Multiple Ligands of von Willebrand Factor-binding Protein (vWbp) Promote Staphylococcus aureus Clot Formation in Human Plasma*

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Lena Thomer, Olaf Schneewind, and Dominique Missiakas1

From the Department of Microbiology, University of Chicago, Chicago, Illinois 60637

Background: Staphylococcus aureus manipulates blood coagulation by secreting von Willebrand factor binding protein (vWbp) and coagulase.

Results: vWbp forms a macromolecular complex with prothrombin, fibrinogen, factor XIII, and fibronectin.

Conclusion: vWbp activates FXIII in a non-proteolytic manner and recruits fibronectin to staphylococcal clots.

Significance: Activation of FXIII by vWbp represents a novel virulence strategy to promote formation of cross-linked fibrin cables in human plasma.

Staphylococcus aureus secretes coagulase (Coa) and von Willebrand factor-binding protein (vWbp) to activate host prothrombin and form fibrin cables, thereby促进ing the establishment of infectious lesions. The D1-D2 domains of Coa and vWbp associate with, and non-proteolytically activate prothrombin. Moreover, Coa encompasses C-terminal tandem repeats for binding to fibrinogen, whereas vWbp has been reported to associate with von Willebrand factor and fibrinogen. Here we used affinity chromatography with non-catalytic Coa and vWbp to identify the ligands for these virulence factors in human plasma. vWbp bound to prothrombin, fibrinogen, fibronectin, and factor XIII, whereas Coa co-purified with prothrombin and fibrinogen. vWbp association with fibrinogen and factor XIII, but not fibronectin, required prothrombin and triggered the non-proteolytic activation of FXIII in vitro. Staphylococcus aureus coagulation of human plasma was associated with the recruitment of prothrombin, FXIII, and fibronectin as well as the formation of cross-linked fibrin. FXIII activity in staphylococcal clots could be attributed to thrombin-dependent proteolytic activation as well as vWbp-mediated non-proteolytic activation of FXIII zymogen.

Staphylococcus aureus is the microbial agent of soft tissue abscesses and bloodstream infections (1), which are frequent causes of infectious disease mortality in the United States (2–4). Antibiotic therapy has been challenged by the emergence of multidrug-resistant strains, designated methicillin-resistant S. aureus or MRSA (5). Currently, there is an unmet clinical need for the development of new therapeutics to treat S. aureus skin and bloodstream infections.

Under physiological conditions, hemostasis is controlled by a cascade of serine proteases with rapid and highly localized activation in response to vascular damage (6). The hemostatic system is also responsible for preventing the dissemination of microbial invaders (7). For example, the intrinsic blood coagulation cascade is activated by negatively charged surfaces, as occurs with many different bacterial species (8). The relevance of this innate defense mechanism has been demonstrated for Streptococcus pyogenes, the causative agent of pharyngitis and skin infections (9, 10). Furthermore, microbial recognition by pathogen-associated molecular pattern recognition receptors triggers the release of cytokines, which activate the extrinsic blood coagulation cascade, thereby depositing fibrin around bacterial invaders (11). Fibrin deposition around microbes generates peptides with antimicrobial activity that attract phagocytes for opsonophagocytic removal of invading pathogens.

S. aureus has evolved a unique virulence strategy that usurps the hemostatic system for pathogen survival and replication in infected tissues. Exploiting the presence of coagulation factors in their zymogen form in the bloodstream, all clinical isolates of S. aureus secrete two hemostasis factors, coagulase (Coa) (12) and von Willebrand factor binding protein (vWbp) (13), that bind to and activate prothrombin in a non-proteolytic manner (14). Two N-terminal residues from Coa or vWbp insert into the activation cleft of the zymogen to form an equimolar complex designated staphylothrombin (Coa-prothrombin and vWbp-prothrombin) (14). Both forms of staphylothrombin display a novel exosite otherwise not found in proteolytically activated thrombin (14, 15). Both complexes cleave fibrinogen and form fibrin cables; however, neither has been reported to cleave or activate the other zymogen substrates of thrombin (15, 16).

Coa and vWbp contribute to the pathogenesis of S. aureus sepsis, endocarditis, and abscess formation in murine infection models (17–19). Furthermore, Coa and vWbp have been implicated as protective antigens (18, 19). McAdow et al. (20)

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1 To whom correspondence should be addressed: Dept. of Microbiology, University of Chicago, 920 East 58th St., CLSC609b, Chicago, IL 60637. Tel.: 773-834-8161; Fax: 773-834-8150; E-mail: dmissiak@bsd.uchicago.edu.

2 The abbreviations used are: Coa, coagulase; FXIII, FXIII A and FXIIIB, factor XIII; A or B; vWF, von Willebrand factor; vWbp, von Willebrand factor binding protein; H6, His6 tag.
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**TABLE 1**

Sequences of oligonucleotides used in this study

| Primer                  | Sequence                                             |
|-------------------------|------------------------------------------------------|
| vWbp_fwd_NcoI_His       | GGAATTCCTAGGCGCTAGGCCATCTCCAGCTCGGTGTTTTGACTCGGGAAG |
| vWbp_rev_XhoI_strep     | CATGCTCGAGTTATTTTGAACTCGGGGAGCACTCACTCATCTATGTTTCTCTGAGG |
| N1_fwd_NcoI_His         | GGAATTCCTAGGCGCTAGGCCATCTCCAGCTCGGTGTTTTGACTCGGGAAG |
| N2_fwd_NcoI_His         | GGAATTCCTAGGCGCTAGGCCATCTCCAGCTCGGTGTTTTGACTCGGGAAG |
| N3_fwd_NcoI_His         | GGAATTCCTAGGCGCTAGGCCATCTCCAGCTCGGTGTTTTGACTCGGGAAG |
| N4_fwd_NcoI_NcoI_His    | GGAATTCCTAGGCGCTAGGCCATCTCCAGCTCGGTGTTTTGACTCGGGAAG |
| C1_rev_XhoI_strep       | CATGCTCGAGTTATTTTGAACTCGGGGAGCACTCACTCATCTATGTTTCTCTGAGG |
| C2_rev_XhoI_strep       | CATGCTCGAGTTATTTTGAACTCGGGGAGCACTCACTCATCTATGTTTCTCTGAGG |
| C3_rev_XhoI_strep       | CATGCTCGAGTTATTTTGAACTCGGGGAGCACTCACTCATCTATGTTTCTCTGAGG |
| C4_rev_XhoI_strep       | CATGCTCGAGTTATTTTGAACTCGGGGAGCACTCACTCATCTATGTTTCTCTGAGG |
| Coa_fwd_NcoI_His        | AAAACATATGATAGTAACAAAGGATTATAGTGGGA |
| Coa_rev_NdeI            | AAAACATATGATAGTAACAAAGGATTATAGTGGGA |
| Coa_rev_XhoI_strep      | AAAACATATGATAGTAACAAAGGATTATAGTGGGA |

reported that immune sera of mice harbored antibodies that were directed mainly against the N-terminal D1-D2 domains and neutralized their ability to bind and activate prothrombin. Nevertheless, the C-terminal domains of Coa and vWbp, when purified and used as vaccine antigens, also raised protective antibody responses (20). We presume that these antibodies may block the association of Coa and vWbp with other host proteins and disrupt the pathogenesis of staphylococcal infections.

Coa and vWbp share significant structural homology in the D1-D2 domains; however, their C-terminal domains are dissimilar (15, 21). The C-terminal domain of Coa is comprised of tandem repeats of a 27-residue peptide also found in the N-terminal domain of Efb (22). The C-terminal domain of vWbp is found in another uncharacterized gene product of *S. aureus*. Presumably, this mosaic structure endows coagulases with specific functions. Indeed, vWbp was initially characterized because of its ability to bind von Willebrand factor (vWF) (13). A 26-amino acid peptide sequence within the C-terminal region of vWbp was identified as the minimal binding region; however, the physiological significance of this interaction has not been established (13). Here we sought to identify the ligands of Coa and vWbp in human plasma and to analyze the role of these complexes in staphylococcal infections with host hemostasis.

**EXPERIMENTAL PROCEDURES**

**Ethics Statement**—Blood donations were obtained from anonymous healthy adult donors. Written, informed consent was obtained from participants at the time of collection. The procedure was reviewed and approved by the Institutional Review Board at The University of Chicago.

**Reagents**—Human fibrinogen (Sigma), prothrombin (Innovative Research), coagulation factor XIII (FXIII; Hematologic Technologies), and von Willebrand factor (Hematologic Technologies) were used for affinity chromatography studies at 9, 1.4, 0.03, and 0.4 μM, respectively. When needed, lepirudin was used at a final concentration of 10 μg ml⁻¹ and CaCl₂ was used at 5 mM. FXIII-deficient plasma was obtained from Affinity Biologicals. Commercial antibodies used in the study included sheep anti-human prothrombin, HRP-conjugated (Affinity Biologicals), mouse anti-human factor XIIIa (FXIIa; Thermo Scientific), rabbit anti-human FXIIIIB (Abcam), rabbit anti-fibronec tin (Abcam), goat anti-human vWF, HRP-conjugated (Thermo Scientific), anti-mouse IgG, HRP-conjugated (Cell Signaling), and anti-rabbit IgG, HRP-conjugated (Cell Signaling).

**Bacterial Strains and Growth**—*S. aureus* Newman (23) was grown in tryptic soy broth at 37 °C. *Escherichia coli* strains DH5α (24) and BL21 (DE3) (25) were grown in Luria-Bertani (LB) broth with 100 μg ml⁻¹ ampicillin at 37 °C. To examine *S. aureus*-mediated coagulation, overnight cultures of Newman (wild-type) and isogenic coa, vwb, or coa/vwb mutants (18) were diluted 1:100 in fresh tryptic soy broth and grown at 37 °C until they reached A₆₀₀ 0.4. One ml of culture was centrifuged at 7000 × g, and bacterial sediment was washed and suspended in 1 ml of sterile PBS to generate a suspension of 1 × 10⁸ cfu ml⁻¹.

**Recombinant Proteins**—For binding studies, coding sequence of full-length mature vWbp or its truncations was extended to encode an N-terminal six-histidyl tag (H6) and a C-terminal strep-tag and cloned into pET22b using the primers listed in Table 1. Recombinant plasmids were transformed into E. coli BL21 (DE3). Overnight cultures of E. coli BL21 (DE3) were diluted 1:100 into fresh media, grown at 37 °C to A₆₀₀ 0.6, induced with 1 mM isopropyl β-D-thiogalactopyranoside, and grown for an additional 3 h. Bacterial cells were sedimented by centrifugation, suspended in column buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA), and disrupted with a French pressure cell at 10,000 p.s.i. Lysates were cleared of membrane and insoluble components by ultracentrifugation at 40,000 × g for 30 min. Proteins in the soluble lysate were subjected to Strep-Tactin affinity chromatography. Proteins were eluted in column buffer containing 2.5 mM d-desthiobiotin and subsequently dialyzed against PBS. Immediately before use, protein concentrations were determined with the BCA protein assay (Thermo Scientific). Activity was monitored by the ability to cleave chromogenic thrombin substrate S-2238 (Chromogenix). Coa_strep was cloned into pET15b using the primers listed in Table 1 and purified as described above. To assess FXIII activity, the coding sequence of full-length mature vWbp_strep with a C-terminal strep-tag was cloned into pET22b using the primers listed in Table 1 and purified as described above.

**Affinity Chromatography of Human Plasma Proteins**—Strep-Tactin-Sepharose (IBA) was equilibrated in PBS buffer and charged with 100 nmol of H₆vWbp_strep. N1–4, C1–4, or Coa_strep. Citrate-plasma from healthy human volunteers (500 μl) was diluted 1:1 in PBS (1.5 mM NaH₂PO₄, 8.5 mM Na₃HPO₄, 67 mM NaCl, pH 7.2, containing only traces of calcium (<0.002%), and magnesium (<0.001%) was applied by gravity flow over the resin followed by extensive washing in PBS.
TABLE 2
vWbp ligands identified during Strep-Tactin affinity chromatography of human citrate-plasma

| Sample number | Electrophoretic mobility | Proteins identified (number of unique peptide matches) |
|---------------|--------------------------|------------------------------------------------------|
| #1            | 200 kDa                  | Isoform 1 of fibrinectin (60)                        |
|               |                          | Isoform 1 of Fibrinogen α-chain (14)                |
|               |                          | Putative uncharacterized protein ALB (12)           |
|               |                          | Fibrinogen β-chain (6)                              |
|               |                          | α-2-Macroglobulin (4)                               |
|               |                          | Isoform 1 of fibrinectin (30)                       |
|               |                          | Isoform 1 of fibrinectin (19)                       |
|               |                          | Fibrinogen β-chain (11)                             |
|               |                          | Putative uncharacterized protein ALB (11)           |
|               |                          | Isoform γ-B of fibrinogen γ-chain (6)               |
|               |                          | 55-kDa protein (4)                                  |
|               |                          | Elongation factor 1-α2 (3)                          |
| #2            | 170 kDa                  | Isoform 1 of fibrinogen α-chain (37)                |
|               |                          | Isoform γ-B of fibrinogen γ-chain (25)              |
|               |                          | Putative uncharacterized protein ALB (7)           |
|               |                          | Isoform 1 of fibrinectin (5)                        |
|               |                          | Fibrinogen β-chain (4)                              |
|               |                          | SNC66 protein (3)                                  |
| #3            | 130 kDa                  | Isoform 1 of fibrinogen α-chain (23)                |
|               |                          | F2 prothrombin (fragment) (14)                      |
|               |                          | Coagulation factor XIII A chain (13)               |
|               |                          | Isoform γ-B of fibrinogen γ-chain (7)               |
|               |                          | Glyceraldehyde-3-phosphate dehydrogenase (4)       |
|               |                          | Putative uncharacterized protein ALB (4)           |
|               |                          | Peroxiredoxin-2 (4)                                |
|               |                          | Peroxiredoxin-1 (4)                                |
|               |                          | Coagulation factor XIII B chain (3)                |
| #4            | 100 kDa                  | Isoform 1 of fibrinogen α-chain (3)                 |
|               |                          | Putative uncharacterized protein ALB (16)          |
|               |                          | Isoform γ-B of fibrinogen γ-chain (6)              |
|               |                          | SNC66 protein (6)                                  |
|               |                          | Fibrinogen β-chain (60)                             |
| #5            | 70 kDa                   | Isoform 1 of fibrinogen α-chain (60)                |
|               |                          | Fibrinogen β-chain (19)                             |
|               |                          | Putative uncharacterized protein ALB (16)          |
| #6            | 60 kDa                   | Isoform 1 of fibrinogen α-chain (32)                |
|               |                          | Isoform γ-B of fibrinogen γ-chain (21)             |
|               |                          | FL100385 protein (fragment) (5)                     |
|               |                          | PRO2275 (4)                                        |
|               |                          | Putative uncharacterized protein DKFZp686N02209 (3) |
| #7            | 55 kDa                   | Isoform 1 of α-1-antitrypsin (3)                    |
|               |                          | Isoform γ-B of fibrinogen γ-chain (52)             |
| #8            | 45 kDa                   | Fibrinogen β-chain (39)                             |
|               |                          | Isoform 1 of fibrinogen α-chain (24)               |
|               |                          | Isoform 1 of fibrinogen α-chain (29)               |
| #9            | 35 kDa                   | Fibrinogen β-chain (14)                             |
|               |                          | Isoform γ-B of fibrinogen γ-chain (13)             |
|               |                          | Isoform 1 of fibrinogen α-chain (36)               |
|               |                          | Fibrinogen β-chain (16)                             |
|               |                          | Isoform γ-B of fibrinogen γ-chain (11)             |

Bound proteins were recovered by boiling the resin in sample buffer. Where indicated, human plasma was substituted with 500 µl of purified human plasma proteins in PBS.

**SDS-PAGE and Immunoblot**—Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed for extracts prepared in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromphenol blue). Gels were cast at 12% polyacrylamide, and proteins in extracts were separated by electrophoresis at 25 mA for 50 min. Proteins were either stained with 0.2% Coomassie Brilliant Blue (Sigma) or electrotransferred to polyvinylidene difluoride (PVDF) membrane (Millipore) for immunoblot analysis. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

**Assessing FXIII Activity**—1.5 µl fibrinogen was incubated with 100 nM thrombin, 200 nM prothrombin, 200 nM vWbP<sub>Strep</sub>, and/or 20 nM FXIII. Lepirudin or CaCl<sub>2</sub> was added to the reaction volume as needed. Insoluble reaction products were isolated by sedimentation at 20,000 × g for 10 min, and the sediments were washed 3 times in PBS and boiled in sample buffer supplemented with 4 M urea. To assess FXIII activity in human plasma, fresh blood was obtained from consenting volunteers and anticoagulated with 10 mM sodium citrate. Bacterial suspension (50 µl) was added to 50 µl of plasma in the presence of 5 mM CaCl<sub>2</sub> and incubated at 37 °C for 2 or 24 h. Insoluble reaction products were isolated as described above.

**Mass Spectrometry**—To identify Coomassie-stained proteins in SDS-PAGE gels, bands were excised, placed into microcentrifuge tubes with double distilled H<sub>2</sub>O, and submitted to the Taplin Mass Spectrometry Facility at Harvard Medical School, Cambridge, MA, for microcapillary LC/MS/MS mass spectrometry. Any protein with three or more unique peptide matches was considered as a hit. The complete list of identified proteins is provided in Table 2.

**RESULTS**

**Identification of vWbp Ligands in Human Plasma**—To identify interacting ligands of vWbp, we generated a non-catalytic variant with an N-terminal six histidyl tag and a C-terminal strep tag: <sup>5</sup>His<sub>vWbp</sub><sup>Strep</sup>. Human plasma represents a heteroge-
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FIGURE 1. Identification of vWbp ligands isolated from human plasma and comparative analysis with Coa. A, human plasma (500 μl) was flowed over Strep-Tactin resin uncharged (control) or charged with H6vWbpstrep (100 nmol). Bound proteins were eluted by boiling the resin in sample buffer separated by SDS-PAGE, and visualized after Coomassie staining. The identity of proteins labeled 1–9 was performed by microcapillary LC/MS/MS techniques. A complete list of protein hits can be found in Table 2. PT, prothrombin; FG, fibrinogen; α, β, and γ, for the comparative analysis of Coa and vWbp ligands, human plasma (500 μl) was flowed over Strep-Tactin resin uncharged (control) or charged with either H6vWbpstrep (100 nmol) or Coastrep (100 nmol). Bound proteins were eluted by boiling the resin in sample buffer and separated by SDS-PAGE. Proteins in gels were visualized by Coomassie staining (B) or after transfer to PVDF membranes for immunoblotting using specific antibodies against prothrombin, FXIII subunit A (FXIII A), and fibronectin (C). A panel of recombinant H6vWbpstrep variants with serial deletions of N- and C-terminal amino acid sequences was generated, and protein products were purified (Fig. 2A). These variants were subjected to affinity chromatography experiments with human plasma and ligand binding monitored via Coomassie-stained SDS-PAGE and immunoblotting (Fig. 2, B and C). Prothrombin and fibrinogen did not bind to vWbp variants with N-terminal truncations (N1–N4), suggesting that the D1 domain of vWbp is necessary for its association with both plasma proteins (Fig. 2B). On the other hand, vWbp variants C1–3, with C-terminal truncations, were able to retain prothrombin and fibrinogen during affinity chromatography; this attribute was abolished after further truncation of the D2 domain (Fig. 2, B and C). Thus, vWbp D1–D2 domains are necessary and sufficient for prothrombin and fibrinogen binding. These data are in accordance with previous studies (15). The A and B chains of FXIII were not retained during affinity chromatography of human plasma with the vWbp N1–N4 variants (Fig. 2, B and C). FXIII bound to the C1 and C2 truncations but not to the C3 no the C4 variants (Fig. 2, B and C). These results suggest that FXIII requires the D1 and D2 domains as well as the central domain of vWbp for binding. In contrast to prothrombin, fibrinogen, and FXIII, fibronectin bound to the vWbp N1–N4 variants but not to C1 or any further truncation at the C terminus of vWbp. Thus, fibronectin binds to the C-terminal domain of vWbp in a manner that does not require prothrombin, fibrinogen, or FXIII (Fig. 2, B and C).

H6vWbpstrep Does Not Retain Plasma-borne vWF—vWbp was initially identified by shotgun phage display of S. aureus genome-encoded peptides panned against recombinant vWF (13). In addition to measuring the binding of recombinant vWbp to both immobilized and soluble forms of vWF, Bjerketorp et al. (13) purified vWF from human serum by chromatography on resin charged with vWbp-part (residues 124–392). These data were interpreted as a demonstration of the specific interaction between vWbp and vWF in human serum (13). Of note, the variant vWbp-part lacks the D1 domain and the C-terminal domain of vWbp and, therefore, cannot bind prothrombin, fibrinogen, FXIII, or fibronectin. Using full-length, catalytically inactive H6vWbpstrep for affinity chromatography of human plasma, we failed to detect an interaction between vWbp and vWF (Fig. 2C), although vWF was clearly present in the plasma sample (Fig. 3A). To test whether full-length vWbp can bind vWF, purified human vWF was subjected to affinity chromatography on H6vWbpstrep-charged resin. vWF was indeed retained on H6vWbpstrep-charged control beads (Fig. 3B).
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Requirements for Prothrombin, Fibrinogen, and FXIII Binding to vWbp—As part of their investigation of vWbp-prothrombin, Kroh et al. reported that neither of the two proteins by themselves associated with fibrinogen (15). However, assembly of the novel exosite on the vWbp staphylothrombin complex allowed for fibrinogen binding, as previously reported for the Coa(1–325)-prethrombin 2 complex (14). To test whether vWbp-prothrombin assembly is required for the fibrinogen binding activity of vWbp in human plasma, we immobilized H6vWbp on Strep-Tactin resin and added fibrinogen in the presence or absence of prothrombin. Samples were analyzed by Coomassie-stained SDS-PAGE, which revealed that fibrinogen occurred with H6vWbp prothrombin complexes but not with H6vWbp alone (Fig. 4, A and B).

The minimal binding site of vWbp for association with FXIII in human plasma encompasses its D1-D2 domains. We wondered whether prothrombin and fibrinogen are required for the association of vWbp with FXIII. To test this, purified FXIII was chromatographed on H6vWbp immobilized on Strep-Tactin beads. When the eluate was analyzed by immunoblotting with antibodies against the A subunit, FXIII binding to vWbp could not be detected (Fig. 4, C and D). The addition of prothrombin to FXIII did not promote vWbp association; however, when subjecting prothrombin, fibrinogen, and FXIII to affinity chromatography, all three plasma proteins were retained on H6vWbp charged Strep-Tactin beads (Fig. 4, C and D). As a control, chromatography of prothrombin, fibrinogen, and FXIII on Coa-strep-charged resin led to the formation of Coa-strep-prothrombin-fibrinogen complexes but not to the retention of FXIII (Fig. 4, C and D). Thus, FXIII associates specifically with vWbp-prothrombin-fibrinogen complexes but not with Coa-prothrombin-fibrinogen.

Non-proteolytic Activation of FXIII by vWbp-Prothrombin—Plasma FXIII represents a heterotrimer composed of two A and two B subunits (28). The A subunit harbors the active site (30), whereas the B subunit fulfills a regulatory function (31).

Under physiological conditions, activation of FXIII involves thrombin-mediated cleavage of the activation peptides from both A subunits to generate A’ products (30). Calcium and fibrin then induce dissociation of the B subunits from the A’ dimer, thereby exposing the active site of the now fully active A* dimer (for review, see Ref. 29). FXIII-mediated cross-linking initially occurs between the properly aligned γ-chains of fibrin cables, resulting in the formation of γ dimers (32). Over time, intermolecular cross-linking between α-chains creates oligomers and α-chain multimers as well as complexes between α- and γ-chains that can be visualized as higher molecular weight species on SDS-PAGE (32).
To examine whether vWbp-prothrombin activates FXIII in vitro, we purified vWbpstrept, which once complexed with prothrombin, cleaves fibrinogen to form fibrin cables (18). Purified human FXIII was incubated with vWbpstrept in the presence or absence of human prothrombin and fibrinogen. Purified proteins and their reaction products were centrifuged, and fibrin cables were solubilized with urea and analyzed by Coomassie-stained SDS-PAGE (Fig. 5A). As controls, incubation of FXIII with fibrinogen did not generate significant amounts of γ-chain dimers or α-chain multimers and did not lead to FXIII A cleavage. Incubation with thrombin converted FXIII A to FXIII A∗ and A∗, which catalyzed the formation of γ-chain dimers and α-chain multimers in polymerized fibrin cables (Fig. 5A). This sequence of fibrin cross-linking reactions was blocked in the absence of calcium ions or in the presence of lepirudin, a recombinant form of hirudin that functions as a direct inhibitor of thrombin (33) (Fig. 5A). The addition of vWbpstrept-prothrombin to fibrinogen and FXIII also led to the sedimentation of fibrin cables and to the formation of γ-chain dimers and α-chain multimers (32). Unlike thrombin, vWbpstrept-prothrombin did not cleave FXIII A and its activation of FXIII was not inhibited by lepirudin (Fig. 5, A and B). vWbpstrept-prothrombin-mediated activation of FXIII required calcium ions, as the formation of γ-chain dimers and α-chain multimers did not occur in samples where the divalent cation had been omitted (34) (Fig. 5, A and B).

The canonical view of FXIII activation centers on the proteolytic removal of the 37-residue activation peptide from the N terminus of the A subunit of FXIII by thrombin (29). Immunoblotting of solubilized fibrin clots revealed the cleavage of the A subunit in samples treated with thrombin as indicated by the appearance of A∗ species (Fig. 5B). No cleavage of FXIII A was detectable in the presence of vWbpstrept-prothrombin despite the presence of cross-linked-fibrin (Fig. 5, A and B). We, therefore, conclude that vWbpstrept-prothrombin-fibrinogen complex can activate FXIII in a non-proteolytic manner as has been reported for coagulase-mediated activation of prothrombin (14) or staphylokinase-mediated activation of plasminogen (35). The B subunit of FXIII was present in all samples in which a fibrin clot had been formed (Fig. 5, A and B).

FXIII and Fibronectin Are Incorporated into Staphylococcal Coagulation Products—We wondered whether the vWbp-mediated clotting reactions observed in vitro occur during infection. To test this, wild-type S. aureus Newman (23) and its variants lacking either coagulase (coa), vWbp (vwb), or both (coa/vwb) (18) were incubated with calcium-supplemented human plasma. In accordance with previously published reports (18), S. aureus as well as the coa and vwb mutants promoted the formation of fibrin cables that sedimented with the bacteria and, after solubilization with urea, were detected via Coomassie-stained SDS-PAGE (Fig. 6A). This was not observed for the coa/vwb mutant (Fig. 6A).

S. aureus-mediated fibrin clots catalyzed the formation of γ-chain dimers and α-chain multimers within polymerized fibrin cables; this did not occur in human plasma inoculated with the coa/vwb mutant strain (Fig. 6A). Immunoblotting experiments revealed that FXIII (A and B) and fibronectin were incorporated in these clots (Fig. 6B). Fibrin clot formation, FXIII and fibronectin recruitment, and formation of γ-chain dimers and α-chain multimers occurred after incubation with...
coa or vwb mutants but not with the coa/vwb strain (Fig. 6, A and B). The formation of fibrin γ-chain dimers and α-chain multimers in clots formed by the vwb mutant, but not in clots generated by the wild-type or coa mutant strains, was reduced when plasma samples were treated with lepirudin (Fig. 6, A and B). Taken together these data suggest that S. aureus promotes fibrin clotting via the secretion of Coa and vWbp. Fibrin cross-linking is promoted by two mechanisms, the thrombin-dependent pathway that can be blocked with lepirudin and the vWbp-mediated recruitment and FXIII activation pathway that cannot be inhibited with lepirudin as it involves the non-proteolytic activation of FXIII A via vWbp-prothrombin-fibrinogen. S. aureus failed to induce fibrin γ-chain dimers and α-chain multimers in FXIII-depleted plasma (Fig. 7). After incubation of staphylococci in plasma for 24 h, cleavage of FXIII A to A’ was readily detected, and this was blocked by treatment with lepirudin (Fig. 6B). Thus, thrombin-mediated activation of FXIII indeed occurs in plasma harboring staphylococci. The conversion of prothrombin to activated thrombin occurs presumably via the contact system (36) and must account for the observed fibrin-cross-linking in plasma inoculated with the vwb mutant strain.

**DISCUSSION**

Unlike non-pathogenic or opportunistic staphylococcal species, the ability to coagulate human blood represents a hallmark of clinical S. aureus isolates (37). Why does S. aureus promote coagulation, a pathway generally appreciated as an innate defense mechanism to limit the dissemination of bacterial invaders (36)? Although the exuberant activation of hemostasis by S. aureus may appear enigmatic, recent results suggest that fibrin clot formation may be a prerequisite for the replication and persistence of staphylococci in host tissues (18). Histopathology of S. aureus abscess lesions revealed the formation of bacterial abscess communities that are enclosed by a pseudocapsule formed via fibrin deposits to form a shield against host immune cells (38). A second layer of fibrin delineates healthy and infected tissues. Purulent lesions with increasing size develop within this second layer and are eventually drained onto the surface of infected organs (18). S. aureus mutants lacking coa and vwb cannot form abscess lesions or persist in organ tissues of intravenously infected mice (18). When analyzed by immunohistochemistry, Coa is found within the pseudocapsule of S. aureus abscess communities, whereas vWbp is distributed throughout the lesion with pronounced accumulation in its periphery (18). S. aureus coagulation has been reconstituted within a three-dimensional collagen matrix supplemented with plasma proteins (39). Under these conditions, staphylococci form two concentric structures, an inner pseudocapsule and an extended, outer microcolony-associated meshwork containing fibrin that shield staphylococci against neutrophils (39). Use of isogenic mutants could attribute the formation of the inner pseudocapsule to Coa, whereas establishment of the outer meshwork required vWbp (39). Together, these observations suggest that Coa and vWbp fulfill partially overlapping yet non-redundant functions during infection (18).

To explore the molecular attributes of vWbp, we employed affinity chromatography of human plasma with vWbp and Coa variants unable to promote staphylothrombin-mediated cleavage of fibrin while retaining the ability to bind both prothrombin and fibrinogen. This approach uncovered prothrombin, fibrinogen, and fibronecintin as ligands of both Coa and vWbp.
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...and FXIII as a specific ligand of vWbp. Fibrinogen and pro-thrombin binding involves the D1-D2 domains of vWbp, whereas FXIII binding involved the D1-D2 domains and its central domain. Fibrinogen binding occurred at the C-terminal domain of vWbp. These data support a model for the assembly of a supramolecular complex initiated via vWbp-prothrombin that first associates with fibrinogen and then with FXIII. Fibronectin association with vWbp-prothrombin-fibrinogen-FXIII occurs independently of other plasma proteins. vWF does not function as a ligand of vWbp-prothrombin in plasma; presumably, fibrinogen and FXIII occupy the vWF binding site (residues 333–358; Ref. 13). Perhaps vWbp binds vWF in specific host tissues where fibrinogen/FXIII is not available. In contrast to vWbp, Coa has evolved designated fibrinogen binding sites (C-terminal tandem repeats) (17). The binding site of Coa for fibronectin is not yet known; however, it must be distinct from that of vWbp, as amino acids 359–482 lack sequence similarity with Coa. Structure predictions of the C-terminal domain of vWbp suggest a helical segment of ~50 residues (40) that may be involved in the recruitment of fibronectin.

Recent work proposed that the vWbp staphylo-thrombin complex displays an exosite with unique substrate specificity as compared with thrombin (14, 15). Indeed, vWbp staphylo-thrombin cleaves fibrinogen but not the other thrombin substrates: protein C, coagulation factors FV, FIX, FX, FXI, anti-thrombin III, or heparin cofactor II (15). We observed that association of vWbp-prothrombin did not trigger cleavage of the FXIII A zymogen. Nevertheless, the vWbp-prothrombin-fibrinogen-FXIII complex activated the FXIII A transglutaminase activity, as judged by the formation of fibrin γ-chain dimers and α-chain multimers. Whereas thrombin cleaves fibrinogen and FXIII A at similar rates (41), vWbp-prothrombin cleaved fibrinogen at a much faster rate than it activated FXIII.

When fibrin clots were formed by live bacteria, FXIII was found associated with the products of staphylococcal coagulation (Fig. 6). Surprisingly, even though Coa did not interact with FXIII in vitro, FXIII was recruited to staphylococcal clots formed by vWbp mutants. This observation hints at either the presence of another, yet unidentified staphylococcal factor capable of binding FXIII, or at the recruitment of FXIII to the products of Coa- and vWbp-mediated coagulation. Under physiological conditions, zymogen FXIII interacts with fibrinogen γ’ through its B subunits (42). Fibrinogen γ’ is a product of alternative splicing of the γ-chain transcript, causing read-through at the exon IX/intron I junction (43). The product of alternative splicing and translation is the replacement of the final four amino acids at the C-terminal end of the γ-chain with 20 distinct residues (43, 44). The possibility that Coa or any other fibrinogen binding factor of S. aureus may selectively recruit γ’ fibrinogen, which accounts for 10% of fibrinogen in human plasma, into the staphylococcal clot together with FXIII has not yet been investigated. Although entirely speculative, such a scenario could explain the recruitment of FXIII to the staphylococcal clot.

Studies with S. pyogenes reported the proteolytic activation of FXIII via the contact system and the conversion of prothrombin to thrombin (36). Because FXIII is activated by vWbp-pro-thrombin even in the absence of thrombin, we chose to investigate the possibility of non-proteolytic activation. Although slower in nature than thrombin-mediated cleavage, activation of FXIII by staphylococcal factors did occur within S. aureus clots. Previous studies have already reported the non-proteolytic activation of FXIII. For example, tetrameric A2B2 zymogen can be dissociated at high calcium ion concentrations, resulting in non-proteolytic activation of FXIII A (45–47). Calcium concentrations required for FXIII A activation can be reduced to a physiological range in the presence of fibrinogen (47). Siebenlist et al. (48) suggested that during the lag phase of the cross-linking reaction, fibrin may bind at or near the active site of FXIII, causing a conformational change that renders the zymogen catalytically active and promotes dissociation of A and B subunits. The recruitment of FXIII into staphylococcal clots via vWbp represents yet another mechanism for non-proteolytic activation of the zymogen. Because it has been shown that fibrin, but not fibrinogen, is a substrate for FXIII cross-linking (48), the incorporation of FXIII into the staphylococcal clot may be sufficient for the non-proteolytic activation of the zymogen.

In summary, S. aureus appears to recruit and activate FXIII within vWbp- and Coa-mediated fibrin clots. FXIII products, fibrin cables with increased stability and resistance to degradation, may fortify the barriers that are formed by staphylococci in abscess communities. Fibronectin, a known substrate of factor XIIIa that is preferentially cross-linked to the α-chain of fibrin (47), was also found associated with staphylococcal clots (Fig. 6). Presumably, fibronectin cross-linking modulates the mechanical and structural properties of the clot (49, 50). If so, vWbp-mediated recruitment of FXIII and fibronectin may favor bacterial escape from innate immune responses and bacterial replication in host tissues.

Finally, fibronectin-binding protein A (FnbpA), a surface protein expressed by most S. aureus strains, also serves as a substrate for factor XIIla (51). At least in vitro, FnbpA is covalently cross-linked to both fibronectin and fibrin/fibrinogen (51). Thus, in addition to stabilizing the fibrin barriers against phagocytes, S. aureus may also utilize the transglutaminase activity of FXIII to deposit its own proteins in the fibrin scaffold, thereby modulating host immune responses to infection.

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