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Clumping Factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of *Staphylococcus aureus*, and decreases complement-mediated phagocytosis.

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Abstract.

The human complement system is important in the immunological control of *Staphylococcus aureus* infection. We have previously shown that the *S. aureus* surface protein clumping factor A (ClfA), when expressed in recombinant form, bound the complement control protein factor I and increased factor I cleavage of C3b to iC3b. In the present study, we show that, when compared to the wild type, isogenic ClfA-deficient *S. aureus* mutants when incubated in serum bound less factor I, generated less iC3b on the bacterial surface, and bound less C3-fragments. It has been shown previously that two amino acids in ClfA (P_{336} and Y_{338}) were essential for fibrinogen binding. However *S. aureus* expressing ClfA P336A Y338S were less virulent than ClfA-deficient strains in animal models. This suggested that ClfA contributed to *S. aureus* virulence by a mechanism different to fibrinogen binding. In the present study, we have shown that *S. aureus* expressing ClfA P336A Y338S was more susceptible to complement-mediated phagocytosis compared to the ClfA-null mutant or wild-type. Unlike ClfA, ClfA P336A Y338S did not enhance factor I cleavage of C3b to iC3b and inhibited the cofactor function of factor H. Fibrinogen enhanced factor I binding to ClfA and the *S. aureus* surface. Twenty clinical *S. aureus* strains all expressed ClfA and bound factor I. High factor I-binding by clinical strains correlated with poor phagocytosis. In summary, these results suggest the interaction of ClfA with factor I contributes to *S. aureus* virulence by a complement-mediated mechanism.

Introduction.

Staphylococcus aureus is a significant cause of morbidity and mortality; methicillin-resistant *S. aureus* (MRSA) caused an estimated 18,650 deaths in the United States in 2005 (19). Antibiotic resistance continues to increase among *S. aureus* isolates including community-associated MRSA (7, 30), healthcare-associated MRSA (12), and *S. aureus* with reduced susceptibility to vancomycin (20). Understanding how this organism avoids host immune defenses is crucial for the development of new strategies to prevent and treat infections.

Complement is a major component of innate immunity and plays a vital role in the control of many bacterial pathogens (28) including *S. aureus* (15, 21, 33). Indeed this organism secretes several small soluble proteins that interfere with normal complement host defense mechanisms, including SCIN and Efb (15, 32). We have previously shown that the human complement regulator factor I is captured on the *S. aureus* cell surface where it is activated and cleaves the crucial opsonin C3b (22) to iC3b (3). This results in decreased phagocytosis by human neutrophils (2). We subsequently showed that the A domain of clumping factor A (ClfA), an important surface-located fibrinogen binding protein, bound factor I and acted as a cofactor to trigger cleavage of C3b to iC3b (13).

The binding to fibrinogen by ClfA involves the C-terminus of the γ-chain binding to a trench located between subdomains N2 and N3 by "dock-lock-latch" mechanism (18). Residues Pro336 and Tyr338 are located in the trench and are crucial for ligand binding - a P336S Y338A mutant (ClfAPYII) is completely defective in fibrinogen binding (23).

Clumping factor A is covalently anchored to the cell wall of *S. aureus* and promotes adhesion of the bacterium to fibrin clots and to thrombi created on heart valves in a rat model of endocarditis (25). In addition, ClfA is required for survival of bacteria following injection into the blood stream of mice (16). This was attributed to the ability of the protein to promote bacterial resistance to phagocytosis by neutrophils. It was proposed that binding to fibrinogen prevented the deposition or recognition of opsonins. However, phagocytosis experiments performed in the absence of fibrinogen demonstrated that the expression of ClfA still contributed an anti-phagocytic, suggesting the existence of another mechanism (14).

In mouse models of *S. aureus* bacteremia and septic arthritis, bacteria expressing the non-fibrinogen binding mutant of ClfA were less virulent than a null mutant that was devoid of the surface protein (17). It was difficult to explain these effects by the loss of fibrinogen binding alone. In the present study, we have analyzed the interaction of ClfA with factor I on the bacterial cell surface and their roles in triggering cleavage of C3b to iC3b. In doing so, we have provided a novel explanation for the role of ClfA in disrupting opsonophagocytosis.

Materials and Methods.

Bacteria and growth conditions. The *S. aureus* strains Newman or Reynolds were used in all experiments. Bacteria were grown in Columbia 2% NaCl broth at 37ºC to mid-logarithmic phase, unless otherwise noted. Two ClfA-deficient strains that are isogenic mutants of strain Newman (9) were tested. ClfA-(2) denotes the isogenic mutant with the genotype *clfA2*::Tn917 (21). ClfA-(5) denotes the isogenic mutant with a frame-shift mutation in *clfA5* (11). The ClfAPYII-expressing strain that expresses a non-fibrinogen binding variant of ClfA (ClfA P336A Y338S) (17). Newman *srtA*::Tc^r is a sortase A-deficient mutant (27).

S. aureus strain Reynolds in mid-logarithmic phase produces undetectable amounts of capsule by rocket immunoelectophoresis (4). To evaluate the role of capsule, a capsule-deficient isogenic mutant of Reynolds strain, JL022 (29), was tested.

Clinical *S. aureus* strains were obtained as discarded de-identified isolates from the Clinical Microbiology Laboratory of The Children's Hospital of The King's Daughters (Eastern Virginia Medical School IRB protocol #06-04-WC-0040). Twenty isolates were tested, 5 CA-MRSA invasive isolates, 5 CA-MRSA non-invasive isolates, 5 methicillin-susceptible *S. aureus* (MSSA) invasive isolates, and 5 MSSA non-invasive isolates. CA-MRSA isolates were defined as MRSA susceptible to clindamycin, while hospital-associated MRSA were defined as resistant to clindamycin. Invasive isolates were recovered from blood, bone, or joint cultures. Non-invasive isolates were recovered from nasopharyngeal cultures, superficial wounds, or colonized tracheostomies of patients without systemic or deep tissue *S. aureus* infection.

Recombinant ClfA. Recombinant ClfA (rClfA) was expressed from pQE30 in *E. coli.* It comprised the full A region (residues 40-599) with an N-terminal His-tag (26). Recombinant ClfAPYII (rClfAPYII, P336S Y338A) is a variant that does not bind fibrinogen. It was also expressed in *E. coli* with a His-tag (17). Recombinant protein expression and purification were performed as previously described (26).

Buffers. *S. aureus* was incubated in serum diluted in 60% $DGVBS^{++}$ buffer (60% Veronal buffered saline [VBS] with 3% dextrose, 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0mM $MgCl_2$), unless otherwise stated. Complement activation was inhibited with $EDTA\text{-}GVBS^{--}$ (VBS with 0.1% gelatin and 0.01 M EDTA).

Serum, hirudin plasma, and factor I. Normal human serum (NHS) was obtained from the blood of healthy human volunteers in accordance with an Institutional Review Board approved protocol (Eastern Virginia Medical School IRB 02-06-EX-0216). The serum from 5 persons was pooled, aliquoted, and frozen at -80ºC, as previously described (4). Heat-inactivated serum was generated by warming NHS to 56ºC for 30 minutes. Hirudin plasma was generated from the blood of human volunteers, as previously described (31). Hirudin plasma was used because it does not alter the function of the complement system unlike other methods of generating plasma like EDTA and heparin. Purified factor I was purchased commercially (CompTech, Tyler TX) and tested for purity and functionality (3). C3-depleted serum was purchased commercially (CompTech).

Cell wall preparations. Cell wall preparations were generated as previously described (13). Briefly, *S. aureus* cell walls were digested with lysostaphin in a 30% raffinose buffer, to stabilize the bacterial protoplasts, containing protease inhibitors and DNAse. The protoplasts were sedimented and the supernatant was recovered as the cell wall preparation. Detection of ClfA in cell wall preparations was performed by Western blot analysis as previously described (13).

Serum factor I binding to *S. aureus*. After washing, mid-logarithmic phase *S. aureus* were adjusted to a concentration of 1×10^9 bacteria per mL. Staphylococcal suspension (0.125mL) was added to 0.1 mL 60% DGVBS buffer and serum to achieve the described final serum concentration and incubated for 5 minutes at 37° C. Bacteria were washed twice in $GVBS^{--}$ buffer and then boiled in 30µL of 2% SDS (0.05 M Tris) buffer for 5 minutes to remove surface bound proteins. Staphylococci were then sedimented and the supernatant recovered for analysis by factor I ELISA (below). Calculation of factor I expressed as molecules/CFU:

$$\text{factor I}\,\frac{\text{molecules}}{\text{CFU}} = \left[\left(\text{X}\,\frac{\text{ng}}{\text{mL}} \div 10^9\,\frac{\text{ng}}{\text{g}}\right) \times \left(\frac{0.24\text{mL}}{10^9\,\text{CFU}} \div 88\,\frac{\text{g}}{\text{mole}}\right) \times \left(6 \times 10^{23}\,\frac{\text{molecules}}{\text{mole}}\right)\right]$$

Factor I detection and quantitation. Factor I quantitation was performed by ELISA as previously described (13). Briefly, ELISA plates were coated with goat anti-human factor I antibody (CompTech, Tyler, TX). Plates were blocked with 3% BSA in PBS/Tween. Test samples were then incubated for 1 hour at room temperature. Factor I was detected with mouse monoclonal anti-human factor I antibody (Quidel, San Diego,

CA) followed by goat anti-mouse horseradish peroxidase-linked antibody. Western blot detection of factor I was performed as previously described (13). Briefly, the membrane was probed with a monoclonal anti-factor I antibody, a polyclonal goat anti-mouse-HRP antibody and then developed by ECL. A modified ELISA was used to quantitate factor I from NHS binding to rClfA. Wells of an ELISA plate were coated with 20 μg/ml of either rClfA or rClfAPYII in carbonate buffer. Wells were washed, blocked, and then incubated with NHS in 3% BSA for 1 hour. Washed wells were then incubated with an anti-factor I monoclonal antibody followed by an anti-mouse HRP antibody as previously described.

Generation of iC3b in the presence of recombinant ClfA and ClfAPYII. In 100 μl of 60% DGVBS^{++}, purified C3b (1 μg) and purified factor I (0.1 μg) were combined with rClfA or rClfAPYII (20, 100, or 200 μg) at 37°C for 16 hr. A control containing C3b, factor I, and factor H was used for these experiments. The samples were measured for iC3b generation by ELISA, as described elsewhere (2).

Bacterial opsonization. *S. aureus* isolates were grown as described, washed with GVBS^{++} and diluted to a concentration of 1×10^9 CFU based on optical density at 600 nm. An aliquot of 1×10^8 CFU was incubated in 1% NHS in a total volume of 0.5 mL of GVBS^{++} for 5 min. at 37° C. The bacteria were then washed twice with GVBS^{--}.

Bacteria were also opsonized with C3b using purified complement components to activate the classical pathway as previously described (3). Briefly, antibody-sensitized *S. aureus* were successively incubated with purified complement components C1, C4, C2,

and C3 to generate the classical pathway C3-convertase and bind C3b to the bacterial surface in the absence of other serum proteins. C3b-coated staphylococci were then incubated with factor I (4μg/mL) or factor H (4μg/mL) and factor I in $GVBS^{--}$.

C3-fragment detection and quantitation. Deposited C3-fragments were stripped from bacteria with 25 mM methylamine as previously described (4). Measurement of iC3b was performed by ELISA using polyclonal anti-C3 antibody for capture and monoclonal anti-iC3b for detection, as previously described (2). Total C3-fragments were measured by ELISA using different polyclonal anti-C3 antibodies for capture and detection, as previously described (2). C3-fragments were also analyzed by Western blot probing with polyclonal anti-C3 antibody that recognizes the peptide chains of both C3b and iC3b, as previously described (5). Images were captured digitally using Versadoc (BioRad).

Phagocytosis. Human neutrophils were prepared from heparinized human blood from healthy human volunteers by Hypaque-ficoll step gradient centrifugation, dextran sedimentation, and hypotonic lysis. *S. aureus* isolates were grown, washed, and diluted as described above. An aliquot of 1×10^8 *S. aureus* was incubated in 1% NHS or 1% heat-inactived serum in a total volume of 0.5 mL of $GVBS^{++}$ for 30 min. at 37°C. These were not washed, but instead an aliquot of 2×10^7 opsonized *S. aureus* was directly incubated with 1×10^6 neutrophils and tumbled for 45 min. at 37°C. A 100 μL aliquot of the mixture was removed, stained with acridine orange (0.01% final) and then quenched with crystal violet (0.03% final) as previously described (2).

Western blot detection of ClfA. The PVDF membrane was blocked in 3% BSA/TBST, which was also used for antibody diluents. The primary probe was a chicken anti-ClfA antibody (1:5000), the secondary probe was a goat anti-chicken HRP antibody (1:5000) (Genway Biotech Inc., San Diego, CA) and then the blot was developed by ECL.

Recombinant ClfA binding to serum factor I. Wells of an ELISA plate were coated with a goat anti-factor I antibody at a 1:1000 dilution in carbonate buffer for 4 hours, washed with PBS (0.5% Tween), as done between each of the following steps, and blocked with 3% BSA/PBST overnight at 4°C. Plates were then incubated with 10% NHS for 1 hour at room temperature to capture the serum factor I onto the plate. Titrating amounts of rClfA or rClfAPYII were then applied to the plate starting at 200 µg/ml diluted in 60% $DGVBS^{++}$ for 3 hours at 37°C. Bound rClfA was detected using a chicken anti-ClfA (1:1000 in 3% BSA/PBST for 1 hour at room temperature) and a goat anti-chicken HRP (1:1000 in 3% BSA/PBST for 1 hour at room temperature). Plates were developed with TMB, stopped with 1 N H_2SO_4 and read at 450 nm.

Statistical analysis. The values of replicate experiments were averaged, from which means and standard errors were calculated (Microsoft Excel XP). Statistical comparisons were made with a Student *t* test with *p* values of < 0.05 considered significant (GraphPad InStat).

Results.

Factor I binding and C3b cleavage by *S .aureus.* Previously we have shown that the soluble A domain of ClfA can bind complement factor I and act as a cofactor (13). In order to analyze the ability of ClfA expressed on the surface of *S .aureus* to promote binding of factor I, the wild type ClfA+ strain Newman was compared with two null mutants lacking ClfA. Bacteria were incubated in human serum and factor I bound to the cells was stripped off by boiling cells in 2% SDS and measured by ELISA (Fig. 1A). In 0.5% serum, the ClfA+ strain bound 112% more factor I than the mutants (mean value for the two null mutants; $P = 0.012$) whereas in 1% serum, the wild-type bound 69% more factor I than the mutants ($P = 0.012$). These results show that expression of ClfA results in higher factor I binding compared to cells lacking the protein, but that some factor I still bound to the ClfA- mutants.

In order to evaluate the effect of ClfA on cleavage of C3b to iC3b on the *S. aureus* cell surface, the wild-type and ClfA-defective mutants were incubated in 1% NHS for 5 minutes and the bound iC3b was stripped off using methylamine and measured by ELISA (Fig. 1B). The level of iC3b on the ClfA+ strain was 125% higher than the mutants (mean value for the two mutants; $P = 0.005$). This showed that expression of ClfA increased the generation of iC3b, but that some C3 degradation occurred in the absence of ClfA. A decrease in iC3b was also detected by Western blotting analysis of C3 fragments solubilized from the cell surface (Fig. 1C).

Cleavage of C3b to iC3b on the *S. aureus* surface should decrease the number of alternative pathway C3-convertases on the bacterial surface and inhibit the deposition of

additional C3b. Therefore, we tested the total amount of C3-fragments deposited by serum on the surface of the wild-type compared with a ClfA-null strain over 60 minutes (Fig. 1D). The differences were most striking at 1 minute with the ClfA-null strain binding 9-fold more C3-fragments compared with the wild-type ($P < 0.001$). The difference in C3-fragment binding narrowed over time, but remained significant. Thus, the presence of ClfA appears to correlate with an overall decrease in the deposition of C3-fragment opsonins.

In order to evaluate whether the residual factor I binding to the ClfA-null strains could be non-specific, we tested a sortase-null mutant (27) of *S. aureus* strain Newman (Fig. 1E). The sortase-null mutant is unable to anchor any LPXTG-motif cell wall associated proteins (24). No significant difference in factor I binding was evident between the ClfA-null strain [ClfA-(2)] and the sortase-null (Sortase-) strain ($P = 0.17$), suggesting that the non-ClfA-dependent factor I-binding is unrelated to covalently anchored surface proteins.

C3b cleavage and ClfAPYII. ClfAPYII is a ClfA mutant with substitutions at positions 336 and 338 (ClfA P336A Y338S) that does not bind fibrinogen (6), the primary function described for ClfA. In order to test the role of ClfAPYII in factor I cleavage of C3b to iC3b on the *S. aureus* surface, a mutant of Newman expressing ClfAPYII was incubated in 1% normal human serum, stripped of C3-fragments and iC3b was measured by ELISA (Fig. 2A). The ClfAPYII expressing strain generated 57% less iC3b compared with wild-type ($P = 0.02$, based on absolute values). This suggested that

the presence of ClfAPYII altered the ability of factor I in serum to cleave C3b to iC3b on the bacterial surface.

In order to further elucidate how the expression of ClfAPYII affects factor I cleavage of C3b to iC3b on the *S. aureus* surface, experiments were conducted using purified complement components. *S. aureus* strains expressing ClfAPYII or ClfA (wild-type) were incubated with purified components to activate the classical pathway and bind C3b to the bacterial surface (3). The C3b-coated bacteria were then incubated with purified factor I or both purified factor I and purified factor H, stripped of C3-fragments using methylamine and iC3b and total C3-fragments measured by ELISA (Fig. 2B). The ClfA-expressing (wild-type) strain incubated with factor I alone showed increased C3b cleavage by iC3b/C3 ratio ($P = 0.05$) compared with control. However, the ClfAPYII-expressing strain incubated with factor I alone showed no increase in C3b cleavage above control, suggesting that ClfAPYII could not act as a cofactor for factor I on the *S. aureus* surface. Curiously, when the C3b-coated ClfAPYII-expressing strain was exposed to both factor I and a strong cofactor, factor H, C3b cleavage was similar to control. This suggests that ClfAPYII interaction with factor I on the *S. aureus* surface may inhibit the normal cofactor activity of factor H.

We then tested whether recombinant ClfAPYII would show differences in cofactor activity for factor I compared with recombinant ClfA. Recombinant ClfA and ClfAPYII were incubated with purified C3b and purified factor I and iC3b generation was measured by ELISA (Fig. 2C). In the presence of 200 μg of recombinant ClfAPYII, factor I produced 57% less iC3b compared with the same amount of recombinant ClfA (*P*

= 0.01). These findings again suggest that ClfAPYII has diminished cofactor activity for factor I compared with wild-type ClfA.

Factor I binding to *S. aureus* expressing ClfAPYII and recombinant ClfAPYII. In order to test factor I binding on the bacterial surface, serum factor I binding to the *S. aureus* expressing ClfAPYII was compared with the wild-type and ClfA-null strains. The bacteria were incubated in serum, stripped of surface proteins by boiling in 2% SDS and factor I was measured by ELISA (Fig. 3A). Minimal factor I could be detected in the stripped surface protein supernatant for the ClfAPYII-expressing strain and was 86% decreased compared with the ClfA-deficient strains ($P < 0.03$). This suggested that either the factor I was not binding to the bacterial surface of the ClfAPYII-expressing strain or that factor I could not be stripped from the ClfAPYII strain.

In order to test these possibilities, we incubated both strains in purified factor I and then solubilized cell-wall proteins with lysostaphin (13). The cell-wall preparations were analyzed by Western blotting and probed for factor I with anti-factor I antibody (Fig. 3B). The factor I content of the cell wall preparations was also measured by ELISA (Fig. 3C). The Western blot analysis showed that factor I was present in the cell wall preparation of both organisms. The ELISA measured similar amounts of factor I in the cell wall preparations of the ClfAPYII-expressing *S. aureus* compared with the wild-type. This demonstrated that similar amounts factor I bound to the cell wall of the ClfAPYII-expressing strain, but could not be removed from the cell wall by boiling in 2% SDS, which readily removed factor I from the surface of the wild-type strain. These results

suggested that ClfAPYII bound factor I, but the binding properties were altered compared to wild-type ClfA.

In order to evaluate further the binding of factor I to ClfAPYII, we then tested the binding of serum factor I to recombinant ClfA (rClfA) or recombinant ClfAPYII (rClfAPYII) in an ELISA-type assay (Fig. 3D). Both rClfA and rClfAPYII bound serum factor I with similar half-maximal binding values of 50nM showing that both forms bind serum factor I effectively.

Phagocytosis of *S. aureus* expressing ClfAPYII. In order to understand better the impact of ClfA on complement-mediated phagocytosis, ClfAPYII-expressing *S. aureus* was compared with the wild-type and ClfA-null strains. The bacteria were incubated with 1% normal human serum (NHS) or heated serum without complement activity and then added to purified human neutrophils in a 20:1 ratio. Bacteria were stained with acridine orange and extracellular bacteria were quenched with crystal violet. With heat inactivated serum minimal phagocytosis occurred for each strain, whether evaluated as the absolute number of bacteria phagocytized per 100 neutrophils (Fig. 4A) or as the percent of neutrophils phagocytizing bacteria (Fig. 4B). In normal human serum, neutrophils phagocytized 3-fold more ClfAPYII-expressing *S. aureus* compared with wild-type ($P < 0.01$) and phagocytized 2-fold more ClfAPYII-expressing *S. aureus* compared with the ClfA-null strain ($P = 0.01$). More neutrophils ingested ClfAPYII-expressing bacteria than either the wild-type ($P = 0.02$) or the ClfA-null strain ($P = 0.03$). Although a trend towards increased phagocytosis was noticed for the ClfA-null strain compared with wild-type, this did not reach statistical significance. These findings show

that ClfAPYII-expressing *S. aureus* are much more susceptible to complement-mediated phagocytosis than the wild-type or the ClfA-null mutant suggesting that the substitutions at positions 336 and 338 of ClfA are important in modulating *S. aureus* susceptibility to phagocytosis.

Effect of complement activation on factor I binding. In order to determine if complement activation and C3b deposition had any effect on the binding of factor I to the *S. aureus* surface, the ClfA+ wild type strain Newman was incubated in normal human serum or in serum where complement was inactivated by heat treatment or with EDTA-GVBS^{--} buffer. Complement proteins were stripped and factor I was measured by ELISA (Fig. 5A). No difference in the level of factor I was detected when comparing samples incubated in NHS or complement-inactivated sera ($P > 0.11$).

In order to test if C3 or C3b was necessary for factor I binding to ClfA, recombinant ClfA was incubated with normal human serum or C3-depleted serum and factor I binding was measured by ELISA (Fig. 5B). Serum factor I binding to rClfA was not significantly different in the presence or the absence of C3 or C3b at 25% serum ($P = 0.07$), but in 50% serum, rClfA bound 16% less factor I in the presence of C3 compared with C3-deficient serum ($P = 0.02$). These findings suggest that C3 is not required for serum factor I binding to ClfA.

Effect of capsule on factor I binding to *S .aureus*. In order to determine if expression of capsular polysaccharide interfered with factor I binding to *S. aureus*, the well characterized capsule type 5 expressing strain Reynolds and an isogenic capsule deficient

mutant were compared. Bacteria were grown in conditions where capsule was expressed strongly (stationary phase) or poorly (mid logarithmic phase) (4). Similar levels of factor I bound to the two strains under both growth conditions (Fig. 6A, $P = 0.17$) suggesting that neither capsule expression nor the phase of growth significantly affected factor I binding. Control experiments showed that ClfA was expressed by bacteria in both phases (Fig. 6B).

Factor I binding to clinical isolates. To evaluate if factor I could bind to clinical isolates of *S. aureus*, five strains from each of four categories (invasive CA-MRSA, non-invasive CA-MRSA, invasive MSSA, non-invasive MSSA) were incubated with serum, surface proteins were stripped and factor I measured by ELISA (Fig. 7A). All 20 strains bound factor I. A three-fold difference was found comparing the strains which bound the most factor I compared with those which bound the least. We calculated that approximately 60,000 molecules of factor I were bound on average to the *S. aureus* isolates. These experiments demonstrate that all clinical strains of *S. aureus* bind factor I. Control experiments showed that ClfA was expressed by all clinical strains; a representative blot is shown in figure 7B.

Clinical strains that bound the most factor I would be expected to cleave more effectively C3b to iC3b and limit opsonic C3-fragment deposition on the *S. aureus* surface by the alternative pathway, as shown in figure 1D. Neutrophil phagocytosis experiments were performed with the two clinical strains that bound the most factor I and the two clinical strains which bound the least factor I (Fig. 7C). A 1.6-fold increase in phagocytosis efficiency was found for clinical isolates that poorly bound factor I

compared with strains that bound the most factor I ($P = 0.003$). These findings support the likely clinical relevance of an association between factor I binding and virulence.

Effect of fibrinogen on factor I binding to ClfA. In order to test whether the presence of fibrinogen interferes with factor I binding to ClfA, we compared factor I binding in serum, where there is minimal fibrinogen, to plasma which has a physiological concentration of fibrinogen (Fig 8A). Use of the anti-coagulant hirudin does not affect activation of complement as occurs in plasma stabilized with EDTA or heparin (1). Factor I binding to wild-type (ClfA+) *S. aureus* was increased in both plasma preparations compared with serum ($P \leq 0.01$). There was no difference in factor I binding to the ClfA-null strain [ClfA-(2)] in the plasma compared to serum ($P \geq 0.1$). These results suggest that the presence of fibrinogen increases factor I binding to wild-type *S. aureus*.

In order to test whether factor I bound to the *S. aureus* continued to cleave C3b to iC3b in the presence of a physiological concentration of fibrinogen (2-4 mg/mL), we compared the generation of iC3b on the surface of bacteria incubated in NHS or hirudin plasma (Fig. 8B). For the wild-type strain (ClfA+), there was no difference in iC3b generated on the bacterial surface in NHS compared to hirudin plasma ($P = 0.63$). This suggests that factor I functions normally on the *S. aureus* surface in the presence of physiologic fibrinogen.

The influence of fibrinogen was also tested using purified factor I and purified fibrinogen in a solid phase binding assay with immobilized rClfA (Fig. 8C). Purified fibrinogen increased purified factor I binding to rClfA in a dose-dependent manner. This

result is consistent with the results of the experiments shown in figure 5A where factor I binding to *S. aureus* was increased in plasma, suggesting that fibrinogen increases the association of factor I with ClfA.

We then tested if fibrinogen in plasma was associated detectably with factor I in a solid phase assay where factor I was captured from plasma with an immobilized monoclonal anti-factor I antibody (Fig. 8D). Factor I was readily captured from plasma, but no bound plasma fibrinogen was detected when probed with an anti-fibrinogen antibody. We also investigated whether fixing fibrinogen to a surface would cause binding by factor I (Fig. 8E). Here an immobilized anti-fibrinogen antibody was used to capture fibrinogen from plasma and bound plasma factor I measured with an anti-factor I antibody. No plasma factor I bound to the solid-phased plasma fibrinogen.

In order to clarify whether fibrinogen is required for ClfA binding to factor I, we performed a solid phase assay to capture factor I from serum followed by incubation with increasing concentrations of rClfA or rClfAPYII (Fig. 8F). Wells were coated with anti-factor I antibody to capture factor I from serum. After washing, rClfA or rClfAPYII were added and binding was detected with anti-ClfA antibody. The rClfA or rClfAPYII bound to serum factor I dose-dependently with similar affinities suggesting that ClfA binding to serum factor I does not require fibrinogen.

Discussion.

The studies described in this paper show that ClfA expression on the surface of *S. aureus* is associated with increased binding by factor I from serum, increased cleavage of surface-bound C3b to iC3b, and decreased C3-fragment binding to the *S. aureus* surface. We have previously shown that factor I-mediated cleavage of C3b to iC3b on the *S. aureus* surface is associated with decreased phagocytosis by human neutrophils (2). Taken together, these findings suggest that ClfA binding of serum factor I and the resultant C3b cleavage to iC3b on the *S. aureus* surface is a plausible mechanism contributing to *S. aureus* evasion of complement host defenses.

Previous studies have shown that ClfA P336A Y338S does not bind fibrinogen (23) and that in mouse models of *S. aureus* bacteremia and septic arthritis a consistent virulence pattern is found with the ClfAPYII mutant being less virulent that the ClfA null mutant (17). It is quite striking that complement-mediated phagocytosis of these strains shows a similar progression in that ClfAPYII-expressing *S. aureus* are more readily phagocytized than the ClfA-null or the wild-type strain. These differences in complement-mediated phagocytosis can be at least partly explained by the failure of ClfAPYII to act as a cofactor for factor I-mediated cleavage of C3b to iC3b. ClfAPYII also appears to inhibit the cofactor activity of factor H. Thus, ClfA has an important interaction with the complement system that likely contributes to the differences in virulence found *in vivo* that could not be attributed to ClfA interaction with fibrinogen. These findings support the hypothesis that factor I binding to ClfA results in C3b cleavage to iC3b on the *S. aureus* surface and contributes to immune evasion and virulence.

The presence of fibrinogen at physiological concentrations increases factor I binding to the *S. aureus* surface, increases factor I binding to ClfA, and does not adversely affect the cleavage of C3b on the bacterial surface. Factor I in serum does not appear to require fibrinogen for binding to ClfA or ClfAPYII (the latter being a molecule that cannot bind fibrinogen).

Previous studies have suggested that upon binding the factor H-C3b complex, factor I undergoes a conformational change that enables it to cleave C3b (8, 10). We speculate that factor I may undergo a similar conformational change upon binding the fibrinogen-ClfA complex that enables it to cleave C3b. We also propose that the 'activating' conformational change for factor I does not occur upon binding to ClfAPYII.

Based on these data, we propose the model illustrated in figure 9. The apo form of ClfA is shown with the latching peptide emanating from the C-terminus of the N3 domain unbound to the N2 domain. When the D domain of fibrinogen contacts ClfA and the gamma-chain peptide inserts into the ligand-binding trench, a conformational change occurs which results in the fibrinogen peptide being locked in place by the latching peptide undergoing beta-strand complementation with two beta strands in N2. Factor I binds the fibrinogen-ClfA complex and undergoes a conformational change into an 'active' form that can cleave C3b to iC3b. ClfAPYII, in contrast, is unable to bind fibrinogen and remains in the apo form with the latching peptide free. Factor I is also able to bind ClfAPYII, but does not undergo a conformational change to an 'active' form. Thus, factor I complexed with ClfAPYII is unable to cleave C3b to iC3b. The stronger binding of factor I to ClfAPYII could be due to local changes in conformation due to the amino acid substitutions.

All 20 clinical isolates expressed ClfA and bound factor I. Clinical isolates with increased factor I binding were poorly phagocytized relative to isolates with decreased factor I binding. These data support the likely physiological relevance of ClfA-mediated factor I cleavage of C3b.

Future studies will continue to investigate the binding and functional interaction between factor I, fibrinogen, ClfA, and ClfAPYII in order to further elucidate this relationship.

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Figure Legends.

FIG. 1. Factor I binding and C3b cleavage to iC3b on wild-type (ClfA+) and ClfA-deficient [ClfA-(5) and ClfA-(2)] *S. aureus* strains. Bacteria were incubated in 1% normal human serum (NHS) for 5 minutes, washed and boiled in 2% SDS to remove bound factor I, which was measured by ELISA (A). Data are mean ± SE for 5 independent experiments. Bacteria were incubated in 1% NHS for 5 minutes, washed, stripped of C3-fragments with methylamine and assayed for iC3b by ELISA (B). Data are mean ± SE for 4 independent experiments. C3-fragments stripped from the surface of serum-incubated *S. aureus* strains were analyzed by Western blot and probed for C3-fragments (C). Total C3-fragment binding to *S. aureus* expressing wild-type ClfA (ClfA+) or ClfA-null [ClfA-(2)] incubated in 1% NHS for increasing lengths of time and assayed by ELISA (D). Data are mean ± SE for 3 independent experiments. Factor I binding was tested for a ClfA-deficient strain [ClfA-(2)] and a sortase-deficient isogenic mutant (Sortase-) (E). Bacteria were incubated in 1% plasma for 5 minutes, washed, stripped and measured for bound factor I by ELISA. Data are mean ± SE for 3 independent experiments.

FIG. 2. C3b cleavage to iC3b for *S. aureus* expressing ClfAPYII and recombinant ClfAPYII. *S. aureus* strains expressing the wild-type ClfA (ClfA+), the mutant ClfAPYII (ClfAPYII+), or ClfA-null [(ClfA-(5) and ClfA-(2)] were incubated in 1% NHS for 5 minutes, washed, stripped of C3-fragments with methylamine and measured for iC3b by ELISA (A). Data are normalized to wild-type and are mean ± SE for 3 independent experiments. *S. aureus* expressing wild-type ClfA (ClfA+) or ClfAPY

(ClfAPYII+) were coated with C3b by complement activation with purified components, incubated with purified factor (fI), purified factors I and H (+fI +fH), or buffer alone (control), stripped of bound C3-fragments and analyzed by ELISA (B). Factor H (fH) was used as a control cofactor. Data are mean ± SE for 3 independent experiments. Recombinant ClfA (rClfA) and recombinant ClfAPYII (rClfAPYII) were incubated with purified C3b and purified factor I and assayed for iC3b by ELISA (C). Data are mean ± SE for 3 independent experiments.

FIG. 3. Factor I binding to *S. aureus* expressing ClfAPYII and recombinant ClfAPYII. *S. aureus* strains expressing the wild-type ClfA (ClfA+), the mutant ClfAPYII (ClfAPYII+), or ClfA-null [ClfA-(5) and ClfA-(2)] were incubated in 1% NHS, washed, and boiled in 2%SDS to remove bound factor I, which was measured by ELISA (A). Data are normalized to wild-type and are mean ± SE for 5 independent experiments. *S. aureus* strains expressing the wild-type ClfA (ClfA+) or the mutant ClfAPYII (ClfAPYII+) were incubated with purified factor I, washed and cell-wall preparations were analyzed by Western blot probing for factor I (B). The cell-wall preparations of *S. aureus* incubated with purified factor I (+fI) or buffer (control) were also measured for factor I by ELISA (C). Data are the means ± SEM of 4 independent experiments. Serum factor I binding to recombinant ClfA (rClfA) or recombinant ClfAPYII (rClfAPYII), measured by ELISA (D). Data are the means ± SEM of 5 independent experiments.

FIG. 4. Phagocytosis efficiency for neutrophil engulfment of isogenic *S. aureus* strains incubated with normal human serum (NHS) or heated serum (heated serum) without

complement activity. Wild-type (ClfA+), ClfA-null [ClfA-(2)], or ClfAPYII-expressing (ClfAPYII+) strains were incubated with 1% serum for 30 minutes, to which were added purified human neutrophils for 45 minutes, then stained with acridine orange, quenched with crystal violet and assayed by fluorescence microscopy. The numbers of bacteria phagocytized per 100 neutrophils were measured (A). The percent of neutrophils phagocytizing bacteria were measured (B). Data are the means ± SEM of 4 independent experiments.

FIG. 5. Factor I binding to ClfA-expressing *S. aureus* and recombinant ClfA in the absence of complement activation or C3. *S. aureus* expressing ClfA (wild-type) were incubated in 10% normal human serum (NHS), heat-inactivated sera (Heat sera) or sera in EDTA-GVBS- - buffer (EDTAsera), washed and boiled in 2% SDS to remove factor I, which was measured by ELISA (A). Data are the mean ± SE for 5 independent experiments. Serum factor I binding to recombinant ClfA was tested for NHS, heat-inactivated sera or C3-depleted sera (C3depl sera) and measured by ELISA (B). Data are the means ± SEM of 6 independent experiments.

FIG. 6. Serum factor I binding to *S. aureus* in the presence of capsule. Isogenic ClfA-sufficient *S. aureus* strains that expressed capsule (capsule+) or was capsule-deficient (capsule-def) were grown to mid logarithmic (log) or stationary phase, incubated in 10% normal human serum, washed, and boiled in 2% SDS to remove bound factor I, which was measured by ELISA (A). Data are the means ± SEM of 4 independent experiments.

ClfA expression for each strain and each growth condition was assayed by Western blotting of cell wall preparations (B).

FIG. 7. Clinical *S. aureus* isolates binding serum factor I, ClfA expression, and phagocytosis efficiency. Five separate isolates were tested for each category: invasive CA-MRSA (MRSA-Inv), non-invasive CA-MRSA (MRSA-Non), invasive MSSA (MSSA-Inv), and non-invasive MSSA (MSSA-Non) (A). A laboratory control strain (Reynolds) was also tested. Bacteria were incubated in 10% normal human serum, washed and boiled in 2% SDS to remove bound factor I, which was measured by factor I ELISA. Data are the mean ± SE of 5 independent experiments. ClfA expression of *S. aureus* clinical isolates was assayed by Western blotting of cell wall preparations (B). Phagocytosis efficiency for the two clinical isolates that bound the least factor I (M25 and S68) and the two clinical isolates that bound the most factor I (M32 and M2) (C). Isolates were incubated with 1% serum for 30 minutes, to which were added purified human neutrophils for 45 minutes, then stained with acridine orange, quenched with crystal violet and assayed by fluorescence microscopy. Data are the mean ± SE of 3 independent experiments.

Figure 8. Factor I binding and function in the presence of fibrinogen. Factor I binding to *S. aureus* wild-type (ClfA+) or ClfA-null strain [ClfA-(2)] incubated in 1% serum (NHS), EDTA-plasma, or hirudin-plasma, measured by ELISA (A). Data are the mean ± SE of 7 independent experiments. Bound iC3b to *S. aureus* wild-type (ClfA+) or ClfAPYII-expressing strain (ClfAPYII+) incubated in 1% serum or hirudin-plasma,

measured by ELISA (B). Data are the mean ± SE of 3 independent experiments. Purified factor I (10 ng/mL) binding to recombinant ClfA (rClfA) in an ELISA in the presence of increasing purified fibrinogen (C). Data are the mean ± SE of 6 independent experiments. Factor I was captured from increasing concentrations of plasma and probed for associated plasma fibrinogen by ELISA (D). Monoclonal anti-factor I was used to capture plasma factor I (control fI) and bound plasma fibrinogen (pfibrinogen/captured fI) was detected with anti-fibrinogen antibody. Data are the mean ± SE of 3 independent experiments. Fibrinogen was captured from increasing concentrations of plasma and probed from associated plasma factor I by ELISA (E). Anti-fibrinogen antibody was used to capture plasma fibrinogen and bound plasma factor I (pfactor I/captured fg) was detected with anti-factor I antibody. Data are the mean ± SE of 3 independent experiments. Serum factor I was captured with anti-factor I antibody and assayed for bound rClfA or rClfAPYII by ELISA (F). Increasing concentrations of rClfA or rClfAPYII were added and detected with anti-ClfA antibody. Data are the mean ± SE of 4 independent experiments.

Figure 9. A scheme to the associations of factor I and fibrinogen with ClfA and ClfAPYII. Fibrinogen (Fg) binds to ClfA and enhances the binding of factor I which is then in an 'active' conformation and able to cleave C3b to iC3b. ClfAPYII, which is unable to bind fibrinogen, is also able to bind factor I, but the factor I is not in an 'active' conformation and unable to cleave C3b to iC3b.