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Membrane-Damaging Activity Against Various Phospholipid Liposomes by γ-hemolysin, Staphylococcal Two-Component Pore-Forming Cytolysin*

Noriko TOMITA**, Yoshiyuki KAMIO*** and Makoto OHTA**

** Institute of Fluid Science, Tohoku University
2-1-1 Katahira, Aoba-ku, Sendai, Japan
E-mail: tomita@biofluid.ifs.tohoku.ac.jp

*** Laboratory of Plant Molecular and Cellular Biology, Graduate School of Life Science, Tohoku University
2-1-1 Katahira, Aoba-ku, Sendai, Japan

Abstract

Staphylococcal γ-hemolysin (Hlg) is a two-component cytolysin, which consists of LukF (Hlg1) and Hlg2. Our previous study has suggested that LukF and Hlg2 are alternatively arranged on the human erythrocyte membrane and form a ring-shaped heterooligomeric transmembrane pore with a functional diameter of approximately 2 nm. Quantitative image analyses using high-resolution transmission electron microscopy have revealed that LukF and Hlg2 tend to be arranged in several mismatch patterns. Several previous studies have reported that the LukF component has a binding pocket for phosphatidylcholine. In the present study, membrane-damaging activities by Hlg were investigated by using carboxyfluoresceine (CF)-loading liposomes consisting of mammalian erythrocyte phospholipids or one kind of phospholipid. Our results revealed that Hlg shows membrane-damaging activities on horse, rabbit and human erythrocyte total phospholipid liposomes. Such activity was closely related to the ratio of phosphatidylcholine. Hlg showed membrane-damaging activity against phosphatidylcholine liposome by formation of ring-shaped pore complex and cluster. Besides, it was revealed that Hlg could target cardiolipin, which is not included in the mammalian erythrocyte membrane, but exists in the bacterial cytoplasmic membrane and in the inner mitochondrial membrane of mammal cell. The results suggest that Hlg has the potential to recognize several phospholipids in both erythrocyte and non-erythrocyte membranes and induce cytolysis not only of mammalian cells but also of bacterial cells by the formation of pores and clusters. These novel findings will contribute to the elucidation of mutual actions between cytotoxicity protein and phospholipids, and eventually lead to the development of a treatment for staphylococcal infection.

Key words: Staphylococcal γ-hemolysin (Hlg), Two-Component Pore-Forming Cytolysin, Heteroorigomeric Transmembrane Pore, Mammalian Erythrocyte Phospholipid, Phospholipid Liposome, Membrane-Damaging Activity, Transmission Electron Microscopy

1. Introduction

Bacterial pore-forming toxins are produced by various bacteria and may play an important role in pathogenesis. Staphylococcal γ-hemolysin (Hlg), leukocidin (Luk), and Pantone-Valentine leukocidin (Luk-PV) are two-component cytolysins secreted by *Staphylococcus aureus*, a common pathogen in hospitals[1-4]. Hlg (Hlg1 of 34 kDa/ Hlg2 of

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32 kDa) effectively lyses erythrocytes of humans and other mammalian species. Luk (LukF of 34 kDa/ LukS of 33 kDa) is cytolytic toward human and rabbit polymorph nuclear leukocytes and rabbit erythrocytes, and LukF-PV (LukF-PV of 34 kDa/ LukS-PV of 33 kDa) has cytolytic activity with a high cell specificity to mammalian leukocytes[5, 6]. Previous studies have revealed that Hlg shares one component with Luk (i.e., Hlg1 is identical to LukF) and that the cell specificities of the cytolysins are determined by the other components, Hlg2 and LukS[7-9]. Therefore, Hlg1 is referred to as LukF.

We have previously studied the pore-forming nature and assembly mechanism of these toxins and revealed that Hlg and Luk form ring-shaped structures with an inner/outer diameter of 3/9 nm on their target membranes and that they act as a transmembrane pore with a functional diameter of ca. 2 nm[10, 11]. High-resolution transmission electron microscopic (TEM) study has elucidated that an Hlg transmembrane pore has a heteroheptameric structure with an alternative arrangement of LukF and Hlg2 at molar ratios of 3:4 and 4:3[12]. Additionally, we performed quantitative 2-D image analysis of subunit arrangement in the Hlg pores based on top TEM image of the pore[13-15] and revealed that the differences of binding force between identical and different subunits have the potential to induce mismatched arrangement of subunits with specific patterns. The subunits tend to be polarized into a region in which the distance between subunits is great and a region in which such distance is small. Furthermore, our recent work using TEM suggested a novel and unique property of the Hlg pore, namely Hlg pores gather and form aggregates termed clusters with destruction of the membrane[16]. Quantitative analysis using TEM and image processing revealed that the formation of single pores and clusters is related to the release of potassium ions and of hemoglobin from erythrocytes, respectively. Effective cytolytic activity by cluster formation of Hlg pores is a different mechanism than that by α-hemolysin (Hla) from *Staphylococcus aureus*, which has been intensively studied as a prototype of pore-forming toxin and shown to be a major virulence factor in animal models of infection[17, 18]. These findings indicate that Hlg has potential to facilitate *S. aureus* infection to animals effectively.

Based on these facts, Hlg is regarded as an illustrative molecule for the study of assembly and membrane insertion of transmembrane proteins with a unique characteristic of being composed of two separate proteins different from Hla. To date, it has been revealed that the initial binding of LukF to the membrane is an essential process for the binding of Hlg2, the formation of a transmembrane pore, and the induction of hemolysis[19]. However, a receptor molecule for Hlg has not yet been clarified. Olson et al. suggested that the phosphorylcholine group of phosphatidylcholine (PC) is a binding ligand on the membrane for LukF and that it binds the pocket portion formed by Trp177 and Arg198 of LukF[20]. Yokota et al. proposed that Trp177 and Arg198 of LukF are essential for formation of a LukF-Hlg pore complex having hemolytic activity[21]. In addition, Monma et al. revealed that LukF binds to PC at a molar ratio of 1:1 and that the mutation of Trp177 and Arg198 with replacement of both residues by Thr can bind the erythrocyte membrane and form a pre-pore with Hlg2, but not form a stable transmembrane pore[22]. These results imply that although the binding of LukF to PC is an indispensable process for cytolytic activity, it has not been determined whether PC is an exclusive ligand of LukF.

In this study, we assessed the possibility that a phospholipid acts as a specific receptor for Hlg by analyzing membrane–damaging activities against various kinds of phospholipid liposome, which consist of mammalian erythrocyte total phospholipids or one kind of phospholipid. The membrane-damaging activities were measured by the release of carboxyfluoresceine (CF) from the liposomes, and investigated as to whether the activity is due to the pore and the cluster formation by Hlg. It is hoped that this study contribute for the identification of receptor molecules for Hlg and provide new insights on the interaction of Hlg pores with various kinds of phospholipid.

2. Materials and Methods

Liposome-preparation lysis assay

Mammalian erythrocyte total lipid liposomes were prepared from total phospholipid extracted from human, rabbit, horse, cow, sheep, and pig erythrocyte ghost cells as per previous studies[23, 24]. Total lipids were extracted with a solution consisting of chloroform, methanol and H_2O (volume ratio of 30:60:8) at 55ºC for 60 min. A mixture of each total lipid (0.8 μmol) and cholesterol (0.8 μmol) (Sigma-Aldrich Japan K.K. Tokyo, Japan) was dissolved in chloroform and methanol solution (2:1) and evaporated to form a lipid film on the wall of a conical-bottomed flask. The lipid film was dispersed in 2 ml of 10 mM Tris HCl buffer, pH 7.2, with 140 mM NaCl (Tris-buffered saline, TBS) solution with or without 50 mM CF by vortexing at 45-50ºC. CF-loaded liposomes were collected and washed by centrifugation at 22,000 × g for 15 min at 4ºC three times and finally suspended in 2 ml of 5 mM CF solution. For preparation of phospholipid/cholestrerol liposomes, egg yolk phosphatidylcholine (PC) (Nippon Oil and Fats Co., Tokyo, Japan), bovine brain sphingomyelin (SM), bovine brain phosphatidylserine (PS), bovine cardiac muscle cardiolipin (CL) and egg yolk phosphatidylglycerol (PG) (Avanti Polar Lipids Alabaster, AL) were used after purification as described previously[25]. A mixture of each phospholipid (0.8 μmol and cholesterol (0.8 μmol was dissolved in chloroform and methanol (2:1) and CF-loaded liposome was prepared as described above.

CF release assay

To assay liposome lysis, 200 μL of CF-loaded liposomes (80 nmol of lipid) suspension was incubated with Hlg (LukF: 1.5 nM; Hlg2: 1.5 nM) at 37ºC for 20 min. After unbound toxin was removed by centrifugation at 22,000 × g for 20 min, 100 μL of supernatants was assayed. Fluorescence of the released CF was measured at excitation of 490 nm and emission of 530 nm using a microplate reader MTP32 (Corona Electric, Katsuda, Japan). Zero percent CF release was assayed as described above without Hlg and 100% CF release was defined as the fluorescence intensity obtained upon exposure to 0.5% TritonX-100.

Transmission electron microscopy (TEM)

Two hundred microliters of liposome (80 nmol of lipid) suspension without CF was inculated with Hlg (LukF: 1.5 nM; Hlg2: 1.5 nM). Also, 250 μL of 1% (v/v) human or rabbit erythrocyte suspension was incubated with Hlg (LukF: 0.03 nM; Hlg2: 0.03 nM). Each reaction mixture was incubated at 37ºC for 20 min. After the removal of unbound toxin by centrifugation at 22,000 × g for 20 min, toxin-bounded liposomes or toxin-bounded erythrocytes were suspended in 100 μl of 5 mM sodium phosphate buffer (pH 7.2). Twenty-microliter of liposome suspension was placed onto a carbon-coated grid and stained negatively with 1% (w/v) sodium phosphotungstic acid (pH 7.2). Specimens were examined under an electron microscope H-8100 (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kV.

Assembly of Hlg complex on PC liposome

Two hundred microliters of liposome suspension without CF was incubated with Hlg (LukF: 0.6-6.0 nM; Hlg2: 0.6-6.0 nM) at 37ºC for 20 min. Hlg-treated PC liposomes were collected by centrifugation at 22,000 × g for 20 min and suspended in 5 mM sodium phosphate buffer (pH 7.4). The liposomes were washed twice by centrifugation at 22,000 × g for 20 min, solubilized with 2% SDS at 20°C or 100°C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a linear gradient gel of 3-14% (w/v) acrylamide. Protein bands were detected by using Silver Stain Kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan).

Extract protein band of Hlg complex

PC liposomes without CF were incubated with Hlg (LukF: 3.0 nM; Hg2: 3.0 nM) at 37ºC for 20 min. The liposomes were washed twice by centrifugation at 22,000 × g for 20 min, solubilized with 2% SDS at 20°C for 5 min, and subjected to SDS-PAGE, followed by staining with Coomassie brilliant Blue (CBB) R-250. The migration distances of protein bands detected by CBBR-250 agreed with those detected by silver stain. The protein bands corresponding to > 200 kDa and 140 kDa were cut out from the gel, smeared on the bottom of a small glass tube, and suspended in a small amount of TBS containing 2% SDS. The suspension was centrifuged at 15,000 × g for 10 min. Supernatants obtained were dialyzed against 10 mM Tris-HCl buffer and observed by TEM as described in the section of "TEM".

3. Results and Discussion

As shown in Fig.1, CF release assays using horse, rabbit, and human erythrocyte total phospholipid liposomes showed 31.6%, 27.5% and 14.3% of CF release, respectively, at a total concentration of 3.0 nM of Hlg (LukF: 1.5 nM, Hlg2: 1.5 nM). However, CF release was not detected at all in sheep and cow erythrocyte liposomes. Table 1A shows the percentage of the phospholipids used in mammalian erythrocytes[26, 27]. It is known that most PC and SM are located in the outer leaflet of the lipid bilayer and that most PS, PE, PI and PG are in the inner leaflet [28]. SM accounts for about 50% of the erythrocyte phospholipids of sheep and cows. However, PC is not contained in either of these erythrocyte phospholipids. Meanwhile, lipid components of horse erythrocyte membranes are mainly occupied by PC. Rabbits and humans also have high contents of PC. Table 1B shows the values of slope (a), intercept (b) and correlation coefficient (R^2) in each linear equation representing the correlation between the ratio of phospholipid (x) in the species and the CF release activity (y) at a toxin concentration of 3.0 nM. A strong positive correlation was detected between the ratio of PC and release of CF. Thereby, it is estimated that membrane–

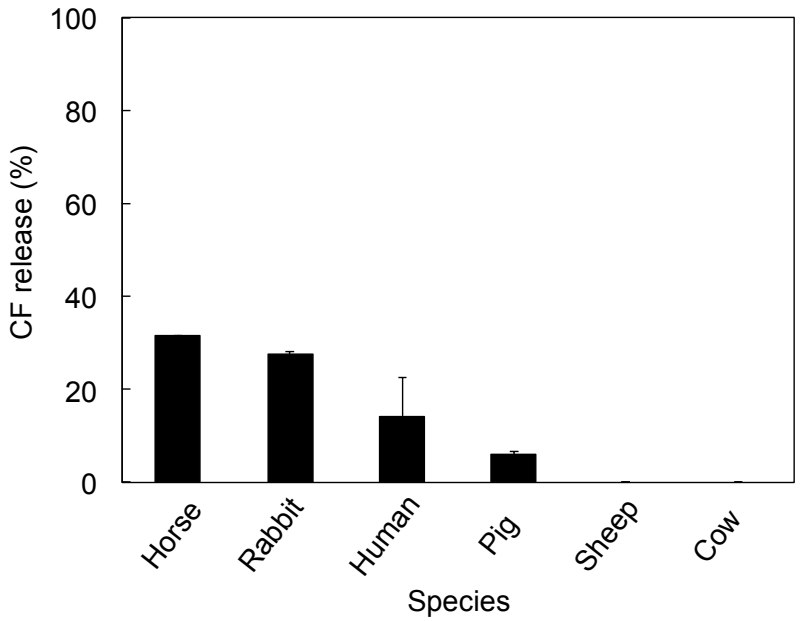

Fig. 1 Hlg-induced CF-release from mammalian erythrocyte total lipid liposome.

The CF-loaded liposome prepared from each animal erythrocyte lipid was incubated with LukF (1.5 nM) and Hlg2 (1.5 nM) at 37ºC for 20 min. The relative amount of CF released from each liposome was analyzed as described in Materials and Methods. Error bars indicate standard deviation. The CF release analysis in each lipid liposome was analyzed by two independent experiments.

Table 1 Phospholipid distribution in various mammalian species and correlation with CF-release activity by Hlg

A

Phospholipid	species					
	Ho	Ra	Hu	Pi	Sh	Co
PC	42.4	33.9	28.3	23.3	0.0	0.0
SM	13.5	19.0	25.8	26.5	51.0	46.7
PS	18.0	12.2	12.7	17.8	14.1	19.3
PE	24.3	31.9	26.7	29.7	26.2	29.1
PI	<0.3	1.6	1.0	1.8	2.9	3.7
PA	<0.3	1.6	1.0	<0.3	<0.3	<0.3

B

Phospholipid	y=ax+b: y:CF release (%), x: percentage of phospholipid (%) in the species, R^2: correlation coefficient between y and x		
	a	b	R^2
PC	0.7	-2.1	0.86
SM	-0.8	38.2	0.82
PS	1.1	31.0	0.06
PE	-0.4	25.4	0.01
PI	-9.2	30.6	0.69
PA	12.7	5.2	0.26

(A) Phospholipid distribution in erythrocytes from various mammalian species presented as weight percent phosphorus of the total phospholipid phosphorus. Each piece of data was cited from the references: Broekhuyse[26] and Nelson[27].

(B) Correlation between Hlg-induced CF-release at the Hlg concentration of 3.0 nM and the percentage of each phospholipid in mammalian erythrocyte total lipid liposome. a, b and R^2 indicate the value of slope (a), intercept (b) and correlation coefficient (R^2), respectively, when the correlation is represented by a linear expression as y=ax +b. y and x indicate CF-release activity (%) and the percentage of each phospholipid (%), respectively.

Ho, horse; Ra, rabbit; Hu, human; Pi, pig; Sh, sheep; Co, cow

PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid

damaging activity by Hlg depends on the ratio of PC. On the other hand, a strong negative correlation was found between the ratio of SM and CF release (Table 1B), and also, a negative correlation was observed between the ratio of PC and SM (data not shown but calculated from the ratio of PC and SM shown in Table 1A) in the species. Because both SM and PC exist in the outer leaflet of the erythrocyte membrane and have a phosphorylcholine group[28], it is still unclear whether CF release activity is due to the high content of PC, the low content of SM or both.

Thus, in this study, CF-release assay was performed with liposome consisting of only a kind of phospholipid. In order to analyze the role of PC and SM in membrane-damaging activity by Hlg, we selected several comparable candidates, namely PS, which is a major acidic phospholipid of the erythrocyte membrane, and CL and PG, which are acidic phospholipids like PS but not included in the erythrocyte membrane. As shown in Fig. 2, 25.9% and 29.4% CF release activities were detected in PC and CL liposomes, respectively, with a total toxin concentration of 3.0 nM. In contrast, low or no activity was found in PS, SM and PG liposomes. Unfortunately, several lipid samples are unavailable and the number of experiment was limited to two to four. However, the result of Fig.1 can show Hlg is able

to interact with lipid having phosphorylcholine group, such as PC and SM. Then, the result of Fig.2 can indicate that Hlg interact with PC as well as CL, but not SM.

In the phospholipid used in this experiment, CL has two negatively charged groups, whereas PS and PG have only one negatively charged group. To the contrary, PC and SM are neutral phospholipids. Previous studies have shown that several positive-charged cytotoxicity proteins interact with acidic phospholipid such as CL and PG and express their toxicities by transmembrane pore formation[29-31]. Because LukF and Hlg2 are basic proteins[32], they have the ability to interact with acidic phospholipids more than with neutral phospholipids and show cytolytic activity. Additionally, Valcarcel et al.[33] have demonstrated that the pore forming activity by sticholysin I and II from *stichodactyla helianthus* is promoted in the presence of CL. They suggested that CL is an inducer of positive and negative curvatures in a membrane bilayer and then forms a toroidal lipid pore surrounding a toxin structure to open the toxin channel readily in the bilayer. Also, Lewis et al.[34] found that in lipid membranes containing mixed cationic and anionic lipids the order of propensity for the nonlamellar phase is PA > CL > PG > PS. With summarizing previous reports, in general, pore-forming cytotoxic proteins are affected strongly by CL. Our result indicates that Hlg will be also affected by CL. It is speculated that the charge and the unique shape of CL will induce the higher activity of Hlg to CL liposome than to other liposomes.

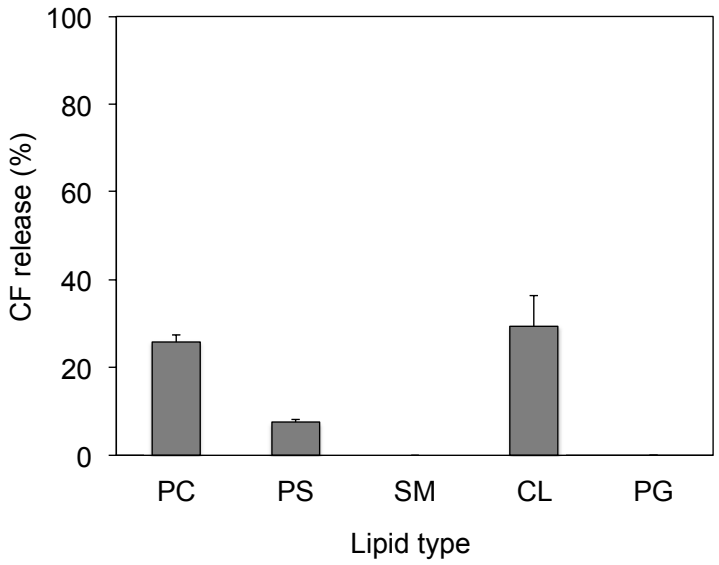

Fig. 2 Hlg-induced Hlg-induced CF-release from phospholipid liposome

The CF-loaded liposome consisting of PC, PS, SM, CL, or PG was incubated with LukF (1.5 nM) and Hlg2 (1.5 nM) at 37ºC for 20 min. The relative amount of CF released from each liposome was analyzed as described in Materials and Methods. Error bars indicate standard deviation. The two independent experiments were performed on the CF release analyses using PC and CL liposomes, and four independent experiments were performed on PS, SM and PG liposomes.

Then, we investigated whether Hlg-induced CF-release is due to pore formation. As described previously, Hlg forms a ring-shaped transmembrane pore complex on the human erythrocyte membrane[10, 12]. Because PC is a major lipid in several kinds of mammalian erythrocytes (Table 1A), in this study, we focused on the structure of the Hlg complex on the PC liposome and human and rabbit erythrocyte membranes, in which PC is contained predominately, and analyzed pore complex formation using TEM and SDS-PAGE. As shown in Fig. 3 panel A, ring-shaped structures with inner and outer diameters of approximately 3 and 9 nm, respectively, were observed on the Hlg-treated PC liposome. Additionally, as shown in panel B, the ring-shaped structures tend to aggregate and form

cluster. The shape of ring structures constituting cluster are almost similar to the pore observed on human and rabbit erythrocyte membranes treated with Hlg (Fig. 3 panels C and D). Such ring-shaped pores could not be detected on the PC liposome nor on human and rabbit erythrocyte membranes without Hlg-treatment (data not shown; see ref. 10).

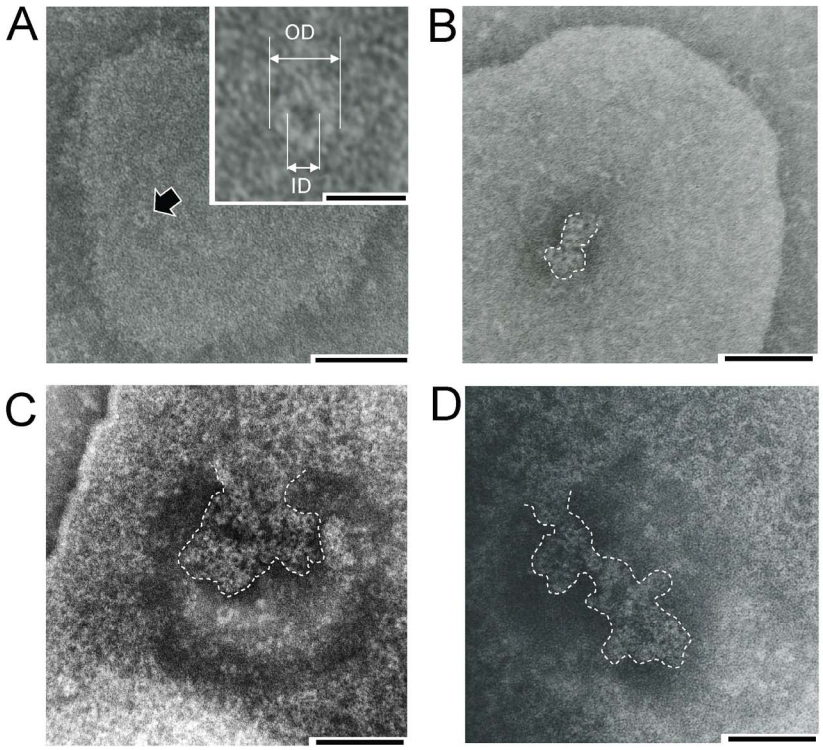

Fig. 3 The formation of transmembrane pores and clusters by Hlg on PC liposomes and mammalian erythrocyte membranes

Panels A and B are the TEM images of the Hlg-treated PC liposomes stained negatively. Panel A shows ring-shaped single transmenbrane pore formed on PC liposome. A top-right image of panel A is an enlarged image of the single transmembrane pore. 'ID' and 'OD' mean inner diameter and outer diameter of the pore, respectively. Panel B shows a cluster consisting of the transmembrane pores. Panels C and D are the TEM image of the Hlg-treated human and rabbit erythrocyte membranes stained negatively, respectively. The single pore and clusters are indicated by arrow and dashed lines, respectively. The areas in the dashed line indicate the parts of clusters. And the clusters anchoring to membrane is the area without dashed line. The cluster formation can be observed on the PC membrane as well as on the erythrocyte membrane. The bars in panel A to D indicate 50 nm. The bar in the top-right image of panel A indicates 10 nm.

Our previous study revealed that the ring-shaped pores formed by Hlg could be detected as large-molecular-sized complexes (≥195 kDa) on human erythrocyte membrane, which is stable in the presence of 2% SDS at 20°C, but dissociated to give monomers of LukF and Hlg2 at 100°C[10]. In this study, we investigated whether such a large-molecular-sized complex can be formed on the PC membrane. As shown in Fig. 4A, protein bands corresponding to toxin complexes of >200 kDa and 140 kDa, and toxin mononers could be detected when PC liposomes were treated with Hlg at a concentration more than 3.0 nM and solubilized with SDS at 20°C (lanes 3 and 5). The toxin complexes of >200 kDa and 140 kDa were dissociated to monomeric LukF and Hlg2 when heated to 100° C in the presence of 2% SDS (lanes 4 and 6). The complexes could not be detected when PC liposomes were treated with LukF or Hlg2 in the absence of their counterpart (lanes 7-10). Also, when

monomeric LukF was mixed with monomeric Hlg2 in the solution without PC liposome, no complex band could be found (lanes 11 and 12). We attempted to extract the toxin complex from the SDS-PAGE gel. As shown in Fig. 4B, clusters of ring-shaped structures were recovered from the bands corresponding to >200 kDa. Thus, LukF and Hlg2 can assemble and form ring-shaped structure, which is similar to Fig.3B, with molecular weight of >200 kDa on PC liposome. In contrast, no ring-shaped structure was visible in the extract from the 140 kDa band (data not shown). These results indicate that LukF and Hlg2 form ring-shaped pore complexes and clusters on the PC liposome as in the case of human and rabbit erythrocyte membranes. The 140 kDa complex could be an intermediary for pore oligomers, or it is perhaps yielded from a ring-shaped complex during the solubilization of PC liposome with 2% SDS and/or SDS-PAGE.

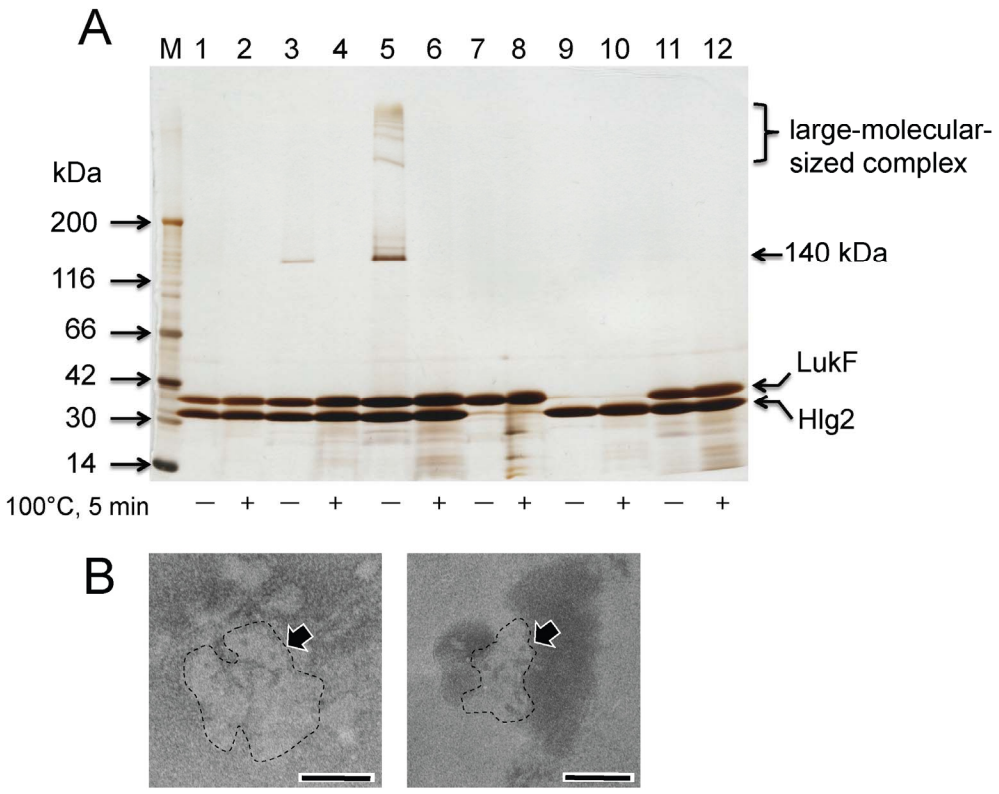

Fig.4 Assembly of LukF and Hlg2 into large-molecular-sized complex on PC liposome

(A) PC liposomes were incubated with LukF and Hlg2 at 37°C for 20 min with the following concentrations: Lanes 1 and 2: 0.6 nM of LukF and 0.6 nM of Hlg2. Lanes 3 and 4: 1.5 nM of LukF and 1.5 nM of Hlg2. Lane 5 and 6: 3.0 nM of LukF and 3.0 nM of Hlg2. Lanes 7 and 8: 6.0 nM of LukF. Lanes 9 and 10: 6.0 nM of Hlg2. Lanes 11 and 12: 0.3 nM of LukF and 0.3 nM of Hlg2 were incubated in the solution without PC liposome. M: Molecular-weight marker. + and −: Treated with 2% SDS at 100°C or 20°C for 5 min, respectively.

(B) Examples of ring-shaped Hlg complex extracted from the protein bands of SDS-PAGE gel. The protein bands corresponding to >200 kDa, which was formed on PC liposome treated with LukF (3.0 nM) and Hlg2 (3.0 nM), were cut out and extracted from the gel. Extracts were observed by TEM as described in Materials and Methods. The arrows and dashed lines show the ring-shaped pore complexes and cluster areas, respectively. The bars indicate 30 nm.

Although it could not be ascertained whether the Hlg-induced releases of CF from CL and PS liposomes were due to the pore formation, the membrane-damage activities by Hlg against phospholipid liposomes and mammalian erythrocyte total phospholipid liposomes

could well be caused by the pore formation. It is still unknown if the differences of CF-release activities by Hlg among the various phospholipid liposomes depend on the cluster formation. Further study to visualize the formation of pore and cluster on various lipid liposomes should be performed.

Potrich et al.[35] have reported that the pore-forming ability of Hlg against a lipid vesicle increased in the presence of a conical-shaped lipid in the vesicle but not a cylindrical-shaped lipid such as PC and SM. They suggested that Hlg could bind to the bilayer only if the phosphocholine head group is freely accessible and that cholesterol in the bilayer helps to achieve a better exposition of phosphocholine head groups only if acyl chains are shorter than 12 carbon atoms or unsaturated. In our study, the membrane-damaging activity against PC liposome could be detected only at a high concentration of toxin (3.0 nM), which indicates that the head of PC would be accessible against toxin and that cholesterol could help to better facilitate its action at such high concentration. SM is characterized by a lower degree of exposure of the phosphocholine head group compared to PC and induces less activity by Hlg. These facts may also support the previous finding that LukF could not bind to a membrane raft containing sphingolipid[36].

This study is the first to suggest that Hlg has potential to bind to several phospholipids in both erythrocyte and non-erythrocyte membranes and induce membrane-damaging activity by transmembrane pore formation. Although our results support that PC is a key factor in the formation of membrane pore as described previously[20, 22], the pore-forming activity against PC liposome is estimated to be lower than that against human erythrocytes because the concentration of Hlg needed on the PC liposome for detection of the pores and clusters was 50-fold that in the case of the human erythrocyte (Fig. 3). Also, the membrane-damaging activities against the mammalian erythrocyte lipid liposomes and the PC liposome reached around 30% at most (Figs. 1 and 2). Therefore, effective cytotoxicity by Hlg requires more critical factors besides PC.

CL is not included in the erythrocyte membrane, but in membranes such as the cytoplasmic membrane of bacteria and in the inner mitochondrial membrane of mammal cells. While it has been revealed that the primary sequence of LukF shares approximately 30% identities with that of S. aureus Hla[37] and that tertiary structures of LukF and Hla monomer are strongly resemble each other[18, 20], it has been not ascertained whether Hla can interact with CL as well as Hlg. Watanabe et al. have demonstrated that Hla has membrane-damaging activity against PC and SM liposomes. However, other liposomes composed of PE, PS, PG or PI with cholesterol were not susceptible to Hla[38]. These reports indicate that the interaction mechanism between Hlg and lipids is different from that between Hla and lipids even if the structures of Hlg and Hla are similar each other. The property that can target non-erythrocyte membrane lipid is specific character in Hlg as is cluster formation. The diversity in interaction mechanism between cytotoxicity proteins and phospholipids should be helpful to elucidate the inherent actions of the cytolysins to the target cells.

Yamashita et al. have succeeded in crystallization of Hlg pore in the presence of 2-methyl-2,4-pentanediol (MPD)[39]. However, MPD does not have a phosphorylcholine-like structure, and the crystalized Hlg pore with MPD has octameric stoichiometry, which is different from the heptameric structure formed on the human erythrocyte membrane[12]. The crystallization study implied two possibilities: One is that MDP can act as an alternative substrate to PC, and the other is that Hlg oligomer has the potential to alter its structure depending on the substrate structure. Actually, several studies on the interaction of proteins with cell membranes have suggested that the activities and the structures of integral membrane proteins such as ion channels may be affected by the lipid bilayer environment, namely properties of the lipid molecules including structure, thickness and charge, and resultant fluidity, elasticity and tension of the lipid membrane surrounding proteins[40-42].

Thus, the differences in responsiveness of Hlg against different types of lipids indicate that the structure of Hlg pore may change along with the structure and charge of the lipid.

Additionally, over the past several years, pore-forming toxins have been developed and engineered as stochastic sensors for the detection of small ions, organic molecules, and DNA[43, 44]. In this method, a single pore is set in a planar lipid bilayer, and a molecule passing through the pore can be detected by the change of current through the pore as a potential is applied. The structure of the pore should be affected by lipid molecules. An Hlg pore consisting of two different subunits is useful as a stochastic sensor because the two subunits enable the distinguishment and the capture of two or more molecules at the same time. If the structure of Hlg pores can be regulated by the kind of lipid, this would contribute to the development of sensing applications and protein engineering technology by using Hlg.

4. Conclusion

The CF-release assay on Hlg-treated liposomes consisting of mammalian erythrocyte total phospholipid revealed that Hlg could show the membrane-damaging activities against horse, rabbit and human erythrocyte lipid liposomes. The ratios of PC and SM in these mammalian erythrocyte lipids have positive and negative correlations with the membrane-damaging activities by Hlg, respectively. Hlg was also able to show the membrane-damaging activity against a liposome consisting of only PC but not against one consisting of only SM. The formations of pore complex and cluster by Hlg on the PC liposome were detected almost the same as those on the human erythrocyte membrane. These results indicate that PC is a one of major candidates for the receptor molecule for Hlg. Besides, a new finding of this study was that Hlg can show membrane-damaging activity against not only mammalian erythrocyte lipids but also CL, which is a phospholipid including bacterial plasma membrane and mitochondrial inner membrane of mammal cells. The relatively effective membrane-damaging activities of Hlg against PC and CL liposomes are due to the mutual action between Hlg and the lipids, which will be closely related to the shape and charge of the lipid.

The diversity in membrane-damaging activity of Hlg found in this study can make a contribution to the understanding of the interaction mechanism of Hlg with various lipids structurally and chemically, and it has the potential to expand the advantages of Hlg as a nanodevice such as a stochastic sensor and a key molecule for progress in the treatment of staphylococcal infection.

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