Staphylococcus aureus Clumping Factor A Binds to Complement Regulator Factor I and Increases Factor I Cleavage of C3b

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The human complement system plays an important role in the control of *Staphylococcus aureus* infection. For instance, we previously demonstrated that the central complement component deposited on the organism's surface, C3b, can be cleaved by the host complement control protein, factor I, resulting in diminished phagocytosis of *S. aureus*. In the present study, we have identified clumping factor A (ClfA) from cell wall proteins of *S. aureus* as a specific protein bound by factor I. Recombinant ClfA (rClfA) containing the full-length A region (peptides 40–559) also bound factor I. We identified an ~50-kDa fragment of ClfA that is shed by *S. aureus* into growth medium. The shed ClfA fragment was derived from the A region of ClfA and bound factor I. rClfA and the shed ClfA fragment increased factor I cleavage of C3b into inactive C3b. Our findings describe a new *S. aureus* mechanism for modification of host complement activities.

Staphylococcus aureus is a highly successful pathogen, a common cause of community-associated infection, and the most common cause of severe nosocomial infection [1–3]. *S. aureus* infections cause considerable morbidity, mortality, and medical expense [4, 5]. Antibiotic resistance continues to increase for *S. aureus* isolates, and the rapid worldwide spread of community-associated methicillin-resistant *S. aureus* infection is particularly disconcerting [6–8]. New approaches to prevent and treat *S. aureus* infection are needed, and immune-directed strategies appear to have great potential for the development of novel therapeutics against this pathogen.

Evasion of host defenses is necessary for pathogens to replicate, establish infection, and produce disease. Complement-mediated opsonization is a critical host defense against many pathogenic bacteria, and C3, the central complement component, plays an important role in host defense against *S. aureus* infection in humans and animal models [9, 10]. Activation of complement pathways leads to the conversion of C3 to C3b, which binds to *S. aureus* as a highly potent opsonin [11, 12]. In vitro and in vivo data suggest that surface-bound C3b is important for immunologic control of *S. aureus* [13, 14]. We have shown elsewhere that much of the C3b bound to the surface of *S. aureus* is rapidly cleaved into inactive C3b (iC3b) and that this cleavage is mediated by complement regulatory protein factor I [15]. Known properties of C3b cleavage into iC3b include diminishment of binding to complement receptor 1 (CD35) on phagocytes, prevention of activation of the alternative complement pathway, and prevention of activation of the terminal complement cascade [16]. Binding to CD11b/18 (CR1) is increased for iC3b [17]. We have shown previously that factor I cleavage of *S. aureus*–bound C3b diminished phagocytosis by neutrophils [18]. Taken together, these data suggest that factor I affects immunologic control of *S. aureus* by cleaving C3b.

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Pathogen utilization of host complement-regulatory proteins has been described for several agents [19]. *Streptococcus pyogenes* and *Streptococcus pneumoniae,* which, like *S. aureus,* are gram-positive cocci, express surface proteins that bind complement factor H, a cofactor for factor I [20–23]. We hypothesized that *S. aureus* may enhance the activity of factor I, specifically by localizing factor I to its surface and providing a staphylococcal cofactor to facilitate efficient cleavage of bound C3b. We have shown elsewhere, using Western blotting and SDS-PAGE, that purified factor I will bind to a well-characterized laboratory strain of *S. aureus* [15]. In the present study, we extend our support of this hypothesis by identifying a *S. aureus* cell wall component that binds factor I and a shed staphylococcal protein with cofactor activity for factor I.

METHODS

Bacteria. *S. aureus* strain Reynolds or Newman was grown to midlogarithmic phase, as described elsewhere [11]. *S. aureus* strain Reynolds in midlogarithmic phase produces undetectable amounts of capsule, as shown by rocket immunoelectrophoresis [11].

Buffers, serum, and factor I. Normal human serum (NHS) was diluted in 60% dextrose-gelatin veronal-buffered saline $(DGVBS)^{2+}$ buffer [11], unless otherwise stated. NHS was obtained from the blood of healthy human volunteers (Eastern Virginia Medical School Institutional Review Board 02-06-EX-0216) and pooled. Purified factor I was purchased commercially (CompTech) and tested for purity by SDS-PAGE with SYPRO Ruby staining (Invitrogen) [15] and Western blot analysis with anti–factor H antibody detection.

S. aureus *cell wall lysate and supernatant from growth medium.* Midlogarithmic-phase *S. aureus* were washed with PBS, and cell wall preparations were generated as described elsewhere [24]. *S. aureus* growth medium supernatant was generated from bacterial culture grown overnight via sedimentation and supernatant recovery.

Recombinant ClfA (rClfA). rClfA was expressed as a His-tagged protein containing the A region (peptides 40–559) of ClfA in an *Escherichia coli* expression system, as described elsewhere [25].

***Factor I binding to and release from* S. aureus.** Washed midlogarithmic-phase *S. aureus* (1×10^9 cfu/mL) were incubated with 10% NHS in 60% $DGVBS^{2+}$ buffer for 5 min, unless otherwise noted. Washed bacteria were boiled in 30 μL of 2% SDS (0.5 mol/L Tris) for 5 min to remove surface-bound proteins and then sedimented, and the supernatant was recovered for analysis by factor I ELISA.

S. aureus (5×10^8 cfu/mL) were incubated with purified factor I (0–40 μg/mL) in 250 μL, washed, and incubated in 25 μL of PBS for 16 h. Bacteria were sedimented and the supernatants recovered. Sedimented bacteria were boiled in 25 μL of 2% SDS for 5 min, and supernatants containing surface proteins were recovered. Both samples were analyzed by Western blotting for factor I.

Factor I ELISA. Flat-bottom Immulon 2 plates (Thermo Labsystems) were coated with 50 μL (1.0 μg/mL) of monoclonal anti–human factor I antibody (Quidel) in carbonate buffer (overnight at 4°C). Washed wells were blocked with 3% bovine serum albumin (BSA; overnight at 4°C), washed, incubated with samples (1 h at room temperature), and washed again. The primary probe was goat anti–human factor I antibody (1:500 in 3% BSA for 1 h at room temperature), and the secondary probe was rabbit anti–goat horseradish peroxidase (HRP) antibody (1:4000 in 3% BSA for 1 h at room temperature), developed with tetramethylbenzidine, stopped with 1N H_2SO_4, and read at 450 nm. Purified factor I was used for standard curves.

SDS-PAGE and C3 Western blotting. SDS-PAGE, total protein staining, and Western blotting for C3 fragments were performed as described elsewhere [18].

Chromatography. A HiPrep 16/60 Sephacryl column (GE Healthcare) was used for size-exclusion column chromatography. The column was equilibrated with distilled water and then 60% VBS^{2+}; 5.0-mL fractions were collected at a rate of 1.0 mL/min. A 5-mL HiTrap Q HP column (GE Healthcare) was used for ion-exchange column chromatography. The column was equilibrated with 20 mmol/L Tris buffer (pH 8.6), and the sample was loaded and washed. The column was eluted with a salt gradient, using 20 mmol/L Tris and 20 mmol/L Tris plus 1 mol/L NaCl buffers.

Dot blot detection of factor I binding. Column fractions (400 μL) were added to dot blot wells and drained through a polyvinylidene fluoride (PVDF) membrane. The washed membrane was incubated in 20% heat-inactivated human serum/Tris-buffered saline (TBS; overnight at 4°C). The primary probe was goat anti–human factor I (1:4000 in 5% heat-inactivated serum), and the secondary probe was rabbit anti–goat HRP antibody (1:4000 in 5% heat-inactivated serum), developed by enhanced chemiluminescence (ECL). Quantitation was done by optical densitometry (Quantity One; Bio-Rad).

Western blot detection of factor I and factor I overlay blot. PVDF was blocked in 3% BSA/TBS-Tween (TBST; overnight at 4°C). The primary probe was anti–factor I monoclonal antibody (1:2000 in 3% BSA), and the secondary probe was goat anti–mouse HRP antibody (1:1000 in 3% BSA), developed by ECL. Purified factor I was used as a control in these assays.

PVDF for factor I overlay blot was incubated in 20% heat-inactivated human serum/TBST (overnight at 4°C). The membrane was probed with anti–factor I monoclonal antibody (1:2000 in 5% heat-inactivated serum) and then with goat anti–mouse HRP antibody (5% heat-inactivated serum) and was developed by ECL.

Western blot detection of ClfA and rClfA overlay blot. The PVDF membrane for Western blot detection of ClfA was blocked in

3% BSA/TBST (overnight at 4°C). The primary probe was rabbit anti-ClfA antibody (1:5000 in blocking solution), and the secondary probe was goat anti–rabbit HRP antibody (1:5000 in 3% BSA), developed by ECL. The PVDF membrane for the rClfA overlay blot was blocked with 3% BSA/TBST, and then 60 μg of rClfA was added (overnight at 4°C). The primary probe was rabbit anti-ClfA antibody (1:5000 in 3% BSA), the secondary probe was goat anti–rabbit HRP antibody (1:4000 in 3% BSA), and development was done by ECL.

Staphylococcal supernatant overlay blot. One microliter of NHS and 0.01 mg of factor I were separated on an SDS-PAGE gel (nondenaturing) and transferred to a PVDF membrane. The membrane was blocked with 3% BSA/TBS/Tween and then incubated with 75 μL of *S. aureus* growth medium supernatant (overnight at 4°C). The primary probe was rabbit anti-ClfA antibody (1:5000 in 3% BSA), the secondary probe was goat anti–rabbit HRP antibody (1:4000 in 3% BSA), and development was done by ECL.

Factor I–binding rClfA. For a modified ELISA, wells of an ELISA plate were coated with rClfA at 1–10 μg/mL in carbonate buffer (overnight at 4°C). Washed wells were blocked with 3% BSA/PBS/Tween, washed again, and incubated with NHS in 3% BSA for 1 h. Washed wells were incubated with 0.1 mL of anti–factor I monoclonal antibody (1:1000 in 3% BSA for 1 h) and then 0.1 mL of anti–mouse HRP antibody (1:500 in 3% BSA for 1 h), washed, and developed as described above. Half-maximal binding was calculated as described elsewhere [26]. Similar to the rClfA-coated microtiter plate assay described below, biotinylated (EZ-Link; Pierce) purified factor I (50 nmol/L) was incubated with increasing amounts of unlabeled purified factor I in the presence of C3b; residual binding was measured using streptavidin-HRP and developed as described above.

Factor I–mediated cleavage of C3b to iC3b. In 100 μL of 60% DGVBS^{2+}, purified C3b (1 μg) and purified factor I (1 μg, unless otherwise noted) were combined with concentrated *S. aureus* growth medium supernatant, column fractions, column-purified secreted ClfA (sClfA; ~50-kDa fragment), or rClfA (16 h at 37°C) and then measured for C3b cleavage by iC3b ELISA, as described elsewhere [18]. A C3b plus factor H plus factor I control was used for these experiments. Where noted, microtiter plate wells were coated with rClfA (5 μg) and then incubated with 10 μg of purified C3b and 0.25 μg of purified factor I; supernatants were analyzed by Western blot for C3 fragments.

Mass spectrometry (MS) identification. Protein bands were excised from 1-dimensional PAGE gels. Gel slices were cut into 1–2-mm cubes, washed 3 times with 500 μL of ultrapure water, and incubated in 100% acetonitrile for 45 min. Samples were reduced with 50 mmol/L dithiothreitol at 56°C for 45 min and then alkylated with 55 mmol/L iodoacetamide for 1 h at room temperature. The material was dried in a SpeedVac (Savant), rehydrated in 12.5 ng/μL modified sequencing-grade trypsin solution (Promega), and incubated in an ice bath for 40–45 min. The excess trypsin solution was then removed and replaced with 40–50 μL of 50 mmol/L ammonium bicarbonate and 10% acetonitrile (pH 8.0), and the mixture was incubated overnight at 37°C.

Elastase digests were performed as described for trypsin at an enzyme concentration of 15 ng/μL without acetonitrile in the reaction buffer. Peptides were extracted twice with 25 μL of 50% acetonitrile and 5% formic acid and dried in a SpeedVac. Digests were resuspended in 20 μL of buffer A (5% acetonitrile, 0.1% formic acid, and 0.005% heptafluorobutyric acid), and 3–6 μL was loaded onto a 12-cm by 0.075-mm fused silica capillary column packed with 5-μm-diameter carbon 18 beads (The Nest Group), using a nitrogen pressure vessel at 1100 psi. Peptides were eluted over 55 min by applying a 0%–80% linear gradient of buffer B (95% acetonitrile, 0.1% formic acid, and 0.005% heptafluorobutyric acid) at a flow rate of 150 μL/min with a precolumn flow splitter, resulting in a final flow rate of ~200 nL/min directly into the source. In some cases, the gradient was extended to 150 min, to acquire more tandem MS (MS/MS) spectra.

An LTQ Linear Ion Trap (ThermoFinnigan) was used in an automated collection mode with an instrument method composed of a single segment and 5 data-dependent scan events with a full MS scan, followed by 4 MS/MS scans of the highest-intensity ions. The normalized collision energy was set at 35 and the activation *Q* was 0.250, with minimum full-scan signal intensity at 1×10^5 and no minimum MS/MS intensity specified. Dynamic exclusion was turned on, with a 3-min repeat count of 2 and the mass width set at a mass to charge ratio of 1.0. Sequence analysis was performed with Mascot (Matrix Sciences), using an indexed bacterial subset database of the nonredundant protein database from the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/).

Statistical analysis. Results of independent replicate experiments were averaged, and SEs were calculated. Statistical comparisons were made using Student's *t* test.

RESULTS

***Serum factor I binding to* S. aureus.** To test the rapidity of factor I binding to *S. aureus,* a time course using midlogarithmic-phase bacteria incubated in 10% serum was analyzed(figure 1*A*). Maximal factor I binding was observed by 1 min, with no increase in binding observed at subsequent time points. Increased factor I binding to *S. aureus* was observed with increasing serum concentration up to 10% NHS (figure 1*B*). Persistence of factor I binding to *S. aureus* was tested using bacteria incubated in purified factor I, washed, and then incubated in PBS for 16 h. The amount of factor I released into the buffer was minimal, whereas *S. aureus*–associated factor I signal remained strong in the Western blot analysis (figure 1*C*).

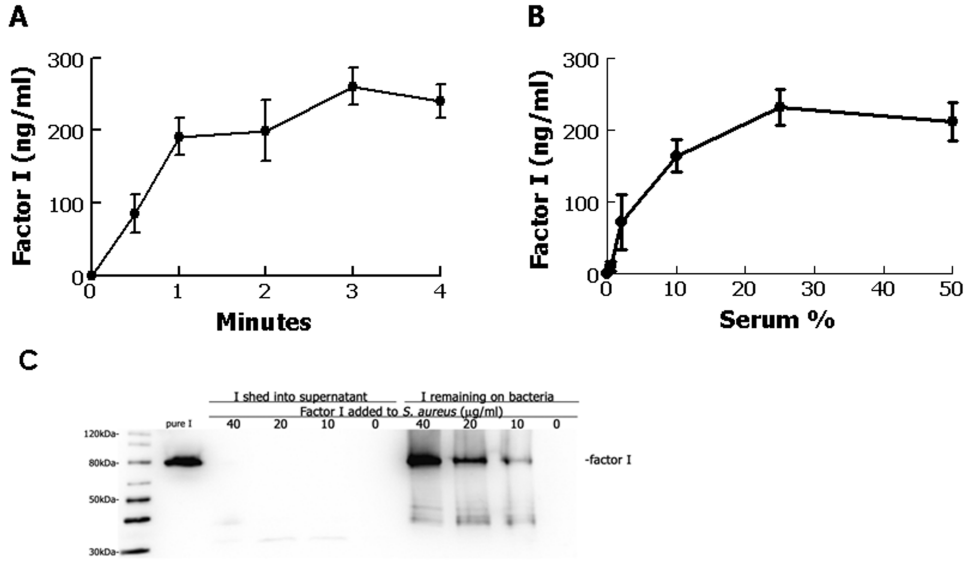

Figure 1. Serum factor I binding to *Staphylococcus aureus* in normal human serum (NHS), as measured by ELISA, for increasing time in 10% NHS *(A)* and for increasing serum concentration *(B)*. Panel C shows a Western blot analysis of *S. aureus*–bound factor I shed into surrounding PBS or remaining on the bacterial surface over 16 h. Data are mean ± SE results of ⩾3 independent experiments.

Identification of factor I binding to a cell wall protein. To identify cell wall components that bind to factor I, *S. aureus* cell wall proteins were prepared using 30% raffinose to stabilize the protoplasts and lysostaphin to digest the cell wall. Cell wall proteins were then fractionated by alternating size-exclusion chromatography on sepharose columns and ion-exchange column chromatography. Fractions were then dot blotted and tested by factor I overlay for factor I binding (figure 2*A*). Fractions dem-

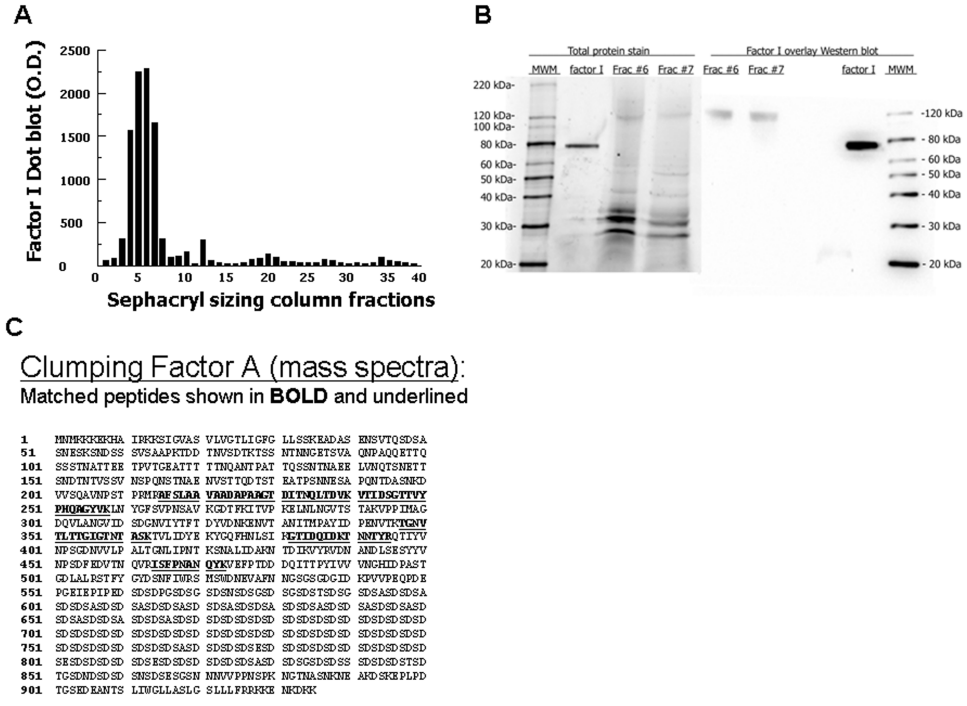

Figure 2. *A, Staphylococcus aureus* cell wall protein preparation fractionated by size-exclusion chromatography, with serum factor I binding detection by dot blot analysis and quantitation by optical densitometry. *B,* Fractions with maximal binding to factor I, as analyzed by SDS-PAGE, and serum factor I overlay Western blot identifying a band of ~130 kDa. Sizes were estimated using molecular weight markers (MWMs). *C,* Map of clumping factor A peptides, identified by mass spectrometry of the ~130-kDa band.

Table 1. Mass spectrometry of ~130-kDa factor I–binding protein identifying clumping factor A peptides.

Expected M_r	Calculated M_r	Peptide score	Expectation score	Peptide sequence
1180.68	1180.59	30	0.019	R.ISFPNANQYK.V
1635.73	1634.84	44	0.0086	K.TGNVTLTTGIGTNTASK.T
888.50	888.50	44	0.01	K.GTIDQIDK.T
1638.60	1637.80	47	0.004	K.GTIDQIDKTNNTYR.Q
1935.34	1934.97	44	0.0083	K.VTIDSGTTVYPHQAGYVK.L
2530.70	2530.30	137	5×10^{-12}	R.AFSLAAVAADAPAAGTDITNQLTDVK.V

onstrating high factor I binding were separated by SDS-PAGE, and a band of ~130 kDa was identified that bound to factor I by overlay Western blot (figure 2*B*). The band was cut from the gel and digested for MS analysis. Two independent cell wall protein preparations led to MS identification of the ~130-kDa band as ClfA (figure 2*C* and table 1).

Factor I interaction with ClfA. To test the binding of ClfA and factor I, a solubilized cell wall preparation was separated by SDS-PAGE and blotted onto a membrane. The band at ~130 kDa bound to serum factor I by overlay Western blot analysis and was detected with anti-ClfA antibody (figure 3*A*). A modified ELISA was then performed using rClfA (peptides 40–559) bound to the bottom of the ELISA plate to capture serum factor I, with detection by use of anti–factor I monoclonal antibody (figure 3*B*). A dose response was noted for increasing amounts of rClfA and increasing amounts of factor I. Half-maximal binding for factor I was observed at 50 nmol/L, where rClfA was coated at 10 μg/mL. NHS was then analyzed by rClfA overlay Western

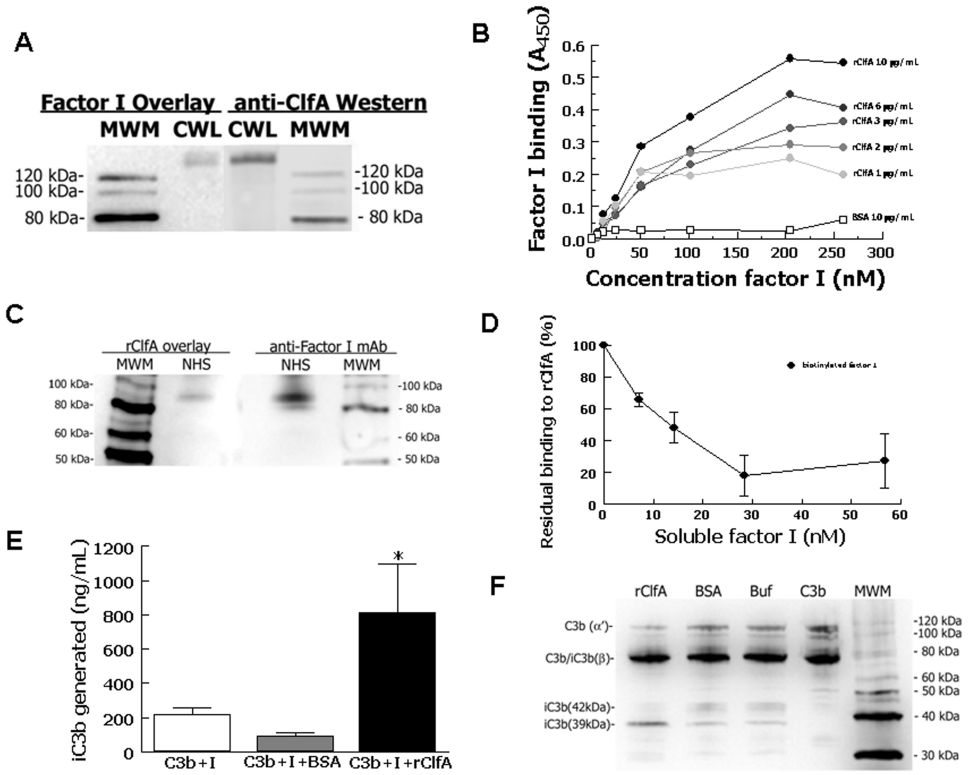

Figure 3. *A,* Serum factor I overlay Western blot (anti–factor I monoclonal antibody) of *Staphylococcus aureus* cell wall protein preparation (cell wall lysate [CWL]) showing binding to ~130-kDa ClfA. Sizes were estimated using molecular weight markers (MWMs). *B,* Serum factor I binding to recombinant clumping factor A (rClfA), as determined by a modified ELISA using rClfA to capture factor I and anti–factor I monoclonal antibody for detection. *C,* rClfA overlay Western blot of normal human serum (NHS) showing binding to ~88-kDa factor I. *D,* Residual binding of biotinylated factor I to rClfA-coated microtiter plates in the presence of increasing amounts of unlabeled purified factor I, demonstrating competitive inhibition. *E,* rClfA (3 μg) incubated with purified C3b and purified factor I, showing increased generation of inactive C3b (iC3b), as measured by ELISA. Data are mean ± SE results of 4 independent experiments. *F,* Results from microtiter plates coated with rClfA and incubated with purified C3b and purified factor I (ratio, 40:1). The supernatants, analyzed for C3 fragments by Western blotting, showed decreased C3b (104 kDa; α'-chain) and increased iC3b (39 kDa; α'_2-chain), compared with control values. BSA, bovine serum albumin; buf, buffer.

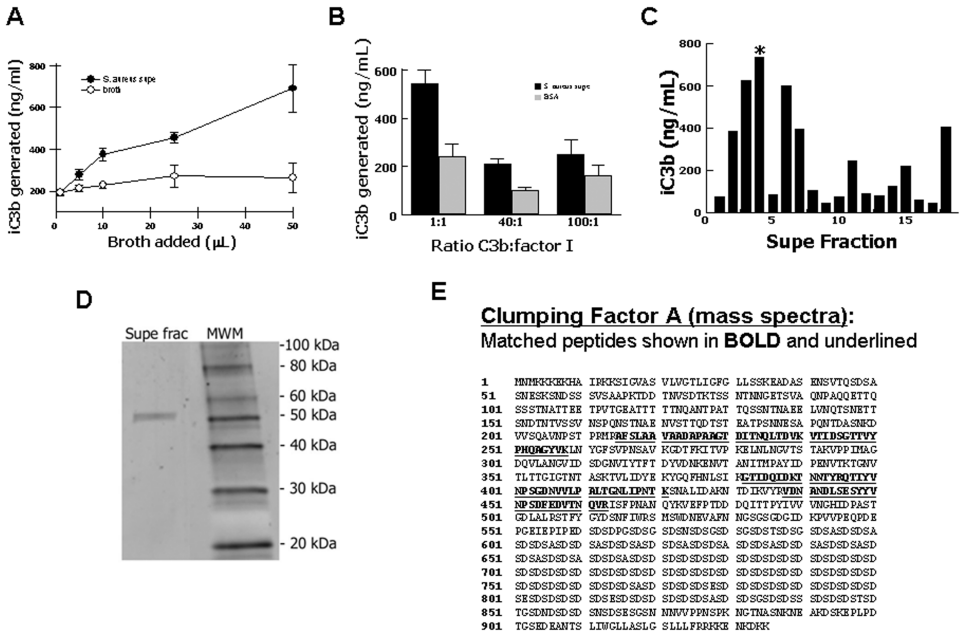

Figure 4. *A, Staphylococcus aureus* growth medium supernatant incubated with purified C3b and purified factor I, with generated inactive C3b (iC3b) measured by ELISA. *B,* Generation of iC3b in the presence of growth medium supernatant, tested for different ratios of C3b to factor I while holding the amount of C3b constant. Data are mean ± SE results of 3 or 4 independent experiments. *C, S. aureus* growth medium supernatant fractionated by ion-exchange column chromatography and then incubated with purified C3b and factor I, with generated iC3b measured by ELISA. *D,* SDS-PAGE analysis of fraction 4 (asterisk in panel C) showing maximal cofactor activity for factor I. *E,* Map of ClfA peptides, identified by mass spectrometry of the band. BSA, bovine serum albumin.

blotting (figure 3*C*). rClfA bound to an ~88-kDa band that was consistent with factor I by Western blot analysis. Binding of soluble biotinylated purified factor I to rClfA-coated microtiter plates was inhibited by increasing amounts of soluble unlabeled purified factor I (figure 3*D*).

Given that ClfA appeared to bind to factor I, it was of interest to determine whether ClfA could act as a "cofactor" for factor I–mediated cleavage of C3b into iC3b, a phenomenon noted to occur on the surface of *S. aureus* in the absence of a known cofactor for factor I [15]. rClfA (3 μg) was incubated with purified C3b and purified factor I, and the increased iC3b was measured by ELISA ($P < .05$) and compared with control values (figure 3*E*). C3b incubated with rClfA in the absence of factor I generated no measurable iC3b. rClfA was used to coat microtiter plates and incubated with purified C3b and purified factor I (ratio, 40:1). C3 fragments were analyzed by Western blot (figure 3*F*). Optical densitometry measurements, normalized for the β-chain, showed that rClfA plus factor I decreased C3b (α'-chain) by 60% and increased iC3b (α'_2-chain) by 287%, compared with control values. These findings suggest that human factor I binds to the *S. aureus* surface protein ClfA and can increase factor I–mediated cleavage of C3b into iC3b.

Identification of a shed protein with cofactor activity for factor I. To test whether shed or secreted staphylococcal components could act as a cofactor for factor I, *S. aureus* growth medium supernatants were prepared from overnight growth in liquid medium to recover secreted and shed proteins. The growth medium supernatant was incubated with purified factor I and purified C3b and was found to increase factor I cleavage of C3b into iC3b, as detected by ELISA (figure 4*A*). A dose response was noted for increasing amounts of supernatant used. Staphylococcal growth medium incubated with purified C3b without factor I did not result in iC3b generation (data not shown). Generation of iC3b was also tested for growth medium supernatants at different ratios of C3b to factor I, including 40:1, the ratio in serum (figure 4*C*). Growth medium supernatants were then fractionated by alternating size-exclusion column chromatography and ion-exchange column chromatography. Fractions were incubated with purified C3b and purified factor I to detect iC3b generation or cofactor activity for factor I (figure 4*B*). Fractions with cofactor activity were consistently found to contain a 50–55-kDa band that could be highly purified, as determined by SDS-PAGE with total protein staining (figure 4*D*). From this fraction, peptides from the A region of ClfA were identified by MS (figure 4*E* and table 2). This suggests that an ~50-kDa fragment containing part of the A region of ClfA is shed from the *S. aureus* surface into the surrounding environment.

Interaction of sClfA with factor I. To elucidate the interaction of ClfA and factor I, *S. aureus* growth medium supernatant was analyzed by serum factor I overlay Western blot and detected with an anti–factor I monoclonal antibody. Factor I was noted to bind to an ~50-kDa band in the growth medium su-

Table 2. Mass spectrometry of shed ~50-kDa protein identifying clumping factor A peptides.

Expected M_r	Calculated M_r	Peptide score	Expectation score	Peptide sequence
888.49	888.45	44	0.01	K.GTIDQIDK.T
1638.59	1637.80	47	0.004	K.GTIDQIDKTNNTYR.Q
1935.34	1934.97	44	0.0083	K.VTIDSGTTVYPHQAGYVK.L
2530.70	2530.29	135	5×10^{-12}	R.AFSLAAVAADAPAAGTDITNQLTDVK.V
2738.46	2737.46	33	0.083	R.QTIYVNPSGDNVVLPALTGNLIPNTK.S
2989.90	2989.32	36	0.038	R.VDNANDLSESYYVNPSDFEDVTNQVR.I

pernatant, and anti-ClfA antibody also detected an ~50-kDa band (figure 5*A*). NHS and purified factor I were separated by SDS-PAGE, blotted to a membrane, and probed with staphylococcal growth medium supernatant and then anti-ClfA antibody (figure 5*B*). An ~88-kDa band was detected for both conditions, consistent with factor I. These findings suggest that *S. aureus* sheds a fragment of ClfA (sClfA) that binds to factor I.

sClfA purified by column chromatography of growth medium supernatants was incubated with purified C3b and purified factor I, resulting in C3b cleavage into iC3b, as determined by ELISA (figure 5*C*). sClfA (2 μg) increased factor I–mediated cleavage of C3b into iC3b 9-fold, compared with control values ($P < .01$). The sClfA cofactor activity for factor I increased in a dose-dependent manner (data not shown). sClfA alone did not cleave C3b (data not shown). These samples were then subjected to Western blot analysis to identify all C3 fragments present (figure 5*D*). The 42-kDa fragment of iC3b was clearly increased for sClfA, compared with control values. These samples were also analyzed by SDS-PAGE to quantify cleavage of the α'-chain of C3b (figure 5*E*). Optical densitometric measurements of the α'-chain, normalized for the β-chain, showed a 38% decrease in C3b for sClfA, compared with control values. These findings

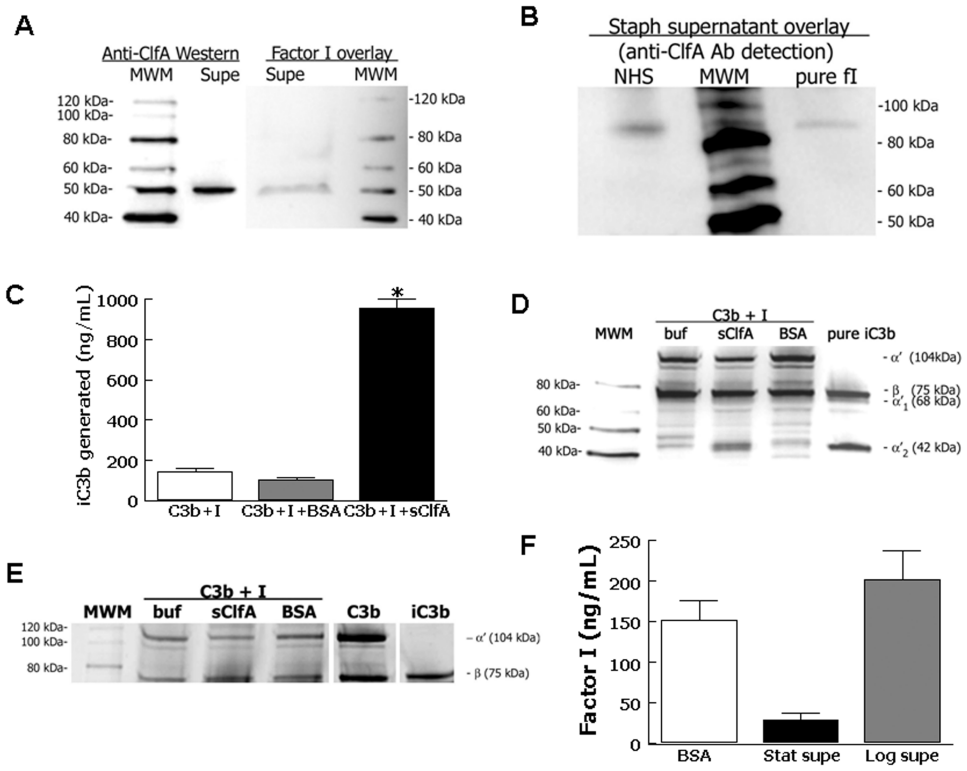

Figure 5. *A,* Serum factor I overlay Western blot of *Staphylococcus aureus* growth medium supernatant showing binding to an ~50-kDa shed clumping factor A fragment (sClfA). *B,* Normal human serum (NHS) and purified factor I analyzed by overlay Western blot of *S. aureus* growth medium supernatant, with detection by use of anti-ClfA antibody. *C–E,* sClfA (2 μg) incubated with purified C3b and purified factor I. sClfA showed cofactor activity, increasing the generation of inactive C3b (iC3b), as measured by ELISA *(C)* and as shown by Western blot analysis *(D)* and total protein–stained SDS-PAGE of C3 fragments *(E). F,* Inhibition of factor I binding to the bacterial surface by *S. aureus* growth medium supernatant. Data are mean ± SE results of ⩾3 independent experiments. BSA, bovine serum albumin; buf, buffer; Log sup, logarithmic-phase supernatant; MWM, molecular weight marker; Stat supe, stationary-phase supernatant.

suggest that an ~50-kDa fragment of ClfA is shed from *S. aureus,* binds to factor I, and increases factor I–mediated cleavage of C3b into iC3b. Factor I binding to the *S. aureus* surface was also tested in the presence of overnight growth medium supernatant containing sClfA, which showed diminished factor I binding ($P < .03$) to the bacterium (figure 5*F*).

DISCUSSION

ClfA is a 933-aa, cell wall, surface-expressed protein that is composed of a distal A region (peptides 40–559) and a proximal SD–repeat R region, relative to the cell wall [27]. The A region binds to fibrinogen and contributes to the ability of *S. aureus* to clump when incubated in serum [28]. Interestingly, ClfA has been shown to contribute to platelet aggregation by a fibrinogen-mediated as well as a complement-mediated mechanism [29]. Also of interest is the observation that ClfA has been found to have an antiphagocytic effect, only part of which can be attributed to interaction with fibrinogen [30].

We believe that this is the first description of a fragment of ClfA being shed from *S. aureus*. The full-length ClfA is attached to the cell wall by the LPXTG motif and is ~130 kDa by SDS-PAGE [27]. The fragment of sClfA present in staphylococcal growth medium supernatant appears to be ~50 kDa. The peptide sequences identified by MS are all present in the A region. sClfA appears to bind to factor I, similar to both the full-length ClfA and rClfA (peptides 40–559). rClfA contains the entire A region of ClfA (~80 kDa), thus encompassing much, if not all, of the sClfA fragment. These findings suggest that factor I binds in the A region of the molecule and that this binding domain is also present in the sClfA fragment.

We believe that this is the first description of a bacterial protein that binds to factor I and has cofactor activity for factor I, enhancing cleavage of C3b into iC3b. Pox and herpes family viruses express viral cofactors for factor I, suggesting that enhancing factor I activity is important in the pathogenesis of the disease produced by these viruses in humans [31–34]. We have shown elsewhere that factor I–mediated cleavage of C3b into iC3b on the surface of *S. aureus* results in diminished efficiency of phagocytosis [18], Thus, it is plausible that the ability of ClfA to localize factor I to the *S. aureus* surface and increase factor I–mediated cleavage of C3b into iC3b contributes to immune evasion and pathogenesis.

S. aureus appears to employ multiple strategies that modify complement activities, such as that of capsule, which has anti-opsonophagocytic activities for solid medium–grown organisms [11, 13]. Chemotaxis inhibitory protein of *S. aureus* (CHIPS), a secreted protein, inhibits C5a-mediated chemotaxis by phagocytes [35]. Extracellular fibrinogen-binding (Efb) protein is a secreted protein reported to inhibit C3 activation [36, 37]. Staphylococcal complement inhibitor (SCIN) is a secreted *S. aureus* protein that adversely affects C3 convertases [38, 39]. The interaction between ClfA and factor I appears to be a new mechanism whereby *S. aureus* modifies complement activity.

References

1. National Nosocomial Infections Surveillance (NNIS) System report, data summary from January 1992 through June 2004, issued October 2004. Am J Infect Control **2004**; 32:470–85.
2. Chesney JP. *Staphylococcus aureus*. In: Long SS, Pickering LK, Prober CG, eds. Principals and practice of pediatric infectious diseases. New York: Churchill Livingstone, **2003**: 694–707.
3. Diekema DJ, Pfaller MA, Schmitz FJ, et al. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin Infect Dis **2001**; 32(Suppl 2):S114–32.
4. Mylotte JM, McDermott C, Spooner JA. Prospective study of 114 consecutive episodes of *Staphylococcus aureus* bacteremia. Rev Infect Dis **1987**; 9:891–907.
5. Noskin GA, Rubin RJ, Schentag JJ, et al. The burden of *Staphylococcus aureus* infections on hospitals in the United States: an analysis of the 2000 and 2001 Nationwide Inpatient Sample database. Arch Intern Med **2005**; 165:1756–61.
6. Fridkin SK, Hageman JC, Morrison M, et al. Methicillin-resistant *Staphylococcus aureus* disease in three communities. N Engl J Med **2005**; 352: 1436–44.
7. Goetz A, Posey K, Fleming J, et al. Methicillin-resistant *Staphylococcus aureus* in the community: a hospital-based study. Infect Control Hosp Epidemiol **1999**; 20:689–91.
8. Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. J Clin Invest **2003**; 111:1265–73.
9. Reis ES, Falcao DA, Isaac L. Clinical aspects and molecular basis of primary deficiencies of complement component C3 and its regulatory proteins factor I and factor H. Scand J Immunol **2006**; 63:155–68.
10. Takahashi K, Shi L, Gowda LD, Ezekowitz RA. Relative roles of complement factor 3 and mannose-binding lectin in host defense against infection. Infect Immun **2005**; 73:8188–93.
11. Cunnion KM, Lee JC, Frank MM. Capsule production and growth phase influence binding of complement to *Staphylococcus aureus.* Infect Immun **2001**; 69:6796–803.
12. Gordon DL, Rice J, Finlay-Jones JJ, McDonald PJ, Hostetter MK. Analysis of C3 deposition and degradation on bacterial surfaces after opsonization. J Infect Dis **1988**; 157:697–704.
13. Cunnion KM, Zhang HM, Frank MM. Availability of complement bound to *Staphylococcus aureus* to interact with membrane complement receptors influences efficiency of phagocytosis. Infect Immun **2003**; 71: 656–62.
14. Cunnion KM, Benjamin DK Jr, Hester CG, Frank MM. Role of complement receptors 1 and 2 (CD35 and CD21), C3, C4, and C5 in survival by mice of *Staphylococcus aureus* bacteremia. J Lab Clin Med **2004**; 143: 358–65.
15. Cunnion KM, Hair PS, Buescher ES. Cleavage of complement C3b to iC3b on the surface of *Staphylococcus aureus* is mediated by serum complement factor I. Infect Immun **2004**; 72:2858–63.
16. Lambris JD, Sahu A, Wetsel RA. The chemistry and biology of C3, C4, and C5. In: Volanakis JE, Frank MM, eds. The human complement system in health and disease. New York: Marcel Dekker, **1998**:83–118.
17. Ross GD, Polley MJ, Rabellino EM, Grey HM. Two different complement receptors on human lymphocytes: one specific for C3b and one specific for C3b inactivator-cleaved C3b. J Exp Med **1973**; 138:798–811.
18. Cunnion KM, Buescher ES, Hair PS. Serum complement factor I decreases *Staphylococcus aureus* phagocytosis. J Lab Clin Med **2005**; 146: 279–86.
19. Kraiczy P, Wurzner R. Complement escape of human pathogenic bac-

teria by acquisition of complement regulators. Mol Immunol **2006**; 43: 31–44.
20. Horstmann RD, Sievertsen HJ, Knobloch J, Fischetti VA. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. Proc Natl Acad Sci USA **1988**; 85:1657–61.
21. Janulczyk R, Iannelli F, Sjoholm AG, Pozzi G, Bjorck L. Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function. J Biol Chem **2000**; 275:37257–63.
22. Neeleman C, Geelen SP, Aerts PC, et al. Resistance to both complement activation and phagocytosis in type 3 pneumococci is mediated by the binding of complement regulatory protein factor H. Infect Immun **1999**; 67:4517–24.
23. Pandiripally V, Gregory E, Cue D. Acquisition of regulators of complement activation by *Streptococcus pyogenes* serotype M1. Infect Immun **2002**; 70:6206–14.
24. McCrea KW, Hartford O, Davis S, et al. The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology **2000**; 146:1535–46.
25. O'Connell DP, Nanavaty T, McDevitt D, Gurusiddappa S, Hook M, Foster TJ. The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca2+-dependent inhibitory site. J Biol Chem **1998**; 273:6821–9.
26. O'Seaghdha M, van Schooten CJ, Kerrigan SW, et al. *Staphylococcus aureus* protein A binding to von Willebrand factor A1 domain is mediated by conserved IgG binding regions. FEBS J **2006**; 273:4831–41.
27. McDevitt D, Frančois P, Vaudaux P, Foster TJ. Identification of the ligand-binding domain of the surface-located fibrinogen receptor (clumping factor) of *Staphylococcus aureus*. Mol Microbiol **1995**; 16:895–907.
28. McDevitt D, Francois P, Vaudaux P, Foster TJ. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol Microbiol **1994**; 11:237–48.
29. Loughman A, Fitzgerald JR, Brennan MP, et al. Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by *Staphylococcus aureus* clumping factor A. Mol Microbiol **2005**; 57:804–18.
30. Higgins J, Loughman A, van Kessel KP, van Strijp JA, Foster TJ. Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes. FEMS Microbiol Lett **2006**; 258:290–6.
31. Rosengard AM, Liu Y, Nie Z, Jimenez R. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. Proc Natl Acad Sci USA **2002**; 99:8808–13.
32. Liszewski MK, Leung MK, Hauhart R, et al. Structure and regulatory profile of the monkeypox inhibitor of complement: comparison to homologs in vaccinia and variola and evidence for dimer formation. J Immunol **2006**; 176:3725–34.
33. Sahu A, Isaacs SN, Soulika AM, Lambris JD. Interaction of vaccinia virus complement control protein with human complement proteins: factor I–mediated degradation of C3b to iC3b1 inactivates the alternative complement pathway. J Immunol **1998**; 160:5596–604.
34. Mark L, Spiller OB, Villoutreix BO, Blom AM. Kaposi's sarcoma-associated herpes virus complement control protein: KCP—complement inhibition and more. Mol Immunol **2007**; 44:11–22.
35. de Haas CJ, Veldkamp KE, Peschel A, et al. Chemotaxis inhibitory protein of *Staphylococcus aureus,* a bacterial antiinflammatory agent. J Exp Med **2004**; 199:687–95.
36. Lee LY, Hook M, Haviland D, et al. Inhibition of complement activation by a secreted *Staphylococcus aureus* protein. J Infect Dis **2004**; 190:571–9.
37. Lee LY, Liang X, Hook M, Brown EL. Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb). J Biol Chem **2004**; 279:50710–6.
38. Rooijakkers SH, Ruyken M, Roos A, et al. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol **2005**; 6:920–7.
39. Rooijakkers SH, Ruyken M, van Roon J, van Kessel KP, van Strijp JA, van Wamel WJ. Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus*. Cell Microbiol **2006**; 8:1282–93.