Assembly of Staphylococcal Leukocidin into a Pore-Forming Oligomer on Detergent-Resistant Membrane Microdomains, Lipid Rafts, in Human Polymorphonuclear Leukocytes

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Staphylococcal leukocidin (Luk) consists of LukS and LukF, which cooperatively lyse human polymorphonuclear leukocytes (HPMNLs), monocytes, and macrophages. Here we found that LukS and LukF assembles into hetero-oligomeric pore complexes on the detergent-resistant membrane microdomains, lipid rafts of HPMNLs. When HPMNLs were treated with LukS alone, 24% of the added LukS was localized in lipid rafts. Furthermore, in HPMNLs treated with both LukS and LukF simultaneously, about 90% of high molecular-mass complexes of 100 kDa, which consists of LukS and LukF, were detected in the lipid raft fractions. In contrast, in HPMNLs treated with LukF alone, LukF was not localized in lipid rafts despite binding to the target cell membranes. Ten mM methyl-cyclodextrin, a dysfunctioning agent of lipid rafts, completely inhibited assembly of Luk on lipid rafts, and resulted in null leukocytolytic activity of Luk. Hence, we concluded that assembly of LukS and LukF into the pore-complex occurs in lipid rafts in HPMNLs and that LukF can bind to LukS, which had already bound to lipid rafts, to assemble into hetero-oligomers.

Key words: staphylococcal leukocidin; LukS; pore-forming toxin; assembly of toxin components; detergent-resistant membrane microdomains, lipid rafts

The staphylococcal leukocytolytic toxin, leukocidin (Luk), has been isolated as a two-component pore-forming cytolysin from Staphylococcus aureus, consisting LukF of 34 kDa and LukS of 32 kDa, which cooperatively lyse human and rabbit polymorphonuclear leukocytes.¹ We found previously that Luk shares LukF with the staphylococcal two-component hemolysin γ-hemolysin (Hlg), which consists of LukF and Hlg2,² and that the cell specificities of Luk and Hlg are decided by LukS and Hlg2 respectively.¹ Recently, we resolved the three-dimensional structure of LukF and clarified the assembly of LukF and Hlg2 monomers into hetero-oligomers on membranes of human erythrocytes using a single-molecule fluorescence imaging technique.³,4 Eleven sequential equilibrium constants for the assembly pathway, which includes initial membrane binding of monomers, single pore oligomerization, and the formation of clusters of pores, were estimated. Furthermore, we found that Trp177 and Arg198 residues of LukF are essential for proper pore-formation by staphylococcal γ-hemolysin.⁵ In Luk, LukF and LukS assemble on membranes of human polymorphonuclear leukocytes (HPMNLs) into ring-shaped hetero-oligomers of more than 100-kDa forming a transmembrane pore with a functional diameter of 1.9–2.1 nm.⁶,⁷ The binding of LukS to the HPMNL plasma membrane is prerequisite for sequential binding of LukF to express the leukocytolytic activity of Luk.¹ Nevertheless, interaction between Luk components and HPMNLs to form hetero-oligomers has not yet been studied. Up to now, most studies on Luk have focused on the functions of the LukS component. For example, (i) LukS binds to monosialoganglioside GM₁ (GM₁) on the leukocyte membrane;¹⁰ (ii) Trp²⁷⁵ residue in LukS is an essential amino acid residue for LukS binding to GM₁ and this binding is accompanied with a conformational change in LukS;¹¹,¹² (iii) the 5-residue segment Ile²⁴²-Lys-Arg-Thr-Thr²⁴⁶ is a minimum segment responsible for the LukS specific function of Luk; and (iv) phosphorylation of LukS at Thr²⁴⁶ residue in the 5-residue segment on HPMNLs as well as pore-formation of Luk on HPMNLs is essential for expression of the leukocytolytic activity of Luk, besides its pore formation on HPMNLs.¹³–¹⁵ These findings suggest that a certain signaling system plays a role in the leukocytolytic activity of Luk and that the interaction of LukS with a leukocyte surface

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protein(s) as well as Gm1 is crucial for the complete leukocytolytic activity of Luk. Therefore, it is of interest to know if there are keys for such interaction between LukS and membranes of leukocyte. For better understanding of the mechanism of oligomerization on the HPMNLs surface, if there is any, entities on the leukocyte surface directly involved in the assembly of Luk into hetero-oligomer forming transmembrane pore, should be found.

Recently, detergent-resistant membrane micro lipid domains/rafts (lipid rafts), which are rich in cholesterol and glycosphingolipids, were found on the plasma membrane of mammalian cells.16,17 Gm1 is also one of the major components of lipid rafts.16 Moreover, it has also been demonstrated that glycosylphosphatidylinositol (GPI)-anchored proteins and other specific signaling molecules are localized in lipid rafts and that they play a pivotal role in signal transduction and endocytosis.16,18,19 It has been reported that several bacterial toxins, such as cholera toxin B subunit,20,21 Shiga toxin,22 and pertussis toxin23 specifically bind to lipid rafts for oligomerization into the membrane pore and then internalize into the target cells by endocytosis. A recent study by us suggested the presence of a protease-sensitive LukS receptor(s) on HPMNLs surface, causing a lack of assembly of Luk components into hetero-oligomeric pore complexes by treatment of HPMNLs with protease (unpublished data). These findings strongly suggest that LukS interacts with a certain cell surface protein as well as Gm1 on HPMNLs surface and that interaction of Luk components with HPMNLs to form pore complex occurs in lipid rafts. Here, we identified MD-2, an accessory protein of the extracellular domain of Toll-like receptor 4 in lipid rafts, as the cell surface molecule associated with LukS by yeast two-hybrid screening, and demonstrated that LukS and LukF assemble into hetero-oligomeric pores on lipid rafts in HPMNLs.

Materials and Methods

Toxin components, reagents, and antibodies. LukS and LukF were purified from the culture supernatant of S. aureus Smith 5R, as described previously.24 Methyl-\(\beta\)-cyclodextrin (MBCD) was purchased from Sigma (St. Louis, MO). Sheep polyclonal anti-human CD14 antibody was purchased from Genzyme Technica (Minneapolis, MN). Other chemicals, including detergents, were obtained from Wako Pure Chemicals (Osaka, Japan).

Yeast two-hybrid screening. Vectors, yeast strains, a human leukocyte cDNA library, and reagents were purchased from Clontech (Palo Alto, CA). To express the GAL4 DNA binding domain (GAL4-BD) fused to the C-terminal 57-residue segment (between the Glu228 residue and the C-terminus) of LukS in yeast, a bait plasmid was constructed by the following procedure: A DNA fragment coding for the C-terminal 57-residue segment of LukS was amplified by PCR using a pair of primers, LS228-F 5'-GATAACGCGAATTCGAAA-TTACTTACG-G-3' and LS286-R 5'-TGACTGTGTC-GACTTAATTCTGTCCTTTACC-3' (single and double underlined sequences represent the EcoRI and SalI restriction sites respectively) with the full length of the lukS gene as a template. The amplified fragment was cleaved at primer-derived EcoRI and SalI sites, and inserted into the EcoRI and SalI sites of a plasmid, pGBT9. The nucleotide sequencing of the inserted DNA fragment was done, and found to be identical with corresponding region of lukS.25 This plasmid was designated pGBD-228LS286, and was used as a bait plasmid. Saccharomyces cerevisiae AH109, which has a reporter plasmid, was transformed with both pGBD-228LS286 and the MATCHMAKER human leukocyte cDNA library fused to the GAL4 activation domain (GAL4-AD), according to the standard yeast transformation protocols.26 Detergent treatment of cell membranes from Luk-treated HPMNLs. Cell membranes from HPMNLs (1 \(\times\) 10\(^6\) cells/ml), which were treated with LukS (30 pmol/1 \(\times\) 10\(^6\) cells) and LukF (30 pmol/1 \(\times\) 10\(^6\) cells) for 10 min at 37\(^\circ\)C, were incubated with either 1% Brij 35, 1% Brij 58, or 1% Triton X-100 on ice for 30 min in TBS buffer containing 5 mM EDTA, 1 mM PMSF, 20 microgram/ml leupeptin hemisulfate (buffer A), and centrifuged at 15,000 \(\times\) g for 5 min to obtain supernatant and pellet. The supernatant and pellet fractions were individually mixed with 2% SDS, heated at 100\(^\circ\)C for 5 min, and analyzed by SDS–PAGE using 12.5% polyacrylamide gel. The proteins in the gel were blotted onto a poly-vinylidene difluoride membrane for 1 h and immunostained using anti-LukS antibodies raised against LukS.

Isolation of HPMNL lipid rafts. Plasma membrane preparation from HPMNLs was solubilized on ice for 30 min with buffer A containing 1% Brij 35. The cell lysate was brought into 40% sucrose (w/v) in a final volume of 2 ml, placed at the bottom of a 5-ml
ultracentrifuge tube, and overlaid with 2 ml of 36% and 1 ml of 5% sucrose in lysis buffer without detergent at 4 °C. Gradients were centrifuged to equilibrium at 200,000 × g for 20 h at 4 °C using a Hitachi RPS85T-2 rotor to separate the lipid raft fractions from non-lipid raft fractions, and 400-μl fractions were collected from the top of the gradient. Part of each fraction was incubated with 1% SDS and analyzed by SDS-PAGE and Western immuno-blotting for detection of CD14. Low-density fractions, which were rich in CD14, were defined as lipid raft fractions. The lipid raft fractions were collected and dialyzed against TBS buffer without detergent. The blotting efficiency of the ring-shaped hetero oligomers of more than 100 kDa was calculated to be about 20%, using an isolated single pore with hetero oligomer.7)

**Assembly of Luk on HPMNL lipid rafts.** Lipid rafts and non-lipid raft fractions were prepared from Luk-treated HPMNLs as described above. Both fractions were incubated with 1% SDS and analyzed by SDS-PAGE and Western blotting, using anti-LukS and anti-LukF antibodies after incubation with 1% SDS for 5 min at 100 °C for monomers and 20 °C for oligomer complex detection.15) CD14 was also detected as a marker of lipid rafts.

**Measurement of leukocytolytic activity.** Assay of leukocytolytic activity of Luk on HPMNLs was done essentially according to the methods described previously.12) HPMNLs (1 × 10⁶ cells/ml) were treated with LukS and LukF (30 pmol/1 × 10⁶ cells each) on a slide glass at 37 °C for 30 min under 100% humidity. After trypan blue staining, blue cells were defined as lysed cells. To determine the effects of MBCD on the leukocytolytic activity of Luk, the cells were treated with several concentrations of MBCD at 37 °C for 30 min under 100% humidity, and both LukS and LukF were added to each well, followed by incubation at 37 °C for 30 min. The percentage of Luk activity in the absence of MBCD was calculated from the ratio of Luk activity in the presence of MBCD.

**Isolation and observation of ring-shaped LukS and LukF complexes from the cell membrane preparation.** Luk complexes from cell membranes of HPMNLs were isolated essentially as described previously.7) Cell membranes and lipid raft fractions obtained from the Luk-treated HPMNLs were negatively stained with 1% sodium phosphotungstic acid, pH 7.4, and examined under a Hitachi electron microscope H-8100 (Hitachi, Tokyo) at an acceleration of 100 kV.7)

**Miscellaneous.** Protein was measured by the method of Bradford using bovine serum albumin as a standard.28) Unless otherwise stated, the chemicals used in this study were of the best grade commercially available.

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**Results**

**Identification of a protein potentially bound to LukS by yeast two-hybrid screening.** To identify the proteins that associate with LukS on the cell surface, yeast two-hybrid screening was done using the C-terminal segment between the Glu²⁷⁸ residue and the C-terminus of LukS, which was designated 228LS286, as a screening bait, because the C-terminal region of LukS contains a Lys²⁴⁴-Arg-Ser-Thr²⁵⁰ segment, which is crucial for LukS-specific function, and in which Thr²⁴⁶ residue is phosphorylated when LukS binds onto HPMNL plasma membrane.13,14) and Trp²⁷⁵ residue, an essential amino acid residue for the association of LukS with GMI.11,12) From the crystal structure of LukS-PV water-soluble monomer,13) which shows 85% identity and 91% similarity in primary structure with that of LukS,1) the 228LS286 segment of LukS might span the cap and rim domains (extracellular domain localized close to plasma membrane) of the LukS monomer. Furthermore, the Lys-Arg-Ser-Thr sequence in this segment might be localized at the bottom of the rim domain.4,15,30) Therefore, a plasmid, pGBD-228LS286, which contains the C-terminal 57-residue segment of LukS fused to the GAL4-DNA binding domain, was constructed, and the Saccharomyces cerevisiae AH109 was transformed with this plasmid and the MATCHMAKER human leukocyte cDNA library fused to the GAL4-activation domain. The 1 × 10⁷ independent clones were applied for the screening, and two positive clones, which grew on SD/-Leu, -Trp, -His, and -Ade plates and formed blue colonies in β-galactosidase colony-lift assay, were obtained (data not shown). S. cerevisiae AH109 transformed with either pGBD-228LS286 or cDNA library plasmids obtained from the positive clones grew neither on SD/-Leu, -Trp, -His, and -Ade plates, nor did they form blue colonies in β-galactosidase colony-lift filter assay. Sequencing analysis and a GenBank database search revealed that two cDNA clones coded for MD-2.

**Effect of dysfunctioning agents to lipid raft on the leukocytolytic activity of Luk.** It is known that MD-2/TLR4 complex interacts with CD14/LPS complex.31,32) CD14 is one of the residents of lipid rafts, and MD-2/TLR 4 complex moves into lipid rafts from detergent-soluble membrane in response to LPS.31) In addition, GMI, which can be associated with LukS both on the cell surface and in cell-free systems, is also known to be one of the major molecules of lipid rafts.10-12) These findings suggest that lipid rafts play a crucial role in the leukocytolytic activity of Luk. To clarify this hypothesis, the effect of MBCD, which is known to be a disruptive agent on lipid rafts by extraction of cholesterol from lipid rafts, on the leukocytolytic activity of Luk was examined. Five mM or 10 mM MBCD inhibited the cytolytic activity of Luk by 24% and 90% respectively.
Assembly of LukS and LukF into pore complex on lipid rafts of HPMNL plasma membranes

In previous studies, we reported that the pore complex of Luk from the membrane of Luk-treated HPMNLs was detected by SDS–PAGE after the membrane was solubilized with 1% SDS at room temperature. If the pore complex of Luk interacts with lipid rafts, Luk can be separated as pore-lipid raft complexes from HPMNL membranes. Solubility of Luk on Luk-treated HPMNLs by Brij 35, Brij 58, or Triton X-100 (1% each), all of which are known to be detergents for recovery of lipid rafts in pellet fraction upon centrifugation of lysed cells, was investigated. The solubilized cells were centrifuged to obtain supernatant and pellet fractions, and then the Luk components in both fractions were analyzed by SDS–PAGE and Western blot. About 90%, 86%, and 68% of LukS and LukF were detected in the pellet fractions in the Brij 35-, Brij 58-, and Triton X-100-treated samples respectively. These findings strongly suggest that Luk associates with lipid rafts in plasma membrane. To prove this, plasma membranes were prepared from HPMNLs treated with LukS alone, LukF alone, or both, and incubated with Brij 35. The incubation mixture was subjected to a sucrose-density gradient centrifugation, fractionized, and then analyzed by Western blotting for the Luk components, as described above. In HPMNLs which were treated with LukS alone, about 35% and 65% of the membrane-bound LukS distributed into the lipid rafts and non-lipid rafts fractions respectively (Fig. 1, panels A and E). In contrast, in HPMNLs treated with LukF alone, LukF was detected in the non-lipid raft fraction but never detected in the lipid raft fraction (Fig. 1, panels B and F). In HPMNLs incubated with both LukS and LukF, 60% of each component was detected in lipid raft fractions (Fig. 1, panels C, E, and F), and the relative percentages of LukS and LukF distributed in non-lipid raft fractions were deduced by 50% and 37% of the total amount of membrane-bound LukS and LukF respectively. Taken together with the finding that LukS binding is a prerequisite for the sequential binding of LukF to the leukocytolytic activity of Luk, the data suggest that binding of LukS to the specific receptor on the lipid rafts is prerequisite for the sequential binding of LukF to the assembly of Luk into pore-complexes on lipid rafts in HPMNLs. Accordingly, we examined for assembly of LukS and LukF into hetero-oligomer complexes in lipid rafts fractions from HPMNLs treated with Luk. The Brij 35-treated membrane preparation from Luk-treated HPMNLs was subjected to a sucrose-density gradient centrifugation and fractionated. Fractions were then treated with 1% SDS at 20 °C, and analyzed for LukS and LukF by SDS–PAGE, followed by Western immunoblot with both anti-LukS and -LukF antibodies. As shown in Fig. 2, more than 90% of high molecular-mass complexes of 100 kDa, which consists of LukS and LukF, were detected in the lipid raft fractions (Fig. 2, panels A and B), while no LukF monomer was detected in lipid raft fractions. As the lipid raft fractions (nos. 2–5 in Fig. 2) were combined and subjected to electron microscopic analysis, ring-shaped pore complexes with inner and outer diameters of 2 and 9 nm respectively were clearly observed (data not shown). In contrast, both LukS and LukF, which were distributed in non-lipid raft fractions, were recovered as monomers (nos. 6–12 in Fig. 2). The data indicated that almost all of both LukS and LukF, which existed in the lipid raft fractions, assemble into hetero-oligomers forming membrane pores, and that LukF can bind the non-lipid raft area of membrane but cannot assemble with LukS into hetero-oligomers for pore-formation. Hence we concluded that assembly of LukS and LukF into the pore-complex occurs in lipid rafts in HPMNLs, and that LukF can bind to LukS, already bound to lipid rafts, to assemble into hetero-oligomers.

Discussion

In this study, we assayed association of LukS and LukF with lipid rafts for the leukocytolytic activity of Luk. Our data indicate that when HPMNLs are treated with LukS alone, a small part of LukS associates with lipid rafts on HPMNL plasma membrane (Fig. 1). Both LukS and LukF were detected in lipid rafts as pore complex when HPMNLs were treated with both LukS and LukF (Figs. 1 and 2). But no LukF associated with lipid rafts when HPMNLs were treated with LukF alone. Recently, it has been reported that many bacterial toxins utilize lipid rafts as sites for initial recognition, oligomerization, and/or internalization. The cholera toxin B subunit, whose receptor is GM1, is known to bind to lipid rafts and to be internalized in the target cell. Lipid rafts are domains of 50 to 100 nm diameter which are rich in glycosphingolipids and cholesterol. Cholesterol and saturated fatty acid chains of glycosphingolipids make the lipid bilayer of lipid rafts tight in comparison with that of the surrounding membrane. This might allow the molecules trapped in these domains to stay in such a narrow area for relatively long times, and appears to allow the molecules in lipid rafts to assemble into high molecular weight complexes or functional signaling complexes. In addition, we found that MD-2, which is localized on the leukocyte cell surface by association with the extracellular domain of TLR4, associates with the C-terminal domain of LukS by yeast two-hybrid analysis. TLR4/MD-2 complex specifically associates with LPS-CD14 complex on lipid rafts and participates in LPS-induced signaling. The profile of sucrose density-gradient centrifugation of MD-2/TLR4 complex under LPS-stimulation is similar to that of LukS obtained when HPMNLs are treated with LukS alone. Therefore, it is reasonable that LukS associates with lipid rafts and assemblies into pore-complex with LukF there. As Figs. 1 and 2 show, in sucrose density-gradient
centrifugation, a major portion of LukS was detected in high density fractions in which detergent-soluble membrane components when HPMNLs were treated with LukS alone. On the other hand, the amounts of LukS and LukF detected in lipid raft fractions increased, and most of the pore-complexes consisting of LukS and LukF were detected in lipid raft fractions when HPMNLs were treated with both LukS and LukF. This pore-complex accumulation into lipid rafts was also observed when LukS was washed out from the reaction mixture prior to treatment of LukS-bound HPMNLs with LukF (data not shown). Furthermore, our previous data showed that binding of LukS to Gmt induced conformational change of LukS in vitro. These findings suggest the following mechanism of target-cell recognition and pore-formation by Luk: LukS can bind directly to lipid rafts through

Fig. 1. Distribution of LukS and LukF in Lipid Rafts Prepared from HPMNLs Treated with LukS Alone, LukF Alone, or Both.

HPMNLs were incubated with LukS alone (A), LukF alone (B), or both (C) at 37 °C for 30 min, and solubilized with Brij 35. The cell lysates were subjected to sucrose-density gradient centrifugation, as described in “Materials and Methods.” The fractions were boiled with 1% SDS and analyzed for LukS and LukF by SDS–PAGE and Western immunoblot using specific antibodies raised against LukS (A and C), LukF (B and C), and CD14, a marker protein of lipid rafts (D). Panel E shows the schematic distribution of LukS in sucrose density-gradient fractions in the sample treated with LukS alone (white bars) and both LukS and LukF (black bars). Panel F represents the schematic distribution of LukF in sucrose-density gradient fractions in the sample treated with LukF alone (gray bars) and both LukS and LukF (black bars). Relative intensities (%) of LukS or LukF in the sucrose-density gradient fractions were calculated from the ratio of the intensity of LukS or LukF in the fractions to the total intensities of each component.
GM1, or LukS can be recruited to GM1 via the TLR4/MD-2 complex in lipid rafts. Interaction of GM1 and LukS induces conformational change in LukS, and conformation-changed LukS can act as a high-affinity receptor for LukF. This situation might allow further accumulation of LukS and LukF, resulting in pore formation and accumulation of pore-complex of Luk in lipid rafts.

In addition to pore-formation, phosphorylation of LukS on the HMPNL surface is an event essential to Luk activity. In our previous studies, we found that phosphorylation of LukS at the Thr246 residue on the HMPNL surface is essential for Luk function to cause leukocytolysis when HPMNLs are treated not only in lipid rafts, since LukS is phosphorylated on the HMPNL surface when HPMNLs were treated with Luk (data not shown), but it is still unknown whether LukS actually binds to the TLR4/MD-2 complex on the HMPNL surface. For further elucidation, the binding of LukS to functional MD-2 and the relationship between LukS and the MD-2 mediated signaling mechanism for induction of inflammatory cytokines, must be examined.

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References

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