Short Communication

Identification of the Minimum Segment Essential for the H7II-Specific Function of Staphylococcal γ-Hemolysin

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Staphylococcal γ-hemolysin consists of H7I (or LukF) of 34 kDa and H7II of 32 kDa, which cooperatively lyse human erythrocytes. Our previous data showed that the N-terminal 57-residue segment of H7II is the essential region for the H7II function [11. Nariya and Y. Kamio, Biosci. Biotech. Biochem., 59, 1603-1604 (1995)]. To identify the minimum amino acid residues in the 57-residue segment responsible for the specific hemolytic activity, a series of mutant genes were constructed and expressed in Escherichia coli. The mutant proteins were purified and assayed for their hemolytic activity. The results indicate that the 5-residue segment (K188R189L253A254F255) of H7II is the minimum region essential for the H7II function.

Key words: staphylococcal leukocidin; γ-hemolysin; bi-component cytolytin; pore-forming toxin

Staphylococcal γ-hemolysin has been isolated as a bi-component hemolysin from the culture fluids of Staphylococcus aureus. It consists of H7I (or Hlg1) of 34 kDa and H7II (or Hlg2) of 32 kDa, which cooperatively lyse erythrocytes from mammalian species.1) We have demonstrated in previous studies that γ-hemolysin shares one component with the staphylococcal bi-component leukocytolytic toxin, leukocidin, which consists of LukF and LukS (i.e., H7I is identical with LukF), and that LukS and H7II decide the specificities of these toxins towards their target cells. 2) - 5) What regions of LukS and H7II are pivotal for the cell specificities? Previously, we produced 12 kinds of chimeric proteins (LukS/H7II) and assayed these chimeric proteins for their specific cytolytic activity, and obtained the following results; (i) substitution of the N-terminal 59 amino acid residues of LukS for that of H7II resulted in 10% less hemolytic activity compared with that of intact H7II, irrespective of 72% amino acid sequence identity of the 59-residue segment between LukS and H7II, 6) and (ii) in contrast, the substitution of the N-terminal 57-residue segment of H7II for that of LukS showed full hemolytic activity (see Fig. 2, lane 6). Thus, we concluded that the essential region for H7II functions is located within the N-terminal 57-residue segment of H7II. 6) In this report, we identify the minimum amino acid residues in the N-terminal 57-residue segment of H7II responsible for the hemolytic activity.

In the 59-residue and 57-residue segments of the LukS and H7II, respectively, we found six regions which contain different amino acid residues between LukS and H7II (Fig. 1), i.e., (i) A1-NDT1/E1-NK14 (Box A), (ii) K1-GSDI13/Q1-GA11- (deletion, Box B), (iii) E12-DK12/Q18-DI17 (Box C), (iv) N23-KWGV29/K23RLAI27 (Box D), (v) T34/K39 (Box E), and (iv) I49L50/V47/Y48 (Box F).

To identify the segment(s) responsible for the γ-hemolysin activity, we constructed plasmids containing a series of mutant genes for each Box by an overlapping-extension method, 7) and inserted them into pUC119 to have a high level of lac-inducible expression in E. coli. The following three pairs of 54-nucleotide oligonucleotides, [A-1 and A-2], [D-1 and D-2], and [D-1 and D-2] were synthesized and used as primers for amplifying the segments using PCR. (i) A-1, 5'-GTCTTCATATCTTATTTTCAGTTGGCATTTCTAAATACGGAATTTGGAAGGG-3' and A-2, 5'-AAAGCTGGAAATAAACGGAATACGGAATTTGGAGAAGG-3'; (ii) A-1 and A-2, 5'-AAAGCTGAAATAAACGGAATACGGAATTTGGAGAAGG-3' and A-2, 5'-AAAGCTGGAAATAAACGGAATACGGAATTTGGAGAAGG-3'; and (iii) A-1 and A-2, 5'-AAAGCTGAAATAAACGGAATACGGAATTTGGAGAAGG-3' and A-2, 5'-AAAGCTGAAATAAACGGAATACGGAATTTGGAGAAGG-3'.

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Abbreviations: LukS, LukS component of leukocidin; LukF, LukF component of γ-hemolysin.

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2) Dissimilarities between them are boxed. Asterisks and dashed lines in Box D indicate the deleted amino acids and nucleotides, respectively.

Fig. 1. Comparison of the Amino Acid Sequence Analyzed Chemically in the N-Terminal 59-Residue Segment of LukS with That of H7II and the Position of XbaI Restriction Site. 2)
ment of A^1NDT^2 of LukS with E^1NKI^4 of HylII in Box A (underlined nucleotide sequences in both primers correspond to the 4-residue, E^1NKI^4 in Box A; (ii) D1-I, 5'-AGTCACGCTAACTTGTTTTTACTCTCTCTGCTCATTGTAAATTTCT-3' and D1-II, 5'-AAAA-CAAGTTAAACGATAGGCGGTACCTCAAATATTCTAATTTGATTTGTGGAGGATA-3' for the replacement of N^23KWG^26 of LukS with K^23RLAI^27 of HylII in Box D1 (underlined nucleotide sequences in the primers correspond to 3-residue, K^23RLI^2 in Box D1; and (iii) D1-I, 5'-TGTGAGTTATAGTAATCGTTATCTGTTTATCTCTTGCTCTTTTGATAATTTCTAT-3' and D2-1, 5'-ACAAGGAACGTTTAACCTCAAAAATATTTCAATTGATTTGTGGAGGATA-3' for the replacement of N^23KWG^26 of LukS with K^23RLAI^27 of HylII in Box D (underline indicates the nucleotide sequences corresponding to K^23RLAI^27 in Box D in Fig. 1). The resulting 1.5 Kbp [HindIII–HindIII] fragments were ligated into the HindIII site of pUC119. The mutagenesis and orientation of the fragments was confirmed by DNA sequencing and restriction endonuclease analysis, respectively. As a result of these manipulations, four different plasmids that contained mutant genes were obtained and they were designated as pMLS-A, pMLS-D1, pMLS-AD1, and pMLS-D (Fig. 2). The mutant protein expressed in E. coli harboring the appropriate plasmid was prepared from the sonicated extract of the cells from one liter of culture, and purified to the degree of electrophoretic homogeneity using hydroxylapatite and HPLC with a TSKgel SP-5PW column (Tosoh, Tokyo) by the method described previously (data not shown). The amino acid sequences from 1st to 35th of all mutant proteins coincided with that predicted from the nucleotide sequences found. When leukocytolytic and hemolytic activities of the purified mutant proteins were measured in the presence of LukF, the following findings became evident: (I) Mutant proteins MLS-A, MLS-D1, MLS-AD1, and MLS-D had full leukocytolytic activity as well as intact LukS (Fig. 2, lanes 2–5). It is reasonable that they have the leukocidin activity, because they have the C-terminal 121-residue segment from Ser^164 responsible for the leukocytolytic activity of intact LukS and the chimeric protein MHL5S (Fig. 2, lanes 1 and 6). (II) Mutant protein MLS-A has no hemolytic activity (Fig. 2, lane 2). (II) Mutant MLS-D1 and MLS-AD1, in which the entire 4-residue in Box A and 3-residue in Box D1 of the LukS were replaced by that of HylII, showed 5.3% and 4.2% hemolytic activity, respectively, compared with that of intact LukS (Fig. 2, lanes 3 and 4). (IV) Mutant protein MLS-D had full hemolytic activity (Fig. 2, lane 5). The findings clearly indicate that the 5-residue segment K^23RLAI^27 in Box D of HylII is necessary for the HylII function.

If γ-hemolysin-specific activity is decided by the 5-residue segment K^23RLAI^27 in Box D of HylII, γ-hemolysin activity should be lost or weakened by substituting the 5-residue segment N^23KWG^26 of LukS for K^23RLAI^27 of HylII. Accordingly, we created the mutant plasmid pmHS-D using the following primers for construction of the mutant protein MHS-D, in which the 5-residue segment K^23RLAI^27 of HylII was replaced by N^23KWG^26 of LukS (Fig. 2, lane 7). The primers for pmHS-D are 5'-TGAATGCTTGGGCATCCGGATTTGCTAGTAAATGTCTGGTCTTCTTTGATATTTCTTGCAGTTTATTTTGAGAGTTTACATGACTCAAAAA- CATTCAATTGTGGTTTTGAAAAGATAA-3' (underlined nucleotide sequences in both primers correspond to the 4-residue, N^23KWG^26). The MHS-D was expressed in E. coli DH5× (pMHS-D) and then purified from the sonicated extracts from the cells by the methods described above. The MHS-D lost almost all the hemolytic activity (Fig. 2, lane 7). Thus, we concluded that the 5-residue K^23RLAI^27 in Box D of HylII is the minimum segment essential for the HylII-specific function of staphylococcal γ-hemolysin.

It is interesting to note a computer analysis of the predicted secondary structures of the N-terminal 59-residue and 57-residue segments of LukS and HylII, respectively, and the N-terminal 59-residue segments of the mutant proteins MLS-A, MLS-D1, MLS-AD1, MLS-D, and MHS-D. The analysis was done by the computer program designed by Garnier et al. An imaginary mutant, MLS-D2, in which gly^28 and Val^29 of LukS in Box D were replaced by Ala^26 and Ile^27, respectively, was also analyzed. In intact LukS and HylII, a striking dissimilarity in the predicted secondary structure was observed in a region from the 21st to 34th amino acid residues. Among these segments, random coiled structure appears to be formed in LukS.

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Fig. 2. Schematic Representation of LukS, HylII and Mutant Proteins (A), and the Resulting Leukocidin and γ-Hemolysin Activities. Black and white boxes in (A) indicate the HylII- and LukS-segments, respectively. The activities of leukocidin and γ-hemolysin were measured by the methods described previously. Percentage leukocytolysis and hemolysis indicated the activities compared with that of intact LukS (lane 1) and HylII (lane 8). A minus indicates no detectable activity.
the case of HylII, the random coiled and $\alpha$-helix structure are observed between the 21st and 27th residues, and between the 27th and 34th residues, respectively. On the other hand, MLS-D showed a very similar profile of the secondary structure to that of the HylII. However, the mutants MLS-D1 and MLS-AD1, both of which showed a lower hemolytic activity, could form $\alpha$-helix structure between the 21st and 23rd residues. However, random coiled structures still remain in the region between the 23rd and 34th residues of both mutant proteins. The MHS-D and MLS-D2 showed a similar predicted secondary structure as that in LukS. These results suggest that the presence of the $\alpha$-helix structure in the region between the 21st and 34th residues is important for the HylII activity.

Our previous studies have demonstrated that the binding of LukF is a prerequisite for the subsequent binding of HylII to human erythrocytes to form [LukF–HylII] complex of approximately 200 kDa on the surface of human erythrocytes with a functional diameter of approximately 2.5 nm.\textsuperscript{11–13} In contrast, initial binding of LukS is a prerequisite for the subsequent binding of LukF to the human leukocytes in leukocidin.\textsuperscript{14} The finding that the MLS-D has both leukocytolytic and hemolytic activities led us to identify the order of binding to human erythrocytes and leukocytes. The binding order of the mutant protein MLS-D and LukF to the human erythrocytes was identified by the methods described previously.\textsuperscript{11,12} The following findings are evident. [1] The initial binding of MLS-D as well as LukS is essential for the leukocytolytic activity. [2] In contrast, the binding of LukF is a prerequisite for the subsequent binding of MLS-D to human erythrocytes forming the [F-MLS-D] complex of about 200 kDa on the human erythrocytes for the hemolytic activity. [3] However, MHS-D could not bind to LukF that had been initially bound to the erythrocytes. Thus, it is demonstrated that the 5-residue segment K$^{23}$RALI$^{27}$ of HylII has binding activity to LukF on the erythrocytes.

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References