Preliminary Communication

Phosphorylation of LukS by Protein Kinase A is Crucial for the LukS-Specific Function of the Staphylococcal Leukocidin on Human Polymorphonuclear Leukocytes

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Staphylococcal leukocidin (Luk) consists of two protein components, LukF and LukS, which cooperatively lyse human and rabbit polymorphonuclear leukocytes. Here, we demonstrate that the phosphorylation of LukS by protein kinase A is crucial for the LukS-specific leukocytolytic function of Luk on HPMNLs by using N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), which is a potent and selective inhibitor of protein kinase A. At 0.5 μM H-89 completely prevented the Luk-induced cell lysis accompanied by blocking of the incorporation of exogenous 32P-H2PO4 into LukS on HPMNLs. However, with LukS and LukF together, 0.5 μM H-89 did not inhibit the cell swelling which takes place before the cell lysis. HPMNLs also became swollen upon treating with both LukF and LukS mutants which could not be phosphorylated.

Key words: staphylococcal leukocidin; bi-component cytolsin; LukS; toxin phosphorylated by protein kinase A; protein kinase A inhibitor H-89

Staphylococcal leukocidin (Luk) consists of two protein components, LukF of 34 kDa and LukS of 32 kDa, which cooperatively lyse human and rabbit polymorphonuclear leukocytes. Luk shares one component with the staphylococcal bi-component hemolysin, γ-hemolysin, which consists of Hlg1 and Hlg2 (i.e. Hlg1 is identical with LukF), and the cell specificities of Luk and γ-hemolysin are decided by LukS and Hlg2, respectively. The amino acid sequences of LukS and Hlg2 indicate 72% identity between them. 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-functions for Luk and γ-hemolysin activities. In our previous report, we identified a 5-residue sequence K[243]R[244]S[245]T[246] in which a 4-residue segment K[243]R[244]S[245]T[246] is identical with the phosphorylated site of the protein phosphorylated by protein kinase A as a minimum segment responsible for the LukS specific function of Luk. LukS denatured by heating at 100°C, but not intact LukS, was strongly phosphorylated by [γ-32P] ATP in the presence of protein kinase A in a cell-free system. However, neither of the mutants MLS-TY and MLS-TA in which T[246] was replaced by Y or A residue, respectively, nor Hlg2 itself, in which the 5-residue segment of LukS was deleted, had leukocytolytic activity. They were not phosphorylated by protein kinase A in the cell-free system, even though they were denatured by heating at 100°C. The Hlg2 mutant MHS-Z, in which the 5-residue segment was inserted at the position that the segment is deleted in Hlg2, was phosphorylated by protein kinase A and conferred leukocytolytic activity cooperatively with LukF. The results obtained indicate that the 5-residue segment IKRTS is the pivotal segment of LukS responsible for the LukS function of staphylococcal leukocidin. The data also suggest that the phosphorylation of LukS occurs in HPMNLs and this reaction is crucial for the LukS-specific function of staphylococcal leukocidin. However, no direct evidence of the phosphorylation of LukS in HPMNLs is available. Here, we demonstrate that the phosphorylation of LukS occurs on HPMNLs and it is a crucial event for the leukocytolytic activity of LukS in the presence of LukF in the toxin, by using H-89, which has been shown to be a potent and selective inhibitor of protein kinase A in forskolin-induced neurite outgrowth from PC12D cells.

We examined the phosphorylation of LukS in HPMNLs using 32P-H2PO4. HPMNLs were treated with Luk components in the presence of 32P-H2PO4 and then analyzed directly by SDS-PAGE. As shown in Fig. 1A, a radioactive band corresponding to LukS appeared in HPMNLs treated with LukS alone or both LukS and LukF (Fig. 1A, lanes 1 and 2), but no radioactive band at the area corresponding to MLS-TA was detected in the cells treated with MLS-TA or without the toxin components (Fig. 1A, lane 3 and data not shown). The radioactive products in lanes 1 and 2 of Fig. 1A were immunoprecipitated by anti-LukS antibodies and migrated at the same position as LukS did in SDS-PAGE as a single radioactive band each (Fig. 1B, lanes 1 and 2). Thus, we conclude that LukS is phosphorylated by exogenous 32P-H2PO4 on HPMNLs. It is of interest to note that a radioactive band was detected at the position where LukF migrated in SDS-PAGE in the cells treated with..
Fig. 1. SDS-PAGE (A) and Immunoblot (B) Analyses of the Phosphorylated Product of LukS and Its Mutant MLS-TA by \(^{32}\)P-H\(_3\)PO\(_4\) on HPMLNs Using Anti-LukS Antibodies.

Recombinant LukF, LukS, and its mutant MLS-TA protein were purified from the sonicated extracts from *Escherichia coli* DH5\(\alpha\) harboring plasmids pSF\(_1\),\(^6\) pLS4,\(^9\) and pMLS-TA,\(^6\) respectively, as described previously.\(^8\) HPMLNs (1 x 10\(^6\) cells) were incubated with 300 pmol of LukS alone or 300 pmol each of LukS and LukF in the presence of \(^{32}\)P-H\(_3\)PO\(_4\) (1 mCi, 1.15 \(\mu\)Ci) in 1 ml of PKA-Glc buffer, which was composed of 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 5 mM MgCl\(_2\), and 5.6 mM glucose for 1 hr at 37°C. The lysed or non-lysed cells were collected by centrifugation at 10,000 \(\times\) g for 5 min, washed three times with 1 ml of ice-cold PKA-Glc buffer, and then solubilized with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5% SDS, 50 mM NaF, 1 mM PMSF, and leupeptin (20 \(\mu\)g/ml) at 20°C.

**Panel A:** The solubilized samples were heated at 100°C for 5 min and run on SDS-PAGE using 12.5% acrylamide. The gel was dried and scanned by an image scanner for detection of the radioactivity. Lane 1, the cells treated with LukS alone; Lane 2, the cells treated with LukS and LukF; Lane 3, the cells treated with LukS mutant MLS-TA and LukF.

**Panel B:** The solubilized samples were immunoprecipitated with anti-LukS antibodies for 2 hr. The resultant precipitates were heated at 100°C in the presence of 1% SDS, and run on SDS-PAGE using 12.5% polyacrylamide gel. The proteins in the gel were blotted onto a polyvinylidene difluoride membrane for 2 hr. After the membrane was treated with anti-LukS antibodies, the LukS-antibody complexes were treated with anti-rabbit IgG (Fc)-alkaline phosphatase conjugate (Seikagaku Kogyo, Tokyo). The LukS was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrate (lane S). The membrane was also scanned by the image scanner (lanes 1–3). Molecular marker standards used were rabbit muscle myosin (200 kDa), *E. coli* \(\beta\)-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), rabbit muscle aldolase (42.4 kDa), bovine carbonic anhydrase (30 kDa), and horse muscle myoglobin (17.2 kDa). Lane S represents immunostained proteins in the sample from the cells treated with LukS. Lanes 1–3 represent the radioactive products in immunoprecipitates from samples of lanes 1–3 of panel A, respectively.

Both LukF and LukS but not with LukS alone (white arrow in lane 2 of Fig. 1A). This band was immunoprecipitated by neither anti-LukF nor LukS (data not shown), suggesting that this is derived from HPMLNs.

H-89 is a potent and selective inhibitor of protein kinase A with an inhibition constant of 0.048 \(\mu\)M.\(^3\) Accordingly, the effects of H-89 on the phosphorylation of LukS both *in vitro* and *in vivo* was examined. An experiment in the cell-free system was done as described previously.\(^9\) The reaction mixture for assaying the phosphorylation of LukS and its mutants by protein kinase A contained 20 mM Tris-HCl buffer (pH 7.5), 1 mM EGTA, 5 mM MgCl\(_2\), 0.2 mM [\(\gamma\)-\(^{32}\)P]ATP (20 \(\mu\)Ci), 30 pmol denatured LukS, and 5 ng of protein kinase A (catalytic subunit) from bovine heart (Upstate Biotechnology, Lake Placid, NY) in a total volume of 10 \(\mu\)l. The reaction mixture was incubated at 37°C for 20 min, and stopped by adding 2 \(\mu\)l of sample application buffer, which was composed of 125 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol 1.28 mM mercaptoethanol, and 0.125% bromo-phenol blue. After heating at 100°C for 5 min, the sample was run on SDS-PAGE using 12.5% acrylamide. After PAGE, the gel was dried and scanned by an image scanner (Fuji photo film, BAS-2000). Addition of 0.05 \(\mu\)M H-89 to the reaction mixture in the cell-free system caused 50% inhibition of the transfer of \(^{32}\)P from [\(\gamma\)-\(^{32}\)P]ATP to LukS, and 0.5 \(\mu\)M H-89 completely prevented the enzyme reaction (data not shown). These results indicate that H-89 is a potent inhibitor of the phosphorylation of LukS by protein kinase A in the cell-free system. Therefore, the effects of H-89 over a range of 0.005 to 0.5 \(\mu\)M on *in vivo* phosphorylation reaction in HPMLNs using \(^{32}\)P-H\(_3\)PO\(_4\) were examined. Exposure of the cells to 0.05 \(\mu\)M and 0.5 \(\mu\)M H-89 caused 50% and 100% inhibition of \(^{32}\)P-H\(_3\)PO\(_4\) incorporation into LukS, respectively, compared to that in the unexposed cells (Fig. 2, lanes 4 and 5, and Fig. 2B). The IC\(_{50}\) of H-89 in the incorporation of \(^{32}\)P-
H$_2$PO$_4$ into LukS coincided with its inhibition constant of 0.048 μM reported. These results indicate that LukS is phosphorylated by protein kinase A on HPMNLs. This suggested that H-89 should inhibit the leukocytolytic activity, if the phosphorylation of LukS by protein kinase A is crucial for the LukS-specific function of leukocidin. Accordingly, we examined the effects of H-89 on the leukocytolytic activity of leukocidin by observing the leukocidin-induced leukocytolysis of HPMNLs microscopically after staining the cells with trypan blue. The intact cells (Fig. 3A) were exposed to H-89 at the concentrations indicated at the top of panel of Fig. 3. The cells that were exposed to 50 μM H-89 without leukocidin components showed no change of their morphology (Fig. 3, panel B). The addition of H-89 at the concentration over a range of 50 to 0.5 μM to the reaction mixture containing LukS and LukF caused the cells to be swollen but not lysed for at least 20 min observed at 37°C (Fig. 3, panels C-E). The cells exposed to 0.05μM H-89, that led to 50% inhibition of the phosphorylation of LukS, began to lyse them with a loosened edge. However, the cell lysis remained incomplete for 10 min. However, 0.005 μM H-89 had no inhibitory effect on leukocytolysis by leukocidin (Fig. 3, panel G). Thus, it was concluded that the phosphorylation of LukS is crucial for the LukS-specific function of leukocidin.

Gouaux et al. have analyzed the aligned sequences of staphylococcal α-hemolysin and Luk components in the context of the α-hemolysin heptamer structure found by the crystallographic analysis and suggested that even though the level of sequence identity among α-hemolysin and Luk components, LukF and LukS is distant, the three-dimensional structures of the protomers are conserved. Based on their suggestion, we might propose that the phosphorylated residue T$^{246}$ of LukS is at the bottom of the rim domain that spans the hydrophobic domain of membrane bilayer of HPMNLs and that the T$^{246}$ residue is phosphorylated by protein kinase A at the contact point of inner leaflet of the membrane and cytoplasm of HPMNLs.

Although the mechanism of the lysis of HPMNLs by Luk remains to be discovered, it is feasible that the modification of LukS to the phosphorylated one by protein kinase A on HPMNLs after its initial binding to HPMNLs followed by the subsequent binding of LukF is essential for inducing the cell lysis, because H-89 did not inhibit HPMNLs to be swollen, but prevented lysis. We observed the same phenomena in HPMNLs which were treated with both LukF and the mutant of LukS which had no phosphorylated site in itself. We could propose two possibilities of the involvement of the phosphorylation of LukS in its leukocytolytic activity. One is the fragility of the membrane of HPMNLs that was caused by the induction of some lytic enzyme, such as an autolytic enzyme or phospholipase that degrades the membrane. Previously, Noda et al. reported that the
Fig. 3. Light Micrographs of the HPMNLs Which Were Treated with Luk (LukF + LukS) in the Presence or Absence of H-89.

The preparation of HPMNLs was done by using Mono-Poly Resolving Medium (Dainippon Pharmaceutical Co. Ltd., Tokyo). The cells of HPMNL (2.5 x 10⁶) in 25 μl of Mono-Poly Resolving Medium were exposed to H-89 at 37°C for 10 min at the concentration indicated in this figure, and then treated with LukS (7.5 pmol) and LukF (7.5 pmol) were added. After 10 min of incubation, the morphological observation was done under a light microscope after staining the cells with trypan blue. Panels A and B represent intact HPMNL treated with neither leukocidin nor H-89, and treated with 50 μM H-89 alone, respectively.

binding of LukS to rabbit polymorphonuclear leukocytes induced an activation of phospholipase A2 in the leukocytes. Taken together with our data, if any, the phosphorylated LukS-mediated signal transduction might be required for an activation of phospholipase A2. The other is the possibility that the membrane pore consisting of LukF and phosphorylated LukS on HPMNLs might regulate some ion such as a calcium current through the membrane. Finck-Barbancon et al. previously reported that staphylococcal leukocidin induces an increase in the free intracellular Ca²⁺ under the physiological conditions ([Ca²⁺] = 1 to 1.5 mM) in HPMNLs.

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