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*J Immunol* 2014; 193:6161-6171; Prepublished online 7 November 2014; doi: 10.4049/jimmunol.1401600

http://www.jimmunol.org/content/193/12/6161
The Extracellular Adherence Protein from *Staphylococcus aureus* Inhibits the Classical and Lectin Pathways of Complement by Blocking Formation of the C3 Proconvertase

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The pathogenic bacterium *Staphylococcus aureus* actively evades many aspects of human innate immunity by expressing a series of small inhibitory proteins. A number of these proteins inhibit the complement system, which labels bacteria for phagocytosis and generates inflammatory chemotaxants. Although the majority of staphylococcal complement inhibitors act on the alternative pathway to block the amplification loop, only a few proteins act on the initial recognition cascades that constitute the classical pathway (CP) and lectin pathway (LP). We screened a collection of recombinant, secreted staphylococcal proteins to determine whether *S. aureus* produces other molecules that inhibit the CP and/or LP. Using this approach, we identified the extracellular adherence protein (Eap) as a potent, specific inhibitor of both the CP and LP. We found that Eap blocked CP/LP-dependent immune evasion. The Journal of Immunology, 2014, 193: 6161–6171.

The complement system serves as a critical hub in the human innate immune and inflammatory system, and fulfills numerous roles in homeostasis, defense, repair, and disease (1). Despite its diverse list of functions, complement remains best known for its ability to opsonize and eliminate invading microorganisms. To achieve this most efficiently, the microbial surface must first be recognized by one of a series of pattern–recognition proteins (1). These ligand-bound sensors can then trigger one of three canonical activation routes (the classical pathways [CP], lectin pathways [LP], and alternative pathways [AP]), which all result in cleavage of the abundant plasma protein C3 into its bioactive C3a (chemoattractant) and C3b (covalent opsonin) fragments. Although such activation of C3 may occur at a low level spontaneously, this central process is catalyzed at the bacterial surface through the function of two transiently stable, multisubunit proteolytic complexes known as C3 convertases. In the case of the CP or LP, the initiating complexes of surface Ig-bound C1 or carbohydrate-bound MBL/MASPs trigger proteolytic activation of C4 to produce C4b. Surface-bound C4b then binds C2 to form the CP/LP C3 proconvertase, which, when proteolytically activated by the same initiation complexes named above, gives rise to the fully active CP/LP C3 convertase, C4b2a. C4b2a converts native C3 into C3b, and ultimately gives rise to the AP C3 convertase (C3bBb). In this scenario, surface-deposited C3b, along with the proenzyme factor B and factor D, cooperate to generate the C3bBb complex. It is this surface-bound AP C3 convertase that activates massive amounts of C3 into C3b, thereby being responsible for the self-amplifying nature of the complement response, and that stimulates efficient opsonization of bacteria and production of powerful inflammatory mediators like C3a and C5a (2). Furthermore, deposited C3b molecules also activate the terminal pathway of complement that results in the formation of the membrane attack complex (C5b-9) that can directly kill Gram-negative bacteria.

The pathogenic bacterium *Staphylococcus aureus* has evolved a diverse and multifaceted approach to successfully evade the human innate immune response (3–5). Central to this global strategy is its ability to manipulate the human complement system to a greater extent than perhaps any other pathogen studied to date.
(3, 4, 6). Whereas studies from the last decade have revealed much on the diverse nature of S. aureus complement evasion, the large number of C3 convertase inhibitors that act on the AP suggests that a conceptually similar mechanism(s) that affects the CP and/or LP might be manifested by a component of the S. aureus immune evasion arsenal. In this regard, the fact that CP and LP share the same C3 convertase, C4b2a, raises the intriguing possibility that a single inhibitor might effectively block C3b deposition and downstream anaphylatoxin production via both of these pathways simultaneously. Although staphylococcal complement inhibitor (SCIN) proteins have been reported to inhibit the CP and LP at the level of C3b deposition, their activities against these pathways are only partial and are substantially weaker than they are against the AP (7, 8). Thus, we hypothesized that S. aureus might express and secrete an as yet unidentified inhibitor of CP and LP C3 convertase formation and/or activity.

To this end, we screened a collection of recombinant secreted S. aureus proteins to examine whether any of these molecules had inhibitory activities on the CP/LP. In so doing, we identified the staphylococcal extracellular adherence protein (Eap) as a potent, specific inhibitor of both the CP and LP. We found that Eap, but not its structural homologs EapH1 and EapH2 (9), inhibits the CP/LP in a dose-dependent manner by forming a nanomolar affinity complex with C4b. This C4b/Eap complex inhibits binding of C2 to C4b, and therefore impedes formation of the CP/LP C3 convertase. From a broader perspective, the studies we present in this work suggest that the effects of Eap on the CP/LP in many respects mirror those of the staphylococcal complement inhibitor Efb-C, which inhibits AP C3 proconvertase formation by binding C3b (10). In sum, this work provides new insight into staphylococcal immune evasion, and also describes an entirely novel mechanism of CP/LP regulation that may hold significant implications for future design of therapeutic CP/LP inhibitors.

Materials and Methods
Preparation of native and recombinant proteins
Human serum proteins C3, C3b, C4, C4b, C1s, C4b-binding protein (C4BP), and factor I (F) were obtained in purified form from Complement Technologies (Tyler, TX). Recombinant forms of C2 and C2b were expressed and purified from the conditioned culture medium of transiently transfected human embryonic kidney-293 cells according to the general methods described previously (11). All recombinant S. aureus proteins were overexpressed and purified according to the general methods described previously (12), with the exception that recombinant, full-length Eap was prepared according to the published protocol of Xie et al. (13).

Human-derived materials
Blood was drawn from healthy adult volunteers after obtaining informed consent and approval of the protocol by the medical-ethical committee of the University Medical Center Utrecht. Normal human serum (NHS) was isolated, as described before (14), and frozen at –80°C until needed for further use. For neutrophil preparation, heparinized vacutainers (BD Biosciences) were used, and neutrophils were isolated over a Ficoll/Hypaque gradient, as described previously (15).

Complement pathway activity on an artificial surface
Functional activity of the CP, LP, and AP was determined essentially as described (16). In short, Nunc-Maxisorb ELISA plates were coated overnight to specifically activate the CP (human IgM [Calbiochem]), LP (coated with 20 μg/ml Saccharomyces cerevisiae mannan [Sigma-Aldrich]), or AP (coated with 20 μg/ml Salmonella enteritidis LPS [Sigma-Aldrich]). Plates were blocked with 1% (w/v) BSA in PBS with 0.05% (v/v) Tween 20 (Merck). The indicated percentages of NHS or mouse serum (Innovative Research) were incubated with 1 μM recombinant S. aureus proteins in the appropriate assay buffers for a maximum of 5 min at 25°C (veronal-buffered saline at pH 7.5 with 0.1% [w/v] gelatin, 500 μM CaCl2, and 250 μM MgCl2 for CP and LP; veronal-buffered saline at pH 7.5 with 0.1% [w/v] gelatin, 5 mM MgCl2, and 10 mM EGTA for AP). Deposited C3b, C4b, and C5b-9 were detected with specific Abs (0.1 μg/ml α-human Cα γ-WM 1 clone digoxigenin labeled or 1 μg/ml rat α-mouse Cα mouse (Hycult; HM1078). American Type Culture Collection; 1 μg/ml αC4d, Quidel; 1 μg/ml αC5b-9 eB; Santa Cruz Biotechnology, respectively). Second, HRP-labeled Abs were detected with 100 μg/ml tetramethylbenzidine and 60 μg/ml uraeum peroxide in 100 mM sodium acetate buffer at pH 6.0. The reaction was stopped by adding an equal volume of 2 M H2SO4, and the absorbance at 450 nm was measured using a Bio-Rad microplate reader.

Complement deposition on S. aureus
S. aureus Newmann wild-type (WT) or Δeap (MR1811) (17) was grown on a blood-agar plate overnight at 37°C. Bacteria were resuspended in assay buffer (20 mM HEPES [pH 7.4] with 140 mM NaCl, 0.5 mM CaCl2, 0.25 mM MgCl2, and 0.1% [w/v] BSA). Eap (1 μM) was added to the indicated concentrations of NHS, and this mixture was added directly to the bacteria (8 × 106 CFU) in a total volume of 100 μl and incubated at 37°C with shaking for 10 min. Unbound components were washed away with assay buffer, and deposited C3b was quantified by flow cytometry (FACS Verse; BD Biosciences) by using the specific FITC-conjugated goat anti-human C3 (Proto Immunoresearch, Burlingame, CA).

Phagocytosis assays
S. aureus Newmann WT or Δeap transformed with pCM29 (18), a vector inducing constitutive expression of superfolded GFP under the sarA promoter, was grown in Todd-Hewitt broth until an OD600 of 0.5. Bacteria were washed with RPMI 1640 (Invirotong) supplemented with 0.05% (w/v) human IgM (Sigma-Aldrich), algin (Sigma-Aldrich) and stored at –80°C until use. Eap, EapH1, or EapH2 (1 μM) was added to the indicated concentrations of NHS in RPMI 1640/HSA and directly added to the thawed bacteria (2.5 × 106 CFU). Then 2.5 × 105 isolated neutrophils were added to obtain a total volume of 250 μl and incubated at 37°C with shaking at 600 rpm for 15 min. The reaction was stopped by adding 100 μl ice-cold 2% (v/v) paraformaldehyde. Phagocytosis was assessed by flow cytometry (FACS Verse; BD Biosciences). Graphs show the percentage of GFP-positive neutrophils. The fluorescent signal originated exclusively from intracellular bacteria, as verified by confocal microscopy.

Neutrophil-mediated killing
S. aureus Newmann WT or Δeap was grown in Todd-Hewitt broth to OD600 of 0.5 (corresponding to 2 × 106 CFU/ml). Eap was added in 1 μM, and 10% NHS was added for 15 min at 25°C in RPMI 1640/HSA to allow for opsonization. Opsonized bacteria (5 × 106 CFU) were incubated with 9 × 106 neutrophils in 100 μl RPMI 1640/HSA. The reaction was stopped at the indicated time points with 900 μl ice-cold 0.1% saponin (w/v). After 15 min, the samples were resuspended via a 25-gauge needle, to assure lysis of the neutrophils (19). Surviving bacteria were enumerated by plating serial dilutions on Luria broth-agar plates.

Eap affinity isolation of human serum proteins
A recombinant form of Eap that harbored a single, N-terminal cysteine was expressed and purified from Escherichia coli similarly to WT Eap (13). Eap produced in this manner was site-specifically biotinylated using EZ-link maleimide–polyethylene glycol2000–biotin reagent according to manufacturer’s suggestions (ThermoFisher). Following derivatization, 2 μg Eap biotin was added to samples containing either 20 μl EDTA serum or C4-depleted serum (Complement Technologies), and an appropriate quantity of PBS to give a final volume of 100 μl. The samples were incubated for 1 h at room temperature, after which time 30 μl of a 50% (v/v) slurry of magnetic streptavidin-coated Dynabeads was added (Invitrogen). Following an additional 15-min incubation, the beads were isolated via a magnet and washed three times with 100 μl PBS, and all remaining liquid was removed by aspiration. A total of 15 μl nonreducing Laemmli buffer was added to each sample, and 5 μl of each sample was analyzed by SDS-PAGE, followed by staining with Coomassie brilliant blue.

Stoichiometry and m.w. estimations
The apparent m.w. and stoichiometry of the C4b/Eap complex were determined by a combination of size-exclusion chromatography and sedimentation equilibrium analytical ultracentrifugation. For chromatographic analysis, samples consisting of either C4b or C4b/Eap (20 μM final concentration) were prepared, and 100 μl was injected at 0.75 ml/min onto a Superdex S200 Increase 10/30 column that had been previously equili- brated at 1°C in a buffer of PBS. The contents of peak fractions were analyzed by Coomassie-stained SDS-PAGE of samples that had been prepared under nonreducing conditions. For sedimentation equilibrium analysis, all experiments were performed using a Beckman XL-I ultra-
centrifuge with a four-position analytical, titanium rotor. Protein solutions (1.6 μM C4b or C4b/Eap complex in PBS; 110 μl) and dialysate buffer (PBS; 120 μl) were placed in the double-sector centrifuge cells. The samples were equilibrated at 4°C at 6000 rpm, and the approach to equilibrium was monitored by repetitive absorption scans at 280 nm every 6 h. The apparent equilibrium was reached after ~60 h. After the final data collection, the rotor was accelerated to 42,000 rpm for ~2 h and the cells were scanned to obtain the baseline absorption value. Data were analyzed with the software supplied with the instrument (Beckman-Coulter). Both the protein partial specific volume and buffer density were calculated using Sednterp software (http://biowiki.sr.unh.edu/index.php/Main_Page).

**Biotinylation of C4b**

Biotinylated C4b was prepared by overnight, room-temperature incubation of native C4 (1 mg/ml final concentration) with C1s (5 μg/ml final concentration) in the presence of EZ-link maleimide-polyethylene glycol2000-biotin reagent, according to manufacturer’s suggestions (ThermoFisher). PBS-T (PBS [pH 7.0] with 0.005% [v/v] Tween 20) was used as the running buffer throughout the entire set of experiments. C4b biotin was captured on a streptavidin sensor chip (GE Life Sciences) to a density of ~5000 resonance units. Ligands were diluted to their working concentrations in PBS-T. For comparison of C4b binding to C4b, samples were prepared at 1 and 10 μg/ml, and the evolving AlphaScreen signal was measured. AlphaScreen acceptor beads, and 20 μM C4b biotin donor beads. A dilution series was prepared for each unlabeled complement protein of interest. Reactions were performed over 2.5 h and were begun by incubating the C4b biotin, myc-Eap, and a given concentration of each complement protein for 1 h. Following this, the acceptor beads were added, incubated for 1 h, and then the donor beads were added and incubated for an additional 0.5 h. At that point, the donor beads were excited at 680 nm, and the evolving AlphaScreen signal (photons per second) for each data point was measured using an EnSpire multimode plate reader (Perkin Elmer Life Sciences). Data analysis and curve fitting were carried out as previously described (20).

**Surface plasmon resonance experiments**

Interactions between C4b biotin and the S. aureus proteins Eap, EapH1, and EapH2 were measured by surface plasmon resonance (SPR) using either a Biacore X or BiaCore 3000 instrument (GE Life Sciences) at room temperature. PBS-T (PBS [pH 7.0] with 0.005% [v/v] Tween 20) was used as the running buffer throughout the entire set of experiments. C4b biotin was captured on a streptavidin sensor chip (GE Life Sciences) to a density of ~5000 resonance units. Ligands were diluted to their working concentrations in PBS-T. For comparison of EAP-domain proteins’ binding to C4b, samples were prepared at 1 and 10 μg/ml and injected for 1 min at a flow rate of 20 μl/min, followed by a 2 min dissociation phase of 20 μl/min. Injections were performed at concentrations: 50 nM myc-Eap, 5 nM C4b biotin, 20 μg/ml anti-c-myc antibodies, and 20 μg/ml C4b biotin acceptor beads. A dilution series was prepared for each unlabeled complement protein of interest. Reactions were performed over 2.5 h and were begun by incubating the C4b biotin, myc-Eap, and a given concentration of each complement protein for 1 h. Following this, the acceptor beads were added, incubated for 1 h, and then the donor beads were added and incubated for an additional 0.5 h. At that point, the donor beads were excited at 680 nm, and the evolving AlphaScreen signal (photons per second) for each data point was measured using an EnSpire multimode plate reader (Perkin Elmer Life Sciences). Data analysis and curve fitting were carried out as previously described (20).

**AlphaScreen-binding assays**

An AlphaScreen equilibrium competition assay was used to derive both positional information and apparent dissociation constants for C4b binding to various complement components and staphylococcal inhibitors. This assay system is based upon modification of a previously published protocol (20) and is established via the following principle: a luminescence signal is generated by laser excitation of a streptavidin-coated donor bead, which recognizes a C4b biotin that binds directly to a second target protein (in this case myc-Eap), which itself can be adsorbed to an acceptor bead coated with anti-c-myc monoclonal IgG. C4b/Eap competition-binding assays were carried out in a total volume of 25 μl by adding each assay component to the following final concentrations: 50 nM myc-Eap, 5 nM C4b biotin, 20 μg/ml anti-c-myc antibodies, and 20 μg/ml C4b biotin acceptor beads. A dilution series was prepared for each unlabeled complement protein of interest. Reactions were performed over 2.5 h and were begun by incubating the C4b biotin, myc-Eap, and a given concentration of each complement protein for 1 h. Following this, the acceptor beads were added, incubated for 1 h, and then the donor beads were added and incubated for an additional 0.5 h. At that point, the donor beads were excited at 680 nm, and the evolving AlphaScreen signal (photons per second) for each data point was measured using an EnSpire multimode plate reader (Perkin Elmer Life Sciences). Data analysis and curve fitting were carried out as previously described (20).

**Statistics**

All analyses were performed in GraphPad Prism 6.0.

**Results**

Identification of Eap as an inhibitor of the CP and LP of complement

We screened a library of ~30 secreted staphylococcal proteins to test whether any of these molecules were capable of inhibiting the CP and LP on the surface of pathway-specific activator/acceptor-coated ELISA plates (16). In doing so, we discovered that a recombinant form of Eap inhibited both pathways at the level of terminal complement complex (C5b-9) deposition (Fig. 1A). This effect was specific for the CP and LP, because Eap did not block the AP (data not shown). A gene encoding Eap is found in 98% of all clinical isolates of S. aureus (24). Although there is some variability in the molecular mass of Eap protein produced by different strains, characterization of Eap from S. aureus strain Mu50 (~50 kDa) and Newman (~63 kDa) in various assays suggests that these two isoforms retain similar functions (13, 17). Consistent with this, Eap proteins from both S. aureus Mu50 and Newman are equally potent in their ability to inhibit the CP and LP (data not shown). Eap from S. aureus strain Mu50 is comprised of four tandem repeats of an ~100 residue motif known as the EAP domain (9). These repeats share between 40 and 80% identity with one another, and show ~25−50% identity to the structurally related S. aureus proteins, EapH1 and EapH2, which themselves consist of little more than a single EAP domain (9). Although Eap potently inhibited the CP and LP at the level of C5b-9, neither EapH1 nor EapH2 had any significant impact on either pathway. Thus, the inhibitory effect on the CP and LP is specific to Eap, and not a general feature of EAP domain-containing proteins. To determine the specific steps in the CP and LP that are inhibited by Eap, we investigated whether Eap could block C4b or C3b deposition. We found that Eap inhibited C3b deposition by the CP and LP both in human and mouse serum (Fig. 1B, Supplemental Fig. 1A), but Eap failed to block C4b deposition (Fig. 1C). Together, these results indicate that Eap blocks activation of C3, but not C4, via both the CP and LP of complement.

**Eap inhibits deposition of C3b at the S. aureus surface and blocks phagocytosis and killing**

The results described above revealed that Eap specifically inhibits C3 activation via the CP and LP. Nevertheless, one limitation of these experiments is that they employed artificial activator and acceptor surfaces to study the complement response. To test whether Eap could impact an experimental system that more...
closely reflects the situation found in vivo, we examined the effect of Eap on C3b opsonization of the *S. aureus* cell surface. Although Eap is a secreted protein, ∼30% of Eap rebinds the bacterial surface after secretion (25). To address the role of surface-bound Eap in complement inhibition, we analyzed C3b deposition and subsequent phagocytosis for both the *S. aureus* Newman WT strain and an isogenic eap-mutant strain (*Deap*) in parallel (17) (Supplemental Fig. 1B). Neither assay revealed any difference in the level of C3b deposition or phagocytosis between the WT and mutant strain in the absence of exogenous Eap, suggesting that surface-retained Eap does not contribute significantly to *S. aureus* complement evasion (Fig. 2A, 2B). However, exogenously added Eap (1 μM) blocked deposition of C3b on both strains by nearly 50% across four different serum concentrations that ranged from 1 to 8% (v/v) (Fig. 2A). As expected, this diminished level of C3b opsonization in the presence of Eap significantly inhibited the efficiency with which neutrophils phagocytosed both strains (Fig. 2B).

We then used the *Deap* strain to conduct several additional experiments aimed at assessing the significance of Eap’s effects on phagocytosis and its impact on bacterial survival. To begin, we found that inhibition of phagocytosis by increasing concentrations of Eap was both dose dependent and saturable (Supplemental Fig. 1C). Importantly, the concentration of Eap found in stationary liquid cultures of *S. aureus* [up to 10 μg/ml, or ∼200 nM (26)] resulted in >50% inhibition of phagocytosis by human neutrophils. Although the concentration of Eap secreted into the human body during *S. aureus* infections remains uncertain, these data suggest that Eap-dependent inhibition of the CP/LP, and subsequently of phagocytosis, is most likely physiologically relevant. Along these lines, and in concordance with the ELISA data presented above, this antiphagocytic effect was not observed when either control protein EapH1 or EapH2 was added at the same exogenous concentration that resulted in potent inhibition of phagocytosis by Eap (Fig. 2C). Finally, we observed that diminished levels of phagocytosis likewise resulted in significantly diminished killing of *S. aureus* by human neutrophils (Fig. 2D).

Together, these results indicate that secreted Eap specifically blocks CP/LP-mediated opsonization of *S. aureus* with C3b and subsequent phagocytosis of *S. aureus* by neutrophils.

**Eap binds with nanomolar affinity to complement component C4b**

We observed that Eap bound an ∼200-kDa protein present in EDTA-treated human serum, but not in C4-depleted serum (Supplemental Fig. 2A). While this result provided evidence that Eap binds to native C4, the functional studies presented above indicated that Eap inhibits an event within the CP and LP that mediates activation of C3, but leaves C4 activation intact. We...
therefore predicted that Eap must act on either the fully assembled CP/LP C3 convertase (C4b2a) or an isolated component thereof. As a first test of this hypothesis, we examined the behavior of purified C4b and a mixture of C4b and Eap by analytical size-exclusion chromatography (Fig. 3A, left panel). Inclusion of equimolar amounts of Eap in the C4b sample resulted in a pronounced shift of the peak to a larger apparent molecular mass that eluted as a single species. Indeed, bands corresponding to both Eap and C4b were present when the peak fractions were analyzed by Coomassie-stained SDS-PAGE (Fig. 3A, right panel). Due to potential inaccuracy of molecular mass estimates obtained from size-exclusion chromatography, we also used analytical ultracentrifugation to provide further characterization of the C4b/Eap complex. Sedimentation equilibrium data for both C4b/Eap and C4b alone were obtained at one concentration and were well described by a single particle model (Supplemental Fig. 2C). Whereas the molecular mass for C4b itself was estimated at 268 kDa, the apparent molecular mass of C4b/Eap was estimated at 308 kDa. Because previous sedimentation equilibrium studies of Eap yielded an apparent molecular mass of 51 kDa for this protein (27), these data strongly suggest that Eap forms a 1:1 complex with C4b.

FIGURE 2. Eap inhibits opsonization, phagocytosis, and killing of *S. aureus*. The impact of recombinant Eap on complement deposition and phagocytosis of *S. aureus* Newman strains was assessed using flow cytometry. (A) C3b deposition on the surface of *S. aureus* Newman WT or Δeap in the presence of 1 μM Eap or a buffer control. Legend is inset. (B) Phagocytosis of *S. aureus* Newman WT or Δeap in the presence of 1 μM Eap or a buffer control. Legend is inset in the adjacent panel. (C) Extent of phagocytosis of *S. aureus* Newman Δeap using 1% (v/v) NHS in the presence of 1 μM Eap, EapH1, or EapH2, or a buffer control. (D) Neutrophil-mediated killing of *S. aureus* Newman Δeap opsonized in the presence of 1 μM Eap or a buffer control. Error bars represent the mean ± SD of three independent experiments and at least two different donors. Legend is inset. Measures of statistical significance were determined by an unpaired t test of each experimental series versus the corresponding buffer control for each strain and serum concentration, as appropriate. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ns, not significant.

We next used a bead-based AlphaScreen assay to explore both the affinity and specificity of the C4b/Eap interaction in greater detail (20, 28). Whereas untagged Eap itself could diminish the luminescence signal generated by interaction between myc-tagged Eap and C4b biotin in a dose-dependent manner, neither EapH1 nor EapH2 had any competitive effect even at the highest concentrations tested (Fig. 3B). Nonlinear curve fitting of the C4b/Eap competition data revealed an apparent $K_d$ of 185 ± 14 nM for this complex. Saturable binding of similar affinity was also observed when either native C4 or C4c was used as the competitor, and fit to apparent $K_d$ values of 45 ± 2 and 138 ± 16 nM, respectively (Supplemental Fig. 2B). To study the C4b/Eap interaction through an independent approach, we constructed a SPR biosensor wherein C4b biotin was uniformly immobilized on a streptavidin-coated surface similarly to what we have previously reported for C3b biotin (10, 20, 21, 28). Significantly, neither EapH1 nor EapH2 bound the C4b surface even at concentrations 10-fold higher than those that showed clear evidence of C4b binding by Eap (Fig. 3C). Thus, the ability of Eap to bind C4b and the lack of C4b binding by EapH1 and EapH2 are in agreement with the inhibition of CP/LP function by Eap and the lack thereof by its homologs.
Eap binding to C4b and subsequent inhibition of the CP/LP require the third domain of Eap

The modular architecture of the Eap protein raised questions as to whether a discrete combination of these domains is responsible for binding to C4b and, furthermore, whether that domain drives inhibition of CP/LP activity. To address these issues simultaneously, we overexpressed and purified a series of Eap fragments consisting of adjacent pairs of domains (i.e., Eap12, Eap23, and Eap34) as well as the individual Eap repeats themselves (i.e., Eap1, Eap2, Eap3, and Eap4) (Fig. 4A). Like EapH1 and EapH2, an equimolar mixture of the individual Eap repeats did not compete the luminescence signal generated by myc-Eap binding to C4b biotin at concentrations up to 10⁻⁸ M (Fig. 4B).

Similarly, competition by Eap12 was detected only at the highest concentrations examined. By contrast, saturable binding of nearly equivalent affinity to Eap was observed in the same assay system for both Eap23 (Kₐ = 2.93 ± 0.38 nM) and Eap34 (Kₐ = 525 ± 65 nM) (Fig. 4B). Consistent with this, Eap23 and Eap34 both inhibited C5b-9 deposition via the CP and LP, although Eap34 did so at levels closer to Eap in both assays (Fig. 4C). In summary, the facts that 1) none of the individual Eap domains manifested clear C4b binding or inhibitory properties against either the CP or LP, 2) Eap12 bound C4b nearly 100-fold more weakly than Eap itself and failed to inhibit both the CP and LP on its own, and 3) Eap23 and Eap34 both bind C4b and inhibit the CP and LP indicate that domain Eap3 is necessary, but not sufficient for Eap binding to C4b and inhibition of the CP and LP.

Eap blocks binding of C2 to C4b by interfering with the initial C4b2 interaction

Formation of the CP/LP C3 convertase is a stepwise process that starts with the deposition of surface-bound C4b. Although C4b has no enzymatic activity of its own, it serves as a molecular platform first for binding of C2 to yield the C4b2 proconvertase and then for C1s/MASP-dependent cleavage of C2 to generate a fully active C4b2a convertase (1). We examined whether Eap binding to C4b would inhibit binding of C2 to C4b, and thus disrupt formation of the C4b2 proconvertase. Indeed, C2 efficiently diminished the luminescence signal in the AlphaScreen assay generated by myc-Eap and C4b biotin with an apparent IC₅₀ of 460 ± 32 nM (Fig. 5A).

The C2 proprotease is comprised of two functionally discrete regions. Whereas the larger C2a region houses its serine protease module, the smaller C2b fragment provides the molecular basis for its initial interaction with C4b (29, 30). We therefore tested whether Eap inhibition of C2 binding to C4b might arise from disrupting the C4b2b interaction using the same AlphaScreen assay system described above. Although C2b bound to C4b with ~7.6-fold lower affinity than full-length C2 (IC₅₀ = 3.5 ± 0.6 μM), it still effectively competed with Eap for C4b binding (Fig. 5A). Together, these data show that Eap shares a common binding site on C4b with C2 and, further, that this C4b site is also important for forming the initial interaction that gives rise to the CP/LP C3 proconvertase, C4b2.

To test this inhibitory mechanism through an alternative approach, we devised a SPR strategy to investigate the outcome of increasing Eap concentrations on the ability of a C4b biotin surface
to bind C2 (Fig. 5B). In this experiment, if C4b were capable of binding Eap and C2 independently of one another, then the sensorgrams characteristic of the specific concentrations for each analyte alone would be strictly additive. Injection of 200 nM C2 in the presence of Eap resulted in a diminished response from what would be expected from two independent analytes, however. When the residual C2 contribution to the SPR signal from six different observations was fit to a dose–response curve as a function of Eap concentration, we obtained an IC50 value of ∼50 nM (Fig. 5C). Because this figure is in reasonably good agreement with the Kd of the C4b/Eap interaction (185 nM, as determined by AlphaScreen [Fig. 3B]), the outcome of this set of experiments provided an independent confirmation of the results presented in Fig. 5A above.

The requirement of Eap3 for both C4b binding (Fig. 4B) and inhibition of CP/LP activity (Fig. 4C) suggested that the ability to inhibit C4b2 binding should also be intrinsic to a minimal functional region of the Eap protein. To test this hypothesis, we established another AlphaScreen assay system in which the ability to inhibit C4b binding to C4b biotin could be assessed quantitatively. Using this approach, we determined that full-length C2 competing the myc–Eap C4b biotin pair (460 nM) would be expected from two independent analytes, however. When the residual C2 contribution to the SPR signal from six different observations was fit to a dose–response curve as a function of Eap concentration, we obtained an IC50 value of ∼13 nM for the C4b/Eap interaction, which represents ∼9-fold tighter binding when compared with the C4b/Eap34 interaction (525 nM, as determined by AlphaScreen [Fig. 4B]). Thus, the observation that Eap34 on its own competes with C2 for C4b binding demonstrates that disruption of the initial proconvertase assembly event is necessary for Eap-mediated inhibition of the CP/LP.

The Eap binding site on C4b represents a functional hot spot within the CP/LP

The CP/LP C3 convertase is only transiently stable when formed and decays with a t1/2 of ∼60 s at 37˚C (31). This rate of spontaneous decay is greatly enhanced in the presence of C4BP, which irreversibly dissociates C2a from its C4b scaffold and in addition serves as a cofactor for FI-mediated degradation of C4b to iC4b and C4c. Because the results presented in this work demonstrated that Eap effectively inhibits C4b2 binding (Fig. 6), we examined whether Eap might also disrupt the interaction of C4BP with C4b. Through use of the AlphaScreen assay, we found that C4BP also competed with Eap for binding to C4b (Fig. 5A). Nonlinear curve fitting of the competition data revealed an apparent IC50 of 21 ± 3 nM for the C4b/C4BP interaction, which represents ∼9-fold tighter binding when compared with the C4b/Eap interaction (Fig. 3B). This suggests that Eap would not disrupt the inhibitory function of C4BP when both are present in equimolar concentrations. We obtained a similar result when C4BP was used to compete the luminescence signal generated by myc–Eap34 binding C4b biotin (Fig. 5B), in which nonlinear curve fitting revealed an apparent IC50 of 13 ± 2 nM. It has to be noted, however, that this IC50 value reflects only the apparent affinity, and does not represent that of the individual C4b binding sites present within the polyvalent C4BP assembly (32).
Because Eap binds C4b at a similar site as C4BP, we tested whether Eap itself might display intrinsic cofactor activity to stimulate FI-mediated proteolysis of C4b. When purified C4b was incubated with both C4BP and FI, we were able to show rapid degradation of C4b into iC4b and then C4c, as judged by SDS-PAGE and liquid chromatography–coupled tandem mass spectrometry of tryptic peptides derived from various bands on gel (Supplemental Fig. 3A, top panel, left). However, substitution of Eap for C4BP in an otherwise identical assay provided no evidence for proteolysis of C4b by FI (Supplemental Fig. 3A, top panel, right). Thus, although Eap has no intrinsic FI cofactor activity, it shares a C4b binding site with multiple factors critical to the function and regulation of the CP/LP. We therefore propose that the Eap binding site on C4b represents a functional hot spot within the CP/LP, and that this hot spot is analogous to what we previously described for the binding site of the SCIN family of AP inhibitors on C3b (6, 20, 21, 33).
Despite its potent effects on the CP and LP, it is perhaps not surprising that Eap has been shown to promote staphylococcal immune evasion. But, the possibility that *S. aureus* also absorbs native host regulators FH (36) and C4BP (37) cannot be discounted, an overwhelming amount of structural, biochemical, functional, and immunological evidence in the literature strongly suggests that direct inhibition of convertase assembly, dynamics, and function, rather than indirect disruption via adsorption of fluid-phase regulators, is of paramount importance to *S. aureus* pathogenesis.

Our functional data demonstrate that Eap blocks both the CP and LP at the level of C3 activation. This inhibition arises from impaired formation of the CP/LP C3 proconvertase, C4b2a, which would subsequently reduce formation of the active CP/LP C3 convertase, C4b2a. The mechanistic basis of CP/LP inhibition by Eap therefore appears very similar to AP inhibition by Efb-C, which itself binds to C3b and reduces formation of the AP C3 proconvertase, C3bB, by nearly 80% (10) (Fig. 6). An important distinction between Eap and Efb-C, however, is that the latter has been shown to act in an allosteric manner (10). Our observation of direct competition for C4b binding between Eap and both C2 and C2b seems to favor a purely steric mechanism for Eap inhibition of CP/LP C3 convertase formation, although the possibility of Eap-dependent “action-at-a-distance” type effects on C4b cannot be dismissed without significantly more structural insight into these interactions. Still, the fact that Eap also blocks the C4b/C4BP interaction strongly suggests that the Eap binding site on C4b constitutes a functional hot spot for CP/LP C3 convertase formation, dynamics, and function. This raises some attractive conceptual analogies between Eap and the SCIN family of C3b-binding AP C3 convertase inhibitors (6, 20, 21). Furthermore, it also suggests that effective targeting of these functional hot spots that exist in various host response pathways might be a central theme behind pathogen-specific evolution of innate immune evasion mechanisms.

Given its potent effects on the CP and LP, it is perhaps not surprising that Eap has been shown to promote staphylococcal virulence in rodent models of acute peritonitis (38), as well as chronic arthritis, osteomyelitis, and abscess formation (39). Although the precise contributions of the CP and LP remain to be fully evaluated in each of these experimental systems, the con-
Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Supporting Information on Eap Inhibition of CP/LP and Phagocytosis. (a) Eap inhibits the murine CP and LP. The effect of 1 µM Eap, EapH1, or EapH2 on CP (left) and LP-mediated (right) complement activation was measured across a dilution series of serum concentrations. Activation was detected as C3b deposition on an ELISA plate surface. Legends are inset. (b) Eap is present on the surface of cells of a WT but not a Δeap S. aureus strain. Bacteria were grown to stationary phase in liquid culture and harvested by centrifugation. After washing, Laemmli sample buffer with DTT was added to an equal volume of cell suspension and the samples were analyzed on a 12.5% (w/v) glycine polyacrylamide gel. Eap was detected by Western blot with 1:20,000 diluted rabbit-α-Eap serum and an HRP-labeled secondary antibody. A control experiment was performed to ensure that the same amount of bacteria were present in each sample by plating serial dilutions of each sample immediately prior to adding the Laemmli buffer. Note that proteolytic degradation of Eap into various combinations of adjacent subdomains has been reported elsewhere, and is due to protease sensitivity of the interdomain linkers (27). (c) Phagocytosis of S. aureus Newman Δeap using 1% (v/v) NHS as a source of complement components and the indicated concentrations of Eap.
Supplemental Figure 2. Supporting Information on Eap Binding to C4 and its Derivatives. (a) Affinity isolation of C4 from human serum. Biotinylated Eap was used as an affinity reagent to identify potential binding partners in both normal and C4-depleted human serum. Following capture of Eap-biotin by magnetic streptavidin beads and a series of washes, the bound proteins in both samples were separated by SDS-PAGE under non-reducing conditions. A band of approximately 200 kDa was found in the lane corresponding to normal but no C4-depleted serum, strongly suggesting that Eap binds to native C4. (b) The ability of C4, C4b, and C4c to compete the AlphaScreen signal generated by myc-Eap and C4b-biotin was assessed over a logarithmic dilution series. While three independent trials were carried out, the data presented here are from a single representative titration. The smooth line indicates the outcome of fitting all points to a dose-response curve. (c) Sedimentation equilibrium analytical ultracentrifugation analysis of C4b and C4b/Eap. Experimental data were obtained as described in Materials & Methods for C4b (left panel) and an equimolar mixture of C4b/Eap (right panel). Equilibrium profiles were fit to a single particle model to yield the observed molecular weight for both C4b (268 kDa) and C4b/Eap (308 kDa). The top plots in both panels show the random residuals for the respective fits.
Supplemental Figure 3. Eap Lacks FI-cofactor Activity and Only Weakly Impacts C4BP Cofactor Activity. (a) The effect(s) of including Eap, C4BP, both, or neither on FI-mediated proteolysis of C4b was investigated. Aliquots of each reaction series (indicated) were withdrawn over the course of 20 min and the proteins were analyzed by SDS-PAGE. (b) The effect of varying the molar ratio of Eap to C4BP on FI-mediated proteolysis of C4b was investigated. Aliquots of each reaction series (indicated) were withdrawn at 0 and 2 min, and the proteins were analyzed by SDS-PAGE. The identity of various bands, as determined by mass-spectrometry, is indicated. The ability of Eap to inhibit C4BP-dependent cleavage of C4b by FI was quantified by densitometry of the α’ chain bands before and after the reaction. Addition of equimolar concentrations of both C4BP and Eap to this assay appeared to slow the rate of FI proteolysis (a, bottom panel left). This likely resulted from competition between Eap and C4BP for the same binding site on C4b, which consequently reduced the effective concentration of substrate available to FI since Eap does not have intrinsic cofactor activity (a, top panel right). To explore the functional consequences of Eap competition with C4BP in more detail, we carried out a set of studies wherein the molar ratio of Eap to C4BP was varied between 0, 0.1, 1.0, and 10 (b, top panel). Significant inhibition of FI-mediated proteolysis appeared to occur only when Eap was present at 10-fold higher concentration than C4BP (b, bottom panel). The requirement of such a high level of Eap to disrupt C4BP activity was most likely due to the weaker affinity of Eap for C4b and its lack of polyvalency, as it forms equimolar complexes with C4b. Thus, while Eap competes with C4BP for the same recognition site on C4b, it has no cofactor activity of its own, nor does competition with C4BP appear to be essential to its effects on the CP/LP of complement.