CHARACTERIZATION OF FIBRINOGEN-BINDING SURFACE PROTEIN B AND STAPHYLOCOAGULASE IN HUMAN BLOOD FIBRINOLYSIS AND COAGULATION

By

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Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Pathology

August, 2011

Nashville, Tennessee

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To my fiancé and best friend, Steve,

for the happiness, love, and

never-ending laughter he brings to my life

and

To my sweet “little girl,” Harley,

for her unconditional love and companionship

ACKNOWLEDGEMENTS

I would like to recognize my advisor, Dr. Paul E. Bock, for sharing his scientific knowledge and encouraging me to undertake the biomedical research program. I would like to thank my thesis committee, Ingrid Verhamme, Ph.D., and Richard L. Hoover, Ph.D., for their academic and personal support. I would also like to thank past and present members of the laboratory including Malabika Laha, M.S., who taught me the basics and how to make my first buffer, Karen Wiles, Ph.D., Jonathan Creamer, Ph.D., Anthony Tharp, Ph.D., Ashok Maddur, Ph.D., Jennifer Newell-Caito, Ph.D., and Samantha Bouldin, Ph.D., for their scientific support and camaraderie. I owe special gratitude to Heather K. Kroh, Ph.D. for spending time on a daily basis to teach me the techniques I have learned, answer every question I could think of, and ensure that I not only knew how to perform the experiments involved in my research, but that I understood why. She has constantly tested my knowledge and consequently helped me become a more confident scientist. Lastly, I would like to thank the National Institutes of Health (NIH) for the financial support of this work through NIH Grant 5-R37 HL071544, and the Training Grant in Mechanisms of Vascular Disease, 2-T32 HL07751, from the National Heart, Lung, and Blood Institute.

I would like to acknowledge one of my long-time support systems, Dr. Joe Wolf of Peace College, for being a phenomenal undergraduate professor and mentor, and for his ongoing guidance. I am indebted to my parents, Dr. Henry W. Davis, Jr. and Ceily M. Davis, as well as my sisters, Allison Glasson and Sarah

Caudle, for listening to my scientific jargon and encouraging me even when they didn’t comprehend my research. My final acknowledgment is for Steve Janson, my better half. Meeting him brought me to Nashville and gave me the opportunity to further my education. I will forever appreciate him being my stability, comedian, chef, and therapist throughout my graduate career. I wouldn’t have made it this far without his love and support.

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CHAPTER I

INTRODUCTION

Blood Coagulation and Fibrinolysis

The process of hemostasis involves a delicate balance between procoagulant and anticoagulant states. This balance is regulated by the coagulation and fibrinolytic systems that maintain normal blood fluidity, if thrombotic or bleeding disorders are not present. In the absence of vascular injury, the endothelial cells that line the lumen of vessel walls inhibit undesired coagulation and platelet adhesion by blocking the interaction of blood components with the extracellular matrix and tissue factor. Upon vessel injury, collagen and von Willebrand factor, proteins normally found in the subendothelial matrix, are exposed to the blood, and platelets quickly adhere to these proteins through membrane glycoprotein receptors. Primary hemostasis includes events such as platelet activation, secretion, and aggregation. During platelet activation, cytoskeleton-dependent shape changes occur and phosphatidylserine, a negatively charged phospholipid found in the inner leaflet of resting platelets, is exposed on the outer surface. This negatively charged phospholipid provides the appropriate trigger needed for the blood coagulation components to localize for activation (1-3).

The process of secondary hemostasis culminates in the formation of fibrin by the coagulation cascade. Activation of a series of serine protease zymogens,

the coagulation factors, and their cofactors occurs through step-wise proteolytic cleavage, resulting in a “cascade.” With the exception of tissue factor (TF), the enzymes and cofactors responsible for coagulation circulate in their inactive forms. The end result of the coagulation cascade is formation of thrombin, which in turn cleaves fibrinogen (Fbg) into fibrin. Once fibrin is produced, the transglutaminase factor XIIIa cross-links the fibrin polymers, and an insoluble fibrin mesh is formed that stabilizes the platelet plug formed during primary hemostasis. It is exposure of TF to the circulation after vascular injury that triggers activation of the coagulation cascade through the extrinsic pathway (4,5).

The extrinsic pathway of coagulation begins with the formation of a TF-factor VIIa complex. This complex activates factor IX and factor X, which together with its cofactor factor VIIIa, sustains factor X activation (6). The intrinsic pathway of coagulation is an alternate pathway that converges with the extrinsic pathway at the level of factor X activation. The intrinsic system is triggered when factor XII is activated on a charged surface, such as glass or collagen, through a process known as contact activation. After contact activation occurs, factor XIIa activates factor XI, and factor XIa goes on to activate factor IX. Subsequently, factor IXa in association with factor VIIIa converts factor X to factor Xa. Factor Xa and its non-enzymatic cofactor, factor Va, assemble on a negatively charged phospholipid surface, such as that of activated platelets, in the presence of Ca^{2+} to form the prothrombinase complex. The prothrombinase complex proteolytically converts prothrombin (ProT) to thrombin. It remains unclear as to the importance of the contact activation pathway in normal coagulation, but it is possible that it may be

necessary for the amplification phase of coagulation and may regulate fibrin formation during certain pathologic processes (7-9).

Once the platelet and fibrin-rich clot has been formed, the process of wound healing and eventual clot lysis begins. The key enzyme in fibrinolysis is plasmin (Pm), the active serine protease produced after activation of its zymogen precursor, plasminogen (Pg). Pm proteolytically cleaves insoluble fibrin, producing soluble degradation products that are easily cleared. The two major physiological Pg activators are tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), which use fibrin-dependent and fibrin-independent activation mechanisms, respectively (10) (Fig. 1).

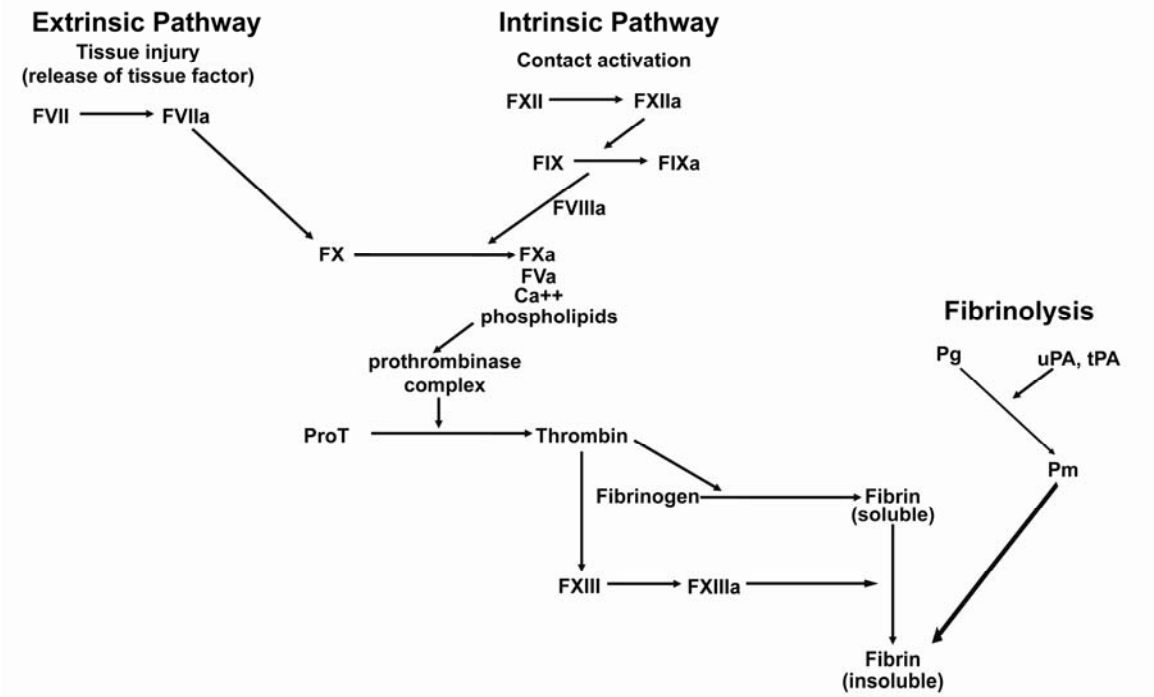

Figure 1. Schematic of blood coagulation and fibrinolysis pathways. After vascular injury, the factor VII-TF complex initiates the extrinsic pathway, resulting in thrombin production and fibrin formation. Contact activation triggers the intrinsic pathway with factor XIIa, also leading to factor X activation and thrombin formation. Once a clot is formed, Pg is converted to Pm by uPA and tPA, and Pm cleaves insoluble fibrin to soluble fibrin degradation products.

The intimate relationship between coagulation and fibrinolysis, and the ordered interactions and feedback mechanisms involved is what allows the hemostatic balance to maintain normal blood fluidity and integrity of the vasculature.

Prothrombin Activation and Staphylocoagulase

The enzymes involved in human blood coagulation and fibrinolysis belong to the serine proteases. All serine proteases exist in an inactive zymogen precursor form, and under physiological conditions they must be activated by proteolytic cleavage. Formation of a new NH_2-terminus through cleavage of the Arg^{15}-Ile^{16} (chymotrypsinogen numbering) peptide bond occurs; subsequently, Ile^{16} is inserted into the NH_2-terminal binding pocket where its α-ammino group forms a vital salt bridge with the carboxylate side chain of Asp^{194}, folding the activation domain of the zymogen and forming the substrate binding site and oxyanion hole necessary for proteolytic cleavage (11).

ProT, the zymogen precursor of thrombin, is composed of a fragment 1 domain spanning from its NH_2-terminus to residue 155, a fragment 2 domain composed of residues 156-271, and the catalytic domain from residues 272-581. Physiological ProT activation occurs through proteolytic cleavage by the serine protease of the prothrombinase complex, factor Xa, at the Arg^{320}-Ile^{321} and Arg^{271}-Thr^{272} peptide bonds. Cleavage at Arg^{320} alone produces meizothrombin, a proteolytically active intermediate, and subsequent cleavage at Arg^{271} releases fragments 1 and 2 of ProT. This process liberates a new Ile^{321} NH_2-terminus that can insert into the Ile binding pocket and form active thrombin (Fig. 2A). After

cleavage at Arg^{320}, thrombin contains an A chain and B chain that are linked by disulfide bonds.

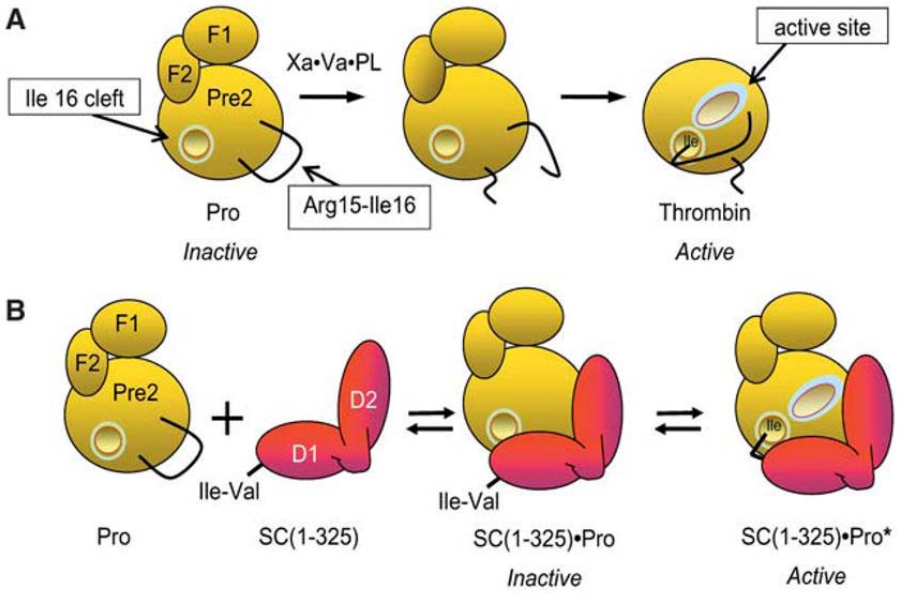

Figure 2. Physiological vs. staphylocoagulase-induced ProT activation. (*A*) The physiological mechanism of activation of ProT to thrombin. Thrombin is produced by cleavage of the Arg^{15}-Ile^{16} peptide bond in ProT and insertion of the newly liberated NH_2-terminus into the NH_2-terminal binding pocket. The active site and oxyanion hole are formed after folding of the activation domain occurs. ProT consists of the prethrombin 2 (*Pre 2*) catalytic domain, fragment 1 (*F1*), and fragment 2 (*F2*), and thrombin is liberated from F1 and F2 during activation. (*B*) The molecular sexuality mechanism of ProT activation by SC(1-325). SC forms a stoichiometric complex with ProT and inserts its own NH_2-terminus into the NH_2-terminal binding pocket of ProT, forming a critical salt bridge with Asp^{194} and triggering conformational activation. **(16)**

ProT peptide bond cleavage can occur in the reverse order when factor Va is absent from the prothrombinase complex. If Arg^{271} is cleaved prior to Arg^{320}, fragment 1.2 forms a non-covalently bound complex with prethrombin 2 (Pre 2) that is inactive (13,14) (Fig. 3).

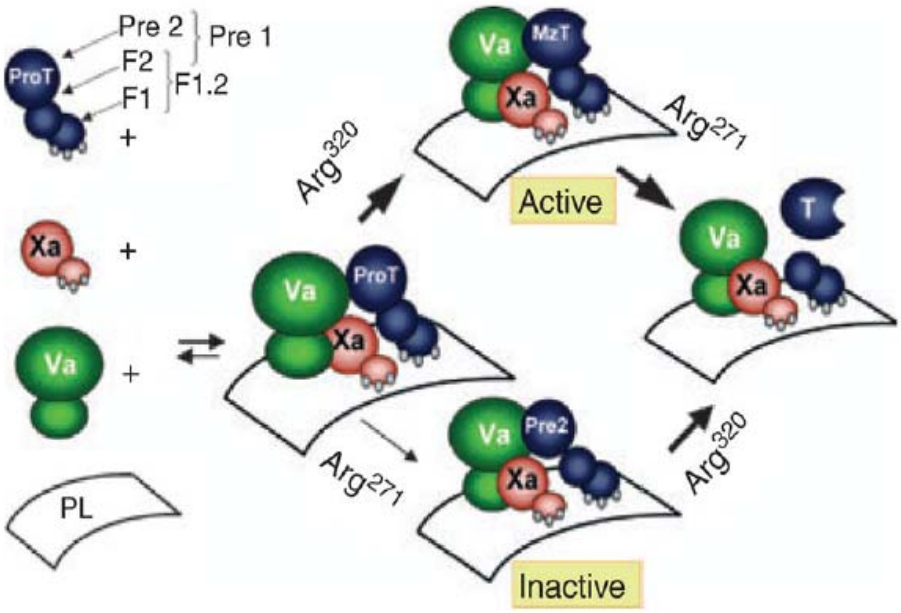

Figure 3. Activation of ProT by the prothrombinase complex. Initial cleavage at Arg^{320} by factor Xa generates the active intermediate meizothrombin (*MzT*). Alternate cleavage at Arg^{271} produces inactive prethrombin 2 (*Pre 2*) and fragment 1.2 (*F1.2*). **(18)**

There are also two thrombin-sensitive cleavage sites within ProT at Arg^{155} and Arg^{284}. Cleavage at these peptide bonds gives rise to prethrombin 1 that lacks fragment 1, and prethrombin 2', which is prethrombin 2 missing residues 272-284 (Fig. 4) (15). Anion-binding exosites I and II exist on the active thrombin molecule and are important for allosteric regulation of thrombin activity through substrate, effector, and inhibitor binding. Fibrin formation by thrombin is mediated by binding of Fbg through exosite I of thrombin (12).

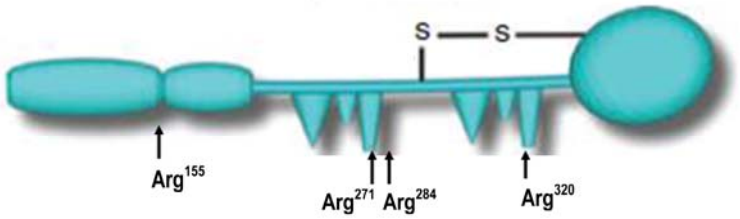

Figure 4. Cleavage sites in ProT. Initial ProT cleavage at Arg^{320} by factor Xa gives rise to meizothrombin. Subsequent cleavage at Arg^{271} releases fragment 1.2. Initial cleavage at Arg^{271} by factor Xa gives rise to fragment 1.2 and prethrombin 2. Two thrombin-sensitive cleavage sites exist at Arg^{155} and Arg^{284}. Cleavage at Arg^{155} produces prethrombin 1 that lacks F1 and thrombin cleavage at Arg^{284} gives rise to prethrombin 2'. **(17)**

Staphylocoagulase (SC), a 74-kDa, 660-residue protein secreted by *Staphylococcus aureus*, is also able to activate ProT and induce blood clot formation. SC contains NH_2-terminal D1-D2 domains that are responsible for binding and conformationally activating ProT, as well as a COOH-terminal region that contains seven 27-amino acid repeats with a 32-residue pseudorepeat that are able to adhere to Fbg (19). In contrast to prothrombinase, SC activates ProT through a non-proteolytic activation pathway known as the "molecular sexuality" mechanism. The NH_2-terminal dipeptide of SC, Ile-Val, mimics the NH_2-terminal Ile-Val-Gly/Asn residues conserved among most vertebrate serine proteases. SC exploits the physiological mechanism of serine protease activation by forming a stoichiometric complex with ProT (20), and then it inserts its own NH_2-terminus into the ProT NH_2-terminal binding pocket and forms a critical salt bridge with Asp^{194}, triggering conformational activation (Fig. 2B). Early fragmentation studies with full-length SC(1-660) determined that SC(1-325) (38-kDa) had the same activity as the full-length protein (21). Mutagenesis studies with NH_2-terminal SC(1-325) mutants reported that addition of an NH_2-terminal Met or deletion of

the first (Ile) and/or second (Val) residue(s) significantly reduced the ability of SC to bind and activate ProT, showing that the NH_2-terminal dipeptide of SC is imperative for conformational activation of ProT (16). The mechanism of conformational ProT activation by SC and the role that the NH_2-terminal SC residues play in ProT activation are not well understood.

SC•ProT is hypothesized to bind Fbg through a novel substrate recognition exosite expressed by the complex, and it directly cleaves Fbg into fibrin, inducing the formation of fibrin clots (16,22,23). The SC•ProT complex is not inhibited by physiological ProT inhibitors, including hirudin, antithrombin III, and heparin, even in the presence of 5-fold molar excess. Exosite I is blocked by the D2 domain of SC in the SC•ProT complex, yet Fbg is still cleaved with high specificity, further supporting the hypothesis that a new Fbg substrate recognition exosite is expressed on the SC•ProT complex (23). SC is the prototype for a group of proteins called Zymogen Activator and Adhesion Proteins (ZAAPs), which bind plasma or extracellular matrix proteins and possess homologous D1-D2 domains of similar structure and fold to SC(1-325). Other proteins that belong to this family include SfbX from *Streptococcus pyogenes*, vWbp from *Staphylococcus aureus,* and the novel protein NP_687847 from *Streptococcus agalactiae*. The protein FbsB, or fibrinogen-binding surface protein B, that corresponds to the CAD46494 gene of the NEM316 strain of *Streptococcus agalactiae* was also initially hypothesized to be a member of the ZAAP family (Fig. 5) (16).

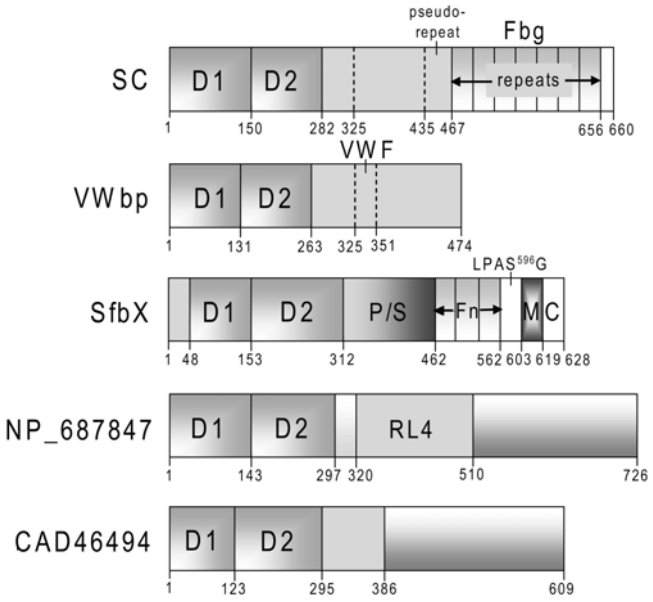

Figure 5. Domain organization of ZAAPs. All members of the ZAAP family contain homologous NH_2-terminal D1-D2 domains and COOH-terminal adhesion protein-binding domains. (Adapted from (16))

Staphylococcus aureus Pathogenesis and Acute Bacterial Endocarditis

Staphylococcus aureus is a commensal gram-positive organism that can colonize the nose and skin of healthy individuals. It is when the barriers of the skin and mucosa are breached that *S. aureus* can invade and enter the tissue and bloodstream and potentially cause illness. Pathologies caused by colonization of *S. aureus* range from meningitis, sepsis, and pneumonia to endocarditis and septic arthritis in high risk populations, such as infants, immunocompromised adults, and intravenous drug users. The presence of foreign materials in the body, including intravenous catheters, greatly increases the risk of developing *S. aureus*-induced endocarditis because the catheters become coated with Fbg and fibronectin, to which the bacterium can easily adhere (24). Methicillin-resistant *S. aureus* (MRSA) strains have evolved to

produce β-lactamases that break down penicillin and methicillin, and it is estimated that only 5% of *S. aureus* isolates are susceptible to penicillin treatment (25). Another common antibiotic used to treat these infections is vancomycin; however, *S. aureus* has also developed resistance to this antibiotic through a currently unknown mechanism. In light of the overwhelming number of antibiotic resistant strains of *S. aureus*, the need for new treatments for staphylococcal infections is integral to the survival of infected individuals.

Acute bacterial endocarditis (ABE), one of the illnesses caused by *S. aureus*, is a life-threatening disease characterized by inflammation of the inner lining of the heart and heart valves. Vegetations can form at sites of endothelial injury caused by turbulent blood flow, the presence of intravascular catheters, intravenous drug use, or physiological stress from hypersensitivity states, hormonal changes, or exposure to high altitudes. After the injury occurs, coagulation is initiated and a sterile thrombus composed primarily of fibrin and platelets forms. Bacteria can then enter the bloodstream and adhere to the sterile thrombus through binding Fbg and fibronectin. The vegetation undergoes a "maturation" process where it is layered with more fibrin, bacteria, and platelets. As it matures and grows in size, the vegetation is at risk of embolization, which can lead to abscesses, heart failure, myocardial infarction, and stroke (Fig. 6). The interaction of SC with Fbg may contribute to the virulence of acute bacterial endocarditis by allowing the pathogen to elude the host immune system by forming protective fibrin-platelet-bacteria vegetations (11,26-28).

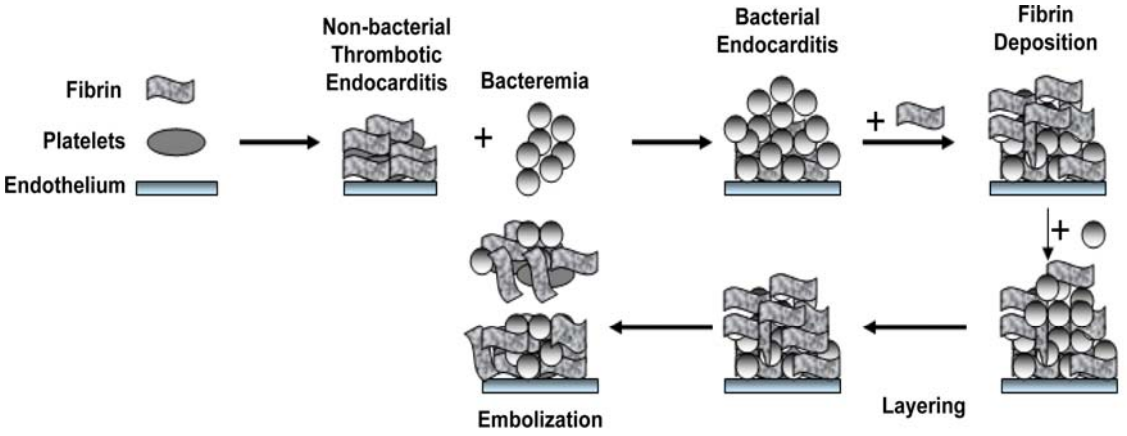

Figure 6. Schematic of the pathogenesis of acute bacterial endocarditis. (Adapted from (29))

Plasminogen and Plasmin

Pg is a 791-residue, 92-kDa zymogen form of Pm comprised of an NH_2-terminal PAN (Pg/apple/nematode) module, five kringle domains, and a COOH-terminal serine protease catalytic domain. Pg is present in two major forms, Glu-plasminogen ([Glu]Pg) and Lys-plasminogen ([Lys]Pg), which differ according to their NH_2-terminal residues, and both forms have two isoelectric variants, Pg I and Pg II, that vary by the number of glycosylation sites. Pg I has N-linked (Asn^{278}) and O-linked (Thr^{345}) polysaccharides whereas Pg II only contains the O-linked polysaccharide at Thr^{345} (30-32). Native Pg, [Glu]Pg, circulates in the bloodstream and its two carbohydrate variants are referred to as Glu-plasminogen I ([Glu]Pg I) and Glu-plasminogen II ([Glu]Pg II). After proteolytic cleavage by Pm releases the NH_2-terminal 77-residue PAN module from [Glu]Pg, the second major form of Pg, [Lys]Pg, is formed (33). The carbohydrate variants of [Lys]Pg are referred to as [Lys]Pg I and [Lys]Pg II. [Glu]Pg and Pm contain important binding sites for *L*-lysine and 6-aminohexanoic acid (6-AHA) (34),

which allow the proteins to bind fibrin and Fbg (35) and Pm to interact with its major inhibitor α_2-antiplasmin (36). [Glu]Pg contains lysine-binding sites (LBS) in kringles 1, 4, and 5 (31), and in its native form exists in a compact α-conformation that is slowly activated by physiological Pg activators and stabilized by the interaction between its NH_2-terminal PAN module LBS in kringles 4 and 5 (37-39). Once [Glu]Pg is cleaved by Pm, [Lys]Pg is generated, exhibiting an extended β-conformation that allows for the rate of Pg activation by uPA and tPA to increase. [Glu]Pg also converts to an extended β/γ conformation when effectors are bound to its LBS (34,40-46).

Pg is physiologically activated by uPA and tPA through proteolytic cleavage of the Arg^{561}-Val^{562} peptide bond in the catalytic domain. uPA is a 411-amino acid, 54-kDa protein that contains an NH_2-terminal epidermal growth factor-like domain, a finger-like domain, a single kringle domain, and a COOH-terminal serine protease catalytic domain. It activates Pg through a fibrin-independent mechanism, because it lacks a LBS and, therefore, lacks the ability to bind fibrin (31). Conversely, tPA activates Pg through a fibrin-dependent mechanism. The structure of tPA differs from uPA, because it contains a second kringle domain with a LBS that has high affinity for not only fibrin, but also lysine-sepharose, and the lysine analog, 6-aminohexanoic acid (6-AHA) (Fig. 7). There are two forms of tPA: the 70-kDa low-activity, zymogen-like form called single-chain tPA (sctPA), and the more active form generated by Pm cleavage of the Arg^{275}-Ile^{276} peptide bond called two-chain tPA (tctPA) (44).

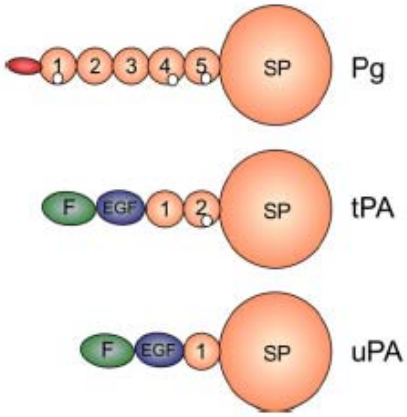

Figure 7. Domain structure of native Pg, tPA, and uPA. Serine protease (*SP*), kringles (*1-5*), epidermal growth factor (*EGF*), and finger-like (*F*) domains with LBS marked by *white circles* and PAN (Pg/apple/nematode) module in *red* (31,47).

Pm, uPA, and tPA are targets of the major inhibitors of the fibrinolytic system. Serine protease inhibitors (serpins) comprise a class of inhibitors that are responsible for regulating most plasma proteases. Serpins act through a mechanism that involves formation of a stoichiometric complex with the plasma protease. After a complex is formed, the reactive center loop of the serpin is cleaved by the protease, resulting in a terminal covalent complex which traps the acyl-enzyme intermediate in its inactive form (48). Free, unbound Pm is inhibited by α_2-antiplasmin, and binding of fibrin protects Pm from the action of α_2-antiplasmin and allows appropriate fibrin proteolysis. The main physiological inhibitor of Pg activation by the activators tPA and uPA is plasminogen activator inhibitor-1 (PAI-1). When tPA is bound to fibrin, it is protected from inhibition by PAI-1. Thrombin-activatable fibrinolysis inhibitor (TAFI) is unique in that it does not belong to the serpin family. TAFI can cleave COOH-terminal Lys residues from fibrin, thus inhibiting fibrin proteolysis by preventing Pg/Pm binding (49,50).

These fibrinolytic inhibitors are necessary to restrict clot lysis only until an insult to the vessels has been repaired by the coagulation cascade.

Bacterial Plasminogen Activators

One of the most highly studied bacterial Pg activators is streptokinase (SK), a 47-kDa protein produced by Group A, C, and G streptococci. SK binds and conformationally activates Pg through the "molecular sexuality" mechanism, forming a reversible SK•Pg* active complex that can proteolytically cleave a second Pg molecule to Pm. SK can also bind to Pm and alter its substrate specificity, so that it becomes a potent Pg activator (Fig. 8) (27,51,52).

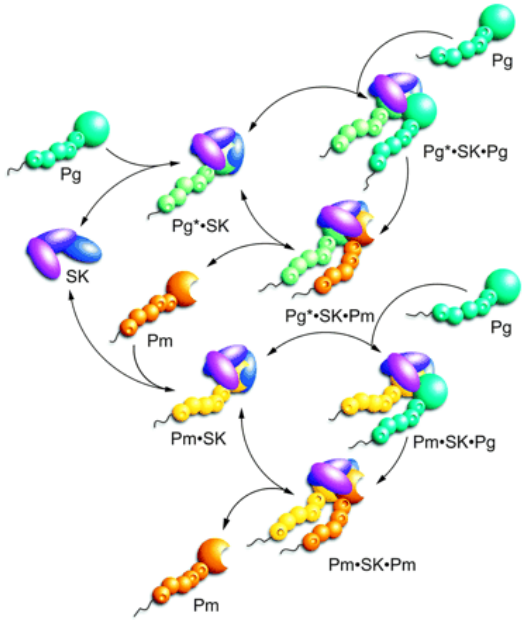

Figure 8. Mechanism of Pg activation by SK•Pg* complex. SK binds Pg and forms a reversible SK•Pg* active complex. This complex then forms a ternary Pg*•SK•Pg complex from which Pg is cleaved into Pm. Free Pm can bind SK and form an SK•Pm complex that then binds Pg, forming Pm•SK•Pg, which generates free Pm (60).

The molecular sexuality mechanism of Pg activation involves insertion of the NH_2-terminal Ile^1 residue of SK into the NH_2-terminal binding cleft of Pg. Ile^1 forms a salt bridge with Asp^{194} (chymotrypsinogen numbering), inducing a conformational change that forms the oxyanion hole and substrate binding site of Pg (53-56). SK binding to Pg is regulated by interactions of kringle 5 of Pg with Arg^{253}, Lys^{256}, and Lys^{257} of the SK 250-loop (57). The COOH-terminal Lys^{414} of SK is also important for enhancing Pg and Pm binding affinity and subsequent Pg formation (58). The activity of SK-bound Pm is protected from inhibition by α_2-antiplasmin, like the protection seen with fibrin-bound Pm (59). A recently characterized bacterial cofactor for Pg activation, skizzle (SkzL), binds both [Lys]Pg and [Glu]Pg in a LBS-dependent manner, and enhances [Glu]Pg activation by uPA, as well as [Glu]Pg and [Lys]Pg activation by tPA (Fig. 9). Binding of Pg by SkzL is facilitated by the COOH-terminal Lys^{415} residue of SkzL, and it is hypothesized that this binding may convert [Glu]Pg from its compact α-conformation to its extended γ-conformation, rendering Pg more susceptible to the action of uPA. To date, SkzL is the only known protein secreted by *Streptococcus agalactiae* that contributes to human fibrinolytic activity.

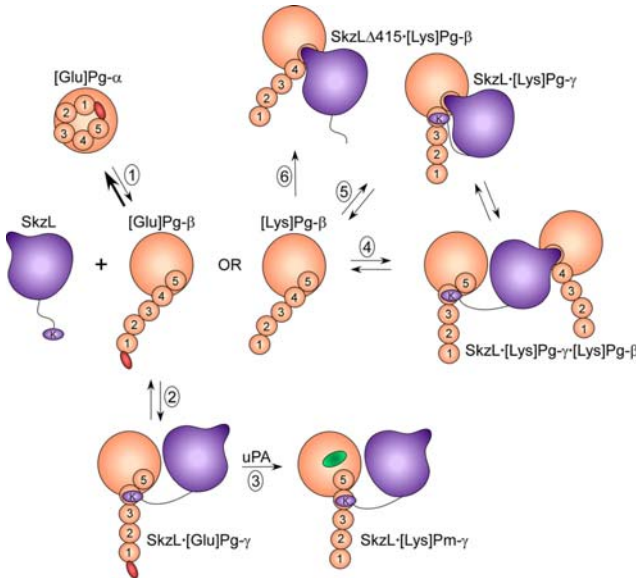

Figure 9. Mechanism of SkzL-mediated [Glu]Pg activation by uPA, and hypothesized LBS-dependent complexes formed with [Lys]Pg and Pm mediated by SkzL. The proposed mechanism of SkzL-dependent [Glu]Pg activation by uPA is shown in Reactions 1, 2, and 3. SkzL binds to the compact β-conformation of [Glu]Pg, mediated by binding of Lys^{415} of SkzL to the LBS of kringle 4 of Pg. This converts the SkzL•[Glu]Pg complex to the extended γ-conformation, thus accelerating the rate of [Glu]Pg activation by uPA. The proposed mechanism for SkzL and [Lys]Pg or Pm complex formation is shown in Reactions 4, 5, and 6. Reaction 4 shows formation of a ternary SkzL•$[Lys]Pg_2$ complex in which one Pg molecule is bound through the Lys^{415}-kringle 4 interaction in the γ-conformation and binding of the second Pg molecule is mediated by the SkzL internal motif-kringle 5 interaction, with Pg in the β-conformation; Reaction 5 illustrates formation of an SkzL•[Lys]Pg complex in the γ-conformation with the internal motif and Lys^{415} bound to their respective kringle domains; Reaction 6 displays complex formation between SkzLΔK415 and [Lys]Pg or Pm mediated by the internal motif in SkzL (61).

Streptococcus agalactiae Pathogenesis

Streptococcus agalactiae (Group B streptococci, GBS) can cause meningitis, sepsis, pneumonia, and endocarditis in neonates and immunocompromised patients. Several streptococcal surface proteins, including α-enolase and glyceraldehyde-3-phosphate-dehydrogenase, can interact with the human fibrinolytic system by binding Pg (40,62). *S. agalactiae* does not express

SK, and SkzL is currently the only protein secreted by GBS that has been characterized to influence fibrinolytic activity (61). Fibrinogen-binding surface protein A (FbsA) is a protein from *S. agalactiae* that binds to human Fbg through multiple (3-30 total) NH_2-terminal 16-amino acid (GNVLERRQRDAENRSQ) repeat sequences (40,63). A second protein thought to bind human Fbg, fibrinogen-binding surface protein B (FbsB), was discovered through genomic sequencing of the serotype III strain NEM316 of *S. agalactiae* (64,65). FbsB does not share sequence homology with FbsA, but does share minor 22% identity with SfbX, a fibronectin-binding protein from *Streptococcus pyogenes* (66,67). FbsB was hypothesized to activate one of the coagulation serine protease zymogens and possibly be a member of the SC-like ZAAP family, due to its potential binding of Fbg and predicted homologous D1-D2 domains (16). However, initial studies presented here suggest that FbsB is not a ZAAP, but rather it interacts with Pg and Pm and may be only the second protein from *S. agalactiae* with the ability to enhance tPA-dependent Pg activation, potentially contributing to *S. agalactiae* pathogenesis.

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CHAPTER II

FIBRINOGEN-BINDING SURFACE PROTEIN B IS A NOVEL PROTEIN FROM *STREPTOCOCCUS AGALACTIAE* THAT INTERACTS WITH PROTEINS INVOLVED IN HUMAN FIBRINOLYSIS

Introduction

Streptococcus agalactiae, commonly referred to as Group B streptococci (GBS), causes human illnesses including meningitis, sepsis, and pneumonia in neonates as well as endocarditis, cellulitis, and arthritis in immunocompromised and elderly individuals (1,2). Serotype III of GBS initiates most cases of neonatal meningitis induced by *S. agalactiae* (3), and there is a need to elucidate further the molecular mechanisms contributing to its virulence to enable prevention and therapeutic intervention. *S. agalactiae* infects its host through several pathways that involve evasion of the immune system and infiltration and colonization of epithelial and endothelial surfaces (4). Group B streptococci do not express streptokinase (SK), a virulence factor expressed by Group A, C, and G streptococci (5), but they can usurp the human fibrinolytic system through other mechanisms. *S. agalactiae* can elude host immune defenses and exploit the fibrinolytic system by adhering to fibrinogen (Fbg) and either directly or indirectly interacting with plasminogen (Pg) and/or plasmin (Pm) (6,7). Fibrinogen-binding surface protein A (FbsA) is a recently characterized GBS protein that binds to human fibrinogen through its 3 to 30 NH_2-terminal 16-amino acid repeat sequences (GNVLERRQRDAENRSQ) (8,9). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and α-enolase are proteins expressed by *S. agalactiae*

that enhance GBS virulence and contribute to the activation and coating of Pg and active Pm on the bacterial surface (8,10). To date, the only known protein secreted by *S. agalactiae* that contributes to human fibrinolytic activity is skizzle (SkzL). SkzL has been shown to form complexes with both human Pg and Pm in a lysine-binding site (LBS)-dependent manner. Binding of SkzL to Pg and Pm amplifies activation of native human Glu-plasminogen ([Glu]Pg) by the Pg activators, urokinase-type plasminogen activator (uPA) and single-chain tissue-type plasminogen activator (sctPA) (11).

Fibrinogen-binding surface protein B (FbsB) is a novel, 70-kDa protein that was discovered upon genomic sequencing of the serotype III strain NEM316 of *S. agalactiae*, isolated from a case of fatal septicemia (3,12). FbsB is hypothesized to be an anchorless adhesin (13) and lacks any sequence homology with FbsA or its Fbg-binding repeats (14), yet it shares 22% identity with Sfbx, a fibronectin-binding protein from *Streptococcus pyogenes* (15). FbsB has a conserved COOH-terminal region of 223 residues that are almost identical to the residues responsible for Fbg binding characterized in fgags, a novel family of Fbg-binding proteins from a bovine strain of *S. agalactiae* (16). Other than the similarity between FbsB and its bovine homologs the COOH-terminal region of FbsB shows no sequence homology with any previously characterized protein (14). Several studies reported that the NH_2-terminal portion of FbsB binds human Fbg and not the COOH-terminal conserved region, which was found to bind only bovine Fbg, although the protein is from a human strain (12,16). Circular dichroism spectra from a recent study indicate that FbsB is composed primarily

of alpha helices and random coils with few beta strands, although the structure of most Fbg-binding proteins consists of mainly beta sheets. For these reasons, further characterizing the structure and function of FbsB, especially the conserved COOH-terminal region, may be useful in developing therapeutic agents to treat GBS infections (14).

Another protein capable of forming a complex with fibrinogen and subsequently hijacking the human coagulation cascade is staphylocoagulase (SC). SC from *Staphylococcus aureus* induces coagulation by conformationally activating prothrombin, the zymogen form of the active serine protease, thrombin. SC is a bifunctional protein with the ability to adhere to Fbg through its COOH-terminal 7, 27-residue repeat sequences, and to bind and activate prothrombin through its NH_2-terminal D1-D2 domains. SC is the first member of a group of proteins called Zymogen Activator and Adhesion Proteins (ZAAPs). Proteins including Sfbx from *S. pyogenes*, VWbp from *S. aureus,* and the novel protein NP_687847 from *S. agalactiae* were assigned to the ZAAP family because they adhere to plasma or extracellular matrix proteins and possess homologous D1-D2 domains of similar structure and fold to the active SC fragment, SC(1-325). The novel protein, FbsB, was hypothesized to be a member of the ZAAP family with the potential to activate prothrombin or one of the other serine protease zymogens of the human coagulation cascade (17).

Pg, a component of the fibrinolytic system, is present in at least four major forms that can be isolated from human plasma and vary according to their NH_2-terminal residues and number of glycosylation sites. The native form of

plasminogen, [Glu]Pg, circulates in the bloodstream and has two carbohydrate variants referred to as Glu-plasminogen I ([Glu]PgI) and Glu-plasminogen II ([Glu]PgII). [Glu]Pg is composed of an NH_2-terminal PAN (Pg/apple/nematode) module and five kringle domains, and a COOH-terminal serine protease zymogen catalytic domain. The second major form of Pg, Lys-plasminogen ([Lys]Pg), also contains two variants based on their pattern of glycosylation and is formed when the catalytic action of Pm releases the NH_2-terminal PAN module from [Glu]Pg (7,11,18). [Glu]Pg and Pm contain important binding sites for *L*-lysine and 6- aminohexanoic acid (6-AHA) (19) that allow the proteins to bind fibrin (Fbn) and Fbg (20), and for Pm to interact with its major inhibitor α_2-antiplasmin (21). [Glu]Pg contains LBS in kringles 1, 4, and 5 (7), and in its native form exists in a compact conformation that is stabilized by the interaction between its NH_2-terminal PAN module LBS in kringles 4 and 5 (22-24) . Once [Glu]Pg is cleaved by Pm, [Lys]Pg is generated, which exhibits an extended conformation that allows Pg to be proteolytically cleaved and converted to the active enzyme Pm at an accelerated rate (8,19,25-30).

Physiologically, activation of Pg is achieved by proteolytic cleavage of the Arg^{561}-Val^{562} peptide bond in its catalytic domain, resulting in formation of the serine protease, Pm. The conversion of Pg to Pm is catalyzed by the Pg activators, uPA and tPA (31,32). There are two forms of tPA, the low-activity zymogen-like form, single-chain tPA (sctPA), and the more active two-chain form (tctPA) that is formed following cleavage of the Arg^{275}-Ile^{276} peptide bond by Pm (7,28). Though uPA and tPA are both serine proteases proteolytically activated

by Pm, they encompass one major structural difference. Urokinase contains a single kringle domain that lacks a LBS, whereas tPA also has a second kringle domain containing a LBS with affinity for not only *L*-lysine and 6-AHA, but also Fbn (33,34). Pathophysiologically, Pg can be converted to Pm in the presence of SK, and Pm generation is greatly enhanced. The active SK•Pg* complex binds a substrate Pg molecule through kringle 5 of Pg with Arg^{253}, Lys^{256}, and Lys^{257} of the SK 250-loop and proteolytically converts it into Pm. Pg is conformationally activated by insertion of its NH_2-terminal Ile-Ala-Gly into the Pg NH_2-terminal activation pocket, inducing conformational activation of the zymogen catalytic site (35). The COOH-terminal Lys^{414} of SK also plays a critical role in enhancing Pg and Pm binding affinity and subsequent Pg formation (36). SkzL enhances Pg activation by binding [Glu]Pg, likely through kringle 4, in a LBS-dependent manner and shifting Pg to its extended conformation, making it more susceptible to activation by uPA. [Glu]Pg activation by single-chain tPA (sctPA) is also enhanced by SkzL through a currently unknown mechanism (11).

With FbsB hypothesized to bind human Fbg and to contain a D1-D2 domain homologous to SC, it was thought that FbsB may be a member of the bifunctional ZAAP family with the ability to conformationally activate a serine protease zymogen. Based on studies performed in this chapter, FbsB neither bound Fbg nor activated any of the zymogens of the human coagulation cascade tested. However, it was discovered that FbsB is able to bind Pm in a LBS-dependent manner and significantly inhibit the rate of Pm substrate hydrolysis. In preliminary experiments, FbsB appeared to enhance Pg activation in the

presence of tPA, similar to the action of SkzL. It is possible that FbsB may be only the second characterized protein secreted from *S. agalactiae* capable of contributing to the spread of GBS infections through its human fibrinolytic activity.

Materials and Methods

Cloning, expression, and purification of FbsB constructs

The FbsB gene was PCR-amplified from *S. agalactiae* NEM316 strain (ATCC) genomic DNA and cloned into a modified pET30b(+) vector (Novagen) (37) using *Nco*I and *Xho*I restriction sites. FbsB was expressed with an NH_2-terminal His_6-tag and tobacco etch virus (TEV) proteinase cleavage site in Rosetta 2 (DE3) pLysS *E. coli* (Novagen) with lactose induction. Recombinant FbsB protein was purified from the soluble fraction by Ni^{2+}-iminodiacetic acid chromatography. The His_6-tag was removed by overnight incubation with a 1:10 molar ratio of TEV proteinase to fusion protein (36). Protein expression with the native FbsB construct was minimal and unproductive, therefore an optimized gene construct was made by OptimumGene™ Codon Optimization Analysis (GenScript), using the following parameters to increase the efficiency of gene expression: codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature PolyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motif, inhibition sites, and repeat sequences. Post-optimization subcloning of the altered FbsB gene was performed as above. Optimized FbsB was expressed in

BL21 (DE3) pLysS *E. coli* (Novagen) and purified as described. The FbsBΔK607ΔE608 mutant was generated by QuikChange site-directed mutagenesis (Stratagene). FbsB concentration was determined using the following calculated absorption coefficient ($(mg/ml)^{-1}$ cm^{-1}) at 280 nm (38) and molecular weight: 1.28, 70,224.

Kinetic screening of FbsB with coagulation proteins

Kinetic assays were performed with 100 nM of the following coagulation zymogens: factor X, [Lys]Pg, [Glu]Pg, prothrombin, protein C, factor IX, factor XI, factor XII, and prekallikrein. Assays were performed in the absence of FbsB and as a function of FbsB concentration. The proteases respective chromogenic substrate (200 μM) was added after 10 min incubation of FbsB and the coagulation proteins at 25 ºC.

Kinetic assays were also performed in the presence and absence of FbsB and the following active serine proteases: factor Xa, Pm, thrombin, activated protein C (APC), factor IXa, factor XIa, factor XIIa, and kallikrein. As before, 200 μM of the respective chromogenic substrate was added after a 10 minute incubation at 25ºC with FbsB and the active enzyme. The initial rate of chromogenic substrate hydrolysis measured at 405 nm was compared with and without FbsB for each assay.

Factor V clotting activity assays

Clotting activity assays were done with a fibrometer. Equal volumes (100 µl) of factor V-deficient plasma, diluted normal plasma (1:10 to 1:1000), and 20 mM Hepes, 0.15 M NaCl, 1 mg/ml PEG 8000, pH 7.4 were incubated at 37 ºC. Increasing concentrations of FbsB were added (1-5 µM), with thromboplastin and 5 mM $CaCl_2$ added to initiate clotting. Clotting times with FbsB were compared to a standard curve of log clotting time *versus* log dilutions of normal plasma in the absence of FbsB.

Pm-Sulfolink affinity chromatography

FbsB was applied onto a Pm-Sulfolink affinity column (5 mL) equilibrated in 0.1 M Hepes, 10 mM NaCl, 1 mM EDTA, pH 7.4. The column was washed with equilibration buffer and eluted with 50 mM 6-AHA step-elution in the same buffer.

Fbg and fibronectin affinity chromatography

FbsB in 100 mM Hepes, 10 mM NaCl, 1 mM EDTA, pH 7.4 was loaded onto a 25 ml human Fbg-agarose affinity column containing 6 mg/ml Fbg coupled to the matrix, or a 14 ml bovine Fbg-agarose affinity column containing 8.2 mg/ml Fbg coupled to the matrix, or an 8 ml human fibronectin-agarose column containing 3.8 mg/ml fibronectin coupled to the matrix. The loaded column was washed with the equilibration buffer.

Superdex 200 size-exclusion chromatography

Identical concentrations (17 μM) of FbsB and Fbg in 50 mM Hepes, 125 mM NaCl, pH 7.4 were first chromatographed alone on a Superdex 200 gel filtration column. To detect any interaction, a reaction containing 17 μM FbsB and 17 μM Fbg in the same buffer was incubated at 25ºC for 30 min and the mixture was chromatographed under the same buffer conditions.

FbsB kinetic titrations of Pm

Pm (10 nM) was titrated with FbsB and Pm inhibition was measured by the decrease in the initial rate of hydrolysis of 200 μM H-*D*-Val-Leu-Lys-*p*NA at 405 nm and 25 ºC. FbsB was incubated with Pm for 5 min in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml polyethylene glycol (PEG) 8000, pH 7.4, before initiating the assay by substrate addition. Assays with 6-AHA were performed with 5 min incubation of Pm in 50 mM Hepes, 125 mM NaCl, 10 mM 6-AHA, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4, followed by 5 min incubation with FbsB before substrate addition. The titrations were analyzed by nonlinear least-squares fitting of a hyperbola.

Pm chromogenic substrate hydrolysis

The initial rate of hydrolysis by Pm of varying concentrations of different chromogenic substrates (Table 1) was measured at 405 nm and 25 ºC, in the presence and absence of 1 μM FbsB. FbsB was incubated with Pm for 5 min in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4, before

substrate addition. k_{cat} and K_M were determined by fitting of the substrate dependence by the Michaelis-Menten equation in the absence and presence of FbsB.

Pg kinetic titrations with FbsB and Pm

Titrations of [Lys]Pg and [Glu]Pg in the presence of 10 nM Pm and 500 nM FbsB were performed, and the rate of Pm formation was measured by an increase in the rate of hydrolysis of 200 μM H-*D*-Val-Leu-Lys-*p*NA. Pg was incubated with Pm and FbsB for 5 min in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4 before chromogenic substrate addition. Assays with 6-AHA were performed with 5 min incubation of plasmin in 50 mM Hepes, 125 mM NaCl, 10 mM 6-AHA, 1 mM EDTA, 1 mg/ml polyethylene glycol (PEG) 8000, pH 7.4, followed by 5 min incubation with FbsB and Pg before substrate addition. The maximum velocity was determined by nonlinear least-squares analysis of the hyperbolic titrations.

[Lys]Pg activation kinetics

The kinetics of activation of 100 nM [Lys]Pg by 2 nM tctPA, as a function of FbsB concentration, were performed by discontinuous measurements of the linear initial rates of hydrolysis of 200 μM H-*D*-Val-Leu-Lys-*p*NA at 405 nm and 25 ºC. Assays were performed in 100 mM Hepes, 100 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4. In assays with FbsB, FbsB and tctPA were incubated for 10 min; [Lys]Pg was then added and incubated with FbsB and tctPA for 10

min prior to chromogenic substrate addition. The results were analyzed by nonlinear least-squares fitting of the hyperbolic titrations.

Western blots with Fbg

Samples of 0.05 μg staphylocoagulase and 25 μg FbsB were electrophoresed on 4-15% Tris-glycine gradient gels, and transferred onto Immobilin-FL polyvinyldiene difluoride (PVDF) membranes in Tris/glycine western transfer buffer containing 10% methanol. Membranes were blocked for 1, 2, or 5 hours in 50 mM Tris, 150 mM NaCl, pH, 7.5, 0.1% Tween-20 (TBS-T) + 3% bovine serum albumin (BSA), TBS-T containing 5% dry milk, TBS-T containing 5% casein, or Odyssey blocking buffer (LI-COR). Fbg (2 μg/ml) was added to the respective blocking buffer and incubated with the membrane for 1 h for the experiments in the presence of Fbg. An overnight incubation was performed with a primary goat anti-human Fbg antibody (Abcam, ab6666). The membranes were washed with TBS-T and incubated for 2 h with a donkey anti-goat secondary LI-COR IR 800 antibody. Bands were visualized using a LI-COR Odyssey Imaging System.

FbsB and Pm solubility reactions

Native or FFR-CH_2Cl active-site blocked Pm (4.5 μM) and FbsB (10 μM) were incubated for up to 60 min. At each time point tested, 40 μl of the reaction was removed and quenched with 2 μl FFR-CH_2Cl and 10 μl of hot sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) treatment

buffer, boiled for 2 min, and electrophoresed on 4-15% Tris-glycine gradient gels. Protein bands were Coomassie-stained.

FbsB turbidity assays

Precipitation of FbsB by 5 nM Pm was monitored from the increase in turbidity at 350 nm at 25 ºC under various buffer conditions. The buffer used for kinetic assays (50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4) was altered in the following ways: Hepes concentration (50-500 mM), replacing Hepes with 100 mM Na_2HPO_4, Tris, or Bis-Tris propane, NaCl concentration (125-500 mM), replacing NaCl with 500 mM KCl, addition of 10 mM $CaCl_2$, or addition of 2-10% glycerol.

NH_2-terminal sequencing of FbsB degradation products

FbsB (10 μM) and Pm (25 nM) were incubated for 20 min in 50 mM Hepes, 125 mM NaCl, 1 mM EDTA, 1 mg/ml PEG 8000, pH 7.4. A 40 μl sample was quenched with 10 μl hot SDS-PAGE treatment buffer (non-reducing), electrophoresed on 4-15% Tris-glycine gradient gel, and transferred onto an Immobilin-FL PVDF membrane in 0.2 M 3-[cyclohexylamine]-1-propane sulfonic acid (CAPS), pH 11 containing 10% methanol. The membrane was stained with Ponceau S, and major degradation bands were excised. NH_2-terminal sequencing by Edman degradation was performed by the Molecular Structure Facility at the University of California, Davis.

Results

Effect of FbsB on proteins of the human coagulation cascade

To determine whether FbsB was able to interact with proteins involved in human blood coagulation, chromogenic substrate kinetic assays with the following serine protease zymogens and active proteases were completed: factor X, factor Xa, [Lys]Pg, [Glu]Pg, Pm, prothrombin, thrombin, protein C, activated protein C, factor IX, factor IXa, factor XI, factor XIa, factor XII, factor XIIa, prekallikrein, and kallikrein. Assays were carried out in the presence and absence of FbsB to observe any change in the rate of substrate hydrolysis resulting from FbsB addition. FV-dependent plasma clotting assays were performed in the presence and absence of FbsB. A substantial decrease in the rate of chromogenic substrate hydrolysis by Pm was measured in the presence of FbsB. The rate of hydrolysis of 200 μM H-*D*-Val-Leu-Lys-*p*NA by Pm (10 nM) was 0.11 μM *p*NA/s, compared to a rate of 0.03 μM *p*NA/s in the presence of FbsB (1 μM). This indicated that FbsB may inhibit Pm activity. No change in the rate of substrate hydrolysis was measured for the serine protease zymogens and their corresponding proteases in the presence of FbsB (data not shown).

Binding and inhibition of Pm by FbsB

Because the zymogen and enzyme screen demonstrated that FbsB inhibits the rate of Pm substrate hydrolysis, chromogenic substrate kinetic assays were carried out with varying concentrations of FbsB and Pm (10 nM) to determine the degree of inhibition of Pm substrate hydrolysis in the presence of FbsB. In the absence of FbsB, the initial velocity (v_o) of substrate hydrolysis was

0.11 ± 0.005 μM *p*NA/s, compared to a limiting velocity (v_{lim}) of 0.022 ± 0.003 μM *p*NA/s with saturating concentrations of FbsB, representing 80% inhibition. To examine whether the interaction between FbsB and Pm was LBS-dependent and whether the COOH-terminal lysine residue of FbsB was largely responsible for the interaction between FbsB and Pm, a titration with an FbsBΔK607ΔE608 mutant, lacking two COOH-terminal residues, and the effect of the lysine analog, 6-AHA, was examined (Fig. 1). There was a ~4-fold decrease in the affinity (K_D) with the FbsBΔK607ΔE608 mutant (182 ± 47 nM) compared to wild-type, (62 ± 14 nM), but the v_{lim} for the mutant was similar to that of wild-type (0.018 ± 0.005 μM *p*NA/s). Addition of 6-AHA (10 mM) to saturating concentrations of either wild-type or mutant FbsB (1 μM) caused the rate of Pm substrate hydrolysis to increase to 0.091 μM *p*NA/s and 0.095 μM *p*NA/s respectively, approximating the rate in the absence of FbsB, and suggesting that the interaction between FbsB and Pm was largely LBS-dependent.

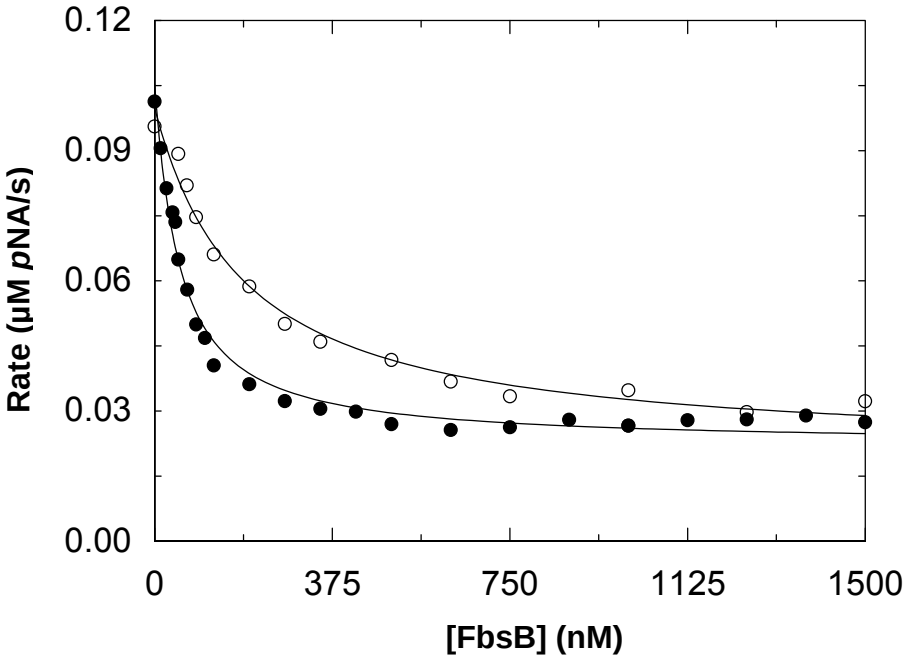

Figure 1. Kinetic titrations of Pm with FbsB. Initial velocities of hydrolysis of 200 μM H-*D*-Val-Leu-Lys-*p*NA are shown for mixtures of 10 nM Pm as a function of wild-type FbsB (●) or FbsBΔK607ΔE608 (○) concentration. The *lines* represent the least-squares fits by the quadratic binding equation.

To establish whether FbsB binds Pm, purified FbsB was chromatographed on a human Pm-Sulfolink affinity column. There was no protein absorbance measured during the load and wash steps of the procedure, indicating that FbsB bound the column. FbsB was subsequently eluted from the Pm column with 50 mM 6-AHA elution buffer (Fig. 2), but elution with 20 mM 6-AHA was unsuccessful. These results confirm that FbsB binds human Pm and suggests that the interaction is likely LBS-dependent because 6-AHA eluted the bound FbsB.

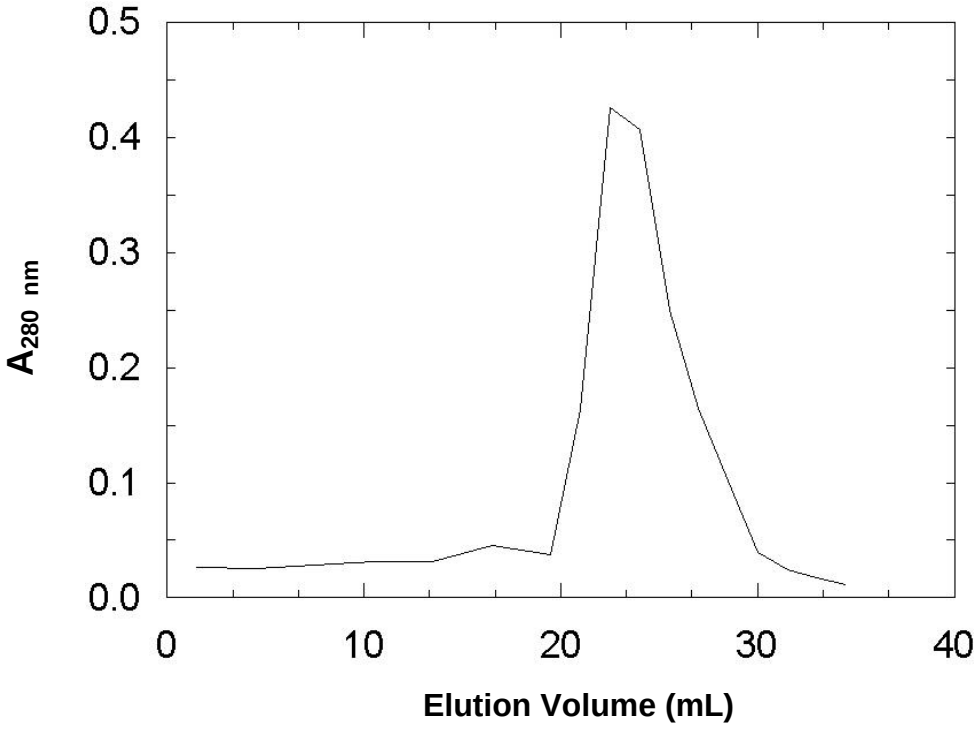

Figure 2. Pm-Sulfolink affinity chromatography of FbsB. The chromatogram shows the absorbance at 280 nm of fractions (1.5 ml) for chromatography of FbsB (12 µM) on a Pm-Sulfolink column (5 mL). FbsB was eluted from the affinity column with a 50 mM 6-AHA step-elution.

Effect of FbsB on Pm chromogenic substrate hydrolysis

Because FbsB significantly inhibits the rate at which Pm hydrolyzes H-*D*-Val-Leu-Lys-*p*NA (D-VLK-*p*NA), other chromogenic substrates were tested to determine the effect of FbsB on the turnover rates (k_{cat}) and Michaelis-Menten constant (K_M) for Pm (Table 1). The results in Table 1 indicate that FbsB affects the bimolecular specificity constant (k_{cat}/K_M) of Pm for all substrates tested, indicating a change in enzyme specificity. The k_{cat} was increased by 47%, 44%, and 24% for *D*-VLK-*p*NA, *D*-VLR-*p*NA, and *D*-IPR-*p*NA, and decreased by 42% and 46% for pyro-EPK-*p*NA and pyro-EPR-*p*NA respectively. FbsB affected the

K_M for the five tripeptide-*p*NA substrates screened by ~5 to ~20-fold increases. FbsB had the most significant effect on D-VLK-*p*NA as it drastically increases the K_M and decreases k_{cat}/K_M by ~20-fold and ~12-fold, respectively (Table 1, Fig. 3).

Table 1. Kinetic parameters for Pm substrate cleavage in the presence of FbsB. Catalytic rate constant (k_{cat}) and Michaelis-Menten constant (K_M) for Pm with various chromogenic substrates in the absence and presence of FbsB were calculated from titrations of the initial rate of substrate hydrolysis as a function of substrate concentration, fit by the Michaelis-Menten equation. Experimental error represents 2 x SD.

	Pm			FbsB•Pm		
Substrate	k_{cat} (s-1)	K_M (μM)	k_{cat}/K_M (μM-1 s-1)	k_{cat} (s-1)	K_M (μM)	k_{cat}/K_M (μM-1 s-1)
D-VLK-*p*NA	15 ± 1	85 ± 14	0.18 ± .06	22 ± 3	1750 ± 465	0.013 ± 0.007
D-VLR-*p*NA	16 ± 1	414 ± 96	0.04 ± 0.01	23 ± 3	1954 ± 464	0.012 ± 0.006
pyro-EPK-*p*NA	53 ± 3	45 ± 10	1.0 ± 0.6	31 ± 3	506 ± 137	0.06 ± 0.03
pyro-EPR-*p*NA	57 ± 4	94 ± 20	0.6 ± 0.3	31 ± 2	800 ± 135	0.04 ± 0.01
D-IPR-*p*NA	25 ± 1	247 ± 32	0.10 ± 0.03	31 ± 4	2593 ± 448	0.012 ± 0.004

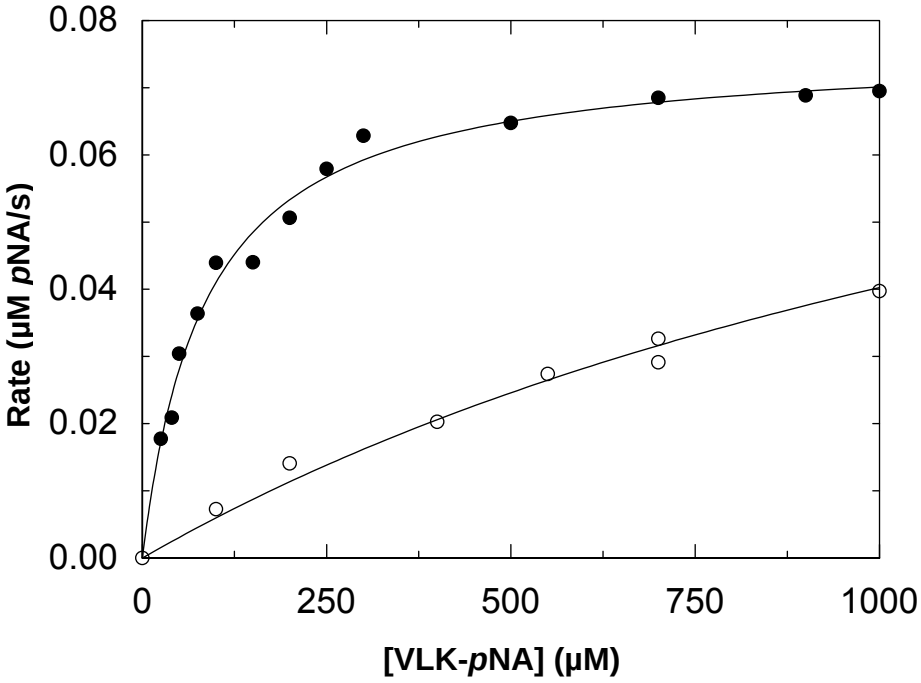

Figure 3. Effect of FbsB on H-*D*-Val-Leu-Lys-*p*NA hydrolysis by Pm. Initial velocity (μM *p*NA/s) of 5 nM Pm as a function of total VLK-*p*NA concentration in the absence (●) and presence (○) of 1 μM FbsB. The *lines* represent the fit by the Michaelis-Menten equation. K_M and k_{cat} in the absence and presence of FbsB were 85 ± 14 μM, 15 ± 0.7 s^{-1} and 1750 ± 465 μM, 22 ± 3 s^{-1} respectively.

Interaction of FbsB with Pg

Chromogenic substrate kinetic assays of mixtures of FbsB (500 nM) and Pm (10 nM), titrated with [Lys]Pg and [Glu]Pg, were performed. The purpose was to determine whether the addition of Pg can alter the affinity of FbsB for Pm, as indicated by a change in the affinity (K_D) of the interaction between FbsB and Pm alone. The titration with [Lys]Pg weakened the K_D ~6-fold to 384 ± 126 nM, compared to 65 ± 14 nM for FbsB and Pm in the absence of Pg (Fig. 4). Preliminary results with [Glu]Pg (data not shown) also indicate a ~2-fold increase in the K_D. When 6-AHA (10 mM) was added to an assay containing 10 nM Pm,

500 nM FbsB, and 1 μM [Lys]Pg, the rate of substrate hydrolysis increased to 0.098 μM *p*NA/s, close to the control rate of Pm alone. These results suggest that FbsB interacts with Pm and Pg and the interaction is inhibited by Pg, suggesting that Pm and Pg bind to the same site on FbsB. The results also suggest that both Pm and Pg binding to FbsB is LBS-dependent.

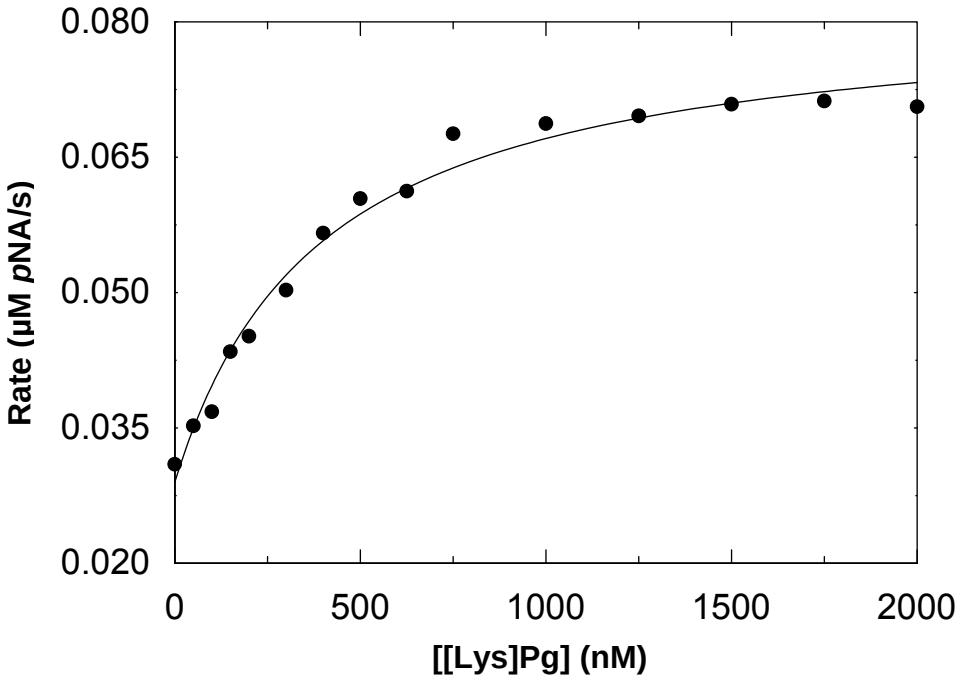

Figure 4. Kinetic titration of a mixture of FbsB and Pm with [Lys]Pg. Initial velocities of hydrolysis of 200 μM H-*D*-Val-Leu-Lys-*p*NA for mixtures of 10 nM Pm and 500 nM FbsB as a function of [Lys]Pg concentration. The *line* represents the least-squares fit by the quadratic binding equation. K_D is 384 ± 126 nM.

Kinetic assays of Pm formation were performed to examine the effect of FbsB on activation of 100 nM [Lys]Pg by 2 nM two-chain tPA (tctPA) (Fig. 5). The rate of Pm generation from [Lys]Pg by tctPA was enhanced ~6-fold from 0.018 μM *p*NA/s (v_o) to 0.10 ± 0.01μM *p*NA/s (v_{lim}) in the presence of FbsB, indicating

that FbsB may act as a cofactor for tPA-catalyzed Pg activation.

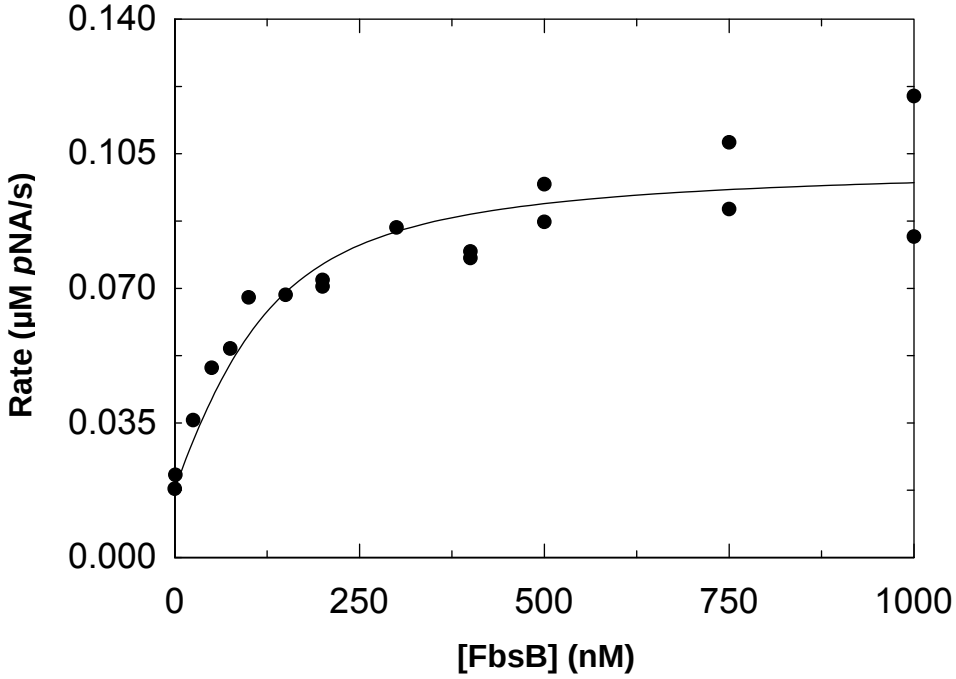

Figure 5. The rate of [Lys]Pg activation by tctPA enhanced by FbsB. Initial rates of Pm hydrolysis of 200 μM *D*-VLK-*p*NA for activation of 100 nM [Lys]Pg by 2 nM tctPA as a function of total FbsB concentration. The *line* represents the least-squares fit of the data by the quadratic binding equation. K_D is 59 ± 50 nM.

FbsB and Fbg binding experiments

Because FbsB was reported to bind to human Fbg (12,16), purified FbsB was subjected to chromatography on bovine or human Fbg-agarose columns. Even under low ionic strength conditions (10 mM NaCl), FbsB did not bind to the human or bovine Fbg columns. The same procedure was used to test FbsB binding to a human fibronectin-agarose column, again with no binding.

Another attempt was made to show FbsB-Fbg binding by subjecting the proteins to size-exclusion chromatography on Superdex 200 after a 30 min incubation period. If complex formation occurred, a shift in the elution peaks

measured from chromatograms of FbsB and Fbg alone would be anticipated. However, two separate peaks were measured that mimic the sum of the elution profiles of the respective proteins alone, indicating that a FbsB•Fbg complex was not formed (Fig. 6).

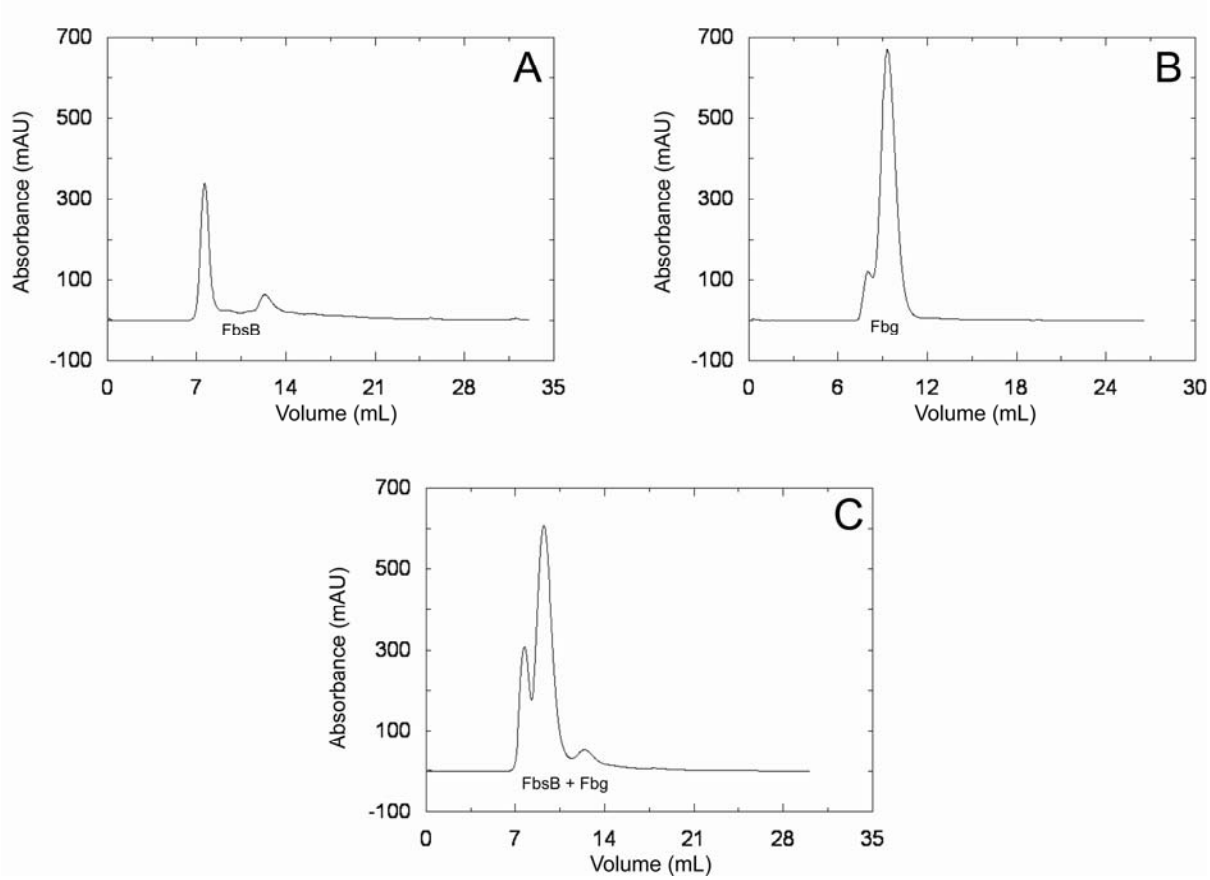

Figure 6. Evaluation of complex formation between FbsB and Fbg. The chromatograms of FbsB (*A*), Fbg (*B*), and an equimolar mixture of FbsB and Fbg incubated for 30 min (*C*) on Superdex 200 equilibrated with 50 mM Hepes, 125 mM NaCl, pH 7.4.

Western blot experiments were designed to replicate the binding of FbsB to Fbg reported in previously published studies (12,14). Each blot contained full-length SC(1-660) (0.05 µg), a known Fbg-binding protein, as a positive control and FbsB (25 µg). Membranes were blocked for various times with 5% dry milk

(Fig. 7, *A*-*F*), Odyssey (LI-COR) blocking buffer (Fig. 7, *G* and *H*), 3% bovine serum albumin (BSA) (Fig. 7, *I* and *J*), or 5% casein (Fig. 7, *K* and *L*), with one membrane from each blocking condition incubated with Fbg (2 μg/ml) in the respective blocking buffer. When FbsB was blocked with 5% dry milk for 2 or 5 h, FbsB tests positive for Fbg binding, even in the absence of Fbg (Fig. 7, *C* and *E*). The SC control also tests positive for Fbg binding after 5 h of blocking in the absence of Fbg (Fig. 7, *E*). The Odyssey blocking buffer and 3% BSA blots also show false positives for FbsB•Fbg complex formation. However, when the blots were blocked with a 5% casein solution, Fbg-binding was only reported with SC, not FbsB, on the blot that was incubated with Fbg (Fig. 7, *K* and *L*). The blots, as well as the Fbg chromatography experiments, suggest that FbsB may not bind Fbg and experiments performed by other researchers where FbsB•Fbg binding is reported may be a result of false positive results from blocking buffer conditions.

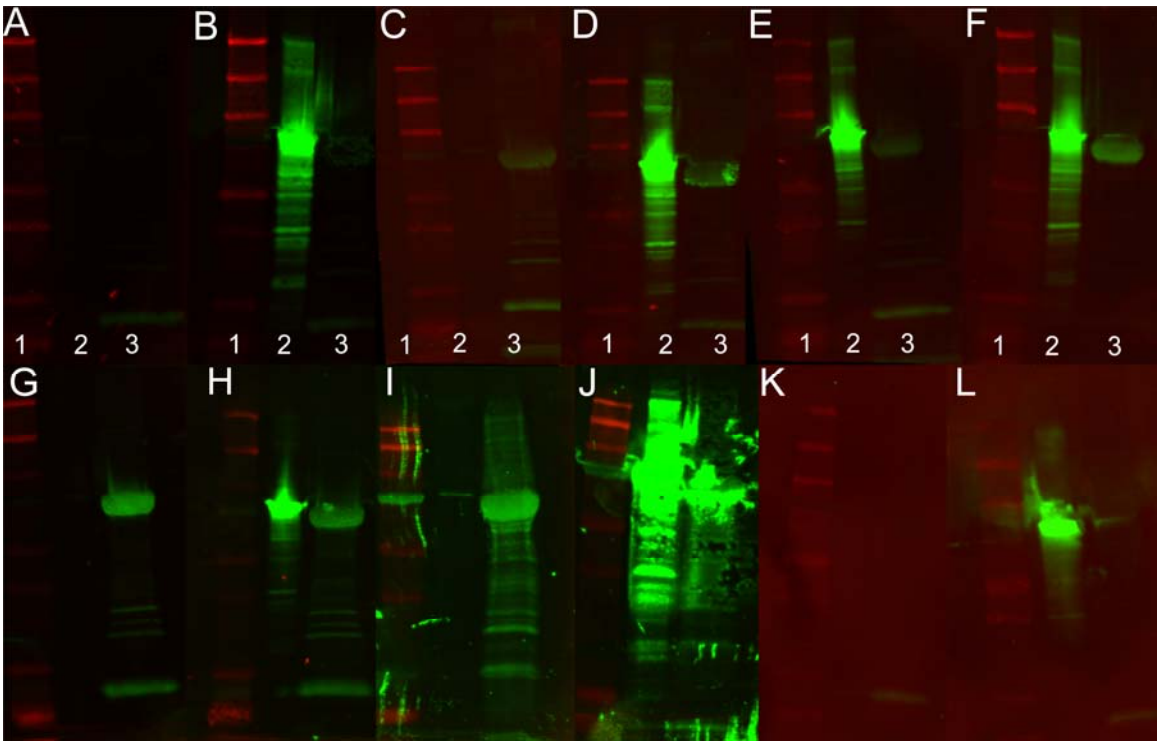

Figure 7. Western blot analysis of FbsB and SC for Fbg binding. Molecular mass markers (*lane 1*), 0.05 µg SC (*lane 2*), and 25 µg FbsB (*lane 3*) were separated by SDS-PAGE, transferred onto a PVDF membrane, and tested for fibrinogen binding. Bound Fbg was detected with a goat anti-human fibrinogen primary antibody followed by a donkey anti-goat secondary that was visualized with a LiCor near-infrared imaging system. 1 h block in 5% dry milk without (*A*) and with 2 µg/ml Fbg (*B*). 2 h block in 5% dry milk without (*C*) and with 2 µg/ml Fbg (*D*). 5 h block in 5% dry milk without (*E*) and with 2 µg/ml Fbg (*F*). 1 h block in Odyssey buffer (LI-COR) without (*G*) and with 2 µg/ml Fbg (*H*). 5 h block in 3% BSA without (*I*) and with 2 µg/ml Fbg (*J*). 5 h block in 5% casein without (*K*) and with 2 µg/ml Fbg (*L*).

FbsB stability and solubility assays in the presence of Pm, and NH_2-terminal sequencing of Pm cleavage products

To investigate the stability of FbsB in the presence of Pm, time-course reactions of FbsB (4.5 µM) incubated with either native Pm or D-Phe-Phe-Arg-CH_2-Cl (FFR-CH_2Cl) active site-blocked Pm (10 nM) were analyzed by SDS-

PAGE (Fig. 8). FbsB shows degradation starting at 2 min incubation with Pm (Fig. 8, *A*); however, at times up to 60 min with FFR-Pm (Fig. 8, *B*), FbsB is not degraded.

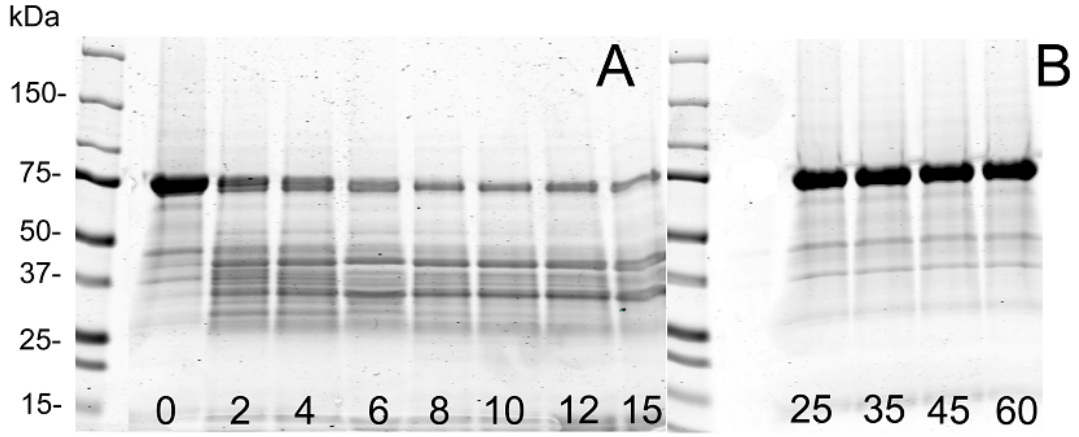

Figure 8. Proteolysis of FbsB by Pm. Coomassie-stained SDS-PAGE of non-reduced samples of FbsB and native Pm (*A*) and FbsB and FFR-Pm (*B*) incubations at 0, 2, 4, 6, 8,10,12,15, 25, 35, 45, or 60 min.

Turbidity assays with 5 nM Pm and different FbsB concentrations were carried out under various buffer conditions to confirm whether degradation and subsequent precipitation caused by Pm could be avoided. FbsB (1 µM) did not precipitate after a 30 min incubation with Pm (5 nM) in the following buffer: 500 mM Hepes, 300 mM NaCl, 1 mg/ml PEG 8000, 1 mM EDTA, 5% glycerol, pH 7.4. Kinetic assays were subsequently performed with FbsB (1 µM) and Pm (5 nM) in this buffer. Pm cleaved 400 µM *D*-VLK-*p*NA with a rate of 0.036 µM *p*NA/s in the presence of FbsB, compared to 0.031 µM *p*NA/s in the absence of FbsB. Taking these results and the rate of hydrolysis of 200 µM *D*-VLK-*p*NA by 5 nM Pm in the absence of FbsB (0.11 µM *p*NA/s) into consideration, it appears that precipitation does not occur because FbsB may not interact with Pm in the

glycerol and high salt buffer conditions. It is also probable that Pm may not cleave its chromogenic substrate well under these buffer conditions, since the rate of substrate hydrolysis in the absence of FbsB is ~4-fold lower than expected.

To determine where Pm cleaves FbsB, NH_2-terminal sequencing of the major degradation products of FbsB formed by incubation with Pm was completed. The results of Edman degradation propose two 5-residue peptides, LKNEV and IRLNG, indicating that FbsB is cleaved after the Lys residues 337 and 378 (Fig. 9).

Figure 9. Amino acid sequence of FbsB. Lysine residues within the FbsB sequence are highlighted in *yellow*, and the peptides reported by NH_2-terminal sequencing of FbsB major degradation products are outlined in *red*.

Discussion

FbsB from *Streptococcus agalactiae* is a novel protein with weak, 22% sequence identity to the fibronectin-binding protein from *S. pyogenes*, Sfbx, and a conserved COOH-terminal 223-residue region almost identical to that found in the fgag family of bovine Fbg-binding proteins in *S. agalactiae*. FbsB was initially hypothesized to be a zymogen activation and adhesion protein (ZAAP) because it contains a D1-D2 domain that is homologous to that of SC, and it was reported to bind Fbg and hypothesized to activate one of the serine protease zymogens of the human coagulation cascade. However, the data presented herein indicates that FbsB neither activates any of the coagulation zymogens, nor binds fibronectin or Fbg. FbsB does, however, appear to bind Pm and likely Pg in a LBS-dependent manner. It also enhances tctPA catalyzed [Lys]Pg activation. Therefore, FbsB may be the second characterized protein secreted by *S. agalactiae* that is able to enhance Pg activation by one of the endogenous Pg activators.

Kinetic assays and affinity chromatography experiments indicate that FbsB binds Pm and inhibits its ability to hydrolyze one of its tripeptide-*p*NA substrates by 80%. This interaction is eliminated in the presence of 6-AHA, indicating that the interaction between FbsB and Pm is LBS-dependent. Because the COOH-terminal Lys^{414} residue of SK is important for Pg and Pm binding and subsequent Pg activation, it was possible that FbsB may interact with Pm through a similar mechanism. Therefore, assays with the FbsBΔK607ΔE608 mutant were performed and confirm that the COOH-terminal lysine residue of

FbsB is not primarily responsible for the interaction with Pm, unlike what is seen with SK and Pg (36), because deleting this residue only weakens the dissociation constant ~4-fold. Kinetic assays that evaluate the specificity of Pm for various chromogenic substrates in the presence and absence of FbsB verify that FbsB greatly reduces catalytic efficiency of Pm for all five substrates tested. Experiments with [Glu]Pg and [Lys]Pg suggest that FbsB may bind Pg at the same site that binds Pm. The affinity of FbsB for Pm is weakened ~6-fold in the presence of [Lys]Pg and ~2-fold with [Glu]Pg. The interaction between [Lys]Pg, Pm, and FbsB was eliminated in the presence of 6-AHA, indicating that LBS are involved in both interactions. In Pg activation experiments, FbsB may act as a cofactor of tPA activation of [Lys]Pg, enhancing the rate of Pm generation ~6-fold. Although the experiments are preliminary, it is possible that FbsB alters Pg conformation, making it more accessible for the action of tctPA, or that both tPA and [Lys]Pg bind to FbsB to form a ternary complex (11).

Even though there is 22% homology between FbsB and the fibronectin-binding protein Sfbx, FbsB does not bind to fibronectin. This is likely because the region of Sfbx that FbsB shares is not a fibronectin-binding region. Experiments with FbsB and Fbg failed to corroborate reports that FbsB is a Fbg-binding protein. FbsB did not bind to bovine or human Fbg affinity columns, nor did it form a complex with Fbg, as shown in size-exclusion chromatography experiments. Western blots were performed using 5% dry milk, 3% BSA, Odyssey (LI-COR), and 5% casein blocking buffers with varying blocking times. It was concluded that FbsB tests positive for Fbg-binding, even in the absence of

Fbg, except with the 5% casein blocking buffer which shows Fbg-binding only for the SC positive control. Researchers who reported Fbg-binding of FbsB, as shown by western and/or dot blots, used a proprietary blocking reagent (Roche) with unknown components (12), or a 3% BSA blocking buffer (14) that is shown in the present experiments to produce false positive results. Therefore, FbsB is likely not a Fbg-binding protein, and reports of Fbg-binding activity may be a result of poorly controlled western/dot blot experiments.

Solubility experiments with FbsB in the presence of Pm indicate that Pm degrades FbsB and induces precipitation, thus complicating kinetic experiments. The fact that there are over 60 lysine residues in the FbsB construct may render FbsB to be easily degraded by the Pm. Active-site-blocked Pm (FFR-Pm) does not degrade FbsB and may be used to perform more in-depth binding studies in the future. NH_2-terminal sequencing of the major FbsB degradation products was performed, identifying the major cleavage sites, and mutant FbsB constructs that are terminated prior to these Pm cleavage sites could be generated. Studies with truncated FbsB may eliminate precipitation issues seen with full-length FbsB, if the mutant exhibits binding and activity similar to that measured with wild-type.

The data presented show that FbsB is a novel Pm and Pg-binding protein that significantly inhibits the activity of Pm, and that it may act as a cofactor for tctPA in the enhancement of [Lys]Pg activation. FbsB may have a synergistic effect with SkzL, GAPDH, α-enolase, and other GBS proteins that contribute to the virulence of *S. agalactiae* infections by binding and/or activating Pg and Pm, and thereby exploiting the human fibrinolytic system. FbsB has a high affinity for

Pm, as-yet- undetermined affinity for Pg, and both interactions are LBS-dependent. Preliminary data suggests that FbsB is a novel effector of tPA-mediated [Lys]Pg activation, and this interaction is also LBS-dependent. FbsB, along with the recently characterized SkzL, may contribute to the virulence of GBS infections that are responsible for illness and death in elderly, immunocompromised, and neonatal patients.

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CHAPTER III

ROLE OF THE NH_2-TERMINAL DIPEPTIDE OF STAPHYLOCOAGULASE IN CONFORMATIONAL PROTHROMBIN ACTIVATION

Introduction

Staphylococcus aureus is a highly virulent human pathogen responsible for pathologies ranging from minor skin infections and abscesses to life-threatening cases of meningitis, toxic shock syndrome, bacteremia, sepsis, and acute bacterial endocarditis. With the increasing incidence of methicillin and vancomycin-resistant strains of *S. aureus*, development of new treatment methods is vital for the survival of infected patients (1). Staphylocoagulase (SC), an extracellular protein produced by *S. aureus*, contributes to the severity of acute bacterial endocarditis by hijacking the human coagulation cascade and producing unwanted thrombi and vegetations, primarily on heart valves (2).

Thrombin is a serine protease that is responsible for cleaving fibrinogen (Fbg) to fibrin, and it is generated after activation of its zymogen precursor, prothrombin (ProT), by proteolytic cleavage of the Arg^{320}-Ile^{321} and Arg^{271}-Thr^{272} peptide bonds by the prothrombinase complex. The prothrombinase complex is composed of factor Xa, factor Va, and a phospholipid membrane surface, such as that of platelets, and is responsible for physiological ProT cleavage and activation (3). The prothrombinase complex initiates ProT peptide bond cleavage first at Arg^{320} and second at Arg^{271}. Initial cleavage at Arg^{320} of ProT by

prothrombinase gives rise to an active intermediate, meizothrombin (MzT), and subsequent cleavage at Arg^{271} produces active thrombin and fragment 1.2 (4,5). Conversely, ProT peptide bond cleavage occurs in the reverse order in the absence of factor Va. In this alternative pathway, initial cleavage at Arg^{271} gives rise to inactive prethrombin 2 (Pre 2), which is non-covalently bound to fragment 1.2, and thrombin is then formed after cleavage at Arg^{320} (6-9).

Under physiological conditions, ProT is activated by cleavage of its Arg^{320}-Ile^{321} peptide bond (Arg^{15}-Ile^{16} chymotrypsinogen numbering) following the classical proteolytic activation mechanism. Cleavage of this peptide bond releases a new Ile-Val NH_2-terminus that inserts into the NH_2-terminal binding pocket and forms a critical salt bridge with Asp^{194}. Consequently, folding of the activation domain of the zymogen occurs and the substrate binding site and oxyanion hole are formed (10-12). In comparison, SC is able to activate ProT through a non-proteolytic, bacterial cofactor-induced activation pathway known as the “molecular sexuality” mechanism. The NH_2-terminal dipeptide of SC, Ile-Val, imitates the conserved Ile-Val-Gly/Asn trypsin-like catalytic domain sequence found in almost all vertebrate serine proteases (13). SC usurps the coagulation cascade by forming a tight stoichiometric complex with ProT and inserting its NH_2-terminus into the NH_2-terminal binding pocket, thus forming the required salt bridge with Asp^{194} and initiating conformational activation. The SC•ProT complex is hypothesized to bind Fbg through a novel substrate recognition exosite because SC binding blocks proexosite I of ProT and thrombin and inhibits recognition of various substrates and effectors, including Fbg.

Interactions of SC with proexosite I are also important for protection of the SC•ProT complex from inhibition by serine protease inhibitors (serpins) (2,10,14,15). SC contains a series of seven, 27-amino acid repeats and a 32-residue pseudorepeat at its COOH-terminus that are able to bind Fbg in a different way, and the SC•ProT complex directly cleaves Fbg into fibrin, inducing the formation of fibrin clots (16). The interaction of SC with Fbg contributes to the virulence of acute bacterial endocarditis by allowing the pathogen to elude the host immune system by forming protective fibrin-platelet-bacteria vegetations (17).

Despite extensive studies on the structure and function of SC, there remains a significant gap in knowledge about the mechanistic importance of its NH_2-terminal dipeptide in conformational ProT activation. The impact of the conserved Ile-Val NH_2-terminal dipeptide in physiological serine protease activation is also not well understood. Early studies with the NH_2-terminal dipeptide of trypsinogen reported that although Ile^{16} has high affinity for the NH_2-terminal binding cleft and greatly contributes to binding affinity, the second residue also has a strong influence due to its placement at the entrance of the binding pocket during insertion. The Val^{17} side-chain makes van der Waals' contacts with neighboring amino acids, thus providing conformational stability (13,18). The importance of the Ile-Val NH_2-terminus of SC in zymogen activation is made clear by the fact that Met-SC(1-325), SC(2-325), and SC(3-325) mutants have 60 fold-lower activity, 6-fold lower affinity, and <2% activity respectively as compared to the fully active SC(1-325) fragment (2). The present study defines

the significance of the promiscuity of the NH_2-terminal dipeptide in conformational ProT activation by SC.

Materials and Methods

Cloning, expression, and purification of SC(1-246) contructs

SC(1-246) was cloned by PCR from the full-length SC(1-660) construct, previously cloned from genomic DNA of *S. aureus* Newman D2, strain Tager 104 (19), and inserted into a modified pET30b(+) vector (Novagen) (20). SC(1-246) was expressed with an NH_2-terminal His_6-tag and tobacco etch virus (TEV) proteinase cleavage site in Rosetta 2 (DE3) pLysS *E. coli* (Novagen) with 50 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Recombinant SC(1-246) protein was isolated from inclusion bodies and purified by Ni^{2+}-iminodiacetic acid chromatography. The His_6-tag was removed by overnight incubation with a 1:10 molar ratio of TEV proteinase to fusion protein (21) to give the native NH_2-terminus of the protein. The NH_2-terminal dipeptide SC(1-246) mutants were generated by QuikChange site-directed mutagenesis (Stratagene) using degenerate primers in the first and second amino acid positions. SC(1-246) concentration was determined using the following calculated absorption coefficient ($(mg/ml)^{-1}$ cm^{-1}) at 280 nm (22) and molecular weight: 1.18; 29,145.

ProTQQQ expression and purification

A recombinant human prothrombin construct, ProTQQQ, with the Arg at two thrombin-sensitive and one factor Xa-sensitive cleavage sites mutated to Gln (R155Q, R284Q, R271Q) was obtained from the laboratory of Dr. Sriram Krishnaswamy at the Children's Hospital of Philadelphia. ProTQQQ expression and purification were performed essentially as previously described (9) with minor modifications. The cDNA for ProTQQQ was transfected into HEK 293 cells and stable cell lines were expanded into cell factories. Protein production was conducted in DMEM/F12 without phenol red, 10% FBS, L-Glutamine, G-418 and Pen-Strep at 5% CO_2, 37 °C, 85% relative humidity. After a 5 day incubation period, conditioned media was harvested daily and stored at -20 °C.

Conditioned media were thawed, pooled, and run at room temperature on a Q-Sepharose column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.4. The column was washed with the same buffer and bound ProTQQQ eluted with 20 mM HEPES, 1.0 M NaCl, pH 7.4. Eluted protein was treated with 11 mM sodium citrate, then 1 M $BaCl_2$ was added over 15 min to a final concentration of 74.1 mM. The precipitate was collected by centrifugation, dissolved in 0.5 M EDTA, 5 mM benzamidine, pH 8.0. The protein was run on a Resource Q column (GE Healthcare) equilibrated in 20 mM HEPES, pH 7.4. Bound ProTQQQ was eluted with a 1.0 M NaCl gradient in the same buffer. Fractions containing ProTQQQ were pooled and dialyzed against 1 mM sodium phosphate, pH 6.8, applied to a ceramic hydroxyapatite matrix, CHT5-1 (Bio-Rad), equilibrated in the same buffer, and eluted with a gradient of 500 mM sodium phosphate, pH 6.8.

Fully carboxylated ProTQQQ was dialyzed against 5 mM MES, 150 mM NaCl, pH 6.0 and stored at -80 °C.

Western blot time course

Reactions containing SC(1-246) (10 nM) and ProTQQQ (1 nM) were incubated for 2 hr. At each time point, 40 µl was quenched with 10 µl of hot sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) treatment buffer, boiled for 2 min, and electrophoresed on 4-15% Tris-glycine gradient gels. Samples were transferred onto Immobilin-FL polyvinyldiene diflouride (PVDF) membranes (Millipore) in Tris/glycine transfer buffer containing 10% methanol. Membranes were blocked for 2 h with 50 mM Tris, 150 mM NaCl, pH, 7.5, 0.1% Tween-20 (TBS-T) containing 5% dry milk, then incubated overnight with a primary rabbit anti-human ProT antibody (ab48627). The membranes were washed with TBS-T and incubated for 2 h with a goat anti-rabbit secondary LI-COR IR 680 antibody. Bands were visualized using a near-infrared LI-COR Odyssey Imaging System.

ProT activation kinetics

The rates of hydrolysis of 100 µM or 600 µM H-*D*-Phe-Pip-Arg-*p*NA at 405 nm by ProTQQQ (1 nM) activated by SC(1-246) mutants were measured as a function of SC(1-246) concentration. Assays were performed at 25 °C in 50 mM Hepes, 110 mM NaCl, 5 mM $CaCl_2$, 1 mg/ml PEG 8000, pH 7.4. ProTQQQ and SC(1-246) were pre-incubated for 2 min prior to chromogenic substrate addition.

The maximum velocity (v_{LIM}) and affinity (K_D) were determined by nonlinear least-squares analysis of the hyperbolic titrations with the quadratic binding equation with stoichiometry fixed at 1. The Gibbs free energy (ΔG) for each SC mutant was calculated, using the equation $\Delta G = RT\ln(K_D)$, where R = 1.98 cal•mol^{-1}•degree^{-1} and T = 298.15 K (25 ºC).

Results

SC(1-246)•ProT time courses

The affinity of the active SC(1-325) fragment for ProT is extremely tight (17-72 pM) (23); consequently, any change in affinity (K_D) between SC and ProT from mutants on this background may not be measurable. For this reason, the SC(1-246) fragment, which displays a ~10-fold weaker K_D, was used. When bound to SC(1-246), native ProT is autocatalytically cleaved to prethrombin 1 (Pre 1), prethrombin 2' (Pre 2'), fragment 1 (F1), and fragment 2 (F2) (Fig. 1) due to cleavage of the Arg^{155} and Arg^{284} thrombin-sensitive peptide bonds. To evaluate unwanted autocatalysis of the SC(1-246)•ProT complex, SC(1-246) was incubated with ProT for 0 to 240 min, and samples from each time point were analyzed by SDS-PAGE (Fig. 1).

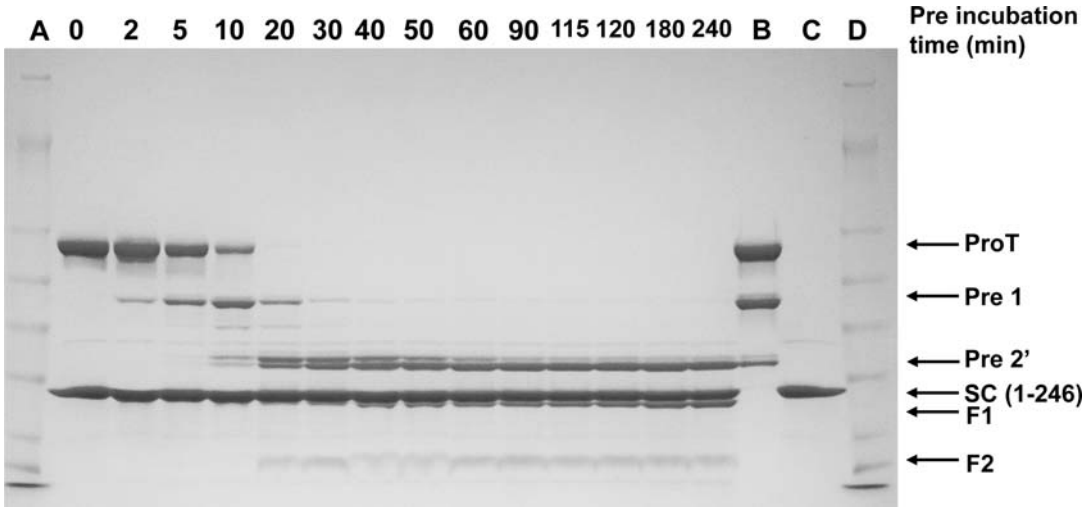

Figure 1. Autocatalysis of SC(1-246)•ProT. (Maddur, A., *unpublished data, 2009*). SDS-PAGE time-course of varying the preincubation times of ProT and SC(1-246). Lanes *A* and *D* are molecular weight markers, lane *B* is a control sample containing ProT, Pre 1, and Pre 2, and lane *C* is SC only.

To inhibit the production of ProT intermediates in the SC(1-246)•ProT* experiments, a recombinant human ProT construct (ProTQQQ) was used that was mutated at the two thrombin-sensitive and one factor Xa (FXa)-sensitive cleavage sites (R155Q, R284Q, R271Q), leaving only the remaining Arg^{320} FXa cleavage site. A western blot time-course was performed with SC(1-246) (10 nM) and ProTQQQ (1 nM) to mimic the concentrations used in kinetic experiments (Fig. 2).

After 0 to 120 min incubation, samples from each time point were subjected to SDS-PAGE and incubated with a polyclonal anti-ProT antibody that can detect native ProT and its intermediates. It was determined that SC(1-246)•ProTQQQ showed no autocatalysis; therefore, ProTQQQ was used in subsequent experiments.

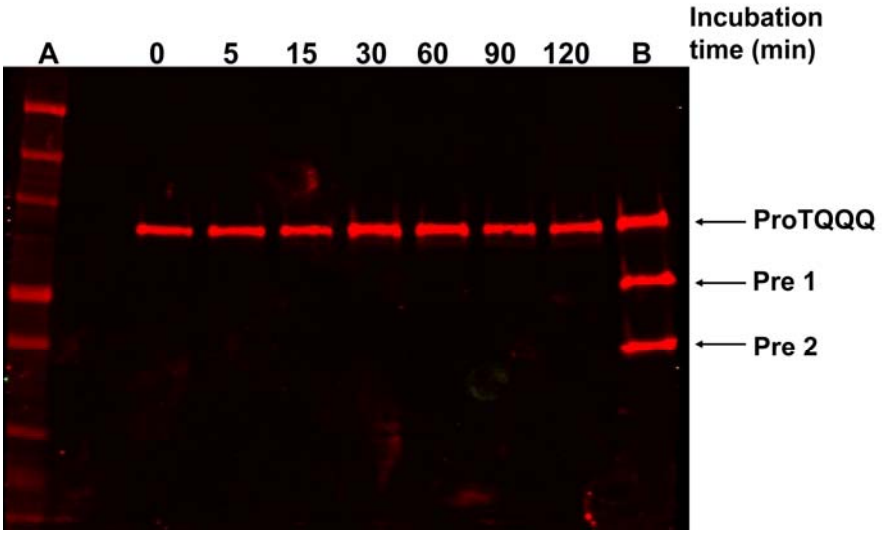

Figure 2. Western blot analysis of SC(1-246) and ProTQQQ time course. Molecular mass markers (*lane A*), a control sample containing 1 nM ProTQQQ, Pre 1, and Pre 2 (*lane B*), and samples at various time points (*0, 5, 15, 30, 60, 90, and 120 min*) of a 10 nM SC(1-246) and 1 nM ProTQQQ reaction were size separated by SDS-PAGE, transferred onto a PVDF membrane, and tested for the presence of ProT and its intermediates. ProT was detected with a polyclonal rabbit anti-human ProT primary antibody followed by a goat anti-rabbit secondary that was visualized with a near-infrared LI-COR Odyssey Imaging System.

ProT activation by NH_2-terminal dipeptide SC(1-246) mutants

To examine the specificity of the NH_2-terminal residues of SC in ProT activation, titrations were performed with 29 SC(1-246) mutants and wild-type SC(1-246) in the presence of ProTQQQ (1 nM). ProT activity was measured by an increase in the initial rate of hydrolysis of 100 μM H-*D*-Phe-Pip-Arg-*p*NA measured at 405 nM and 25 ºC. Although wild-type SC(1-246) has the highest affinity (K_D) for ProT (0.33 ± 0.06 nM), there are 7 mutants with activity equal to or greater than that of SC(1-246) (MK, ML, AS, LQ, LT, LK, and VG). NH_2-terminal dipeptide mutant activity ranged from <1% up to 153% activity (Table 1 and Fig. 3). Gibbs free energy changes (ΔG) only modestly vary between the

mutants (-8.9 to -12.0 kcal/mol), and wild-type SC(1-246) has the largest ΔG (-12.9 kcal/mol), in spite of not having the highest ProT activation activity (Fig. 4).

Table 1. Kinetic parameters of ProTQQQ in complex with NH_2-terminal dipeptide SC(1-246) mutants. Dissociation constants (K_D) and limiting velocities (v_{LIM}) (± 2 SD) were determined by the least-squares fits by the quadratic binding equation. The Gibbs free energy change (ΔG) was calculated from the K_D for each mutant as described in "Material and Methods."

NH_2-terminus	K_D ± 2 SD (nM)	ΔG (kcal/mol)	Normalized V_{LIM} ± 2 SD
VG	2.5 ± 0.3	-11.7	1.53 ± 0.03
LK	1.4 ± 0.1	-12.0	1.40 ± 0.01
LT	2.2 ± 0.3	-11.8	1.37 ± 0.03
LQ	1.4 ± 0.1	-12.0	1.36 ± 0.02
AS	7.8 ± 0.6	-11.0	1.14 ± 0.01
ML	7.2 ± 0.6	-11.1	1.06 ± 0.01
IV (Wt)	0.33 ± 0.06	-12.9	1.05 ± 0.01
MK	11 ± 1	-10.8	1.05 ± 0.01
AT	11.2 ± 0.5	-10.8	0.976 ± 0.008
AK	27 ± 4	-10.3	0.52 ± 0.01
LP	43 ± 4	-10.0	0.35 ± 0.01
GG	51 ± 4	-9.9	0.199 ± 0.003
TD	83 ± 7	-9.6	0.172 ± 0.003
MW	31 ± 3	-10.2	0.169 ± 0.002
GA	82 ± 11	-9.6	0.159 ± 0.005
SK	62 ± 4	-9.8	0.122 ± 0.002
AW	44 ± 5	-10.0	0.117 ± 0.003
RR	24 ± 4	-10.0	0.109 ± 0.003
CA	180 ± 18	-9.2	0.099 ± 0.003
ME	76 ± 12	-9.7	0.057 ± 0.002
ET	85 ± 6	-9.6	0.0340 ± 0.0006
GD	83 ± 6	-9.6	0.0255 ± 0.004
ND	74 ± 4	-9.7	0.0251 ± 0.003
QK	45 ± 11	-10.0	0.024 ± 0.001
GP	45 ± 4	-10.0	0.0213 ± 0.0003
QL	297 ± 48	-8.9	0.017 ± 0.001
GH	147 ± 14	-9.3	0.0117 ± 0.0003
ES	237 ± 29	-9.0	0.007 ± 0.003
KA	116 ± 25	-9.4	0.0034 ± 0.0002
RQ	149 ± 13	-9.3	0.0031 ± 0.0001

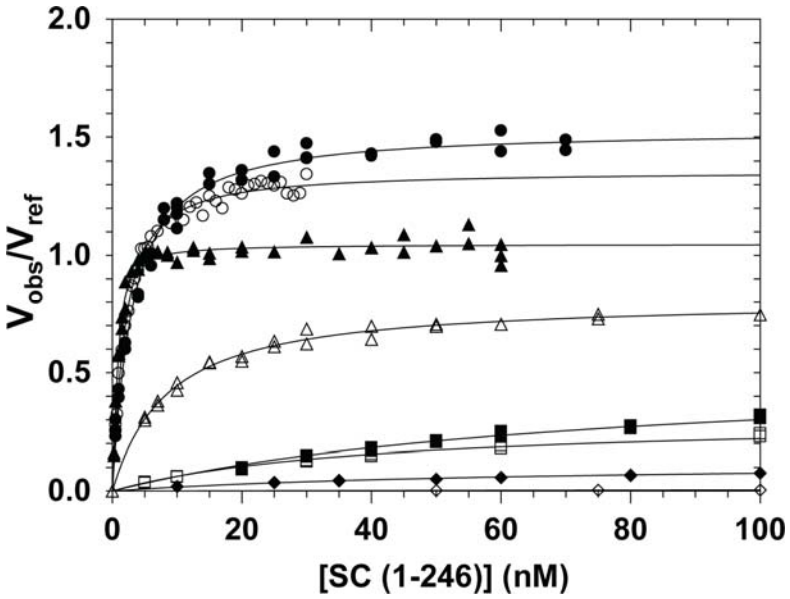

Figure 3. Kinetic titrations of selected NH_2-terminal dipeptide SC(1-246) mutants and ProTQQQ. Initial velocities of hydrolysis of 100 μM H-*D*-Phe-Pip-Arg-*p*NA are shown for mixtures of 1 nM ProTQQQ as a function of VG (●), LQ (○), wild-type IV (▲), AS (Δ), ND (■), RR (□), SK (♦), and QL (◊) SC(1-246) concentration. The *lines* represent the least-squares fits by the quadratic binding equation.

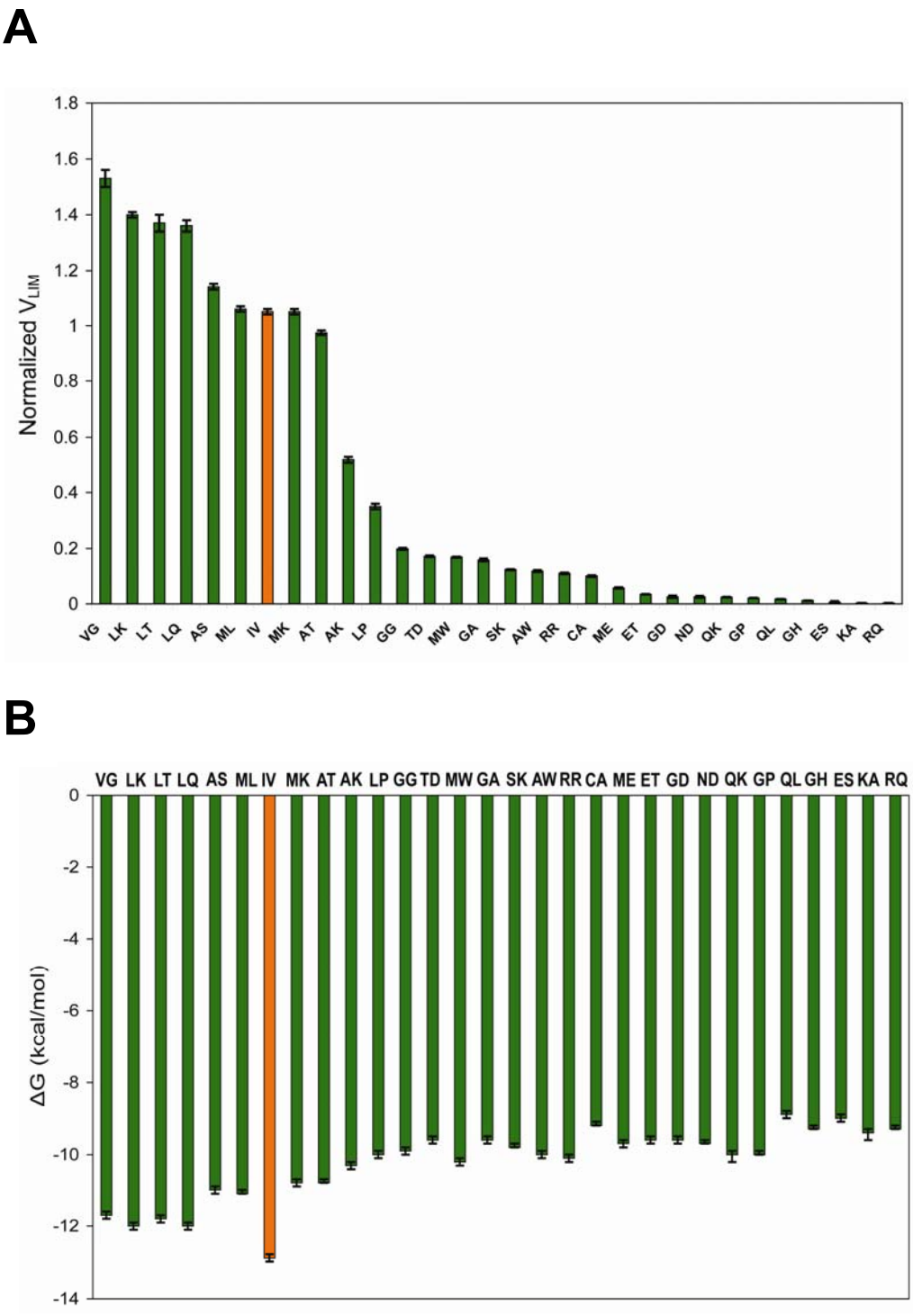

Figure 4. Limiting velocities (v_{LIM}) and changes in Gibbs free energy (∆G) of ProTQQQ with SC mutants. Limiting velocities (*A*) and calculated ∆G (kcal/mol) (*B*) for NH_2-terminal SC(1-246) mutants. Wild-type IV SC(1-246) is in *orange*, dipeptide mutants are in *green*. Error bars represent K_D ± 2 SD (listed in Table 1), calculated by least-squares fitting of the quadratic binding equation (*A*).

As a control, additional titrations with 15 NH_2-terminal mutants and wild-type SC(1-246) were completed to determine whether the assays were performed under saturating substrate conditions. When 600 μM H-*D*-Phe-Pip-Arg-*p*NA was present, the affinity of SC(1-246) for ProTQQQ (1 nM) was tighter for more than half of the mutants, but overall the activity of the mutants remained within the experimental error (Table 2). In the future, the remaining 14 mutants will also be re-screened at higher chromogenic substrate concentration.

Table 2. Kinetic parameters of ProTQQQ with selected NH_2-terminal dipeptide SC(1-246) mutants. Dissociation constants (K_D) and limiting velocities (v_{LIM}) (± 2 SD) were determined by the least-squares fits by the quadratic binding equation. The ΔG was calculated as before.

NH_2-terminus	K_D ± 2 SD (nM)	ΔG (kcal/mol)	Normalized V_{LIM} ± 2 SD
VG	2.3 ± 0.3	-11.7	1.60 ± 0.04
LQ	0.9 ± 0.3	-12.3	1.30 ± 0.06
IV (Wt)	0.45 ± 0.06	-12.7	1.01 ± 0.02
AT	4.1 ± 0.2	-11.4	1.00 ± 0.01
LP	17 ± 2	-10.6	0.306 ± 0.004
GG	25 ± 2	-10.3	0.260 ± 0.004
MW	12 ± 2	-10.8	0.246 ± 0.005
SK	39 ± 2	-10.1	0.159 ± 0.002
CA	100 ± 14	-9.5	0.098 ± 0.003
ME	48 ± 6	-9.9	0.059 ± 0.001
ND	75 ± 6	-9.7	0.0229 ± 0.0004
QL	210 ± 18	-9.1	0.024 ± 0.001
GH	361 ± 128	-8.8	0.024 ± 0.003
ES	163 ± 12	-9.2	0.0087 ± 0.002
KA	58 ± 16	-9.8	0.0063 ± 0.0003
RQ	97 ± 11	-9.5	0.0040 ± 0.0001

Discussion

Staphylococcus aureus is a human pathogen that usurps the human coagulation cascade by SC-dependent conformational activation of ProT through the “molecular sexuality” mechanism. The NH_2-terminal dipeptide of SC inserts into the NH_2-terminal binding pocket, activating ProT. Little is known about the specificity of the NH_2-terminal SC residues and the role they play in conformational ProT activation. Because the native NH_2-terminal residues are conserved among all serine proteases, mutagenesis studies of SC may also provide insight into the mechanism of serine protease zymogen activation, as well as that of ProT.

Kinetic assays revealed that MK, ML, AS, LQ, LT, LK, and VG NH_2-terminal SC(1-246) mutants can activate ProT to rates equal to or even higher than wild-type SC. Relative activity between the mutants varied from 0.3% to 160%, likely due to improper insertion and/or orientation of the NH_2-terminal residues in the NH_2-terminal binding pocket of ProT. When comparing the activity of the mutants, the highest rates are measured when the first amino acid is a non-polar, hydrophobic residue. It is difficult to make assumptions about the preference for the second residue; however, the mutants that contain a large, bulky residue or an aromatic residue in the second position have significantly lower activity. This may be because insertion and orientation of the first residue in the NH_2-terminal binding pocket of ProT is vital for activation, and a bulky residue in the second amino acid position may hinder proper insertion of the first residue.

Although wild-type SC(1-246) has the highest affinity for ProT, four mutants displayed 36-60% higher activity than wild-type. It is unclear why IV-SC(1-246) would be evolutionarily selected for over residues with higher activity, or why these residues are conserved among the serine protease family. One possible explanation is that these specific residues allow for protection from aminopeptidases. Leucine aminopeptidase studies have reported that the first and second NH_2-terminal residues of the substrate influence the specificity and rate of hydrolysis, although the first residue is of the most importance (24). Compared to other amino acids, reactions involving Val and Ile are greatly impeded, and this is likely due to the branching at the β-carbon atom. Peptides that contain Ile or Val in the D-configuration are completely resistant to leucine aminopeptidase activity, and these amino acids are also highly resistant in the L-configuration (25). This evidence suggests that because Ile and Val are highly resistant to aminopeptidase activity individually, as a dipeptide NH_2-terminus they may protect SC and serine proteases from detrimental hydrolysis.

The data presented demonstrate that the NH_2-terminal dipeptide of SC is promiscuous, and other branched, hydrophobic amino acids in the first position can activate ProT with activity up to 60% greater than that of wild-type SC(1-246). SC and the family of serine proteases may have separately evolved to contain Ile-Val at their NH_2-terminus as a mechanism for protection against hydrolysis by aminopeptidases. This hypothesis needs to be explored further to provide new insight into the mechanism of ProT (and serine protease zymogen) activation.

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CHAPTER IV

SIGNIFICANCE AND FUTURE DIRECTIONS

Identification of Fibrinogen-Binding Surface Protein B as a Plasminogen- and Plasmin-Binding Protein and Plasmin Inhibitor

The research within this thesis identifies fibrinogen-binding surface protein B (FbsB) as a *Steptococcus agalactiae* protein that binds human plasminogen (Pg) and plasmin (Pm), and it also may enhance [Lys]Pg activation by the physiological Pg activator, tissue-type plasminogen activator (tPA). It was further determined that FbsB significantly inhibits the rate at which Pm hydrolyzes a synthetic substrate. *S. agalactiae* is the only species of streptococci that does not express the Pg activator streptokinase (SK), but it does express two Pg-binding proteins, glyceraldehyde-3-phosphate dehydrogenase and α-enolase, which interact with the human fibrinolytic system (1,2). Skizzle (SkzL) is a recently characterized Pg-binding bacterial cofactor of urokinase-type plasminogen activator (uPA) and tPA-catalyzed Pm formation. SkzL is credited as the first characterized *S. agalactiae*-secreted protein known to target the human fibrinolytic system (3), and the data in this study suggests that FbsB may be the second.

Like SkzL, FbsB binds Pg and Pm in a lysine-binding-site-(LBS) dependent manner. Kinetic assays with an FbsBΔK607ΔE608 truncation mutant, lacking two COOH-terminal residues, demonstrated that the COOH-terminal most lysine is not primarily responsible for the interaction of FbsB with Pg and

Pm, contrary to the Lys^{415} residue of SkzL and the Lys^{414} residue of SK (4). It is known that FbsB binds Pm with high affinity, but further studies are needed to elucidate the mechanism responsible for Pm inhibition. Future equilibrium binding studies are needed to determine the affinity of FbsB for [Lys]Pg, and site-directed mutagenesis studies can determine which Lys residue(s) are responsible for [Lys]Pg and Pm binding. The interaction of FbsB with [Glu]Pg will also be explored.

Significance of FbsB Enhancement of tPA-catalyzed [Lys]Pg Activation

This thesis provides preliminary evidence that FbsB enhances tPA-catalyzed [Lys]Pg activation. However, the mechanism thorough which this occurs is currently unknown. tPA, unlike uPA, contains a LBS, and based on evidence of a LBS-dependent interaction between FbsB and Pg, it is possible that FbsB also binds tPA in a LBS-dependent manner (5). Therefore, the hypothesized mechanism of enhancement of Pm formation by SkzL may also explain the enhancement of tPA-dependent [Lys]Pg activation by FbsB. In this mechanism, a ternary [Lys]Pg•FbsB•tPA complex may be formed in which FbsB acts as a bacterial cofactor for Pg activation. If FbsB does not bind tPA, it may bind and form a complex with [Lys]Pg and alter its conformation such that [Lys]Pg is more readily activated by tPA (3). FbsB binding of tPA and the effect of FbsB on uPA-catalyzed [Lys]Pg activation and both tPA and uPA-dependent [Glu]Pg activation have not been studied. The experiments will be necessary to determine the exact mechanism of enhancement of Pm formation by FbsB.

Influence of FbsB in Evasion of Host Immune Defenses and Streptococcus agalactiae *Pathogenesis*

Streptococcus agalactiae can cause meningitis, sepsis, pneumonia, endocarditis, cellulitis, and arthritis in neonates, the elderly, and immunocompromised patients. The ability of *S. agalactiae* to usurp the human fibrinolytic system is important in pathogenesis, and the existence of cell-surface Pm and Pg-binding proteins is common among different species of *Streptococcus*. Streptococci can enhance Pm generation by several different cell-surface bound proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-enolase. Once activated, Pm can degrade extracellular matrix proteins such as fibrin, collagen, and fibronectin, and enhance the spread of bacteria throughout the tissues and bloodstream (1,5,12-16). Inhibition of Pm and tPA by the serine protease inhibitors (serpins) α_2-antiplasmin and plasminogen activator inhibitor-1 (PAI-1) respectively are vital for controlling the fibrinolytic system when the processes involving wound healing and vascular repair are inactive. Under normal conditions, tPA activates Pg through a fibrin-dependent solid-phase mechanism, and uPA activates Pg through a fibrin-independent fluid-phase mechanism. Binding of fibrin protects tPA and Pm from the action of their respective serpins (5-7). Physiologically, fibrin protection of tPA and Pm allows for successful clot lysis when a thrombus is formed. It was hypothesized that binding of Pm by SK or SkzL also protects Pm from inactivation by α_2-antiplasmin in the absence of fibrin, and that SkzL-binding protects tPA from inactivation by PAI-1 (3,8,9). If FbsB binds tPA, it may also inhibit inactivation of tPA by PAI-1.

Unlike SkzL, FbsB significantly inhibits the rate of hydrolysis of a synthetic substrate by Pm. If this occurs with natural substrates, it could inhibit degradation of tissues that are colonized by *S. agalactiae*. FbsB could contribute to the invasiveness of *S. agalactiae* infections through enhancing [Lys]Pg activation by tPA, creating a burst of Pm formation and local degradation of extracellular matrix proteins. However, FbsB could alternately inhibit the action of Pm as a means to evade host immune defenses once the bacteria has taken up residence in specific tissues. Pm not only dissolves fibrin clots and extracellular matrix proteins, but it also activates some mediators of the complement system, potentially playing a role in innate immunity. For this reason, it is possible that after *S. agalactiae* enters the tissues or bloodstream, FbsB may down-regulate Pm activity to protect the bacteria from clearance by the complement system. This breach in the regulation of fibrinolysis by *S. agalactiae* could render an infected patient susceptible to various life-threatening illnesses (10,11).

These studies characterize FbsB as a potential Pm inhibitor and bacterial cofactor for tPA-dependent [Lys]Pg activation. FbsB may contribute to *S. agalactiae* virulence through a bifunctional mechanism. To enhance the spread of infection, FbsB may assist in Pm generation and local degradation of extracellular matrix proteins. It may also help degrade thrombi vital for the repair of vascular injury, allowing bacteria to enter the bloodstream. Once the bacteria have entered the tissue or bloodstream, FbsB may inhibit the activity of Pm, potentially down-regulating complement activation and allowing evasion of host immune defenses. More extensive studies on the interactions of FbsB with Pm

and Pg are needed to determine the exact role of FbsB in fibrinolysis and the relevance of Pm inhibition in *S. agalactiae* pathogenesis.

Prothrombin Activation by NH_2-terminal Dipeptide Staphylocoagulase Mutants

The goal of the staphylocoagulase (SC) research within this thesis was to characterize NH_2-terminal dipeptide SC(1-246) mutants based on their ability to conformationally activate prothrombin (ProT). The mutant constructs generated in this study can be used as a tool to investigate the molecular sexuality mechanism of activation of ProT triggered by the insertion of Ile^1-Ile^2 of SC into the NH_2-terminal binding pocket of ProT (17). The structure and function of SC has been extensively studied, but little is known about the specificity of its NH_2-terminus and how this relates to the mechanism of ProT activation. Because the NH_2-terminal dipeptide (Ile-Val) is conserved among most vertebrate serine proteases, this project may also provide insight into the classical mechanism of serine protease zymogen activation.

Site-directed mutagenesis generated 29 mutants that were kinetically screened for ProT activation. This study revealed that 7 of the 29 mutants tested were able to activate ProT with 100-160% of the activity of wild-type SC(1-246). It was concluded that mutants with a non-polar, hydrophobic residue in the first amino acid position showed the highest ProT activity, and mutants with a bulky residue in the second position displayed significantly lower activity, likely due to inhibition of proper insertion of the first residue into the NH_2-termnial binding pocket. It was also hypothesized that Ile-Val may have been evolutionarily

selected for as the NH_2-terminus of SC due to the high resistance of these residues to hydrolysis by aminopeptidases (18,19). This study provides preliminary information about how various NH_2-terminal residues influence ProT activity; however, specific binding studies need to be performed. A more inclusive group of mutants needs to be tested to provide conclusive evidence of the residues preferred in the first and second amino acid positions of SC and how they affect conformational ProT activation and SC•ProT* function.

Significance of the NH_2-terminus of SC in Serine Protease Activation

The serine proteases involved in human blood coagulation and fibrinolysis exist in an inactive zymogen precursor form, and under physiological conditions, are activated by proteolytic cleavage. Cleavage of the Arg^{15}-Ile^{16} (chymotrypsinogen numbering) peptide bond gives and a newly liberated Ile^{16} that inserts into the NH_2-terminal binding pocket, where the α-ammino group of Ile^{16} forms a salt bridge with the carboxylate side chain of Asp^{194}. This induces folding of the zymogen activation domain and formation of the substrate binding site and oxyanion hole (20). Under pathologic conditions, SC activates ProT through a non-proteolytic, conformational mechanism. The NH_2-terminal dipeptide of SC, Ile-Val, imitates the conserved sequence found in almost all vertebrate serine proteases. Trypsinogen was used as an early tool to investigate the importance of the NH_2-terminus in serine protease activation; these studies concluded that both the first and second residues play a critical role (17,21). The importance of the NH_2-terminus of SC in ProT activation was initially evaluated

through mutagenesis studies in which Met-SC(1-325), SC(2-325), and SC(3-325) were screened for ProT activation, revealing that they had significantly lower activity compared to wild-type SC(1-325) (22). Characterization of the role of the NH_2-terminus of SC in conformational ProT activation may help to further elucidate the classical mechanism of serine protease zymogen activation.

Proteases play key roles in physiological and pathophysiological processes in the human body, including regulation of growth factors, cytokines, and chemokines, blood coagulation, fibrinolysis, and complement. For this reason, interest in the field of protease engineering, in which exploitation of the properties of enzymes can be used for therapeutic purposes, is on the rise. Further elucidation of the mechanism of ProT activation by SC and its relationship with serine protease activation may aid in the production of serine protease therapeutics (23). This may contribute to the treatment of a plethora of disease processes that involve *S. aureus* pathogenesis, including acute bacterial endocarditis, and pathologies that are characterized by unregulated serine protease activity.

SC in Staphylococcus aureus *Pathogenesis*

Pathologies caused by *Staphylococcus aureus* range from minor skin infections to deadly cases of meningitis, toxic-shock syndrome, sepsis, and acute bacterial endocarditis. Various *S. aureus* proteins, including SC, can bind and interact with proteins of the human coagulation and fibrinolytic pathways and contribute to the invasiveness of *Staphylococcus* infections. Areas of damaged

vasculature and artificial surfaces, including intravenous catheters, stents, and prosthetic devices, are easy targets for adhesion of proteins such as fibrinogen and fibronectin. When Fbg adheres to damaged areas within the heart or on damaged heart valves, patients are at greater risk of thrombus formation and subsequent acute bacterial endocarditis if *S. aureus* enters the bloodstream (22,24).

Acute bacterial endocarditis (ABE) is a disease characterized by inflammation of the inner lining of the heart and heart valves. In ABE, sterile fibrin-platelet vegetations form at sites of endocardial injury, initiating coagulation. If bacteria enter the bloodstream, they can bind Fbg and fibronectin within the sterile thrombus and form a fibrin-platelet-bacteria vegetation. Mature vegetations are at risk of embolization, which can lead to complications, including abscesses, heart failure, myocardial infarction, and stroke (20,25-27). SC influences the progression and invasiveness of ABE by conformationally activating ProT through the molecular sexuality mechanism. The SC•ProT complex readily cleaves Fbg to fibrin, thereby contributing to formation of characteristic ABE vegetations. *S. aureus*-induced ABE is almost always fatal without immediate treatment, and the mortality rate of treated patients is still 25-40% (28). If more were known about the mechanism of non-proteolytic ProT activation by SC and the role that the NH_2-terminus of SC plays, drugs could be developed that target pathological ProT activation, providing new treatments for acute bacterial endocarditis.

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