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Serratia marcescens Hemolysin (ShlA) Binds Artificial Membranes and Forms Pores in a Receptor-independent Manner

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Abstract. Both the inactive and active conformations of the hemolysin/cytolysin of *Serratia marcescens* (ShlA) binds membranes of erythrocytes, but only active ShlA is able to form pores. ShlA is unable to lyse prokaryotic membranes. To determine the receptors of the binding and pore-forming domains of active cytolysin on eukaryotic membranes, artificial large unilamellar vesicles (LUVs) of various membrane compositions were examined. In the current study, it is shown that significant pore formation and lysis was achieved with binary phosphatidylcholine/phosphatidylserine (PS) liposomes. No proteinaceous receptor was needed for either binding or pore formation by ShlA. Membrane integration and pore-forming activity were enhanced by addition of phosphatidylethanolamine. Phosphatidylserine is negatively charged at physiologic pH and is almost absent in prokaryotic membranes. Hence, membrane binding and insertion of ShlA are highly dependent on phosphatidylserine, which targets the toxic activity to eukaryotic cell membranes without any need of a proteinaceous receptor. This may explain why prokaryotic membranes were found to be resistant against ShlA in a previous study.

Key words: Hemolysin — Membrane — Phosphatidylserine — Phospholipid — Pore-forming toxin — Tryptophan — Fluorescence — Vesicle

Introduction

The opportunistic pathogen *Serratia marcescens* secretes a number of exoenzymes, toxins, proteases, chitinases, a lipase, and a nuclease (Hines et al., 1988). One important pathogenic factor secreted is the hemolysin/cytolysin (ShlA), which is responsible for several effects on infected tissue culture cells (Hertle et al., 1999). ShlA is activated and secreted by an outer membrane protein (ShlB) in a phosphatidylethanolamine (PE)-dependent manner (Hertle et al., 1997). Without ShlB in the outer membrane, inactive ShlA (termed ShlA*) remains unprocessed in the periplasm. Inactive ShlA* could either be activated in vitro by purified ShlB or complemented by N-terminal (and ShlB-secreted) fragments of ShlA (Ondraczek et al., 1992). In vitro activated or complemented ShlA was indistinguishable from originally secreted ShlA in hemolytic (Ondraczek et al., 1992) and cytolytic activity (Hertle et al., 1999). It has been shown that the toxic and lytic effects of ShlA were caused initially by pore formation in the eukaryotic membrane (Schiebel & Braun, 1989; Schönherr et al., 1994). Specific binding sites have been described for other pore-forming toxins. For example, the family of oxygen-sensitive thiol-activated toxins, which include established virulence factors such as perfringolysin (theta toxin), pneumolysin, and listeriolysin O (Alouf & Alouf, 1991; Berry et al., 2001; Cossart et al., 1989), all interact with cholesterol in target cells to form multi-subunit pores. These toxins are active on a number of eukaryotic cell types, including erythrocytes, leukocytes, macrophages, platelets, and various cell culture lines. Aerolysin, produced by *Aeromonas hydrophila*, is activated by furin cleavage, binds to a GPI-anchored membrane protein and heptamerizes upon pore formation (Lesieur et al., 1999; Abrami et al., 1998). Up until now, no evidence for a specific receptor, as discussed for the cytolysins above, was found for ShlA.

However, *Serratia* hemolysin was found to be lytic only with eukaryotic, but not prokaryotic cells (Sieben et al., 1998). This specificity indicated a binding site unique for eukaryotic cell membranes. Human erythrocytes contain approximately 17%

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phosphatidylcholine (PC), 7% phosphatidylserine (PS), 18% phosphatidylethanolamine (PE), 23% cholesterol, 18% sphingomyelin, 3% glycolipids and 13% of other lipids. In eukaryotic cells, PS is found exclusively on the cytoplasmic side of intact membrane bilayers (Bergelson & Barsukov, 1977). In contrast, *E. coli* cytoplasmic membranes (as a reference for prokaryotic membranes) are composed of 70% PE, 29% phosphatidylglycerol (PG) and cardiolipin (CL), trace amounts of PS, but no PC, cholesterol, glycolipids or sphingolipids (Lugtenberg & Peters, 1976). The outer membrane composition differs from the bacterial cytoplasmic membrane mainly in the PE content, which is extremely high, comprising about 90% of total lipids. This membrane composition is also true for total *S. marcescens* phospholipids (Etienne et al., 1965). Concerning the differences between eukaryotic and prokaryotic membranes, various membrane compositions were examined to explain the different susceptibility of eukaryotic and prokaryotic membranes to ShlA. PS, PC, cholesterol, glycolipids and sphingolipids, lacking in prokaryotic membranes, were examined for susceptibility to ShlA action. In addition, we took into consideration that the mixing properties of PS or PE in a PC matrix are not random, and that phase separation and microdomain formation occur in the liquid-crystalline phase of artificial liposomes (Ahn & Yun, 1998). In this study, it was found that the membrane composition, with negatively charged phosphatidylserine, is sufficient for membrane binding and pore formation of ShlA. However, PS was not strictly necessary because a negative charge, elicited by other negatively charged lipids in the membrane (dicetylphosphate), also rendered liposomes susceptible to ShlA-mediated lysis. It was clearly shown in this artificial system that ShlA-mediated lysis does not require a proteinaceous receptor. However, the existence of such proteinaceous receptors on certain cells and used by ShlA in vivo can not be excluded.

Hence, studies on ShlA toxin provide a deeper insight into the basic interactions of bacterial protein toxins with eukaryotic membranes. The lack of a specific receptor implies an archaic and simple system of protein-membrane interaction and integration.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strain BL21 (DE3) (Studier & Moffatt, 1986) was used for T7-based expression of pRO3 (pT7-5 *shlA shlB*) (Schönherr et al., 1993), pES15 (pT7-5 *shlA*) (Poole et al., 1988) and pRO2 (pT7-6 *shlB shlA* (Δ256–1578)) (Schönherr et al., 1993). Cells were grown in TY medium consisting of 0.8% tryptone (Difco Laboratories), 0.5% yeast extract, 0.5% NaCl, pH 7.0 at 37°C with aeration. Ampicillin was added in a final concentration of 100 μg/ml.

Expression of *shlA* and *shlA** and Purification of Recombinant Proteins

Expression of proteins on the plasmids pRO3, pES15 and pRO2 via the T7-system was induced with IPTG as described (Hertle et al., 1997). ShlA and ShlA255 were isolated from the culture supernatant and ShlA* from the periplasm. Detailed purification protocols were previously described (Hertle et al., 1997; Hertle et al., 2000). ShlA and ShlA255 were in precipitated, spent media; ShlA* was isolated from the periplasm.

Activation of *ShlA** by Complementation with *ShlA255*

Aliquots (20 μg) containing ShlA* were mixed with ShlA255 (5 μg) and incubated 5 min on ice prior to further manipulations.

Preparation of Artificial Liposomes and Lysis Assay

Phospholipids L-α-phosphatidylcholin (from bovine brain), L-α-phosphatidylcholin, -distearoyl (C18:0), -dipalitoyl (C16:0), or -dioleoyl (C18:l,[cis]-9), L-α-phosphatidylethanolamine, distearoyl (C18:0), -dipalmitoyl (C16:0), -dimyristoyl (C14:0), or -dioleoyl (C18:1,[cis]-9), L-α-phosphatidylethanolamine from bovine brain (L-α-cephalin, type I), L-α-phosphatidyl-DL-glycerol, -distearoyl (C18:0), -dipalmitoyl (C16:0), -dimyristoyl (C14:0), or -dioleoyl (C18:1,[cis]-9), L-α-phosphatidyl-L-serin distearoyl (C18:0), -dipalmitoyl (C16:0), -dimyristoyl (C14:0), or -dioleoyl (C18:1,[cis]-9), L-α-phosphatidylinositol (from bovine liver), L-α-phosphatidylcholin β-(pyren-1-YL)decanoyl-γ-palmitoyl (C:16:0), cardiolipin (from bovine heart), sphingomyelin from bovine brain, liposome kit positive (L-α-phosphatidylcholine (egg yolk) 63 μmol, stearylamine 18 μmol, cholesterol 9 μmol) and liposome kit negative (L-α-phosphatidylcholin (egg yolk) 63 μmol, dicetylphosphate 18 μmol, cholesterol 9 μmol) (all phospholipids from Sigma Chemicals, St. Louis, MO) were solubilized in chloroform and 10 mg in total were mixed in a 100-ml round flask with 1 ml chloroform/methanol (9/1; v/v) and dried under a stream of nitrogen with constant turning to yield an evenly distributed layer of lipids. The phospholipids were suspended in 1 ml calcein (from Sigma) solution (50 mM calcein, 50 mM HEPES, 100 mM NaCl, pH 7.5). This suspension was forced 3 times through a 0.4-μm nitrocellulose filter in a LIPEX extruder (Lipex, Vancouver, BC, Canada) according to the instructions of the manufacturer and then forced 5 times through a 0.2-μm nitrocellulose filter and at least 7 times through a 0.1-μm nitrocellulose filter membrane. Excess calcein was removed from the extruded vesicle preparation by gel filtration through a PD10 column (Pharmacia, Uppsala, Sweden). This resulted in an opaque suspension of large unilamellar vesicles (LUVs) including calcein with an average diameter of about 0.1 μm (Mayer, Hope & Cullis, 1986). Release of calcein yielded an increase of fluorescence intensity due to the dequenching of the dye. Relative lysis was determined by the increase of fluorescence at emission wavelengths 520 nm (Em) with a fluorescence spectrometer (FluoroMax-2, Jobin Yvon Spex, Edison NY) at 494-nm excitation wavelength (Ex). Complete lysis (F_{max}) was achieved with desoxycholate (DOC), 1 mM final concentration. Percentage of calcein release (Rel.lysis %) was calculated as follows:

$$Rel.lysis\ \% = (F_0 - F)/(F_{max} - F) \times 100, \quad (1)$$

where F_0 represents the initial fluorescent value of LUVs alone and F the final fluorescent value at given time points.

Isolation of Total Erythrocyte Lipids

Thirty milliliters of human erythrocytes were lysed by addition of a 10-fold amount of distilled water for 10 min on ice. Membrane ghosts were pelleted by centrifugation, washed again with water and suspended in 50 ml water. Lipids and phospholipids were extracted by chloroform:methanol (2:1; v/v) in a shaking funnel. The lipid-containing phase was evaporated with rotation and lyophilized. Nitrogen gas was used for all ventilation steps to minimize lipid oxidation. This preparation was used to constitute LUVs as described above.

Binding and Integration of *ShlA* or *ShlA** on LUVs

LUVs were prepared as described above without calcein with 50 mM Tris-HCl, 1 mM EDTA, pH 7.4. A LUV preparation was mixed with ShlA or ShlA* in the fluorescence spectrometer at 22°C. The fluorescence of tryptophan residues of ShlA was recorded from 310 to 460 nm at an excitation wavelength of 295 nm. The integration of ShlA domains containing tryptophan residues was measured by the energy transfer to pyrene-PC in PC LUVs (2.5 mol/100 mol). After incubation for 10 min at 27°C, the fluorescence intensity of tryptophan residues in ShlA was measured (with Ex 295 nm, Em 347 nm). Excitation at 295 nm was used to minimize the fluorescence of tyrosine.

Modulation of Ionic Strength for pH-dependent Lysis

LUVs were prepared as described above with the calcein loading buffer. 20 μl of LUV preparations were suspended in 1 ml PBS ranging from pH 5 to 10 and incubated at 22°C for 10 min. To modulate the pH inside of LUVs, the phospholipids were extruded with calcein loading buffer in the appropriate pH range. The lysis assay was performed as described. Washed erythrocytes were incubated in the same buffers in parallel.

Tryptic Digestion of Membrane-bound Proteins

Erythrocytes or liposomes were incubated with 3 μg/ml ShlA or ShlA* at 22°C for 15 min. Liposomes were centrifuged and the pellet and supernatant were examined by SDS-PAGE and western blot. Concentrated human erythrocytes (20 μl) were incubated with 25 μg trypsin in HBSS for 20 min at 37°C, washed two times with PBS (with or without 100 μg/ml trypsin inhibitor), and then incubated with given amounts of ShlA. Ghosts of erythrocytes were prepared according to Tomoda et al. (Tomoda et al., 1984) and sealed ghosts as described (Whitlow, Ramm & Mayor, 1985). Ghosts and LUVs, pretreated with 3 μg/ml ShlA, were incubated with 5 μg/ml trypsin 30 min on ice. Then 100 μg/ml trypsin inhibitor was added before further manipulations.

Quantification of Lipid Binding

Transil^R-particles (Nimbus, Leipzig, Germany) coated with 100% PC and PC/PS (90/10; w/w) were prepared in logarithmic dilution steps. The particles were mixed with a defined amount of ShlA or ShlA* according to the instructions of the manufacturer. After centrifugation, supernatants were analyzed by SDS-PAGE. Relative protein concentrations were determined by densitometric analysis of the stained bands (Image Master, Pharmacia, Uppsala, Sweden). In parallel, the residual hemolytic activity in the supernatant was determined.

Hemolysis Assay

Hemolysis was determined as described previously (Hertle et al., 1999). In brief, serial dilutions of hemolytic samples were prepared with PBS or U-buffer (6 M urea in 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 6.8. Aliquots of each dilution were incubated with 1 ml of erythrocyte suspension for 15 min at 22°C and then centrifuged for 1 min in a microcentrifuge. The absorbance at 405 nm (A_{405}) of released hemoglobin was measured spectroscopically. Hemolytic activities are presented either as the percentage of the total erythrocytes lysed (percent hemolysis), or as hemolytic units (HU) per ml, determined as described previously (Hertle et al., 1999). The hemolytic unit (HU = release of 50% of the total hemoglobin) is defined as

$$\frac{A_{405}(\text{sample with hemolysin}) - A_{405}(\text{control without hemolysin}) \times 100}{A_{405}(\text{total lysis caused by SDS}) - A_{405}(\text{control})} \quad (2)$$

Results

Activity of ShlA on Trypsinized and Non-trypsinized Human Erythrocytes

The kinetics of hemolytic activity of ShlA on human erythrocytes was not altered by erythrocyte trypsination (Fig. 1*A*). Receptor-independent binding and lysis by ShlA was indicated. Efficient trypsination of erythrocytes was shown by SDS-PAGE (Fig. 1*B*) and the binding of ShlA to erythrocytes and trypsinized erythrocyte membranes is shown in lanes *7* and *9*, respectively. However, certain proteins in the erythrocytes were obviously not affected by trypsin (lower bands in Fig. 1*B*), and they may still serve as binding sites for ShlA.

A western blot of ShlA bound to erythrocytes and PC/PE/PS (75/18/7) LUVs shows the binding of ShlA to these membranes (lanes *1* and *5*). As shown before (Schiebel & Braun, 1989), by trypsinizing sealed and unsealed erythrocyte "ghosts," ShlA exposed the C-terminal end on the inner leaflet of the erythrocyte membrane. The residual N-terminal 138 kDa-fragment of ShlA remained resistant to trypsin, indicating that it is embedded in the membrane. A similar digestion of artificial sealed (Fig. 1*C*, lanes *2* and *6*) and open vesicles (lanes *3* and *7*) confirmed the transmembrane location of ShlA in PC/PE/PS LUVs to be similar to that in erythrocytes. The additional bands in lanes *2* and *3* appeared after trypsinization, indicating two possible orientations of ShlA (the C-terminus being exposed on either side of the bilayer membrane) in artificial LUVs (lanes *2* and *3*), in contrast to erythrocytes (lanes *6* and *7*).

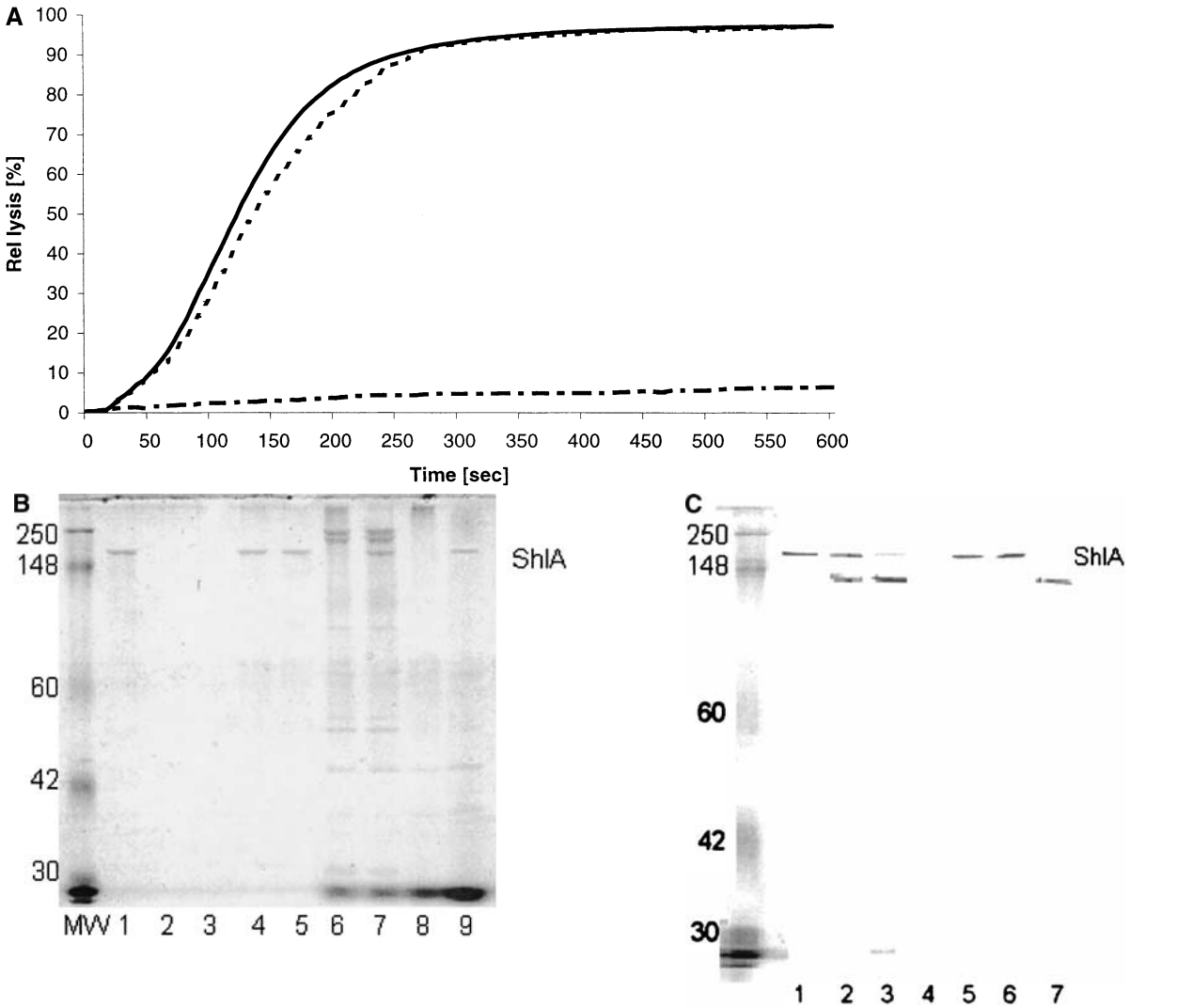


Fig. 1. (*A*) Lysis kinetics of human erythrocytes (*solid line*) and trypsinized human erythrocytes (*dotted line*) with 2 HU ShlA. Baseline of untreated erythrocytes as control (dot-dash-dot). Curves represent the median out of three experiments. (*B*) SDS-PAGE of ShlA bound to erythrocytes and liposomes; lane *1*: 5 μg ShlA; lane *2*: PC/PE/PS (75/18/7) LUVs; lane *3*: LUVs from erythrocyte membranes (*see* Fig. 4A); lane *4*: 5 μg ShlA + PC/PE/PS LUVs; lane *5*: 5 μg ShlA + erythrocyte membrane LUVs; lane *6* shows erythrocyte ghosts and lane *7* ghosts with ShlA; lanes *8* and *9* show the same with trypsinized erythrocyte ghosts. (*C*) blot with antibodies against ShlA on PC/PE/PS LUVs (lane *1*), erythrocytes (lane *5*), from-outside-trypsinized PC/PE/PS (lane *2*) and erythrocytes (lane *6*), trypsinized opened PC/PE/PS LUVs (lane *3*) and erythrocytes (lane *7*). Lane *4* was blank. MW, molecular weight marker.

Activity of ShlA on LUVs with Two-Component Phospholipid Composition

Calcein-containing liposomes consisting of 100% PC, 90% (80%) PC plus 10% (20%) of either PE, PS, PG, cholesterol or PI were tested for osmotic lysis caused by ShlA. PC, PE, PS and PG with fatty acid chain lengths of C18 were compared to PC, PE, PS and PG isolated from natural sources, which contained the natural distribution of different fatty acid chain length and saturation. ShlA (2 HU/ml) exhibited the highest lytic activity on PC/PS (90/10) or PC/PS (80/20) vesicles (Fig. 2). But the activity on PC/PS (80/20) LUVs were slightly reduced compared to PC/PS (90/10) LUVs. LUVs containing only PC were resistant to ShlA. Addition of cholesterol had some effect in increasing ShlA-induced lysis, but not more than 10% of total lysis was reached. In brief, there was no significant difference between LUVs made with undefined phospholipids obtained from natural sources and LUVs made from chemically defined C-18 phospholipids. Even addition of PC-, PE-, or PS-dioleoyl (C18:1,[cis]-9) did not alter the results obtained with saturated derivatives. In addition, different fatty acid chain lengths of PE, PS, PG (C14 and C16) or derivatives with unsaturated fatty acid chain lengths (which influence the membrane fluidity) were tested but had no significant effect in comparison to lysis with C18

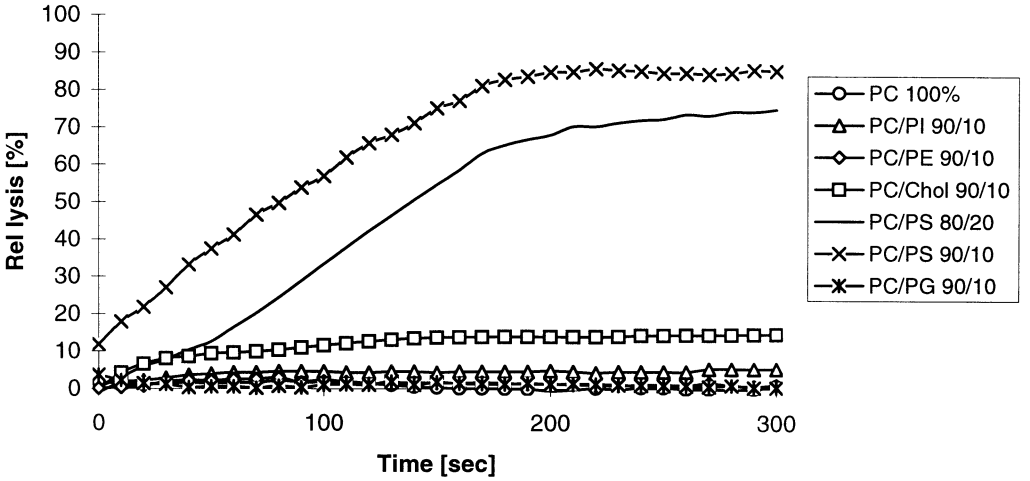


Fig. 2. Lysis kinetics of different artificial LUVs loaded with calcein in the presence of 2 HU ShlA. Curves represent the median out of three experiments.

phospholipids (*data not shown*). Accordingly, the same results were obtained with 2 hemolytic units of ShlA* complemented with ShlA255 (N-terminal 255 amino acids of mature ShlA) (*data not shown*).

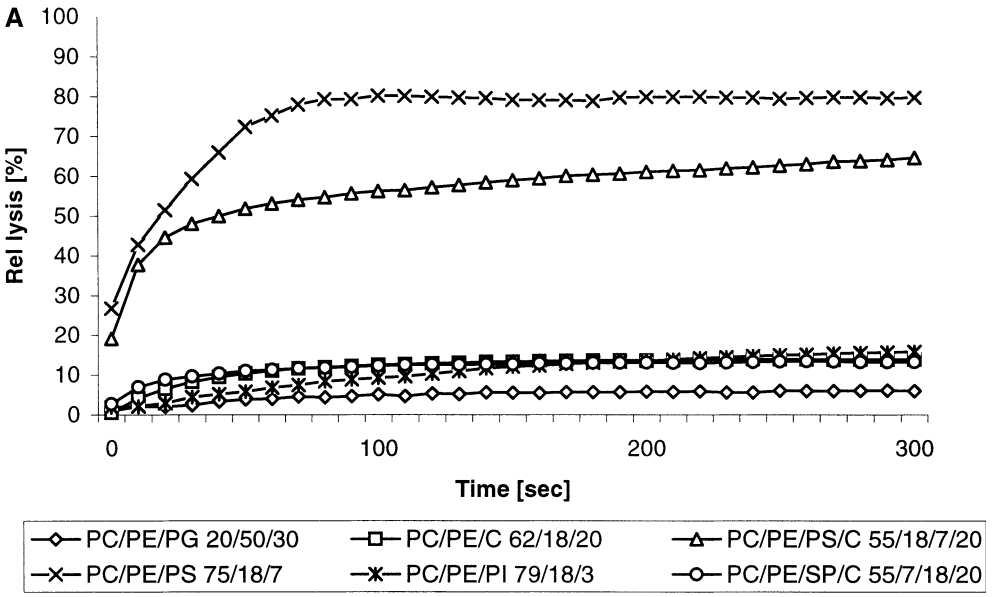
Activity of ShlA and ShlA* on LUVs with Complex Phospholipid Composition

LUVs with three or more different phospholipids from natural sources were compared to LUVs with phospholipids of defined fatty acid chain length with regard to ShlA-mediated lysis. To mimic erythrocyte membranes, LUVs were first made with 62% PC, 18% PE and 20% cholesterol. Then, the corresponding amount of PC was replaced by 3% PI, 18% sphingomyelin and finally 7% PS in different mixtures. Only the addition of PS rendered the LUVs susceptible for ShlA-mediated lysis (Fig. 3*A*). The lysis kinetics of artificial LUVs consisting of 55% PC, 18% PE, 7% PS and 20% cholesterol were significantly slower than the one obtained with LUVs consisting of 75% PC, 18% PE and 7% PS with no added cholesterol. However, the addition of cholesterol increased the background level of lysis to 12%. ShlA* had no effect on any of the liposomes tested, whereas complementation with ShlA255 showed the same specificity as ShlA (*data not shown*). An unexpected finding was that PC/PE/PS (75/18/7) LUVs were most sensitive to ShlA (more than PC/PS (90/10) LUVs), showing lysis kinetics similar to human erythrocytes (*see* Fig. 1*A*). Lysis data obtained with LUVs containing phospholipids of defined fatty acid chain length from C14 to C18 were indistinguishable from LUVs made of partially unsaturated natural phospholipids, indicating that membrane fluidity does not influence the results.

To mimic prokaryotic membranes, LUVs with 20% PC, 50% PE and 30% PG were generated by extruding all phospholipids together. The small amount of PC was needed to get stable liposomes. Little activity was recorded with 2 HU/ml ShlA and no activity was seen with ShlA* (*data not shown*). Doubling the hemolytic activity used to 4 HU/ml had no effect on the LUVs (*data not shown*). Considering that PS was probably negatively charged under the condition used, negatively and positively charged artificial LUVs (from SIGMA liposome kit) were prepared as a control to see if charge played a role in ShlA-mediated lysis. Fig. 3*B* depicts the relative lysis of different LUVs, showing clearly that the negatively charged LUVs (with dicetylphosphate) were significantly lysed by ShlA. This indicates that negatively charged membranes are sufficient for efficient pore formation by ShlA, but to a lesser extent than with PS.

Susceptibility of Erythrocyte-derived LUVs to ShlA

All the lipids of erythrocyte ghosts were isolated and used to prepare calcein-containing liposomes in order to compare susceptibility to ShlA lysis with that of artificially composed LUVs. The lysis kinetics of these erythrocyte membrane-derived vesicles were virtually the same as those obtained with intact erythrocytes (Fig. 1*A*), but faster than LUVs containing PC/PS (90/10) or PC/PE/PS (75/18/7) (Fig. 4*A*). A thin layer chromatography (TLC) of isolated total phospholipids of erythrocytes showed the phospholipid composition to be rather similar but not identical to PC/PE/PS 75/18/7 LUVs in terms of PC, PE and PS content (Fig. 4*B*). Compared to the R_f values of reference substances, PC (*4*), PE (*5*), and PS (*3*) could be determined, but the differences in R_f values may reflect the difference in fatty acid chain length and saturation. Additional spots referred to sphingolipids (*6*), various lyso-phospholipids (*1,2*)



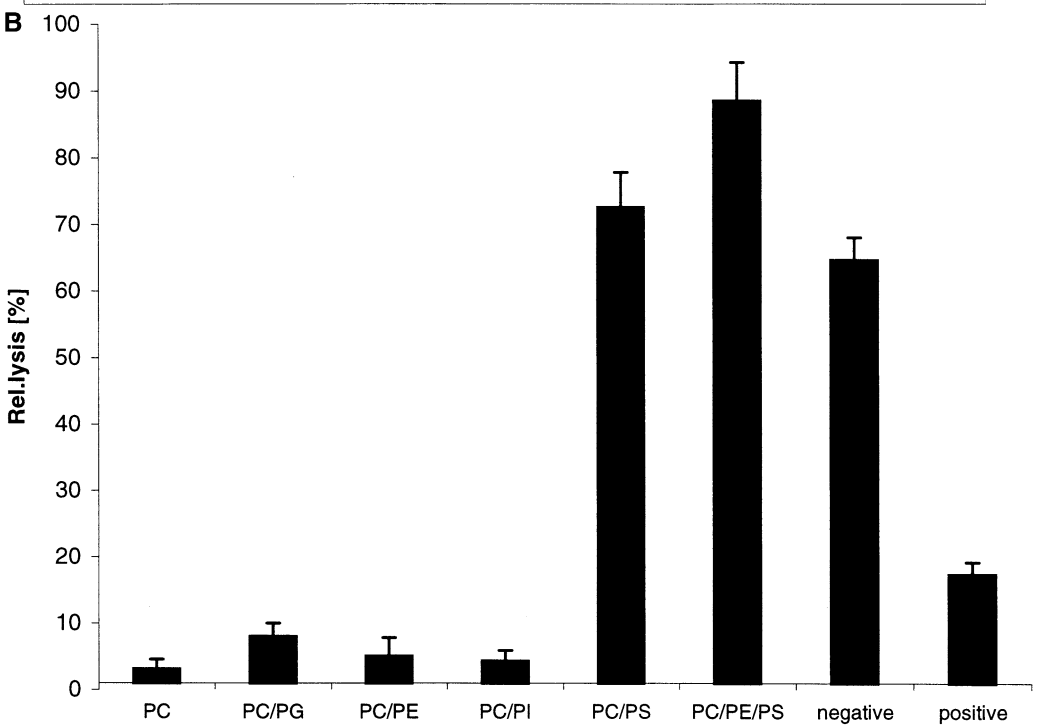
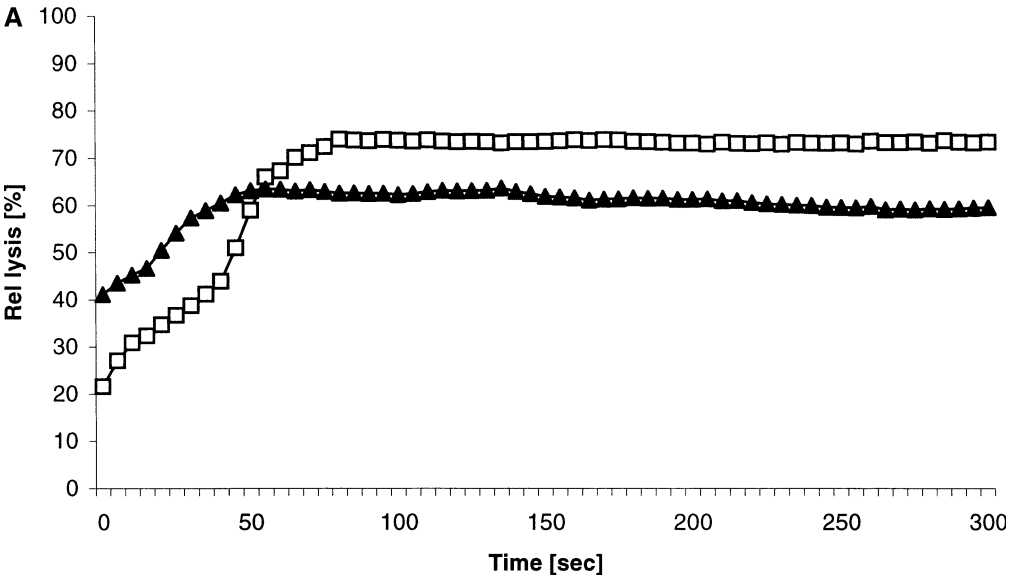


Fig. 3. (*A*) Lysis kinetics of complex artificial calcein-loaded LUVs with PE and 2 HU ShlA. Curves represent the median out of three experiments. (*B*) Mean relative lysis of seven independent measurements of different LUVs after 10 min incubation with 2 HU ShlA. negative: liposome kit (L-α-phosphatidylcholine (egg yolk) 63 μmol, dicetylphosphate 18 μmol, cholesterol 9 μmol); positive: liposome kit (L-α-phosphatidylcholine (egg yolk) 63 μmol, stearylamine 18 μmol, cholesterol 9 μmol).

and minor uncharacterized derivatives. Artificial liposomes were included as a control (Fig. 4*B*).

pH-Dependent ShlA-induced Lysis of Phosphatidylserine-containing LUVs

The net charge of the acidic phospholipid PS was modulated by changing the pH outside the vesicles. LUVs with 75% PC, 18% PE and 7% PS showed a slight decrease in ShlA-mediated lysis of about 12% when the pH was increased from 5 to 10. The most effective lysis was obtained at pH 7 and 8. A similar decrease was obtained with human erythrocytes under the same conditions. LUVs consisting of 100% PC were not altered or affected within this pH range in their resistance to ShlA. Changing the pH inside the



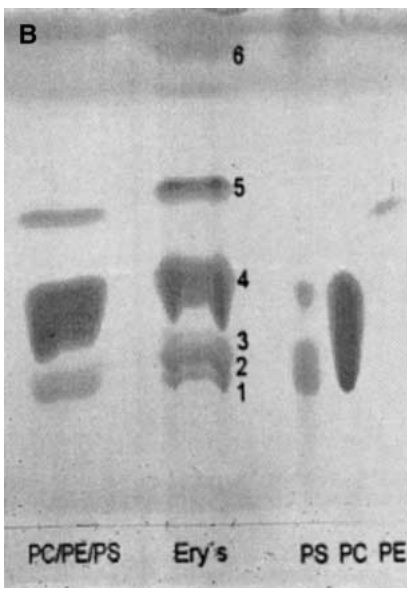


Fig. 4. (*A*) Lysis kinetics of PC/PE/PS (75/18/7) LUVs (*open squares*) and erythrocyte membrane-derived LUVs (*filled triangles*) with 2 HU ShlA. (*B*) TLC of LUVs derived from distinct phospholipids and isolated erythrocyte membrane phospholipids, (*1* and *2*) lyso-phospholipids and uncharacterized derivatives; (*3*) PS; (*4*) PC; (*5*) PE; (*6*) sphingolipids. Iodine stain.

vesicles showed about the same range of lysis as observed above. But the decrease in activity of about 20% from pH 5 to 10 was more striking. However, it was evident that the activity of ShlA on LUVs was not significantly affected by the pH range from 5 to 10.

Time- and Concentration-dependent Lysis

2 HU/ml of either active ShlA or ShlA* that had been complemented with ShlA255 showed up to 80% lysis within 1.5 to 3 min on PC/PS 90/10 and PC/PE/PS 75/18/7 (w/w/w) LUVs, followed by a slowly increasing plateau (see Figs. 2 and 3). LUVs without PS were not significantly lysed by ShlA after 10 min. Concentration-dependent lysis (measured at 10 min) was observed with ShlA and PC/PS (90/10), PC/PE/PS (75/18/7) LUVs at doses as low as 0.1 HU/ml (Fig. 5). If data were taken after 10 min of incubation, PC/PS (90/10) and PC/PE/PS (75/18/7) LUVs showed no significant difference in total lysis. PC/PE (90/10) LUVs remained stable in the presence of 2 HU/ml ShlA (Fig. 5).

Binding of Inactive ShlA* versus Active ShlA on Defined Liposomes

Binding of ShlA and ShlA* to PC/PS (90/10) Transil^R-particles has shown a significant binding efficiency between active ShlA (Fig. 6*A*) and inactive ShlA* (Fig. 6*B*). ShlA bound to PC and to PC/PS (90/10) particles. While ShlA* also bound to PC/PS particles, but to a 10-fold less extent. However, binding of ShlA or ShlA* to PC particles was lower than binding of ShlA or ShlA* to PC/PS (90/10) particles, by a factor of 10 (Fig. 6). This made clear that PC/PS (90/10) particles provided the best binding properties for active ShlA only.

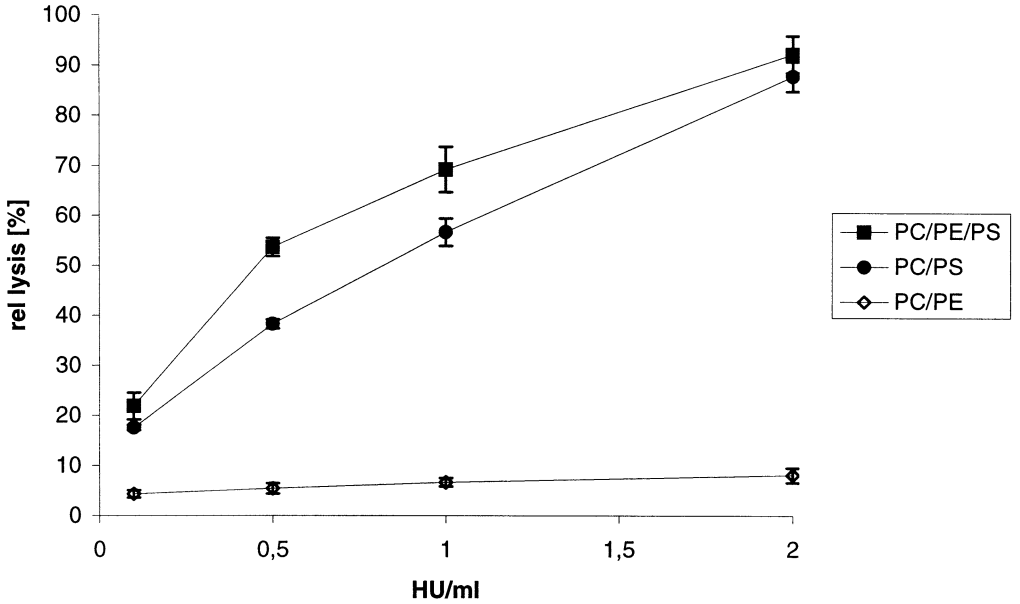


Fig. 5. Concentration dependent lysis of calcein-loaded LUVs with ShlA.

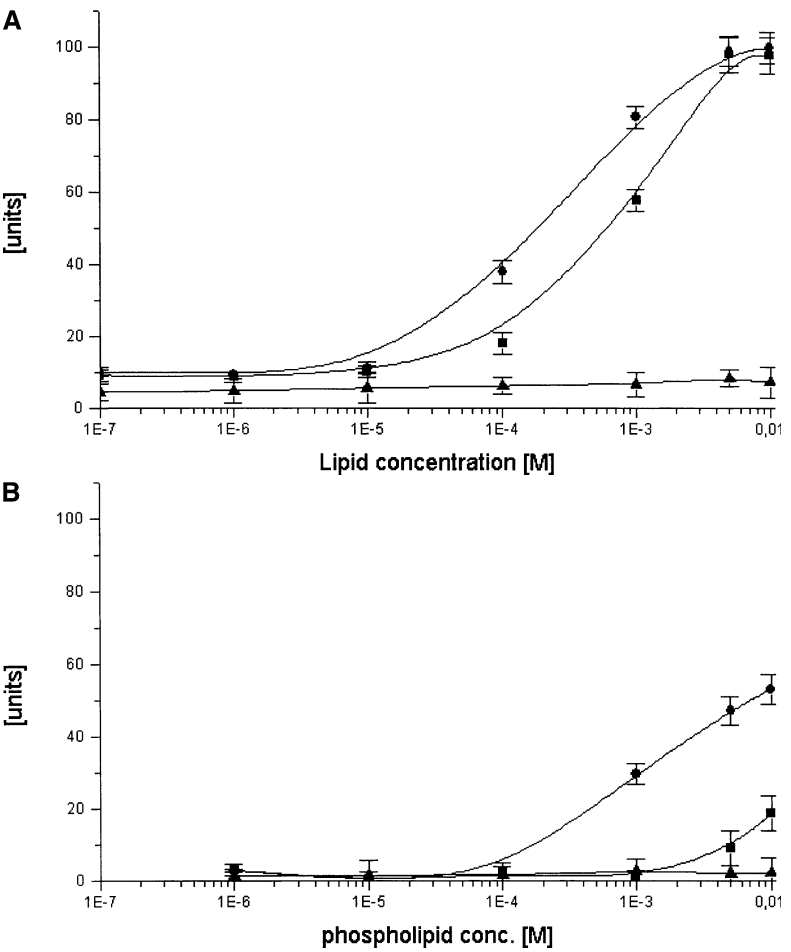


Fig. 6. (*A*) Binding of ShlA on TRANSIL^R particles coated with 100% PC (■) and PC/PS (●) 90/10. Baseline represents uncoated TRANSIL particles (▲). (*B*) Binding of ShlA* on TRANSIL^R particles coated with 100% PC (■) and PC/PS 90/10 (●). Baseline represents uncoated TRANSIL^R particles (▲). Error bars represent the deviation of five independent experiments. The scale is from (E) exponent −7 (= 0.1 μM) to 1E-2 (= 0.01 M) in phospholipid concentration.

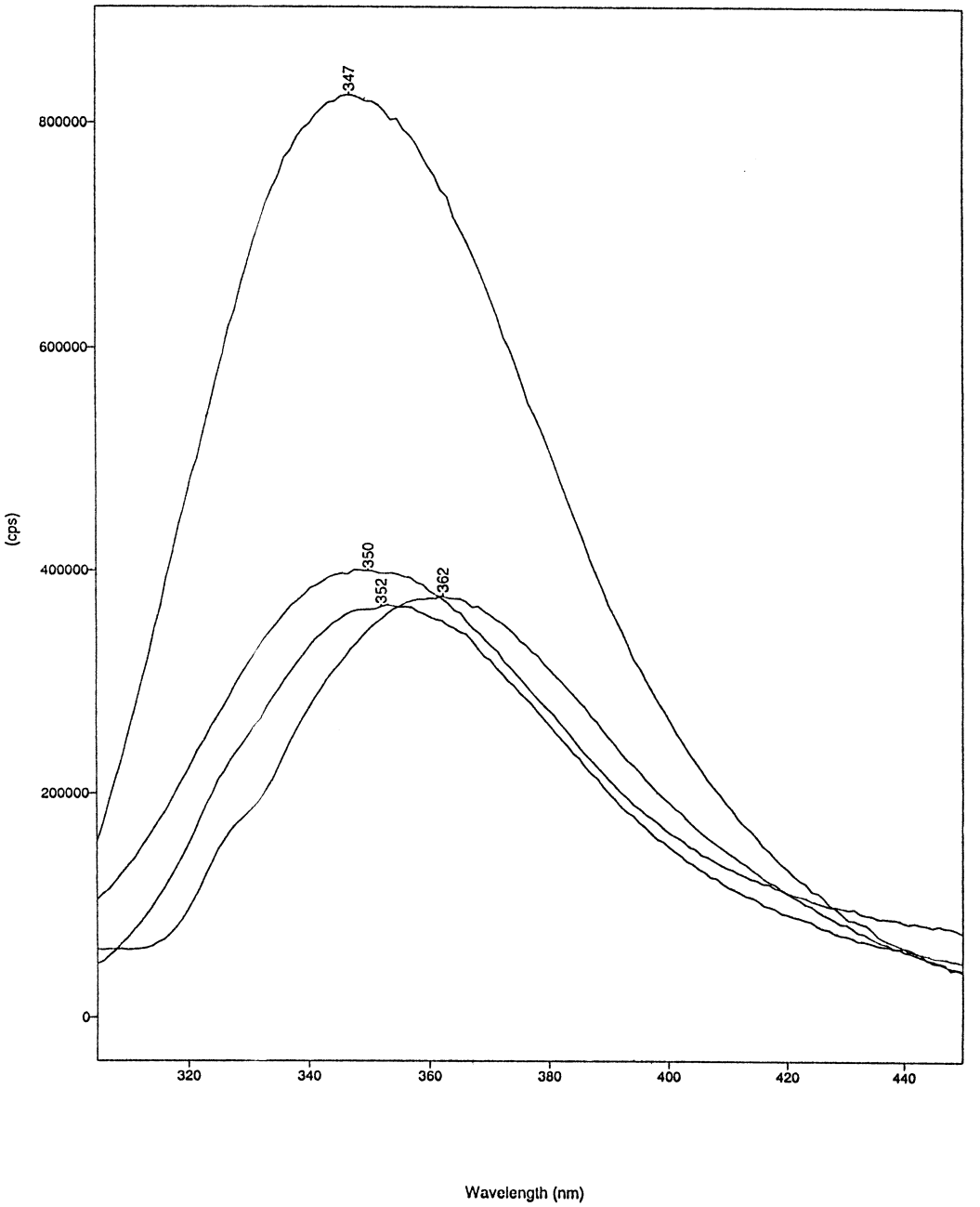


Fig. 7. Tryptophan fluorescence spectra of ShlA in 6 M urea (peak 362 nm), in buffer (352 nm), with PC vesicles (350 nm) and PC/PS (90/10) LUVs (347 nm). Curves depict single representative measurements.

Osmotic Protection of ShlA-mediated Lysis

The monosaccharide sorbitol (M_r 182 Da) and the oligosaccharides sucrose (342 Da), maltotriose (504 Da), maltopentaose (828 Da), dextrin 20 (900 Da), maltoheptaose (1152 Da), dextran 15 (1400 Da), and dextran 4 (4000 Da) were solubilized in PBS at a concentration of 10 mM. PC/PE/PS (75/18/7) LUVs were treated with a lytic dose of ShlA (3 HU/ml) in PBS containing the oligosaccharides as described above. Dextrin 20 partially reduced ShlA-mediated lysis by approximately 15.3% ± 1.6. Maltoheptaose nearly inhibited lysis within 5 min up to 68.6% ± 2.4. Both dextrans (15 and 4) were completely protective (more than 95% inhibition in all experiments), indicating a small pore size ranging from 1.5–2.0 nm. As a positive control, all samples containing LUVs, the saccharides described above and ShlA were diluted 1:10 in PBS, resulting in total lysis in all cases after 10 min at 22°C. The negative control with PC-LUVs showed no lysis with or without oligosaccharides after dilution.

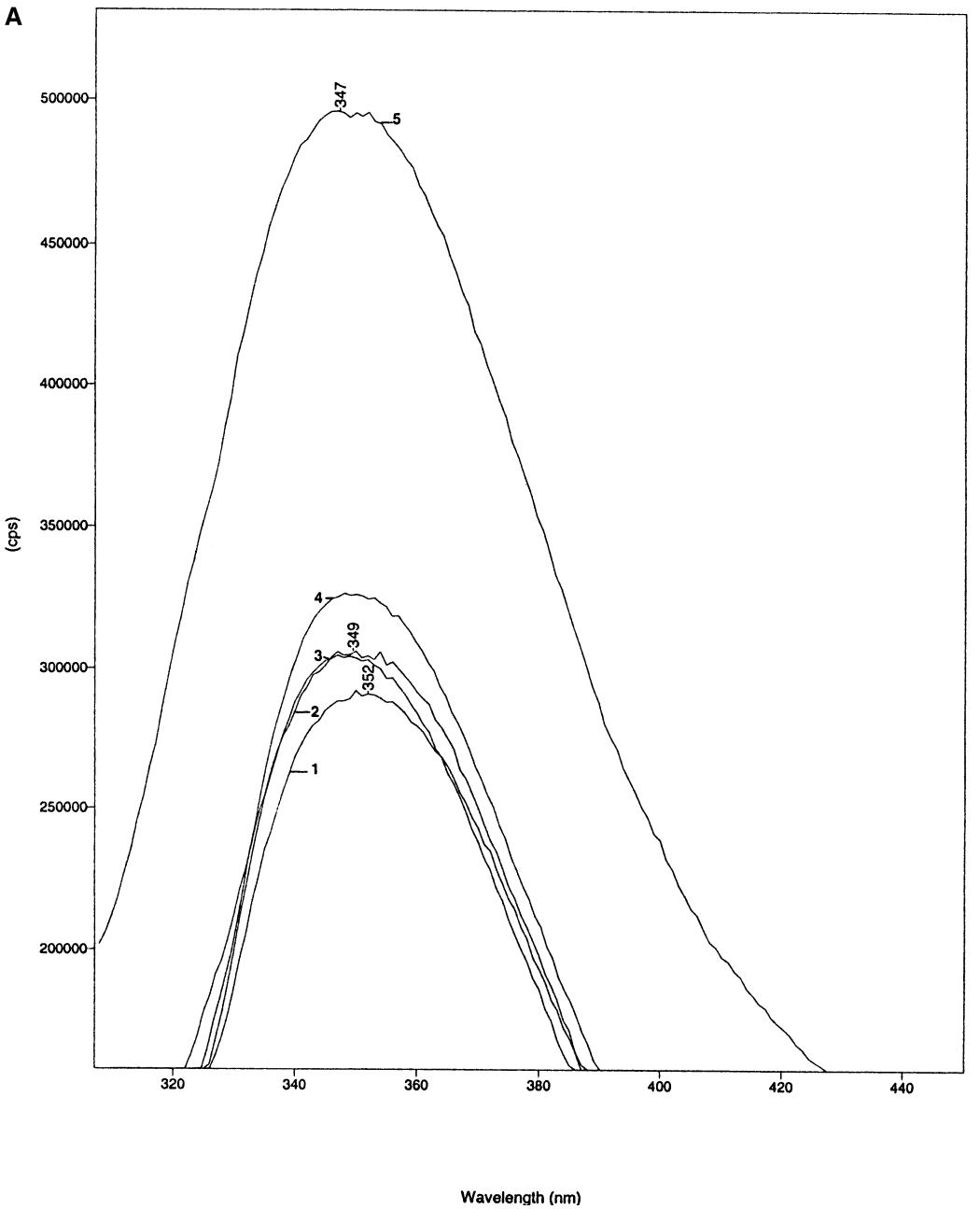


Fig. 8. (*A*) Tryptophan fluorescence spectra of ShlA with (*1*) PC vesicles; (*2*) PC/PE (90/10) vesicles; (*3*) PC/PI (90/10) vesicles ; (*4*) PC/PG (90/10) vesicles; and (*5*) PC/PE/PS (75/18/7) vesicles. (*B*) Tryptophan fluorescence spectra of ShlA* with (*1*) PC vesicles; (*2*) PC/PE (90/10) vesicles; (*3*) PC/PI (90/10) vesicles; (*4*) PC/PG (90/10) vesicles; and (*5*) PC/PE/PS (75/18/7) vesicles.

Intrinsic Fluorescence of ShlA and ShlA*

Constant (after 5 min, *see* Fig. 4*B*) intrinsic fluorescence spectra of the active and inactive toxin excited at 295 nm were taken (Fig. 7). The 1578 amino acids of the full-length mature ShlA contain 6 tryptophan residues clustered at positions 384, 388, 471 and 540 in the membrane binding domain and at positions 1276 and 1470 in the C-terminus. The emission spectra of an isomolar mixture of free tryptophan and tyrosine amino acids in the same molarity as found in the toxin, resembled those of denatured ShlA or ShlA* (362 nm in 6 M urea) with a maximum slightly shifted to 366 nm. Dilution of ShlA or ShlA* from 6 M to 0.06 M urea in 50 mM Tris buffer, pH 7.5 resulted in an emission peak blue-shift from 362 nm to 352 nm immediately upon mixing (Fig. 7). The fluorescence intensity was slightly increased, indicating that ShlA was undergoing a conformational change while certain tryptophan residues were transferred into a more hydrophobic environment.

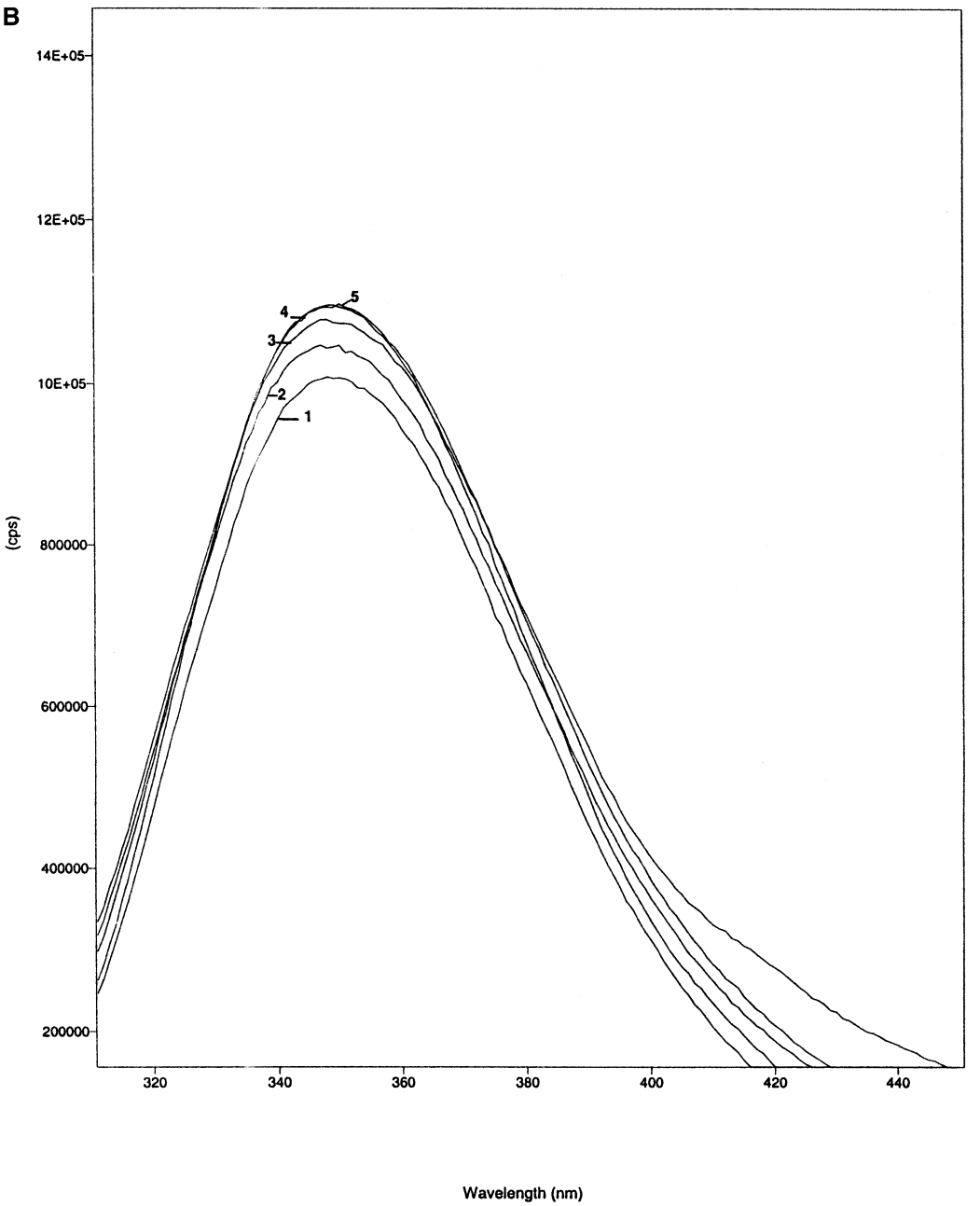


Fig. 8. Continued

Changes in Intrinsic Tryptophan Fluorescence upon Binding and Membrane Integration of ShlA

Transferring tryptophan residues into a more hydrophobic environment, either by changing the solute or by translocation into a membrane, results in an increase of Trp fluorescence intensity and a blue-shift of the emission maximum (Lakowicz, 1983). Addition of LUVs consisting of PC, PC/PE (90/10), PC/PI (90/10) and PC/PG (90/10) yielded only a slight increase in Trp-fluorescence intensity at 350 nm by both ShlA and ShlA* (Fig. 8), which indicates binding of the proteins to the liposomes. Trp-fluorescence intensity of ShlA was markedly increased with PC/PS (90/10) and additionally enhanced by PC/PE/PS (75/18/7) liposomes, whereas ShlA* showed only a slight increase (Fig. 8). In addition, with PC/PE/PS (75/18/7) the emission peak of ShlA shifted to 347 nm, indicating the integration of certain tryptophans into this membrane bilayer, in contrast to vesicles not containing PS.

Membrane Quenching of Tryptophan Fluorescence

The well-known quenching of Trp-fluorescence by energy transfer from tryptophan to the pyrene group of pyrene-PC (Somerharju, 1985) was used to assess the membrane integration of ShlA into LUVs with or without PS (and PE, respectively). F/F_0 of ShlA with 100% PC LUVs was 0.95 ± 0.008, with PC/PE (90/10) it was 0.92 ± 0.012, with PC/PS (90/10) it was 0.88 ± 0.014, and with PC/PE/PS (75/18/7) it was 0.84 ± 0.007. The Trp-fluorescence intensity was quenched significantly only when PS was present in the liposome membranes. This indicated the integration of certain tryptophans into the hydrophobic environment of the membrane bilayer.

Discussion

S. marcescens is an opportunistic pathogen and shows tissue-damaging capacity in vivo (König et al., 1987; Marre, Hacker & Braun, 1989). It has been shown recently that cells in tissue culture were affected by the hemolysin/cytolysin (ShlA) produced by *S. marcescens* (Hertle et al., 1999). It has also been shown that lysis of human epithelial cells in culture was due to pore formation by ShlA in the plasma membrane (Hertle et al., 1999). In addition, ShlA was shown to be hemolytic on erythrocytes from various species and on artificial "black lipid" (DOPC) bilayer membranes (Schönherr et al., 1994). In these previous studies of measuring conductivity of pores formed by ShlA in black lipid membranes (Schönherr et al., 1994), a membrane potential of 20 mV was applied, which may have unspecifically driven ShlA into the diphtanoylglycerolphosphocholine/*n*-decane membrane. This bypassed the need for the presence of a specific receptor in the membrane. Hence, these data are not comparable with the data obtained from liposomes. The data presented here are closer to the in vivo situation and show that LUVs consisting of 100% PC are resistant to ShlA-mediated lysis and that ShlA is unable to form pores in these artificial LUVs. Therefore, PC vesicles were used as an experimental platform to which other lipids could be added to form LUVs. In addition, the ShlA-induced lysis kinetics of trypsinized erythrocytes showed no difference when compared to native erythrocytes. Because some proteins were unaffected by trypsin digestion, it is possible they could serve as receptors. However, these could also be integral membrane proteins or proteins exposed on the inner leaflet of the erythrocyte membrane. There are two possible explanations for the resistance (Sieben et al., 1998) of prokaryotic membranes to ShlA: (i) there is no specific receptor for ShlA, or (ii) the phospholipid composition of the prokaryotic membrane rendered it resistant to ShlA. Potential candidates for making the eukaryotic membrane susceptible for ShlA were phosphatidylcholine (PC), ceramides, cholesterol, sphingolipids and phosphatidyl serine (PS). These phospholipids are found in eukaryotic membranes and absent in prokaryotic membranes.

To confirm that the source of phospholipids had any influence on susceptibility to ShlA, PC isolated from bovine brain (with various fatty acid chain lengths) or chemically synthesized PC (C18:0) were used but no difference in resistance was found. By substituting parts of total PC in pure PC-LUVs with the membrane compounds discussed above (either from natural sources or synthesized), it became evident that only PS promoted pore formation by ShlA. These data indicate that ShlA forms pores exclusively in PS-containing vesicles. When PC vesicles contained 7% PS (approximately the same level found in human erythrocytes), pore formation was observed. In natural membranes the fatty acid chain length and saturation of phospholipids vary. In PC/PS combinations isolated from natural sources, we found no significant difference in lysis compared to chemically defined phospholipids. Variation of the fatty acid chain length of chemically synthesized phospholipids did not alter the ShlA-mediated lysis of LUVs. This raised the hypothesis that membrane fluidity and fatty acid chain saturation may not be involved in efficient binding and pore formation of ShlA in these liposomes. At the physiological pH of 7.4, PS exhibits a negative charge. To determine if the entire structure of the charged PS head group or only the negative charge is essential for pore formation, negatively charged LUVs were measured (*see* Fig. 3). Indeed, the negative charge appeared to be the driving force for ShlA insertion. This may also explain the partial lysis of PG-containing vesicles, as PG is also capable of forming negatively charged liposomes. Positively charged LUVs were also lysed by ShlA, but to a much lesser extent. This may reflect better binding of ShlA to membranes via ionic interactions; however, this remains to be elucidated.

In eukaryotic membranes, PS is located at the cytoplasmic side of the membrane bilayer and is partially enriched in clusters (Ahn & Yun, 1998). By adding PE to the LUVs, an increased clustering of PS was observed, which was shown to be a prerequisite for integration of apocytochrome c into membrane bilayers (Ahn et al., 2000; Ahn & Yun, 1999). In a recent study, the extent of membrane insertion of cytochrome P450 1A2 was strictly dependent on acidic phospholipids as PS or phosphatidic acid (Ahn, Guengerich & Yun, 1998). The membrane integration mechanism of ShlA is believed to be similar. It is proposed that the negative charge of the cytoplasmic side of the eukaryotic membrane is necessary for efficient pore formation by ShlA. Reaching the negatively charged inside of eukaryotic plasma

membranes, ShlA must in fact undergo a conformational change in the transition from the aqueous to the lipid phase. Indeed, ShlA did undergo a conformational change upon transfer from 6 M urea to a urea-free buffer and upon membrane integration, indicated by the blue-shift of the intrinsic Trp-fluorescence maximum from 362 to 352 nm and the increase in emission maximum at 347 nm. Data presented here and elsewhere (Poole, Schiebel & Braun, 1988; Hertle et al., 1997) show that dilution of ShlA (from 6 M urea to 0.3 M urea) led to spontaneous refolding into an active conformation.

Fluorescence intensity was only slightly increased by liposomes without PS. PS-containing liposomes nearly doubled the Trp-fluorescence intensity with ShlA in contrast to inactive ShlA*, indicating the exclusive integration of the active toxin into the membrane bilayer.

It is known that ShlA becomes trypsin-resistant upon membrane integration (Schiebel & Braun, 1989), which suggests that integration involves the entire molecule. The additional emission shift from 354 nm to 347 nm of ShlA with PC/PS LUVs supported this assumption of membrane integration. Integration of ShlA into PS-containing LUVs was shown by the blue shift and the increase of Trp-fluorescence (*see* Fig. 8) and the membrane-specific reduction of fluorescence intensity by an energy transfer (pyrene-PC) probe in the membrane.

Once ShlA is bound to the membrane by hydrophobic interaction, it should integrate into the membrane, undergoing a conformational change. De Kruijff et al., (Rietveld, Koorengevel & de Kruijff, 1995; de Kruijff, 1997) provided a similar model for SecA membrane integration, in which the protein-membrane transition is supported by ionic interaction with charged groups in the membrane. Thus, after a portion of ShlA (specifically, the C-terminus) has reached the cytoplasmic side of the membrane, a stable pore will be established by interaction with the negatively charged PS heads on the cytoplasmic side. It was shown previously that ShlA exposes the C-terminus on the inside of human erythrocyte membranes, contributing to insertion and pore formation (Schiebel & Braun, 1989). Membrane-inserted ShlA was cleavable with trypsin, yielding a C-terminal 19.47 kDa fragment and both ShlA fragments remained membrane-bound. This C-terminal 19.47 kDa oligopeptide has a pI of 9.45 and a positive charge at physiological pH. Hence, this C-terminal domain of ShlA is likely to stabilize the ShlA pore by anchoring the polypeptide via ionic interaction with the negatively charged PS head groups.

In artificial membranes, PS is equally distributed on both sides of the LUVs, and therefore the ShlA pore can be orientated in both directions: one transmembrane structure anchored with its C-terminus at the outside and one towards the inside. The similar partial trypsin cleavage from right-side-out and inside-out LUVs in Fig. 1*B* reflected this possibility. These findings also confirmed that the penetration of PS-containing LUVs by ShlA is similar to the penetration of ShlA in erythrocytes.

In contrast to ShlA, the inactive form ShlA* had a diminished membrane-binding capacity of a factor of 10 and did not show a change in intrinsic tryptophan fluorescence intensity upon membrane binding. ShlA* was also completely digested by trypsin even in the membrane-bound state. This indicates that unlike the active ShlA, the inactive form of ShlA is not capable of undergoing the proposed conformational changes necessary for integration into the membrane.

In conclusion, the evidence shows that pore formation of ShlA is receptor independent in artificial, phosphatidylserine-containing LUVs (negatively charged).

The following model is proposed for ShlA pore formation: ShlA is first targeted to membranes by the presence of non-covalently bound PE molecules (Hertle et al., 1997) and by the conformation imposed by ShlB during secretion. Once in tight contact with the target membrane, in terms of integration, a stable pore will be established by ionic interaction of the positively charged C-terminus of ShlA with the negatively charged PS clusters on the inside of eukaryotic cell membranes. From the data discussed earlier, however, it cannot be excluded that a proteinaceous receptor for ShlA in certain eukaryotic cells exists. The proposed membrane-integration mechanism may be an archaic mechanism by which proteins interact with and integrate into membranes. Specific receptor- binding mechanisms may have evolved later.

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