Identification of Serine138 Residue in the 4-residue Segment K135K136I137S138 of LukS-I Component of *Staphylococcus intermedius* Leukocidin Crucial for the LukS-I-specific Function of Staphylococcal Leukocidin

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Received August 13, 2001; Accepted October 4, 2001

Luk-I produced by *Staphylococcus intermedius* was found to be a new member of the staphylococcal bi-component pore-forming toxin family, in which staphylococcal leukocidin, Panton-Valentine leukocidin, and γ-hemolysin are included. Luk-I consists of LukS-I and LukF-I. From the deduced amino acid sequence of LukS-I, a 4-residue sequence, K135K136I137S138, at the root of the stem region was found to be identical with that of the phosphorylated segment of a protein phosphorylated by protein kinase A. A mutant of LukS-I (MLSI-SA), in which the Ser138 residue was replaced by an alanine residue, was created, purified, and assayed for its leukocytolytic and pore-forming activities with LukF-I. Both LukS-I and MLSI-SA formed a ring-shaped complex with LukF-I on rabbit erythrocytes and human polymorphonuclear leukocytes (PMNs) membrane. However, MLSI-SA showed no leukocytolytic activity with LukF-I. LukS-I was phosphorylated by protein kinase A in the presence of [γ-32P] ATP in a cell-free system, but MLSI-SA was not phosphorylated significantly. A potent and selective inhibitor of protein kinase A (N- [2(p-bromocinnamylamino) ethyl]-5-isouquinolinesulphonamide (H-89)) showed 50% inhibition of the Luk-I-induced cell lysis at 0.5 μM. Thus, it is concluded that the phosphorylation of the Ser138 residue in the 4-residue segment K135K136I137S138 of LukS-I is important for the leukocytolysis of PMNs.

Key words: staphylococcal leukocidin; *Staphylococcus intermedius*; LukF-I; LukS-I; protein kinase A inhibitor H-89

Staphylococcal leukocidin (Luk) has been isolated as a bi-component leukocidin from the culture fluid of *Staphylococcus aureus*. It consists of LukF of 34 kDa and LukS of 32 kDa, which cooperatively lyse human and rabbit polymorphonuclear leukocytes, and rabbit erythrocytes.⁵ LukF and LukS assemble into a ring-shaped oligomer of 200-kDa in a molar ratio of 1:1 on the target cells, forming a transmembrane pore with a functional diameter of 1.9-2.1 nm.¹ Luk shares one component with the staphylococcal bi-component and pore-forming hemolytic toxin, γ-hemolysin (Hlg), which consists of Hlg1 and Hlg2 (i.e., Hlg1 is identical with LukF) and LukS and Hlg2 decide the specificities of these toxins towards their target cells, although approximately 80% amino acid sequence identity of the entire amino acid sequences exist between them.¹ Luk causes cell lysis after swelling of human erythrocytes. However, it leads to only swelling of human polymorphonuclear leukocytes (PMNs) without lysis.⁵ Our previous reports demonstrated that the 4-residue segment, K243R244S245T246 of LukS, is the pivotal region of LukS, responsible for the LukS-specific function of the staphylococcal leukocidin.⁶ The 4-residue segment is deleted in Hlg2.⁷ A mutant of Hlg2 in which the 4-residue segment KRST was inserted at the position that the segment is deleted, was endowed with the leukocytolytic activity in the presence of LukF.⁸ We also demonstrated that the phosphorylation of the Thr246 residue in the 4-residue segment in LukS by protein kinase A in PMNs is essential for the leukocytolysis as well as the pore-formation in leukocidin on PMNs.⁹

Recently, a new bi-component leukocidin, Luk-I, which consists of LukS-I and LukF-I and was cloned from the genome of *Staphylococcus intermedius*, was characterized and sequenced.⁰ The apparent molecu-
lar masses of LukF-I and LukS-I were 33 kDa and 32 kDa, respectively. Like Luk, Luk-I lyses rabbit erythrocytes as well as HPMNLs. The amino acid sequences of LukF-I and LukS-I revealed 73% and 67% identity with that of LukF and LukS, respectively.\(^6\) However, like Hig2, Luk-S-I has a deletion of the 4-residue segment for the recognition of protein kinase A at the position which it was found in LukS (Fig. 1). Accordingly, the specificity of Luk-I to the leukocytes raised the question of what region(s) of LukS-I play a pivotal role in the LukS-I-specific function for leukocytolytic activity. In this report, we identified the 4-residue segment K135K136I137S138 for the recognition site of protein phosphorylated by protein kinase A, which is 104 amino acid residues away from the 4-residue sequence of LukS. Our results demonstrated that the phosphorylation of Ser138 residue in the 4-residue KKIS segment by protein kinase A is crucial for Luk-S-I-specific function of Luk-I.

### Materials and Methods

**Bacterial strains.** *S. intermedius* ATCC 51874 was used as the source of the lukS-I gene, cloned in pTrc99A. *Escherichia coli* DH5\(\alpha\) was used as the host strain for the cloning of lukS-I and its mutant, and the expression of the recombinant proteins.

**Cloning of LukS-I gene (lukS-I) and construction of a mutant protein of LukS-I.** The lukS-I was amplified from the chromosomal DNA of *S. intermedius* ATCC 51874 by PCR, using the primers 5'-TTCT CCATGG TAAAAAATAAATTATAGCCGC-3' and 5'-ATTAAGCTTTAATTGCCCCTTTAC-TTAT-3' (single and double underlined sequences represent the NcoI and HindIII restriction sites, respectively).\(^6\) A 933-bp fragment was amplified. The amplified HindIII-NcoI fragment was then inserted into hindIII and the NcoI site of pTrc99A. The nucleotide sequencing of the inserted DNA fragment was done, and found to be identical with that reported previously by Prevost et al.\(^6\) Thus the amplified DNA fragment was used for the construction of a mutant gene of lukS-I. The resulting plasmid was designated as pLSI. The mutant gene of Luk-S-I, in which the Ser138 residue was replaced by an alanine residue, was constructed by an overlapping-extension method,\(^7,8\) using a pair of primers, SIS138A 5'-ATTCAGGAGAAAAATGGCCTATCTCAAAAA-3' and SIS138Ac 5'-TTTTGAGTATA GCGAATTCTTTGGAAT-3' (underlined nucleotide sequences in both primers correspond to the 4-residue sequence K135KIA138 in the segment). Following successful mutagenesis (as confirmed by DNA sequencing), the HindIII-NcoI fragment was ligated into the HindIII-NcoI site of pTrc99A. The resulting plasmid was designated as pMLSI-SA.

**Purification of LukF-I, LukS-I and the mutant protein of LukS-I.** *S. intermedius* ATCC 51874 was grown in 2.5% heart infusion broth (Difco, Detroit, USA) at 37°C for 20 h under aeration until the cell density reached 9×10^6 cells/ml. The culture were centrifuged at 5,500×g for 10 min at 4°C and the supernatant was collected. For the purification of LukS-I, 25 grams of hydroxylapatite (HA) powder (100–350 mesh, Nacalai Tesque Inc., Kyoto, Japan) were added to 2.7 liters of the culture supernatant and kept at 4°C with constant stirring for 2 h. After separating the supernatant from HA by centrifugation, on which Luk-S-I but not LukF-I was adsorbed, the supernatant containing LukF-I was diluted 4-fold with distilled water. Twenty-five grams of HA was added to the supernatant for adsorbing LukF-I. LukF-I and LukS-I were then eluted from each HA with 0.4 M and 0.6 M potassium phosphate buffer (pH 6.8), respectively and dialyzed against 10 mM HEPES buffer (pH 6.5). Each dialysate was chromatographed with a Hi-Trash SP-Sepharose column (Amersham Pharmacia, Biotech) using a linear gradient of 0–0.8 M NaCl in 10 mM HEPES buffer (pH 6.5). LukF-I and LukS-I fractions that were eluted with 0.2 M and 0.4 M, respectively, were collected. MLSI-SA was purified from a sonicated extract from *E. coli* DH5\(\alpha\) harboring pMLSI-SA according to the methods described previously.\(^6\) Purity of the intact LukF-I and LukS-I, and the recombinant MLSI-SA preparations were examined by SDS-PAGE. All preparations were electrophoretically homogeneous.

**Assay of hemolytic and leukocytolytic activities.** Rabbit erythrocytes (1×10^8 cells/ml) were incubated with several concentrations of the toxin components at 37°C for 30 min in 100 μl of BSS (KH_2PO_4, 7.6 mM; Na_2HPO_4, 2 mM; NaCl, 137 mM; pH 6.8). The assay of hemolytic activity was done as described previously.\(^9\) For the assay of leukocytolytic activity, HPMNLs were freshly isolated with Mono-Poly Resolving Medium (Dai-Nippon Pharmaceuticals, Osaka, Japan), according to the protocol of the manufacturer. The leukocytolytic activity was measured by the methods described previously.\(^10\) The cells of HPMNLs (2.5×10^7) in 25 ml of Mono-Poly Resolving Medium were treated with LukS, LukS-I, or MLSI-SA (7.5 pmol) and LukF or LukF-I (7.5 pmol). After 30 min of incubation, morphological observation was done under a light microscope. The percentages of leukocytolysis and hemolysis indicate the activities compared with that of LukS-I.

**Assay of phosphorylation of LukS-I and its mutant by ATP in the presence of protein kinase A in a cell-free system.** This was done essentially as described previously.\(^6\) The reaction mixture for the phosphorylation of each toxin component by protein kinase A contained 20 mM Tris-HCl buffer (pH 7.5),
1 mM EGTA, 5 mM MgCl₂, 30 pmol of toxin component, 0.2 µM [γ-32P]ATP (Amersham, 37 kBq) and 5 ng of protein kinase A (catalytic subunit) from bovine heart (Upstate Biotechnology, Lake Placid, NY) in a total volume of 10 µl. The reaction mixture was incubated at 30°C for 20 min, and stopped by adding 5 × SAB loading buffer. After being heated at 100°C for 5 min, the sample was analyzed by SDS-PAGE using 12.5% acrylamide. After being stained with Coomassie brilliant blue R-250, the gel was scanned by an image scanner and measured by FLA-200 (Fuji photo film, Tokyo).

**Inhibition assay of leukocytolytic activity by H-89.**
The inhibition assay was done essentially as described previously. Isolated HPMNLs (2.5 × 10⁶) from fresh human blood were suspended in TBS-Mg-Glc (Tris-HCl, 10 mM; NaCl, 132.5 mM; MgCl₂, 5 mM; glucose, 0.1%; pH 7.4) and incubated with 25 µl of varying concentrations of H-89 (Seikagaku Kogyo, Tokyo) at 37°C for 10 min. After the addition of LukS-I and LukF-I to the reaction mixture, it was incubated again at 37°C for 30 min. The effects of H-89 on the leukocytolytic activity of leukocidin were measured by observing the leukocidin-induced leukocytolysis of HPMNLs microscopically after staining the cells with trypan blue.

**Electron microscopy of ring-shaped structures of Luk-I.** This was done essentially as described by Sugawara et al. Rabbit erythrocytes (1 × 10⁶ cells/ml) were incubated with LukS-I (60 µg/ml) and LukF-I (60 µg/ml) or MLSI-SA (60 µg/ml) and LukF-I (60 µg/ml) in TBS (Tris-HCl, 10 mM; NaCl, 140 mM; pH 7.2) at 37°C for 30 min. Erythrocyte membranes from the lysed cells were collected by centrifugation at 8,000 × g for 2 min at 4°C and washed twice with TBS. The membrane preparations were placed onto carbon-coated grids, washed briefly with a small amount of 5 mM sodium phosphate buffer (pH 7.4), and negatively stained with 1% (w/v) sodium 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.

**Results**

**Identification of the crucial amino acid residue(s) in LukS-I for its function**

Figure 1 shows an alignment of the deduced amino acid sequences of Luk family proteins. In the sequence of LukS-I, we found a unique 4-residue sequence, K135K136I137S138, that is identical to the recognition site of protein phosphorylated by protein kinase A (Fig. 1, box I). The segment was 104 amino acid residues away from the 4-residue sequence of LukS (Fig. 1, box Z located in the arrow 14). This segment was predicted to be located in the stem region of LukS-I, based on the 3-dimensional (3-D) structure of LukF and LukF-PV and the analysis of the aligned sequence of the components showed in Fig. 1 (Fig. 1, box I located in the arrow 8,12,13). To examine if the 4-residue segment of LukS-I (KKIS) is essential for the LukS-I function, a mutant of LukS-I (MLSI-SA), in which the Ser138 residue was replaced by an alanine residue, was created. MLSI-SA was prepared from the sonicated extract of E. coli DH5α harboring pMLSI-SA from 1 liter of culture and purified to electrophoretic homogeneity. MLSI-SA was assayed for hemolytic and leukocytolytic activities to rabbit erythrocytes and HPMNLs, respectively, in the presence of LukF-I, and each activity of MLSI-SA was compared with that of LukS-I. LukS-I and MLSI-SA had almost the same hemolytic activity on rabbit erythrocytes (Fig. 2), indicating that the change of the Ser138 residue to an alanine residue had no effect on its hemolytic activity. On the other hand, Luk-I had twice stronger leukocytolytic activity than that of Luk (Fig. 2, lanes 1 and 2). However, MLSI-SA with LukF-I had no leukocytolytic activity (Fig. 2, lane 3). The cells treated with MLSI-SA and LukF-I became swollen but did not reach lysis (Fig. 4, panel G). The findings clearly indicated that the Ser138 residue of the 4-residue segment of LukS-I is important for the leukocytolytic activity of LukS-I, and strongly suggested that the phosphorylation of LukS-I at this Ser138 residue is essential for the leukocytolytic function of LukS-I.

**Phosphorylation of LukS-I and MLSI-SA by protein kinase A and inhibition of leukocytolytic activity of Luk-I with H-89**

The findings described above suggest that LukS-I is phosphorylated by protein kinase A at the Ser138 residue. We examined phosphorylation of LukS-I and MLSI-SA by protein kinase A in a cell-free system. LukS-I and MLSI-SA were incubated with protein kinase A from bovine heart in the presence of [γ-32P] ATP and MgCl₂, and reaction mixtures were analyzed by SDS-PAGE and the gel was scanned by the image scanner. The native LukS-I and MLSI-SA were slightly phosphorylated (Fig. 3, lanes 1 and 3). If they were boiled for 5 min and used as substrates, the phosphorylation of LukS-I was increased about 50 times higher than that of the native one (Fig. 3, lanes 1 and 2), but the intensity of the phosphorylation of MLSI-SA slightly increased regardless of its denaturing by boiling (Fig. 3, lanes 3 and 4). The radioactivity of LukS-I was 10 times higher than that of MLSI-SA. The data indicate that LukS-I can also be phosphorylated by protein kinase A at the Ser138 residue in the 4-residue segment on HPMNLs and also suggested that a phosphorylation of LukS-I by protein kinase A at the Ser138 residue on HPMNL requires a conformational change of LukS-I induced...
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Fig. 1. Alignment of the Sequences of LukS-I, LukS, LukF, LukS-PV, LukF-PV, Hlg2, and Hla.

The corresponding elements of LukF secondary structure are given above the sequence, using the same color code as in Fig. 6. The numbers on the arrows are indicated in reference 12. Residues that are shaded in gray are conservative substitutions in more than 4 of the sequences. The asterisks indicate residues that are conserved among all of the sequences. The recognition sites of protein kinase A in LukS-I and LukS are indicated by boxes I and Z, respectively.

by binding onto HPMNL membrane.

To confirm whether protein kinase A participates in expression of leukocytolytic activity by Luk-I, H-89, which had been known to be a potent and selective inhibitor of protein kinase A with an inhibition constant of 0.048 μM, was used. It was found that HPMNLs exposed to 50 μM H-89 (Fig. 4, panel C) without Luk-I components showed no change in their morphology, but exposure of the cells to 0.5 nM H-89 caused 50% inhibition of Luk-I induced leukocytolysis (Fig. 4, panel A). The concentration of H-89 for 50% inhibition of Luk-I was 100 times lower as that of Luk. The addition of H-89 concentrations over a range of 50 to 0.5 nM H-89 to the reac-
Fig. 2. Schematic Representation of LukS, LukS-I, and Mutant Proteins, and Resulting Leukocytolytic Activity on HPMNLs and Hemolytic Activity on Rabbit Erythrocytes.

Black and gray boxes indicate the LukS and LukS-I segments, respectively. White boxes indicate KRST in LukS, KKIS in LukS-I, and KKIA in MLSI-SA. Percentages of leukocytolysis and hemolysis activities indicate the relative activities compared with that of LukS-I. Absence of leukocytolytic activity is shown by a minus.

Fig. 3. Phosphorylated Products of LukS-I and MLSI-SA by [γ-32P] ATP in the Presence of Protein Kinase A.

The reaction was done as described in Materials and Methods. Reaction mixtures contained 20 mM Tris-HCl buffer (pH 7.5), 1 mM EGTA, 5 mM MgCl₂, 0.2 mM [γ-32P] ATP (37 kBq), 5 ng of protein kinase A, and 30 pmol of toxin component in a total volume of 10 μL. Toxin components are indicated above the panel. Lanes 1, 2, 3, and 4 represent native LukS-I, boiled LukS-I, native MLSI-SA, and boiled MLSI-SA, respectively.

Discussion

In this study, we assayed leukocytolytic activity and phosphorylation of LukS-I and its mutant, MLSI-SA. Our data demonstrated that the phosphorylation of LukS-I at the Ser138 residue in the 4-residue segment K135K136I137S138 by protein kinase A is essential for the expression of the leukocytolytic activity of Luk-I. This finding suggested that the leukocytolytic activity of Luk-I is expressed by the same mechanism as with Luk and Panton-Valentine leukocidin (PVL), which consists of LukF-PV and LukS-PV, except for differences in the intensity of leukocytolytic activity and the location of...
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phosphorylation sites between them. As mentioned above, Luk-I had twice higher leukocytolytic activity on HPMNLs than that of Luk (Fig. 2, lanes 1 and 2). It is known that, for the recognition site motifs of the protein phosphorylated by protein kinase A, a Ser residue is more suitable than a Thr residue as the final amino acid residue in the 4-residue sequence R/K-R/K-X-S/T.15 Indeed, the mutant of LukS (MLS-TS) in which the Thr residue of the 4-residue segment KRST of LukS was replaced by a Ser residue showed 10 times higher leukocytolytic activity than that of intact LukS.6 Taken together, there might be some relationship between the phosphorylation of the Ser residue in the segment, if any, and the leukocytolytic function of Luk-S-I. Of the Luk family toxin components, 3-D structures of α-hemolysin (Hla) heptamer, LukF water-soluble monomer, and LukF-PV water-soluble monomer have been published.12,13,16 Gouaux *et al.* have analyzed the aligned sequences of Hla, Luk, Hlg, and Luk-I components in the context of the 3-D structure of Hla heptamer and found that even though the level of sequence conservation is low, most of the conserved residues are clustered in a few key groups, located in the interior of the Hla protomer structure which is a constituent of Hla heptamer, therefore reinforcing the conclusion that the protomeric structure taken from the Hla heptamer, Luk oligomer, and Hlg oligomer are similar.17 Indeed, the 3-D structures of Hla protomer and LukF water-soluble monomer are identical except for the channel region (stem region) of LukF folding into an amphipathic, three strand β-sheet.12 Figure 6 shows 3-D structure models of LukS or LukS-I based on the 3-D structure of LukF, LukF-PV, and Hla protomer.12,13,16 In the previous report, we proposed a mechanism of assembly of the staphylococcal pore-forming toxins on the target cells.12 In this model, S component monomer binds onto the target cell mem-

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**Fig. 4.** Schematic Representation of Relative Leukocytolytic Activity of Luk-I in the Presence of H-89 and Light Micrographs of HPMNLs that Were Treated with Luk-I in the Presence or Absence of H-89.

HPMNLs (2.5 × 10^6) in TBS-Mg-Glc were exposed to H-89 at 37°C for 10 min at the concentration indicated in the panel A, and then treated with LukS-I (37.5 pmol) and Luk-F-I (37.5 pmol). After 30 min of incubation, the morphological observation was done under a light microscope. Results in panel A represent the percentage of leukocytosis of Luk-I on H-89-exposed cells compared with that on H-89-unexposed cells. Panels B and C represent intact HPMNL and HPMNL treated with 50 μM H-89 alone, respectively. Panels D and G represent HPMNLs treated with Luk-I alone and [MLSI-SA + LukF-I], respectively. The bars on panels B to G represent 5 μm.

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**Fig. 5.** Ring-shaped Structure on the Luk-I-treated Rabbit Erythrocytes.

Panels A and B show rabbit erythrocytes that were exposed to LukS-I and LukF-I (60 μg/ml each) or MLSI-SA and LukF-I (60 μg/ml each), respectively. The lysed erythrocytes were washed and negatively stained with 1% sodium phosphotungstic acid, as described in Materials and Methods. Panel C shows rabbit erythrocytes without treatment by the toxin.
brane through a bottom of the rim domain, assembles into a pre-pore complex with the F component, and, finally, the pre-stem of each component is stretched into a membrane to form a water-soluble channel. The 3-D locations of KKIS in LukS-I, KRST in LukS, and RRTT in LukS-PV in a ring-shaped complex are similar in location near the target cell membrane. Accordingly, it is reasonable that LukS-I is phosphorylated by the same protein kinase A as LukS and LukS-PV. Herein, the location of the 4-residue segment in LukS and LukS-I might be important for its interaction to protein kinase A. If the oligomeric state of Hla on the target cells is applicable to Luk-I and Luk, the phosphorylated residues of LukS-I is located at the root of the stem region that spans the hydrophobic domain of the membrane bilayer of HPMNLs and can easily interact with protein kinase A which might be localized on the target cell membrane. On the other hand, the Thr246 residue in KRST segments of LukS is located at the bottom of the rim domain and might be just in contact with the surface of the target cells. The evidence, that the H-89 concentration for 50% inhibition of leukocytolytic activity of Luk-I was 100 times lower than that of Luk, can be explained by the location of the 4-residue segment between them.

Recently, we discovered a pre-pore of leukocidin of which the pre-stem is still included within the lumen of the cap domain of the toxin (to be published). The results clearly indicate that the location of KKIS in LukS-I of the pre-pore is distant from the membrane of HPMNLs. Moreover, MLSI-SA caused the HPMNLs to be swollen but not lysed (Fig. 2, lane 3 and Fig. 4, panel G), and was not phosphorylated by protein kinase A (Fig. 3, lane 4). Furthermore, MLSI-SA formed a ring-shaped complex with LukF-I (Fig. 5, panel B). Taken together, it is suggested that the phosphorylation of LukS-I occurs after the pre-stem of the pre-pore is stretched into the membrane of the target cells and that the phosphorylation of LukS-I by protein kinase A at the Ser138 residue is not required for the Luk-I assembly or the conformational change of pre-stem region of LukS-I during the lytic pore formation.

The intensity of phosphorylation of the 4-residue segment of the native LukS-I at Ser138 residue in the cell-free system was 1/50 of that of the boiled one by protein kinase A in the presence of ATP (Fig. 3, lanes 1 and 2). The low phosphorylation of the native LukS-I might be due to the conformational difference of the pre-stem region between native and boiled ones. The conformational change of the pre-stem region by boiling might cause the Ser138 residue of the 4-residue segment that is masking in pre-stem region, to be exposed on the surface where it is easily accessible to protein kinase A.

Acknowledgments

The authors thank Mr. Tsuruji Sato of Tohoku University for their help of the electron microscopical study. This work was supported in part by a Grant-in Aid for Scientific Research (Nos. 11460034 and 13460034) from Japan Society for the Promotion of Sciences. A. N. was supported by the Japan Society for the Promotion of Sciences with Predoctoral
Fellowships. M. A. R. V. G. was a student of the 27th International Post-Graduate University Course in Microbiology of UNESCO.

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