The Fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* Is a Bifunctional Protein That Also Binds to Fibrinogen*

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*1 The abbreviations used are: MSCRAMM, microbial surface component recognizing adhesive matrix molecules; Fn, fibronectin; Fg, fibrinogen; ClfA and ClfB, clumping factors A and B; FnbpA and FnbpB, fibronectin-binding proteins A and B; PFR, peroxidase; PAGE, polyacrylamide gel electrophoresis.

*2 The Fiberlink-binding MSCRAMPs, FnbpA and FnbpB, have been previously identified. The FnbpA and FnbpB, have been previously identified. The

*3 Staphylococcus aureus* is an important pathogen capable of causing a wide spectrum of diseases in humans and animals. This bacterium expresses a variety of virulence factors that participate in the process of infection. These include MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that mediate the adherence of the bacteria to host extracellular matrix components, such as collagen, fibronectin (Fn), and fibrinogen (Fg). Two Fn-binding MSCRAMMs, FnbpA and FnbpB, have been previously identified. The Fn binding activity has been localized to the ~40-amino acid residue D repeats in the C-terminal part of these proteins. However, no biological activity has yet been attributed to the N-terminal A regions of these proteins. These regions exhibit substantial amino acid sequence identity to the A regions of other staphylococcal MSCRAMMs, including ClfA, ClfB, and SdrG (Fbe), all of which bind Fg. This raises the question of whether the Fn-binding MSCRAMMs can also bind specifically to Fg. In this report, we show that a recombinant form of the A region of FnbpA does specifically recognize Fg. We localize the binding site in Fg for recombinant FnbpA to the γ-chain, in particular to the C-terminal residues of this polypeptide, the site also recognized by ClfA. In addition, we demonstrate that recombinant FnbpA can compete with ClfA for binding to both immobilized and soluble Fg. By the use of surface plasmon resonance spectroscopy and fluorescence polarization, we determine the dissociation equilibrium constant for the interaction of recombinant FnbpA with intact immobilized Fg and with a synthetic C-terminal γ-chain peptide, respectively. Finally, by overexpressing FnbpA in a mutant strain of *S. aureus* that lacks the expression of both ClfA and ClfB, we show that native FnbpA can mediate the interaction of *S. aureus* with soluble Fg.

*Staphylococcus aureus* is an important opportunistic Gram-positive bacterial pathogen of humans and animals. It is a highly versatile bacterium that is capable of causing a wide spectrum of diseases. These range from superficial skin infections to more serious and potentially fatal illnesses such as endocarditis, septic arthritis, pneumonia, and septicemia. It is also a major cause of infections associated with indwelling medical devices, such as catheters and prostheses (1). *S. aureus* produces a variety of cell surface-associated and extracellular factors that enable the bacteria to colonize and multiply within the host, evade host defenses and destroy host tissue, causing the symptoms of disease. Colonization of host tissue is considered a critical first step in the infection process. A family of cell surface adhesins, called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), that specifically bind to host extracellular matrix components, are involved in this colonization (2, 3). *S. aureus* MSCRAMMs that bind to collagen, fibronectin (Fn), and fibrinogen (Fg) have been identified and characterized in detail (reviewed in Ref. 4).

Fg is a 340-kDa glycoprotein that is found at a concentration of ~9 μM in the blood. It is composed of six polypeptide chains, two Aα-, two Bβ-, and two γ-chains, that are arranged in a symmetrical dimeric structure. Fg is an important clotting protein that participates in controlling blood loss following vascular injury. It acts as an adhesive protein that mediates platelet adherence and aggregation at sites of injury by interacting with the integrin γ<sub>1bβ<sub>3</sub> on the platelet surface. In addition, it is cleaved by thrombin to form fibrin, which is the major component of blood clots. Various cleavage products of Fg and fibrin, released during coagulation and fibrinolysis, respectively, also exhibit biological activities. These include the regulation of cell adhesion, cell spreading, and vasocostrictive and chemotactic activities (reviewed in Ref. 5). Fg is also one of the main proteins deposited on implanted biomaterials.

*S. aureus* expresses a large number of proteins that can bind specifically to Fg. Some of these proteins are MSCRAMMs, such as the clumping factors A and B (ClfA and ClfB) (6, 7). Others are largely secreted proteins, such as coagulase, Efb, and Map (8–12). Whereas the Fg-binding MSCRAMMs are important adhesins, mediating the adherence of *S. aureus* to host tissues and biomaterials, the role of the secreted Fg-binding proteins in the infection process is unclear (6, 8, 13–16). ClfA is the prototype Fg-binding MSCRAMM of *S. aureus*, having been studied in the greatest detail. The Fg binding activity has been localized to residues 221–559 in the N-terminal A region of this protein (Fig. 1, ClfA1) (17). It has been determined that ClfA binds to the γ-chain of Fg, in particular to the C-terminal residues of this polypeptide (18, 19). This site is also recognized by the platelet integrin γ<sub>1bβ<sub>3</sub> and ClfA can inhibit Fg-mediated platelet aggregation and the adherence of platelets to immobilized Fg under conditions of fluid shear stress (18). Furthermore, the Fg binding activity of both ClfA

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and ε_{0,2} β_{2} is regulated by divergent cations, such as Ca^{2+} and Mn^{2+} (19).

Two Fn-binding MSCRAMMs have been identified in *S. aureus*, FnbpA and FnbpB, and the corresponding genes (fnbA and fnbB, respectively) have been cloned and sequenced (20–23). Whereas some strains of *S. aureus* have both fnb genes, such as strains 8325-4, Newman, and P1, others carry only fnbA. The Fnbps have structural features that are common to other surface proteins expressed by Gram-positive bacteria, including ClfA (Fig. 1). A signal sequence (region S), involved in Sec-dependent transport of the proteins across the cytoplasmic membrane, is located at the N terminus of these proteins. At the C terminus are features that are required for anchoring the proteins to the cell wall. These include a stretch of hydrophobic residues (region M), thought to span the cell membrane, and an LPXTG motif that is the target of a specific enzyme (“sortase”). This enzyme cleaves the motif between the threonine and glycine residues and subsequently covalently anchors the protein to the peptidoglycan cell wall (24–26). The Fn binding activity of the Fnbps has been localized to the −40-amino acid residue repeat units (D repeats) at the C terminus of these proteins. FnbpA and FnbpB of *S. aureus* strain 8325-4 both contain four tandemly repeated units (D1–D4), and a fifth repeat (Du) is found at −100 amino acid residues N-terminal to D1 (22, 23, 27). These repeats are highly conserved between the two proteins (~94% amino acid identity), and homologous repeats are also found in the Fn-binding MSCRAMMs expressed by several streptococcal species (28). The N-terminal A regions of the Fnbps are ~500 amino acid residues long and are less similar, sharing ~40% amino acid identity. In the case of FnbpA, two further repeats (B repeats) of ~30 amino acid residues, of unknown function, are located at the C-terminal end of the A region.

No biological activity has yet been attributed to the A regions of the Fnbps. However, it has been noted previously that the A region of FnbpA exhibits substantial amino acid sequence similarity with the A region of ClfA (6). This raises the question of whether this Fn-binding MSCRAMM can also bind specifically to Fg. In this report, we investigate this possibility and show that a recombinant form of the A region of FnbpA does specifically recognize Fg. We localize the binding site in Fg for recombinant FnbpA to the γ-chain, in particular to the C-terminal residues of this polypeptide. In addition, we demonstrate that recombinant FnbpA can compete with ClfA for binding to both immobilized and soluble Fg. By the use of surface plasmon resonance spectroscopy and fluorescence polarization, we determine the dissociation equilibrium constant for the interaction of recombinant FnbpA with intact immobilized Fg and with a synthetic C-terminal γ-chain peptide, respectively. Finally, by overexpressing FnbpA in a mutant strain of *S. aureus* that lacks the expression of both ClfA and ClfB, we show that native FnbpA can mediate the interaction of *S. aureus* with soluble Fg.

**EXPERIMENTAL PROCEDURES**

**Bacterial Culture Conditions—** *Escherichia coli* strains carrying plasmids were routinely cultured in Lennox L broth (Sigma) or on Lennox L agar (Sigma), containing ampicillin at 100 μg/ml. *S. aureus* was cultured in trypticase soy broth (Difco), containing tetracycline at 2 μg/ml or erythromycin at 10 μg/ml for the selection of chromosomal markers, and chloramphenicol at 10 μg/ml for plasmid selection, when appropriate.

**Manipulation of DNA—** DNA restriction and modification enzymes were purchased from Life Technologies, Inc. and were used according to the manufacturer’s instructions. All DNA manipulations were performed using standard procedures (29).

**Transformation and Transduction—** *E. coli* strain JM101 (Stratagene) and strain Topp3 (Stratagene) were made competent by CaCl\(_2\) treatment (29). The clfA2/Topp17 and clfB/Tc\(_6\) chromosomal mutations were transduced from *S. aureus* strain DUL5876 and strain DUL5943 (6, 7), respectively, into *S. aureus* strain P1 (30), using bacteriophage 85, yielding strain TAMU3 (31). The recombinant plasmid pFNBA4, expressing FnbpA of *S. aureus* strain 8325-4, was transduced from strain DUL5876(pFNBA4) (32) into strain TAMU3, using bacteriophage 85. The *S. aureus* strain TAMU3(pFNBA4). Suc cationic proteins of *S. aureus* strain DUL5876, DUL5943, P1, and DUL5838(pFNBA4) were kindly provided by Professor Timothy J. Foster (Trinity College, Dublin, Ireland).

**PCR Amplification of fnbA Gene Fragments—** A DNA fragment encoding the A region and two B repeats of FnbpA (residues 37-605) was amplified by PCR from plasmid pFNBA3 (Ref. 32; kindly provided by Professor J. Foster) using bacteriophage Pfu DNA polymerase. The oligonucleotide primers used were 5'-AAAGGTATCCGGCTAGAACAAGACAAACAA-3' and 5'-AGAGTCGAC-CTACAGAGAATTTCACGTTGAGAATGTA-3'. A DNA fragment encoding the full-length FnbpA (residues 37-881, which excludes regions W and M) was amplified from plasmid pFNBA6 using the oligonucleotide primers 5'-AAAGGTATCCGGCTAGAACAAGACAAACAA-3' and 5'-GGGTCGAC-TTATGGCGTTGGTGGCACGAT-3'. Restriction enzyme cleavage sites were incorporated at the 5' ends of the primers (underlined) to facilitate directional cloning of the PCR fragments. The PCR reactions were carried out using a Perkin-Elmer Cetus DNA thermocycler. The reactions contained 50 ng of template DNA, 100 pmol of forward and reverse primers, 10 mM KCl, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 20 mM Tris-HCl (pH 8.0), 2 mM MgSO\(_4\), 0.1% Triton X-100, and 2 units of Pfu DNA polymerase (Life Technologies, Inc.). The reactions were overlaid with 100 μl of mineral oil and amplified for 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 5 min. The reactions were incubated at 72 °C for an additional 10 min upon completion of the 25 cycles. After amplification, the products were analyzed by agarose gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s instructions.

**Construction of FnbpA Expression Plasmids—** Amplified fnbA fragments were cloned into the expression plasmid pQE30 (Qiagen), generating constructs pFnbpA2 (37-605) and pFnbpA5 (37-881). The recombinant proteins, called rFnbpA3 (37-605) and rFnbpA5 (37-881), respectively, contain an N-terminal extension of six histidine residues (His tag).

**Expression and Purification of Recombinant Proteins—** Recombinant plasmids were cloned in *E. coli* strain JM101 and subsequently transformed into strain Topp3 for expression of recombinant proteins. Bacterial cell lysates containing recombinant FnbpA and ClfA proteins were prepared as described previously (19, 28). Fusion proteins containing the N-terminal His tag were purified by immobilized metal chelate affinity chromatography. A 5-ml HiTrap column (Amersham Pharmacia Biotech) was connected to a fast protein liquid chromatography system and charged with 97.5 mM Ni\(^{2+}\) equilibrated with buffer A (0.1 mM NaCl and 10 mM Tris-HCl, pH 7.9). The cleared and filtered bacterial cell lysate was applied to the column, and the column was washed with 10 bed volumes of buffer A containing 5 mM imidazole. The bound protein was eluted with a continuous linear gradient of imidazole (5–120 mM; total volume of 160 ml) in buffer A. Flow-through containing recombinant protein, as determined by absorbance at 280 nm, was pooled, dialyzed against 25 mM Tris-HCl, pH 8.0, and applied to a 5-ml HiTrap Q-Sepharose column (Amersham Pharmacia Biotech) that had been equilibrated with the same buffer. Bound protein was eluted with a continuous linear gradient of NaCl (0–0.5 M; total volume of 160 ml) in buffer A. Eluted fractions containing recombinant protein, as determined by absorbance at 280 nm, were pooled, dialyzed against 25 mM Tris-HCl, pH 8.0, and applied to a 5-ml HiTrap Q-Sepharose column (Amersham Pharmacia Biotech) that had been equilibrated with the same buffer. Bound protein was eluted with a continuous linear gradient of NaCl (0–0.5 M; total volume of 160 ml) in buffer A. Eluted fractions containing recombinant protein, as determined by absorbance at 280 nm, were pooled, dialyzed against 25 mM Tris-HCl, pH 8.0, and applied to a 5-ml HiTrap Q-Sepharose column (Amersham Pharmacia Biotech) that had been equilibrated with the same buffer. Bound protein was eluted with a continuous linear gradient of NaCl (0–0.5 M; total volume of 160 ml) in buffer A. Eluted fractions containing recombinant protein, as determined by absorbance at 280 nm, were pooled, dialyzed against 25 mM Tris-HCl, pH 8.0, and applied to a 5-ml HiTrap Q-Sepharose column (Amersham Pharmacia Biotech) that had been equilibrated with the same buffer. Bound protein was eluted with a continuous linear gradient of NaCl (0–0.5 M; total volume of 160 ml) in buffer A.

**Purification of Fg and Fn for Binding Studies—** For enzyme-linked immunosorbent assays (ELISAs), human Fg (Chromogenix AB) was made free of contaminating Fn by purification over a gelatin-Sepharose column. The purity of the Fg was then assessed by Western immunoblot analysis using anti-human Fg polyclonal antibodies (Abs, ICN Biomedicals, Inc.). The concentration of the purified Fg solution was determined by absorbance at 280 nm, using an extinction coefficient (1%) of 15.1. Fn was purified from human plasma using a gelatin-Sepharose column, as described by Vuento and Vaheri (33), and the concentration was determined by absorbance at 280 nm, using an extinction coefficient (1%) of 12.8. The purity of the Fn was assessed by SDS-PAGE, followed by staining with Brilliant Blue R (Sigma). For surface plasmon resonance (SPR) and the bacterial clumping assays, a commercial source of Fn-depleted human Fg was used (Enzyme Research Laboratories).

**Analysis of the Fg Binding Activity of Recombinant Proteins: Western Blot Assay—** Proteins were fractionated on 10% polyacrylamide gels according to standard procedures (34). The proteins were either stained with Brilliant Blue R or transferred to a nitrocellulose membrane.
(Proteus, Schleicher & Schuell) using a semidyed blotter (Bio-Rad) and 48 mm Tris-HCl, 39 mM glycine (pH 9.2), and 20% methanol for 20 min at 15 V. Remaining protein binding sites were blocked by incubating the membrane for 18 h at 4 °C in 10% (v/v) nonfat dry milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.5). The E. coli strain used was strain 168, in F. coli, all proteins were labeled using sulfo-NHS-biotin (Pierce), according to the manufacturer's instructions (Bio-Rad). For the ligand affinity blots, membranes were incubated with the recombinant FnbpA and C1f proteins (20 μg/mL) for 1.5 h at room temperature, followed by incubation with a 1:5000 dilution of anti-protein A-his monoclonal antibody (mAb) (Clontech) in TBS, 0.1% (v/v) nonfat dry milk and a 1:20,000 dilution of goat anti-mouse HRP-conjugated polyclonal Ab in Ab-Rad in TBS, 0.1% (v/v) nonfat dry milk for 1 h each at room temperature. Bound HRP-conjugated Abs were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

ELISA—An ELISA was used to analyze the ability of recombinant proteins to bind to immobilized Fn-depleted human Fg and human Fn. Wells of a microtiter plate (Immulon 4, Dynatech Laboratories Inc.) were coated with 1 μg of Fg or Fn in TBS, pH 7.5, for 18 h at 4 °C. Plates were washed three times with TBS, and remaining protein binding sites were blocked with 5% (w/v) bovine serum albumin (BSA) in TBS for 2 h at room temperature. The wells were then washed three times with TBS, purified recombinant proteins, diluted in TBS, 0.1% (v/v) BSA, were added; and the plates were incubated for 1 h at room temperature. The plates were again washed with TBS, and bound protein was detected by incubation with a 1:3000 dilution of anti-His tag mAb (Clontech) in TBS, 0.1% (v/v) BSA for 1 h at room temperature. After three further washes with TBS, a 1:2000 dilution of goat anti-mouse AP-conjugated polyclonal Ab (Bio-Rad) in TBS, 0.1% (v/v) BSA was added to the wells and the plates were incubated for 1 h at room temperature. Finally, bound AP-conjugated Abs were detected by the addition of p-nitrophenyl phosphate/nitroblue tetrazolium chloride color development solution, according to the manufacturer's instructions (Bio-Rad). For the ligand affinity blots, membranes were incubated with the recombinant FnbpA and C1f proteins (20 μg/mL) for 1.5 h at room temperature, followed by incubation with a 1:5000 dilution of anti-protein A-his monoclonal antibody (mAb) (Clontech) in TBS, 0.1% (v/v) nonfat dry milk and a 1:20,000 dilution of goat anti-mouse HRP-conjugated polyclonal Ab in Ab-Rad in TBS, 0.1% (v/v) nonfat dry milk for 1 h each at room temperature. Bound HRP-conjugated Abs were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

For the inhibition ELISAs, the recombinant proteins were pre-incubated for 1–1.5 h at room temperature in TBS, 0.1% (w/v) BSA, soluble recombinant Fn-depleted human Fg (FGd), were analyzed using the same method as described above. For the inhibition ELISAs using biotinylated proteins, the recombinant proteins were labeled using sulfo-NHS-biotin (Pierce), according to the manufacturer’s method. Biotinylated proteins were added to the Fn-coated wells together with the unlabelled proteins in TBS, 0.1% (w/v) BSA for 1 h at room temperature. Bound biotinylated protein was detected by incubation with a 1:10,000 dilution of AP-conjugated streptavidin (Roche Molecular Biochemicals) in TBS, 0.1% (w/v) BSA for 30 min at room temperature. Development was carried out as described above.

Positive and negative controls for the interaction of FnbpA with immobilized Fg were determined by SPR using the Blacore 1000 system (Pharmacia Biosensor AB). Fn-depleted human Fg was covalently immobilized on CM5 sensor chips, as described previously (18). A uncoated “blank” chip was also prepared by activating the dextran surface of the chip, followed by blocking of the activated sites with 1M ethanolamine hydrochloride. Increasing concentrations of the recombinant Fg (2000–4000 resonance units (RU)), at a flow rate of 5 μl/min, were injected over the immobilized Fn-depleted human Fg. The Fn-depleted human Fg-coated chips (rFnbpA) were coated with 1 μg of Fg in the dark. To determine the effect of divalent cations on the interaction of FnbpA with immobilized Fg, rFnbpA was dialyzed extensively from HBS-EDTA into HBS (without EDTA). CaCl₂, MgCl₂, or MgCl₂ was then added to the binding assay at concentrations ranging from 0.01 mM to 20 mM. Polarization measurements were taken with an LS50B luminescence spectrometer (Perkin Elmer) using FL-WinLab software (Perkin Elmer). Binding data were analyzed by nonlinear regression used to fit a binding function as defined by Equation 1.

Bacterial Clumping Assay—Bacterial clumping assays were carried out in flat-bottomed microtiter plates (Immulon 4, Dynatech Laboratories, Inc.). Serial twofold dilutions of Fn-depleted human Fg were made in TBS, pH 7.5, from a starting concentration of 2 mg/mL. S. aureus strain DSM943, a CIB-negative mutant of strain Newman (7), was used to analyze the ability of recombinant FnbpA to inhibit CIB-mediated cell clumping. An 18-h culture of this strain was diluted 1:50 into fresh trypticase soy broth and grown to postexponential phase (6 h) with aeration. The cells were washed once in TBS and resuspended in one-fifth of the original culture volume. Then, 20 μl of the concentrated cell suspension (~2.5 × 10⁶ colony-forming units) was added to 25 μl of each Fg dilution and 25 μl of TBS in the wells of the plate. For the inhibition assays, 25 μl of the test protein was substituted for the TBS in each well. The plate was agitated vigorously for 5 min and visually examined for clumping. The clumping titer was taken as the lowest concentration of Fg at which clumping was still visible.

To analyze the ability of cell surface-expressed FnbpA to mediate cell clumping, S. aureus strains P1, TAMU3 (a CIA- and CIB-negative mutant of strain P1), and TAMU3(pFNBA4) were used. An 18-h culture of these strains was diluted 1:50 into fresh trypticase soy broth and grown to early exponential phase (1.5 h) with aeration. The cells were washed once with TBS and resuspended in one-fortieth of the original culture volume. The clumping assays were then performed as described above, adding ~5 × 10⁶ colony-forming units to each well.

RESULTS

Specific Binding of Recombinant FnbpA to Fg—A homology search of the GenBank data bank with the amino acid sequence of the A region of FnbpA, using BLASTP software, revealed that this protein exhibits homology to several other staphylococcal MSCRAMMs (data not shown). In particular, this sequence was found to share ~25% amino acid identity with the ligand-binding regions (A regions) of CIA and CIB from S. aureus and SdrG (Fhe) from S. epidermidis, all of which are known to bind Fg (4, 36).² This raised the possibility that FnbpA may also bind to Fg. To address this question, two recombinant proteins with N-terminal His tags, encompassing the A region and two B repeats of FnbpA, rFnbpA(37-605) and the full-length protein (rFnbpA(37-881)), were expressed (Fig. 1). The purity of the isolated recombinant proteins was verified by SDS-PAGE. In this system, the rFnbpA(37-605) and rFnbpA(37-881) proteins migrated slower than predicted from

² S. Davis, K. McKee, T. J. Foster, and M. Höök, manuscript in preparation.
their molecular masses of 64 and 94 kDa, respectively, (data not shown). The acidic nature of these polypeptides (pI of 4.7 and 4.4, respectively) may explain this observation.

The Fg binding activity of the recombinant FnbpA proteins was assessed by ELISA and compared with that of Clf40, a recombinant protein encompassing the Fg-binding region (A region) of ClfA (Fig. 1) (19). Both rFnbpA(37-881) and rFnbpA(37-605), as well as Clf40, bound to the immobilized Fg in a dose-dependent, saturable manner in this assay (Fig. 2A). However, only rFnbpA(37-881), which contains the C-terminal Fn-binding D repeats, bound to immobilized Fn (Fig. 2B). This suggests that recombinant FnbpA can bind specifically to immobilized Fg and that this activity is associated with the A region of the protein.

It was possible to inhibit the binding of rFnbpA(37-605) and Clf40 to immobilized Fg with excess soluble Fg but not with BSA (Fig. 3). This further demonstrates the specificity of the rFnbpA(37-605)-Fg interaction and also indicates that recombinant FnbpA, like ClfA, can bind both immobilized and soluble forms of Fg.

Localization of the Binding Site in Fg for Recombinant FnbpA—To identify which Fg polypeptide chain contains the binding site for rFnbpA(37-605), a Western ligand affinity blot assay was used. Fg is composed of three types of polypeptide chain, Aα, Bβ, and γ, which can be separated on a polyacrylamide gel under reducing conditions (Fig. 4A, lane 2). The binding site for ClfA has been localized to the γ-chain (18, 19). Therefore, Clf41, a recombinant protein encompassing the minimum Fg-binding region of ClfA (Fig. 1) (19), was included in the assay for comparison purposes. Similar to Clf41, rFnbpA(37-605) was found to bind to the γ-chain of Fg (Fig. 4A, lanes 3 and 4). To further localize the binding site in the γ-chain, E. coli cell lysates expressing a normal recombinant γ-chain (γ411) and two mutant recombinant γ-chains (γ’411 and γ407) were used. One of the mutant γ-chains was a substitution mutant in which the four C-terminal amino acid residues were replaced by four unrelated residues (γ’411). The other mutant γ-chain was a deletion mutant in which the four C-terminal amino acid residues were removed (γ407) (Fig. 4B). Clf40 was not found to bind to any Fg polypeptide chain (Fig. 4A, lanes 5 and 6). Similar to Clf41, rFnbpA(37-605) bound to the normal γ-chain (Fig. 4A, lanes 7 and 8) but failed to bind to the mutant γ-chains (Fig. 4A, lanes 9 and 10 and lanes 12 and 13). This suggests that, as for ClfA, the integrity of the C-terminal residues of the γ-chain is essential for rFnbpA(37-605) binding.

As both recombinant FnbpA and ClfA appeared to bind to the middle region of Fg, which is transcribed from the recombinant plasmids. The recombinant proteins (with N-terminal His tags) used in these experiments were each expressed in E. coli cell lysates. In addition, the recombinant γ-chains were of lower molecular mass than the non-recombinant γ-chain (Fig. 4A, lanes 2 and 5–7). This is consistent with the observations of Hettasch et al. (37), who suggested that the heterogeneity results from the presence of internal translational initiation sites in the γ-chain mRNA which is transcribed from the recombinant plasmids. Hettasch et al. also showed that the recombinant γ-chains were unglycosylated, explaining their lower molecular mass compared with the non-recombinant γ-chain (37). Similar to Clf41, rFnbpA(37-605) bound to the normal γ-chain (Fig. 4A, lanes 7 and 11) but failed to bind to the mutant γ-chains (Fig. 4A, lanes 9 and 10 and lanes 12 and 13). This suggests that, as for ClfA, the integrity of the C-terminal residues of the γ-chain is essential for rFnbpA(37-605) binding.

Fig. 1. Structural organization of the Fnbps and ClfA of S. aureus. S, signal sequence; A, unique N-terminal region that is known to bind Fg in the case of ClfA; B1 and B2, homologous repeats of unknown function; Du–D4, Fn-binding repeat units; R, serine-aspartate repeat region that forms a putative stalk, extending the A region of ClfA from the cell surface; W, wall-spanning region; M, membrane-spanning region; +, positively charged tail. The LPXTG motifs, involved in anchoring the proteins to the cell wall peptidoglycan, are indicated. The recombinant proteins (with N-terminal His tags) used in this study are also shown. The amino acid residues contained in each construct are indicated in parentheses.

Fig. 2. Binding of recombinant FnbpA and ClfA to immobilized Fg and Fn. Microtiter wells were coated with 1 µg (A) human Fg and (B) human Fn. Increasing concentrations of rFnbpA(37-605) (∆), rFnbpA(37-881) (■), and Clf40 (○) were incubated in the wells for 1 h at room temperature. Bound protein was detected with anti-His tag mAb and goat anti-mouse AP-conjugated polyclonal Abs, followed by development with p-nitrophenyl phosphate substrate. Values represent the means of duplicate wells. This experiment was repeated three times with similar results.
same region of the Fg γ-chain, the ability of rFnbpA(37-605) and Clf40 to compete with each other for binding to immobilized Fg was assessed. Each protein could inhibit the binding of the other to immobilized Fg, whereas a recombinant protein (rFNBD-D) composed of three of the Fn-binding D repeats of FnbpA (Fig. 1; Ref. 28) had no inhibitory effect (Fig. 5, B). From the four experiments, the \( K_d \) of the interaction was calculated to be 11.0 ± 1.2 \( \mu \)M. The number of binding sites (\( n \)) in Fg for rFnbpA(37-605) was calculated to be 0.9 ± 0.1. As Fg is a dimeric molecule with equivalent γ-chains, this value was expected to be 2. This discrepancy was also observed when the interaction of recombinant CIFA with immobilized Fg was analyzed by SPR (18). It is possible that a portion of the binding sites were destroyed or obscured during immobilization of the Fg onto the chip.

To determine the \( K_d \) for the interaction of rFnbpA(37-605) and rFnbpA(37-881) with intact immobilized Fg. Increasing concentrations of rFnbpA(37-605) were flowed over four flow cells containing chips that had been coated with different amounts of Fg. A representative sensorgram is shown in Fig. 7A. Scatchard analysis of the equilibrium binding data obtained for each experiment yielded a linear plot, indicating the presence of a single class of binding site in Fg for rFnbpA(37-605) (Fig. 7B). From the four experiments, the \( K_d \) of the interaction was calculated to be 11.0 ± 1.2 \( \mu \)M. The number of binding sites (\( n \)) in Fg for rFnbpA(37-605) was calculated to be 0.9 ± 0.1. As Fg is a dimeric molecule with equivalent γ-chains, this value was expected to be 2. This discrepancy was also observed when the interaction of recombinant CIFA with immobilized Fg was analyzed by SPR (18). It is possible that a portion of the binding sites were destroyed or obscured during immobilization of the Fg onto the chip.

To determine the \( K_d \) for the interaction of rFnbpA(37-605) and rFnbpA(37-881) with the 17-mer γ-chain peptide, the binding of increasing concentrations of each protein to fluorescein-labeled peptide was measured by fluorescence polarization. Both rFnbpA(37-605) and rFnbpA(37-881) bound to the peptide in a dose-dependent saturable fashion (Fig. 8, A and B, respectively). The binding of rFnbpA(37-605) to the peptide could be inhibited with excess unlabeled peptide but not with excess unlabeled scrambled peptide, demonstrating the specificity of
the interaction (Fig. 8A, inset). In the absence of divalent cations, the binding of rFnbpA(37-605) and rFnbpA(37-881) to the fluorescein-labeled peptide was very similar, with a $K_D$ of 2.461.2 mM and 3.560.2 mM, respectively. This is almost 10-fold lower than the $K_D$ previously determined for the binding of Clf40 to this peptide ($K_D$ of 20.862.5 mM) (19). The addition of Ca$^{2+}$, Mn$^{2+}$, or Mg$^{2+}$ to the assay did not affect the binding of rFnbpA(37-605) to the γ-chain peptide (data not shown). This contrasts with the Clf40-γ-chain peptide interaction that is inhibited by both Ca$^{2+}$ and Mn$^{2+}$ (19).

**Fg Binding Activity of Cell Surface-expressed FnbpA**—To determine whether cell surface-expressed FnbpA can bind Fg, the clumping titers of *S. aureus* strain P1, strain TAMU3 (an isogenic ClfA- and ClfB-negative mutant of strain P1) and strain TAMU3(pFNBA4) (a derivative of strain TAMU3 that overexpresses FnbpA from a multicopy plasmid) were compared. Wild-type strain P1 expresses ClfA, ClfB, FnbpA, and FnbpB. Strain TAMU3 was constructed by transducing the clfA2::Tn917 and clfB::TcR mutations from strain DU5876 and DU5943, respectively, into strain P1. The genotype of strain TAMU3 was verified by PCR analysis of the clfA and clfB loci (data not shown). A multicopy shuttle plasmid carrying the fnbA gene of strain 8325-4, pFNBA4, was transduced from strain DU5883(pFNBA4) into strain TAMU3, yielding strain TAMU3(pFNBA4). Overexpression of FnbpA in strain TAMU3(pFNBA4) was confirmed by Western immunoblot analysis of cell wall extracts using anti-rFnbpA(37-605) polyclonal Abs (data not shown). As the Fnbps are maximally
expressed early in the growth cycle (38, 39), the three strains were grown to early exponential phase for the clumping assays. Although the clumping titer of strain TAMU3 was dramatically reduced compared with wild-type strain P1, a low level of cell clumping was still observed for this mutant (Table II). When FnbpA was overexpressed in strain TAMU3, the clumping titer was restored to wild-type levels (Table II). This indicates that cell surface-expressed FnbpA can mediate the interaction of S. aureus with soluble Fg.

DISCUSSION

The Fn-binding MSCRAMMs, FnbpA and FnbpB, of S. aureus have been studied in detail by our laboratory and other investigators. These studies have largely focused on the C-terminal D repeat units of these proteins, in particular on the mechanism by which these units recognize and bind to Fn (reviewed in Ref. 40). However, the N-terminal A regions of the Fnbps have received little attention and have previously not been attributed any biological activity. In contrast, the ligand binding activity of other staphylococcal MSCRAMMs, such as ClfA, ClfB, and Cna, has been localized to the N-terminal A regions of these proteins (reviewed in Ref. 4).

In this study, we describe a second binding activity for FnbpA of S. aureus. We show that recombinant FnbpA binds specifically to Fg, in particular to the C terminus of the γ-chain. Both the recombinant full-length FnbpA (rFnbpA(37-881)) and the recombinant A region (including the D repeats) of FnbpA (rFnbpA(37-605)) bound to the synthetic C-terminal γ-chain peptide with a similar affinity (KD of 2.4 μM and 3.5 μM, respectively; Fig. 8). In addition, both proteins were equally potent inhibitors of Fg-mediated staphylococcal cell clumping (Table I). This suggests that the D repeat region does not contribute to or influence the Fg binding activity of FnbpA. We have further localized the Fg binding activity to the A region alone, suggesting that the B repeats also do not contribute to this activity.3

It appears that FnbpA targets the same site in Fg as ClfA. Consistent with this finding, the A region of FnbpA (residues 194–515) exhibits ~25% amino acid identity and ~46% amino acid similarity with the minimum Fg-binding region of ClfA (residues 221–559). Interestingly, the A region of FnbpB (residues 155–478) is even more homologous to ClfA (residues 221–559), exhibiting ~29% amino acid identity and ~49% amino acid similarity. Indeed, we have found that the A region of FnbpB also binds to Fg and targets the C-terminal residues of the γ-chain.2 However, as the homology between the Fnbps and ClfA is spread out over ~300 amino acid residues (data not shown), it is difficult to further narrow down the ligand-binding regions in these proteins from the alignment of the three sequences.

There have been reports of other MSCRAMMs that bind both Fg and Fn. These include protein F1, FBP54, and the M3 protein, of Strepococcus pyogenes (41–43). Protein F1 has a similar structural organization to the Fnbps of S. aureus, including C-terminal Fn-binding repeats that are homologous to the D repeats of the Fnbps (44, 45). The Fg binding activity has been mapped to the N terminus of the A region of this protein, a region that exhibits little amino acid similarity to the Fnbps or ClfA of S. aureus (41). Furthermore, the site in Fg to which protein F1 binds has not been reported. The interactions of FBP54 and the M3 protein with Fg have not been studied in detail, and these proteins also exhibit little amino acid similarity to the staphylococcal Fg-binding proteins.

Although FnbpA and ClfA both bind to the C terminus of the γ-chain, the interaction of ClfA with Fg appears to be more complex than for FnbpA. In this study, rFnbpA(37-605) bound to intact immobilized Fg and the C-terminal γ-chain peptide with similar affinities, as determined by SPR and fluorescence polarization, respectively (KD of ~11 μM and ~2.4 μM, respectively; Figs. 7 and 8A). In addition, the binding of rFnbpA(37-605) to intact Fg was completely inhibited by the γ-chain peptide (Fig. 6A). These findings suggest that the only binding site for rFnbpA(37-605) in Fg is contained in the γ-chain peptide. In contrast, the γ-chain peptide only partially inhibited the binding of ClfA to intact Fg, raising the possibility that this protein may recognize an additional site in Fg (Fig. 6B). Furthermore, although rFnbpA(37-605) bound to the γ-chain peptide with a ~10-fold higher affinity than that previously determined for ClfA (19), several observations suggest that ClfA may bind to intact Fg with a higher affinity than rFnbpA(37-605). Unlabeled ClfA was a more potent inhibitor than unlabeled rFnbpA(37-605) of the binding of either biotinylated ClfA or biotinylated rFnbpA(37-605) to immobilized Fg (Fig. 5). In addition, ClfA was also a more potent inhibitor than rFnbpA(37-605) of ClfA-mediated bacterial cell clumping (Table I).

The interaction of rFnbpA(37-605) with the fluorescein-labeled C-terminal γ-chain peptide was unaffected by the presence of Ca2+, Mn2+, or Mg2+ (data not shown), suggesting that the Fg binding activity of recombinant FnbpA is not regulated by divalent cations. This is consistent with the lack of any potential metal ion-binding motifs within the A region of this protein. In contrast, the A region of ClfA contains a putative metal ion-binding motif that is essential for ClfA-mediated cell clumping and the interaction of ClfA with the γ-chain peptide is inhibited by Ca2+ or Mn2+ (19). This reflects a difference in the Fg-binding mechanism of FnbpA and ClfA that may be important in vivo.

The question arises of whether cell surface-expressed FnbpA can mediate the interaction of S. aureus with Fg. Two other Fg-binding MSCRAMMs of S. aureus, ClfA and ClfB, have been previously identified and characterized in detail (reviewed in Ref. 4). When the genes encoding both of these MSCRAMMs were inactivated in strain Newman, the mutant bacteria failed to clump in the presence of soluble Fg and failed to adhere to immobilized Fg (7). However, it is possible that the Fg binding activity of FnbpA (and FnbpB) was overlooked in this strain due to low expression levels. In fact, when the clfA2::Tn917 and clfB::Tn5 mutations were transduced from strain Newman into strain P1, a low level of cell clumping was still observed in the resulting mutant (strain TAMU3, Table II). It has been previously noted that strain P1 binds to Fn more efficiently than strain Newman, suggesting that strain P1 may express higher

3 E. R. Wann and M. Höök, unpublished observations.
4 E. R. Wann and M. Höök, manuscript in preparation.
levels of the Fnbps. It is thus possible that the residual cell clumping observed for strain TAMU3 may be due to the Fg binding activity of FnbpA (and FnbpB). Furthermore, overexpression of FnbpA in strain TAMU3 restored the clumping titer to wild-type levels, demonstrating that native FnbpA can bind Fg and mediate bacterial cell clumping in vitro (Table II). Whether cell surface-expressed FnbpA can also mediate bacterial adherence to immobilized Fg in vitro is currently under investigation. The significance of the Fg binding activity of FnbpA in vivo remains to be determined. Investigation of the role of the Fnbps in animal models of staphylococcal infection, using isogenic mutants, has been limited and the results obtained are contradictory (46, 47). The development of any infection is likely to be a multifactorial process, involving a multitude of virulence factors. Thus, elucidation of the role of a single factor may be difficult and the results obtained may vary depending on the bacterial strain and animal model used. In addition to the Fg binding activity attributed to FnbpA in this study, it has recently been demonstrated that the Fnbps mediate the invasion of S. aureus into cultured mammalian cells in vitro (48–51). In

| S. aureus strain | Clumping titer µg/ml Fg |
|------------------|-------------------------|
| P1               | 16                      |
| TAMU3 (P1 clfA2::Tn917 clfB::TeR) | 1000                   |
| TAMU3(pFNBA4)    | 16                      |

5 T. J. Foster, personal communication.

6 T. Fowler, E. R. Wann, D. Joh, S. Johansson, and M. Höök, manuscript in preparation.
light of these new findings, further investigation into the role of these proteins in different animal infection models seems warranted.

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The Fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* Is a Bifunctional Protein That Also Binds to Fibrinogen

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