FOR THE RECORD

α -Hemolysin, γ -hemolysin, and leukocidin from *Staphylococcus aureus*: Distant in sequence but similar in structure

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Abstract: α -Hemolysin from *Staphylococcus aureus* assembles from a water-soluble, monomeric species to a membrane-bound heptamer on the surface of target cells, creating water-filled channels that lead to cell death and lysis. *Staphylococcus aureus* also produces the γ -hemolysin and leukocidin toxins, which function as two component toxins in the disruption and lysis of erythrocytes and leukocytes. Analysis of the aligned sequences of α -hemolysin, γ -hemolysin, and leukocidin in the context of the α -hemolysin heptamer structure supports the conclusion that even though the level of sequence identity between α -hemolysin and the γ -hemolysin and leukocidin toxins is in the so-called twilight zone, the three-dimensional structures of the protomers are probably conserved. By analogy with α -hemolysin, γ -hemolysin and leukocidin may also form oligomeric, transmembrane channels in which an anti-parallel β -barrel constitutes the primary membrane-embedded domain.

Keywords: α -hemolysin; γ -hemolysin; heptameric channels; leukocidin; pore-forming bacterial toxins; *Staphylococcus aureus*; transmembrane β -barrel

Staphylococcus aureus, one of the most common human pathogens, secretes an arsenal of toxins that include α -hemolysin (α HL; Bhakdi & Trantum-Jensen, 1991; Bayley, 1995; Bhakdi et al., 1996; Gouaux, 1997a, 1997b; Lesieur et al., 1997), γ -hemolysin (γ HL), and leukocidin (Luk; see Table 1 and a recent review by Tomita & Kamio, 1997). These toxins have hemolytic, leukotoxic, dermonecrotic and cytotoxic properties and are associated with disease states that range from superficial skin infections to severe septicemias (Bhakdi & Trantum-Jensen, 1991; Cribier et al., 1992). α HL attacks human platelets (Bhakdi et al., 1988), peripheral blood

monocytes (Bhakdi et al., 1989), keratinocytes (Walev et al., 1993), and endothelial cells (Suttorp et al., 1988). The cell damaging form of α HL is the membrane-associated homoheptamer, which assembles from the secreted, water-soluble monomer on the surface of sensitive cells (Fig. 1; Gouaux et al., 1994; Song et al., 1996; Fang et al., 1997). γ HL and Luk are leukotoxic, hemolytic, and they target polymorphonuclear cells, monocytes, and macrophages (Smith & Price, 1938; Prévost et al., 1995a; Tomita & Kamio, 1997). The γ HL and Luk toxins consist of a group of proteins (HlgA, HlgB, HlgC, LukF, LukS, etc.; see Table 1), each belonging to a class F or class S subtype, so named because of their relative elution positions on an ion exchange column. The active γ HL or Luk toxins function via the synergistic combination of a class F and a class S component. Thus, γ HL and Luk have been deemed “two-component” toxins (Woodin, 1960). Evidence accumulated to date suggests that γ HL and Luk form heterooligomeric complexes, which may be transmembrane channels, on the surfaces of susceptible cells (Finck-Barbançon et al., 1993; Sugawara et al., 1997).

Comparison of the sequences corresponding to the mature α HL, γ HL and Luk polypeptides shows that the pairwise sequence identity between α HL and the other toxins ranges from 23% to 32%, using the FASTA alignment program (GCG, 1996). As described previously (Cooney et al., 1993), γ HL is more closely related to Luk by primary sequence than α HL is related to γ HL and Luk. Indeed, the level of sequence conservation between α HL and the γ HL and Luk proteins borders on the so-called twilight zone, indicating that conservation of three-dimensional structure is not unambiguous. Multiple-sequence alignments of α HL and nine representative sequences of γ HL and Luk using Clustal W (version 1.6; Thompson et al., 1994), MAP (Huang, 1994), and PIMA (Smith & Smith, 1990, 1992), operating from the web site <http://dot.imgen.bcm.tmc.edu:9331/multi-align> at Baylor College of Medicine, gave similar results, although there were some specific differences between the alignments (Fig. 2). Relative to the α HL sequence, the sites of variation in the multiple sequence alignments using the three programs were located (1) at the amino terminus; (2) near residue 75; (3) at the glycine-rich region, between residues

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Abbreviations: α HL, α -hemolysin; γ HL, γ -hemolysin; Luk, leukocidin.

Table 1. α -Hemolysin homologs

Protein ^a	Class ^b	Strain ^c	Accession number ^d	Mature N-terminus ^e	No. A.A. ^f	MW ^g	pI ^h	References
α HL	—	<i>S.a.</i> , Wood 46	152953	*ADSDIN...	293	33.25	7.94	(Walker et al., 1992)
HlgB	F	<i>S.a.</i> , ATCC 49775	550424	*AEGKIT...	300	34.18	9.17	(Prévost et al., 1995a)
HlgB	F	<i>S.a.</i> , Smith 5R	295156	AEGKIT...	300	34.12	9.18	(Cooney et al., 1993)
HlgB (LukF)	F	<i>S.a.</i> , MRSA	484391	*AEGKIT...	298	33.98	9.25	(Rahman et al., 1992)
LukF-R	F	<i>S.a.</i> , P83	477911	*AEGKIT...	300	34.18	9.17	(Supersac et al., 1993)
LukF-I	F	<i>S.i.</i> , ATCC 51874	628931	*ANQITP...	300	33.86	7.25	(Prévost et al., 1995b)
LukF-PV	F	<i>S.a.</i> , ATCC 49775	288292	*AQHITP...	301	34.38	8.96	(Prévost et al., 1995a)
HlgA	S	<i>S.a.</i> , ATCC 49775	550422	*ENKIED...	280	31.81	9.52	(Prévost et al., 1995a)
HlgA (γ HLII)	S	<i>S.a.</i> , MRSA	484389	ENKIED...	280	31.92	9.53	(Rahman et al., 1993)
HlgA	S	<i>S.a.</i> , Smith 5R	295154	ENKIED...	280	31.92	9.53	(Cooney et al., 1993)
LukM	S	<i>S.a.</i> , P83 ATCC 31890	577649	TTNAED...	280	32.01	9.17	(Choorit et al., 1995)
LukS-PV	S	<i>S.a.</i> , ATCC 49775	551670	*DNNIEN...	284	32.39	8.77	(Prévost et al., 1995a)
LukS-R	S	<i>S.a.</i> , P83	477585	*ANDTED...	286	32.69	9.23	(Supersac et al., 1993)
HlgC	S	<i>S.a.</i> , ATCC 49775	550423	*ANDTED...	286	32.54	9.32	(Prévost et al., 1995a)
HlgC	S	<i>S.a.</i> , Smith 5R	295155	*ANDTED...	286	32.55	9.18	(Cooney et al., 1993)
LukS-I	S	<i>S.i.</i> , ATCC 51874	628930	*ANTIEE...	281	32.14	9.37	(Prévost et al., 1995b)
HlgC (LukS)	S	<i>S.a.</i> , MRSA	400204	*ANDTED...	286	32.53	9.10	(Rahman et al., 1991)

^aProtein names with synonyms in parenthesis. The γ -hemolysins are HlgA, HlgB, and HlgC. The leukocidins are LukF, LukF-R, LukF-I, LukF-PV, LukM, LukS-PV, LukS-R, and LukS-I.

^bF and S, fast and slow eluting from cation exchange chromatography resin, respectively.

^c*S.a.*, *Staphylococcus aureus*; MRSA, methicillin resistant *Staphylococcus aureus*; *S.i.*, *Staphylococcus intermedius*.

^dAccession number is the National Center for Biotechnology Information sequence identification number.

^eSequence of the amino terminal 6 residues of the mature protein, after cleavage of the signal sequence. Sequences that are preceded by an asterisk have been determined by amino acid sequencing.

^fNo. A.A., the number of amino acid residues in the mature protein.

^gMW, the molecular weight of the mature protein (kDa) calculated from the amino acid sequence.

^hpI, the isoelectric point of the mature protein calculated from the amino acid sequence (Bjellqvist et al., 1993, 1994).

120–148; (4) between residues 171–190; and (5) around residues 250–270. The sites of variability in the sequence alignments were located in either non- α -helical, non- β -strand regions of the α HL protomer structure, or they were confined to the glycine-rich seg-

ment. γ HL and Luk proteins isolated from different *Staphylococcal* strains that were >95% identical to each other were purposefully excluded from the multiple sequence alignments. Inspection of the alignment shows that there are only 28 strictly conserved residues,

Fig. 1. (A) View of the α HL heptamer, drawn in a C_α ribbon representation, perpendicular to the sevenfold axis of molecular symmetry and approximately parallel to the putative membrane plane. Each protomer is in a different color. The Cap, Rim, and Stem domains are labeled. The heptamer is approximately 100 Å in height and measures up to 100 Å in diameter. (B) View of the heptamer down the sevenfold axis, from the top of the Cap domain. The transmembrane channel spans the length of the molecule and varies in diameter from 26 to 46 Å, measured from C_α to C_α . (C) A ribbon representation of a protomer removed from the heptamer structure. The amino latch is in purple, the β -sandwich domain is in blue, the triangle region is in yellow, the stem is in green, and the rim domain is in red. Tyrosine and tryptophan residues are drawn in a ball-and-stick representation and the locations of glycine residues are indicated by white swatches on the ribbon.

Fig. 2. Alignment of the sequences of α -hemolysin (α HL), γ HL (HlgA, HlgB, and HlgC) and Luk (LukF-PV, LukF-P83, LukF-I, LukS-I, and LukM) proteins. Clustal W (version 1.6) was used to perform the alignment. The corresponding elements of α HL secondary structure are given above the sequences, using the same color code as in Figure 1C. Residues shaded in black are identical in one half or more of the sequences, while residues that are shaded in gray are conservative substitutions in one-half or more of the sequences. The asterisks indicate residues that are conserved among all of the sequences. Amino acid names are abbreviated using the single-letter code.

out of a total of ~ 285 (Fig. 2). Although β -toxin from *Clostridium perfringens* (Hunter et al., 1993) and hemolysin II (HL II) from *Bacillus cereus* (Sinev et al., 1993; Budarina et al., 1994) also have low-level sequence identity to α HL, γ HL, and Luk, we will limit our analysis and discussion to the Staphylococcal pore-forming, cytolytic toxins.

Overall, the sequences of α HL, γ HL, and Luk share a number of features in common, such as similarity in length and isoelectric point. In addition, both γ HL and LukF have glycine-rich regions that correspond to the glycine-rich, membrane-spanning region of α HL. In α HL, this region is bounded by the conserved residues Y118 and Y148. However, for the class F and class S toxins the glycine-rich segment is two and four residues shorter than the glycine-rich region of α HL, respectively. Thus, the γ HL and Luk proteins are predicted to have a slightly shorter stem domain. Even though the putative stems of γ HL and LukF may be shorter than the α HL stem, the sequences have an alternating hydrophobic/hydrophilic pattern that would allow for a nonpolar, membrane-embedded stem exterior and a polar channel interior, as is found in α HL (Song et al., 1996; Valeva et al., 1996). Whether the glycine-rich regions of the γ HL and Luk toxins actually traverse the mem-

brane remains to be determined. A region of α HL that is close to the membrane, if not partially inserted into it, is the loop between W260 and W265 at the base of the rim domain (Valeva et al., 1996). Interestingly, these two tryptophan residues are conserved in the class F toxins but are absent in the class S toxins.

The conserved residues are distributed in a relatively uniform fashion throughout the primary sequences, with the exception of the amino termini. In the first ~ 10 residues of α HL, which constitutes a segment essential for lytic activity (Walker et al., 1992), there are no residues conserved with the LukF proteins. Of the 28 strictly conserved residues, 13 are aromatic and only 4 are charged. The site of the single *cis* peptide bond in the α HL structure is P103 and this residue, along with the flanking Y101 and N105 residues, are conserved in the γ HL and Luk family of proteins. Interestingly, H35 is not conserved within this group of toxins, even though H35 plays a critical role in the assembly and function of α HL (Jursch et al., 1994; Krishnasastri et al., 1994; Menzies & Kernodle, 1994; Walker & Bayley, 1995a, 1995b). H35 is located at a protomer-protomer interface and is buried in a crevice defined by strand 6 and the loop between strands 9 and 10 (Song et al., 1996). Because the γ HL and Luk proteins function as two-component toxins, and

the functional species is probably a heterooligomer (Sugawara et al., 1997), it is likely that the γ HL and Luk protomer–protomer interfaces will differ from the corresponding α HL interfaces. The interactions between the amino latch regions and the adjacent protomers, as well as the structures of the amino latches in the γ HL and Luk oligomers, may also diverge from the corresponding elements of the α HL heptamer structure.

Most of the 28 conserved residues are located in the interior the α HL protomer rather than on the surface, supporting the notion that the folds of α HL, γ HL and Luk are conserved and that the surfaces and perhaps the modes of protomer–protomer association are distinct. If the conserved residues are grouped by domain or region, we see that 15 are in the β -sandwich, 6 are in the rim, 4 are in the stem, and 3 are in the triangle. Although the conserved residues are dispersed relatively uniformly throughout the primary sequence, they are clustered together in three-dimensional space and they form three main groups (Fig. 3). One group, located in the β -sandwich domain and proximal to the rim, consists of residues G59, I61, P103, W167, L219, F224, P226, F228, and Y249. A second group, also in the β -sandwich interior but proximal to the top of the heptamer cap, is made up of F42, L53, Y282, and W286. A third group is defined by residues W80, F196, and F210, and is located in the rim domain. Residues F224, P226, and F228 constitute a striking element of structure in that the two phenyl rings are situated underneath the proline residue, one ring pointing in a N $\rightarrow$ C direction and the other in a C $\rightarrow$ N orientation.

Fig. 3. Ribbon representation of three protomers from the heptamer structure, viewed from the lumen of the channel. The strictly conserved residues between the α HL, γ HL, and Luk toxins are shown in ball-and-stick representation, with the exception of residues Y118, G122, and G134 in the stem domain. For clarity, distinct clusters of conserved residues are depicted on different protomers.

Aromatic residues are frequently found on the exterior surface of membrane proteins at the membrane/aqueous solution boundary (Deisenhofer et al., 1985; Weiss & Schulz, 1992; Wimley & White, 1996). In α HL, as determined from the heptamer structure (Song et al., 1996), residues Y118 and F120 form an aromatic belt that may define the interface between the membrane bilayer and the extracellular solution. In the γ HL and Luk proteins, Y118 is conserved and F120 is either phenylalanine or tyrosine in the class F proteins or isoleucine or valine in the class S proteins. On the second transmembrane segment of α HL, which corresponds to strand 8 in the heptamer structure, there is another conserved tyrosine at position 148. However, this site is near the top of the stem and is predicted to not interact with the membrane, at least in the fully assembled, heptameric state.

By studying the location of conserved residues, one can gain insight into residues that occupy essential structural and/or functional roles. Consider, for example, the single amino acid mutant H35N (histidine 35 to asparagine), a species that shows reduced hemolytic and heptamer-forming activity (Panchal & Bayley, 1995). One group of second site revertants that recover substantial hemolytic and heptamer-forming activity comprise the double mutants H35N/S217C, H35N/L219P, H35N/ Δ 222 (deletion of residue 222), H35N/F224Y, H35N/D227N, H35N/D227A, and H35N/F228L. Although at first glance these sites may appear remote from the location of the primary mutation (H35), the residues in this region make up a central element of the β -sandwich and, through van der Waals contacts with conserved residues I61, W167, and Y249, are in a position to influence the properties of the H35N binding site on an adjacent protomer by their proximity to the crevice occupied by residue 35.

Conserved and partially conserved amino acids may also play a role in membrane binding. Residues R200 and D254 were shown by site-directed mutagenesis and chemical modification to participate in the binding of α HL to the erythrocyte membrane (Walker & Bayley, 1995a). R200 is conserved in the class F toxins while D254 is conserved in both the class F and S toxins. R200 is located in a crevice between the rim and stem domains and may bind to the head groups of phosphatidyl choline lipids (Song et al., 1996). The role of D254 in membrane binding is not clear from the structure of α HL heptamer, which shows D254 occupying a position ~ 20 Å from the putative membrane surface. Nevertheless, D254 may participate in “early” membrane-associated events, such as the binding of the monomer or the prepore to the bilayer.

In conclusion, alignment of the primary sequences of the α HL, γ HL, and Luk proteins shows that even though the level of sequence conservation is low, most of the conserved residues are clustered in a few key groups, located in the interior of the α HL protomer structure, therefore reinforcing the conclusion that the protomeric structures of α HL, γ HL, and Luk are similar. By contrast, the residues at the protomer–protomer interfaces in the α HL heptamer structure are poorly conserved, suggesting that the nature of the interactions between subunits in γ HL and Luk will be different and that the structure of an assembled oligomeric complex may diverge from that of the α HL heptamer.

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