

Interaction of Purified Tear Lipocalin with Lipid Membranes

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PURPOSE. The principal lipid-interacting protein in human tears has been reported to be tear lipocalin (Tlc). Tlc has been suggested to scavenge harmful lipophilic substances from the corneal epithelium and to maintain the integrity of the anterior tear film lipid layer by binding and releasing lipid(s) that are accommodated within the protein. Although lipids can be extracted from Tlc, it is still unclear whether Tlc can actually bind to lipid membranes and accept membrane lipids and whether it possesses lipid transfer activity. The purpose of this study was to explore the interaction of Tlc with neutral, anionic, and cationic lipid membranes and to assess the potential of Tlc to facilitate the transfer of either polar or neutral lipids in a lipid transfer assay.

METHODS. The binding of Tlc to lipid membranes was assessed by a monolayer technique and fluorescence spectroscopy. The polar lipid transfer activity of Tlc was assessed with a radiometric assay based on the transfer of ¹⁴C-phosphatidylcholine (PC) from PC-liposomes to HDL₃. The neutral lipid transfer activity of Tlc was assayed by measuring the transfer of radioactive cholesteryl ester from LDL to HDL₃.

RESULTS. Purified Tlc showed significant surface activity as evidenced by an increase in surface pressure at the air–buffer interface. Likewise, it interacted actively with neutral, anionic, and cationic lipid monolayers, as evidenced by an equal increase in surface pressure despite the surface charge. Enhanced quenching of the single tryptophan residue of Tlc by pyrene and I[−] anion suggested that different protein domains are involved in the interaction of Tlc with oppositely charged lipid membranes. Finally, radiometric assays revealed that Tlc does not possess any neutral or polar lipid transfer activity between lipid vesicles or/and lipoproteins.

CONCLUSIONS. Tlc interacted with lipid membranes composed of neutral, cationic, or anionic membranes, which supports a role for Tlc in the maintenance of the tear film interfaces. Tlc did not show any neutral or polar lipid transfer activity what-

soever. The findings suggest that the notion of the role of Tlc as the major lipid-transferring protein in human tears should be revised. (*Invest Ophthalmol Vis Sci.* 2005;46:3649–3656) DOI: 10.1167/iovs.05-0176

The tear film is a complex three-dimensional (3-D) aqueous fluid composed of a complex mixture of carbohydrates, proteins, and lipids.¹ Recent reports using interferometry and optical coherence tomography have shown that the thickness of the human tear film ranges between 2.3 and 3.3 μm.^{2–4} A thorough protein composition of the tear film is yet to be revealed. Likewise, the function and composition of the carbohydrate layer remains to be established. Analyses of meibomian gland secretions have shown that it is composed of wax esters, sterol esters, and polar lipids.^{5,6} Yet the tear fluid lipids are composed of both meibomian secretions and sebum. No thorough studies of the chemical composition of the tear film lipids are available. Although a comprehensive list of the constituents exists, the broad function of this complex fluid is still poorly understood. There is no information on the complex interactions among the proteins, carbohydrates, and lipids. To begin with, we studied the interaction of purified tear lipocalin (Tlc) with lipid membranes. The motivation for this study derives from the proposed physiological function of Tlc in human tear film.⁷ In brief, the conventional structure of the human tear film consists of three layers: a superficial lipid layer, an aqueous middle layer, and a precorneal mucin layer.^{8–10} It appears, however, that the two latter compartments form a somewhat homogenous, mucin-enriched fluid.^{1,11} Phospholipids are found adjacent to the aqueous–mucin layer and external to this a layer composed of nonpolar lipids, such as cholesteryl esters and triglycerides, face the tear–air interface.^{6,12–14} This type of structure is vulnerable to destabilization if the composition of the lipid layer is changed or if lipids contaminate the mucin layer. Accordingly, some mechanism(s) to prevent contamination of the ocular surface, such as removing meibomian lipids, is necessary. Tlc has been suggested to be such a protein in tear fluid.^{7,15} In essence, this would necessitate that Tlc interact with lipid membranes and furthermore that Tlc bind and release (i.e., transport) new lipid molecules to and from its surface.

The protein superfamily of lipocalins consists of small secretory proteins that partly share conserved amino acid sequence motifs. Functionally, they show common properties including the ability to bind an array of small hydrophobic molecules, to bind to specific receptors, and to form oligomeric complexes.¹⁶ Despite weak amino acid sequence homology among lipocalins, their overall folding pattern is highly conserved. The 3-D structure of apo-Tlc consists of an eight-stranded, nonparallel β-barrel with a loop scaffold at the entrance of the β-barrel and a C-terminal α-helix.^{17,18} The core of the β-barrel cavity in apo-Tlc is extremely wide and is believed to be the ligand-binding site.¹⁷ In human tears, Tlc is secreted by the lacrimal glands¹⁹ and comprises 15% to 33% of the protein content of tears.²⁰ Tlc is not unique to tears, and it is expressed in such tissue as the sublingual glands, the prostate, the mucosal glands of the tracheobronchial tree, and nasal

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mucosa.²¹ Tlc has been suggested to be the major lipid-binding protein in human tears and is known to bind fatty acids, fatty alcohols, phospholipids, glycolipids, and cholesterol.¹⁵ Several lines of evidence suggest that Tlc acts as a physiological protector of epithelia by scavenging harmful hydrophobic molecules and by controlling inflammatory processes by its protease inhibition activity.^{22,23} Both of these functions require interactions with lipid membranes and/or other proteins that are bound to the cellular membranes. Considering the former, it has remained unclear whether Tlc shows any lipid transfer activity—that is, lipid binding and releasing. When Tlc is expressed in *Escherichia coli*, purified, and analyzed for its ligands, it appears that the lipids are derived from bacterial membranes.²⁴ This finding implies that the lipids may be necessary for maintaining the proper conformation of the protein.

MATERIALS AND METHODS

POPG (1-palmitoyl-2-oleoyl-3-phosphatidylglycerol) and sphingosine (Sph) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and POPC (1-palmitoyl-2-oleoyl-3-phosphatidylcholine) from Avanti (Birmingham, AL). PPDPC (1-palmitoyl-2-[6-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine) was obtained from K&V Bioware (Espoo, Finland) and DPH (1,6-diphenyl-1,3,5-hexatriene) from Molecular Probes (Eugene, OR). Phospholipid stock solutions were made in chloroform. Thin-layer chromatography assessed the purity of lipids and fluorescent probes on silicic-acid-coated plates (Merck, Darmstadt, Germany) using chloroform-methanol-water (65:25:4, vol/vol/vol) as a solvent. Examination of the plates after iodine staining or, when appropriate, under fluorescent illumination revealed no impurities. Concentrations of the nonfluorescent phospholipids were determined gravimetrically with a high-precision electrobalance (Cahn Instruments, Inc., Cerritos, CA), and those of the fluorescent phospholipid analogues were determined spectrophotometrically with appropriate molar extinction coefficients provided by the manufacturer. Human Tlc was prepared as described previously.²⁵ This study was performed according to the principles of the Declaration of Helsinki, and approved by the Ethics Review Committee of the Department of Ophthalmology (University of Helsinki). Informed consent was obtained from each subject.

Tear Fluid Samples

Tear fluid samples (approximately 20 μL , tear fluid flow rate was 2.9 \pm 3.6 $\mu\text{L}/\text{min}$) were collected from the lower conjunctival sac from six healthy subjects by means of 10- μL micropipettes (Blaubrand Intramark; Brand GMBH, Wertheim, Germany). The samples were immediately cooled to +4°C, pooled, and stored at -70°C until analyzed.

Blood Samples

Blood samples ($n = 6$) were collected from healthy volunteers into sterile heparin-containing tubes. The samples were kept at room temperature for 15 minutes followed by centrifugation at 3000g for 15 minutes at +4°C. The plasma was separated, pooled, and stored at -70°C until analyzed.

Adsorption Isotherm of Tear Lipocalin

Penetration of the Tlc into the air-buffer (5 mM HEPES, 0.1 mM EDTA [pH 7.4]) interface was measured in magnetically stirred circular wells (subphase volume, 1200 μL). The buffer was allowed to equilibrate for 15 minutes before the addition of Tlc (7 μL , 4.7 mg/mL in Na-phosphate buffer 150 mM NaCl [pH 7.5]; final concentration, 1.6 μM) into the subphase. Experiments were performed at ambient room temperature \sim 24°C. Surface pressure (π) was monitored with a Wilhelmy wire attached to a microbalance ($\mu\text{TroughS}$; Kibron Inc., Helsinki, Finland) and connected to a computer.

Interaction of Tear Lipocalin with Lipid Monolayers

Lipid monolayers residing on an air-buffer interface provided a convenient means to assess lipophilicity of Tlc by monitoring the increase in surface pressure caused by insertion of the protein into the film. Penetration of Tlc into neutral POPC, anionic POPC-POPG (8:2 molar ratio), and cationic POPC-Sph (8:2 molar ratio)²⁶ films was measured using magnetically stirred circular wells. Surface pressure (π) was monitored as described earlier. Lipid was spread on an air-buffer (5 mM HEPES, 0.1 mM EDTA, [pH 7.4]) interface in chloroform (1 mg/mL) to different initial surface pressures (π_0), and the resultant monolayer was allowed to equilibrate for 15 minutes before the addition of Tlc (7 μL , 4.7 mg/mL in Na-phosphate buffer 150 mM NaCl, [pH 7.5], final concentration 1.6 μM) into the subphase. The increment in π from the initial surface pressures (π_0) after the addition of Tlc was complete in less than 45 minutes, and the difference between π_0 and the value observed after binding of Tlc into the film was taken as $\Delta\pi$. All the measurements were performed at the ambient temperature (\sim 24°C). The data are represented as $\Delta\pi$ versus π_0 .

Preparation of Liposomes

Lipid stock solutions were mixed in chloroform to obtain the desired compositions. The solvent was removed under a gentle stream of nitrogen, and the lipid residue was subsequently maintained under reduced pressure overnight. Multilamellar vesicles (MLVs) were formed by hydrating the dry lipids at 65°C with 1 mL of 5 mM HEPES and 0.1 mM EDTA (pH 7.4), to a lipid concentration of 1 mM. Multilamellar liposomes were freeze-thawed five times and large unilamellar vesicles (LUVs) were obtained by extruding multilamellar dispersions 19 times through a 100-nm pore-size polycarbonate membrane (Nuclepore, Pleasanton, CA) with a gas-pressure, small-volume homogenizer (LiposoFast Pneumatic; Avestin, Ottawa, Ontario, Canada).^{27,28} The pressure used for extrusion of vesicles through the filters was 25 psi (\sim 170 kPa).

Fluorescence Spectroscopy

The environments of the tryptophan (Trp) residues of Tlc in the presence of liposomes were studied by fluorescence spectroscopy. The center of the Trp fluorescence peak is at \sim 350 nm when in water, whereas in a hydrophobic environment, the emission is centered near 330 nm. Accordingly, changes in the microenvironment of Trp can be monitored by measuring I_{350}/I_{330} , the ratio of the emission at 350 nm to that at 330 nm. Trp fluorescence was recorded with a spectrofluorometer (model LS 50 B; PerkinElmer, Wellesley, MA) equipped with a magnetically stirred and thermostated cuvette compartment. All the measurements were performed at 25°C in 5 mM HEPES and 0.1 mM EDTA (pH 7.4) buffer. Excitation and emission band passes were both 10 nm. Excitation wavelength was 295 nm, and emission spectra were recorded in the range of 300 to 400 nm. Trp emission spectra of Tlc were recorded in both the absence and presence of POPC, POPC-POPG (8:2 molar ratio), and POPC-Sph (8:2 molar ratio) LUVs, yielding final Tlc and lipid concentrations of 5 and 50 μM , respectively. All experiments were performed in triplicate.

Steady State Fluorescence Anisotropy

Fluorescence anisotropy of the Trp residue of Tlc (5 μM) as a function of increasing concentration of LUVs (\leq 50 μM) was measured with rotating polarizers in the excitation and emission beams (LS 50 B; PerkinElmer), with a band pass of 10 nm. The results are expressed as emission anisotropy (r) calculated as follows:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}},$$

where I_{\perp} and I_{\parallel} are the emission intensity perpendicular and parallel, respectively, to the excitation polarizer. The experiments were performed in triplicate.

Trp Emission Quenching by Pyrene

To assess the binding of Tlc to lipid bilayers we measured the quenching of Trp fluorescence by pyrene-containing liposomes. Excitation of Trp at 295 nm yields a wide emission peak that can be used to excite pyrene. As a result, the emission intensity at 334 nm decreases. In brief, if Tlc does not bind to LUVs the emission intensity at 334 nm with and without liposomes remains unchanged. In case of interaction, the Trp emission intensity at 334 nm decreases when PPDPC-containing liposomes are present. These types of experiments are very sensitive to the distance of the two fluorophores, and thus even small changes can be observed.²⁹ Typically, the distance of the two fluorophores should not exceed 2 to 10 nm for fluorescence resonance energy transfer (FRET) to occur. All the measurements were performed at 25°C in triplicate in 5 mM HEPES and 0.1 mM EDTA (pH 7.4) buffer with the spectrofluorometer (LS 50 B; PerkinElmer) and with a Tlc concentration of 5 μ M and an LUV concentration of 50 μ M.

Collisional Quenching of Trp Emission

The extent of exposure of Trp to the aqueous phase was assessed using Γ^{-} as a collisional quencher. The extent of quenching was calculated by using the equation

$$\frac{F_0}{F} = 1 + k(Q) = 1 + k_q \tau_0(Q),$$

where F_0 and F are Trp fluorescence intensities at 345 nm in the absence and presence of the quencher Q , and τ_0 is fluorescence lifetime of Trp in the absence of the quencher k , the Stern-Volmer constant (obtained from the slope of the linear fit of the data). Fluorescence emission of Trp in Tlc (5 μ M) and its quenching were measured in the presence of POPC, POPC-POPG (8:2 molar ratio), or POPC-Sph (8:2 molar ratio) LUVs (final lipid concentration of 50 μ M). The results were corrected for scattering caused by liposomes in the buffer. To ensure reproducibility, the experiments were repeated three times.

Measurement of Phospholipid Transfer Activity

Phospholipid transfer activities of plasma and tear fluid samples of the study subjects ($n = 6$) and Tlc were obtained by measuring the transfer of [¹⁴C]-DPPC (dipalmitoylphosphatidylcholine) from radiolabeled PC-liposomes to high-density lipoprotein-3 (HDL₃) acceptors. The Tlc concentration per assay varied from 0.047 to 0.47 mg/mL. Essentially, the assay was performed as described elsewhere,³⁰ with minor modifications.³¹

Cholesterol Ester Transfer Activity

Plasma, tear fluid, and Tlc cholesterol ester transfer activities were determined from the transfer rate of radiolabeled cholesterol ester from low-density lipoprotein (LDL) to HDL, as described.³²

RESULTS

Surface Activity of Purified Tlc

We first assessed the surface activity of purified Tlc in Langmuir troughs. After injection of Tlc into the subphase, a lag time of approximately 100 seconds was observed followed by a monotonous increase in surface pressure up to 5.1 ± 0.3 mN/m in approximately 1800 seconds (Fig. 1). A very slow subsequent increment was observed that may have been attributable to denaturation of the protein at the air-water interface³³ and/or the higher activation energy of protein adsorp-

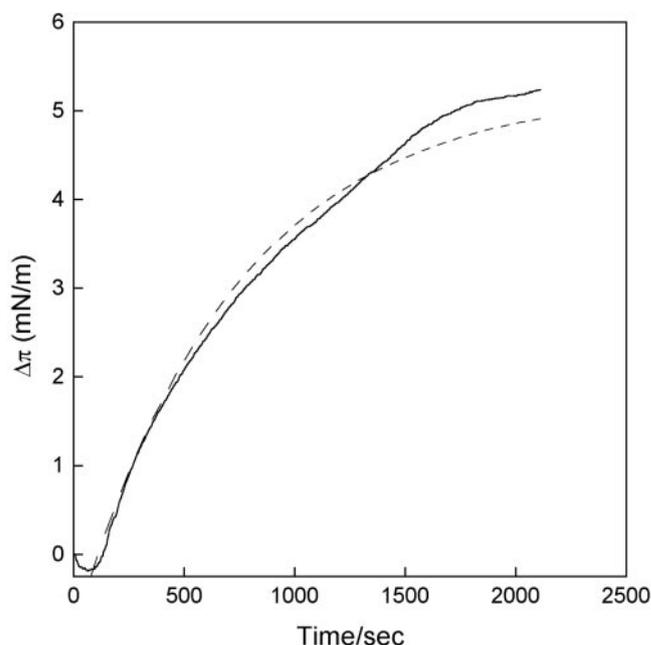


FIGURE 1. Adsorption of purified tear lipocalin (Tlc) into an air-buffer interface. Adsorption (*dashed line*) is shown as an increase in surface pressure ($\Delta\pi$) after the addition of Tlc (final concentration, 1.6 μ M) to the aqueous interface (5 mM HEPES and 0.1 mM EDTA [pH 7.4]). The increase in surface pressure followed first-order reaction kinetics, denoted by the *solid line*.

tion to the air-water interface at high protein surface concentrations.³⁴ The increase in surface pressure followed first-order reaction kinetics (Fig. 1, dashed line; coefficient of determination $r^2 = 0.993$). The behavior resembles the results obtained by Glasgow et al.,⁷ except that in our experiments the increase in surface pressure was much more rapid (approximately seven times faster).

Interaction of Tlc with Phospholipid Monolayers

The molecular interactions between the tear film lipid layer and soluble proteins embedded in the tear fluid have not been published previously. For this purpose, we used phospholipid monolayers residing on an air-buffer interface, a model biomembrane that has been widely used to study lipid-protein interactions (e.g., Ref. 35). Penetration of proteins or peptides into the lipid monolayer after injection underneath the lipid film increases the surface pressure π ,^{36,37} whereas peptides and proteins that do not bind to lipids cause no changes in π .³⁴ Initial surface pressure (π_0) of neutral POPC, anionic POPC-POPG (8:2 molar ratio), and cationic POPC-Sph (8:2 molar ratio) monolayers varied between 4 and 42 mN/m, and increments in surface pressure ($\Delta\pi$) attributable to the addition of Tlc (final concentration, 1.6 μ M) to the subphase were measured (Fig. 2A). The penetration of Tlc into the lipid monolayers was not dependent on the electric charge of the monolayer, and the slopes of $\Delta\pi$ versus π_0 were qualitatively similar in all three lipid films studied. At initial monolayer packing pressures exceeding 34, 32, and 34 mN/m for POPC, POPC-POPG, and POPC-Sph, respectively, the membrane penetration of Tlc was abolished. The increase in surface pressure was significantly higher for lipid monolayers (at $\pi < 15$ mN/m) than for pure air-buffer interfaces, suggesting that Tlc has a substantial affinity to interact with membranes. At higher surface pressures, the monolayers became either unstable or collapsed, as evidenced by a subsequent decrease in surface pressure (data not shown). At present, we lack sophisticated methodology to

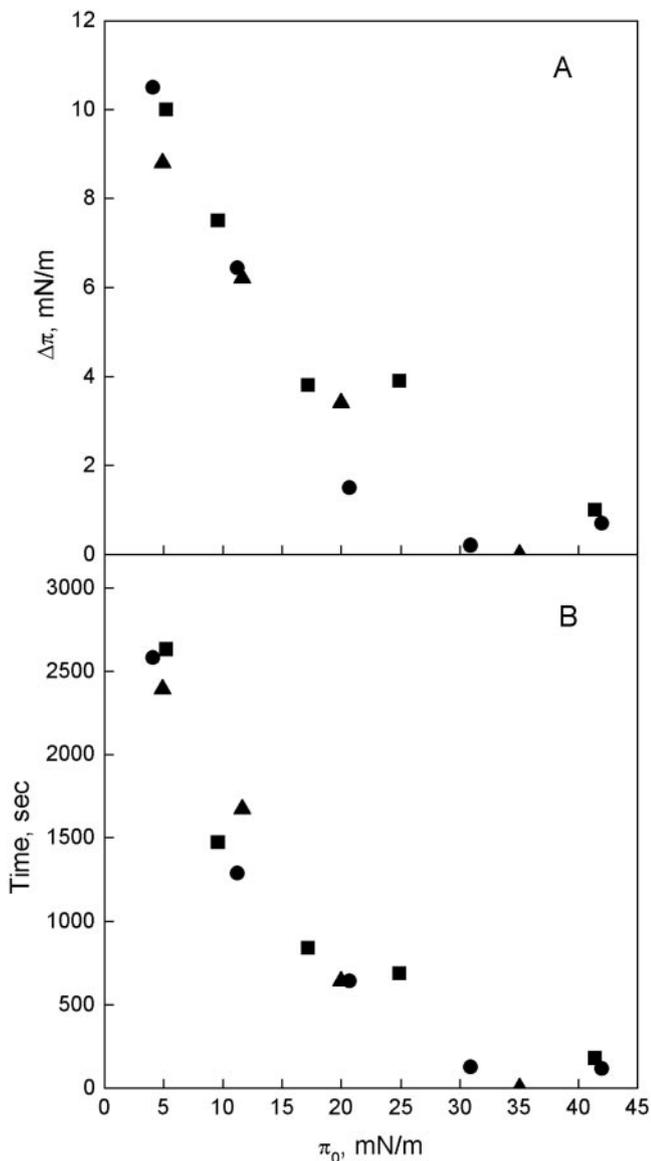


FIGURE 2. Penetration of purified tear lipocalin into POPC (■), POPC-POPG (8:2 molar ratio, ●), and POPC-Sph (8:2 molar ratio, ▲) monolayers. (A) Penetration is shown as an increase in surface pressure ($\Delta\pi$) after the addition of Tlc (final concentration, 1.6 μM) to the aqueous interface (5 mM HEPES and 0.1 mM EDTA [pH 7.4]). Data are shown as a function of the initial surface pressure (π_0). (B) Time needed to reach the maximum surface pressure as a function of π_0 .

assess this effect further. From the isotherms, it is also evident that the time needed to reach the maximum pressure is longer for lipid monolayers than with a pure air-buffer interface at $\pi < 10$ mN/m and is again independent of the surface electric charge of the monolayer (Fig. 2B).

Interactions of Tlc with Liposomes

In the foregoing experiments with neutral, cationic, and anionic lipid monolayers, Tlc interacted avidly with these hydrophobic membranes. However, the monolayer experiments did not reveal any significant differences between the studied lipid membranes. This was further studied by measuring Trp fluorescence emission anisotropy (r) for Tlc in the presence of increasing concentrations of liposomes (Fig. 3). Accordingly, in the absence of liposomes, r was 0.34 ± 0.01 , reflecting very

slow Brownian rotational diffusion of Tlc in solution. This high value for r is in keeping with previous results showing that Trp is located at the tip of the calyx.¹⁷ Increasing concentrations of POPC liposomes caused progressively increasing r , up to 0.51 ± 0.01 measured at 50 μM POPC. Approximately one-half the maximum effect was evident at a Tlc-POPC molar ratio of $\sim 1:4$. The anionic liposomes (POPC-POPG, 8:2 molar ratio) behaved very similarly to the POPC liposomes, except that the maximum r was 0.47 ± 0.005 . We were surprised, however, that on incubation of Tlc with cationic liposomes (POPC-Sph, 8:2 molar ratio) we observed only a very marginal increase in r from 0.34 ± 0.005 to 0.37 ± 0.005 (Fig. 3). The theoretical maximum for r is 0.40.³⁸ However, if light-scattering occurs, r can be much closer to 1.0. Likewise, fluorescence anisotropy is very sensitive to the lifetime of the fluorophore, and a decrease in lifetime would profoundly increase measured anisotropy.³⁸ To overcome these uncertainties with the use of steady state anisotropy we used fluorescence spectroscopy to characterize further the interaction of Tlc with lipid membranes.

Intrinsic Trp fluorescence allows the estimation of possible changes in the microenvironment of this fluorophore on association of Tlc with liposomes.³⁸ Considering Tlc, the method is very sensitive, as only one Trp is found in the protein. The I_{350}/I_{330} ratio measured in buffer for the Trp residue of Tlc was 0.631 ± 0.007 , whereas in the presence of LUVs (50 μM total phospholipid), this ratio slightly increases to 0.666 ± 0.005 . Surface charge of the liposomes did not have any apparent effect on the I_{350}/I_{330} ratio (data not shown). Trp, thus, resides in a highly hydrophobic environment in the absence and presence of liposomes. This is in keeping with the known position of the Trp in the calyx of the protein.¹⁷

Measuring the quenching of Trp fluorescence by pyrene-containing liposomes was performed as a further assessment of the interaction of Tlc with lipid membranes. For neutral POPC liposomes an approximately linear decrease in Trp fluorescence was observed on increasing the concentration of Tlc (Fig. 4). Likewise, in the presence of anionic POPC-POPG

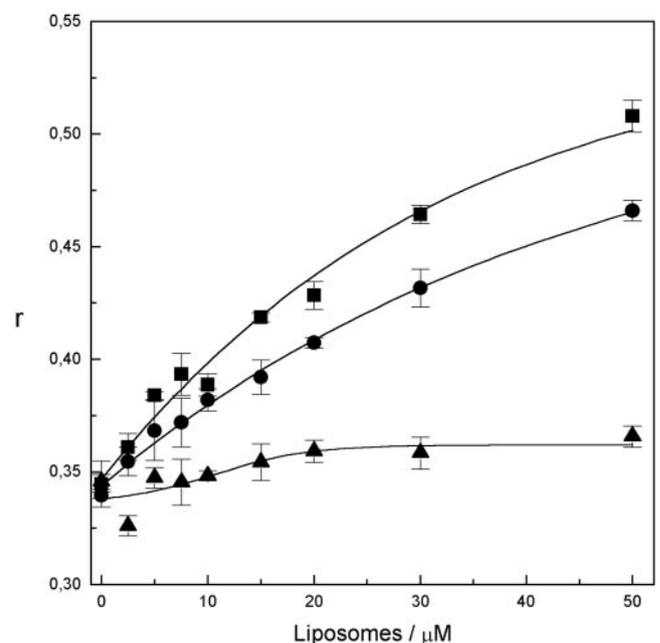


FIGURE 3. Anisotropy (r) for the Trp residue of Tlc as a function of POPC (■), POPC-POPG (8:2 molar ratio, ●), and POPC-Sph (8:2 molar ratio, ▲) concentration. Final lipid concentration was 50 μM in 5 mM HEPES and 0.1 mM EDTA (pH 7.4), and the Tlc concentration was 5 μM . Temperature was maintained at 25°C.

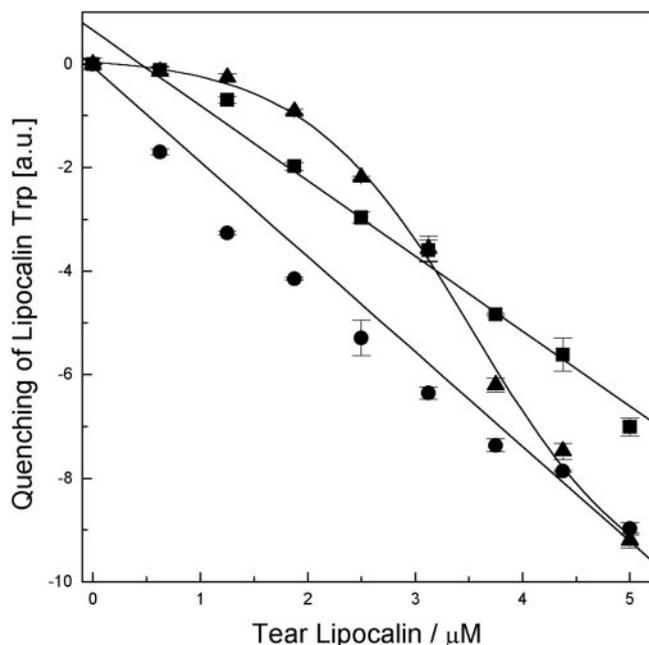


FIGURE 4. Binding of Tlc to pyrene-containing liposomes. The interaction of Tlc with liposomes composed of POPC-PPDPC (99:1 molar ratio ■), POPC-POPG-PPDPC (molar ratio, 79:1:20, ●), and POPC-Sph-PPDPC (79:1:20 molar ratio, ▲) was assessed by measuring the Trp fluorescence emission intensity at 334 nm in the absence and presence of liposomes. Total lipid concentration was 50 μM in 5 mM HEPES and 0.1 mM EDTA (pH 7.4), and the final Tlc concentration was 5 μM . Temperature was maintained at 25°C.

liposomes, a linear decrease in Trp fluorescence was observed. In the latter case, the decrease was more pronounced. However, when using cationic POPC-Sph (8:2 molar ratio) liposomes, no decrease was observed in the intensity of Trp fluorescence up to 2 μM Tlc, whereas on exceeding this concentration, a rapid decrease in the fluorescence intensity was seen. The results thus suggest that for the neutral and anionic liposomes, the Trp residue is positioned so that resonance energy transfer can occur immediately on addition of the protein, whereas the positive charge of Sph-containing membranes initially prevents binding of Tlc to these cationic membranes or the protein is oriented so that resonance energy transfer cannot occur.

The binding of Tlc to lipid bilayers was considerably faster than to monolayers and followed first-order exponential decay kinetics (Fig. 5). We did not observe differences in the binding kinetics between neutral, anionic, and cationic liposomes (data not shown).

The collisional quenching of Trp by I^- was assessed in the presence of neutral (POPC), anionic (POPC-POPG, 8:2 molar ratio), and cationic (POPC-Sph, 8:2 molar ratio) liposomes. The water soluble and small I^- anion would have to enter the hydrophobic cavity of Tlc before it could quench Trp. Accordingly, this method allows exploration of the orientation of the calyx mouth of Tlc-lipid complexes. The results explicitly show that in the presence of neutral and anionic liposomes, the Trp residue in Tlc was significantly less exposed to the collisional quencher I^- than were the cationic liposomes (Fig. 6). The Stern-Volmer constants were 0.0002 M^{-1} , 0.0001 M^{-1} and 0.0005 M^{-1} in the presence of POPC, POPC-POPG, and POPC-Sph liposomes, respectively. Further proof of the more open exposure of Trp in the presence of cationic liposomes is that the Trp quenching for these liposomes followed closely linear relationship in the presence of I^- , whereas a linear fit for

POPC and POPC-POPG liposomes showed only low correlation (Pearson's correlation coefficients).

Lipid Transfer Activity

Finally, the lipid transfer activity of Tlc was assessed. The total polar lipid transfer activity in tear fluid was investigated from pooled tear fluid samples using a radiometric assay based on the transfer of [^{14}C]-dipalmitoylphosphatidylcholine (DPPC) from liposomes to HDL₃. Although tear fluid does not contain lipoproteins, we have used this method previously with success to measure the lipid transfer activity of other tear fluid proteins,³⁹ and thus we consider it applicable to assess lipid transfer activity of Tlc as well. The mean PC transfer activity in tear fluid was 14.8 ± 1.9 micromoles/mL per hour, and that from plasma was 8.3 ± 2.3 micromoles/mL per hour, which is in good accordance with our previous results.³⁹ Tlc (final concentration varied between 0.047 and 0.47 mg/mL) displayed no phospholipid transfer activity even at the highest concentration (data not shown). The presence of neutral lipid transfer activities in tear fluid was studied by a radiometric assay, which measures the transfer of radioactive cholesteryl ester (CE) from LDL to HDL₃. The mean CE transfer activity in human plasma was 31.6 ± 3.6 nanomoles/mL per hour, whereas no CE transfer activity was observed in human tear fluid. Purified Tlc (final concentration varied between 0.047 to 0.47 mg/mL) showed no cholesteryl ester transfer activity in this assay.

DISCUSSION

The present results show that pure Tlc was eminently surface active, as previously demonstrated.⁷ The surface adsorption kinetics, however, between Glasgow et al.⁷ and our study vary considerably. In our experiments, the kinetics were approxi-

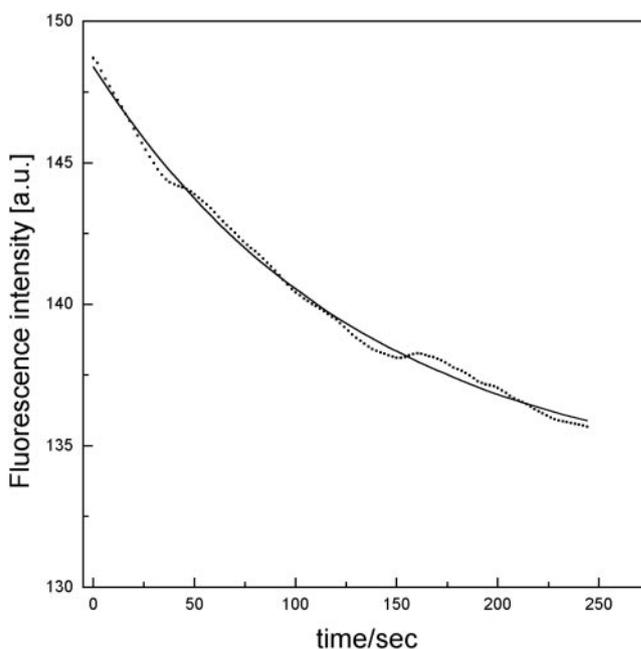


FIGURE 5. Time course of binding of Tlc to POPC-PPDPC (99:1 molar ratio) LUVs. Binding (dotted line) was quantified as Trp emission intensity quenching at 334 nm (excitation, 295 nm). The binding followed first-order exponential decay kinetics as shown by the fitted solid line. Total lipid concentration was 50 μM in 5 mM HEPES and 0.1 mM EDTA (pH 7.4), and the Tlc concentration was 5 μM . Temperature was maintained at 25°C. The measured data represent all different types of vesicles.

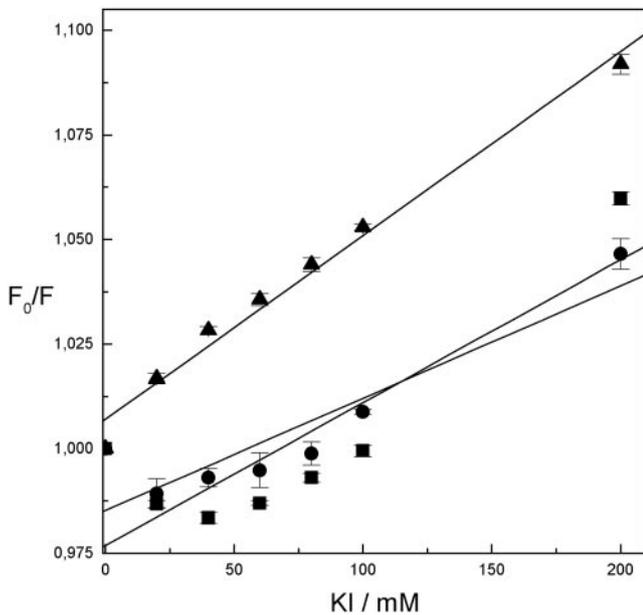


FIGURE 6. Trp emission intensity of Tlc in complexes with POPC (■), POPC-POPG (8:2 molar ratio; ●), and POPC-Sph (8:2 molar ratio, ▲) LUVs with Γ^- as a water-soluble collisional quencher. Total lipid concentration was $50 \mu\text{M}$ in 5 mM HEPES and 0.1 mM EDTA (pH 7.4), and the Tlc concentration was $5 \mu\text{M}$. Temperature was maintained at 25°C .

mately seven times faster. Such kinetics would allow for establishment of the protein on the tear film *in vivo* after a blink where it could release lipids to maintain the integrity of the superficial lipid layer. However, as will be discussed herein, this view is not supported by the lipid transfer data. Purified Tlc attached to phospholipid membranes despite the surface electric charge. This effect could be demonstrated by surface pressure studies on lipid monolayers as well as fluorescence spectroscopy measurements with lipid bilayers. The critical surface pressure of monolayers, 32 to 34 mN/m , needed to prevent the penetration of Tlc into these lipid films, is high and reflects the amphiphilic nature of the protein. Similar critical surface pressures have been observed for other small lipophilic peripheral membrane proteins (e.g., Ref. 40). The approximately 1.5-fold increment in the Trp emission anisotropy of Tlc measured in the presence of neutral and anionic liposomes suggests that Tlc binds to these bilayers or that the lifetime of the excited Trp decreases significantly on binding. Nevertheless, in essence, this implies that the environment of Trp is changed, confirming binding to these membranes. Finally, resonance energy transfer experiments from Trp to pyrene demonstrates that Tlc avidly interacts with neutral and anionic lipid bilayers and also with cationic membranes with higher concentration of the protein. The latter finding suggests that re-orientation or refolding of the protein on this lipid surface takes place, allowing for resonance energy transfer. The fluorescence resonance energy transfer technique used is extremely sensitive to the distance and lifetimes of the two fluorophores.²⁹ Accordingly, when the distance exceeds $\sim 10.0 \text{ nm}$, no resonance energy transfer can occur. Collisional quenching of the Trp residue by water-soluble Γ^- was significantly hindered in the presence of neutral and anionic liposomes compared with cationic liposomes, suggesting that Tlc possess at least two membrane-interacting sites. This is illustrated in Figure 7, which shows that whereas for neutral and anionic membranes reciprocal changes in both Trp quenching by pyrene and anisotropy were measured, only a change in Trp quenching was observed for cationic liposomes.

The interaction of Tlc with lipid membranes was mainly determined by hydrophobic interactions with electrostatic interactions modifying protein-lipid association. This is evident from both surface penetration studies and fluorescence spectroscopy studies and also from the amino acid sequence of Tlc. Altogether, five Tyr residues are found in the vicinity of the protein surface and possibly mediate hydrophobic interaction with lipid membranes.⁴¹ The membrane interface in this study consists of a complex set of water, phosphocholine, Sph, glyceryl, carbonyl, and methylene groups. Accordingly, these regions are suitable for noncovalent hydrophobic-ionic interactions with proteins. Considering small structureless peptides, the largest gain in free energy is obtained when Phe, Trp, and Tyr are transferred from water into the lipid bilayer interface.⁴¹ Although the system is significantly more complex when taking into account proteins that possess a defined secondary structure, this gain in free energy may allow for penetration of the protein into the membrane interface. Unfortunately, at present we cannot unambiguously identify the interacting residues. The pI for Tlc is 4.8 ⁴² and accordingly, electrostatically it would prefer interaction with positively charged proteins or lipid membranes. In concordance with this, it has been shown that Tlc interacts with positively charged lysozyme and lactoferrin.⁴³ The crystal structure of apo-Tlc was very recently published¹⁷ and revealed that the mouth of the proposed lipid-binding cavity was lined with negatively charged Glu-34 and Asp-80, which could enhance interaction of Tlc with positively charged membranes. If so, it seems feasible to suggest that the mouth of the hydrophobic cavity would be buried in the cationic membrane, and thus the entry of Γ^- into the protein hydrophobic cavity would be hindered, but such is not the case (Fig. 6). Instead, it seems likely that, in the presence of neutral and anionic membranes, the mouth of the cavity is buried in the membrane and Γ^- cannot enter the cavity. We suggest that the electrostatic interaction of the protein with the lipid membrane determines

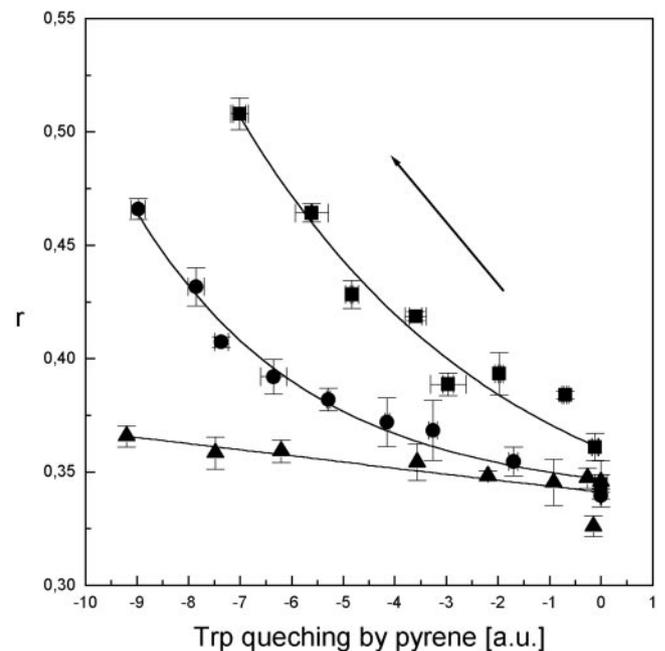


FIGURE 7. Trp quenching by pyrene as a function of Trp fluorescence anisotropy (r) for POPC (■), POPC-POPG (8:2 molar ratio; ●), and POPC-Sph (8:2 molar ratio, ▲) LUVs. *Arrow:* direction of increasing lipid concentration. Data are taken from measurements shown in Figures 3 and 4.

interacting domain of Tlc. In concordance with this, the crystal structure of Tlc reveals that the protein is highly polarized.¹⁷

Tlc is an abundant protein in human tears and has been reported to bind an array of various lipids.¹⁵ It has been suggested that Tlc may scavenge contaminating lipids from the corneal surface and transport them to the anterior lipid layer or to the nasolacrimal system.⁷ This study demonstrates that although Tlc avidly associates with lipid membranes, it does not possess any lipid transfer activity in the assays in which lipids are transferred between liposomes and/or lipoproteins. Furthermore, although in vivo lipids are bound to Tlc, it remains unclear what is the origin of the lipids. In other words, it is possible that when Tlc is excreted from the lacrimal gland¹⁹ lipids are already bound to Tlc and thus it may not be able to adsorb new lipids that are contaminating the ocular surface. A drawback of our lipid transfer assay is that it may not be optimal to detect lipid transfer from systems that are more or less unstable. These could include systems such as lipid-contaminated mucins. To this end, we have recently characterized in human tear fluid another protein, phospholipid transfer protein (PLTP), which shows very efficient lipid transfer activity.³⁹ In contrast to Tlc, which binds lipids at 1:1 protein-lipid ratio,^{7,15} PLTP can accommodate up to 43 moles of PC and 13 moles of cholesterol/mole of PLTP⁴⁴ although only two specific lipid-binding pockets have been identified in PLTP.⁴⁵ These pockets might be needed for specific lipid transfer of this protein, and the rest of the associated phospholipids may be needed for proper folding of the protein and stabilizing of the enzymatic activity. We have suggested that PLTP may play an integral role in tear lipid trafficking and in the protection of the corneal epithelium.⁵⁹ Possible cross-talk between Tlc and PLTP remains to be studied.

It seems that Tlc displays a very high thermal stability,^{17,42} and that the reason for this is the association of hydrophobic molecules within the protein. The bound lipid(s) may be necessary for proper folding of the protein. To this end, on lipid binding, the conformation of Tlc has been shown to change⁴⁶ and the denaturation temperature of Tlc suggested to increase.⁴² The hydrophobic cavity of Tlc has been recently demonstrated to be exceptionally large,¹⁷ and Breustedt et al. suggested that even several molecules could be accommodated within the protein. This may be true; or, alternatively, the conformation of the lipid ligand may be unconventional, as has been demonstrated for endothelial protein C, for example.⁴⁷ In this protein, the phospholipid adopts an extended configuration,⁴⁸ in which the acyl chains extend in opposing directions with the lipid head group protruding out of the protein, as also observed in Tlc.^{47,49} Based on the lack of lipid transfer activity, we suggest that the principal function of Tlc is not related to its ability to accommodate lipid molecules, but it may rather stabilize the oil-water interface as suggested previously.⁵⁰ Yet our study highlights the fact that Tlc interacts avidly with membranes, and this interaction may facilitate its biological function. Interfacial activation of proteins, probably best recognized for phospholipase A₂, states that the activity of the enzyme is intensely (up to 10⁴-fold for phospholipase A₂) enhanced on binding to membranes (for a review, see Ref. 51). Although the molecular mechanism of this phenomenon remains poorly understood, it is obvious that the physicochemical parameters of the membrane play an important role in determining the catalytic activity of the enzyme. One possibility is that on binding to lipid membranes the protein adopts a more active conformation.⁵² In keeping with this, we are currently analyzing possible changes in the Tlc conformation on membrane binding. Finally, if in vivo Tlc is bound to membranes and the biological actions it catalyzes, no matter what, takes place in a two-dimensional platform compared to a

3-D space such as the tear fluid, the catalyzing function can be expected to be several orders of magnitude higher.

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References

- Chen HB, Yamabayashi S, Ou Y, et al. Structure and composition of rat precorneal tear film: a study by an in vivo cryofixation. *Invest Ophthalmol Vis Sci.* 1997;38:381-387.
- King-Smith PE, Fink BA, Fogt N, Nichols KK, Hill RM, Wilson GS. The thickness of the human precorneal tear film: evidence from reflection spectra. *Invest Ophthalmol Vis Sci.* 2000;41:3348-3359.
- Nichols JJ, King-Smith PE. Thickness of the pre- and post-contact lens tear film measured in vivo by interferometry. *Invest Ophthalmol Vis Sci.* 2003;44:68-77.
- Wang J, Fonn D, Simpson TL, Jones L. Precorneal and pre- and postlens tear film thickness measured indirectly with optical coherence tomography. *Invest Ophthalmol Vis Sci.* 2003;44:2524-2528.
- Nicolaides N, Kaitaranta JK, Rawdah TN, et al. Meibomian gland studies: comparison of steer and human lipids. *Invest Ophthalmol Vis Sci.* 1981;20:522-536.
- McCulley JP, Shine WE. The lipid layer of tears: dependent on meibomian gland function. *Exp Eye Res.* 2004;78:361-365.
- Glasgow BJ, Marshall G, Gasymov OK, et al. Tear lipocalins: potential lipid scavengers for the corneal surface. *Invest Ophthalmol Vis Sci.* 1999;40:3100-3107.
- Wolff, E. The muco-cutaneous junction of the lid margin and the distribution of the tear fluid. *Trans Ophthalmol Soc UK* 1946;66:291-308.
- Brauninger GE, Shah DO, Kauffman HE. Direct physical demonstration of oily layer on tear film surface. *Am J Ophthalmol.* 1972;73:132-134.
- Tran CH, Routledge C, Miller J, Miller F, Hodson SA. Examination of murine tear film. *Invest Ophthalmol Vis Sci.* 2003;44:3520-3525.
- Greiner JV, Glonek T, Kord DR, Leahy CD. Meibomian gland phospholipids. *Curr Eye Res.* 1996;15:371-375.
- McCulley JP, Shine WE. A compositional based model for tear film lipid layer. *Trans Am Ophthalmol Soc.* 1997;95:79-88.
- McCulley JP, Shine WE. Meibomian secretions in chronic blepharitis. *Adv Exp Med Biol.* 1998;438:319-326.
- Glasgow BJ, Abduragimov AR, Farahbakash Z, Faull KF, Hubbel WL. Tear lipocalins bind a broad array of lipid ligands. *Curr Eye Res.* 1995;14:363-372.
- Flower DR. The lipocalin protein family: structure and function. *Biochem J.* 1996;318:1-14.
- Breustedt DA, Korndörfer IP, Redl B, Skerra A. The 1.8 Å crystal structure of human tear lipocalin reveals an extended branched cavity with capacity for multiple ligands. *J Biol Chem.* 2005;280:484-493.
- Gasymov OK, Abduragimov AR, Yusifov TN, Glasgow BJ. Site-directed tryptophan fluorescence reveals the solution structure of tear lipocalin: evidence for features that confer promiscuity in ligand binding. *Biochemistry* 2001;40:14754-14762.
- Glasgow BJ. Tissue expression of lipocalins in human lacrimal and von Ebner's glands: colocalization with lysozyme. *Graefes Arch Clin Exp Ophthalmol.* 1995;233:513-522.
- Fullard RJ. Identification of proteins in small tear volumes with and without size exclusion HPLC fractionation. *Curr Eye Res.* 1988;7:163-179.
- Redl B. Human tear lipocalin. *Biochim Biophys Acta.* 2000;1482:241-248.
- van't Hof W, Blankenvoorde MF, Veerman EC, Amerongen AV. The salivary lipocalin von Ebner's gland protein is a cysteine proteinase inhibitor. *J Biol Chem.* 1997;272:1837-1841.

23. Redl B, Merschak P, Abt B, Wojnar P. Phage display reveals a novel interaction of human tear lipocalin and thioredoxin which is relevant for ligand binding. *FEBS Lett.* 1999;460:182-186.
24. Gasymov OK, Abduragimov AR, Yusifov TN, Glasgow BJ. Binding studies of tear lipocalin: the role of the conserved tryptophan in maintaining structure, stability and ligand affinity. *Biochim Biophys Acta.* 1999;1433:307-320.
25. Redl B, Holzfeind P, Lottspeich F. cDNA cloning and sequencing reveals human tear prealbumin to be a member of the lipophilic-ligand carrier protein superfamily. *J Biol Chem.* 1992;267:20282-20287.
26. Mustonen P, Lehtonen JYA, Kõiv A, Kinnunen PKJ. Effects of sphingosine on peripheral membrane interactions: comparison of cytochrome c, adriamycin, and phospholipase A₂. *Biochemistry.* 1993;32:5373-5380.
27. MacDonald RC, MacDonald RI, Menco BP, Takeshita K, Subbarao NK, Hu LR. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim Biophys Acta.* 1991;1061:297-303.
28. Wiedmer SK, Hautala J, Holopainen JM, Kinnunen PK, Riekkola ML. Study on liposomes by capillary electrophoresis. *Electrophoresis.* 2001;22:1305-1313.
29. Selvin PR. The renaissance of fluorescence resonance energy transfer. *Nat Struct Biol.* 2000;7:730-734.
30. Damen J, Regts J, Scherphof G. Transfer of ¹⁴C-phosphatidylcholine between liposomes and human plasma high density lipoprotein: partial purification of a transfer-stimulating plasma factor using a rapid transfer assay. *Biochim Biophys Acta.* 1982;712:444-452.
31. Jauhiainen M, Metso J, Pahlman R, et al. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J Biol Chem.* 1993;268:4032-4036.
32. Groener JE, Pelton R, Kostner GM. Improved estimation of cholesteryl ester transfer/exchange activity in serum or plasma. *Clin Chem.* 1986;32:444-452.
33. Cheesman DF, Davies JT. Physicochemical and biological aspects of protein at interfaces. *Adv Protein Chem.* 1954;9:439-501.
34. Graham DE, Phillips MC. Proteins at liquid interfaces. *J Colloid Interface Sci.* 1979;70:403-414.
35. Ellison EH, Castellino FJ. Adsorption of vitamin K-dependent blood coagulation proteins to spread phospholipid monolayers as determined from combined measurements of the surface pressure and surface protein concentration. *Biochemistry.* 1998;37:7997-8003.
36. Dahim M, Brockman H. How colipase-fatty acid interactions mediate adsorption of pancreatic lipase to interface. *Biochemistry.* 1998;37:8369-8377.
37. Brockman H. Lipid monolayers: why use half a membrane to characterize protein-membrane interactions? *Curr Opin Struct Biol.* 1999;9:425-427.
38. Lakowicz JR. *Principles of Fluorescence Spectroscopy.* London, UK: Plenum Press; 1999:111-153.
39. Jauhiainen M, Setälä N, Ehnholm C, et al. Phospholipid transfer protein is present in human tear fluid. *Biochemistry.* 2005;44:8111-8116.
40. Verger H, Rietsch J, Van Dam-Mieras MC, de Haas GH. Comparative studies of lipase and phospholipase A₂ acting on substrate monolayers. *J Biol Chem.* 1976;251:3128-3133.
41. White SH, Wimley WC. Hydrophobic interactions of peptides with membrane interfaces. *Biochim Biophys Acta.* 1998;1376:339-352.
42. Burova TV, Rabesona H, Choiset Y, et al. Why has porcine VEG protein unusually high stability and suppressed binding ability? *Biochim Biophys Acta.* 2000;1478:267-279.
43. Gasymov OK, Abduragimov AR, Yusifov TN, Glasgow BJ. Interaction of tear lipocalin with lysozyme and lactoferrin. *Biochem Biophys Res Commun.* 1999;265:322-325.
44. Nishida HI, Nishida T. Phospholipid transfer protein mediates transfer of not only phosphatidylcholine but also cholesterol from phosphatidylcholine-cholesterol vesicles to high density lipoproteins. *J Biol Chem.* 1997;272:6959-6964.
45. Huuskonen J, Wohlfahrt G, Jauhiainen M, et al. Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis. *J Lipid Res.* 1999;40:1123-1130.
46. Gasymov OK, Abduragimov AR, Yusifov TN, Glasgow BJ. Structural changes in human tear lipocalins associated with lipid binding. *Biochim Biophys Acta.* 1998;1386:145-156.
47. Oganessian V, Oganessian N, Terzyan S, et al. The crystal structure of the endothelial protein C receptor and a bound phospholipid. *J Biol Chem.* 2002;277:24851-24854.
48. Kinnunen PKJ. Fusion of lipid bilayers: a model involving mechanistic connection to H_{II} phase forming lipids. *Chem Phys Lipids.* 1992;63:251-258.
49. Abduragimov AR, Gasymov OK, Yusifov TN, Glasgow BJ. Functional cavity dimensions of tear lipocalin. *Curr Eye Res.* 2000;21:824-832.
50. Nagyova B, Tiffany JM. Components responsible for the surface tension of human tears. *Curr Eye Res.* 1999;19:4-11.
51. Verheij HM, Slotboom AJ, de Haas GH. Structure and function of phospholipase A₂. *Rev Physiol Biochem Pharmacol.* 1981;91:91-203.
52. Roberts MF, Deems RA, Dennis EA. Dual role of interfacial phospholipid in phospholipase A₂ catalysis. *Proc Natl Acad Sci USA.* 1977;74:1950-1954.