Review

Defense against own arms: staphylococcal cysteine proteases and their inhibitors

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Staphylococcus aureus is a human pathogen causing a wide range of diseases. Most staphylococcal infections, unlike those caused by other bacteria are not toxigenic and very little is known about their pathogenesis. It has been proposed that a core of secreted proteins common to many infectious strains is responsible for colonization and infection. Among those proteins several proteases are present and over the years many different functions in the infection process have been attributed to them. However, little direct, in vivo data has been presented. Two cysteine proteases, staphopain A (ScpA) and staphopain B (SspB) are important members of this group of enzymes. Recently, two cysteine protease inhibitors, staphostatin A and staphostatin B (ScpB and SspC, respectively) were described in S. aureus shedding new light on the complexity of the processes involving the two proteases. The scope of this review is to summarize current knowledge on the network of staphylococcal cysteine proteases and their inhibitors in view of their possible role as virulence factors.

Staphylococcus aureus is a main cause of nosocomial infections of all kinds. It colonizes and infects virtually every tissue of the body (Lowy, 1998). To encounter the environmen

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Abbreviations: SspA, V8 protease; SspB, staphopain B; SspC, staphostatin B; ScpA, staphopain A; ScpB, staphostatin A
tal diversity faced, the pathogen is well endowed with a broad spectrum of secreted proteins (Arvidson, 2000). Unlike those caused by most bacteria, staphylococcal infections are not only unassignable to any particular organ, but are generally not of an obviously toxigenic nature. Rather, a core of secreted proteins common to all strains has been proposed to represent the minimal pathogenic unit responsible for the majority of tissue infections. However, since the secretion profile differs significantly between strains (Zieblandt et al., 2001), the contribution of particular proteins to the growth and survival of S. aureus remains poorly understood (Massimi et al., 2002).

Proteases of three different catalytic classes, including metallo-, serine- and cysteine enzymes are found among the secreted staphylococcal proteins (Drapeau et al., 1972; Arvidson, 1973; Arvidson et al., 1973; Reed et al., 2001; Rice et al., 2001). A variety of different functions including, but not limited to, tissue degradation (Potempa et al., 1988; Travis et al. 1995), defense against host immune response (Arvidson, 2000), interception of host enzymes (Potempa et al., 1986; Maeda & Yamamoto, 1996) and bacterial adhesion regulation (McGavin et al., 1997; McAleese et al., 2001) have been attributed to these proteins. Recently, a direct evidence for the importance of the ssp operon (encoding the SspA serine and the SspB cysteine protease and the SspC cysteine protease inhibitor) in pathogenesis was obtained. The virulence of a mutant strain deficient in these proteins was attenuated (Coulter et al., 1998), however, a strain deficient only in SspA showed no visible changes in virulence (Rice et al., 2001). It has also been demonstrated that strains with deregulated expression of exoproteins (including but not limited to proteases) show greatly attenuated virulence (Abdelnour et al., 1993). Considering this data and the growing amount of information available on the complicated maturation cascades of the proteases it is clear that we are still far from understanding the unquestionable role of these enzymes in the infection process. To further complicate the picture it has been recently shown that staphylococci produce potent inhibitors of some of the proteases they secrete (Massimi et al., 2002; Rzychon et al., 2003a).

Here, a brief review on the operons encoding staphylococcal cysteine proteases is presented, with the focus on a recently discovered group of their inhibitors — staphostatins.

SECRETED PROTEASES OF STAPHYLOCOCCUS AUREUS

Tight control and orchestrated expression of secreted enzymes is vital for the growth and survival of Staphylococci (Abdelnour et al., 1993; Novick, 2000). To allow for an easy and precise control of secreted proteases expression their genes are organized on the bacterial chromosome into four distinct operons: the staphylococcal serine protease (ssp) operon, serine protease like proteins (spl) operon, staphylococcal cysteine protease (scp) operon and a gene of aureolysin (aur) (Fig. 1). The ssp and scp operons encode two cysteine proteas-

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**Figure 1. Genomic organization of S. aureus protease genes.**
es (SspB and ScpA, respectively) and are discussed below in detail with the exception of SspA, a serine protease that is only briefly mentioned (Table 1). The serine proteases encoded in the spl operon and the metalloprotease aureolysin are beyond the scope of this review (for detailed reviews on all classes of staphylococcal proteases see Barrett et al., 1998; Dubin, 2002).

**ssp operon**

The staphylococcal serine protease (ssp) operon groups V8 protease (SspA), staphopain B (SspB) and staphostatin B (SspC). Transcription is driven by a single promoter producing polycistronic mRNA which encodes all three proteins.

The SspA serine protease, previously referred to as V8 protease, has been thoroughly studied being the first purified and characterized S. aureus proteolytic enzyme (Drapeau et al., 1972). It is synthesized in a preproform. The prefragment is a typical signal peptide (Carmona & Gray, 1987) cleaved off during secretion to yield an inactive proenzyme. The zymogen undergoes proteolytic activation by a staphylococcal extracellular metalloprotease (Drapeau, 1978). SspA has narrow substrate specificity, cleaving preferentially after glutamic acid and to a lesser extent after aspartic acid (Drapeau et al., 1972; Houmard & Drapeau, 1972; Björklind & Jörnvall, 1974).

The physiological role of the enzyme remains unclear. In vitro experiments suggested its deleterious role in the infection process. It has been shown to interfere with the host defense mechanisms (Arvidson, 2000). The protease efficiently inactivates α₁-proteinase inhibitor, thus deregulating the host derived proteolytic activity (Potempa et al., 1986). Generation of kinin directly from the high molecular mass kininogen may be responsible for the infection associated pain and edema and might help in the transfer of bacteria into systemic circulation (Molla et al., 1989; Maeda & Yamamoto, 1996). Moreover, SspA alters the overall bacterial surface protein composition, among others affecting the adhesion functions (McGavin et al., 1997), and processes the precursor forms of other secreted enzymes, including prosthaphopain B. However, efficient inhibition of SspA protease by plasma α₂-macroglobulin as well as the results of a study on a knock-out strain, whose virulence in the murine abscess infection model was not impaired (Rice et al., 2001), seem to contradict the previous suggestions about the importance of the enzyme in pathogenesis. Clearly further studies are needed to clarify this issue (for detailed reviews on SspA protease see Qasim, 1998; Dubin, 2002).

The sspB gene encodes the preproform of a cysteine protease — staphopain B (SspB). The secretory prefragment is most probably cleaved off during the process yielding prosthaphopain B, which, as already mentioned, undergoes proteolytic maturation by SspA protease. The proenzyme as well as mature staphopain B are able to degrade gelatin but not casein (Rice et al., 2001; Massimi et al., 2002). As suggested by amino-acid sequence similarity, the overall fold of the enzyme is similar to staphopain A and therefore to papain (Hofmann et al., 1993; Filip et al., 2003).

### Table 1. Nomenclature of selected staphylococcal proteases and their inhibitors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Protein function</th>
</tr>
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<tbody>
<tr>
<td>scpA</td>
<td>staphopain A (ScpA)</td>
<td>cysteine protease</td>
</tr>
<tr>
<td>scpB</td>
<td>staphoatin A (ScpB)</td>
<td>inhibitor of ScpA</td>
</tr>
<tr>
<td>sspA</td>
<td>V8 protease (SspA)</td>
<td>serine protease</td>
</tr>
<tr>
<td>sspB</td>
<td>staphopain B (SspB)</td>
<td>cysteine protease</td>
</tr>
<tr>
<td>sspC</td>
<td>staphoatin B (SspC)</td>
<td>inhibitor of SspB</td>
</tr>
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Regardless of rather poor functional characterization interesting data on the role of the SspB protease in pathogenesis is available. It has been observed that transposon insertion into the sspA gene results in attenuation of virulence in three different animal infection models, which was originally attributed to a lack of the SspA protease activity (Coulter et al., 1998). In a later study, however, it was shown that inactivation of SspA protease does not affect the virulence of the mutant strain. The apparent contradiction was explained by the fact that transposon insertion into sspA exerted a polar effect on the sspB and sspC gene expression and that the attenuated virulence was due to the loss of function of all three genes (Rice et al., 2001). Together, these data seem to point to SspB as a virulence determinant. Several questions concerning the importance of staphopain B, however, remain. In the SspA protease nonpolar mutant only the pro-form of staphopain B is present due to impaired processing. Moreover, the nonpolar mutation in the setup used by Rice et al. (2001) induces constitutive expression of the sspB and sspC genes that is no longer subject to agr control (agr – accessory gene regulator; a global virulence factor expression regulator, for details see Novick, 2000). In addition only a single murine tissue abscess model was used in the latter study, different from the models used by Coulter et al. (1998) making a direct comparison of the two sets of data impossible. These issues set some doubt as to whether a strain with a proper, nonpolar inactivation of sspA would retain the virulence of the wild type. Therefore, the individual importance of SspA and SspB in staphylococcal pathogenesis remains unresolved.

The sspC gene encodes staphostatin B – an inhibitor of the SspB cysteine protease. Unlike the two proteases encoded in the operon, the third protein – the SspC protease inhibitor, is localized inside the cell. Recent achievements in studies on staphostatins are discussed below.

### scp operon

The staphylococcal cysteine protease operon (scp) encodes two proteins: ScpA and ScpB. Purification of staphopain A (ScpA) was initially reported by Arvidson et al. (1973). The protease has broad substrate specificity (Björklind & Jörnvall, 1974) and unlike SspA and aureolysin is able to degrade elastin. The latter feature led to the hypothesis that the enzyme is responsible for the development of S. aureus infection-associated ulceration (Potempa et al., 1988). The importance of ScpA in staphylococcal pathogenesis was further demonstrated by showing that colony formation on sphingosine-treated skin (resulting in a marked decrease in phosphorylated cystatin α, a cysteine protease inhibitor specifically located in the epidermis) is enhanced compared to that on normal skin. Unlike other cystatins, phosphorylated cystatin is efficient against staphopain A (ScpA). Moreover, it has been shown that the level of cystatin A is decreased in lesions of atopic dermatitis, which are frequently complicated by S. aureus infections (Takahashi et al., 1994, 1999). Unfortunately, staphopain B (SspB) has not been tested for elastolytic activity or for inhibition by cystatins, therefore no comparison can be made and no clues as to the role of this protease in the described processes are available.

Staphopain A is able to inactivate human α1-proteinase inhibitor. Although inefficient in this respect compared to SspA protease, the two may act in co-operation to strengthen the effect (Potempa et al., 1986). In human plasma staphopain A is efficiently inhibited by α2-macroglobulin and other unidentified inhibitor(s) since methylamine-treated plasma still reduces the activity of this protease (Potempa et al., 1988).

The three-dimensional crystal structure of staphopain A in complex with E-64 inhibitor reveals an overall folding pattern close to papain (Hofmann et al., 1993), although the
amino-acid sequences of these enzymes are only negligibly similar. Particularly the topology of the active site and the binding mode of the inhibitor are alike in both structures.

Recently, it has been demonstrated that similar to the ssp operon, the gene following the protease in the scp operon (scpB) encodes its inhibitor, designated staphostatin A (Rzychon et al., 2003a).

STAPHOSTATINS

Staphostatins constitute a novel class of cysteine protease inhibitors. The first protein belonging to this group was described by Massimi et al. (2002) during studies on the maturation mechanism of staphopain B. The authors discovered that in contrast to other profragment containing proteases the profragment of SspB is not the enzyme’s inhibitor and they hypothesized that SspC, the third protein encoded in the ssp operon might play this role. This was proven in further experiments, which not only showed that staphostatin B is an inhibitor of mature staphopain B, but also of the proenzyme. Based on this observation, a hypothesis on the maturation of SspB was formulated where the proenzyme inhibited by SspC is processed by the SspA serine protease, which cleaves off the profragment and then liberates mature SspB from the inhibitor control by degrading the latter. However, the cascade has only been presented in vitro with the use of purified recombinant proteins and no data on the in vivo localization of the inhibitor has been shown. At the same time the authors mention that the inhibitor has no obvious secretory sequences, which makes its contact with the extracellular staphopain very improbable. Indeed, a following study by Rzychon et al. (2003a) not only demonstrated the exclusively intracellular localization of SspC, but also argues against the staphostatin–prostaphopain complex formation.

The second known staphylococcal cysteine protease, staphopain A, is also expressed from an operon. The scp operon contains two genes only, scpA (encoding cysteine protease staphopain A, ScpA) and scpB (encoding protein ScpB). The latter protein has only low sequence similarity to SspC, but when overexpressed in Escherichia coli and purified is able to inhibit ScpA, which makes it the second member of the staphostatin class of cysteine protease inhibitors. Interestingly, in spite of the evident sequence similarity of two staphopains and two staphostatins, respectively, ScpB is not an inhibitor of SspB and SspC does not inhibit ScpA (Rzychon et al., 2003a).

Figure 2. Amino-acid sequences of staphostatins.

Staphostatin homologues are found in the genomic sequences of *S. epidermidis* and *S. warnerii* (Fig. 2). Most probably these putative proteins will prove to be inhibitors of either staphopain A or B or of similar enzymes produced by the mentioned species. This assumption is further supported by a closer analysis of the coding regions, which resemble the operons found in *S. aureus*, also encoding a cysteine protease in the case of *S. epidermidis* and a serine and cysteine protease in the case of *S. warnerii*. Moreover, ScpB inhibits a cysteine protease produced by *S. epidermidis*. It is likely that more staphostatin homologues will be found as genomic studies continue.

Staphostatins form noncovalent stable 1:1 complexes with their corresponding enzymes totally abolishing their peptidase and esterase activities and the ability of compounds such as E-64 or its derivative DCG-64 to label the enzyme. A properly formed active site of the enzyme is not necessary for the binding since the proteolytically inactive mutant of the catalytic triad cysteine of staphopain B still binds the inhibitor. Upon binding to the active enzyme a fraction of the inhibitor (less than 5%) is proteolytically cleaved. Most probably it does not remain bound to the enzyme and is replaced by uncleaved molecules. Data exist that support the hypothesis that the cleaved inhibitor molecules are incorrectly folded in the inhibitory loop making the cleavage possible.

Staphostatin B localization studies conducted with specific antibodies showed the presence of the inhibitor only intracellularly, whereas even highly concentrated culture supernatants were deprived of it. This is consistent with gene analysis revealing a lack of secretory signals in staphostatin B sequence (Rzychon *et al.*, 2003a). These findings are contradictory to the processing hypothesis presented by Massimi *et al.* (2002). Several lines of evidence seem to favour another assumption. First, the quantities of staphostatins are much lower than those of the corresponding enzymes. Extracellular inhibition, if it takes place at all, is therefore negligible, unless the production (and secretion by an unknown mechanism) of staphostatins can be efficiently upregulated. There is, however, no evidence for the latter. Second, all attempts to express mature staphopains in *E. coli* failed, probably due to a high cytoplasmatic toxicity of the enzymes. Taking into account the results of localization studies it has been postulated that staphostatins are intracellular inhibitors used for the defense against autoproteolysis caused by misdirected cysteine proteases which, instead of being secreted, remain inside the cell (Rzychon *et al.*, 2003a).

The three-dimensional crystal structure of staphostatin B as well as the NMR structure of staphostatin A have been solved (Filipek *et al.*, 2003; Rzychon *et al.*, 2003b; Dubin *et al.*, 2003a; 2003b). In spite of the rather low amino-acid sequence similarity (Fig. 2) the overall fold of both inhibitors is the same (Fig. 3). The main part of the polypeptide chain forms an 8-stranded β-barrel covered on one side by a short helix, the other side being open to solvent. Protease-inhibitor interactions and the mechanism of inhibition were clarified by the crystal structure of the inactive C243A staphopain B mutant complexed to staphostatin B (Filipek *et al.*, 2003). The inhibitor occludes the enzyme active site in a substrate-like fashion, a mechanism previously not known for cysteine protease inhibitors. In the case of a substrate the peptide bond G98–T99 would be the site of proteolytic attack, however, the unusual conformation adopted by glycine efficiently prevents the cleavage making the molecule a potent inhibitor. This observation was further supported by construction of the G98A mutant. Since alanine can not, for steric reasons, adopt the conformation taken by glycine, the molecule is efficiently cleaved at the A98–T99 peptide bond, therefore losing its inhibitory functions. The same is true for staphostatin A where the exchange of G98 to A converts the inhibitor into a good substrate of staphopain...
A. Studies on short peptides spanning the active site of the inhibitors yield similar results. Such compounds are good substrates of the corresponding enzymes since the glycine (G98) is not forced by the surrounding protein to adopt the special inhibitory conformation. Further mutational studies are under way to pinpoint the determinants responsible for the limited specificity of SspC towards SspB and ScpB for ScpA without crossinhibition (personal communication).

NMR polypeptide chain dynamics studies of staphostatin A show that the inhibitory loop is rather flexible. This may explain the previously mentioned fact that a small fraction of the inhibitor is cleaved upon binding and subsequently released. The high flexibility provides a way for the inhibitor to fit into the protease active site at the cost, however, of a percentage of wrong conformations being processed in a substrate-like fashion. Properly bound, the loop becomes rigid and the molecule is not digested even over extended incubation times (Dubin et al., 2003a). Similar conclusions are favoured by studies on staphostatin B. Conformation of the active site glycine differs significantly between the free and complexed inhibitor suggesting a dynamic rearrangement upon binding (Filipek et al., 2003; Rzychon et al., 2003b).

Cysteine residues present in both staphostatins are not necessary for the inhibition as determined from the structure of the enzyme-inhibitor complex and S-carboxymethylation studies in the case of staphostatin B, and site directed mutagenesis in the case of staphostatin A. In the latter protein the two cysteines may form a disulfide bridge which is, however, not necessary for inhibition and only stabilizes the protein structure (Rzychon et al., 2003a; Dubin et al., 2003a; 2003b).

Why are the two distinct, very specific inhibitors needed? Since we are only beginning to uncover the functions of staphostatins, it is hard to speculate whether the tight and independent regulation of their expression might have called for two proteins. In addition, it has been shown by many authors that extracellular protein expression is strictly controlled by Staphylococci and the scp and ssp operons are subject to such control. Independent regulation of these two operons would call for two distinct inhibitors. Finally, both inhibi-

Figure 3. Comparison of staphostatins structures.
Stereo view of the best backbone fit of the mean structure of staphostatin A (blue, calculated for a family of 20 NMR structures, 1OH1) and of staphostatin B (yellow, 1NYC). Cα traces are shown. The inhibitor reactive site is highlighted in red. The disulfide bridge present in staphostatin A is colored green. The N and C termini are indicated.
itors are very specific in action for their target proteases, therefore, we may not exclude that it was impossible to develop single potent inhibitor of both staphopains. To convincingly answer the question further studies are needed on the mechanism of enzyme-inhibitor recognition, operon expression control and above all, the functions of the inhibitors.

CONCLUSIONS

No consistent and experimentally proven theory as to the exact role of secreted proteins in nontoxigenic staphylococcal infections has been put forward and the set of these proteins constituting the minimal pathogenic unit remains to be defined. Staphopains have been studied for quite a long time in this respect and many functions in pathogenesis of staphylococcal diseases have been attributed to these enzymes. Some genetic studies performed in animal models in vitro also seem to point to their importance in the infection process. However, although these studies complement each other, there is still no unquestionable evidence as to the real role and necessity of staphopains for the virulence of S. aureus.

Considering staphostatins, the in vitro structural and functional studies are very advanced and already much is known about the inhibition mechanisms, nevertheless the actual role of the inhibitors and their importance to Staphylococci remains poorly understood. The hypothesis of their protective role against self-proteolysis seems interesting, but there is little evidence to support it, while the role in proenzyme maturation seems very unlikely in the light of recent data. Knowledge gained up to now allows the design of low-molecular-mass inhibitors of staphopains which in connection with the widely discussed role of these proteins as virulence factors seems promising as a new strategy for treatment of staphylococcal infections. However, since the indispensability of staphopains in pathogenesis is still under discussion, much remains to be done to prove or reject this strategy.

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