Multiple Binding Sites in the Interaction between an Extracellular Fibrinogen-binding Protein from Staphylococcus aureus and Fibrinogen*

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Efb (previously Ftb) is a fibrinogen-binding protein secreted by Staphylococcus aureus. It has previously been shown that it plays a role in a wound infection model in the rat and that antibodies against Efb reduce the number of recovered bacteria from the mammary glands in a mouse mastitis model. Efb binds to the α-chain of fibrinogen and does not participate in bacterial adherence to fibrinogen. The binding of Efb to fibrinogen is divalent, with one binding site within the two repeat regions in Efb at the N terminus and one binding site at the C terminus. The divalent binding nature leads to precipitation of Efb-fibrinogen complex when the proteins are added to each other at a 1:1 molar ratio. The interaction between Efb and fibrinogen is strongly enhanced by Ca2+ or Zn2+ but not by Mg2+.

Five different fibrinogen-binding proteins (FgbPs) from Staphylococcus aureus have been described (1–4), implying a high complexity in the interaction between Fgb and S. aureus. The importance of the interaction between S. aureus and Fgb has been clearly demonstrated in an experimental model of endocarditis in the rat (5). Three different FgbPs that are secreted into the media have been purified from strain Newman (1); one of these is a coagulase of 87 kDa, and one is a 60-kDa protein also able to bind to prothrombin. The gene encoding the third secreted FgbP, a 15.6-kDa FgbP previously designated Efb, was cloned, and the nucleotide sequence was determined (6). These proteins are added to each other at a 1:1 molar ratio. The interaction between Efb and fibrinogen is strongly enhanced by Ca2+ or Zn2+ but not by Mg2+.

The fourth FgbP has been shown to be the major cause of adhesion of S. aureus to Fgb and is termed the clumping factor (Clf) (3, 7). It is cell surface-associated as a result of an LPXTG motif anchoring Clf to the peptidoglycan (8). The fifth FgbP is a cell surface-associated coagulase with unknown function (4).

The nucleotide sequence of Efb revealed two highly homologous 22-amino acid repeats in the N-terminal region separated by a 9-amino acid spacer. These repeats are homologous to the five (9) or eight (10) 27-amino acid repeats found at the C-terminal end of coagulase. In coagulase, these repeats have been suggested to be responsible for the Fgb binding activity, whereas the N-terminal end of coagulase is responsible for the coagulase activity and also includes a prothrombin binding domain (9, 11–13).

An allele replacement mutant of Efb has been constructed where the efb gene was replaced with a gene encoding gentamicin resistance (14). The adherence of the efb negative mutant strain to immobilized Fgb was unaltered compared with the parental strain, indicating that adherence is not directly dependent on Efb.

The Efb negative mutant was used in an experimental wound infection model in the rat. Compared with the isogenic parental strain, the Efb negative mutant resulted in significantly reduced severity of signs of infection (14). Furthermore, in a vaccination study using a mouse mastitis experimental infection model, antibodies against Efb were shown to significantly reduce the number of bacteria recovered from the mammary glands, and the level of histopathological signs of infection was reduced (15). These two findings together and the high incidence of the Efb protein among S. aureus isolates suggest that Efb is an important virulence factor worth further investigation.

The biological function of Efb is not known, but the observation that the severity of S. aureus-infected wounds is influenced by Efb implies that wound healing is delayed due to its binding to Fgb, probably affecting the clotting process.

We have here compared Efb and Clf and found that Efb binds to Fgb in a different way than Clf and that Efb has two binding sites for Fgb leading to precipitation of the Efb-Fgb complex.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Culture Conditions—S. aureus strain Newman was radiolabeled by growing in Luri-Bertani (LB) medium for 5 h at 37 °C in the presence of 50 μCi of [3H]thymidin (specific activity 80 mCi/mmol). The cells were washed with phosphate-buffered saline (PBS) and then resuspended in PBS containing 0.05% Tween 20 (PBST) to an A600 = 1.0.

Purification of Efb and Clumping Factor—One liter of S. aureus strain Newman was grown for 19 h at 37 °C in LB. The culture was centrifuged, and FgbPs from the supernatant were isolated by affinity
chromatography on Fg-Sepharose (Amersham Pharmacia Biotech) as described before (16). Proteins were eluted with 0.7% acetic acid, dialyzed against 40 mM phosphate buffer, pH 6.5 (buffer A), and subjected to fast protein liquid chromatography on a Mono S column using a gradient of 0 to 100% buffer B (1 M NaCl in buffer A). Three peaks of proteins were eluted, and the second one, which eluted at a salt concentration of 0.35–0.45 M NaCl, contained Efb.

Clf was purified from Escherichia coli XL-1-harbor ing plasmid pCF33, derived from pQE30 (Qiagen, Basel, Switzerland), expressing a His6 fusion protein comprising residues 221–550 of the Clf protein. This plasmid was kindly supplied to us by T. J. Foster (Dublin, Ireland) (7). The His6_Clf fusion protein was purified using nickel chelator according to the instructions provided by Qiagen.

**Construction of GST-RR and Efb210 Protein**—The 5’ part of the efb gene encoding the two repeat regions (amino acids 8–69) was amplified by PCR using oligonucleotide primers, efb R.U. with the sequence 5′-TGAAGGGATCCCACAATATCGTAGAG-3′ and efb R.L. 5′-AGTTCCCGGGGTTCGCTGCTGGTT-3′ (obtained from CytoGen AB, Hudding, Sweden). The plasmid pBFibIII (2) was used as a template, and the reaction contained 5 pmol of each primer, 0.2 mM dNTPs, 0.5 units Taq polymerase, and the thermal step program included denaturation at 94 °C for 10 s, annealing at 50 °C for 20 s, and extension at 72 °C for 30 s. The DNA fragment was digested with BamHI and SmaI (efb R.U. has the BamHI site and efb R.L. has the SmaI site incorporated into the BamHI-SmaI sites of the vector pGEX (Amersham). For cloning, e. coli strain XL1-Blue was used, and colonies with a recombinant plasmid containing the two repeat regions were detected by PCR using the primers efb R.U. and efb R.L. For expression of the recombinant protein (GST-RR), the e. coli strain BL21 was used. GST-RR protein was affinity-purified by glutathione-Sepharose using the procedure recommended by the manufacturer (Amersham).

A recombinant protein containing the C-terminal part of Efb (Efbc210; in ref. 2, called Fb210) (amino acids 70–136) was purified from e. coli using the previously described plasmid pFbC210 derived from pQE12 (Qiagen) (2). The pQE12 vector contains an affinity tag of His6 fused to the C-terminal end of the recombinant protein. Efbc210 has been purified both on the nickel nitriotriacetic acid resin and on Fg-Sepharose. Both the Efb210 peptide and the GST-RR were analyzed by SDS-PAGE and by immunoblot probed with Fg (Sigma) followed by horseradish peroxidase (HRP)-conjugated anti-Fg antibodies (DAKO, Glostrup, Denmark).

**Adherence of S. aureus to Fg**—A 96-well mitrotiter plate (Falcon; Becton Dickinson and Co., New Jersey) was coated with 100 μl of 2 μg/ml Fg at room temperature overnight. The wells were blocked by incubation with 2% bovine serum albumin (Sigma) in PBS for 1 h at 37 °C. After washing three times with PBST, 50 μl of a 3H-labeled s. aureus suspension (106 cells in PBST) and 50 μl of a solution containing the protein used as an inhibitor (Efb or Clf) were added. After a 2-h incubation at 37 °C, the wells were washed three times with PBST, and bound bacteria were released by the addition of 2 × 105 μl of SDS for 2 × 30 min at RT. The amount of bound bacteria was measured by scintillation counting.

**Binding of Fg to Clf and Efb**—Microwell plates (96 wells, Falcon) were coated with Efb or Clf (10 μg/ml) in 100 μl of PBS at room temperature overnight, and nonspecific binding sites were blocked with 2% bovine serum albumin for 1 h at 37 °C. A series of different amounts of Clf in 50 μl of PBST (0.01–30 μg) and a constant amount of Fg (10 ng) in 50 μl of PBST were added to the wells followed by a 2-h incubation at room temperature. Bound Fg was subsequently detected by HRP-conjugated rabbit immunoglobulins against Fg diluted 1:1000 in PBST. The plate was washed with PBST after every incubation.

**Precipitation Assay of Efb-Fg**—Efb-Fg precipitation in solution was measured in microplate wells. One hundred μl of PBS solutions with Fg (8.6, 4.3, 2.1, or 0 μg/ml) were added to columns 1, 2, 3, and 4. One hundred μl of PBS with Efb (600, 300, 150, 75, or 37 μg/ml) were added to wells in rows A, B, C, D, and E, respectively. The plate was incubated 30 min at room temperature, and the increase in absorbance caused by the precipitation was measured at 405 nm using a microplate reader.

**Comparison of Binding Characteristics of Clf and Efb**—The Fg-binding protein clumping factor (Clf), which is present on the bacterial cell surface, has been shown to mediate adherence of S. aureus to Fg (3). In contrast, a mutant strain lacking the efb gene has been shown to have unaltered adherence properties (14). This result was not surprising, since the Efb protein is secreted into the medium and is not present on the bacterial cell surface. However, it seemed possible that Efb might influence adherence by competition with Clf for a common binding site on Fg. The adherence of radiolabeled S. aureus to immobilized Fg was measured in the presence of Clf or Efb. The presence of Clf decreased bacterial adherence by up to 50%. In contrast, the presence of Efb did not reduce the adherence as shown in Fig. 1, implying different binding sites for Efb and Clf on Fg.

**Independent binding of Clf and Efb to Fg** was verified by a capture inhibition ELISA. Purified Clf or Efb were immobilized on microtiter wells, and Fg was captured to these molecules in the presence of soluble Clf at various concentrations. The presence of Clf in the assay had no influence on the interaction...
between Fg and immobilized Efb. In contrast, soluble Clf inhibited up to 90% of the binding of Fg to immobilized Clf as shown in Fig. 2. In a similar experiment where various concentrations of Efb were added to assess its inhibitory activity on Efb to Fg binding, a precipitate was formed between Efb and Fg (see below).

To further establish that Clf and Efb have different binding sites on Fg, the α-, β-, and γ-chains of Fg were separated by SDS-PAGE and subjected to a Western affinity blot using Efb or Clf as probes followed by the respective antibodies. Fig. 3 shows that Clf bound to the γ-chains, as shown before (17, 18), whereas Efb bound to the α-chain.

Precipitation of Efb and Fg—Precipitation of antibody-antigen complexes is often visualized in a double diffusion test in agarose. A precipitation line is formed between the wells containing antibody or antigen at a position where the concentrations of each are optimal for precipitation. The same method was used here to demonstrate that the interaction between Fg and Efb leads to precipitation (Fig. 4).

Precipitation could also be visualized in solution. Efb was added at various concentrations to solutions of Fg in microtiter wells using three different concentrations of Fg. A precipitate was formed that could be determined by the increased light absorbance. In Fig. 5A it is seen that the higher the Fg concentration that was used, the higher the Efb concentration that was required to obtain maximal precipitation; increasing the Fg concentration twice or four times gave the most precipitation at twice or four times the concentration of Efb. An excess of either Efb or Fg resulted in less precipitation. It was calculated that maximal precipitation was obtained at a molar ratio close to 1:1. The precipitate formed was threadlike, and the solution never became solid as is the case in coagulation. Purified coagulase and purified Fg gave only weak coagulation after a long time, most likely due to trace amounts of contaminating prothrombin. No optimal ratio for clotting was found for coagulase and Fg. Instead, less coagulase just required more time to produce clotting. Furthermore, the Efb/Fg precipitate was washed extensively in PBS, boiled in loading buffer, run on SDS-PAGE, and Comassie-stained. The three α-, β-, and γ-chains were seen together with Efb in what appeared to be equimolar amounts (data not shown).

Each molecule of Fg has been shown to bind three Ca²⁺ ions, which is important for the structure and functional role of Fg during coagulation (19). Therefore, the interaction between Fg and Efb was tested for its dependence on divalent cations. Fg and Efb were dialyzed separately against EDTA to remove metals associated with the proteins, and a determination of the effect of various concentrations of divalent cations on the precipitation of Fg/Efb mixtures was tested in microtiter wells. Enhancement of precipitation was obtained by the addition of Ca²⁺ and Zn²⁺ but not by Mg²⁺ (Fig. 5B).

Fg Interaction with the C- and N-terminal Portions of Efb—To cause precipitation of Fg, multiple binding sites in Efb would be required. To test this, the N-terminal part of Efb containing the two repeat regions was expressed as a fusion protein with glutathione S-transferase. This protein was designated GST-RR. Also the C-terminal part was expressed separately as a His₉ fusion protein, Efb210, as described previously (2). Fig. 6 shows that Fg was able to bind to each of these fusion proteins in a capture ELISA. As negative controls, GST alone and another His₉ fusion protein were tested for capture of Fg. Fg could not bind to any of these proteins (data not shown).

Both Clf, GST-RR (the Efb repeat regions) and the Efb210 protein (the C-terminal portion) were tested in both precipitation assays, but no precipitation was seen with these proteins (data not shown).

Both the purified Efb210 protein and GST-RR could be further purified by fibrinogen-Sepharose, demonstrating affinity for both parts of Efb for fibrinogen. This confirms earlier findings (2) (not shown here).

DISCUSSION

We have shown here that the binding of Efb to Fg involves two separate binding sites on Efb. The two repeat regions at the N terminus of Efb (amino acids 8–69), which are homologous to the repeat regions at the C terminus of coagulase, constitute one binding region. This is in agreement with suggestions that
A

B

the repeat regions in coagulase bind to Fg. Another binding domain is located at the C terminus of Efb (amino acids 70–136). The Fg binding to the C- and N-terminal ends of Efb was demonstrated by capture ELISA and by the ability to purify the protein on Fg-Sepharose.

Binding of Efb to Fg clearly takes place to the \( \alpha \)-chain of Fg as compared with the binding of Clf, which is to the \( \gamma \)-chain, shown here and previously (18). It is not yet clear if the binding of Efb to the \( \alpha \)-chain is due to the binding of the repeat regions or due to the C-terminal part or if both binding domains of Efb recognize the \( \alpha \)-chain. The exact binding site(s) on Fg is presently under investigation.

Efb was unable to compete with S. aureus for adherence to Fg. Combined with our previous finding that a mutant lacking the \( efb \) gene retained wild type adherence properties (14), it is likely that Efb is not involved in adherence. No enzymatic function of Efb has been shown, and its binding to Fg seems, therefore, to be the only function of Efb. In the case of coagulase, the binding to Fg is logical, since coagulase forms a complex with prothrombin that binds and converts Fg to fibrin, resulting in clot formation. A biological function of Efb, with no other activity than the ability to bind to Fg, is not obvious and can only be speculative.

We have demonstrated earlier that Efb plays an important role in a wound infection model in the rat. Fibrin is a major component of blood clots in wounds. A possible beneficial effect of Efb for the bacterium, by binding to Fg and affecting the conversion of Fg to fibrin, thereby delaying the wound healing process, is under investigation.

Fg is a symmetric protein with two chains each of \( \alpha \), \( \beta \), and \( \gamma \), and each \( \alpha \)-chain has a recognition site for Efb. Therefore, there are at least two recognition sites for Efb on each Fg molecule. Also, both the N- and C-terminal halves of Efb can bind Fg. This would permit the potential formation of three different combinations of two Efb with one Fg and also the formation of larger aggregates of these complexes. The resulting aggregate might be the precipitate that is observed during Efb-Fg interactions. Such a precipitate structure is supported by the finding that the optimal molar ratio between Efb and Fg to get precipitation is 1:1.

In addition, the presence of Ca\(^{2+}\) or Zn\(^{2+}\) enhances the precipitation of the proteins from equimolar mixtures of Efb and fibrinogen. Ca\(^{2+}\) is known to bind to Fg (19, 20) and is involved in blood clotting and wound healing. Fg and possibly also Efb contain Ca\(^{2+}\) binding sites, Fg at the C-terminal part of the \( \gamma \)-chain (DNDNDKFEGNC), and a putative Ca\(^{2+}\) binding site is found in the second repeat region of Efb. Ca\(^{2+}\) may facilitate the interaction between Fg and Efb, possibly by causing some conformational change in one or both of the molecules.

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