Identification of the minimum segment in which the threonine^{246} residue is a potential phosphorylated site by protein kinase A for the LukS-specific function of staphylococcal leukocidin

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Abstract **Staphylococcal leukocidin and γ-hemolysin consist of LukF and LukS for leukocidin and LukF and Hlg2 for γ-hemolysin. In this report, we identify the minimum segment responsible for the LukS-specific function of leukocidin. After chemical analysis and homology study of the amino acid sequence of the C-terminal region between LukS and Hlg2, we found a unique 5-residue sequence $I^{242}K^{243}R^{244}S^{245}T^{246}$ in LukS in which the 4-residue KRST is identical with that of the phosphorylated segment of a protein phosphorylated by protein kinase A. To elucidate whether the 5-residue segment is essential for the LukS function, we created plasmids containing a series of mutant genes corresponding to the 5-residue sequence and expressed them in *Escherichia coli*. The mutant proteins were purified and assayed for their leukocytolytic activity with LukF. The mutant MLS-TS, in which the T^{246} in the 5-residue sequence was replaced by S, showed leukocidin activity 10 times higher than that of the intact LukS. However, neither mutant MLS-TY nor MLS-TA, in which T^{246} was replaced by Y or A, respectively, showed leukocidin activity. The 5-residue segment was found to be deleted in Hlg2. The mutant of Hlg2, in which the 5-residue segment was inserted at the position that the segment is deleted, showed leukocidin activity. The boiled LukS, MLS-TS, and MHS-Z were strongly phosphorylated with [γ-^{32}P]ATP in the presence of protein kinase A in a cell-free system. Thus, we conclude that the 5-residue segment $I^{242}K^{243}R^{244}S^{245}T^{246}$ is the pivotal segment of LukS responsible for the LukS function of staphylococcal leukocidin.**

Key words: Staphylococcal leukocidin; γ-Hemolysin; Bi-component cytolysin; LukS; Hlg2; Toxin phosphorylated by protein kinase A

1. Introduction

Staphylococcal leukocidin has been isolated as a bi-component leukocidin from culture fluids of *Staphylococcus aureus*. It consists of LukS of 32 kDa and LukF of 34 kDa, which cooperatively lyse human and rabbit polymorphonuclear leukocytes [1]. We have demonstrated in previous studies that leukocidin shares one component with the staphylococcal bi-component hemolytic toxin, γ-hemolysin, which consists of Hlg1 and Hlg2 (i.e. Hlg1 is identical with LukF), and that cell specificities of leukocidin and γ-hemolysin are decided by LukS and Hlg2, respectively [1–3]. The deduced amino acid sequences from the genes encoding LukS and Hlg2 indicated 72% identity between them (Fig. 1) [3]. The specificities of the two toxins towards the target cells raise the question of what region(s) of LukS or Hlg2 plays a pivotal role in the LukS- and Hlg2-specific function for leukocidin or γ-hemolysin activities. To answer this question, previously, we created a series of chimeric genes (*lukS/hlg2*) and expressed them in *Escherichia coli*. From the results obtained, we concluded that there is an essential region for LukS-specific function within the C-terminal 122-residue segment (between S^{164} and the C-terminus) of LukS [4] (see Fig. 3, lane 1). Here, we identify the minimum amino acid residues in the C-terminal 122-residue segment of LukS responsible for the leukocytolytic activity. This report indicates that the 5-residue segment $I^{242}K^{243}R^{244}S^{245}T^{246}$, in which the T^{246} residue was phosphorylated by protein kinase A in a cell-free system, is the pivotal region of LukS responsible for the LukS-specific function of staphylococcal leukocidin.

2. Materials and methods

2.1. Staphylococcal leukocidin and γ-hemolysin

LukF, LukS, and Hgl2 were purified from *S. aureus* strain 5R Smith as described previously [5].

2.2. Assay of leukocidin activity

The leukocytolytic activity for human polymorphonuclear leukocytes was measured according to the methods described previously [6,7].

2.3. Construction of plasmids containing a series of mutant genes

To determine the segment(s) responsible for leukocidin activity, plasmids containing a series of mutant genes of *lukS* and *hlg2* were constructed by the overlapping-extension method [7] using the primers described in Section 3. Following successful mutagenesis (as confirmed by DNA sequencing), the *Hin*dIII-*Hin*dIII fragment was ligated into the *Hin*dIII site of pUC119.

2.4. Purification of mutant proteins of LukS and Hlg2

The mutant protein of LukS was purified from the sonicated extract from *E. coli* DH5α harboring the appropriate plasmid according to the methods described previously [5]. The cells were grown in 2 liters of 2×TY medium (Difco) containing ampicillin (100 μg/ml) at 37°C until the cell density reached 9×10^8 cells/ml. The cells were harvested, suspended into 80 mM potassium phosphate buffer (pH 6.8), and sonicated. The supernatant from the sonicated extract after centrifugation at $16000\times g$ was used as starting material for the purification of the mutant proteins.

2.5. Assay of phosphorylation of LukS and its mutants by ATP in the presence of protein kinase A in a cell-free system

This was performed essentially as described by Kennelly and Krebs [8]. The reaction mixture for assaying the phosphorylation of LukS and its mutant by protein kinase A contained 20 mM Tris-HCl buffer

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Abbreviations: LukS, LukS component of leukocidin; LukF, LukF component of leukocidin/γ-hemolysin; Hlg2, Hlg2 component of γ-hemolysin; PMSF, phenylmethylsulfonyl fluoride

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(pH 7.5), 1 mM EGTA, 5 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP (Amersham, 20 μCi), 30 pmol toxin component, 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 37°C for 20 min, and stopped by adding 5×SAB loading buffer. After heating at 100°C for 5 min, the sample was subjected to SDS-PAGE using 12.5% acrylamide. After staining with Coomassie brilliant blue R-250, the gel was scanned by an image scanner (Fuji photo film, BAS-2000).

2.6. Complex formation of leukocidin and γ-hemolysin on human polymorphonuclear leukocytes

Human polymorphonuclear leukocytes (1×10^5 cells) in Locke's solution [9] were incubated with LukF and either LukS, its mutants, or Hlg2 (30 pmol each) at 37°C for 10 min. The toxin-leukocytes complex was collected by centrifugation at 5000×*g* for 1 min at 4°C, and was washed 3 times with 1 ml of ice-cold Locke's solution. The toxin-leukocytes complex was then solubilized in 10 μl of lysis buffer containing 1% SDS, leupepsin hemisulfate (20 μg/ml), and 1 mM PMSF at 20°C for 5 min and subjected to SDS-PAGE at 4°C using 8% acrylamide. Protein bands in the gel were electroblotted onto a polyvinylidene difluoride sheet for 2 h and immunostained using specific antisera raised against LukS and LukF.

3. Results and discussion

3.1. Amino acid homology study

After homology study of the deduced amino acid sequence from the structure genes for LukS and Hlg2, we found a unique 5-residue sequence $I^{242}K^{243}R^{244}S^{245}T^{246}$ of which the 4-residue KRST is identical to a recognition site of protein phosphorylated by protein kinase A. This segment was deleted in Hlg2 (see box in Fig. 1). To obtain direct evidence for the presence and absence of the 5-residue segment in LukS and Hlg2, respectively, LukS and Hlg2 were degraded with CNBr by the method described previously [10] and an Asp^{237}–Asn^{286} fragment (fragment A) in LukS and an Asp^{235}–Lys^{280} fragment (fragment B) in Hlg2 were isolated using HPLC. The N-terminal 12-residue segments of fragments A and B were determined to be D^{237}VTHAIKRSTHY^{248} and D^{235}ATVAYVTRHRL^{246}, respectively. The data reconfirm that the 5-residue segment in LukS is deleted in Hlg2.

3.2. Identification of the minimum segment in LukS for its function

To study whether or not the 5-residue segment (IKRST) is essential for the LukS function, we created a series of mutant genes in the segment by the overlapping-extension method, and inserted them into pUC119 vector DNA. The following three pairs of 53- or 54-oligonucleotide sequences for replacing the T^{246} residue in the segment by S, Y, or A (TS-1 and TS-2; TA-1 and TA-2; and TY-1 and TY-2), respectively, were synthesized and used as primers for amplifying the segments using PCR. (i) TS-1, 5′-GCCATTAAAAGATCATCACATTATGGCAACAGTTATTTAGACGGACATAGAGTC-3′ and TS-2, 5′ACTGTTGCCATAATGTGATGATCTTTTAATGGCATGAGTGACATCCATGTTTC-3′ (underlined nucleotide sequences in both primers correspond to the 5-residue sequence IKRSS in the segment; (ii) TA-1, 5′-GCCATTAAAAGATCAGCCCATTATGGCAACAGTTATTTAGACGGACATAGAGTC-3′ and TA-2, 5′-ACTGTTGCCATAATGGGCTGATCTTTTAATGGCATGAGTGACATCCCATGTTTC-3′ (underlined nucleotide sequences in both primers correspond to the 5-residue sequence IKRSA), and (iii) TY-1, 5′-GCCATTAAAAGATCATATCATTATGGCAACAGTTATTTAGACGGACATAGAGTC-3′ and TY-2, 5′-ACTGTTGCCATAATGATATGATCTTTTAATGGCATGAGTGACATCCATGAATC-3′ (underline nucleotide sequences in both primers correspond to the 5-residue sequence IKRSY). The resulting 1.5 kbp *Hin*dIII-*Hin*dIII fragments were ligated into the *Hin*dIII 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, three different plasmids that contained mutant genes corresponding to the 5-residue segment were obtained, designated pMLS-TS, pMLS-TA, and pMLS-TY. The mutant proteins expressed in *E. coli* harboring the appropriate plasmid were prepared from the sonicated extract of the cells from 1 liter of culture and purified to electrophoretic homogeneity (Fig. 2A,B). After measuring leukocytolytic activity of the purified mutant compo-

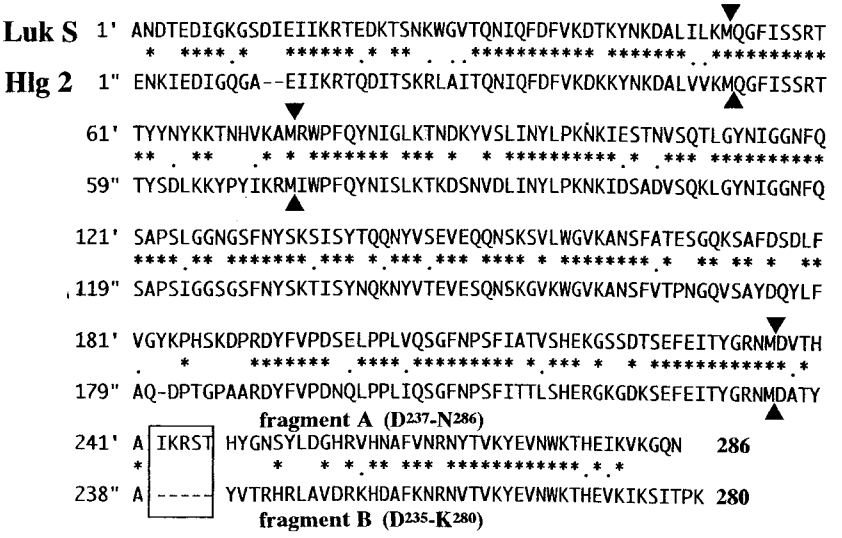

Fig. 1. Comparison of the deduced amino acid sequence of LukS with that of Hlg2. Gross dissimilarity between them is indicated by the box. Identical and related residues are indicated by stars and dots, respectively. Dashed lines indicate deleted amino acids. Arrowheads indicate the cleavage site of CNBr.

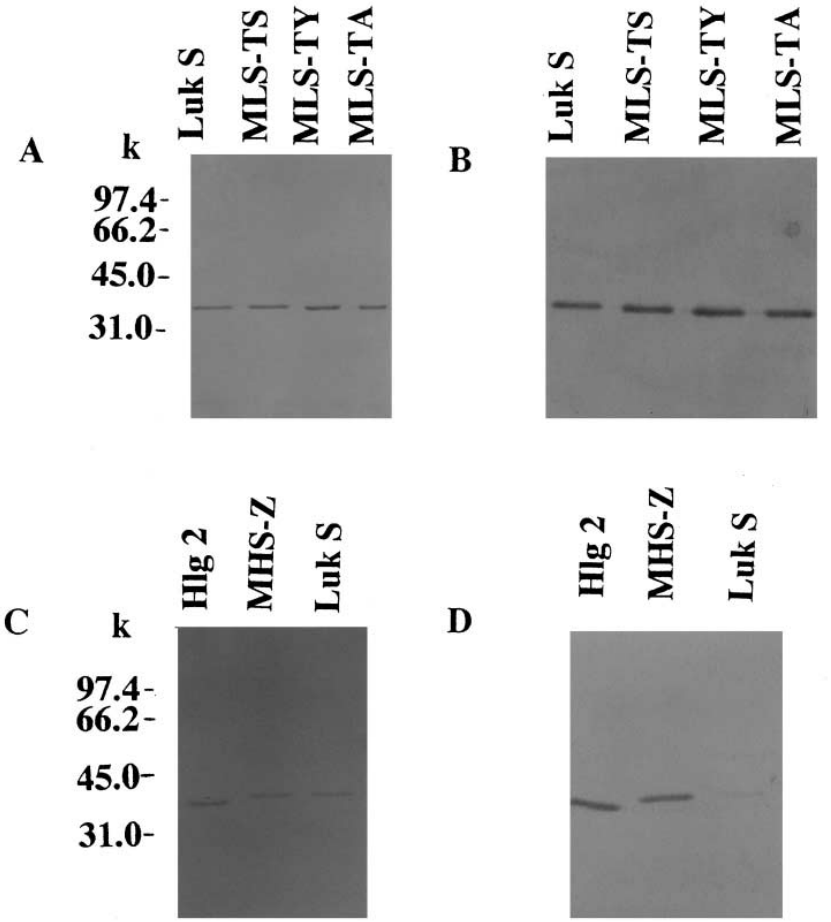

Fig. 2. SDS-PAGE (A and C) and Western immunoblotting (B and D) analyses of the purified LukS, MLS-TS, MLS-TY, MLS-TA, Hlg2, and MHS-Z. Gels A and C were stained with Coomassie brilliant blue R-250. Anti-LukS and anti-Hlg2 antibodies were used for B and D, respectively. Molecular mass standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa) and bovine carbonic anhydrase (31.0 kDa).

nents in the presence of LukF, the following findings became evident. (i) Mutant protein MLS-TS showed 10 times higher leukocytolytic activity than intact LukS (Fig. 3, lanes 4 and 5). (ii) Neither mutant protein MLS-TA nor MLA-TY has any leukocidin activity (Fig. 3, lanes 6 and 7). The findings clearly indicate that the T^{246} residue of the 5-residue segment of LukS is pivotal for the LukS function. It is known that, for the recognition site motifs of the protein phosphorylated by protein kinase A, a serine residue is more suitable than threonine as the final amino acid residue in the 4-residue sequence

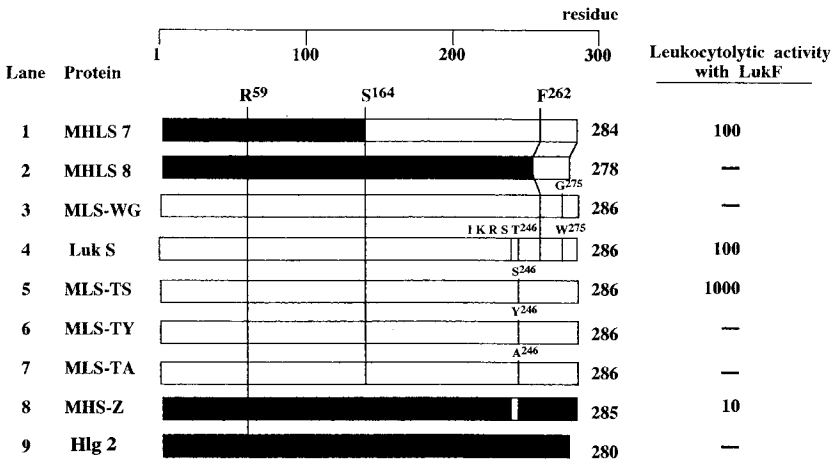

Fig. 3. Schematic representation of LukS, Hlg2 and mutant proteins, and the resulting leukocidin activity. White and black boxes indicate the LukS and Hlg2 segments, respectively. The white box in MHS-Z (lane 8) represents the inserted 5-residue $I^{242}K^{243}R^{244}S^{245}T^{246}$ segment. Percentage leukocytolysis indicates the activity compared with that of intact LukS (lane 4). A minus indicates no detectable activity.

R-R/K-X-S/T [11]. Taken together, there might be some relationship between the phosphorylation of the T residue in the segment, if any, and the leukocytolytic function of LukS.

If leukocidin-specific activity is decided by the 5-residue segment IKRST of LukS, leukocidin activity might be conferred to Hlg2 by inserting the 5-residue segment of LukS between A^{238} and Y^{239} of Hlg2 (Fig. 1). Accordingly, we created the mutant plasmid pMHS-Z using the following primers, 5′-TACATATGCTATTAAAAGATCAACGTACGTGACAAGACATCGTTTAGCCGTTGATAGAAAACATGATGC-3′ and 5′-TTGTCACGTACGTTGATCTTTTAATAGCATATGTAGCATCCATGTTTCTGCCGTAAGTGATTTCAAACTCGC-3′ (underlined nucleotide sequences in both primers correspond to the 5-residue sequence IKRST).

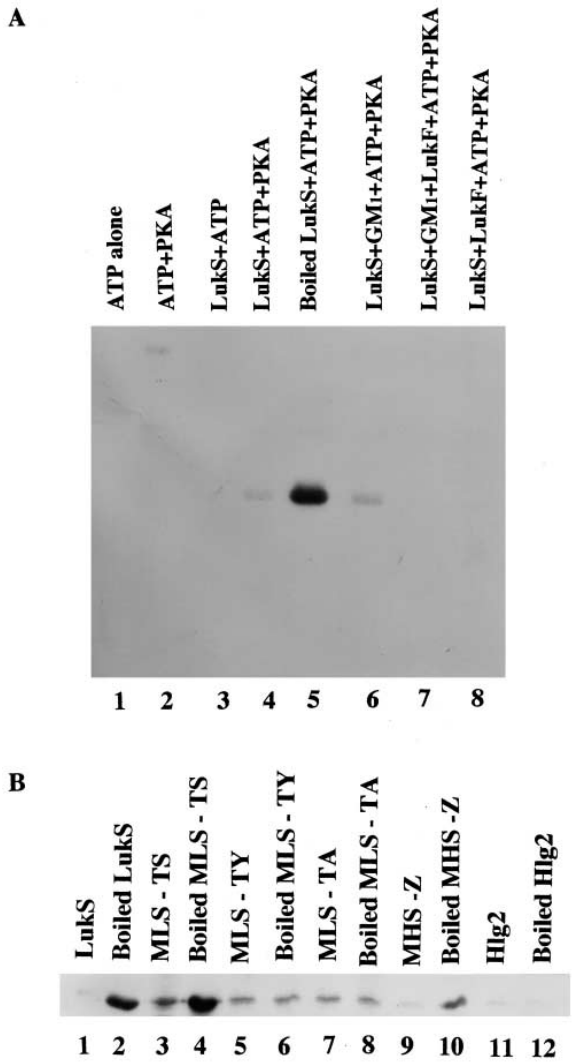

Fig. 4. Phosphorylated products of LukS (A) and the mutants of LukS and Hlg2 (B) by [γ-^{32}P]ATP in the presence of protein kinase A in a cell-free system. The reaction was carried out as described in Section 2. The basal reaction mixture (Base) contained 20 mM Tris-HCl buffer (pH 7.5), 1 mM EGTA, 5 mM $MgCl_2$. A: lane 1, base+[γ-^{32}P]ATP; 2, base+[γ-^{32}P]ATP+protein kinase A; 3, base+LukS+[γ-^{32}P]ATP; 4, base+LukS+[γ-^{32}P]ATP+protein kinase A; 5, base+LukS boiled for 5 min+[γ-^{32}P]ATP+protein kinase A; 6, 4+G_{M1}; 7, 6+LukF; 8, 4+LukF. B: lanes 1–12, base+[γ-^{32}P]ATP+protein kinase A+the toxin component which was indicated at the top of the panel. Odd and even numbers represent native and boiled ones, respectively.

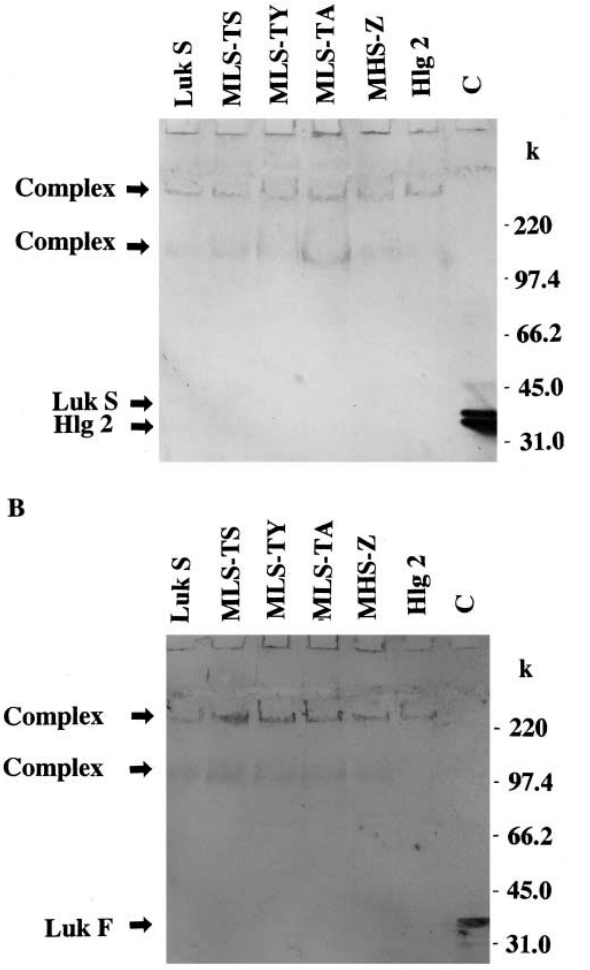

Fig. 5. Immunoblot analysis of LukS, the mutants of LukS, Hlg2, or MHS-Z on human polymorphonuclear leukocyte membranes using both anti-LukS and anti-Hlg2 (A), and anti-LukF (B) antibodies. The conditions for the formation of the toxin complex(es) on the cell surface were described in Section 2. Human polymorphonuclear leukocytes (1×10^5 cells) in Locke's solution [9] were incubated with LukF and the toxin component (30 pmol each) which was indicated at the top of the panels at 37°C for 10 min. Lane C in panels A and B represents the mixture of LukS and Hlg2, and LukF as standards, respectively.

MHS-Z was expressed in *E. coli* DH5α (pMHS-Z) and then purified from the sonicated cell extracts according to the methods described above (Fig. 2C,D). MHS-Z showed leukocytolytic activity with LukF, although not full activity (Fig. 3, lane 8). Thus, we concluded that the 5-residue sequence IKRST is the minimum segment essential for the LukS-specific function of staphylococcal leukocidin.

3.3. *Phosphorylation of LukS and its mutants by ATP in the presence of protein kinase A in a cell-free system*

The findings described above suggest that LukS and the mutant proteins MLS-TS and MHS-Z possessing the 5-residue segment are phosphorylated by protein kinase A. Accordingly, we examined the possibility using a cell-free system. LukS or mutant protein MLS-TS was incubated with protein kinase A from bovine heart in the presence of [γ-^{32}P]ATP, $MgCl_2$, and EGTA, and the reaction mixture was applied to SDS-PAGE. The gel was scanned by the image scanner. The native LukS, MLS-TS, and MHS-Z were slightly phosphoryl-

ated (Fig. 4A, lane 4 and Fig. 4B, lanes 1, 3, and 9). If they were boiled for 5 min and used as substrates, their phosphorylation was increased about 50–100 times higher than that of native ones (Fig. 4A, lane 5 and Fig. 4B, lanes 2, 4, and 10). The boiled MLS-TS was phosphorylated 4 times higher than the boiled LukS (Fig. 4B, lanes 2 and 4). The mutant proteins MLS-TA and MLS-TY, in which T as a phosphorylated residue in the recognition site motif had been replaced by A and Y, respectively, were also slightly phosphorylated. However, the intensity of their phosphorylation did not increase regardless of their denaturing by boiling (Fig. 4, lanes 5–8). It is known that LukS binds specifically to monosialoganglioside G_{M1} (G_{M1}) on the leukocyte membrane [12]. We previously clarified that residue W^{275} of LukS is an essential amino acid for its binding to G_{M1} and that this binding is accompanied by a conformational change of LukS [4,6] (see Fig. 3, lane 3). Accordingly, we examined whether the phosphorylation of native LukS was activated by adding G_{M1} to the reaction mixture. However, its phosphorylation was not activated by G_{M1}. These findings indicate that residue T^{246} in the segment of native LukS and MLS-TS is not exposed to the protein surface even upon binding to G_{M1} in the reaction mixture. The data also strongly suggest that the phosphorylation of residue T^{246} in the 5-residue segment of LukS is important for the LukS function of leukocidin, and that the lack of leukocidin activity in Hgl2 is due to the absence of the 5-residue segment itself.

3.4. *Complex formation of LukF with LukS or its mutants on the cell surface of human polymorphonuclear leukocytes*

Fink-Barbacon et al. reported that leukocidin forms pores inducing an increase in the free intracellular Ca^{2+} which may activate human polymorphonuclear leukocytes [13]. However, no direct evidence of complex formation of the leukocidin components on human leukocytes is available. Recently, we reported that LukF and Hgl2 of γ-hemolysin assemble into a ring-shaped 195 kDa complex in a molar ration of 1:1, which form a membrane pore with a functional diameter of 2.1–2.4 nm [14]. Using our established systems, we examined complex formation of LukF with either LukS, MLS-TS, MLS-TA, MLS-TY, or MHS-Z on human polymorphonuclear leukocytes. Our data show that all of the mutants as well as LukS in combination with LukF assembled into a >100 kDa complex, which may form a pore on the surface of human polymorphonuclear leukocytes (Fig. 5). LukS alone did not assemble on the cell surface (data not shown). From these findings, we could distinguish the leukocytolytic function from the complex formation which forms the membrane pore on the surface of human polymorphonuclear leukocytes.

We monitored the change of morphology of the cells of human polymorphonuclear leukocytes under a phase contrast microscope. Intact cells became swollen after incubation with LukF and Hlg2 at 37°C for 10 min. However, lysed cells were not observed after incubation for more 20 min. These data indicate that LukF and Hlg2 cooperatively caused only swelling of human polymorphonuclear leukocytes without lysis.

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