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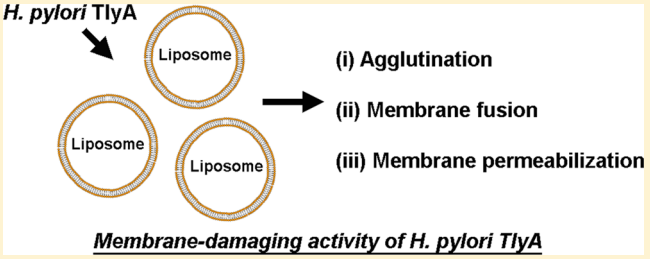
Helicobacter pylori TlyA Agglutinates Liposomes and Induces Fusion and Permeabilization of the Liposome Membranes

Kusum Lata and Kausik Chattopadhyay*

Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Sector 81, SAS Nagar, Manauli 140306, Punjab, India

ABSTRACT: *Helicobacter pylori* TlyA is a pore-forming hemolysin with potent cytotoxic activity. To explore the potential membrane-damaging activity of *H. pylori* TlyA, we have studied its interaction with the synthetic liposome vesicles. In our study, *H. pylori* TlyA shows a prominent ability to associate with the liposome vesicles without displaying an obligatory requirement for any protein receptor on the liposome membranes. Interaction of TlyA triggers agglutination of the liposome vesicles. Such agglutinating activity of TlyA could also be observed with erythrocytes before the induction of its pore-forming hemolytic activity. In addition to its agglutinating activity against liposomes, TlyA also induces fusion and disruption of the liposome membranes. Altogether, our study highlights novel membrane-damaging properties of *H. pylori* TlyA that have not been documented previously with any other TlyA family protein.



Membrane-damaging activity of H. pylori TlyA

Gram-negative bacterium *Helicobacter pylori*[1,2] is a human-specific gastric pathogen that can form colonies in the human stomach to cause inflammation of the gastric mucosa.[3,4] *H. pylori* infection has been shown to be associated with various disorders of the upper gastrointestinal tract,[4] like chronic gastritis, peptic ulcer, gastric mucosa associated lymphoid tissue lymphoma, and gastric cancer.[4−7] The bacterium employs a battery of well-characterized virulence factors[8] that include the "vacuolating cytotoxin" (VacA), "cytotoxin-associated gene A" (CagA),[2,9−12] and the urease.[13] Analysis of the *H. pylori* genome sequencing data,[14,15] however, suggests the existence of additional potential virulence factors that may be implicated in the pathogenesis process of the organism. *H. pylori* has been reported to harbor a gene[14] that encodes a protein with sequence similar to that of the TlyA hemolysins of several pathogenic bacteria, including *Serpulina hyodysenteriae* and *Mycobacterium tuberculosis*.[16−18] Previous studies have indicated that the *tlyA* gene product could potentially contribute to the *in vitro* hemolytic activity of *H. pylori*.[19] Mutation in the *tlyA* gene in *H. pylori* has been shown to compromise the *in vitro* hemolytic activity of the organism. Introduction of the *H. pylori tlyA* gene into the nonhemolytic *Escherichia coli* has also been shown to produce hemolytic activity in the recombinant *E. coli* cells.[19] A more recent study of the purified form of the recombinant *H. pylori* TlyA protein has established its role as a pore-forming hemolysin with potent cytotoxic activity.[20] On the basis of its pore-forming hemolysin/cytotoxin property, TlyA may be considered as a potential virulence factor of *H. pylori*.

In this study, we have explored the membrane-damaging activities of the *H. pylori* TlyA protein by testing its ability to interact with the membrane lipid bilayer of the synthetic lipid vesicles or liposomes. Our study shows that TlyA associates with the membrane lipid bilayer of the liposomes without showing a critical requirement for any protein receptor present on the liposome membranes. Our data further show that the lipid-dependent interaction of TlyA triggers potent agglutination of the liposome vesicles. Such agglutinating activity of TlyA is also observed on the human erythrocytes when they are tested under this condition, before the onset of its pore-forming hemolytic activity. In addition to its agglutinating activity toward the liposomes, TlyA also induces the fusion and subsequent disruption of the liposome membranes. Overall, our study provides new insights regarding the membrane-damaging activities of *H. pylori* TlyA hemolysin.

MATERIALS AND METHODS

Purification of *H. pylori* TlyA. The recombinant form of the *H. pylori* TlyA protein was expressed and purified following the method described previously.[20] Briefly, *E. coli* Origami B cells (Novagen) harboring a recombinant pET14b plasmid containing the nucleotide sequence of *H. pylori* TlyA were induced for protein overexpression with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 30 °C. His-tagged TlyA protein was purified from the bacterial cell lysates by Ni-NTA agarose (QIAGEN) affinity chromatography, followed by cation-exchange chromatography on SP Sepharose (Sigma-Aldrich). Purified protein was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Coomassie staining. The His tag was removed by treatment with thrombin (Sigma-Aldrich) as described previously.[20] Both

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recombinant forms of TlyA, with and without the His tag, were used in this study, as the presence or absence of the His tag did not affect the functionalities of the protein to any noticeable extent. His-tagged proteins, wherever used in the experiments, are specifically mentioned in the subsequent sections of the Materials and Methods.

Experiments with Human Erythrocytes. Experiments using human erythrocytes have been approved by the Bioethics Committee of Indian Institute of Science Education and Research Mohali.

For the qualitative estimation of the hemagglutinating activity[21,22] of *H. pylori* TlyA, human erythrocytes suspended in PBS [20 mM sodium phosphate and 150 mM NaCl (pH 7.0)] (corresponding to an A_{600} of 1.5) were incubated with TlyA in a 100 μL reaction volume in a V-bottom microtiter plate for 1 h at 25 °C. Agglutination of human erythrocytes was monitored visually.[21,22] In the absence of any agglutination, erythrocytes formed a dotlike button at the bottom of the V-shaped well. In the case of positive agglutination, erythrocytes exhibited diffuse and confluent settling. As per the convention,[21,22] the lowest concentration of TlyA showing hemagglutination was taken and reported as the end point. For a positive control of the hemagglutinating activity, we used 40 nM *Vibrio cholerae* cytolysin oligomer.[22]

Agglutinating activity of *H. pylori* TlyA against human erythrocytes was also monitored using flow cytometry. Human erythrocytes suspended in PBS (corresponding to an A_{600} of 1.5) were incubated with TlyA in a 100 μL reaction volume for 2 h at 25 °C. Subsequently, cells were washed with PBS, resuspended in 500 μL of PBS, and analyzed for size [forward scattering (FSC-H)] and complexity [side scattering (SSC-H)] using a FACSCalibur (BD Biosciences) flow cytometer. An increase in the scattering parameters was interpreted as agglutination of the human erythrocytes.[23] Data were analyzed using the FlowJo software (www.flowjo.com).

Experiments with Liposomes. *Preparation of Liposomes.* Asolectin (mixture of soybean phospholipids) and Asolectin/cholesterol liposomes (Asolectin:cholesterol weight ratio of 1:1) with or without trapped calcein were prepared as described previously.[24] Phosphatidylcholine (PC)/cholesterol liposomes (PC:cholesterol weight ratio of 1:1) were also prepared following the method described previously.[24]

Enzyme-Linked Immunosorbent Assay (ELISA). Interactions of His-tagged TlyA with Asolectin and Asolectin/cholesterol liposomes were monitored using an ELISA-based approach using the anti-TlyA antiserum, following the method described previously.[24]

Liposome Pull-Down Assay. Association of His-tagged TlyA with the liposomes was tested by employing a pull-down method. Briefly, His-tagged TlyA (10 μM) was incubated with liposomes (100 μg) in a reaction volume of 500 μL in PBS at 25 °C for 2 h. Subsequently, liposomes were pelleted by ultracentrifugation at 105000*g*, and the pellet was washed with PBS, dissolved in SDS−PAGE sample buffer, and analyzed by SDS−PAGE/Coomassie staining, either after the sample had been boiled or without boiling.

Surface Plasmon Resonance-Based Assay for Monitoring Binding of TlyA with the Membrane Lipid Bilayer of Liposomes. Binding of *H. pylori* TlyA with Asolectin/cholesterol liposomes was analyzed by the surface plasmon resonance (SPR)-based assay. SPR measurements were performed on a BIAcore 3000 platform (GE Healthcare Life Sciences) using L1 sensor chips (GE Healthcare Life Sciences). L1 sensor chips were prepared with the Asolectin/cholesterol liposomes following the method described in ref 25. Briefly, the L1 sensor chip flow cell was injected with 10 μL of Asolectin/cholesterol liposomes (0.5 mM) at a flow rate of 1 μL/min. The flow cell was then injected with 20 μL of 20 mM NaOH at a flow rate of 100 μL/min, followed by an injection with 30 μL of BSA (0.1 mg/mL) at a flow rate of 10 μL/min. The L1 sensor chip flow cell was then washed with running buffer [10 mM HEPES buffer containing 150 mM NaCl (pH 8.0)]. Binding of TlyA with Asolectin/cholesterol membranes was studied with three concentrations (12.5, 25, and 50 nM) of the protein. For each concentration, 25 μL of TlyA in running buffer was injected into the flow cell at a flow rate of 5 μL/min. Between the injections at each concentration, the flow cell was washed with 20 μL of 20 mM NaOH at a flow rate of 100 μL/min.

Assay of Agglutination of Liposomes. TlyA-mediated agglutination of Asolectin, Asolectin/cholesterol, and PC/cholesterol liposomes was monitored as described in ref 22. Briefly, the liposome suspension was adjusted to a turbidity with an A_{500} of ∼0.25 in PBS and incubated in the presence of TlyA at 25 °C. Agglutination of liposomes upon TlyA treatment was monitored by calculating the relative change in turbidity, with respect to the blank containing a liposome suspension without TlyA. The A_{500} values of the liposome suspension were recorded spectrophotometrically. The TlyA protein solution alone did not contribute any turbidity in terms of the A_{500} value over the protein concentration ranges employed in this assay. Relative changes in turbidity of the liposome suspension were calculated using the expression (A_{500} of TlyA-treated liposome suspension at any time point − A_{500} of liposome suspension without TlyA)/A_{500} of liposome suspension without TlyA.

Fluorescence Resonance Energy Transfer (FRET)-Based Assay for Monitoring the Fusion of the Liposome Membranes. TlyA-mediated membrane fusion was monitored by a fluorescence resonance energy transfer (FRET)-based lipid mixing assay using liposomes labeled with NBD-PE and Rhodamine-PE.[26−28] Asolectin/cholesterol liposomes containing 0.5% (w/w) NBD-PE [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Molecular Probes)] and 0.5% (w/w) Rhodamine-PE [Lissamine Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Molecular Probes)] were prepared. Labeled and unlabeled liposomes at a ratio of 1:10 (w/w) were incubated in the presence of TlyA in a reaction volume of 2 mL in PBS at 25 °C. The total lipid concentration in the reaction mixture was adjusted to ∼175 μg/mL. Fluorescence measurements were taken on a PerkinElmer LS 55 spectrofluorimeter. NBD and Rhodamine fluorescence emission were recorded at 536 and 590 nm, respectively, upon excitation of the NBD fluorophore at 463 nm. Excitation and emission slit widths were kept at 5 nm. Relative changes in fluorescence at any time point (t) with respect to the intensity at the initial time point (t_o) were calculated using the expression (fluorescence intensity at time t − fluorescence intensity at time t_o)/fluorescence intensity at time t_o. The maximal extent of lipid mixing was induced by treatment with 0.01% Triton X-100.

FRET-Based Assay for Monitoring the Fusion of the Liposome Membranes with Human Erythrocyte Ghosts. TlyA-induced fusion of the liposome membranes with human

erythrocyte ghosts was also estimated by the FRET-based lipid mixing assay.

Human erythrocyte ghosts were prepared as described previously.[24] Briefly, human erythrocytes were washed five times with PBS, and cells were lysed by incubation in the presence of 5 mM sodium phosphate buffer (pH 7.5) containing 1 mM $MgSO_4$ and pelleted by ultracentrifugation at 105000*g* for 20 min. Pellets were washed extensively with 5 mM sodium phosphate buffer (pH 7.5) containing 1 mM $MgCl_2$. Erythrocyte ghosts were resuspended in 5 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. The protein content of the erythrocyte ghost preparation was estimated by a Bradford assay.[29]

Asolectin/cholesterol liposomes (30 μg/mL) labeled with 0.5% (w/w) NBD-PE and 0.5% (w/w) Rhodamine-PE were incubated with unlabeled human erythrocyte ghosts (corresponding to the protein content of 800 μg) in the presence of His-tagged TlyA in a 2 mL reaction volume in PBS at 25 °C. Fluorescence measurements were recorded on a PerkinElmer LS 55 spectrofluorimeter. Rhodamine fluorescence emissions were recorded at 590 nm upon excitation of the NBD fluorophore at 463 nm, with excitation and emission slit widths of 5 nm. Relative changes in fluorescence at any time point (t) with respect to the intensity at the initial time point (t_o) were calculated using the expression (fluorescence intensity at time t − fluorescence intensity at time t_o)/fluorescence intensity at time t_o. The maximal extent of lipid mixing was induced by treatment with 0.01% Triton X-100.

Dequenching of the Rhodamine-PE Fluorescence for Monitoring Membrane Fusion. Lipid mixing between liposome membranes was monitored by estimating the release of self-quenching of Rhodamine fluorescence in the liposomes labeled with Rhodamine-PE.[30] Asolectin/cholesterol and PC/cholesterol liposomes were labeled with 10% (w/w) Rhodamine-PE at a self-quenching concentration.[30] To monitor the fusion of the liposome membranes, labeled and unlabeled liposomes were mixed at a ratio of 1:10 (w/w) and incubated in the presence of His-tagged TlyA in 2 mL of PBS at 25 °C. The lipid concentration in the reaction mixture was adjusted to 175 μg/mL. For monitoring fusion of the liposome membranes with human erythrocyte ghosts, asolectin/cholesterol liposomes (15 μg/mL) labeled with Rhodamine-PE [10% (w/w)] were incubated with the unlabeled ghost (800 μg of protein) in the presence of His-tagged TlyA in a reaction volume of 2 mL in PBS at 25 °C. Fluorescence measurements were taken on a PerkinElmer LS 55 spectrofluorimeter. Rhodamine fluorescence was recorded at 590 nm upon excitation at 560 nm. Relative changes in fluorescence at any time point (t) with respect to the intensity at initial time point (t_o) were calculated using the expression (fluorescence intensity at time t − fluorescence intensity at time t_o)/fluorescence intensity at time t_o.

Content Mixing and Leakage of Liposomes. Asolectin and asolectin/cholesterol liposomes containing encapsulated ANTS [8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (Molecular Probes)] and DPX [*p*-xylylene bis(pyridinium) bromide (Molecular Probes)] were prepared following the method described previously.[31] Liposomes were prepared with the following compositions of ANTS and DPX: (1) 25 mM ANTS and 40 mM NaCl, (2) 90 mM DPX alone, and (3) 12.5 mM ANTS, 45 mM DPX, and 20 mM NaCl. All ANTS/DPX solutions were prepared in 10 mM Tris-HCl buffer (pH 7.5). Free ANTS and DPX were removed by extensive washing of the liposomes by ultracentrifugation at 105000*g*, using 10 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.5).

For the content mixing assay,[27,31,32] ANTS-containing liposomes were mixed with DPX-containing liposomes (1:1 weight ratio of liposomes) in the presence of TlyA in a reaction volume of 2 mL in 10 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.5) at 25 °C. Total lipid concentrations of the liposomes were adjusted to 100 μg/mL in the final reaction mixture. The ANTS fluorescence was monitored at 520 nm upon excitation at 350 nm, with excitation and emission slit widths of 5 nm. Relative changes in fluorescence at any time point (t) with respect to the intensity at the initial time point (t_o) were calculated using the expression (fluorescence intensity at time t − fluorescence intensity at time t_o)/fluorescence intensity at time t_o.

For the liposome leakage assay,[27,31,32] ANTS/DPX co-encapsulated liposomes were incubated with TlyA in a 2 mL reaction volume in 10 mM Tris-HCl buffer containing 100 mM NaCl (pH 7.5). Total lipid concentrations of the liposomes were adjusted to 50 μg/mL in the final reaction mixture. All the fluorescence measurements were taken on a PerkinElmer LS 55 spectrofluorimeter. The ANTS fluorescence was monitored at 520 nm upon excitation at 350 nm, with excitation and emission slit widths of 2.5 and 5 nm, respectively; 100% liposome leakage was induced upon treatment with 0.01% Triton X-100.

Calcein Release Assay. TlyA-induced permeabilization of the liposome membranes was quantitatively estimated by monitoring the release of calcein from the liposomes.[24] His-tagged TlyA was incubated with 20 μg of liposomes containing trapped calcein in a reaction volume of 500 μL in 20 mM HEPES buffer (pH 8.0) containing 150 mM sodium chloride for 2 h at 25 °C. Subsequently, the reaction mixture was diluted to 2 mL with the same buffer, and the calcein fluorescence was monitored using a Fluoromax-4 (Horiba Scientific, Edison, NJ) spectrofluorimeter equipped with a Peltier-based temperature controller, using a cuvette with a path length of 1 cm. The calcein fluorescence was monitored at 520 nm, upon excitation at 488 nm, with excitation and emission slit widths of 0.25 and 5 nm, respectively. Fluorescence data obtained from the sample treated with 6 mM sodium deoxycholate corresponded to the 100% calcein release, whereas the sample without TlyA treatment was taken as the negative control.

Fluorescence Microscopy. Lipid mixing of the liposome membranes with the human erythrocyte ghosts was also monitored by fluorescence microscopy. Human erythrocyte ghosts (corresponding to 80 μg of protein) were mixed with Rhodamine-PE-labeled [10% (w/w)] asolectin/cholesterol liposomes in the presence of 10 μM His-tagged TlyA in a reaction volume of 100 μL in PBS at 25 °C for 15 min. Liposomes were adjusted to a concentration of 60 μg/mL in the final reaction mixture. Twenty microliters of the reaction mixture was placed on a clean glass slide and covered with a coverslip. The coverslip was sealed on all sides with transparent nail polish. Slides were examined by phase contrast and fluorescence microscopy on an EVOS FL Imaging System (Life Technologies).

Far-UV Circular Dichroism (CD) Spectroscopy. Far-UV CD spectroscopy was used to assess the interaction of TlyA with the asolectin/cholesterol liposomes. For this, TlyA (in the protein concentration range of 2 μM) in 1 mM Tris-HCl buffer (pH 8.0) was incubated with asolectin/cholesterol liposomes (20 μg) in a reaction volume of 1 mL at 25 °C, and the far-UV

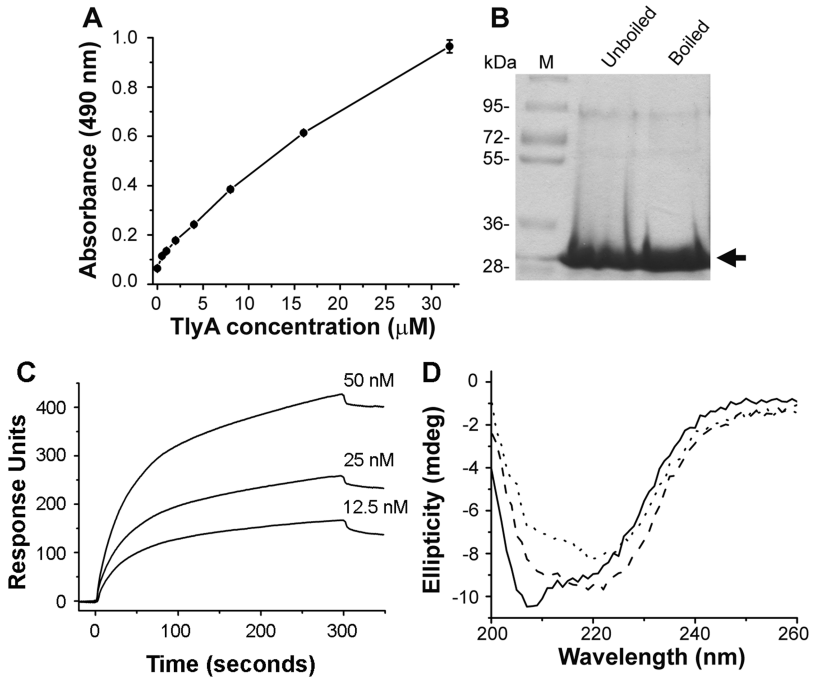

Figure 1. (A) Interaction of TlyA with asolectin/cholesterol liposomes (●), as monitored by the ELISA-based method. Wells of a 96-well microtiter plate were coated with an asolectin/cholesterol liposome suspension (10 μg/mL), and binding of His-tagged TlyA was estimated by an ELISA-based approach using the anti-TlyA antiserum. Each datum represents the average ± the standard deviation, determined from three independent readings. (B) Association of TlyA (10 μM) with asolectin/cholesterol liposomes (200 μg/mL), as monitored by the pull-down-based method coupled with SDS−PAGE and Coomassie staining. Protein standards are shown in lane M. (C) Binding of TlyA with the asolectin/cholesterol membrane lipid bilayer as determined by the SPR-based assay. The SPR sensor chip was coated with asolectin/cholesterol liposomes by injecting 10 μL of the liposome suspension (0.5 mM) at a flow rate of 1 μL/min. Binding sensorgrams are shown for three different concentrations of the protein as indicated. (D) Far-UV CD spectra (200−260 nm) of TlyA in the presence of asolectin/cholesterol liposomes (20 μg/mL): (—) TlyA in the absence of liposomes, (– – –) TlyA in the presence of liposomes at 1 min, and (···) TlyA in the presence of liposomes at 10 min.

CD spectra were recorded on a Chiracsan spectropolarimeter (Applied Photophysics, Leatherhead, U.K.) equipped with a Peltier-based temperature controller, using a quartz cuvette with a path length of 5 mm. Each spectrum was corrected for the baseline by subtracting the appropriate buffer spectrum containing the liposome alone.

RESULTS AND DISCUSSION

TlyA Associates with the Membrane Lipid Bilayer of Asolectin/Cholesterol Liposomes. We tested interaction of the *H. pylori* TlyA protein with the synthetic lipid vesicles or liposomes constituted from asolectin and cholesterol. First, we monitored the binding of TlyA with the asolectin/cholesterol liposomes using an ELISA-based method. The protein displayed significant concentration-dependent interaction with the asolectin/cholesterol liposomes, when tested over a broad protein concentration range of 0.5−30 μM (Figure 1A). It is important to note that even at a protein concentration of 30 μM TlyA−liposome association did no approach saturation. Binding with liposomes, however, was not tested beyond this protein concentration range in the ELISA-based assay. A pull-down-based assay showed that TlyA cosedimented with the asolectin/cholesterol liposomes, thus suggesting a prominent association of the protein with the membrane lipid bilayer of the liposome vesicles (Figure 1B). We further confirmed the binding of TlyA with the asolectin/cholesterol liposomes using an SPR-based assay. In this assay, TlyA exhibited prominent interaction with the membrane lipid bilayer of the asolectin/cholesterol liposomes even over a lower concentration range of 12.5−50 nM (Figure 1C).

We also examined the interaction of purified TlyA with the asolectin/cholesterol liposomes by monitoring the far-UV CD spectra in the wavelength region of 200−260 nm. Incubation of the purified TlyA protein with the asolectin/cholesterol liposomes resulted in significant changes in the far-UV CD spectra; there was a steady decrease in the magnitudes of the overall ellipticity signals (negative ellipticity values of the far-UV CD signal of TlyA were found to decrease in the presence of the liposomes), with a considerable shift in the wavelength of the negative ellipticity minima (Figure 1D). A change in the far-UV CD profile was observed at a time point as early as 1 min (Figure 1D, dashed line), while the process was complete within 10 min of incubation (Figure 1D, dotted line). Such changes in the far-UV CD spectra could be interpreted as the result of alterations in the secondary structural organization in the TlyA protein. Alternatively, the overall distortion of the CD profile could be the result of scattering interference caused by the changes in the liposome structure upon TlyA treatment. With an appreciation of this possibility, more rigorous analysis of the far-UV CD spectra with respect to exploring the structural changes in the TlyA protein in the presence of liposomes was not attempted in this study. Therefore, the data obtained from the far-UV CD spectroscopy experiments conducted with TlyA in the presence of liposomes should be interpreted only for qualitative purposes to assess the TlyA−liposome interaction.

Altogether, these data suggested that the *H. pylori* TlyA protein was potentially capable of associating with the membrane lipid bilayer of the asolectin/cholesterol liposomes, without requiring the stringent involvement of any nonlipid

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membrane component. It is, however, not possible at present to rule out the potential existence of any non-lipid receptor-like molecule, which may act to facilitate interaction of TlyA with the biomembranes. More studies would be required to address this issue in more mechanistic detail.

TlyA Shows Potent Agglutination Activity against Asolectin/Cholesterol Liposomes, a Property That Also Can Be Mimicked against Human Erythrocytes. To explore the effect of association of TlyA with the liposome membranes, we observed that the *H. pylori* TlyA protein could cause clumping or agglutination of the asolectin/cholesterol liposomes. Treatment of the purified TlyA protein, over a concentration range of 0.5−3 μM, with a suspension of asolectin/cholesterol liposomes caused a marked increase in turbidity, suggesting TlyA-induced agglutination of the liposome vesicles (Figure 2A). The data clearly suggested that the purified form of *H. pylori* TlyA could display prominent agglutinating activity against the synthetic liposome system. Also, it appeared that the agglutinating efficacy of TlyA did not critically require any protein receptor on the liposome membranes.

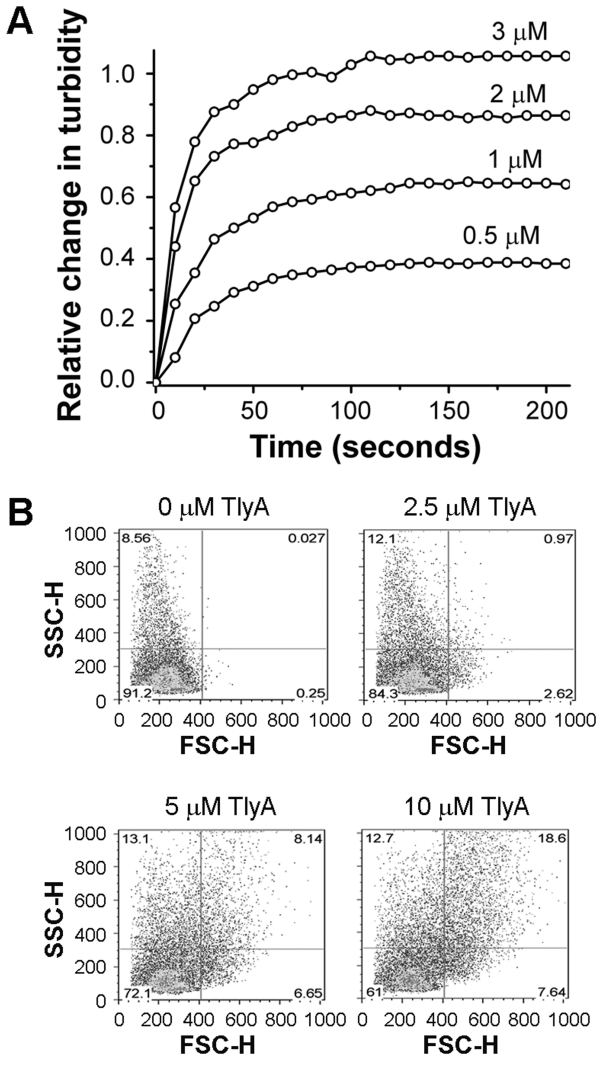

Figure 2. (A) Agglutination of asolectin/cholesterol liposomes (turbidity adjusted to an A_{500} of ~0.25) by TlyA at different protein concentrations. The TlyA protein concentrations are indicated. (B) Flow cytometry-based detection of erythrocyte agglutination mediated by *H. pylori* TlyA. TlyA-treated human erythrocytes were analyzed for an increase in cell size [forward scattering (FSC-H)] and complexity [side scattering (SSC-H)], in comparison to those of the untreated cells.

H. pylori TlyA has been characterized previously as a pore-forming hemolysin.[19,20] The purified form of the recombinant TlyA protein has been shown to cause lysis of human erythrocytes.[20] Profound lysis of erythrocytes has been observed over a period of ~18 h at 25 °C, when tested with protein concentrations in the range of 2.5−20 μM.[20] Now, on the basis of the potent agglutinating activity of TlyA against liposome vesicles, we wanted to test whether such agglutinating activity could also be observed on human erythrocytes. Indeed, purified TlyA was found to display potent concentration-dependent hemagglutinating activity against human erythrocytes. In a microplate-based visual test of hemagglutination, purified TlyA showed hemagglutinating activity at a protein concentration as low as 2.5 μM, upon incubation for 1 h at 25 °C. We also employed a flow cytometry-based assay to monitor agglutination of human erythrocytes upon treatment with TlyA. In this assay, the size and complexities of the TlyA-treated human erythrocytes were analyzed by monitoring the forward scattering and side scattering parameters. An increase in the scattering parameters upon TlyA treatment could be interpreted as being the result of erythrocyte agglutination.[23] Data obtained from this assay clearly showed that the purified form of TlyA, over a protein concentration range of 2.5−10 μM, could display strong agglutinating activity against human erythrocytes, when treated for 2 h at 25 °C (Figure 2B). Consistent with the previous report, prolonged incubation (~18 h or more) of human erythrocytes in the presence of TlyA could induce lysis of the cells (data not shown). Overall, these data established that the TlyA protein could display potent hemagglutinating activity against the human erythrocyte cells. It also appeared that such hemagglutinating activity of TlyA could be documented before the onset of the pore-forming hemolytic activity of the protein.

TlyA Induces Fusion of the Asolectin/Cholesterol Liposome Membranes. After establishing the liposome agglutinating activity of *H. pylori* TlyA, we explored the possibility of whether interaction of the protein with asolectin/cholesterol liposomes could trigger fusion of the liposome membranes. We used a FRET-based lipid mixing assay to monitor the ability of TlyA to induce liposome membrane fusion.[26−28] In this assay, asolectin/cholesterol liposomes labeled with NBD-PE and Rhodamine-PE were mixed with unlabeled liposomes in the presence of TlyA (at a protein concentration of 5 μM). Under such experimental conditions, fusion of the labeled and unlabeled liposome membranes would lead to spatial separation of the donor (NBD) and the acceptor (Rhodamine) fluorophores, which, in turn, would result in a FRET signal with a decreased magnitude (rhodamine emission at 590 nm upon excitation of NBD at 463 nm). Consistent with such a notion, liposome mixing in the presence of the TlyA protein showed a time-dependent decrease in the acceptor (Rhodamine) fluorescence and a dequenching of the donor (NBD) fluorescence (Figure 3A). When analyzed over a period of 10 min, the relative decrease in Rhodamine fluorescence upon TlyA treatment (5 μM) was found to be ~25% of that observed in the case of maximal lipid mixing induced by Triton X-100 treatment (data not shown). These data, therefore, indicated that TlyA was capable of inducing a significant extent

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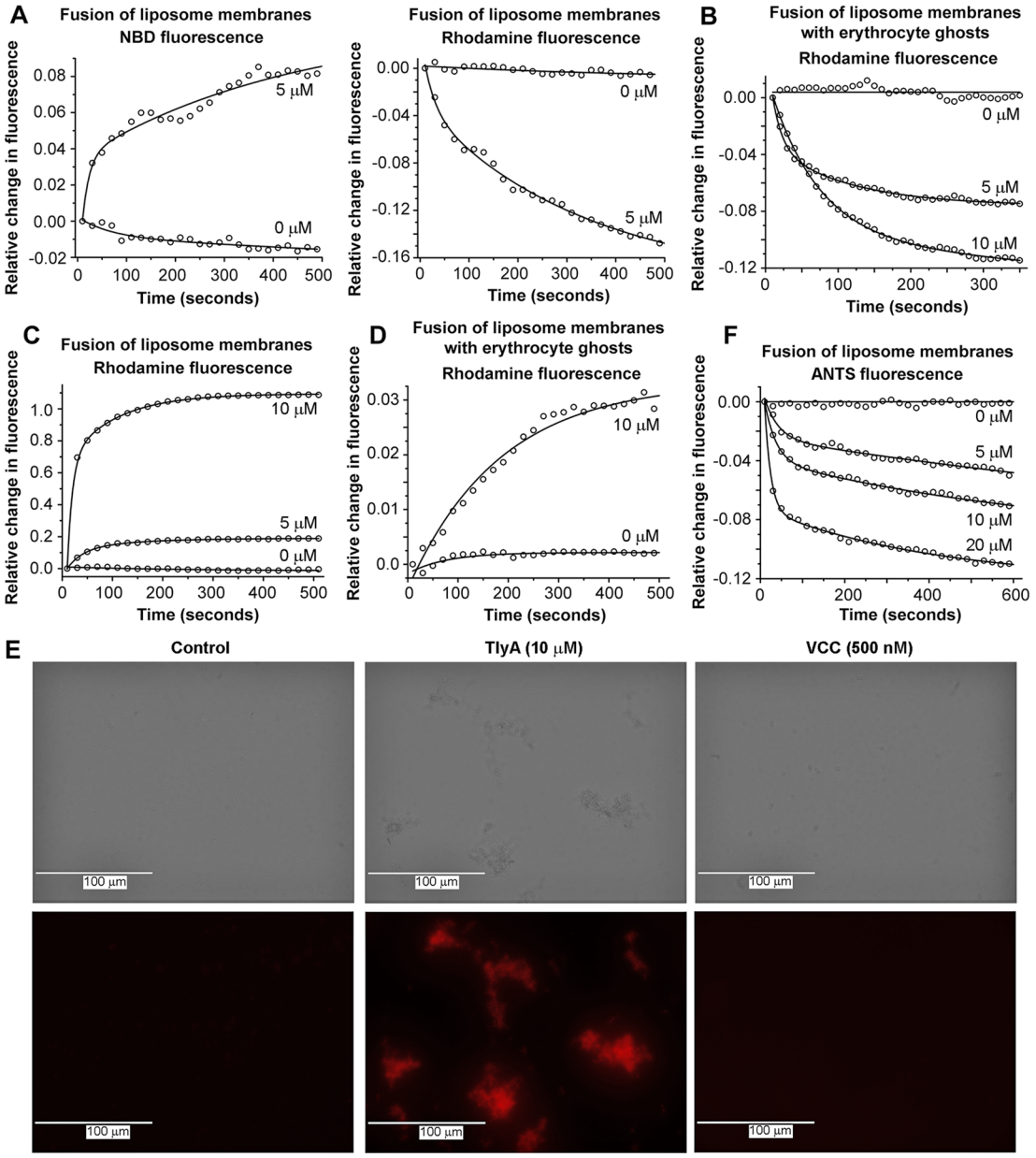

Figure 3. (A) TlyA-mediated fusion of asolectin/cholesterol liposome membranes as monitored by the FRET-based lipid mixing assay. Liposomes containing NBD-PE and Rhodamine-PE were mixed with unlabeled liposomes in presence or absence of 5 μM TlyA, and the changes in the NBD and Rhodamine fluorescence were monitored, upon excitation of NBD. Labeled and unlabeled liposomes were mixed at a ratio of 1:10 (w/w), and the total lipid concentration in the reaction mixture was kept at ~175 μg/mL. TlyA-mediated fusion of the labeled and unlabeled liposome membranes caused physical separation of the NBD and Rhodamine fluorophores, thus resulting in the dequenching of NBD fluorescence (left) and a decrease in Rhodamine fluorescence (right). (B) TlyA-induced fusion of the asolectin/cholesterol liposome membranes with human erythrocyte ghosts monitored by the FRET-based lipid mixing assay as described for panel A. Liposomes (30 μg/mL) containing NBD-PE and Rhodamine-PE were mixed with unlabeled ghosts (corresponding to a protein content of 400 μg/mL) in the presence (5 and 10 μM) or absence of TlyA. Mixing of the membrane lipids resulted in a decrease in Rhodamine fluorescence. (C) Fusion of asolectin/cholesterol liposomes in the presence of TlyA was monitored by recording the dequenching of Rhodamine fluorescence upon lipid mixing. Liposomes containing a self-quenching concentration of Rhodamine-PE were incubated with an excess of unlabeled liposomes in the presence of TlyA. Labeled and unlabeled liposomes were mixed at a ratio of 1:10 (w/w), and the total lipid concentration in the reaction mixture was kept at ~175 μg/mL. Dequencing of Rhodamine fluorescence was observed upon lipid mixing due to the fusion of the liposome membranes in the presence of TlyA. (D) TlyA-mediated fusion of asolectin/cholesterol (15 μg/mL) liposome membranes with human erythrocyte ghosts (corresponding to a protein content of 400 μg/mL) was monitored by documenting the dequenching of Rhodamine fluorescence upon lipid mixing as described for panel C. (E) Fluorescence imaging showing dequenching of Rhodamine fluorescence upon TlyA-induced lipid mixing between asolectin/cholesterol liposomes (60 μg/mL) containing a self-quenching concentration of Rhodamine-PE and human erythrocyte ghosts (corresponding to a protein content of 800 μg/mL) (middle panel in the bottom row). No such effect was observed in a control experiment with either PBS or VCC treatment (left or right panel of the bottom row, respectively). Phase contrast images are shown in the top row. (F) TlyA-mediated fusion of the asolectin/cholesterol liposomes monitored by the liposome content mixing assay. Asolectin/cholesterol liposomes (50 μg/mL) encapsulating ANTS were incubated with the liposomes (50 μg/mL) containing quencher DPX in the presence of varying concentrations of TlyA. Fusion of liposomes was monitored by recording the quenching of ANTS fluorescence by DPX, upon mixing of the liposomes contents in response to TlyA.

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of fusion of the asolectin/cholesterol liposome membrane lipids.

We also wanted to explore whether such membrane fusion activity of TlyA could be observed in the context of biological membrane systems. We used the FRET-based lipid mixing assay to test whether TlyA could induce fusion of the liposome membranes with the human erythrocyte ghosts.[27] For this, asolectin/cholesterol liposomes, labeled with NBD-PE and Rhodamine-PE, were mixed with unlabeled human erythrocyte ghosts in the presence of TlyA. The time-dependent decrease in Rhodamine fluorescence was observed upon TlyA treatment (Figure 3B), suggesting separation of Rhodamine-PE (acceptor fluorophore for the FRET) from NBD-PE (donor fluorophore for the FRET). Consistent with the notion of the FRET-based lipid mixing assay, such a result could be interpreted as the outcome of the mixing of the liposome membrane lipids with those of the erythrocyte ghost membranes, in response to the TlyA treatment. The extent of lipid mixing activity was found to be dependent on the toxin concentrations, as 10 μM TlyA showed more pronounced membrane fusion than 5 μM protein treatments (Figure 3B). Our data showed that 10 μM TlyA induced ~30% membrane fusion activity while 5 μM TlyA triggered ~20% activity over a period of 5 min, as compared to the maximal lipid mixing observed upon Triton X-100 treatment (data not shown).

We further confirmed the membrane fusion activity of TlyA by monitoring the dequenching of the Rhodamine fluorescence upon mixing of the labeled liposomes with unlabeled liposomes or erythrocyte ghosts.[30] Asolectin/cholesterol liposomes containing self-quenching concentrations of Rhodamine-PE were mixed with an excess of unlabeled liposomes or human erythrocyte ghosts in the presence of TlyA, and time-dependent dequenching of the Rhodamine fluorescence was observed as a signature of the membrane fusion activity. The Rhodamine fluorescence dequenching profile confirmed that TlyA, at protein concentrations of 5 and 10 μM, induced fusion of the liposome membranes to a significant extent (Figure 3C). Over a period of 5 min, 10 μM TlyA induced ~30% of the maximal response generated by Triton X-100 treatment (data not shown). A similar trend was also observed in the case of TlyA-mediated fusion of the asolectin/cholesterol liposomes with human erythrocyte ghosts (Figure 3D).

Dequenching of the Rhodamine fluorescence upon TlyA-induced fusion and mixing of the liposome membrane lipids with human erythrocyte ghosts was also visualized by fluorescence microscopy. Asolectin/cholesterol liposomes containing a self-quenching concentration of Rhodamine-PE were incubated with unlabeled human erythrocyte ghosts in the presence of TlyA (10 μM). Consistent with the agglutinating activity of TlyA, clustered aggregates of erythrocyte ghosts were observed in the phase contrast image (Figure 3E). Fluorescence imaging showed a significant extent of dequenching of the Rhodamine fluorescence within the clustered ghosts (Figure 3E). This was presumably the result of mixing and dilution of the Rhodamine-PE from the liposome membranes into the erythrocyte ghosts. In contrast, no such fluorescence dequenching was noticed in the PBS-treated samples used as controls. We also tested the specificity of TlyA in terms of triggering such a lipid mixing effect as observed in this assay. For this, we explored whether another potent membrane-damaging cytotoxic protein, *V. cholerae* cytolysin (VCC), could induce similar dequenching of Rhodamine fluorescence in the lipid mixing assay. VCC has been shown to exhibit potent membrane-damaging pore-forming activity against biomembranes as well as liposomes in the concentration range of 0.1−1 μM.[24] VCC, at a concentration of 500 nM, did not exhibit any lipid mixing or membrane fusion activity, under experimental conditions similar to those used for TlyA. These results, therefore, suggested the specificity of the TlyA-mediated lipid mixing activity observed in our study.

The membrane fusion activity of TlyA was further established in an assay that monitored mixing of the inner contents of the liposome vesicles[31,32] in the presence of the TlyA protein. Liposomes containing an ANTS fluorophore were incubated with the liposomes containing the quencher DPX in the presence of various concentrations of TlyA. Under such conditions, fusion of the liposomes would be expected to allow mixing of their inner contents leading toward quenching of the ANTS fluorescence. Indeed, TlyA was found to trigger time-dependent quenching of the ANTS fluorescence in a dose-dependent manner in this content mixing assay (Figure 3F). When monitored over a period of 10 min, TlyA (at concentrations of 5, 10, and 20 μM) could induce prominent mixing of the liposome contents, thus establishing the membrane fusion property of the protein.

Altogether, the data obtained from our study clearly suggested that TlyA not only resulted in agglutination of the asolectin/cholesterol liposomes but also induced fusion of the liposome membranes. Our data also suggested that TlyA was also capable of triggering fusion of the liposome membranes with that of the human erythrocyte ghosts. It is also important to note here that the membrane fusion property of TlyA did not require the presence of any obligatory protein receptor(s) on the target membrane, as the protein could act upon synthetic lipid vesicles and/or liposomes composed only of lipids. This observation once again emphasizes the notion that the interaction of TlyA with the membrane lipids alone was sufficient for exerting its membrane-damaging functionalities described in this study.

TlyA Induces Permeabilization of the Asolectin/Cholesterol Liposome Membranes. *H. pylori* TlyA has been characterized as a hemolysin protein. The hemolytic activity of TlyA against human erythrocytes has been shown to be inhibited in the presence of osmoprotectants of definite sizes.[20] This observation indicates that the hemolytic activity of TlyA is presumably the result of the pore-forming property of the protein in the target cell membranes. In this direction, we wanted to explore whether TlyA, in addition to its agglutinating activity and membrane fusion property, could also display membrane permeabilization effects against the liposome vesicles.

We monitored the membrane permeabilization efficacy of *H. pylori* TlyA against the asolectin/cholesterol liposomes. TlyA-induced permeabilization of the liposome membranes was quantitatively estimated by monitoring the release of calcein from the asolectin/cholesterol liposomes.[24] It was found that the TlyA protein could trigger a considerable extent of membrane disruption of the asolectin/cholesterol liposomes when they were treated for 2 h at 25 °C (Figure 4A). At a protein concentration of 5 μM, TlyA induced ~25% liposome permeabilization, whereas 10 μM TlyA resulted in ~60% liposome permeabilization.

TlyA-mediated permeabilization of asolectin/cholesterol liposome membranes was also estimated by monitoring leakage of the ANTS−DPX fluorophore−quencher pair from within the liposomes.[31,32] Asolectin/cholesterol liposomes containing

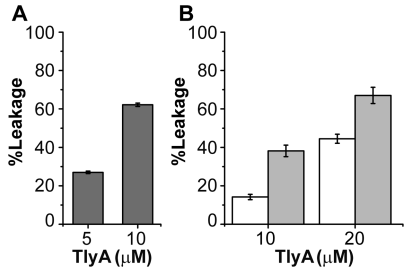

Figure 4. (A) Permeabilization of the asolectin/cholesterol liposome membranes (40 μg/mL) by TlyA, as estimated by the calcein release assay for a period of 2 h. Each datum is the average ± the standard deviation, determined from three independent readings. (B) TlyA-mediated permeabilization of asolectin/cholesterol liposomes (50 μg/mL), as monitored by estimating the leakage of the ANTS−DPX fluorophore−quencher pair. Leakage of the ANTS−DPX fluorophore−quencher pair from within the liposomes resulted in dequenching of ANTS fluorescence: (white bars) incubation for 2 h and (gray bars) incubation for 4 h. Each datum is the average ± the standard deviation, determined from three independent readings.

both ANTS and DPX were incubated in the presence of TlyA. Permeabilization of the liposome membranes would allow leakage of ANTS and DPX, and their dilution would result in the dequenching of the ANTS fluorescence. The results of this assay showed that TlyA induced significant permeabilization of the liposomes when they were treated at protein concentrations of 10 and 20 μM, over a time period of 2−4 h at 25 °C (Figure 4B). TlyA at 10 μM induced nominal, ∼15% leakage at 2 h, while ∼45% activity was observed at 4 h. At a concentration of 20 μM, ∼40 and ∼65% leakage of liposomes was documented over periods of 2 and 4 h, respectively. Overall, these data clearly showed that the purified form of *H. pylori* TlyA displayed prominent membrane permeabilization against the synthetic asolectin/cholesterol liposome vesicles. Also, it appeared that the membrane permeabilization efficacy of TlyA did not critically require any protein receptor on the liposome membranes.

TlyA-Mediated Agglutination, Membrane Fusion, and Permeabilization of the Liposomes Do Not Critically Require the Presence of Cholesterol in the Liposome Membranes. In our study described thus far, we tested the functionalities of TlyA against the liposomes constituted from a mixture of asolectin and cholesterol. Cholesterol was included in the liposome membranes to simulate the physicochemical properties of the lipid bilayers of the biomembranes. However, we explored whether the presence of cholesterol in the membrane lipid bilayer of the asolectin/cholesterol liposomes was critical for the agglutinating activity, membrane fusion, and permeabilization properties of TlyA. ELISA-based and pull-down-based assays showed that TlyA associated efficiently with the liposomes constituted from the asolectin alone (Figure 5A,B). Moreover, binding of TlyA triggered agglutination of the asolectin liposomes, along with fusion and subsequent permeabilization of the asolectin liposome membranes (Figure 5C−E). These data clearly suggested that the activities of TlyA

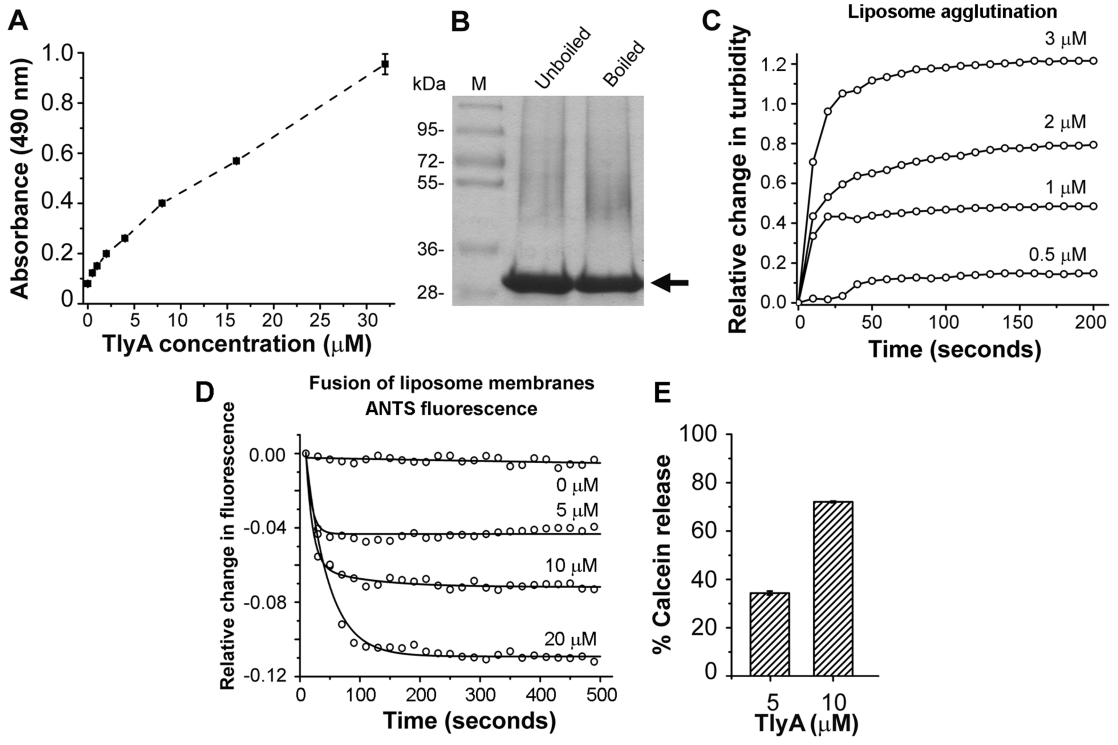

Figure 5. (A) Interaction of TlyA with the asolectin liposomes (■) monitored by the ELISA-based method as described in the legend of Figure 1A. (B) Association of TlyA (10 μM) with the asolectin liposomes (200 μg/mL), as monitored by the pull-down-based method coupled with SDS−PAGE and Coomassie staining. Protein standards are shown in lane M. (C) Agglutination of the asolectin liposomes (turbidity adjusted to an A_{500} of ∼0.25) by TlyA at different protein concentrations. (D) TlyA-mediated fusion of the asolectin liposomes monitored by the liposome content mixing assay. Asolectin liposomes (50 μg/mL) containing ANTS were incubated with the liposomes (50 μg/mL) encapsulating the quencher DPX in the presence of TlyA. Fusion of the liposomes was monitored by recording the quenching of the ANTS fluorescence by DPX, upon mixing of the liposome contents in response to TlyA. (E) Permeabilization of the asolectin liposome membranes (40 μg/mL) by TlyA estimated by the calcein release assay over a period of 2 h. Each datum is the average ± the standard deviation, determined from three independent readings.

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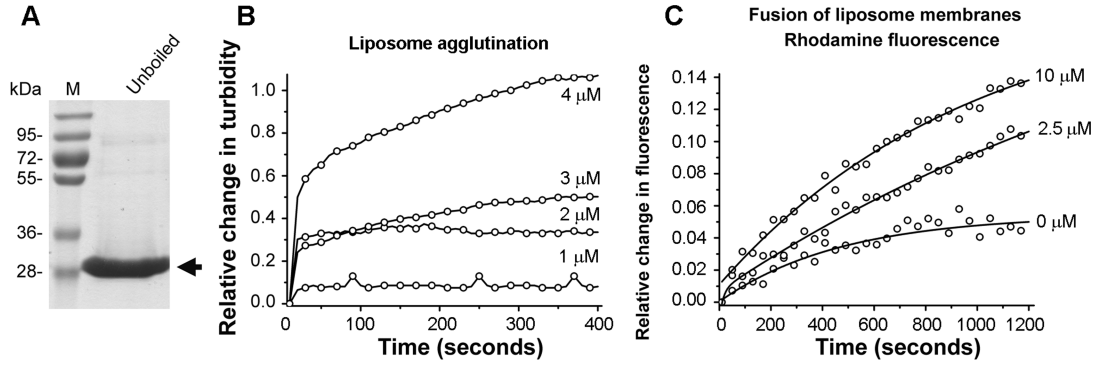

Figure 6. (A) Association of TlyA (10 μM) with the PC/cholesterol liposomes (200 μg/mL), as monitored by the pull-down-based method coupled with SDS−PAGE and Coomassie staining. Protein standards are shown in lane M. (B) Agglutination of the PC/cholesterol liposomes (turbidity adjusted to an A_{500} of ∼0.25) by TlyA at different protein concentrations. (C) Fusion of the PC/cholesterol liposomes in the presence of TlyA was monitored by recording the dequenching of the Rhodamine fluorescence upon lipid mixing, as described in the legend of Figure 3C.

did not critically depend on the obligatory presence of cholesterol in the membrane lipid bilayer of the asolectin liposomes.

TlyA Causes Agglutination and Membrane Fusion of the PC/Cholesterol Liposomes. We also wanted to explore whether the effects of TlyA on the asolectin and asolectin/cholesterol liposomes depended critically on the presence of any distinct class of lipid components present in the soybean phospholipid mixture of the asolectin. In particular, we tested the role(s) of the anionic phospholipids present in the asolectin, as these anionic phospholipids may contribute to facilitated protein−lipid association presumably via the electrostatic interactions. To test this possibility, we monitored some of the functionalities of TlyA against the PC/cholesterol liposomes that lacked the anionic phospholipids of the asolectin. PC is one of the major constituents of asolectin,[33] and altogether, it is a neutral molecule, although it contains a positively charged choline and a negatively charged phosphate moiety within its phosphocholine headgroup.[34] Our data showed that TlyA associated with the PC/cholesterol liposomes (Figure 6A) and triggered their agglutination (Figure 6B) and membrane fusion (Figure 6C), at least to a considerable extent. These data suggested that the presence of any anionic phospholipids was not critically required for the functionalities of the TlyA protein against the liposome vesicles. However, more detailed studies will be required to understand the mechanistic basis of the interactions between TlyA and the membrane lipid components.

CONCLUSION

This study demonstrates the potent agglutinating property as well as membrane fusion and permeabilization activities of *H. pylori* TlyA against the membrane lipid bilayer of the synthetic lipid vesicles. The presence of cholesterol in the liposome membranes does not appear to be critical for such activities of TlyA. Also, it appears that the TlyA-mediated effects do not critically require the obligatory presence of any protein receptor on the liposome membranes. To the best of our knowledge, this is the first report showing such membrane-damaging functions that are relevant in the context of the virulence properties of *H. pylori* TlyA.

On the basis of the results obtained from our study, the agglutinating activity and membrane fusion property of TlyA appear to be more profound than its membrane permeabilization activity, particularly in terms of the kinetics of the processes. While prominent agglutination and membrane fusion activities were observed within minutes of incubation, liposome permeabilization effects were prominent in hours. The slow rate of the liposome permeabilization effect is consistent with the earlier observation that the membrane-damaging hemolytic activity of TlyA against human erythrocytes becomes prominent only over 18 h, when tested at protein concentrations of 2.5−20 μM.[20] Such an extent of membrane permeabilization activity of *H. pylori* TlyA is in agreement with those exhibited by other TlyA family hemolysins.[35] It is also important to mention that the membrane-damaging activities of the TlyA family of hemolysins, including that of *H. pylori* TlyA, are considerably weaker than the potent pore-forming membrane-disrupting actions of conventional hemolysins and cytolysins. Archetypical hemolysins are shown to trigger lysis of erythrocytes and liposomes at nanomolar to micromolar protein concentrations, within a time period of ∼1 h.[24] At present, no experimental data can explain such a discrepancy in the extent of membrane permeabilization effects displayed by *H. pylori* TlyA and related hemolysin proteins.

The ability of *H. pylori* TlyA to trigger agglutination and membrane fusion appears to be quite unique in the TlyA family. No other TlyA-like protein has been reported to display such properties. Membrane fusion properties are widely documented in case of viral fusion proteins that are known to mediate fusion of the viral envelopes with the target cell membranes.[36] Influenza virus hemagglutinin (HA) is one of the best examples of the viral fusion proteins.[37] Another classic example of membrane fusion is documented in the functioning of the SNARE family of proteins that act to mediate cellular membrane fusion.[38,39] Mammalian dynamin family proteins represent another class of archetypical fusion proteins that are implicated in key cellular processes involving membrane fusion.[40] Recently, a bacterial dynamin-like protein has also been reported to display a similar extent of agglutination and membrane fusion properties against liposome vesicles.[41] At present, we do not know the exact implication(s) of such agglutinating and membrane fusion properties of *H. pylori* TlyA for its virulence properties. It is also not clear whether these properties are linked with the pore-forming hemolytic activity of the protein. More studies will be required to address such issues. Regardless, this work extends our insights regarding the mode of action of *H. pylori* TlyA. Also, our study provides novel

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insights into the functionalities of the TlyA family of bacterial protein toxins.

AUTHOR INFORMATION

Corresponding Author

*Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Sector 81, SAS Nagar, Manauli 140306, Punjab, India. Phone: 91-0172-2293147. Fax: 91-0172-2240124. E-mail: kausik@iisermohali.ac.in.

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Notes

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ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; FRET, fluorescence resonance energy transfer; CD, circular dichroism.

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