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Visions & Reflections

The staphylocoagulase family of zymogen activator and adhesion proteins

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Abstract. Staphylocoagulase (SC) secreted by *Staphylococcus aureus* is a potent non-proteolytic activator of the blood coagulation zymogen prothrombin and the prototype of a newly established zymogen activator and adhesion protein (ZAAP) family. The conformationally activated SC•prothrombin complex specifically cleaves fibrinogen to fibrin, which propagates the growth of bacteria-fibrin-platelet vegetations in acute bacterial endocarditis. Our recent 2.2 Å X-ray crystal structures of an active SC fragment [SC(1-325)] bound to the prothrombin zymogen catalytic domain, prethrombin 2, demonstrated that SC(1-325) represents a new type of non-proteolytic activator with a unique fold. The observed insertion of the SC(1-325) N-terminus into the 'Ile 16' cleft of prethrombin 2, which triggers the activating conformational change, provided the first unambiguous structural evidence for the 'molecular sexuality' mechanism of non-proteolytic zymogen activation. Based on the SC(1-325) fold, a new family of bifunctional zymogen activator and adhesion proteins was identified that possess N-terminal domains homologous to SC(1-325) and C-terminal domains that mediate adhesion to plasma or extracellular matrix proteins. Further investigation of the ZAAP family may lead to new insights into the mechanisms of bacterial factors that hijack zymogens of the human blood coagulation and fibrinolytic systems to promote and disseminate endocarditis and other infectious diseases.

Key words. Prothrombin; staphylocoagulase; proteinases; zymogens; blood coagulation; fibrinogen; endocarditis.

Introduction

Pathogenic Gram-positive bacteria, such as streptococcus and staphylococcus, express proteins that specifically activate the human blood coagulation and fibrinolytic systems. Among these factors are two unusual proteins that activate serine proteinase zymogens by inducing a conformational change, without the usual obligatory peptide bond cleavage. Streptokinase (SK) from *Streptococcus equisimilis* and *S. pyogenes* activates fibrinolysis by non-proteolytic activation of plasminogen, and staphylocoagulase (SC) from *Staphylococcus aureus* initiates blood coagulation by conformational activation of prothrombin. SCs are bifunctional proteins that conformationally activate prothrombin through N-terminal domain interactions [1–3], and adhere to fibrinogen through 5–8 tandem 27-amino acid C-terminal repeat sequences [4]. SC binding to prothrombin forms a proteolytically active complex that specifically cleaves fibrinogen to fibrin. Recent structure-function studies of SC have provided new insight into the mechanism by which *S. aureus* activates the human blood clotting system.

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Acute Bacterial Endocarditis

In vivo, SC is not required for the initial infectivity of *S. aureus* [5, 6], but contributes to the pathogenesis of acute bacterial endocarditis (ABE), characterized by formation of vegetations on heart valves consisting of bacteria, platelets and fibrin [7]. Large vegetations are friable and embolize, causing remote abscess formation and ultimately leading to heart failure, myocardial infarction or stroke [7, 8]. Normally, vascular endothelium is resistant to the attachment of bacteria, but blood flow at high shear rates across heart valves can damage the protective endothelial cell layer, exposing blood to extracellular matrix and tissue factor, which initiates blood coagulation. Vascular injury concurrent with *S. aureus* bacteremia can lead to ABE, with infection initiated by microbial attachment to plasma or extracellular matrix proteins, including fibrinogen, fibronectin, von Willebrand factor (vWf), collagen, elastin and laminin [9, 10]. Histology of *S. aureus* vegetations rarely shows leukocyte infiltration, due in part to bacterial secretion of anti-inflammatory factors [11]. In addition, growth and fortification of the vegetation by SC-induced fibrin deposition protects the bacteria in the vegetation from clearance by leukocytes and macrophages [12]. Coagulase-positive *S. aureus* causes 40–50% of neonatal endocarditis and 30–40% of endocarditis in adults between the ages of 16–60 years, with a mortality rate of 25–47%, even with antibiotic therapy [8, 13].

Bacterial Plasminogen Activators

Activation of the fibrinolytic zymogen plasminogen occurs in both streptococcal and staphylococcal infections. SK produced by *Streptococcus equisimilis* and *S. pyogenes* consists of three homologous β-grasp domains (α, β, γ), which bind plasminogen and form a conformationally activated SK•plasminogen complex [14]. SK•plasminogen recognizes free plasminogen specifically as a substrate and proteolytically converts it to plasmin, a capability that plasmin itself does not have [15, 16]. SK also acts as a cofactor of plasmin, redirecting its substrate specificity to that of a specific proteolytic plasminogen activator, a mechanism shared by *S. aureus* staphylokinase [17] and *Streptococcus uberis* plasminogen activator (SUPA) [18]. Plasminogen-activating complexes are localized to bacterial surfaces through plasmin(ogen)-binding proteins, resulting in fibrin degradation and extracellular matrix breakdown that promotes dissemination of the infection [19].

Staphylocoagulase•(pro)thrombin substrate specificity

SC binds to both prothrombin and thrombin, forming complexes that specifically cleave fibrinogen to fibrin, but not the other physiological thrombin substrates, factor V, factor VIII or platelets [20, 21]. There are conflicting results regarding whether the thrombin substrates protein C and factor XIII are activated by SC•(pro)thrombin complexes [20, 22]. Remarkably, there are no known physiological inhibitors of SC•(pro)thrombin complexes, which are resistant to the plasma serpins, antithrombin-heparin and heparin cofactor II [I. Verhamme, P. Panizzi and P. E. Bock, unpublished observations], and inhibition by α_2-macroglobulin [20] and by the thrombin-specific inhibitor from leeches, hirudin [21, 23]. This renders SC•(pro)thrombin complexes resistant to conventional anticoagulant therapy, except for small molecule active site-directed inhibitors, such as argatroban [24]. The unique substrate and inhibitor specificity of SC•(pro)thrombin complexes indicates that during ABE, deposition of fibrin formed by SC•(pro)thrombin complexes would proceed unabated, generating a fibrin barrier that aids *S. aureus* in evading clearance by leukocytes [12].

Crystal structures of SC(1-325) bound to human α-thrombin and prethrombin 2

Our recent crystallographic studies resulted in the first high-resolution structures of a functional N-terminal fragment of SC [SC(1-325)] bound to α-thrombin and to its immediate zymogen precursor, prethrombin 2 (Pre 2). SC(1-325) is a boomerang-shaped molecule composed primarily of two three-helix-bundle domains with the N-terminal domain 1 [SC(1-146); D1] interacting with the autolysis loop (148-loop; chymotrysinogen numbering) of α-thrombin or Pre 2, and domain 2 [SC(147-325); D2] binding to exosite I on thrombin, and the precursor form of exosite I, proexosite I [25], on Pre 2 and prothrombin. The SC(1-325) structure bears no resemblance to the three-domain, primarily β-sheet structure SK, and represents an entirely new class of non-proteolytic zymogen activators. Because SK and SC are the only previously known conformational activators of serine proteinase zymogens, and given the homologous nature of the zymogens they activate, it had been speculated that a similar mechanism might apply to both activators [26].

Proof of the molecular sexuality hypothesis

Physiological prothrombin and plasminogen activation catalyzed by factor Xa and tPA or uPA, respectively, follow the classical mechanism of zymogen activation established in the seminal studies of trypsinogen activation by Wolfram Bode and Robert Huber [27–29]. Trypsinogen is activated by cleavage of the Arg 15-Ile 16 bond, allowing formation of a critical salt bridge between the α-ammonium group of the new N-terminal Ile 16 and the

carboxylate group of Asp 194, which triggers completion of the folding of the zymogen (fig. 1A). The conformational change forms the substrate binding sites and oxyanion hole required for catalysis. Bode and Huber also discovered that the zymogen-proteinase conversion was fundamentally conformational, with a highly unfavorable equilibrium between the active and inactive conformations. The equilibrium for trypsinogen with its catalytic serine acylated by *p*-guanidinobenzoate is shifted toward the active conformation by linked binding to the Ile 16 cleft of Ile-Val or Val-Val dipeptides [29], which correspond to the new N-termini of most serine proteinases. Based on the importance of the Ile 16-Asp 194 salt bridge in zymogen activation, the 'molecular sexuality' mechanism was proposed for non-proteolytic activation of plasminogen by SK [27]. SK was hypothesized to mimic N-terminal insertion and salt-bridge formation by binding to the proteinase domain and inserting its own Ile 1-Ala 2 N-terminus, thereby inducing conformational activation of the active site, as illustrated for prothrombin and SC(1-325) in figure 1B. Some structure-function studies support the N-terminal insertion mechanism for

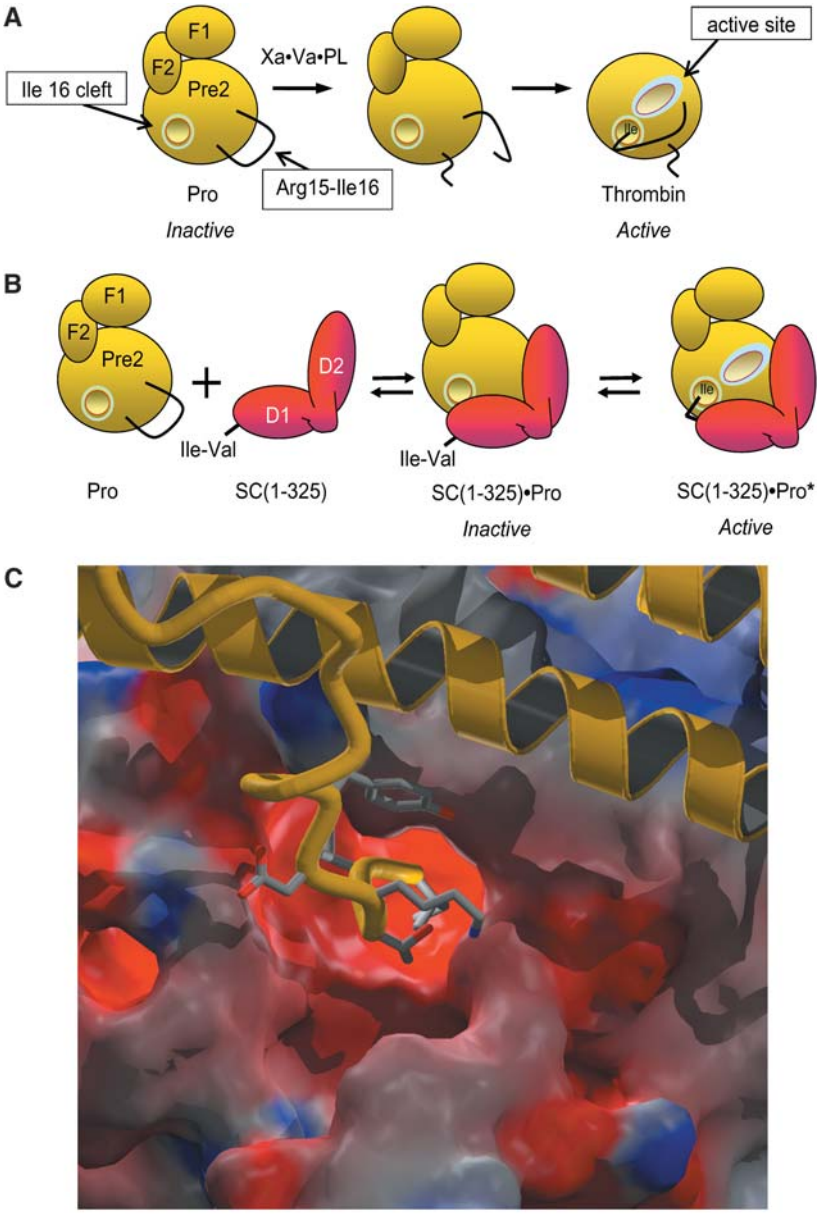

Figure 1. Schematic representation of the mechanisms of prothrombin (*Pro*) activation. (*A*) The classical, physiological mechanism of activation of prothrombin to thrombin catalyzed by the factor Xa•factor Va•phospholipid complex (*Xa•Va•PL*). Prothrombin consists of the catalytic domain prethrombin 2 (*Pre2*), fragment 1 (*F1*) and fragment 2 (*F2*), where thrombin is released from F1 and F2 during activation. (*B*) The pathological, molecular sexuality mechanism of prothrombin activation induced by staphylocoagulase [*SC(1-325)*]. (*C*) SC(1-325) N-terminal residues (*yellow*) inserted into the Ile 16 cleft of Pre 2 (panel C reproduced from [1]).

SK [26, 30], while others support an additional or alternative mechanism [14], and crystal structure evidence for either mechanism is lacking.

The structure of the SC(1-325)•Pre 2 complex provides proof of the cofactor-induced mechanism of prothrombin activation by direct demonstration of insertion of the SC N-terminal segment into the Ile 16 pocket of Pre 2 (fig. 1C). Kinetic studies of prothrombin activation by recombinant SC(1-325) mutants revealed that the presence of a Met residue at the N-terminus of SC(1-325) resulted in 60-fold loss of potency in prothrombin activation. Deletion of Ile 1 in SC(2-325) showed only a ~6-fold loss of apparent affinity, whereas SC(3-325) exhibited <2% prothrombin activator activity. These results demonstrated the specificity of conformational activation for the N-terminal Ile-Val dipeptide of SC, and also highlighted the unexpected tolerance of the Ile 16 cleft of prothrombin for structurally different N-termini, consistent with the recent observation that SC containing an additional N-terminal Ala clots human plasma [31]. This apparent polyandry shown by prothrombin for the altered N-terminus of SC is unlike the strict requirement for the N-terminal Ile 1 residue on SK for conformational activation of plasminogen [26]. Studies of the roles of the two SC domains in prothrombin activation demonstrated that D1 is necessary and sufficient for activation and that N-terminal insertion is facilitated by high affinity binding of D2 to (pro)exosite I on (pro)thrombin. This finding is consistent with the previous observation that deletion of 125 N-terminal amino acids from SC abolished coagulase activity, but the truncated SC retained affinity for prothrombin [2].

Fibrinogen substrate recognition and cleavage

It remains unclear how SC•(pro)thrombin complexes specifically bind and cleave fibrinogen. Fibrin formation by α-thrombin cleavage of fibrinopeptides A and B from fibrinogen is mediated by exosite I. Exosite I is blocked by D2 in SC•(pro)thrombin complexes, yet fibrinogen is still cleaved with high specificity, and Met-SC(1-325)•(pro)thrombin clots fibrinogen with the same potency as α-thrombin [1]. This supports the hypothesis that a new fibrinogen substrate recognition exosite is expressed on the activator-zymogen complex [P. Panizzi and P. E. Bock, unpublished observations]. The existence of the additional binding sites on the C-terminal end of full-length SC suggests that two modes of fibrinogen interactions occur through distinct regions of fibrinogen. The structure of fibrin clots produced by the SC•(pro)thrombin complexes during ABE has not been characterized at the molecular level. Normally, fibrin polymers produced by α-thrombin are stabilized by factor XIIIa crosslinking. The multiple modes of fibrinogen interactions with full-length SC and the unresolved question of whether factor XIII is activated by SC•(pro)thrombin complexes may have important implications for the structure of fibrin clots generated by *S. aureus*, relevant to the tensile strength and stability of vegetations in ABE.

vWf binding protein (vWbp) and the new ZAAP family

Based on the structure and fold of SC(1-325) and the bifunctional nature of full-length SC, we identified and partially characterized proteins containing homologous D1-D2 domains that comprise a new family of bifunctional bacterial zymogen activator and adhesion proteins (ZAAPs; fig. 2). The ZAAP family includes a cell wall-bound *Streptococcus pyogenes* factor with an N-terminal RGD potential integrin binding site and a C-terminal fibronectin binding domain (SfbX); *S. aureus* vWbp; and two proteins of unknown function from the human pathogen *Streptococcus agalactiae* serotype V (NP_687847) and NEM316 (CAD46494).

vWbp is bifunctional, with N-terminal domains [vWbp (1-262)] exhibiting non-proteolytic prothrombin activator activity [P. Panizzi and P. E. Bock, unpublished observations]; [1] and clotting of human plasma [31], and a C-terminal se-

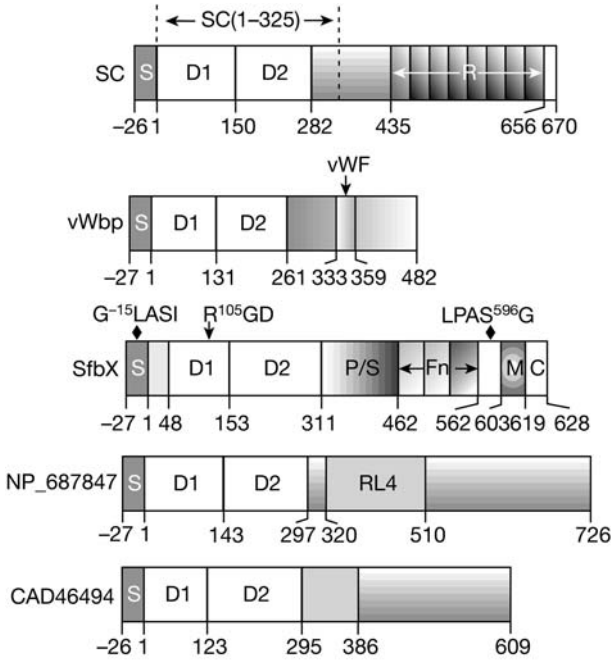

Figure 2. Domain organization of ZAAPs. SC homologs are *S. agalactiae* proteins CAD46494 [34]; NP_687847 [33]; SfbX, *Streptococcus pyogenes* fibronectin (*FN*)-binding protein [37]; and vWbp [32]. The sequence that directs cell-wall sorting of SfbX is indicated by a diamond; the RGD triplet is also indicated. C, cytoplasmic tail; D1/D2, α-helical domains; Fn, FN-binding repeats; M, membrane-spanning peptide; P/S, proline/serine-rich region; R, SC C-terminal repeats; RL4, domain homologous to the large ribosomal subunit L4; S, signal sequence (reproduced with modifications from [1]).

quence that mediates binding to vWf [32]. vWbp is predicted to interact with (pro)exosite I, which may affect the protein substrate specificity of the vWbp•(pro)thrombin complex, as does SC(1-325). The mechanism of fibrinogen cleavage by the vWbp•(pro)thrombin complex is also unknown, but may parallel that of SC.

The ZAAPs, NP_687847 and CAD46494, are produced by strains of *S. agalactiae* which originated from neonatal infections and were initially identified by genome sequencing as novel proteins [33, 34]. Preliminary results indicate that NP_687847 is a prothrombin activator with an unknown mechanism of activation [P. Panizzi and P. E. Bock, unpublished observations]. NP_687847 is identical to secreted fibrinogen-binding protein variant 1 (fgag v1; [35]). Recently, fgag v3 from a bovine mastitis strain was shown to contain a C-terminal 223-amino acid sequence that bound bovine fibrinogen [35], and a nearly identical sequence is present in NP_687847 and fgag v2. On this basis, fibrinogen is likely the adhesion ligand for NP_687847, and the three-member fgag family is a subset of the ZAAP family. Much remains to be learned about the mechanisms and the zymogen, substrate and adhesion protein specificities of ZAAPs. It is interesting to note that homologs of SC were identified in *S. agalactiae* and *S. pyogenes*. It has been well established that plasminogen activators (SK, SUPA, staphylokinase) occur in both staphylococcal and streptococcal strains, but to our knowledge no such procoagulant bacterial activators have been previously identified from streptococci.

The pathology of human ABE caused by coagulase-positive *S. aureus* and results from a murine model of pulmonary *S. aureus* infection [12] indicate clearly that expression of SC is a determinant of the severity of *S. aureus*-related disease. By targeting the last zymogen activation step of the blood clotting system, SC bypasses the normal coagulation reactions and escapes the inhibitory mechanisms that control the hemostatic response, resulting in unregulated fibrin formation. The pathological significance of the novel ZAAPs is unknown. We hypothesize that vWbp and SC may localize prothrombin activation by these zymogen-activator complexes to sites rich in vWf and fibrinogen, which are differentially localized in thrombus formation [36], to promote development of vegetations in ABE. Our finding that prothrombin activation by SC and other ZAAPs occurs via the N-terminal insertion mechanism may lead to the development of novel mechanism-based therapies for Gram-positive ABE.

Concluding remarks

The discovery of the ZAAP family may ultimately lead to a better understanding of the mechanism of bacterial pathogenesis involving zymogen activators that usurp the blood coagulation and fibrinolytic systems of the host to promote and propagate infection. The ZAAP family has expanded the number of non-proteolytic zymogen activators, whose specific functions and mechanisms remain to be determined. Although the N-terminal insertion mechanism of cofactor-induced zymogen activation is used by some ZAAPs, it is possible that other members of the structural family bind active serine proteinases, as SC does thrombin, altering their substrate and inhibitor specificities and localizing these activities to particular physiological sites. Although none have been found yet, it is conceivable that other conformational activators homologous to SC exist naturally and function as non-proteolytic activators of other serine proteinase zymogens, including zymogens of the blood coagulation, fibrinolysis, activated protein C, kallikrein-kinin or complement systems.

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