Assembly of the Bi-component Leukocidin Pore Examined by Truncation Mutagenesis

Received for publication, October 4, 2005, and in revised form, October 28, 2005. Published, JBC Papers in Press, November 3, 2005, DOI 10.1074/jbc.M510842200

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Staphylococcal leukocidin (Luk) and α-hemolysin (αHL) are members of the same family of β barrel pore-forming toxins (βPFTs). Although the αHL pore is a homoheptamer, the Luk pore is formed by the co-assembly of four copies each of the two distantly related polypeptides, LukF and LukS, to form an octamer. Here, we examine N- and C-terminal truncation mutants of LukF and LukS. LukF subunits missing up to nineteen N-terminal amino acids are capable of producing stable, functional hetero-oligomers with WT LukS. LukS subunits missing up to fourteen N-terminal amino acids perform similarly in combination with WT LukF. Further, the simultaneous truncation of both LukF and LukS is tolerated. Both Luk subunits are vulnerable to short deletions at the C terminus. Interestingly, the N terminus of the LukS polypeptide becomes resistant to proteolytic digestion in the fully assembled Luk pore while the N terminus of LukF remains in an exposed conformation. The results from this work and related experiments on αHL suggest that, although the N termini of βPFTs may undergo reorganization during assembly, they are dispensable for the formation of functional pores.

β barrel pore-forming toxins (βPFTs)3 are secreted by bacteria as water-soluble polypeptides that bind to the surfaces of susceptible cells and assemble into oligomeric transmembrane pores. One family of βPFTs includes staphylococcal α-hemolysin (αHL) and the leukocidins (Luk) (1–4). The pathophysiological effects of these proteins have been attributed to pore formation on target cells, leading to cell permeation or lysis. Leukocidin, in particular, has received widespread attention as an important virulence factor in wound and soft tissue infections (5, 6).

αHL, a βPFT comprising a single polypeptide of 293 residues, forms a homo-heptameric pore (7). In contrast, leukocidin is a bi-component toxin (3, 4); two distinct proteins are required to form a functional pore, one component from class F (LukF) and the other from class S (LukS). The leukocidins primarily attack polymorphonuclear cells, monocytes, and macrophages but also assemble on erythrocytes (8, 9), liposomes (10), and planar lipid bilayers (9). There are at least six class-F proteins (LukF-PV, LukF-R, LukD, LukF*-PV, LukF, and LukF-I) and seven class-S proteins (LukS-PV, LukS-R, LukE, LukM, γHLII, LukS, and LukS-I) associated with various strains of Staphylococcus aureus (2, 11, 12). The F and S proteins share a common ancestor (11). Proteins within each class (F or S) share ~70% identity at amino acid level, whereas the identity drops to <27% between members of the two different classes (1, 2). No member of either class is >30% identical to αHL (2, 13). It is believed that the various F and S components are capable of mixing and matching, thereby creating a diverse repertoire of pores (2, 14). Although hexameric and heptameric structures have been proposed for the Luk oligomer (10, 15–18), recent work suggests that Luk pores are octamers with four LukF and four LukS subunits arranged in alternating fashion around the central axis (19, 20).

Although the structure of a Luk oligomer is yet to be solved, the three-dimensional structures of the water-soluble monomeric forms of two class F components, LukF (hlgB gene product) and LukF-PV, and one class S component, LukS-PV, have been determined by x-ray crystallography. The LukF structure has been solved at 1.8-Å and 2.3-Å resolution (Fig. 1) (16). Excluding the amino latches and putative pre-stem regions, the protein displays a fold that is closely similar to the fold of an individual αHL protomer within the structure of the heptameric αHL pore (Fig. 1) (7). The LukF-PV structure has been solved at 2.0-Å resolution and is almost identical to LukF (17). The LukS-PV structure has been determined recently at 2.0-Å resolution and has a similar fold to that of LukF, although the rim domain is in a significantly different conformation (Fig. 1) (12). Together, the structures of the leukocidin monomers and the αHL heptamer represent beginning and end points in βPFT assembly. Additional evidence suggests that these proteins bind to membranes as monomers, associate on the membrane surface to form oligomeric pre pores, and finally insert into the lipid bilayer (7, 16, 21–23) (see Fig. 1B in the preceding paper (36)).

It has been proposed that the N termini of these βPFTs are crucial for the formation of functional pores (21). The N termini are believed to reorganize during the insertion step to form a “latch” that reinforces the interactions between neighboring subunits (7, 16). However, recent work from our laboratory suggests that, whereas this can occur, it is not required for pore formation in the case of αHL (Jayasinghe et al., preceding report (36)). Here, we use truncation mutagenesis to examine the contributions of the N and C termini of both LukF and LukS to the assembly of the leukocidin pore. We find the following: 1) Neither N terminus is required for pore formation; therefore, latch formation is not necessary for assembly of the pore. 2) The N termini of LukF and LukS reside in different conformation in the WT Luk pore: one is in the latch conformation and the other is not. 3) The C termini of LukF and LukS must be intact for efficient oligomerization.
EXPERIMENTAL PROCEDURES

Deletion Mutagenesis—All constructs were made in the pT7-SC1 expression vector (9, 24) and verified by DNA sequencing of the entire genes. Genes encoding a series of N- and C-terminal truncation mutants of LukF (hlgB gene product) (Table 1) and LukS (hlgC gene product) (Table 2) (9) were made by PCR mutagenesis and ligation-free in vivo recombination as described elsewhere (25, 26). Each forward mutagenic primer for N-terminal truncation contained an NdeI site at the initiation codon followed by an alanine codon, except for LukF-N_{H9004} (1) and LukS-N_{H9004} (1). In Vitro Transcription and Translation—All proteins were generated by coupled in vitro transcription and translation (IVTT) by using an Escherichia coli T7-S30 extract optimized for circular DNA (Promega, no. L1130). The complete 1 mM amino acid mixture minus methionine (2.5 μl) or the complete 1 mM amino acid mixture minus cysteine (2.5 μl) that was supplied in the kit was mixed with premix solution (10 μl), [L-35S]methionine (1 μl, ICN Biomedicals, Inc., Irvine, CA, 1175 Ci/mmol) or [35S]cysteine (1 μl, Amersham Biosciences, 1200 Ci/mmol), plasmid DNA (4 μl, 400 ng/μl), and T7-S30 extract (7.5 μl) supplemented with rifampicin (20 μg/ml final) to generate “hot” radiolabeled polypeptides (25 μl) (24). Synthesis was carried out for 60 min at 30 °C, and reactions were terminated with chloramphenicol (100 μM final). The complete amino acid mixture minus cysteine and the complete amino acid mixture minus methionine were mixed in equal volumes and replaced the individual amino acid mixture minus methionine (or cysteine) (2.5 μl) to yield “warm” radiolabeled proteins. Unlabeled (cold) proteins were generated by replacing the [L-35S]methionine or [35S]cysteine (1 μl) with water (1 μl).

Oligomer Formation on Rabbit Erythrocyte Membranes—[35S]Met-labeled truncated polypeptide (IVTT, 25 μl) and its unlabeled wild-type counterpart (IVTT, 25 μl) (e.g. [35S]Met-labeled truncated LukF and unlabeled WT LukS), or in some cases both [35S]Met-labeled truncated components, were mixed and incubated with rRBC membranes (10 μl, 3.0 mg of protein/ml) (27) in MBSA (10 mM 3-N-morpholino)propane sulfonic acid (MOPS), 150 mM NaCl, pH 7.4, containing 1 mg/ml bovine serum albumin), in a final volume of 100
μl. After 1 h at 25 °C, the mixture was centrifuged, and the supernatant discarded. The membrane pellet was washed and resuspended in MBSA (80 μl) prior to solubilization by the addition of 5× Laemmli sample buffer (20 μl) (28). A portion (20 μl) was subjected to electrophoresis in a 10% SDS-polyacrylamide gel. The gel was fixed in destaining solution (40% methanol, 10% acetic acid, 50% H2O) for 1 h prior to drying and autoradiography. Radiolabeled markers (14C-methylated proteins, no. CFA626) were from Amersham Biosciences: myosin (M, 220,000), phosphorylase b (M, 97,400), bovine serum albumin (M, 66,000), ovalbumin (M, 46,000), carbonic anhydrase (M, 30,000), and lysozyme (M, 14,300).

Quantitative Hemolysis Assay—LukF, LukS, and the truncated polypeptides (IVTT, 5 μl of each LukF and LukS component) were diluted into MBSA (90 μl) in the first well of each column or row of a 96-well microtiter plate. The proteins were then subjected to eight or twelve 2-fold serial dilutions from top to bottom are shown, beginning with 5 μl each of truncated LukF and LukS in the first well (see “Experimental Procedures”). Hemolysis was recorded for 2 h at 25 °C by monitoring the decrease in light scattering at 595 nm with a Bio-Rad microplate reader (Model 3550-UV) and the Microplate Manager 4.0 software.

Trypsin Treatment of Polypeptides on Membranes—Trypsin solutions (5.0, 0.5, and 0.05 mg/ml in water, Sigma, T-7309) were prepared by dilution of an enzyme stock (10 mg/ml in water) and used immediately. Wild-type LukF, wild-type LukS, truncated variants, and αHL were allowed bind to rRBC membranes before limited proteolysis was performed. The membranes were resuspended in MBSA (0.19 mg of membrane protein per ml) and divided into four tubes (18 μl in each). Trypsin or water (2 μl) was added to each tube. After 5 min at room temperature, the reactions were stopped by treatment with phenylmethylsulfonyl fluoride (9 mM final, added in 2 μl of isopropanol) for 5 min at room temperature, followed by the addition of 2× Laemmli loading buffer. The samples were subjected to electrophoresis in 10% SDS-polyacrylamide gels.

FIGURE 2. Oligomerization and hemolytic activity of LukF N-terminal truncation mutants. A, oligomer formation by LukF N-terminal truncation mutants examined by SDS-polyacrylamide gel electrophoresis. [35S]Met-labeled, truncated LukF polypeptides and unlabeled WT LukS were synthesized by IVTT. The two proteins were then mixed and incubated in the presence of rRBC membranes. An autoradiogram of a 10% SDS-polyacrylamide gel displaying the membrane-bound proteins is shown. Bands containing the Luk oligomer and the LukF monomer are indicated. B, quantitative hemolysis assays of LukF N-terminal truncation mutants in the presence of WT LukS. Both proteins were synthesized by IVTT. The assay was carried out for 2 h. Two-fold serial dilutions from top to bottom are shown, beginning with 5 μl each of truncated LukF and LukS in the first well (see “Experimental Procedures”).
**Hemolytic Activity and Oligomerization of LukF N-terminal Truncation Mutants**—In a similar fashion, a series of N-terminal truncation mutants of LukS labeled with \[^{35}\text{S}\]Cys (warm, see "Experimental Procedures") were produced with deletions ranging from 1 to 36 amino acid residues (Table 2). The truncated LukS polypeptides were tested for their ability to form SDS-stable oligomers on rabbit erythrocyte membranes with unlabeled WT LukF (9). As in the case of the LukF truncations, all of the LukS mutants were capable of binding to rRBC membranes. SDS-stable oligomers were observed with N-terminal deletions of up to 14 residues, with the exception of LukS-NΔ8 (Fig. 3A). However, no oligomers could be seen with deletions of 15 or more amino acids (LukS-NΔ15 to NΔ18).

Hemolytic activity similar to WT LukS was seen with N-terminal truncations of up to 14 residues, with the exception of LukS-NΔ8, in which case the activity was decreased at least by 15-fold (Fig. 3B). LukS-NΔ15 displayed a more pronounced lag in lysis (−3-fold increase in lag time, defined as the time to lysis of 5% of a red blood cell suspension). No hemolysis was recorded with truncations of 16 or more residues, even when the microtiter plates were examined 24 h later (data not shown).

**Hemolytic Activity and Oligomerization of Mixed LukS and LukF N-terminal Truncation Mutants**—To determine whether leukocidin could tolerate truncations of both components simultaneously, we studied the pore formed by LukF-NΔ16 and LukS-NΔ14. LukF-NΔ16 assembled with LukS-NΔ14 to form stable oligomers, albeit less efficiently than the WT subunits or one truncated subunit with its WT partner (Fig. 4A, lane 2). The hemolytic activity of the pair was at least 8-fold less than that of the WT components and the initial lag time was increased significantly (Fig. 4B).

**Conformations of Leukocidin Polypeptides Probed by Limited Proteolysis**—Trypsin has previously been shown to cleave αHL in solution at Lys-8 near the N terminus and at Lys-131 in the pre-stem domain, and has been used to probe the conformational changes at various stages of pore assembly (30, 31). The N terminus of αHL remains accessible in the membrane-bound monomer and in the non-lytic preprotease state (21, 22). Upon insertion of the stem domain to form the transmembrane β barrel, the N terminus becomes occluded within the heptameric pore and is protease resistant (22).

However, the conformations of the N termini of Luk proteins in the assembled pore have not been studied thoroughly. We therefore probed the LukF and LukS polypeptides by proteolysis with trypsin and compared the results to those obtained with αHL. The N terminus of LukS was labeled by generating the LukS-D3C mutant by IVTT in the presence of \[^{35}\text{S}\]Cys. Although the first trypsin cleavage site of LukF lies before the marker (Lys-4), cleavage sites at positions 11, 12, and 16 are in proximity to the marker (Fig. 5A). Therefore, the radiolabeled \[^{35}\text{S}\]Cys at position three acts as a marker for an intact N terminus of LukS (24). Sequence analysis and comparison of the crystal structures of the monomers revealed that the position equivalent to LukS-D3 is occupied by Ser-9 in LukF (Fig. 5A). Therefore, we also labeled the N terminus of LukF by generating the mutant LukF-S9C by IVTT, in the presence of \[^{35}\text{S}\]Cys. Although the first trypsin cleavage site of LukF lies before the marker (Lys-4), cleavage sites at positions 11, 12, and 16 are positioned after it (Fig. 5A and B). Therefore, the radiolabeled \[^{35}\text{S}\]Cys at position 9 acts as a marker for an intact N terminus in LukF. As a control, we also generated \[^{35}\text{S}\]CysαHL-S3C and observed the cleavage at Lys-8 (Fig. 5A and B). WT αHL, WT LukF, and WT LukS contain no cysteine residues, and cysteines placed at the designated sites have no adverse effects on activity or oligomerization.

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**Truncation Mutagenesis of Staphylococcal Leukocidin**

| TABLE 2 | Nomenclature and sequences of LukS N- and C-terminal truncation mutants |
|---------|--------------------------------------------------------------------------------|
| The polypeptides are deformylated, the initial Ala (in all the N-terminal truncation mutants except LukF-NΔ1) would ensure the efficient co-translational removal of the N-terminal methionine (21). However, we do not know whether our IVTT procedures contain Met or Met at the N terminus. |

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**RESULTS**

**Hemolytic Activity and Oligomerization Properties of LukF N-terminal Truncation Mutants**—Genes encoding truncation mutants of LukF in which the first 22 residues were removed one-by-one (Table 1) were generated by ligation-free in vivo recombination. The corresponding \[^{35}\text{S}\]Met-labeled "warm" proteins were prepared by coupled IVTT by using an S30 extract from *E. coli* (29). The truncated LukF polypeptides were tested for the ability to form SDS-stable oligomers on rRBC membranes with unlabeled (cold) wild-type LukS (9). The lytic activity of leukocidin toward rRBC is normally at least 500-fold lower than that of the WT components and the initial lag time was increased significantly (Fig. 4B).

**Conformations of Leukocidin Polypeptides Probed by Limited Proteolysis**—Trypsin has previously been shown to cleave αHL in solution at Lys-8 near the N terminus and at Lys-131 in the pre-stem domain, and has been used to probe the conformational changes at various stages of pore assembly (30, 31). The N terminus of αHL remains accessible in the membrane-bound monomer and in the non-lytic preprotease state (21, 22). Upon insertion of the stem domain to form the transmembrane β barrel, the N terminus becomes occluded within the heptameric pore and is protease resistant (22).

However, the conformations of the N termini of Luk proteins in the assembled pore have not been studied thoroughly. We therefore probed the LukF and LukS polypeptides by proteolysis with trypsin and compared the results to those obtained with αHL. The N terminus of LukS was labeled by generating the LukS-D3C mutant by IVTT in the presence of \[^{35}\text{S}\]Cys. Although the first trypsin cleavage site of LukF lies before the marker (Lys-4), cleavage sites at positions 11, 12, and 16 are in proximity to the marker (Fig. 5A). Therefore, the radiolabeled \[^{35}\text{S}\]Cys at position three acts as a marker for an intact N terminus of LukS (24). Sequence analysis and comparison of the crystal structures of the monomers revealed that the position equivalent to LukS-D3 is occupied by Ser-9 in LukF (Fig. 5A). Therefore, we also labeled the N terminus of LukF by generating the mutant LukF-S9C by IVTT, in the presence of \[^{35}\text{S}\]Cys. Although the first trypsin cleavage site of LukF lies before the marker (Lys-4), cleavage sites at positions 11, 12, and 16 are positioned after it (Fig. 5A and B). Therefore, the radiolabeled \[^{35}\text{S}\]Cys at position 9 acts as a marker for an intact N terminus in LukF. As a control, we also generated \[^{35}\text{S}\]CysαHL-S3C and observed the cleavage at Lys-8 (Fig. 5A and B). WT αHL, WT LukF, and WT LukS contain no cysteine residues, and cysteines placed at the designated sites have no adverse effects on activity or oligomerization.
With increasing concentrations of trypsin, a gradual loss of radioactivity was observed in the monomeric form of αHL bound to red cell membranes, which is consistent with cleavage near the N terminus (lanes 9–12, Fig. 6). Similarly, both the LukF and LukS membrane-bound monomers were digested with the increasing concentrations of trypsin (lanes 1–8, Fig. 6). In agreement with previous studies, the radiolabel at position 3 was retained in the membrane-bound αHL oligomer, even at a trypsin concentration of 500 μg/ml. This result confirms that occlusion of the N terminus inside the pore (i.e. formation of the amino latch) makes the N terminus resistant to proteolysis (7). However, when LukF-S9C* was assembled with WT LukS on rRBC membranes, the radiolabel at position 9 of LukF was completely lost from the oligomer even at the 5 μg/ml trypsin concentration (lanes 1–4, Fig. 6). By contrast, when LukS-S3C* was assembled with WT LukF, the radiolabel at position 3 of LukS was retained in the oligomeric state even at the 500 μg/ml trypsin concentration (lanes 5–8, Fig. 6). In this case, a shift in the band of oligomers to higher mobility does indicate partial digestion of the assembled pore, which might be attributed to cleavage of the N terminus of LukF. We cannot exclude the possibility that the C terminus of one or both subunits is also digested.

**Properties of LukF and LukS C-terminal Truncation Mutants**—The C terminus of αHL is vital for the stability of the protein assembled on membranes (21). Mutants missing three or five amino acids at the C terminus are inefficient at oligomerization, but do lyse rRBCs, albeit extremely slowly. However, the role of the C terminus of both LukF and LukS has not been investigated in detail. When the sequence and monomeric structures of αHL and LukF are compared, LukF is found to have ten additional residues at the C terminus compared with αHL (Fig. 5, A and B). Therefore, five C-terminal deletion mutants were constructed for LukF involving the removal of 1, 4, 6, 7, and 10 residues (Table 1), and their abilities to form SDS-stable oligomers and to lyse rRBCs were evaluated. A loss of up to 4 residues from the C terminus yielded similar amounts of SDS-stable oligomer as WT LukF, when the truncated LukF subunits were assembled with WT LukS (Fig. 7A, lanes 1–3). Although the mutants had close to WT hemolytic activity, only a trace of the Luk oligomer was observed after the removal of 6 or 7 amino acids (Fig. 7A, lanes 4–5).
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FIGURE 4. Oligomer formation and lytic activity by combined LukF and LukS N-terminal truncation mutants. A, oligomer formation by LukF-NΔ16 with LukS-NΔ14 on erythrocyte membranes. The WT or truncated mutants (both components were labeled with [35S]Met) were assembled in the presence of rRBC membranes and subjected to 10% SDS-polyacrylamide gel electrophoresis and autoradiography. B, 2-h quantitative hemolytic assays of the subunit combinations shown in panel A. The first well contained 5 μl of each LukF and LukS component. Two-fold serial dilutions were carried out from left to right (see "Experimental Procedures").

lanes 4 and 5, Fig. 7B). Neither oligomerization nor hemolytic activity was detected after the deletion of 10 amino acids from the C terminus of LukF (LukF-CΔ291) (data not shown). Although the C terminus of LukF-CΔ291 is the same length as that of αHL, the deletion most likely resulted in an incorrectly folded polypeptide as the mutant was markedly susceptible to proteolysis (data not shown).

The C terminus of LukS is six residues shorter than that of LukF and four residues longer than that of αHL (Fig. 5, A and B). Therefore, three C-terminal deletion mutants were constructed for LukS involving the removal of 4, 6, and 8 residues (Table 2). The removal of 4 amino acids (LukS-CΔ283) produced an SDS-stable oligomer with WT LukF and the hemolytic activity of the pair resembled that of WT leukocidin (Fig. 7A, lane 7 and Fig. 7B). However, after deletions of 6 or more C-terminal residues, SDS-stable oligomers were not detected on rRBC membranes when the LukS mutants were assembled with WT LukF (Fig. 7A, lanes 8 and 9). Despite its inability to form a stable oligomer, LukS-CΔ281 (a deletion of 6 amino acids) did exhibit hemolytic activity of about 15-fold less than WT leukocidin (Fig. 7B). A pronounced lag in lysis, comparable to LukS-NΔ15, was also observed with this mutant. After the removal of eight residues (LukS-CΔ279), only very weak hemolysis was observed. We cannot exclude the possibility that the observed hemolytic activity of the latter two mutants is the result of undetected amounts of SDS-stable oligomers.

Premature Oligomerization and Co-oligomerization of N- and C-terminal Truncation Mutants—In the previous manuscript, we showed that mutants of αHL in part oligomerize in solution in the absence of rRBC membranes when more than three residues are removed from the N terminus. Therefore, we explored the possibility of premature oligomerization with the N- and C-terminal truncation mutants of LukS and LukF and found no detectable spontaneous oligomerization with their respective WT counterparts in the absence of membranes. Like WT LukF and WT LukS, none of the mutants were able, by themselves, to make homo-oligomers on rRBC membranes. In addition, neither the WT Luk polypeptides nor the N-terminal truncation mutants of LukF and LukS were able to co-oligomerize with αHL monomers (32).

DISCUSSION

Staphylococcal αHL and leukocidin are homologous βPFTs with similar structural features. An early analysis of truncation mutants of αHL suggested that the N terminus plays a crucial role in the transformation of the prepro to an active pore (21). However, as documented in the preceding report, by a thorough examination of a series of truncation mutants with up to 22 residues deleted, we demonstrated that the N terminus of αHL is in fact not necessary for pore formation (Jayasinghe et al., preceding paper (36)). During these studies, we found that the N terminus of αHL interacts indirectly with position 217 during the transformation of the prepro to a functional pore. Although a Ser → Asn mutation at this position does not affect pore formation by the full-length protein, the mutation arrested the assembly of the N-terminal truncation mutants at the prepro stage. This mutation was present in the original series of truncations (21).

The role of the N termini of LukF and LukS during pore formation has not been studied fully. The combination of LukF and LukS, as used in the present report, forms the Luk pore, which has leukocytolytic activity toward human and rabbit polymorphonuclear leukocytes and hemolytic activity toward rabbit erythrocytes (2). The combination of LukF and γHIL, a homologous class S component, forms the γ-hemolysin pore, which has hemolytic activity toward human and rabbit erythrocytes (33, 34). In an earlier study, Kaneko and coworkers (34) generated six LukF truncation mutants in which up to 22 residues were deleted from the N terminus. They reported a reduction to <10% leukocytolytic activity toward human leukocytes when a LukF mutant lacking the first
20 residues was assembled with WT LukS. The same LukF mutant also failed to show any detectable hemolytic activity on human erythrocytes with WT γHLII. It was further reported that a mutant lacking the first 22 residues of LukF was unable to bind to human erythrocytes as monomers. However, the study did not include any LukS truncation mutants or examine the hemolytic activity of the combined LukF and LukS subunits on rRBCs.

In the present study, we describe the effects of truncation mutagenesis on the oligomerization and pore-forming activity of the bi-component leukocidin toxin. We demonstrate that large deletions at the N terminus can be made on either component, LukF or LukS, without significantly altering the extent of formation or activity of the oligomer, which is an octamer with alternating F and S subunits (19, 20). The results are in general agreement with the properties of γHL truncation mutants, in which up to 17 N-terminal residues could be removed with retention of hemolytic activity (Jayasinghe et al., preceding paper (36)), although in the case of leukocidin the activity is weaker to begin with and does not drop off so rapidly with truncation. A deletion of 17 residues removes the residues that constitute the N-terminal latch of γHL (Fig. 5, A and B). When mapped onto the structures of monomeric LukF and LukS-PV (LukS-PV has an N terminus of the same length as LukS), deletions of 16 and 10 amino acid residues, respectively, constitute the removal of the N-terminal domains. When assembled with their wild-type counterparts, truncation mutants of up to 19 amino acid residues of LukF and 14 of LukS retained near wild-type lytic activity. Importantly, the leukocidin also tolerated the simultaneous removal of the entire N-terminal domains from both LukF and LukS; although the activity was lowered by at least 8-fold, a truncation mutant missing 16 residues from the N terminus of LukF was able to form a functional pore with a LukS mutant missing 14 residues from its N terminus. Therefore, our results indicate that, as in the case of αHL, the N termini of the Luk proteins are not required for pore formation. Functional pores could be formed after partial or complete loss of the N terminus in one or both of the components. Additionally, the finding that these βPFTs can tolerate large extensions at their N termini (20), without disruption of oligomerization or lytic activity, further strengthens the argument that the N terminus is not required to form an amino latch with the neighboring subunit in the oligomer.

Removal of residues beyond the first 19 of LukF or the first 14 of LukS resulted in significantly diminished extent of oligomer formation and activity. In both proteins, these deletions extend into the β1 strand comprising the inner face of the β-sandwich domain (Fig. 1) and are likely to cause misfolding. We examined this possibility by limited proteolysis and found that LukF mutants with more than 22 N-terminal residues removed and LukS mutants with more than 18 N-terminal residues removed were more susceptible to protease digestion (data not shown). Based on subunit-subunit interactions apparent in the structures of the αHL heptamer, the β1-β2-β3 strands of both LukF and LukS

FIGURE 5. Alignment and structural homology of the staphylococcal βPFTs. A, N- and C-terminal alignments of αHL, LukF, and LukS (12). The N terminus of each polypeptide is colored red, the C terminus is blue. Radiolabeled sites used in the limited proteolysis experiments are colored green. Underlined residues indicate potential trypsin cleavage sites near the N termini. B, structures of βPFTs with corresponding regions colored as in A. The LukS cleavage sites were mapped onto the LukS-PV structure (12). Arrows indicate potential trypsin cleavage sites.
form critical interfaces in the assembled pore, which when disrupted would prevent toxin assembly.

Limited proteolysis has been used to detect changes in the conformation of αHL. Trypsin, which digests polypeptides at the C-terminal side of Lys and Arg, cleaves the N terminus of αHL at Lys-8 in the watersoluble monomer. The cleavage site remains solvent-exposed and susceptible to trypsin until latch formation, which in the WT protein is coincident with membrane insertion of the stem domain (Fig. 8A, stage 4). However, in the preceding report (36), we demonstrated that in various mutants of αHL the formation of a protease-resistant latch from

![Figure 6. Conformational states of αHL and leukocidin examined by limited proteolysis.](image)

Freshly translated LukF-S9C, LukS-D3C, and αHL-S3C labeled with [35S]cysteine were allowed to bind to rRBC membranes. In the cases of the leukocidins, the unlabeled WT counterpart was included to ensure pore formation. The washed membranes were then treated with trypsin as follows (final concentrations): lanes 1, 5, and 9: 0 μg/ml; lanes 2, 6, and 10: 5 μg/ml; lanes 3, 7, and 11: 50 μg/ml; lanes 4, 8, and 12: 500 μg/ml. An autoradiogram of a 10% SDS-polyacrylamide gel is shown.

![Figure 7. Oligomer formation and hemolytic activity of leukocidin C-terminal truncation mutants.](image)

**A**, oligomer formation by [35S]Met-labeled LukF (lanes 1–5) and LukS (lanes 6–9). C-terminal truncation mutants with unlabeled counterpart subunits on rRBC membranes were examined by electrophoresis. An autoradiogram of a 10% SDS-polyacrylamide gel is shown. **B**, Hemolytic activity of C-terminal Luk truncation mutants measured in a 2-h assay. The first well of each row contained 5 μl of each LukF and LukS component. Two-fold serial dilutions are from left to right (see “Experimental Procedures”).
the N terminus is not required to make lytic pores. In the present study, by site-specific radiolabeling and limited proteolysis, we have shown that the N termini of LukF and LukS reside in different conformations within the assembled Luk oligomer: the N terminus of LukS is protease-resistant (Figs. 6 and 8B), whereas the N terminus of LukF is protease-sensitive (Fig. 6). One possibility is that the N terminus of LukS forms a latch while the N terminus of LukF remains exposed to solvent (Fig. 8B). Alternatively, the N terminus of LukF could remain folded onto the β-sandwich domain in a protease-sensitive conformation.

In the previous report (36), we showed that the N terminus of αHL helps to prevent premature oligomerization of the monomer in solution. A gradual increase in the extent of premature oligomerization was observed when residues were sequentially removed from the N terminus. We therefore explored the possibility of premature oligomerization of leukocidin. We generated truncated LukF and LukS mutants by IVTT and analyzed their ability to oligomerize in the absence of membranes with WT LukS and WT LukF, respectively, or by themselves. Unlike αHL, we did not observe premature oligomerization with either LukF or LukS (data not shown). However, even with the WT proteins, the efficiency of oligomerization of leukocidin on membranes is much lower than that of αHL. Therefore, a low relative extent of premature oligomerization would be difficult to detect in the Luk proteins.

Compared with αHL, both Luk F and Luk S have extended C termini. The 10 additional residues of LukF and the 4 of LukS form β strands that are hydrogen-bonded to the backbones of the β-sandwich domains. The functional significance of these extensions remains unclear, but they are likely to contribute to the stability of the β-sandwich domains. LukS-CΔ283, with 4 residues removed, a structural equivalent of wild-type αHL, produced SDS-stable lytic pores, but the removal of an additional 2 residues abolished the SDS-stability of the pore while leaving appreciable hemolytic activity with an increased lag phase. In the case of LukF, up to 7 residues of the extended C terminus could be removed (LukF-CΔ294) without affecting activity. Traces of oligomer were observed, which were no longer detectable after the deletion of 10 C-terminal amino acids (LukF-CΔ290), when activity was completely lost. These results are in keeping with previous studies on αHL, which reported that the removal of 3 or 5 amino acids at the C terminus greatly inhibited both oligomer formation and rRBC lysis (21). A 4-residue C-terminal deletion mutant of αHL (αHL1–289) was extremely susceptible to inclusion body formation and had compromised tertiary structure as deduced from the fluorescence emission of bound 1-anilino-8-naphthalene sulfonate (35). On the other hand, constructs of αHL, LukF, and LukS with very large C-terminal extensions (>94 amino acids) form SDS-stable oligomers and have unaltered hemolytic activity (19).

In summary, we have examined the assembly of leukocidin by truncation mutagenesis and limited proteolysis. Our data show: 1) In the WT proteins, the conformations of the LukF and LukS N termini differ

A mechanism of assembly for αHL. The N terminus is shown as a dashed red line and the (pre)stem as a solid green line. Four principle stages have been defined (16, 21–23) as follows: 1) αHL exists as a monomer in solution. Both the N terminus and the pre-stem are susceptible to proteolysis. 2) Upon membrane binding, the pre-stem becomes resistant to proteolysis (perhaps because the enzyme cannot approach the membrane surface), but the N terminus remains susceptible to cleavage. 3) In the prepore intermediate, the pre-stem domains fill the cavity within the oligomer, where they remain resistant to proteolysis. The N terminus remains accessible, and either it is released as shown or residues such as Lys-8 are still accessible while the strand remains hydrogen-bonded to the β-sandwich domain. 4) In the fully assembled pore, the N terminus is buried and forms a latch within the cap domain (7), where it is protease-resistant. A model for the assembly of leukocidin. In LukS, the N terminus is represented by a dashed red line, and the stem is in solid black. In LukF, the N terminus is a solid red line and the stem is solid green. 1) Both Luk F and Luk S subunits exist as monomers in solution. 2) Little is known about the membrane-bound monomers in the case of leukocidin, although they are sensitive to proteolysis (Fig. 6). 3) In the pre-pore intermediate, the pre-stem domains fill the cavity within the oligomer, where they remain resistant to proteolysis. 4) In the functional pore, the N terminus of LukS becomes protease-resistant and most likely forms a latch within the cap domain. By contrast, the LukF N terminus is protease-sensitive, and either it remains free as shown or cleavage occurs while the strand is hydrogen-bonded to the β-sandwich domain. Although the N termini of LukF and LukS reside in different conformations in the assembled leukocidin oligomer, neither is required for pore formation, as shown in this report.

In summary, we have examined the assembly of leukocidin by truncation mutagenesis and limited proteolysis. Our data show: 1) In the WT proteins, the conformations of the LukF and LukS N termini differ
in the assembled state (trypsin proteolysis experiment). The N terminus of LukF is in a protease sensitive conformation, whereas the N terminus of LukS is protease-resistant. 2) In agreement with work on the αHL pore (see previous report (36)), the N termini of the LukF and LukS subunits are not required for pore formation. This was demonstrated in experiments where both LukF and LukS were truncated, as well as in experiments where a truncated subunit was assembled with its full-length counterpart. 3) The N termini of LukF and LukS are not required to prevent the premature assembly of the Luk pore.

The structures of the leukocidin monomers and the αHL heptamer represent the starting and end points for the assembly of βPFTs. Our new results emphasize the similarities and demonstrate subtle differences between the assembly pathways for the two pores. In the future, several key issues concerning the assembly mechanism remain to be addressed (23). The pathway to the prepore is one unresolved issue. The sequential addition of monomers to the growing pore, random encounters between small oligomers, or a mixed pathway could produce the prepore. Membrane insertion to form the β barrel is another issue that could be a stepwise or concerted process with respect to the individual subunits. A crystal structure of a prepore could provide valuable information about the conformation of the pre-stem domain inside the central cavity, and the observation of pore formation by real-time single molecule fluorescence with labeled proteins could reveal information about transient intermediates (23).

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