Binding of Efb from *Staphylococcus aureus* to Fibrinogen Blocks Neutrophil Adherence

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In addition to its pivotal role in hemostasis, fibrinogen (Fg) and provisional fibrin matrices play important roles in inflammation and regulate innate immune responses by interacting with leukocytes. Efb (the extracellular fibrinogen binding protein) is a secreted *Staphylococcus aureus* protein that engages host Fg and complement C3. However, the molecular details underlying the Efb-Fg interaction and the biological relevance of this interaction have not been determined. In the present study, we characterize the interaction of Efb with Fg. We demonstrate that the Fg-binding activity is located within the intrinsically disordered N-terminal half of Efb (Efb-N) and that the D fragment of Fg is the region that mediates Efb-N binding. More detailed studies of the Efb-N-Fg interactions using ELISA and SPR analyses revealed that Efb-N exhibits a much higher affinity for Fg than typically observed with Fg-binding MSCRAMMs and data obtained from ELISA analyses using truncated Efb-N constructs demonstrate that Efb-N contains two binding sites located within residues 30-67 and 68-98, respectively. Efb-N inhibits neutrophil adhesion to immobilized Fg by binding to Fg and blocking the interaction of the protein with the leukocyte integrin receptor, αMβ2. A motif in the Fg gamma chain previously shown central to the αMβ2 interaction was shown to be functionally distinguishable from the Efb-N binding site suggesting that the Fg-Efb interaction indirectly impedes Fg engagement by αMβ2. Taken together, these studies provide insights into how Efb interacts with Fg and suggest that Efb may support bacterial virulence at least in part by impeding Fg-driven leukocyte adhesion events.

Fibrinogen (Fg) is an acute phase plasma protein that is the fundamental building block of insoluble fibrin clots and is understood to play an important role in hemostasis and inflammatory processes (1). Mice lacking Fg exhibit a delayed inflammatory response and defects in wound-healing (2,3). In addition to providing substrates for bacterial colonization, Fg also participates in the innate immune defense through interactions with leukocytes that support leukocyte activation events and delay apoptosis (4). The interaction of Fg/fibrin with the leukocyte integrin αMβ2 contributes to the development of inflammatory disease processes, including inflammatory joint disease and neuroinflammatory disease (5,6), as well as physiological processes such as the clearance of *Staphylococcus aureus* (*S. aureus*) within the peritoneal cavity (7).

Numerous bacterial proteins interact with Fg, including ClfA (clumping factor A), FnbpA, Eap and coagulase from *S. aureus*, SdrG from *S. epidermidis*, M protein from group A, C and G streptococci and FbsA from *S. agalactiae* (8). In many cases, these Fg-binding proteins act as virulence factors in animal models of infection. For example, ClfA promotes sepsis in a mouse model (9) and the streptococcal M proteins cause lung lesions in mice (10) in part by altering leukocyte function (11). The goal of the present study was to further characterize the interaction of the secreted *S. aureus* extracellular fibrinogen-binding protein (Efb) with Fg and to determine how Efb may alter Fg-mediated leukocyte adhesion events.

*S. aureus* is a versatile, opportunistic Gram-positive pathogen and a leading cause of bacterial infections worldwide (12). It causes a diverse array of diseases ranging from minor skin infections to life-threatening diseases such as pneumonia, endocarditis and sepsis (13). *S. aureus* produces a large arsenal of virulence factors.
including cell surface associated adhesins, and secreted proteins like proteases, toxins and superantigens (14,15). Several secreted staphylococcal proteins, including the extracellular adhesive protein (Eap), the chemotaxis inhibitory protein of staphylococci (CHIPS) and staphylococcus complement inhibitor (SCIN) appear to target and counter the host innate immune response (16) by inhibiting neutrophil trafficking (17), recruitment, chemotaxis (17,18), complement activation and preventing opsonization and phagocytosis of the bacteria (19).

Efb is another innate immune evasion molecule secreted by *S. aureus*, which is reported to inhibit complement activation (20,21), block platelet aggregation (22,23) and delay wound healing in a rat wound infection model (24). The 16 kDa Efb protein has a disordered N-terminal region (Efb-N), spanning the sequence Efb_30-104, and a folded domain in the C-terminal region (Efb-C), spanning the sequence Efb_105-165 (25,26). Earlier studies in our laboratory (20) and those of others (21) established that Efb-C binds to C3 and blocks C3b-containing convertase activities, resulting in decreased C3b deposition on the bacterial surface and down regulation of the neutrophil response mediated by C5a. The crystal structure of the Efb-C/C3d complex revealed that the binding of Efb-C to C3 alters the conformation of C3 such that it cannot be processed into C3b (26). Interestingly, Efb-C also blocks the interaction of C3d with complement receptor 2 (CR2), which plays a critical role in B cell maturation and activation, suggesting an additional role of Efb as an inhibitor of the adaptive immunity (27).

Efb also interacts with Fg (25,28), but the precise motifs on bacterial Efb and host Fg as well as the precise biological significance of this interaction remain unclear (25,29). Our lab previously demonstrated that the intrinsically disordered Efb-N region is important for the Fg binding activity, whereas Efb-C does not contribute to Fg binding (25). Here, we have characterized the interaction of Efb with Fg in detail. Our results demonstrate that Efb binds with unusually high affinity to Fg, and we identify two distinct Fg-binding sequences within the Efb-N domain. Furthermore, we show that this interaction results in an inhibition of neutrophil adherence to immobilized Fg.

**EXPERIMENTAL PROCEDURES**

*Ethics Statement*—For studies with neutrophils from human subjects, written informed consent was obtained from healthy adult individuals with approval of the Baylor College of Medicine Institutional Review Board for Human Subject Research.

*Isolation of neutrophil*—Fresh human peripheral blood neutrophils obtained from healthy adult individuals were isolated from citrate anticoagulated venous blood sedimented in 6% dextran (250.000 MW) and centrifuged over Ficoll-Hypaque gradients at room temperature. Isolated neutrophils were suspended in Dulbecco’s phosphate-buffered saline (D-PBS, Gibco) and maintained at 4°C for up to 4 hours at a concentration of 10⁶/ml.

*Neutrophil adherence assay—*Neutrophil adherence to Fg-coated surfaces was quantitated using a Muntz-static adhesion chamber (30,31), consisting of two metal plates holding two 25-mm round glass cover slips separated by a rubber-O-ring. The cover slips, cleaned with 10% NaOH/57% EtOH Fg for 2 h at room temperature and washed with D-PBS, were coated with Fg (100 µg/ml in D-PBS) over night at 4°C. Freshly isolated neutrophils (10⁶/ml) were injected into the chamber through a 25-gauge needle connected to a 1-ml syringe. Neutrophil adherence was quantified by counting the number of neutrophils that settled and attached during an initial 500 second observation period. The adhesion chamber was then inverted for an additional 500 seconds to allow nonadherent neutrophils to fall off. The remaining adherent neutrophils were counted and expressed as percent adherent cells. All experiments were conducted at 37°C using a Nikon Diaphot inverted phase-contrast microscope. To study the effect of staphylococcus proteins on neutrophil adherence, neutrophils were mixed with 100 µl of 5 µM His-tagged recombinant SdrG or Efb protein (0.5 µM final concentrations) immediately before injection into the adhesion chamber. In the case of preincubation experiments, either neutrophils were preincubated with recombinant Efb for 20 min, collected by centrifugation, washed with PBS and added to a Fg-coated chamber; or Fg-coated glass coverslips were preincubated with Efb for 1 hour and washed with PBS before adding neutrophils.
Cell adherence assay using cell lines-A human embryonic kidney 293 (HEK 293) cell line stably expressing αMβ2 (HEK 293-Mac-1) (kindly provided by Dr. Edward F. Plow) (32) was maintained in DMEM/F-12 (Lonza) supplemented with 10% fetal bovine serum (FBS), 2 µM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 600 µg/ml G418. The HEK 293 cell line was maintained in 5% FBS/DMEM (Thermo scientific). Prior to use, cells were harvested with cell dissociation buffer (Invitrogen), washed and suspended in TBS containing 1mM CaCl₂, 2mM MgCl₂ and 2mM MnCl₂. For cell adherence assays, 96-well plates (Thermo scientific) were coated with 100 µl of Fg (1µg/ml, Enzyme research) overnight at 4 ºC and then post-coated with 0.05% polyvinyl alcohol (PVA 30-70 kDa, Sigma) for 1h at 37 ºC. Subsequently, the cells were seeded at 50,000 cells/well and incubated at 37ºC for 1 h. Non-adherent cells were removed by washing gently twice with PBS. Adherent cells were fixed with 200 µl of 0.25% of glutaraldehyde for 15 min at room temperature followed by 100 µl/well of 100 mM glycine for 10 min. The numbers of adherent cells were determined by staining with 0.2% crystal violet for 15 min (33). The optical density of crystal violet was measured at 590nm after solubilizing the dye in 10% acetic acid.

Cloning of Efb constructs-Genomic DNA isolated from S. aureus strain Newman was used as template for all PCR reactions using the oligonucleotide primers described in supplement data (Supplement Table 1). PCR products were digested with BamH I and EcoR I and ligated into the pGEX-5x-1 vector (GE healthcare) or digested with BamHI and PstI and ligated into the pQE30 (Invitrogen). The ligation mixture was transformed into E. coli XL-1 blue (Stratagene), grown on LB agar plate containing 100 µg/ml ampicillin to select for transformants. Insertions were confirmed by DNA sequencing.

Expression and purification of recombinant Efb-E. coli strain M15 (pREP4) (Qiagen) containing plasmids encoding N-terminal glutathione S-transferase (GST) or N-terminal 6X His-tagged Efb fusion proteins were grown overnight at 37ºC in LB containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The overnight cultures were diluted 1:20 into fresh LB medium and recombinant protein expression was induced with 0.2mM IPTG for 2-3 hours. Bacteria were harvested by centrifugation and lysed using a French press (SLM Aminco). Soluble proteins were purified through glutathione-Sepharose-4B column (GE Healthcare) or by Ni-chelating chromatography (GE Healthcare) according to the manufacturer’s manual. Purified proteins were dialysis into PBS and stored at -20ºC. Protein concentrations were determined by the Bradford assay (Pierce).

Preparation of Fg fragments-Fg D fragments were generated by digestion of Fg (Enzyme research) with plasmin (Enzyme research, 10 ug/15mg Fg, ) in TBS containing 10 mM CaCl₂ for 4 hours at 37ºC as described earlier (34) with modifications. D fragments were obtained by column gel filtration on Sephacryl S-200 (GE Healthcare). Purified D fragments were analyzed on SDS-PAGE with molecular weight of 85 kDa. Fg E fragments were purchased from Calbiochem.

ELISA-type binding assay-96-well immulon 4HBX microtiter plates (Thermo Scientific) were coated overnight at 4 ºC with 100 µl of 2.5 µg/ml full length human Fg (diluted in PBS, Enzyme research) unless otherwise indicated. After blocking the wells with 3% BSA in PBS, recombinant Efb proteins were added and the plates were incubated for one hour. Bound Efb proteins were detected through incubation with horseradish peroxidase (HRP)-conjugated anti-His antibodies (10,000x dilution) or HRP-conjugated anti-GST polyclonal antibodies (5000x dilution) for one hour and quantified after adding the substrate 0-phenylenediamine dihydrochloride (Sigma) by measuring the resulting absorbance at 450 nm in an ELISA microplate reader (Thermomax).

In the case of Efb peptide inhibition assay, various concentration of Efb peptides were mixed with fixed concentration of Efb-GST fusion proteins (10 nM) in PBS and the bound Efb-GST fusion proteins were detected through incubation with HRP-conjugated rabbit anti-GST polyclonal antibodies (Abcam) (5000x dilution) for one hour and quantified after adding the substrate 0-phenylenediamine dihydrochloride (Sigma) by measuring the resulting absorbance at 450 nm in an ELISA microplate reader (Thermomax).
Fg aggregation assay—2 µM of Human Fg (Enzyme research) or isolated Fg-D fragment, generated by plasmin digestion, was incubated with different concentration of Efb-His fusion proteins in 20 mM HEPES buffer (pH 7.4) with 150 mM NaCl and 0.1% Tween 20 in a 96-well assay plate (Falcon3912) for 2h at room temperature. The turbidity of the protein solution mixture was monitored at an optical density of 405 nm.

Isothermal titration calorimetry—The interaction between Efb proteins and the soluble, isolated D fragment of Fg was further characterized by isothermal titration calorimetry (ITC) using a VP-ITC microcalorimeter (MicroCal). The Fg-D fragment used in these studies was generated by digesting full length Fg with plasmin for 4h and fractionating the digestion products by gel filtration chromatography. The ITC cell contained 6 µM Fg-D fragments and the syringe contained 50~70 µM Efb-GST fusion proteins in TBS (25 mM Tris, 3.0 mM KCl and 140 mM NaCl, pH 7.4). All proteins were filtered through 0.22 µm membranes and degassed for 20 min before use. The titrations were performed at 27 ºC using a single preliminary injection of 2 µl of Efb fusion proteins followed by 30~40 injections of 5 µl with an injection speed of 0.5 µl/s. Injections were spaced over 5 min intervals at a stirring speed of 260 rpm. Raw titration data were fit to a one-site model of binding using MicroCal Origin version 5.0.

Surface plasmon resonance (SPR)—analysis of the interactions—SPR-based Biacore binding experiments were performed at 25º C on a Biacore 3000 (GE Healthcare/Biacore, Uppsala, Sweden). Ligand surfaces were prepared using an amine coupling procedure as recommended by the manufacturer. HEPES-buffered saline (HBS-T; 10 mM HEPES, pH7.3, 150 mM NaCl, and 0.005% Tween-20) was used as the running buffer at a flow rate of 5 µl/min. Twenty microliters of Fg-D fragment (15 µg/ml in sodium acetate, pH5.0) were injected to an activated (7 min) flow cell on CM5 chip. After deactivation, the flow cell surface was washed with running buffer until the surface was stable. His-tagged Efb-N, which has a predicted pI of 8.83, was immobilized onto the C1 chip. After 2-min activation, 10 µl of protein (2 µg/ml in water) was injected and the flow cell was subsequently deactivated. The immobilization procedures mentioned above created a ligand density of about 1900 RU for Fg-D, and 50 RU for His-tagged Efb-N. A reference surface lacking coupled protein was prepared on each sensor chip. Binding studies were performed at a flow rate of 30 µl/min with HBS-T as the running buffer. To regenerate the ligand surfaces, bound proteins were removed by 10 or 20 second injections of 5 mM NaOH. All SPR responses were baseline corrected by subtracting the response generated from the reference surface. In order to obtain the binding kinetics data, two-fold increasing concentration of proteins in running buffer were injected over the ligand and reference surfaces. Baseline corrected SPR response curves (with buffer blank run subtracted) were globally fitted using MicroCal Origin version 5.0.

RESULTS

Efb binds to the D-fragment of Fg with high affinity. We initially explored the binding of Efb to immobilized Fg using purified His-tagged full-length Efb, Efb-N and Efb-C in ELISA type binding assays. Binding of full-length Efb and Efb-N, but not Efb-C, to Fg was dose-dependent and exhibited saturation kinetics. Half maximum binding for Efb and Efb-N was observed at concentrations of 1.21 nM and 1.16 nM, respectively (Fig. 1A). These observations corroborate our previous results (25). More detailed studies revealed that Efb binds to both immobilized and soluble forms of Fg (Fig. 1B,C). In a competition ELISA-type assay, soluble Fg efficiently inhibited the binding of Efb to surface-bound Fg with 50% inhibition achieved at 142 ±1 nM (Fig. 1C). Soluble Fg also bound to immobilized Efb coated onto microtiter plates (Fig. 1B). To locate the Efb binding site(s) in Fg we tested isolated D- and E- fragments of Fg in an ELISA-type binding assay. Efb-N bound equally well to the Fg D-fragment (Fg-D) and to intact Fg but did not bind to the Fg E-fragment (Fg-E) (Fig. 1D).

The Efb-Fg interaction was further characterized by surface plasmon resonance (SPR) using a Biacore 3000 with either a CM5 sensor
chip derivatized with Fg-D or a C1 sensor chip derivatized with His-tagged Efb. The results obtained were consistent with our observations from the ELISA-type binding assays; full-length Efb and Efb-N, but not Efb-C, bound to Fg-D (Fig. 2A). We determined that Efb-N bound to Fg-D in a dose-dependent manner with a calculated $K_D$ of 0.23 nM based on kinetic data (Fig. 2B). The interaction of Efb-N with Fg-D exhibits a reasonably fast on rate ($k_o=5.03 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) but a very slow off rate ($k_d = 1.14 \times 10^4 \text{sec}^{-1}$), suggesting that once formed the complex is very stable (Fig. 2B). Interestingly, when the system was reversed and the Efb-N fusion protein was immobilized, Fg-D bound to the Efb-N chip in a dose-dependent manner but with a higher $K_D$ (2.67 nM) due to a faster off rate ($k_d = 7.9 \times 10^4 \text{sec}^{-1}$) (Fig. 2C). The molecular explanation for this difference is presently not clear, but regardless of the experimental design the binding affinities between this bacterial product and host fibrinogen appear to be remarkably high.

**Efb-N contains two Fg binding sites.** We observed that a solution containing an equimolar ratio of Efb and Fg (a symmetrical dimer composed of six polypeptide chains; $\alpha_2\beta_2\gamma_2$) turned cloudy, indicating the formation of aggregates. To elucidate whether this was due to the formation of multimers or precipitation as a result of conformational changes, we incubated Fg, at a set concentration (2 $\mu$M), with increasing amounts of the different Efb proteins and assessed turbidity. Interestingly, combining either full-length Efb or Efb-N with Fg resulted in a highly fast-concentration dependent and bell-shaped change in light scatter, whereby turbidity initially increasing and then subsequently decreasing as the concentration of the Efb proteins increased (Fig. 3A). This behavior is reminiscent of an immunoprecipitation curve, suggesting that the observed turbidity results from the formation of multimers. The addition of Efb-C, which does not bind Fg, did not induce a measurable turbidity change (Fig. 3A). The ability of full-length Efb and Efb-N to induce the formation of multimers suggests that each protein contains at least two Fg binding sites. Notably, incubating the monomeric D-fragment of Fg with either full-length Efb or Efb-N did not yield changes in turbidity (Fig. 3B), suggesting that the two proposed binding sites on each Efb-N molecule do not bind to independent motifs within individual D region.

**Identification the Fg binding sites in Efb.** To identify the two putative Fg binding sites located within Efb, we generated a panel of recombinant truncated Efb-N proteins fused to GST and evaluated their Fg binding ability using the ELISA-type assay. Efb-N contains two nearly identical repeat segments (Efb$_{46-67}$ [Efb-L] and Efb$_{77-98}$ [Efb-I]) composed of 22 residues each. These repeats were previously shown to contribute to Fg binding (35), but neither element appears to be sufficient to support the high affinity Fg interaction. Recombinant forms containing these two segments (Efb-L and Efb-I, Fig. 4A) did not bind to Fg in the ELISA assay (Fig. 4B). However, addition of short N-terminal extensions to generate Efb$_{30-67}$ (Efb-A) and Efb$_{68-98}$ (Efb-O), resulted in proteins with Fg binding activity where half maximal binding was observed at a concentration of 1.1nM and 0.64nM, respectively (Fig. 4B). Interestingly, Efb fragments covering residues 30-45 (Efb-K) and 68-76 (Efb-M) did not show Fg-binding activity (Fig. 4B), suggesting the Fg-binding sites require longer sequences of Efb. Taken together, it appears that the Efb-K and Efb-M segments are insufficient for Fg binding, but residues in these regions are necessary for conferring Fg-binding activity to Efb-A and Efb-O.

**Characterization of the binding affinity of the two Fg-binding sites in Efb.** To characterize the Fg-binding affinity of Efb-A and Efb-O in greater detail, isothermal titration calorimetry (ITC) was conducted with plasmin generated Fg-D fragment and GST-tagged Efb-A and Efb-O. GST fusion proteins were used in these experiments because they are more soluble than the corresponding synthetic peptides. Strikingly, in solution, Efb-O bound to Fg-D with significantly higher affinity than Efb-A with dissociation constants of 4.66 nM and 1.0 $\mu$M, respectively (Fig. 5A, B). Thus the affinity of Efb-O for Fg-D is 200 times higher than that of Efb-A suggesting that Efb-O contains the premiere Fg binding motif in Efb (Fig. 5). Purified GST did not interact with Fg-D and served as the control (data not shown). The remarkable feature of both interactions is the excess favorable binding enthalpy ($\Delta H \sim -27 \text{ kcal/mol}$, Table 1), presumably due to a large number of hydrogen bonds and van der Waals interactions formed in the complex. However, the favorable interaction enthalpy is countered by very large unfavorable...
entropy changes ($\Delta S \sim -30$–$-60$ Calmol$^{-1}$K$^{-1}$, Table 1), particularly for Efb-A ($\Delta S \sim -60$ Calmol$^{-1}$K$^{-1}$, Table 1), indicating a vast loss in the degree of freedom upon binding. This thermodynamic unfavorable entropy change most likely results from reduction of conformational flexibility in the two interacting proteins upon binding (36,37).

In the competition ELISA assays, the synthetic peptide Efb-o (a 31 residue peptide corresponding to Efb amino acids 68-98) effectively inhibited GST-tagged Efb-N protein binding to Fg while synthetic peptide Efb-a (a 38 residue peptide corresponding to Efb amino acids 30-67) had no effect on the Fg-binding activity of recombinant Efb-N even when the peptide was used at a concentration of 100 µM (Fig. 6A and data not shown). These results further demonstrated that Efb-O contains the major Fg binding site. We also compared the ability of peptide Efb-a Efb-o and Efb-h (a 28 residue peptide corresponding to Efb amino acids 77-104) to compete with the Fg-binding activity of GST-tagged Efb-A and Efb-O. Peptide Efb-o successfully inhibited GST-Efb-A (10 nM) binding to Fg with a half maximal inhibitory concentration (IC$_{50}$) of 24 ± 1 nM (Fig. 6B). In contrast, synthetic Efb-a peptide was not able to compete with GST-Efb-O for binding to Fg even at a concentration of 100 µM, which is in 10,000-fold molar excess of the peptide compared to the GST-tagged protein (Fig. 6C and data not shown), again suggesting that the Efb-O binds much stronger to Fg than Efb-A and confirming that Efb-O contains the major binding site for Fg. The ability of Efb-o to compete with Efb-A in Fg binding suggests that these two Efb fragments bind to the same or partially overlapping sites in Fg. Peptide Efb-h did not interfere with the Fg binding of GST-tagged Efb-N (Fig. 6A), Efb-A (Fig. 6B) and Efb-O (Fig. 6C), suggesting that the residues 68-76 are essential for the Fg-binding activity and that the residues 99-104 may not significantly contribute to Fg binding. To further determine the precise sequences in Efb-O that binds to Fg, truncated Efb-o peptides with deletions at either N-terminus or C-terminus were synthesized and their ability to inhibit GST-tagged Efb-N binding to Fg were evaluated. Truncated Efb-o peptides lacking either three N-terminal residues (Efb-o-N3), six N-terminal residues (Efb-o-N6) residues, ten C-terminal residues (Efb-o-C10) or fifteen C-terminal residues (Efb-o-C15) each lost the ability to block GST-Efb-N binding to Fg. However, a five residues deletion at the C-terminal of Efb-o (Efb-o-C5) still effectively inhibits Fg-binding activity of GST-Efb-N, suggesting that the 26 residue peptide Efb-o-C5, corresponding to Efb amino acids 68-93, encompasses the minimal binding site for Fg (Fig. 6D).

**Efb blocks neutrophil adherence to fibrinogen.**

We then examined the effect of Efb on neutrophil adherence to Fg using the static Muntz adhesion chambers described previously (30,31,38). The recombinant full-length Efb effectively inhibited neutrophil attachment to immobilized Fg, reducing the number of attached cells by more than 80% compared to the non-treated control group (Fig. 7A). To determine which segment of Efb is responsible for this inhibitory effect, we examined the ability of purified recombinant Efb-N and Efb-C to inhibit neutrophil attachment. We found that Efb-N reduced neutrophil attachment to Fg to the same extent as full-length Efb whereas Efb-C had no effect (Fig. 7A). A recombinant Fg-binding fragment of SdrG, a cell wall-anchored Fg-binding MSCRAMM from *Staphylococcus epidermidis* (39), did not affect neutrophil attachment to Fg and served as a negative control (Fig. 7A).

The morphology of neutrophils changes from a spherical to a bipolar shape in response to stimulation by chemotactic factors or to adherence to a substratum (40). Microscopic analysis of neutrophils 10 min after treatment with Efb-C exhibited a bipolar morphology similar to the non-treated control group, suggesting that neutrophils that attached to Fg under these conditions also can respond with shape changes and that Efb-C does not interfere with this process (Fig. 7B). However, the few neutrophils attached in the presence of full-length Efb or Efb-N retained a spherical shape (Fig. 7B), indicating that these neutrophils did not progress with the adherence-associated morphological changes.

**Efb blocks neutrophil adherence to Fg by targeting Fg.** To further explore the mechanism by which Efb blocks neutrophil adherence to Fg, human neutrophils were preincubated with recombinant Efb for 20 min, collected by centrifugation, washed with PBS and added to a Fg-coated chamber. Neutrophils pre-incubation with full-length Efb or Efb-N, adhered to Fg to the same extent as the non-treated control group in
which no Efb was added (Fig. 7C). These data suggest that neither Efb nor Efb-N directly engage and stably interfere with the neutrophil surface molecules required for adhesion to Fg.

In contrast, neutrophils were prevented from adhering to Fg when the Fg-coated surface was pretreated with full-length Efb or Efb-N (Fig. 7D), indicating that a direct interaction between Fg and Efb/Efb-N is responsible for inhibiting subsequent neutrophil adherence to immobilized Fg. Pretreatment of the Fg-coated surface with SdrG did not inhibit neutrophil adherence to Fg, which is consistent with the earlier finding that SdrG does not affect neutrophil attachment to Fg (Fig. 7D). Taken together, these results demonstrate that the binding of Efb to Fg interferes with neutrophil-Fg interaction, resulting in an inhibition of neutrophil adherence to Fg. These results do not formally exclude that Efb interacts with a neutrophil surface molecule, but these interactions, if they occur, apparently do not affect neutrophil adhesion to Fg.

**Efb blocks the binding of neutrophil receptor αMβ2 to Fg.** Because the neutrophil-Fg interaction is primarily an αMβ2-dependent process (7), we sought to directly evaluate the role of αMβ2 in Efb-mediated inhibition of neutrophil adherence. Consistent with prior observations, we found that a monoclonal antibody against the αM-I domain effectively blocked the adhesion of HEK293 cells expressing human αMβ2 (kindly provided by Dr. Edward F. Plow) to immobilized Fg (Fig. 8A). Control HEK293 cells lacking αMβ2 cannot attach to immobilized Fg (Fig. 8A). Moreover, two synthetic Fg peptides based on the αM binding sites in Fg (denoted Fg-P1 (41) and Fg-P2 (42)), respectively, abrogated cell attachment to immobilized Fg surface (Fig. 8A). These results were consistent with earlier studies (7) and demonstrated that the attachment of the transfected HEK293 cells to Fg is a specific αMβ2-dependent process. Addition of either full-length Efb or Efb-N to the adhesion assay effectively blocked attachment of the αMβ2 expressing cells to Fg (Fig. 8B), suggesting that the Efb or Efb-N interferes with the αMβ2-Fg interaction. The possibility that Efb competes with αMβ2 for a common site in Fg was investigated. Here, a mutant form of mouse Fg, termed Fgγ390-396A, that retains the capacity to form fibrin in vitro and in vivo, but lacks seven amino acids residues required for αMβ2-mediated cell adhesion (7) was compared to wild-type fibrinogen for its capacity to bind Efb. As shown in Fig. 8C, Fgγ390-396A was indistinguishable from wild-type mouse and human Fg in the Efb binding assay. Consistent with these findings, the synthetic peptides covering the sequences of P1 and P2-C, respectively, do not interfere with Fg binding to Efb (Fig 8D). These results suggest that the Efb binding site is functionally separable from the motifs in Fg critical to αMβ2 binding. These observations also suggest that the mechanism by which Efb blocking neutrophil adherence to Fg is not through a direct competitive interaction.

**DISCUSSION**

The inhibition of neutrophil adherence to Fg described in this study is a novel function for Efb that may be central to overall bacterial evasion of immune surveillance. Neutrophils are professional phagocytes that play a pivotal role in innate immunity and inflammatory responses by virtue of their ability to phagocytose microorganisms, produce inflammatory mediators, and elicit both intracellular and extracellular microbial killing strategies. The leukocyte-specific surface integrin, αMβ2/Mac-1 is one of several β2 integrins known to be central to both leukocyte trafficking and leukocyte activation events that support bacterial clearance. The αMβ2 integrin recognizes numerous ligands including ICAM-1, C3bi, factor X, kininogen and Fg (43). Several ex vivo and in vitro studies have shown that the interaction between Fg and neutrophils promotes neutrophil-endothelial transmigration (44), enhances phagocytosis (45), activates NF-κB signaling (4), promotes degranulation (46), produces chemokines and cytokines (47) and delays apoptosis (45). The biological significance of the Fg-αMβ2 interaction was substantiated in gene-targeted mice expressing Fgγ390-396A, a mutant form of Fg retaining clotting function but lacking the αMβ2 binding motif. Fgγ390-396A mice exhibit a significant diminution in inflammatory disease processes relative to control mice challenged in parallel, including an amelioration in inflammatory joint disease (5) and diminished inflammatory demyelination in the CNS and retained motor function in the context of autoimmune encephalomyelitis (6). Similarly, in a mouse model of *S. aureus* peritonitis Fgγ390-396A showed an impaired bacterial clearance (7). The ability of
Efb to block neutrophil adherence to Fg could have a similar effect on the inflammatory response as that seen in the Fg mutant mice.

Adhesion of neutrophils to immobilized Fg and fibrin is primarily an αβγ-dependent process (32,44,48). We demonstrate here that the binding of Efb to Fg effectively blocks αβγ binding to Fg and strongly impedes neutrophil adhesion to Fg. However, this effect is not the consequence of a direct competition between Efb and αβγ for the same binding sites in Fg (Fig 8C,D) since a) Efb binds equally well to wt Fg and Fgγ390-396A and b) synthetic Fg peptides representing the αβγ binding site(s) do not affect the Efb/Fg interaction. Efb binding is also not dependent on C-terminal binding site(s) to affect the Efb/Fg interaction. 

Furthermore, our studies confirm that Efb-C does not bind Fg, which is consistent with our previous study (25) but is at odds with the report of Palma et al. (29).

Fg binding and aggregation induced by bacterial protein binding is a critical component of the virulence strategy used by Streptococcal M protein, a potent anti-phagocytic molecule (11). Fg aggregated by M protein activates neutrophils and induces vascular leakage and lung damage (10,51). Moreover, Fg-M protein complexes were identified in tissue lesions of patient with necrotizing fasciitis and toxic shock syndrome (10), consistent with a pathogenic significance of the Fg/bacterial protein complex in the disease process. Also, the staphylococcal Fg binding proteins coagulase (Coa) and von Willebrand factor binding protein (vWbp) were recently shown to act as virulence factors in a mouse model of abscess formation and Fg was co-localized with Coa and vWbp proteins in the abscess lesions (52).

The precise role of Efb in the pathogenesis of S. aureus infection and the contribution of the Efb-Fg interaction to virulence are not understood. Efb was previously shown to act as a virulence factor in a wound infection model (24), however the mechanisms of action remain unclear. Our observation that Efb, like streptococcal M protein, can aggregate Fg raises the possibility that the two proteins use similar virulence strategies.

In conclusion, Efb is a powerful evader of human host defense systems. Efb not only blocks neutrophil recruitment to the site of infection by inhibiting C5a generation (21), it also inhibits activation of the adaptive immune system by preventing C3d from binding to CR2 (27). Here we show that Efb also effectively inhibits Fg-neutrophil interactions in vitro and this property may serve the interest of the microbe by impeding neutrophil transmigration and/or impeding fibrin-supported neutrophil activation, resulting in an ineffective antimicrobial response in vivo.
REFERENCES


**FOOTNOTES**

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The abbreviations used are: *Staphylococcus aureus, S. aureus;* Efb, extracellular fibrinogen binding protein; Fg, fibrinogen, Fg-D, D fragment of Fg; Clf, clumping factor; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; PVA, Polyvinyl alcohol; ITC, isothermal titration calorimetry; CNS, central nervous system.
FIGURE LEGENDS

Fig. 1. Efb binds to soluble and immobilized forms of Fg. ELISA-type assays of binding of Efb, Efb-N and Efb-C to immobilized Fg (A), or binding of Fg to immobilized Efb (B). (C) Competition ELISA assay of Fg in solution competing with Fg coated on the surface for binding available Efb. Increasing concentration of Fg was incubated with Efb (25 nM) in microtiter wells containing 0.25 µg/well Fg. (D) The ELISA-type binding assay show that Efb-N binds to the immobilized Fg (squares), Fg-D (triangles) but not to Fg-E (circles). For experimental details see the experimental procedures.

Fig. 2. Efb binds to the D fragment of Fg. (A) SPR sensograms were generated by injecting Efb (0.1 µM), Efb-N (0.1 µM) or Efb-C (0.5 µM) onto a sensory chip with immobilized Fg-D. (B) and (C) Kinetic analyses of Fg-D and Efb-N interactions were examined in both orientations. Two-fold increasing concentrations (0.5 to 32 nM) of analytes (Efb-N or Fg-D) were injected over immobilized Fg-D (B) or Efb-N (C). Baseline corrected SPR response curves (shown in black, with lower curve corresponding to lower concentration of analyte injected) were globally fitted to the 1:1 binding model. The fitted curves are shown in red and the kinetic parameters obtained from the fitting are also listed.

Fig. 3. Efb and Efb-N aggregate Fg. Efb and Efb-N caused aggregation of Fg (A) but not Fg-D (B). Increasing concentration of Efb (squares), Efb-N (triangles) and Efb-C (circles) were incubated with either 2 µM of Fg (A) or Fg-D (B) and the turbidity of the solution was determined by measuring the optical density at 405nm.

Fig. 4. Efb-A (Efb30-67) and Efb-O (Efb68-98) bind to Fg. (A) Schematic presentation of recombinant Efb fragments. (B) ELISA-type assays of GST-tagged Efb fragments binding to immobilized Fg. Efb-A (Efb30-67, filled triangles) and Efb-O (Efb68-98, open squares) bound to Fg but Efb-L (Efb46-67, filled squares), Efb-I (Efb77-98, filled circles), Efb-K (Efb30-45, open triangles) and Efb-M (Efb68-76, open circles) did not interact with Fg.

Fig. 5. Efb-O binds with higher affinity than Efb-A to Fg-D. Characterization of the interaction of Fg-D with Efb by ITC. Binding isotherms for the interaction of Fg-D with GST-tagged Efb-O (Efb68-98) (A) and Efb-A (Efb30-67) (B) were generated by titrating the Efb fragments (~60 µM) into an ITC cell containing 6 µM Fg-D. The top panels showed heat difference upon injection of Efb fragments, and the lower panels showed integrated heats of injection and the best fit (solid line) to a single site binding model using Microcal Origin.

Fig. 6. Efb-O is the major Fg-binding site. The effect of Efb peptides on inhibition of recombinant Efb-N (A), Efb-A (Efb30-67) (B) and Efb-O (Efb68-98) (C) binding to immobilized Fg by the inhibition ELISA-type assays. Increasing concentration of Efb peptides were incubated with 10 nM GST fusion proteins in Fg coated mitrotiter wells. Peptide Efb-a (squares), Efb-o (triangles) and Efb-h (inverted triangles) correspond to Efb residues 30-67, 68-98 and 77-104, respectively. Efb-N corresponds to Efb residues 30-104. (D) The effect of truncated Efb-o peptides on binding of GST-Efb-N to immobilized Fg. Efb-o peptides lacking 3 (-N3) or 6 residues (-N6) at the N-terminus or 10 (-C10) or 15 (-C15) residues at the C-terminus lost the ability to inhibit Efb-N binding to Fg. Peptide Efb-o lacking 5 residues at the C-terminus (Efb-o-C-5) partially inhibited Efb-N binding to Fg compared to the control peptide Efb-o.

Fig. 7. Efb-N inhibits neutrophil binding to immobilized Fg by interacting with Fg. (A) Attachment of neutrophils to Fg immobilized on the glass coverslips was inhibited by addition of Efb and Efb-N but not by Efb-C, SdrG or PBS buffer (control). Error bars, mean ± SD, n=3 (B) Live image of neutrophils attachment to Fg-coated glass coverslips. Arrows indicate the bipolar or spherical neutrophils. (C) and (D) The effect of Efb preincubation with neutrophils (C) or Efb preincubation with Fg-coated coverslips (D) on neutrophil attachment. Attachment of neutrophils to immobilized Fg in PBS buffer (control, C and D) was inhibited by the present of Efb (Efb, C and D) and Efb-N (Efb-N, C and D), as well as by preincubation of Fg-coated glass coverslips with Efb (Fg+Efb, D) or Efb-N (Fg+Efb-N, D), but not by preincubation of neutrophils with Efb (PMN+Efb, C) or Efb-N (PMN+Efb-N, C). Addition of
SdrG (SdrG, D) or preincubation of Fg-coated coverslips with SdrG did not influence neutrophil attachment to Fg. Error bars, mean ± SD, n=3.

**Fig. 8.** Efb and Efb-N block Mac-1 cells adherence to immobilized Fg. (A) Attachment of the HEK293/Mac-1 cells (Mac-1 cells) to microtiter wells coated with Fg (control) was inhibited by anti-αM I domain mAb (44; 10 µg/ml), Fg γ chain peptides corresponded to Mac-1 binding site P1 (P1; 500 µM) and P2 (P2; 50 µM) and Efb-N (0.5 µM). Mac-1 cells did not attach to wells treated with PVA only. Error bars, mean ± SD, n=3. (B) Efb (0.5 µM) and Efb-N (0.5 µM) block Mac-1 cells attachment to immobilized Fg. Parental HEK293 cells did not attach to Fg. Error bars, mean ± SD, n=3. (C) Binding of Efb-N to microtiter wells coated with 0.25 µg/well of wild-type human and mouse Fg and mutant form of mouse Fgγ_{390-396A} lacking αMβ2 binding potential (7). Note the Efb-N binds to Fgγ_{390-396A} to the same extend as wild-type Fg. (D) Competition ELISA. Fg peptide P1, P2-C did not inhibit Fg (10 nM) from binding to immobilized Efb (0.25 µg/well).
Table 1: Thermodynamic parameters for the interaction of Fg-D with Efb-A and Efb-O

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Figure 1

A) Absorbance (450nm) vs Concentration (nM)

B) Absorbance (450nm) vs Fibrinogen concentration (nM)

C) Soluble Fg concentration (10^n nM) vs Absorbance (450nm), IC50 = 142 ± 1 nM

D) Efb-N concentration (nM) vs Absorbance (450nm)
Figure 2

A

Efb
Efb-N
Efb-C

Response (RU)

Time (s)

0 100 200 300 400 500 600

B

Efb-N → immobilized Fg-D

Response (RU)

Time (s)

0 100 200 300 400 500 600

\[ k_a = 5.03 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \]
\[ k_d = 1.14 \times 10^{-4} \text{ s}^{-1} \]
\[ K_D = 0.23 \text{ nM} \]

C

Fg-D → immobilized Efb-N

Response (RU)

Time (s)

0 200 400 600 800 1000

\[ k_a = 2.96 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \]
\[ k_d = 7.90 \times 10^{-4} \text{ s}^{-1} \]
\[ K_D = 2.67 \text{ nM} \]
Figure 3

A

B

Absorbance (405nm)

Efb concentration (µM)

Absorbance (405nm)

Efb concentration (µM)
**Figure 4**

A

<table>
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<th>SP</th>
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<td>165</td>
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**Efb fragments**

- **L** Efb46-67
- **A** Efb30-67
- **I** Efb77-98
- **O** Efb88-98
- **K** Efb30-45
- **M** Efb88-76

B

**Absorbance (450nm)**

**Efb concentration (nM)**

*Graph showing absorbance at 450 nm as a function of Efb concentration for different Efb fragments labeled L, A, I, O, K, and M.*
Figure 5

**A**  
Efb-O

**B**  
Efb-A

$K_D = 4.66$ nM  
$K_D = 1.0$ $\mu$M
Figure 6
Figure 7
Figure 8

A) Mac-1 cells

B) HEK293 vs. HEK293-Mac-1

C) PVA vs. Fg

D) Efb-N concentration vs. Peptide concentration
Binding of Efb from Staphylococcus aureus to fibrinogen blocks neutrophil adherence
Ya-Ping Ko, Xiaowen Liang, C. Wayne Smith, Jay L. Degen and Magnus Hook

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