Further Study on the Two Pivotal Parts of Hlg2 for the Full Hemolytic Activity of Staphylococcal γ-Hemolysin

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Staphylococcal γ-hemolysin consists of LukF of 34 kDa and Hlg2 (or HlyII) of 32 kDa, which cooperatively lyse human and rabbit erythrocytes. Our previous data showed that the 5-residue segment K23RLAI27 of Hlg2 is pivotal for the hemolytic activity [Nariya, H. and Kamio, Y., Biosci. Biotechnol. Biochem., 59, 1603–1604 (1997)]. Here, we identify an additional amino acid residue in Hlg2 necessary for the full γ-hemolysin activity by measuring the toxin activity of Hlg2 mutants in the presence of LukF. The data obtained showed that Arg217 of Hlg2 is an additional pivotal amino acid residue besides the KRLAI segment for the full Hlg2-specific function in γ-hemolysin. We also report evidence that the Hlg2 mutants showing a low or null hemolytic activity in the presence of LukF towards human erythrocytes had low or no binding activity to the cells, resulting in failure of formation of the ring-shaped pore-forming complex on the erythrocytes.

Key words: Staphylococcal γ-hemolysin; bi-component cytotoxin; LukS; Hlg2

Staphylococcal γ-hemolysin has been isolated as a bi-component hemolysin from the culture fluid of Staphylococcus aureus. It consists of Hlg1 (or HlyI) of 34 kDa and Hlg2 (or HlyII) of 32 kDa, which cooperatively lyse erythrocytes from mammalian species. Hlg1 and Hlg2 of γ-hemolysin assemble into a ring-shaped, 195-kDa complex in a molar ratio of 1:1 on the human erythrocyte membrane, forming a transmembrane pore with a functional diameter of 2.1–2.4 nm. γ-Hemolysin shares one component with the staphylococcal bi-component leukocytolytic toxin, leukocidin, which consists of LukF and LukS (i.e., Hlg1 is identical with LukF), and that LukS and Hlg2 decide the specificities of these toxins towards their target cells. Approximately 80% amino acid sequence identity of the entire amino acid sequences exists between LukS and Hlg2. Our previous report showed that the K23RLAI27 segment of Hlg2 is pivotal for the Hlg2-specific hemolytic activity. The Hlg2 mutant protein MHS-D in which K23RLAI27 of Hlg2 is replaced by the 5-residue segment N29KWGV92 of LukS lost almost the hemolytic activity. However, approximately 3% of the Hlg2 activity still remained in this mutant. The data suggest the presence of an additional region(s) responsible for the full function of Hlg2. In this study, we demonstrate that the Arg217 of Hlg2 is an additional amino acid residue, besides KRLAI segment, necessary for the full specific function of γ-hemolysin. We also report evidence that weakened activity of the Hlg2 mutants towards human erythrocytes compared with that of the intact Hlg2 is due to the failure of the binding to LukF on the erythrocytes, resulting in the failure of the formation of the pore-forming complex.

Materials and Methods

Staphylococcal leukocidin and γ-hemolysin. The recombinant LukF, LukS, and Hlg2 of strain ATCC 31189 and Hlg2 (P83) of strain P83 (ATCC 31890) used in this study were purified from sonicated extracts of E. coli DH5α cells containing the plasmids pSF1, pLS4, pHS9, and pHLG2, respectively, by the method described previously.6,7 The recombinant proteins MHS-D, MHS-DRK, and MLSKR were also purified from sonicated extracts of E. coli DH5α cells harboring the plasmids pMHS-D, pMHS-DRK, and pMLSKR, respectively. The purity of the components was examined by SDS-PAGE. All preparations obtained was electrophoretically homogeneous.

Assay of γ-hemolysin activity. The γ-hemolytic activity for human erythrocytes was measured by the method described previously.8 The details are described in the legend of Fig. 2.

Plasmid constructions.

(1) Plasmid pMHS-DRK. By using plasmids pMHS-D and pHLG2, containing the hlg2 (P83) gene from S. aureus P83,5,7 a plasmid, pMHS-DRK, for the MHS-DRK protein in which Arg37 of MHS-D was replaced by a Lys residue was constructed by replacing the XbaI-XbaI fragment of the plasmid pMHS-D by that of pHLG2 (Fig. 1, lanes 2, 3, and 4). The correct DNA sequence of the plasmid was confirmed by DNA sequencing.

(2) Plasmid pMLSKR. This plasmid for the MLSKR protein in which Lys200 of LukS was replaced by an Arg residue was obtained by an over
Lane Plasmid  Protein  1  100  200  300

1  pMIS9  Hlg2  280
2  pHLG2  Hlg2(p83)  280
3  pMHS-D  MHS-D  280
4  pMHS-DRK  MHS-DRK  280
5  pMSLXK  MSLKR  286
6  pL64  LukS  286

**Fig. 1.** Schematic Representation of Hlg2, LukS, and the Mutant Proteins.

Black and white boxes indicate Hlg2 and LukS segments, respectively. XbaI represents an XbaI restriction site.

lapping-extension method, using the primers 5'-ATCTCATGAAAGGTTCAAGC-3' and 5'-GCTTGAACTCTTTCATGAGA-3' (upper double-lined and underlined nucleotide sequences in both primers correspond to Ser237 through Ser238 of LukS and the Arg residue, respectively) (Fig. 1, lane 5).

**Purification of the mutant proteins.** MHS-DRK and MSLKR were purified from the sonicated extract from *E. coli* DH5α containing the appropriate plasmid described above. The cells were grown in 1000 ml of 2×TY medium (Difco) containing ampicillin (100 µg/ml) at 37°C overnight. The cells were harvested at 4°C and suspended into a 80 mM potassium phosphate buffer (pH 6.8) and sonicated on ice. The supernatants from the sonicated extracts after centrifugation at 16,000 × g were used as starting materials for the purification of the mutant proteins. The mutants were purified by HPLC with a hydroxypatite column and TSK gel SP-5PW column by the methods described previously.7 MHS-DRK and MSLKR were eluted as a single peak with 0.3 M and 0.4 M NaCl by TSK gel SP-5PW, respectively. The preparations obtained were electrophoretically homogeneous (data not shown). The N-terminal 217th, and the 220th amino acid residues of MHS-DRK and MSLKR were identified as Lys and Arg, respectively by the method described previously.7

**Complex formation of γ-hemolysin and its mutants on human erythrocytes.** Human erythrocytes (2 × 10^8 cells) in 1 ml of PBS were incubated with LukF and either Hlg2, LukS, or their mutants (30 pmol each) at 37°C for 10 min. The membrane fraction from the cells completely lysed by the toxin was obtained by centrifuging the reaction mixture at 13,000 × g for 2 min at 4°C. In case of the incompletely lysed or non-lysed cells that are treated with the mutant proteins, the cells was lysed osmotically by 5 mM sodium phosphate buffer, pH 7.4, for complete lysis. The membrane preparation was washed 3 times with 1 ml of ice-cold PBS, solubilized in 10 µl of lysis buffer containing 1% SDS at 20°C, and then run on SDS-PAGE at 4°C using linear gradient gels of 3-14% (% T) acrylamide. Protein bands in the gel were electroblotted onto a polyvinylidene difluoride membrane and immuno-stained using specific antisera raised against LukF, Hlg2, or LukS.

**Estimation of the functional diameter of the pore formed by γ-hemolysin.** The value for functional diameter of the pore was calculated using polyethylene glycols with different hydrodynamic diameters by the method described previously.3

**Electron microscopy.** The toxin complexes on human erythrocytes were placed onto a carbon-coated grid, washed briefly with 5 mM sodium phosphate buffer (pH 7.4), and stained negatively with 1% (w/v) potassium phosphotungstic acid, pH 7.4. The specimens were examined under a Hitachi electron microscope H-8100 (Hitachi, Tokyo) at an acceleration voltage of 100 kV as described previously.2

**Results and Discussion**

**Identification of the second pivotal amino acid residue(s) in Hlg2 for its full hemolytic activity on human erythrocytes**

Here, we focused on Arg237 of Hlg2, because of our previous result that a natural-occurring analogous molecule of Hlg2 [Hlg2 (P83)] from *S. aureus* P83, in which only Arg237 is replaced by a Lys residue, had about 50% of the hemolytic activity of normal Hlg2 in the presence of LukF.7 To study whether or not the 3% hemolytic activity in MHS-D is due to the Arg residue, we created a mutant protein having a Lys residue in place of this Arg residue in the 217th position as described in Materials and Methods. Briefly, the XbaI DNA fragment of the plasmid pMHS-D was replaced with an XbaI fragment in which 5'-AGA-3' for Arg is changed to 5'-AAA-3' for Lys residue. The mutant protein expressed in *E. coli* DH5α (pMHS-DRK) was prepared from the sonicated extract of the cells from one liter of culture and purified as described in Materials and Methods. Figure 2 shows the course of the lysis of human erythrocytes in the four possible synergistic combinations of LukF and either Hlg2, Hlg2 (P83), MHS-D, or MHS-DRK. Lysis of the erythrocytes (1 × 10^8 cells) proceeded linearly up to 10 min and reached 100% at 15 min of incubation, when 3 pmol of LukF and Hlg2 were added simultaneously to the reaction mixture. Hlg2 (P83) and MHS-D also acted synergistically with LukF in hemolytic activity. However, the combination [LukF-Hlg2 (P83)] showed 70% activity at 30 min of incubation with a 3-min time lag and a gradual slope, compared with that in the combination [LukF-Hlg2]. The combination [LukF-MHS-D] had only 3% activity at 30 min of incubation with a 10-min time lag and much more gradual slope. These results confirmed that Hlg2 (P83) and MHS-D have 43% and 3.3% hemolytic activity, compared with that of intact Hlg2, respectively (Fig. 2). In contrast to Hlg2 (P83) and MHS-D, MHS-DRK has no hemolytic activity in the presence of an equal dose of LukF (Fig. 2). These data indicate that Arg237 of
Hlg2 is important for the full activity of γ-hemolysin. If hemolysin activity is decided by Arg^{317} besides the 5-residue segment K^{319}RLA^{327} in Hlg2, hemolysin activity might be conferred on LukS by replacing the Lys residue by an Arg residue at the position of the 220th amino acid residue. Accordingly, we created the mutant plasmid pMLSKR and expressed it in E. coli DH5α. The mutant protein MLKR was purified from the sonicated cell extracts and its hemolytic activity was measured. MLKR had no hemolytic activity with LukF in the standard assay system, which was shown in the legend of Fig.2, in 30 min of incubation. However, MLKR with LukF (600 pmol/ml each) lysed 50% human erythrocytes in 2 hr of incubation (data not shown). This finding was confirmed by using an agarose plate containing human erythrocytes (Fig. 3, well 5). The intact LukS never had hemolytic activity with LukF towards human erythrocytes (Fig. 3, well 6). MLKR with LukF had leukocytolytic activity to the same extent of that in the combination [LukF + LukS]. In contrast, no leukocytolytic activity was shown by any mutants of Hlg2 in Fig. 3 in the presence of LukF (data not shown). Thus, we conclude that both the Arg^{317} residue and the 5-residue seg-

![Fig. 2. Course of Hemolysis of Human Erythrocytes by Hlg2 or the Mutant Proteins in the Presence of LukF.](image)

Human erythrocytes (1 x 10^9 cells in 350 μl of PBS) were incubated at 37°C with 3 pmol of LukF and 3 pmol of either Hlg2 (○), Hlg2 (P83) (■), MHS-D (●), or MHS-DRK (●). The concentration of each component in the reaction mixture was 8.6 pmol/ml. At the time indicated, the reaction mixture was taken up and centrifuged at 8000 x g for 1 min. The supernatant was assayed for hemoglobin at 541 nm.

![Fig. 3. Hemolytic Activity of Hlg2, the Mutant Proteins, and LukS in the Presence of LukF on Human Blood Agarose Plate.](image)

Wells 1, 2, 3, 4, 5, and 6 contained Hlg2, MHS-DRK, MHS-D, Hlg2 (P83), MLKR, and LukS, respectively. The concentration of each component was adjusted to 600 pmol/ml in PBS. The amount of each component put in the well was 3 pmol. All wells also contained LukF (3 pmol) of which concentration was also 600 pmol/ml. The plate was incubated at 37°C for 6 hr.

![Fig. 4. Immunoblotting Analysis of the Toxin Complex on the Human Erythrocytes by Using a Mixture of Anti-Hlg2 and Anti-LukS Antibodies (A) and Anti-LukF Antibodies (B).](image)

Lanes 1, 2, 3, 4, 5, and 6 are the membrane fractions from the cells that were treated with LukF and either Hlg2, Hlg2 (P83), MHS-D, MHS-DRK, MLKR, or LukS, respectively. The assay of the toxin complex on the human erythrocytes was done by the method described in Materials and Methods.
ment KRLAI are pivotal for the Hlg2-specific function of staphylococcal γ-hemolysin.

**Complex formation of Hlg2 or its mutants with LukF on the cell surface of the human erythrocytes**

In the previous report, it has been evident that LukF and Hlg2 of γ-hemolysin assemble into an above 195 kDa complex in a molar ratio of 1:1, which forms a membrane pore with a functional diameter of 2.1–2.4 nm. Figure 4 shows the complex formation of LukF with the mutant proteins on the erythrocytes by SDS-PAGE and Western immunoblotting. [Hlg2 (P83)]LukF as well as [Hlg2-LukF] assembled into the complexes of 195–240 kDa on the surface of human erythrocytes (Fig. 4, lanes 1 and 2), although Hlg2 (P83) and LukF formed less of the complex than [Hlg2-LukF]. In contrast, only a small amount of [MHS-D-LukF] assembled on the cells (Fig. 4, lane 3). However, no significant amount of the high-molecular mass complexes of 195–240 kDa could be observed when either MHS-DRK, MLSKR, or LukS with LukF was used (Fig. 4, lanes 4, 5, and 6). Figure 5 shows the measurement of Hlg2, LukS, and the mutant proteins bound to the erythrocytes. The toxin-treated human erythrocytes were heated at 100°C in the presence of 1% SDS and analyzed by SDS-PAGE and Western immunoblotting. The following results were obtained. (i) Binding of LukF to the cells occurred, irrespective of the treatment of the erythrocytes with Hlg2 or the mutant proteins. (ii) The amount of Hlg2 (P83) bound to the erythrocytes was about half that of Hlg2. (iii) The binding of MHS-D to the erythrocytes was only 10% of that of Hlg2. (iv) MLSKR bound to the erythrocytes although the binding activity was about 2% of that of Hlg2. (v) Neither LukS nor MHS-DRK bound to the cells although LukF had bound to the cells. These data clearly indicate that the low or no hemolytic activity of Hlg2 mutants towards human erythrocytes shown in this study is due to the failure of the complex formation with LukF on the erythrocytes, and that manifestation of the hemolytic activity in MLSKR is due to conferring the binding activity to the erythrocytes on it.

We also did an electron microscopical study of the toxin-treated human erythrocytes. A lot of ring-shaped structures, the outer and inner diameter of which were approximately 7 and 3 nm, respectively, were observed in all areas on the membrane surface of the erythrocytes lysed by LukF and Hlg2 (Fig. 6A). In contrast, on the surface of the erythrocytes that were treated with [LukF+Hlg2 (P83)] and [LukF+MHS-D], the number of the rings in unit area of the erythrocyte membrane is approximately 25% and 5% of the intact γ-hemolysin-treated cells, respectively (Fig. 6, B and C). On the cell surface of the cells treated with LukF and MLSKR, the rings structures were detected at the area observed, but their number was less than 1% of the intact γ-hemolysin-treated cells (data not shown). These data were consistent with the results obtained from Figs. 4 and 5.

To estimated the functional diameter of the membrane pore formed by LukF with either Hlg2, Hlg2 (P83), or MHS-D, we assayed toxin-induced hemolysis in the presence of polyethylene glycols of different sizes at a concentration equivalent to 40 mOsm. If hydrodynamic diameters of polyethylene glycols are larger than that of the membrane pore formed by γ-hemolysin, the nonelectrolytes would suppress the toxin-induced lysis by counterbalancing the osmotic pressure endowed by the intracellular hemoglobins (33 mOsm).
The following findings are evident. (1) In the cases of Hlg2 and Hlg2 (P83), polyethylene glycols <1000 did not effectively suppress the toxin-induced hemolysis (Fig. 7, A and B). Polyethylene glycol 1500 effectively inhibited the toxin-induced hemolysis, although its inhibitory effect was not complete (Fig. 7, A and B). No more hemolysis was detected in the presence of polyethylene glycols with an average molecular weight of ≳2000 even at high concentrations of the toxin components (Fig. 7, A and B). (2) In contrast, polyethylene glycol 1000 effectively suppressed the toxin-induced hemolysis and polyethylene glycols ≳1500 entirely suppressed the hemolysis (Fig. 7, C). Since polyethylene glycols 1000, 1500, and 2000 have hydrodynamic diameters of 1.9, 2.1, and 2.4 nm, respectively, these data suggest that Hlg2/ LufK and Hlg2 (P83)/LufK form a hydrophilic pore with a functional diameter of 2.1–2.4 nm in the cell membrane of human erythrocytes. In contrast, MHS-D/LufK forms membrane pores with a functional diameter of 1.9–2.1 nm. The drastic decrease (97%) in the hemolytic activity of MHS-D may be caused by the reduction in pore size as well as the low level of complex formation.

In this study, we assayed hemolytic activity for a series of Hlg2 mutants including the newly constructed null mutant MHS-DRK. Our data demonstrated that Arg217 as well as the 5-residue segment KRLAI of Hlg2 is pivotal for the full Hlg2 activity. We further showed that the weak or null hemolytic activity of the Hlg2 mutants is mainly due to their reduced or null ability to assemble into the ring-shaped pore-forming complex. Recently, Gouaux et al. have analyzed the aligned sequences of staphylococcal α-hemolysin and LufK and Hlg2 of γ-hemolysin in the context of the α-hemolysin heptamer structure found by crystallographical analysis, and suggested that although the level of sequence identity between α-hemolysin and γ-hemolysin is distant, the three-dimensional structures of the protomers are conserved. Gouaux et al. and we also cooperatively succeeded in crystallographical analysis of LufK, and obtained the data which confirmed their suggestion (unpublished data). The comparative analysis of the proximity of the N-terminal 217th amino acid residue among Hlg2, Hlg2 (P83), and MHS-DRK showed the conforma-
tional change from the random coil structure to the α-helix structure by substitution of Lys for Arg in the 217th amino acid residue in Hlg2. Although the relationship between the toxin activity and the substitution of Lys for Arg\textsuperscript{217} of Hlg2 is still unclear, the presence of both the 5-residue segment KRLAI and Arg\textsuperscript{217} in the Hlg2 molecule is important for the binding of Hlg2 to LukF to form the [LukF-Hlg2] complex on the surface of the human erythrocytes.

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