodies against PLTP and tear fluid mucin

Immunoprecipitation was used to confirm or exclude the binding partners for PLTP in human tear fluid. Both tear fluid

samples and pure proteins identified by mass spectrometry were studied. Immunoprecipitation of the PLTP was performed using Protein G. Briefly, anti-PLTP MAb 59 or rabbit polyclonal anti-PLTP (R290) was added to washed Protein G (100 µl) and incubated with gentle mixing for 60 min at room temperature, after which the beads were washed two times with 500 µl of TBS-Tween. Either pooled tear fluid samples (100 µl) or purified active PLTP and candidate proteins were added to the beads and incubated at 4°C overnight with gentle mixing. The beads were recovered, and the supernatants were collected. The beads were washed twice (0.01 M Tris, pH 8.0 and 0.05 M Tris, pH 7.4). Protein G-anti-PLTP IgG bound material and supernatants were analyzed with SDS-PAGE using buffer with or without 2 mM β-mercaptoethanol. Adhered proteins were detected with Western blot analyses. Immunoprecipitation of the tear fluid PLTP-mucin complex was also performed using polyclonal anti-MUC5B antibody. Separation on nonreducing 5% SDS-PAGE was done for mucin. PLTP was analyzed using 12.5% SDS-PAGE under reducing conditions. The mucins were detected with the monoclonal antibody 5AC (see above) and PLTP with MAb 59.

Dot-blot analysis

The dot-blot assay was used to determine the PLTP-mucin interactions. Briefly, increasing concentrations (0–45 µg/well) of either ovomucin (OVM) or BSM were transferred onto nitrocellulose membranes using a Minifold (Amersham Pharmacia Biotech) slot blot apparatus. The membranes were blocked with defatted milk (5% w/v in TBS, 0.1% Tween 20, pH 7.5) and washed twice with TBS-Tween 20. Membranes were incubated with HA-PLTP (25 µg) and controls without PLTP over night at 4°C, and then membranes were washed with TBS-Tween 20. Bound PLTP protein was detected by using monoclonal anti-PLTP MAb 59 antibody as above. Bovine mucin and ovomucin were detected with the monoclonal anti-MUC5AC antibody.

Phospholipidation of BSM

BSM and egg-white OVM were phospholipidated using the cholate dialysis method (19). In brief, 1.27 mg of egg yolk phosphatidylcholine [EggPC, Sigma, 100 mg/ml stock in chloroform methanol, 9:1 (v/v)] and approximately 750 cpm of ^{14}[C]DPPC in toluene (Amersham CFA, UK) per nmol of phospholipids were added to a glass vial. The organic solvent was evaporated under nitrogen at room temperature, 1 ml of BSM or OVM (1 mg/ml in PBS) was added, and the mixture was carefully vortexed. Sodium cholate (stock solution 725 mM in 10 mM Tris buffer containing 1 mM EDTA and 140 mM NaCl, pH 7.4 TBS) was used to solubilize the lipids (final concentration 20 mM), and the mixture was gently vortexed to avoid foaming. After an incubation of 20 min at 24°C with shaking, the mixture was extensively dialyzed to remove cholate using a 12–14 kDa cut-off dialysis membrane. Protein-to-lipid ratio of the preparation was 1:100 (mol/mol).

Size exclusion chromatography of mucin-PC and PLTP

The total volume of mucin-PC after cholate dialysis was 1.32 ml, which was divided into four equal aliquots of 330 ul. Two of the aliquots were incubated together with purified active PLTP (6 µg/ml final concentration) for 30 min at room temperature with occasional mixing. All four samples were subjected to fast-performance liquid chromatography on a Superose 6HR size-exclusion column (Amersham Pharmacia Biotech). The column was equilibrated with PBS or PBS-Tween 20 (0.05%) and calibrated using protein standards (BioRad, Richmond, VA) with molecular weights in 1.35–670 kDa range. Size exclusion chromatography (SEC) was performed at a flow rate of 0.5 ml/min,

and 0.5 ml fractions were collected for radioactivity and phospholipid and protein measurements. Radioactivity of the collected fractions was measured with liquid scintillation counting (Wallac LS Counter, Turku, Finland). Phospholipid concentration was assayed using a commercial kit (DiaSys, Holzheim, Germany). Dot-blot analysis of the proteins was performed as described above using anti-PLTP and anti-mucin antibodies.

Western blot analysis

Purified active PLTP was run on SDS-PAGE and electrically transferred to a nitrocellulose membrane (400 mA for 45 min) using 0.1 M Tris with 0.192 M glycine in 20% methanol as a transfer buffer. The membrane was blocked with milk powder (5% w/v in TBS, 0.05% Tween, pH 7.5) for 1 h at room temperature. Pure candidate proteins revealed by mass spectrometry were then added and incubated overnight at 4°C with gentle mixing. Unbound protein was removed by washing the membrane twice with TBS-Tween buffer. Candidate proteins were separately studied for their possible binding to PLTP with specific antibodies, and the membranes were finally subjected to electrochemiluminescence detection.

RESULTS

PLTP is secreted from the lacrimal gland

Given our observation on the presence of PLTP in human tear fluid (10), we first investigated the cellular origin of PLTP. Sections of isolated lacrimal glands were immunohistochemically stained with monoclonal antibodies to PTLP (MAb59 and MAb66). The staining patterns were similar for all nine lacrimal glands studied and for both antibodies (**Fig. 1A**-C). The basal cells of intraglandular ducts gave a positive, granular staining pattern. Accumulation of secreted PLTP was also seen in the lumen (Fig. 1B, C). Neither control experiments with nonimmunized mouse serum (Fig. 1D) nor the secondary antibody alone (data not shown) show staining.

Heparin affinity separation of PLTP and identification of putative PLTP-binding proteins with MALDI-TOF mass spectrometry

To identify protein-protein interaction partners of PLTP we used heparin affinity chromatography to separate PLTP from the tear fluid. Following two consecutive Heparin-Sepharose (H-S) affinity separations, fractions containing active PLTP were collected and separated on SDS-PAGE gels for MALDI-TOF mass spectrometry (see supplementary data). Immunoblotting using Mab59 confirmed the presence of PLTP in the SDS-PAGE gels (**Fig. 2A**). However, Coomassie blue and silver staining of the SDS-PAGE gel displayed several bands (Fig. 2 and supplementary Fig. I). For a better resolution of the higher molecular weight proteins, such as mucins, we also applied the PLTP-containing H-S fractions on a 4–12% gradient gel. Silver staining revealed a protein band migrating in the high molecular weight position (Fig. 2B, arrow), which was later identified by immunoblotting as mucin (supplementary Fig. II). Bands from 12.5% Coomassie-stained SDS-PAGE (supplementary Fig. I) were excised and subjected to MALDI-TOF analysis. Subsequent database searches yielded
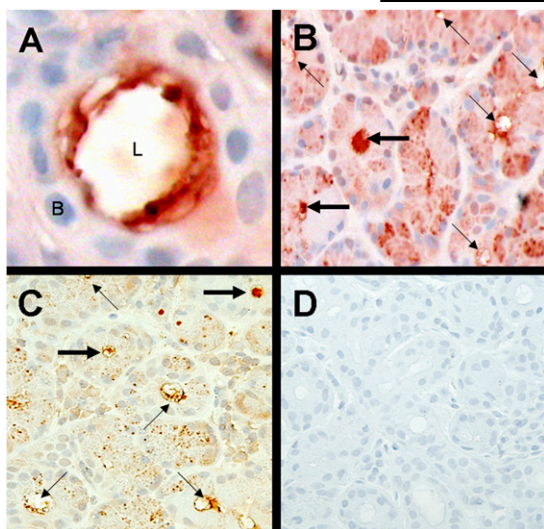
Fig. 1. Production of human tear PLTP in lacrimal glands. A: Histological sections of paraffin-embedded human lacrimal gland stained with hematoxylin-eosin and with MAb59 monoclonal antibody to PLTP. Basal cells of intraglandular ducts display positive staining against PLTP. B and C: Accumulation of secreted PLTP in the lumen (thick arrows) and positively stained basal cells (thin arrows) as demonstrated with strong MAb59 (B) and MAb66 (C) immunostaining. D: Nonimmunized mouse serum showed no staining of the lacrimal glands. At least 10 microscopic fields were visualized for each sample and antibody used. B, basal cell; L, lumen; PLTP, phospholipid transfer protein.

significant hits for lactotransferrin, lactoperoxidase, proline-rich protein 1, and lysozyme C (supplementary Table I). In addition, lipocalin-1 and serum amyloid A were detected in some samples (data not shown). All these proteins have previously been detected in tear fluid and may represent protein-protein interaction partners to PLTP. However, it is also possible that these proteins appear in the PLTP fraction due to direct interaction with the immobilized heparin. To answer this question, we investigated the interactions between these proteins and PLTP by immunoblotting and PLTP immunoprecipitation analysis.

PLTP does not interact with lysozyme-C, lactoperoxidase, lipocalin, or serum amyloid A

We used coimmunoprecipitation and Western blotting to confirm the interaction of PLTP with the proteins coeluted in H-S chromatography and identified by MALDI-TOF mass spectrometry. After several immunoprecipitation experiments both with and without detergent (0.1% Triton X-100), no unambiguous binding was observed between lipocalin or serum amyloid A. A very weak band for lysozyme and lactoperoxidase was observed, and these protein pairs (i.e., PLTP-lysozyme and PLTP-lactoperoxidase) were further analyzed by direct binding assays. In brief, 2 µg of each protein (PLTP and lysozyme or PLTP and lactoperoxidase) were incubated and subjected to PLTP pull-down assay as described above. The results clearly demonstrated that direct binding was not evident (data not shown). Although direct binding between PLTP and the two proteins could not be observed, an interesting

observation was that incubation of PLTP with lysozyme reduced the binding of PLTP to the H-S column (data not shown), suggesting some degree of masking of the heparin-binding domain of PLTP protein or that lysozymes might compete with PLTP for heparin binding sites in the column.

PLTP forms a complex with human tear fluid mucins

Finally, as ocular surface mucins may become contaminated by lipids, the interaction of PLTP with human tear fluid mucins was studied using an immunoprecipitation assay for PLTP. Mucin was not detectable in mass spectrometry analysis (see above) because ocular mucin did not enter the 12.5% SDS-PAGE gel due to its high apparent molecular weight (about 220 kDa). The amount of 50 µl of human tear fluid was incubated with polyclonal anti-PLTP antibody (R 290) (1 mg) in the presence of Protein G. The immunoprecipitate was collected and analyzed by Western blotting using monoclonal antibodies against PLTP and mucin. The results confirmed direct interaction between PLTP and ocular mucins (**Fig. 3**). The existence of a PLTP-ocular mucin complex was also investigated using the polyclonal anti-mucin antibody MUC5B. The results demonstrated that the immunoprecipitate, in addition to mucin, also contained PLTP. However, the commercial antibody used was relatively weak in its ability for immunoprecipitation (data not shown).

PLTP interacts with BSM but not with OVM

Many studies have used commercially available mucins as substitutes for ocular mucins, such as BSM. Prompted by the finding that active PLTP interacted directly with ocular mucins in human tear fluid, we further studied the feasibility of this interaction using purified BSM as well as nonmammalian OVM. To study direct binding of these mucins with PLTP, a dot-blot analysis was carried out. These results demonstrated that purified, active PLTP was directly bound in a concentration-dependent manner to BSM, whereas OVM failed to interact with active PLTP (**Fig. 4**).

Characterization of the mucin-PLTP interaction

To approach possible functional implications for mucin-PLTP interaction in vivo, we designed an in vitro experimental setup in which a phospholipidation protocol was used to generate mucin-phospholipid complex (using both bovine mucin and ovomucin). The complex, also containing trace amounts of ^{14}[C]DPPC, was incubated together with purified PLTP and PLTP distribution was investigated using size exclusion chromatography (SEC). Approximately 80% of the radioactive label was recovered from the BSM-PC complex after cholate dialysis. **Fig. 5** shows the SEC elution profile of the mucins-PC-PLTP samples in PBS (Fig. 5A and 5C for BSM and OVM, respectively) and PBS-Tween 20 (Fig. 5B and 5D). When elution was performed with PBS, PLTP coeluted with BSM-PC in a broad range. However, BSM, ^{14}[C]DPPC, cold PC, and PLTP coeluted in fraction 17, suggesting a complex formation between these components (Fig. 5A). In these ex-
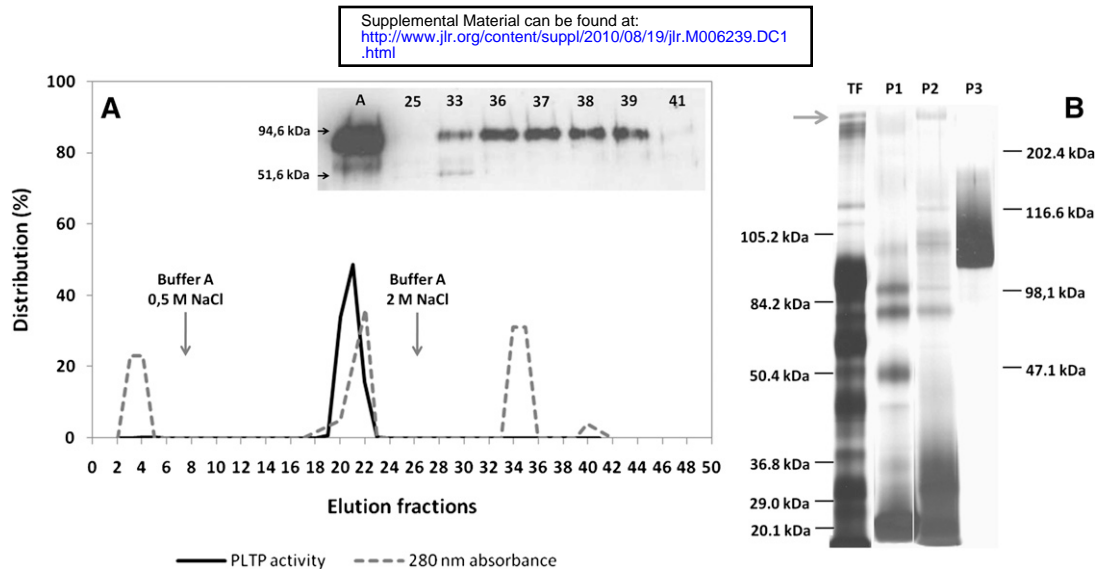
Fig. 2. Analysis of tear fluid PLTP during heparin affinity separation. A: A two-step Heparin-Sepharose affinity chromatography was used for PLTP isolation from tear fluid (see "Experimental Procedures"). Tear fluid (400 μl) was applied to the HiTrap Heparin column. The column was washed with buffer A (25 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA), and 0.5 ml fractions were collected at a flow rate of 0.5 ml/min. Arrows show the start of 0.5 M and 2 M NaCl elution. The dotted gray line represents 280 nm absorbance readings for the collected fractions, and the black line shows PLTP activity from the corresponding fractions. The inset shows a Western blot image using MAb59 of the pooled 20–21 fractions (lane A) and fractions from the second HiTrap Heparin column affinity chromatography where the 20–21 fraction pool was run to the column and then eluted with a linear 0–0.5 M NaCl gradient (flow rate 0.5 ml/min; fraction size 0.5 ml). Fractions that contained active PLTP (36–39) were pooled and used for the proteomic analysis of copurified proteins. PLTP activity was measured using a radiometric method described in "Experimental Procedures." B: Silver stain of an SDS-PAGE gel separation of active PLTP-containing fractions after heparin affinity separation of human tear fluid. The pooled fractions from PLTP purification were loaded on a 4–2% Bis-Tris NuPAGE gel (Invitrogen, CA). P1 represents nonbound material from the washing of the heparin column); P2 is constituted from fractions containing active PLTP; and P3 is the fraction eluted with high-salt concentration. Arrow depicts the migration position of mucins (for immunoblot images of tear fluid mucins and PLTP see supplementary Fig. II). PLTP, phospholipid transfer protein; TF, tear fluid.

periments, lipidated ovomucin behaved similarly with bovine mucin. OVM-PC elutes in two major peaks around fractions 18 and 26. PLTP elutes in the same three peaks observed in the BSM-PC incubation, two of the peaks mirroring the mucin ones. The coelution of all three components—OVM, PLTP, and radiolabeled phospholipids in fractions 17–20—suggests that the lipidated ovomucin can form a complex with PLTP. When 0.05% Tween 20 was used in the elution buffer (PBS), PLTP was released from the complex with mucins (both BSM and OVM) and eluted around the apparent molecular weight of 160 kDa, supporting our earlier observations (15). When used in excess, phospholipids interacting with the mucins could be displaced by Tween 20, a detergent with very high affinity for hydrophobic sites. This result was shown by the change in the phospholipid elution profile (Fig. 5B). When we used lower lipid concentration in the lipidation process, the displacement of excess lipids by the detergent was no longer visible (see OVM in Fig. 5D), but radioactivity in the PLTP fraction 32 doubled compared with the BSM-PC-PLTP sample eluted in PBS. SEC analysis of nonlipidated ovomucin and PLTP interaction was used as a negative control for this experimental setup. Fig. 5E shows elution profile of OVM and PLTP comixture in PBS and PBS-Tween, respectively. PLTP elutes in fractions corresponding to molecular weight of 160 kDa when PBS-Tween is used, whereas this elution profile disappears when we used PBS in the absence of detergent. The same experiments were conducted with nonlipidated BSM, and a 30

min coincubation significantly improved PLTP elution. A longer incubation caused a similar recovery of PLTP to that when PBS-Tween was used as an elution buffer. Our experience in purified PLTP fractionation is that PLTP can nonspecifically interact with the column matrix in the absence of Tween and when an interaction partner is missing (unpublished observations). This finding strongly indicates that PLTP interacts with BSM with or without lipids (interaction with phospholipidated mucin occurs faster and could be more stable). Further, PLTP does not inter-
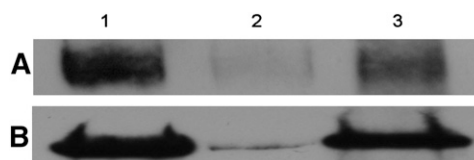

Fig. 3. Interaction of PLTP with human tear fluid mucins. Human tear fluid (50 μl) was incubated with rabbit polyclonal anti-PLTP antibody (R290, 1 mg) in the presence of Protein G. The immunoprecipitate was recovered by low-speed centrifugation. Separation on nonreducing 5% SDS-PAGE was done for mucin. PLTP was analyzed using 12.5% SDS-PAGE under reducing conditions. Lane 1: Human tear fluid. Lane 2: Nonprecipitated supernatant fraction. Lane 3: Immunoprecipitate. A: Western blot analysis of mucin with monoclonal antibody MUC-5AC. PLTP was detected by monoclonal anti-PLTP Mab59 antibody (B). All immunoprecipitation assays were repeated at least three times. PLTP, phospholipid transfer protein.
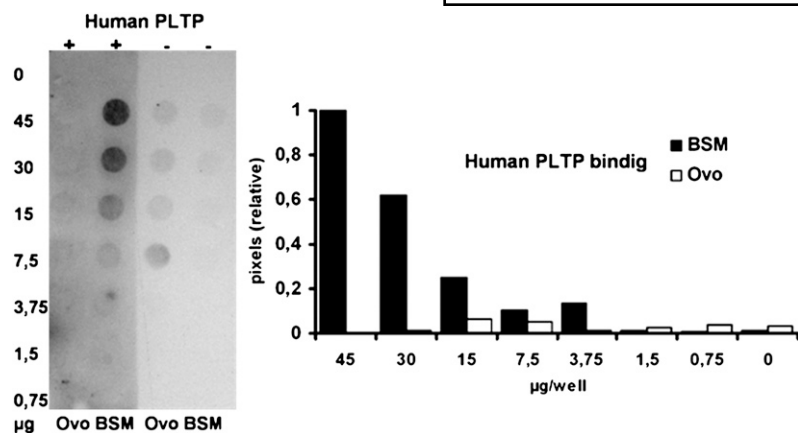
Fig. 4. Binding of PLTP to mammalian-derived mucin BSM but not to avian-derived soluble form of ovomucin. BSM and soluble ovomucin were incorporated with increasing concentrations (0–45 μg/well) to nitrocellulose membranes and incubated with purified PLTP (25 μg). Bound PLTP protein was detected by using monoclonal anti-PLTP Mab59 antibody. The data shown is background-corrected. Binding experiments were repeated at least three times. BSM, bovine submaxillary gland mucin; PLTP, phospholipid transfer protein.

act with OVM in the absence of phospholipids and, therefore, sticks nonspecifically to the column matrix.

DISCUSSION

A recent analysis of the protein composition of the human tear fluid revealed only 54 proteins that were identified with high confidence (11). Yet, de Souza et al. were able to identify nearly 500 proteins in human tear fluid using multiple mass spectrometric approaches (20). It seems that in the latter study, however, the authors may have had significant contaminants from the ocular tissues. This is evidenced by the relatively large number of intracellular proteins as well as the fact that not all of the major proteins, such as phospholipases, were found. The relatively small amount of identified proteins in the study of Li et al. (11) suggests a high stature for these key proteins in human tear fluid. As the composition and functional regulation of tear fluid remains largely unknown, it is important to characterize factors that play a role in the homeostatic processes of tear fluid, such as organization of lipids and viscoelasticity of tear film. We have recently discovered that human tear fluid contains HA-PLTP protein (10). Now we report that human tear fluid PLTP is secreted from the lacrimal gland and that PLTP interacts with human ocular mucins as well as with mammalian-derived mucin (BSM). This finding suggests a possible role for PLTP in the scavenging of lipophilic molecules from the ocular mucins and in maintaining the homeostasis of the anterior tear lipid film. This result does not contradict the recent findings by Glasgow et al. that showed that tear fluid lipocalin can efficiently remove lipids from the ocular surface (9). It seems unreasonable to assume that one protein is solely responsible for removing ocular contaminants in tear fluid. Our current and previous results (10) suggest that, although PLTP is a minor constituent of normal tear fluid, it possesses efficient phospholipid transfer activity and likely aids lipocalin in decontaminating the ocular surface. We suggest that in tear fluid, lipocalin and PLTP may act in concert to ensure efficient lipid transfer. In line with Glasgow et al. (9), our results propose a role for PLTP in the fine-tuning of lipid transfer in tear fluid.

PLTP is a member of the lipid transfer/lipopolysaccharide binding protein family, which also includes CETP, the lipopolysaccharide binding protein (LBP), and bactericidal/permeability increasing protein (BPI). Of these, CETP is absent from tear fluid (10), while BPI is a lacrimal gland-derived, normal component of tear fluid (21). The immunostaining pattern with specific monoclonal antibody against PLTP in lacrimal gland sections was very similar to that demonstrated for BPI (21), and also to that of tear fluid lipocalin (22), suggesting that the lacrimal gland could be one of the sources for the tear fluid PLTP. Indeed, corneal epithelial cells also express PLTP at relatively high levels (unpublished observations).

PLTP plays an essential role for lipid metabolism in the circulation, especially the transfer of postlipolytic phospholipid-enriched surface remnants from triglyceride-rich particles, very low density lipoprotein (VLDL), and chylomicrons (CM) to HDL is of utmost importance for the maintenance of proper HDL levels in the circulation (23). The concentration of PLTP in tear fluid is at least 2-fold higher than PLTP in plasma, while the specific activity of PLTP in tear fluid compared with plasma is significantly lower (10). Both apolipoprotein (apo)A-I and apoE, which are capable of interacting with PLTP in plasma, are absent in tear fluid; the role of these protein-protein interactions have been discussed previously (24). To elucidate other possible PLTP-protein interactions in tear fluid, specific immunoprecipitation and binding assays were carried out. The heparin-bound active PLTP fractions were analyzed by MALDI-TOF mass spectrometry and revealed several candidate proteins: lactoperoxidase, lactotransferrin, proline-rich protein 1, lysozyme C, apoJ (clusterin), serum amyloid A, and lachryglobin. Of these proteins, apoJ and serum amyloid A, as well as PLTP and lipocalin, are known to interact avidly with phospholipids. Thus, complex formation between these proteins seemed to be physiologically reasonable. Yet, further analysis using Western blotting could not confirm binding of any of these proteins to PLTP. Lysozyme and lactotransferrin are major tear fluid proteins and most likely represent contaminants in the sensitive MALDI-TOF analysis. As PLTP does not show interaction with either of these proteins, it seems likely that in tear fluid no other proteins are needed to maintain PLTP capable of transferring phospholipids.
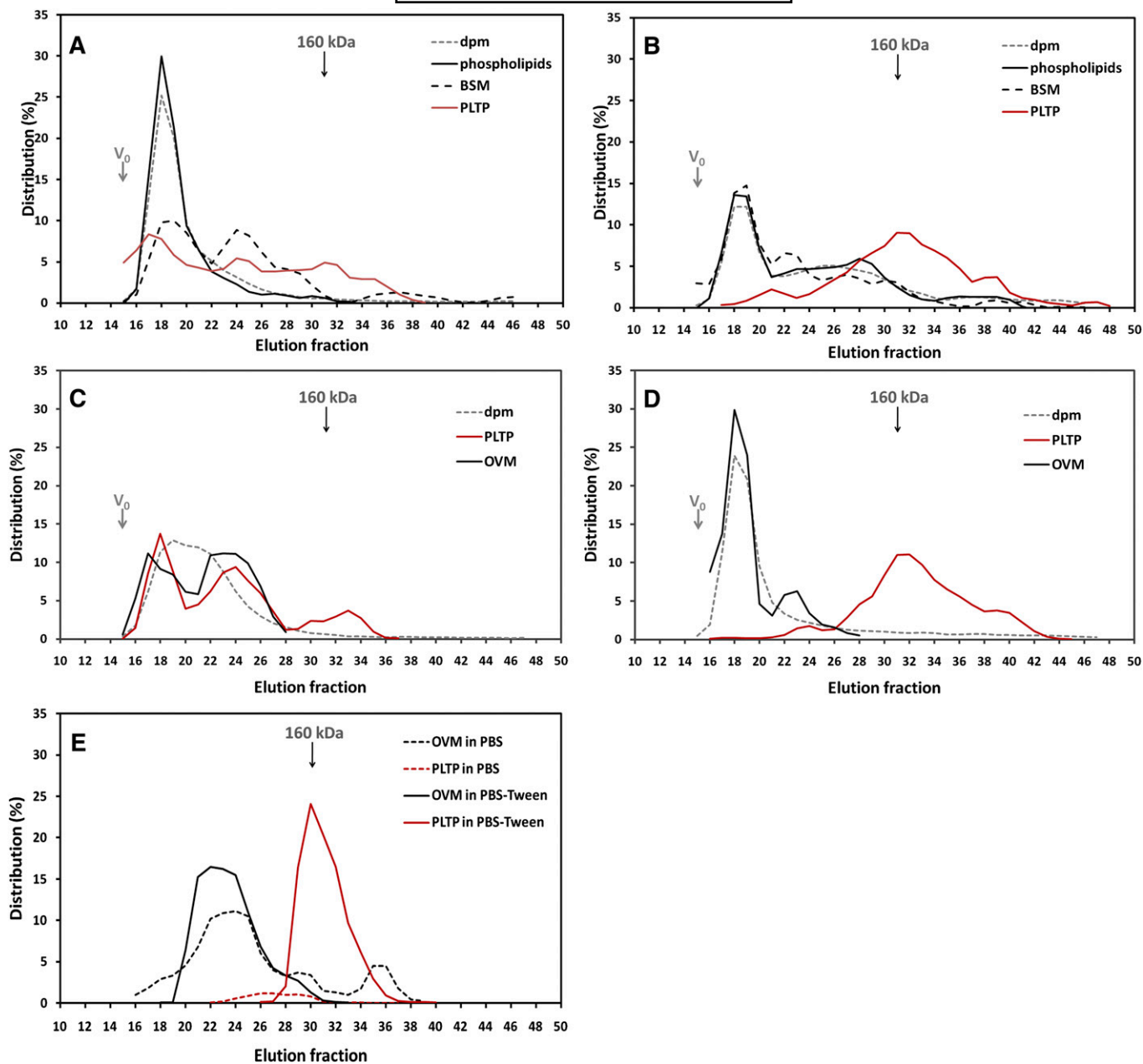
Fig. 5. Size exclusion chromatography of phospholipidated mucins (BSM and OVM) and PLTP. Elution profile of the mucin-PC-PLTP sample in PBS (A and C) and in the presence of Tween 20 (0.05%) (B and D). Panel E represents SEC profile for the nonlipidated OVM and PLTP comixture both in PBS and PBS-Tween. PLTP was incubated with the phospholipidated mucins (final volume of the incubation mix was 0.830 ml) for 30 min at room temperature and applied onto the Superose 6HR column. Fractions (0.5 ml) were collected with a flow rate of 0.5 ml/min. Fractions were analyzed for radioactivity and protein content. Protein data was assessed using immunological dot-blot system (described in "Experimental Procedures") and quantified by densitometry measurements (ImageJ v1.42q software). Results are presented as percentages relative to the total measured value considered as 100%. The arrow at the apparent molecular weight position of 160 kDa depicts the elution position for active PLTP protein (15). SEC runs were repeated three times on average, and a representative run is presented. BSM, bovine submaxillary gland mucin; OVM, ovomucin; PLTP, phospholipid transfer protein; SEC, size exclusion chromatography; V_0, column void volume.

This assumption is also supported by findings that during the normal PLTP purification process from plasma, the final, purified PLTP protein does not contain any other copurified proteins but is nevertheless highly active (16). Furthermore, human seminal plasma PLTP (25) and recombinant PLTP produced by baculovirus protein expression (26) are highly active. Neither of these systems contains apoA-I or apoE, which seems to regulate the activity in plasma. One plausible explanation is that PLTP avidly binds phospholipids and this poorly phospholipidated form could maintain the active conformation.

Mucins are large glycoproteins that have at least 50–80% of their mass as carbohydrate. Mucin polypeptide chains, by definition, have domains rich in serine and threonine (STP, serine, threonine, and proline, tandem repeats), which provide *O*-glycosidic linkage with oligosaccharides

to the protein backbone. Vast polymorphisms are characteristic for mucin molecules, and a unique variable number of tandem repeat lengths are found from one mucin gene to another (27–29). In addition, fatty acids are covalently bound to mucin (30). Ocular mucins are thought to be located at the surface of the tear film, where they cause an increase in surface pressure via lateral reorganization of the lipids and alter surface viscoelastic properties (31). In this study, most importantly, we were able to show by immunoprecipitation assay that PLTP directly interacted with ocular mucins in human tear fluid (Fig. 3). We further examined the interaction of PLTP with mucins in more detail. The collection of human ocular mucins in volumes for in vitro interaction experiments is challenging. Keeping in mind that even within and among individuals each mucin gene has a unique tandem repeat amino acid sequence, length, and variation in allele number of repetitions (32), we decided to use other mammalian mucin (i.e., BSM) and avian-derived soluble OVM. Many studies have used commercially available mucins, such as BSM, and avian-derived mucins, as substitutes for ocular mucins; therefore, they can be considered an adequate model for tear mucins during in vitro experiments. Direct binding assay showed that PLTP interacted with BSM, but not with delipidated avian-derived ovomucin. The likely explanation is that there are certain evolutionary structural differences between these proteins (33).

To further describe the mucin-PC-PLTP interaction in an in vitro experimental setup, we used SEC to verify whether phospholipids are transferred to PLTP following incubation with the phospholipidated mucins. Previous experiments conducted with nonlipidated mucins pointed out that while there appears to be no binding between OVM and PLTP, there is an interaction between lipid-free BSM and PLTP (Fig. 4). Data obtained in the SEC analysis suggested that phospholipids could account as intermediary interaction partners between mucin and PLTP or could strongly promote the interaction between the two proteins. This is very well underlined by the co-elution of phospholipidated OVM with PLTP, which was also demonstrated for BSM. Phospholipids seemed to efficiently bind to mucin and form large complexes. When PLTP was incubated with the phospholipidated mucins, PLTP eluted with mucin in fractions corresponding to a relatively wide molecular size range. Detergent in the buffer significantly changed PLTP elution profile, indicating a disruption of the mucin-PLTP interaction. It has been shown that mucin proteins have hydrophobic regions that can bind lipids (34). PLTP can function as a phospholipid transfer protein both in the presence of apolipoproteins, such as apoAI and apoAII, or when directly bound to phospholipids (24). The displacement of phospholipids and PLTP from phospholipidated mucin by Tween 20 encourages the consideration that phospholipids play a key role in the mucin-PLTP interaction. The broad elution profile of mucins in PBS could be explained by the size heterogeneity of the gel-forming mucin. This explanation was verified by preliminary asymmetric flow field-flow fractionation (AsFlFFF) experiments (24), where mucin formed complexes with a hydrodynamic diameter ranging from 25 to 225 nm (data not shown).

Tear lipocalin, a common tear lipoprotein, has been proposed to serve as a scavenger of lipophilic substances from the corneal surface (35). Lipocalin is capable of binding lipids in tear, and recently its ability to remove fluorescently labeled lipids from the surface of the cornea has been shown (9, 36). Tear PLTP has lipid transfer ability without any acceptor molecule, and it is active without any detectable protein-protein association (26). On the basis of the current data, we suggest that a possible explanation for the presence of PLTP in tear fluid may be a scavenging property of PLTP: the lipid contamination from the surface layer of tear film is directed to the ocular-soluble and membrane-bound mucins. Here PLTP, interacting with these mucins, can remove the harmful overload of lipids and transfer them to the lacrimal drainage system. Alternatively, an intriguing possibility would be that because the anterior tear fluid lipid film possesses high quantities of phospholipids, PLTP could be responsible for transferring all or some of these lipids to the tear fluid lipid layer. At present, we have no instrumentation to study this.

Our findings suggest a notable role for PLTP in lipid transfer in human tears. Accordingly, PLTP could play a significant role in preventing instability of the lipid film and thus in preventing evaporative DES. The precise mechanisms of tear PLTP-facilitated phospholipid transfer and association with mucin subtypes in the tear fluid as well as the effect of PLTP blockage in the development of DES must be further clarified. To this end, we have recently used a PLTP knock-out mouse model to study the role of PLTP in DES. Our preliminary data demonstrate that these mice develop considerable DES with concomitant histological changes and increased corneal epithelial permeability (Setälä et al., unpublished observations). Clarification of the role of PLTP in the development of DES will be the goal of our near-future studies.▮

REFERENCES

1. Moss, S. E., R. Klein, and B. E. Klein. 2008. Long-term incidence of dry eye in an older population. *Optom. Vis. Sci.* **85:** 668–674.
2. Chen, H. B., S. Yamabayashi, B. Ou, Y. Tanaka, S. Ohno, and S. Tsukahara. 1997. Structure and composition of rat precorneal tear film. A study by an in vivo cryofixation. *Invest. Ophthalmol. Vis. Sci.* **38:** 381–387.
3. Gipson, I. K. 2007. The ocular surface: the challenge to enable and protect vision. The Friedenwald lecture. *Invest. Ophthalmol. Vis. Sci.* **48:** 4391–4398.
4. Nicolaides, N., J. K. Kaitaranta, T. N. Rawdah, J. I. Macy, F. M. Boswell 3rd, and R. E. Smith. 1981. Meibomian gland studies: comparison of steer and human lipids. *Invest. Ophthalmol. Vis. Sci.* **20:** 522–536.
5. McCulley, J. P., and W. E. Shine. 2004. The lipid layer of tears: dependent on meibomian gland function. *Exp. Eye Res.* **78:** 361–365.

6. Greiner, J. V., T. Glonek, D. R. Korb, and C. D. Leahy. 1996. Meibomian gland phospholipids. *Curr. Eye Res.* **15:** 371–375.

7. McCulley, J. P., and W. Shine. 1997. A compositional based model for the tear film lipid layer. *Trans. Am. Ophthalmol. Soc.* **95:** 79–88, discussion 88–93.

8. McCulley, J. P., and W. E. Shine. 1998. Meibomian secretions in chronic blepharitis. *Adv. Exp. Med. Biol.* **438:** 319–326.

9. Glasgow, B. J., O. K. Gasymov, A. R. Abduragimov, J. J. Engle, and R. C. Casey. 2010. Tear lipocalin captures exogenous lipid from abnormal corneal surfaces. *Invest. Ophthalmol. Vis. Sci.* **51:** 1981–1987.

10. Jauhiainen, M., N. L. Setala, C. Ehnholm, J. Metso, T. M. Tervo, O. Eriksson, and J. M. Holopainen. 2005. Phospholipid transfer protein is present in human tear fluid. *Biochemistry.* **44:** 8111–8116.

11. Li, N., N. Wang, J. Zheng, X. M. Liu, O. W. Lever, P. M. Erickson, and L. Li. 2005. Characterization of human tear proteome using multiple proteomic analysis techniques. *J. Proteome Res.* **4:** 2052–2061.

12. Tall, A. R., S. Krumholz, T. Olivecrona, and R. J. Deckelbaum. 1985. Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis. *J. Lipid Res.* **26:** 842–851.

13. Huuskonen, J., and C. Ehnholm. 2000. Phospholipid transfer protein in lipid metabolism. *Curr. Opin. Lipidol.* **11:** 285–289.

14. Jiang, X. C., J. D'Armiento, R. K. Mallampalli, J. Mar, S. F. Yan, and M. Lin. 1998. Expression of plasma phospholipid transfer protein mRNA in normal and emphysematous lungs and regulation by hypoxia. *J. Biol. Chem.* **273:** 15714–15718.

15. Karkkainen, M., T. Oka, V. M. Olkkonen, J. Metso, H. Hattori, M. Jauhiainen, and C. Ehnholm. 2002. Isolation and partial characterization of the inactive and active forms of human plasma phospholipid transfer protein (PLTP). *J. Biol. Chem.* **277:** 15413–15418.

16. Jauhiainen, M., J. Metso, R. Pahlman, S. Blomqvist, A. van Tol, and C. Ehnholm. 1993. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem.* **268:** 4032–4036.

17. Hiidenhovi, J., H. S. Aro, and V. Kankare. 1999. Separation of ovomucin subunits by gel filtration: enhanced resolution of subunits by using a dual-column system. *J. Agric. Food Chem.* **47:** 1004–1008.

18. Jauhiainen, M., and C. Ehnholm. 2005. Determination of human plasma phospholipid transfer protein mass and activity. *Methods.* **36:** 97–101.

19. Matz, C. E., and A. Jonas. 1982. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem.* **257:** 4535–4540.

20. de Souza, G. A., L. M. Godoy, and M. Mann. 2006. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. *Genome Biol.* **7:** R72.

21. Peuravuori, H., V. V. Aho, H. J. Aho, Y. Collan, and K. M. Saari. 2006. Bactericidal/permeability-increasing protein in lacrimal gland and in tears of healthy subjects. *Graefes Arch. Clin. Exp. Ophthalmol.* **244:** 143–148.

22. Glasgow, B. J. 1995. Tissue expression of lipocalins in human lacrimal and von Ebner's glands: colocalization with lysozyme. *Graefes Arch. Clin. Exp. Ophthalmol.* **233:** 513–522.

23. Jiang, X. C., C. Bruce, J. Mar, M. Lin, Y. Ji, O. L. Francone, and A. R. Tall. 1999. Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. *J. Clin. Invest.* **103:** 907–914.

24. Setala, N. L., J. M. Holopainen, J. Metso, S. K. Wiedmer, G. Yohannes, P. K. Kinnunen, C. Ehnholm, and M. Jauhiainen. 2007. Interfacial and lipid transfer properties of human phospholipid transfer protein: implications for the transfer mechanism of phospholipids. *Biochemistry.* **46:** 1312–1319.

25. Masson, D., V. Drouineaud, P. Moiroux, T. Gautier, G. Dautin, M. Schneider, J. Fruchart-Najib, M. Jauhiainen, C. Ehnholm, P. Sagot, et al. 2003. Human seminal plasma displays significant phospholipid transfer activity due to the presence of active phospholipid transfer protein. *Mol. Hum. Reprod.* **9:** 457–464.

26. Huuskonen, J., V. M. Olkkonen, M. Jauhiainen, T. Sareneva, P. Somerharju, and C. Ehnholm. 1998. Oxidative modification of HDL3 in vitro and its effect on PLTP-mediated phospholipid transfer. *Biochim. Biophys. Acta.* **1391:** 181–192.

27. Gendler, S. J., and A. P. Spicer. 1995. Epithelial mucin genes. *Annu. Rev. Physiol.* **57:** 607–634.

28. Moniaux, N., F. Escande, N. Porchet, J. P. Aubert, and S. K. Batra. 2001. Structural organization and classification of the human mucin genes. *Front. Biosci.* **6:** D1192–D1206.

29. Hollingsworth, M. A., and B. J. Swanson. 2004. Mucins in cancer: protection and control of the cell surface. *Nat. Rev. Cancer.* **4:** 45–60.

30. Slomiany, A., B. L. Slomiany, H. Witas, M. Aono, and L. J. Newman. 1983. Isolation of fatty acids covalently bound to the gastric mucus glycoprotein of normal and cystic fibrosis patients. *Biochem. Biophys. Res. Commun.* **113:** 286–293.

31. Millar, T. J., S. T. Tragoulias, P. J. Anderton, M. S. Ball, F. Miano, G. R. Dennis, and P. Mudgil. 2006. The surface activity of purified ocular mucin at the air-liquid interface and interactions with meibomian lipids. *Cornea.* **25:** 91–100.

32. Mantelli, F., and P. Argueso. 2008. Functions of ocular surface mucins in health and disease. *Curr. Opin. Allergy Clin. Immunol.* **8:** 477–483.

33. Lang, T., G. C. Hansson, and T. Samuelsson. 2006. An inventory of mucin genes in the chicken genome shows that the mucin domain of Muc13 is encoded by multiple exons and that ovomucin is part of a locus of related gel-forming mucins. *BMC Genomics.* **7:** 197.

34. Smith, B. F., and J. T. LaMont. 1984. Hydrophobic binding properties of bovine gallbladder mucin. *J. Biol. Chem.* **259:** 12170–12177.

35. Glasgow, B. J., G. Marshall, O. K. Gasymov, A. R. Abduragimov, T. N. Yusifov, and C. M. Knobler. 1999. Tear lipocalins: potential lipid scavengers for the corneal surface. *Invest. Ophthalmol. Vis. Sci.* **40:** 3100–3107.

36. Gasymov, O. K., A. R. Abduragimov, P. Prasher, T. N. Yusifov, and B. J. Glasgow. 2005. Tear lipocalin: evidence for a scavenging function to remove lipids from the human corneal surface. *Invest. Ophthalmol. Vis. Sci.* **46:** 3589–3596.