Factor XIIIa Mediated Attachment of *S. aureus* Fibronectin-Binding Protein A (FnbA) to Fibrin: Identification of Gln103 as a Major Cross-Linking Site

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Received October 18, 2005; Revised Manuscript Received December 15, 2005

ABSTRACT: In the present study we investigated the role of factor XIIIa reactive Gln and Lys sites of staphylococcal FnbA receptor in cross-linking reaction with α chains of fibrin. For this purpose we produced two recombinant FnbA mutants in which either a single Gln103 site (1Q FnbA) or all identified reactive Gln103, 105, 783, 830 and Lys157, 503, 620, 762 sites (4Q4K FnbA) were substituted with Ala residues. The results of FXIIIa-catalyzed incorporation of dansylcadaverine and dansylated peptide patterned on the NH_2-terminal segment of fibronectin revealed that the reactivity of Gln substrate sites was drastically reduced in 1Q FnbA and 4Q4K FnbA mutants, while the reactivity of Lys substrate sites was only moderately decreased in 4Q4K FnbA. When it was tested in the FXIIIa-mediated fibrin cross-linking reaction, the 1Q FnbA mutant exhibited about 70−85% reduction in reactivity compared to that of the wild-type FnbA. These results demonstrate that FnbA participates in cross-linking to α chains of fibrin predominantly via its Gln103 reactive site. Several minor sites, including residues replaced in 4Q4K FnbA mutant, contributed to an additional 15−30% of the total fibrin cross-linking reactivity of FnbA. Comparison of amino acid sequences that follow the major reactive Gln site in FnbA and several known substrate proteins revealed that FXIIIa displays a preference for the glutamine residue in an x**Q**AxBxPx sequence, where Q represents reactive glutamine, x is any amino acid residue, A is a polar residue, B is either valine or leucine, and P is proline.

Staphylococcus aureus is a Gram-positive pathogenic bacterium that causes a wide variety of diseases, including serious systemic infections such as sepsis and endocarditis (*1*). One of the most intriguing and potentially lethal properties displayed by *S. aureus* is its ability to colonize human tissues during vascular injury. This property is attributed to the presence on the surface of the organism of several receptors collectively known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (*2*). The MSCRAMM receptors are responsible for the binding of *S. aureus* to human fibrin(ogen) (*3*−*5*), fibronectin (*6, 7*), and collagen (*8*). Such interactions of staphylococci with components of extracellular matrix and coagulation system represent an important first step in the infection process of the wound site. Adhesion of *S. aureus* to fibrin(ogen) is mediated by several fibrinogen-binding receptors, including clumping factor (Clf) (*3, 4*) and multifunctional fibronectin-binding (Fnb) protein, which participates in the adhesion of staphylococci to both fibrin(ogen) (*5, 9*) and fibronectin (*6, 10*). Most *S. aureus* strains express two homologous FnbA and FnbB fibronectin-binding proteins encoded by two linked *fnbA* and *fnbB* genes (*6, 11*). Staphylococcal FnbA and FnbB are single-chain mosaic proteins that are composed of several repetitive segments designated as A, B1, B2, Du, D1, D2, D3, D4, W, and M. Two repeats of the B segment (B1 and B2) are present in FnbA but missing in the FnbB form of the protein (Figure 1). The NH_2-terminal A region of fibronectin binding protein forms the fibrin(ogen) binding site, while the fibronectin binding site predominantly is formed by the COOH-terminal portion of the protein, which is composed of Du and D1−4 repeats. Adhesion of *S. aureus* to fibronectin and fibrin(ogen) via its fibronectin binding protein represents a more complex reaction than simply a high affinity ligand−receptor association. Recently we have demonstrated that FnbA is specifically recognized by and serves as a substrate for coagulation factor XIIIa (*9*). The factor XIII zymogen is composed of two catalytic A and two regulatory B subunits. Thrombin-induced activation of factor XIII in the presence of Ca^{2+} results in release of activation peptides from A subunits followed by dissociation of A and B subunits (*12*). Coagulation factor XIIIa or plasma transglutaminase (EC 2.3.2.13) is a member of the transamidase family of enzymes that catalyze the covalent cross-linking of specific protein substrates through the formation of ϵ-(γ-glutamyl)-lysine isopeptide bonds. The cross-linking mediated by factor XIIIa represents an acyl transfer reaction in which the γ-carboxyamide group of glutamine acts as the acyl donor (amine acceptor) and the ϵ-amino group of lysine acts as the acyl acceptor (amine donor) (*13*). The fibrin γ and α chains serve as the primary physiological substrates for factor XIIIa. Upon catalytic action of factor XIIIa the γ and α chains produce cross-linked γ−γ dimer and α polymer, respectively (*14, 15*). Plasma fibronectin (*16, 17*), plasminogen activator inhibitor 2 (PAI-2) (*18, 19*), and α_2-antiplasmin (*20, 21*) also act as

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10.1021/bi0521240 CCC: $33.50
Published on Web 01/20/2006

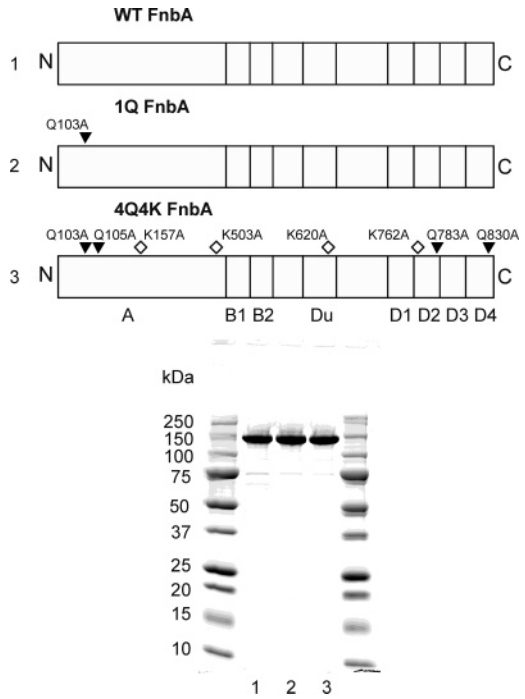

FIGURE 1: Schematic representation of the wild type and mutated FnbA (residues Ala1−Pro839) from *S. aureus* strain ATCC49525 used in this study (top) and SDS-PAGE analysis of isolated proteins (bottom). The top scheme depicts the location of factor XIIIa reactive Gln (filled triangles) and Lys (open diamonds) sites substituted with Ala residues. The drawing also shows the location of the major regions: A, fibrinogen-binding region; B1 and B2, homologous repeats of unknown function; Du, D1, D2, D3, and D4, fibronectin-binding repeats. The schematic representation of proteins at the top and SDS-PAGE analysis of purified proteins at the bottom is shown in the following order: 1, wild type FnbA; 2, 1Q FnbA mutant (Q103A); 3, 4Q4K FnbA mutant (Gln103Ala, Gln105Ala, Gln783Ala, Gln830Ala, Lys157Ala, Lys503Ala, Lys620Ala, Lys762Ala). Electrophoresis was performed using 4−20% gradient gel. The outer lanes in the gel contain molecular mass standards as indicated.

substrates for factor XIIIa and undergo covalent cross-linking to the α chains of fibrin. Overall, only 14 human proteins have been identified as substrates for factor XIIIa ((*22*) and references therein). Our recent findings revealed that pathogenic *S. aureus* might usurp the transglutaminase activity of factor XIIIa for covalent attachment to fibronectin and fibrin. In the presence of factor XIIIa the association of FnbA receptor to fibronectin or fibrin is accompanied by the introduction of intermolecular cross-links and formation of FnbA-fibronectin or FnbA-fibrin α chain heterocomplex, respectively (*9*). Using the amine donor synthetic probe dansylcadaverine (*23*) and a dansylated peptide patterned after the NH_2-terminal sequence of fibronectin (*24*) as an amine acceptor probe, we have identified in FnbA reactive Gln and Lys residues targeted by factor XIIIa. It was found that FnbA from *S. aureus* strain ATCC49525 contains one major (Gln103) and three minor (Gln105, Gln783, and Gln830) amine acceptor sites, and four amine donor sites (Lys157, Lys503, Lys620, and Lys762) (*25*). In the present study we investigated the role of the identified reactive Gln and Lys sites in factor XIIIa catalyzed cross-bridge formation of FnbA with a fibrin α chain. Using site-directed mutagenesis we have replaced the reactive Gln and Lys sites of FnbA with Ala residues and the effect of mutations was evaluated in FnbA-fibrin cross-linking reactions. The major reactive Gln103 site was replaced with Ala in a single residue FnbA mutant, which was designated as 1Q FnbA. All of the identified Gln103, 105, 783, 830 and Lys157, 503, 620, 762 sites were substituted with Ala residues in the FnbA mutant which was designated as 4Q4K FnbA (Figure 1).[1] The factor XIIIa reactivity of both 1Q rFnbA and 4Q4K rFnbA mutants was compared to that of the wild-type FnbA receptor, and the results are discussed with regard to the role of plasma transglutaminase in staphylococcal adhesion and colonization of the human host.

MATERIALS AND METHODS

Site-Directed Mutagenesis: Generation of the 1Q FnbA Mutant (Q103A Mutation). The 1533 region of the *fnbA* gene encoding the NH_2-terminal A region (residues 1−511) of staphylococcal FnbA was produced by PCR amplification using chromosomal DNA from *S. aureus* strain ATCC49525 as the template. Amplification was performed using the following forward 5′-GGCCATGGCATCAGAACAAAAGACAACTACAG-3′ and reverse 5′-CGAGGATCC*TTA*TGTTTCAATTTGCTTGGC-3′ PCR primers. The forward primer incorporated an *Nco* I restriction site (underlined) and ATG initiation codon immediately before the coding region of the mature sequence. The reverse primer included a TAA stop codon immediately after the coding segment, followed by a *Bam* HI site (underlined). The amplified DNA fragment was isolated, treated with *Nco*I and *Bam*HI restriction enzymes, and subsequently ligated into the pET-28a vector (Novagen, Inc.). Mutagenesis of the *fnbA* gene was performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). To synthesize mutant DNA strands, we employed the pET-28a vector containing the 1533 bp fragment of the *fnbA* gene as a template. The synthetic oligonucleotide 5′-GACAATAGCGGAGATGCAAGACAAGTAGATTTAATAC-3′ and its complement were utilized as mutagenic primers. Mutagenic primers contain a GCA codon that replaced CAA to generate the desired Gln → Ala mutation at position 103. Thermal cycling, extension of primers using *Pfu Ultra* DNA polymerase, and digestion of (hemi)methylated template with endonuclease *Dpn* I (Stratagene) were performed according to the manufacturer's instructions. Mutated DNA was transformed into competent XL 10-Gold *E. coli* cells (Stratagene) for nick repair. The resultant plasmid DNA was digested with *Nco* I and *Kpn* I restriction enzymes, and the 680 bp DNA fragment of the *fnbA* gene containing CAA → GCA mutation was subcloned into the pET-28a vector containing the wild type *fnbA* gene (FnbA residues 1−839) (*9*). Ligation of the mutated 680 bp DNA fragment into the pET-28a/*fnbA* gene vector using *Nco* I and *Kpn* I restriction sites resulted in restoration of the 2517 bp *fnbA* gene that encodes mutated FnbA (Q103A FnbA). The resulting pET-28a plasmid (Q103A FnbA mutant) was transformed into BL21(DE3) *E. coli* cells for

[1] Abbreviations: WT FnbA, recombinant wild-type FnbA; 1Q FnbA, recombinant Gln103Ala FnbA mutant; 4Q4K FnbA, recombinant Gln103Ala, Gln105Ala, Gln783Ala, Gln830Ala, Lys157Ala, Lys503Ala, Lys620Ala, and Lys762Ala FnbA mutant; FXIII, factor XIII or plasma transglutaminase; FXIIIa- factor XIII activated; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; TBS, tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl); PBS, phosphate-buffered saline (20 mM Na_2HPO_4, pH 7.4, 150 mM NaCl); DTT, dithiothreitol; EDTA, sodium salt of ethylenediaminetetraacetic acid.

protein expression. The presence of the desired Q103A mutation was confirmed by sequencing the mutated region of *fnbA* gene.

Site-Directed Mutagenesis: Generation of the 4Q4K FnbA Mutant (Gln103Ala, Gln105Ala, Gln783Ala, Gln830Ala, Lys157Ala, Lys503Ala, Lys620Ala, and Lys762Ala mutations). (a) K762A Mutation. The region of the *fnbA* gene encoding the COOH-terminal region (residues 512−839) of staphylococcal FnbA was produced by PCR amplification using chromosomal DNA from the *S. aureus* strain ATCC-49525 as a template. Amplification was performed using the following forward 5′-GAGCCATGGATATTAAGAGT-GAATTAGG-3′ and 5′-CGAGGATCCGGCGTTGTATCT-TCTTCAATC-3′ reverse PCR primers. The forward and reverse primers incorporated *Nco* I and *Bam* HI sites (underlined), respectively. The amplified DNA fragment was isolated, treated with *Nco* I and *Bam* HI restriction enzymes, and ligated into pET-28a vector (Novagen, Inc.). Synthesis of the mutated DNA strand was performed using the pET-28a vector containing the 984 bp fragment of the *fnbA* gene as a template and the synthetic oligonucleotide 5′-GAA-GATACAGAGGCAGACAAACCTAAG-3′ and its complement as mutagenic primers. Mutagenic primers contained the GCA codon that replaced AAA to generate the desired Lys → Ala mutation at position 762. Mutated DNA was transformed into competent XL 10-Gold *E. coli* cells (Stratagene). The resultant plasmid was digested with *Spe* I and *Not* I restriction enzymes, and the 612 bp mutated DNA fragment was subcloned into the pET-28a vector containing full-length wild type *fnbA* gene (FnbA residues 1−839).

(b) Q105A and K157A Mutations. The Q105A and K157A mutations were generated consecutively using a pET-28a template containing single (Q103A) and double (Q103A, Q105A) mutations in the *fnbA* gene, respectively. The synthetic oligonucleotides 5′-GGAGATGCAAGAGCAG-TAGATTTAATAC-3′ and its complement were employed as mutagenic primers for Q105A mutation, while 5′-GTTTCAGAAGTCGCAGGTACAGATGTG-3′ and its complement were utilized for introduction of the K157A mutation. Mutated DNA containing three (Q103A, Q105A, and K157A) mutations was transformed into competent DH 10B *E. coli* cells (Invitrogen). The resultant plasmid was digested with *Nco* I and *Kpn* I restriction enzymes, and the 680 bp mutated DNA fragment was isolated for subsequent subcloning into the pET-28a vector.

(c) K620A Mutation. The K620A mutation was generated using a pET-28a template containing a single (K762A) mutation in the *fnbA* gene. This was achieved using the mutagenic oligonucleotide 5′-CGAAGAGTCTACAGCAG-GTATTGTAACTG-3′ and its complement. Mutated DNA containing two (K620A and K762A) mutations was transformed into competent DH 10B *E. coli* cells (Invitrogen). The resultant plasmid was digested with *Bsr* GI and *Spe* I restriction enzymes, and the 1119 bp mutated DNA fragment was isolated and subcloned into the pET-28a vector containing the *fnbA* gene with single K762A mutation.

(d) K503A Mutation. The K503A mutation was generated using a pET-28a template containing double (K620A, K762A) mutations in the *fnbA* gene. The synthetic oligonucleotide 5′-GCAGTACGATGCCGCGCAAATTATTG-AAAC-3′ and its complement were utilized as mutagenic primers. Mutated DNA was transformed into competent DH 10B *E. coli* cells (Invitrogen). The resultant plasmid was digested with *Kpn* I and *Spe* I restriction enzymes, and the 1256 bp mutated DNA fragment containing K503A and K620A mutations was isolated for subsequent subcloning into the pET-28a vector.

(e) Q783A Mutation. The Q783A mutation was generated using a pET-28a template containing double (K620A, K762A) mutations in the *fnbA* gene. The synthetic oligonucleotide 5′-GACAGTGTGCCAGCAATTCATGGATTC-3′ and its complement were utilized as mutagenic primers. Mutated DNA was transformed into competent DH 10B *E. coli* cells (Invitrogen). The resultant plasmid was digested with *Spe* I and *Not* I restriction enzymes, and the 612 bp mutated DNA fragment containing two (K762A and Q783A) mutations was isolated for subsequent subcloning into the pET-28a vector.

Three DNA fragments containing seven mutations (680 bp with Q103A, Q105A, and K157A; 1265 bp with K503A and K620A; 612 bp with K762A and Q783A) were ligated into the pET-28a vector digested with *Nco* I and *Not* I restriction enzymes, resulting in restoration of the 2516 bp *fnbA* gene that encodes Q103A, Q105A, K157A, K503A, K620A, K762A, and Q783A FnbA mutant.

(f) Q830A Mutation. The Q830A mutation was generated using a pET-28a template containing three (K620A, K762A, and Q783A) mutations in the *fnbA* gene. The synthetic oligonucleotide 5′-CAAAATGAAGGTGCACAAACGAT-TGAAG-3′ and its complement were utilized as mutagenic primers. Mutated DNA was transformed into competent DH 10B *E. coli* cells (Invitrogen). The resultant plasmid was digested with *Spe* I and *Not* I restriction enzymes, and the 612 bp mutated DNA fragment containing K762A, Q783A, and Q830A mutations was isolated for subcloning into the pET-28a vector. This resulted in restoration of the *fnbA* gene that encodes FnbA containing a total of eight mutations (Q103A, Q105A, K157A, K503A, K620A, K762A, Q783A, and Q830A).

The resultant plasmid DNA was transformed into BL21-(DE3) *E. coli* cells for protein expression. Each DNA fragment containing specific mutation was sequenced prior to subcloning into the pET-28a vector. The restored *fnbA* gene was sequenced once more to confirm the presence of desired mutations and the integrity of the entire coding region.

Proteins. Expression and purification of the wild type FnbA and 1Q and 4Q4K FnbA mutants was performed according to procedures described elsewhere (*9*). All FnbA preparations were dialyzed against 20 mM Tris, pH 7.4, 150 mM NaCl, aliquoted, and stored frozen at −20 °C. Labeling of the wild type and mutated forms of FnbA with FITC was accomplished by incubating 1 mg of protein with a 5-fold molar excess of FITC on Celite (Sigma) in 1 mL of 0.1 M $NaHCO_3$ buffer, pH 9.5, for 30 min at 37 °C. The degree of labeling (*26*) was found to be about 1 mol of dye/mol of protein.

Fluorescence Measurements. Fluorescence spectroscopy measurements were performed using an SLM AB2 spectrofluorometer. Fluorescence spectra were recorded at 25 °C in TBS, pH 7.4 or TBS, pH 7.4 buffer containing 5 M urea. Absorbance at 280 nm of all analyzed protein samples was adjusted to 0.08. Urea-induced unfolding of FnbA species was performed by monitoring the ratio of the intrinsic

fluorescence intensity at 350 nm to that at 320 nm with excitation at 280 nm. The ratios of parameter values were then plotted as a function of urea concentration.

Antibodies. Anti-FnbA polyclonal antibodies were generated in rabbits as described earlier (*9*). Monoclonal anti-fibrinogen Aα chain (Aα 529−539, clone 1C2−2) antibody was purchased from Accurate Chemical and Scientific Corp. Monoclonal anti-FITC antibody (clone FL-D6) alkaline phosphatase conjugate was purchased from Sigma. Goat anti-rabbit and anti-mouse IgG alkaline phosphatase conjugates were purchased from BioRad Laboratories.

SDS-PAGE and Western Blot Analysis. SDS-PAGE was carried out using precast 3−8% Tris-Acetate gradient gels (Invitrogen). All SDS-polyacrylamide gels in this study were stained with Coomassie Brilliant Blue R (BioRad Laboratories). For Western Blot analysis protein samples were electroblotted to nitrocellulose membranes and immunostained with the corresponding rabbit polyclonal or mouse monoclonal antibody. The membranes were treated with goat anti-rabbit or anti-mouse alkaline phosphatase conjugated secondary antibody, and the alkaline phosphatase activity was developed with alkaline phosphatase substrate (BioRad Laboratories).

ELISA Binding Assay. Solid-phase binding was performed in plastic microtiter plates (Nunc) using an enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells were coated overnight at 4 °C with 100 μL/well of 3 μg/mL fibrinogen (Calbiochem) in 100 mM Na_2CO_3, pH 9.5 or TBS, pH 7.4 buffer. To convert fibrinogen into fibrin, wells with adsorbed fibrinogen were treated with 100 μL/well of thrombin (1 NIH U/ml) at 37 °C for 1 h in TBS, 5 mM $CaCl_2$, pH 7.4. The wells were then blocked with 5% nonfat milk in TBS for 1 h at 37 °C. After they were washed with TBS containing 0.05% Tween-20 (TBS-Tween), the indicated concentrations of FITC-labeled wild type and mutated FnbA species were added to the wells in TBS, pH 7.4 buffer and incubated for 2 h at 37 °C. Bound wild type and mutated forms of FITC-FnbA were detected with monoclonal anti-FITC antibody alkaline phosphatase conjugate. Phosphatase activity was developed using Alkaline Phosphatase pNPP Substrate (Sigma), and the amounts of bound FITC-WT FnbA, FITC-1Q FnbA, and 4Q4K FnbA were measured spectrophotometrically at 405 nm. The data were fitted by nonlinear regression analysis using eq 1, where A represents

$$A = A_{max}[L]/K_d + [L] \quad (1)$$

absorbance, which is proportional to the amount of bound ligand, A_{max} is the absorbance corresponding to the amount of ligand bound at saturation, [L] is the molar concentration of ligand, and K_d is the dissociation constant .

Fibrin Polymerization Assay. Polymerization of fibrin was measured as an increase of light scattering at 350 nm as a function of time using a Cary 300 Bio spectrophotometer (Varian), equipped with a Multicell Block Peltier temperature controller. Polymerization reactions were performed in 10 mm pathlength quartz cells. Fibrin polymerization was initiated by addition of 0.2 U/mL of thrombin (Sigma) to 1 μM of fibrinogen (Calbiochem) in TBS, pH 7.4 buffer containing 2 mM $CaCl_2$. The effect of WT FnbA, 1Q FnbA, and 4Q4K FnbA on fibrin assembly was evaluated by incorporating increasing concentrations of the wild type or mutated FnbA into the reaction mixture prior to addition of thrombin. Maximum turbidity values (A350 nm/1800 s) were expressed in terms of percent of fibrin polymerization and plotted as a function of different molar concentrations of the WT FnbA, 1Q FnbA, and 4Q4K FnbA. Human fibronectin was utilized in control reactions. All fibrin polymerization experiments were performed at 25 °C.

Activation of Factor XIII. Activation was achieved by treatment of 500 μg/mL of factor XIII (Haematologic Technologies, Inc.) with 0.25 U/mL of thrombin (Sigma) in TBS, pH 7.4 buffer containing 10 mM dithiothreitol and 20 mM $CaCl_2$. After incubation for 20 min at 37 °C, thrombin was inactivated by the addition of a molar excess of hirudin (Sigma) and this mixture was used as factor XIIIa (*27*).

Incorporation of Dansylcadaverine and Dansyl-PGGQQIV Probes. Activated factor XIII was employed to incorporate dansylcadaverine (Sigma) or dansyl-PGGQQIV (New England Peptide, Inc.) in FnbA species. Dansylcadaverine was utilized to probe factor XIIIa reactive glutamines, and the peptide dansyl-PGGQQIV was used to probe reactive lysines. Incorporation was carried out by incubating 2 μM of wild type or mutated rFnbA with 30 μg/mL of factor XIIIa in the presence of either 2 mM of dansylcadaverine or 2 mM of dansyl-PGGQQIV at 37 °C in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM DTT, 5 mM $CaCl_2$ or 20 mM Tris, pH 8.5, 15 mM NaCl, 5 mM DTT, 5 mM $CaCl_2$, respectively. Control reactions were also performed in the same buffers containing 2 mM EDTA. At various times reactions were terminated by addition of 2% SDS and 10% β-mercaptoethanol, heated at 95 °C, and analyzed by SDS-PAGE. Gels were examined under ultraviolet light and then stained with Coomassie brilliant blue.

Cross-Linking to Fibrin. Factor XIIIa catalyzed cross-linking of fibrin in the presence of 2 μM of wild type or mutated FnbA was initiated by addition of 0.5 U/mL of thrombin to a solution containing 5 μM of human fibrinogen (Calbiochem) and 15 μg/mL of factor XIII. Cross-linking reactions were carried out in TBS, pH 7.4 buffer containing 5 mM $CaCl_2$ at 37 °C. At various times the reactions were terminated by addition of 20 mM Tris, pH 7.2, 9 M urea, 40 mM dithiothreitol, 2% SDS. The clots were solubilized at 37 °C for 30 min and heated at 95 °C, and samples were analyzed by SDS-PAGE/Western blotting. The kinetics of factor XIIIa mediated cross-linking of FnbA species to fibrin was examined by densitometric analysis of the gels stained with Coomassie brilliant blue. Laser densitometry was performed using a Personal Densitometer SI (Molecular Dynamics). Each gel was scanned, and the generated images were analyzed using ImageQuant 5.2 software. The rate of reactions was evaluated by the decrease of FnbA upon its cross-linking to fibrin. The relative amount of FnbA in the reaction mixture was determined using the area beneath the peak corresponding to the FnbA band and then plotted as a function of time.

Enzyme-linked immunosorbent binding assay (ELISA) was also utilized for evaluation of factor XIIIa mediated covalent attachment of the wild type and mutated forms of FnbA to fibrin. Microtiter plate wells (Nunc) were coated overnight at 4 °C with human fibrinogen (Calbiochem) at 3 μg/mL in 100 mM Na_2CO_3, pH 9.5 buffer. Fibrinogen was converted into fibrin by treating immobilized fibrinogen with thrombin (1 NIH U/mL) at 37 °C for 1 h in TBS, 5 mM

CaC_2 pH 7.4 buffer. The wells were then blocked by incubation with SuperBlock (Pierce) in TBS for 1 h at 37 °C. After washing with TBS containing 0.05% Tween-20 (TBS-Tween), 300 nM portions of the wild-type FnbA, 1Q FnbA mutant, and 4Q4K FnbA mutant were added to the wells in TBS, pH 7.4 buffer containing either 2 mM CaC_2 or 2 mM EDTA and incubated in the presence of factor XIIIa at 15 μg/mL for 6 h at 37 °C. Bound or cross-linked to fibrin FnbA was detected with rabbit polyclonal antibody to FnbA, followed by goat anti-rabbit IgG horseradish peroxidase conjugate (Promega). Peroxidase activity was developed using ABTS Chromophore substrate (Calbiochem).

RESULTS

Expression and Characterization of the 1Q and 4Q4K FnbA Mutants. The wild type and two mutated (1Q, 4Q4K) forms of FnbA, comprising residues Ala1 through Pro839 (Figure 1, top), were produced in *E. coli* using the pET-28a expression vector and isolated from the soluble fraction of bacterial lysate. Each of the isolated proteins exhibited a single band on SDS-PAGE (Figure 1, bottom) and displayed a single NH_2-terminal sequence starting at ASEQKTTTVE. As was recently reported (*25*), the sequence of the FnbA receptor from *S. aureus* strain ATCC49525 contains a single Trp459 located in the NH_2-terminal fibrinogen-binding A region and 37 Tyr residues distributed throughout the polypeptide chain of the protein. In TBS, pH 7.4 buffer, upon excitation at 280 nm the wild type and mutated forms of FnbA exhibited maximum fluorescence at 304 nm (Figure 2A−C), consistent with the dominant role of the Tyr residues in the intrinsic fluorescence of FnbA receptor. In the presence of 5 M urea, emission spectra of the wild type and mutated forms of FnbA is characterized by the appearance of a shoulder at 350 nm, while the maximum fluorescence remained unchanged (Figure 2A−C). This suggests that 5 M urea induced denaturation of the compact structure, which is accompanied by the exposure of the Trp459 residue to the solvent. To compare the unfolding process of the 1Q FnbA and 4Q4K FnbA mutants to that of the wild type FnbA, proteins were titrated with urea while changes were monitored in the ratio of the fluorescence intensity at 350 nm to that at 320 nm. The results in Figure 2D show that the wild type FnbA and both 1Q and 4Q4K FnbA mutants exhibit identical sigmoidal monophasic transitions in response to urea. The transition midpoint (T_m) observed for all FnbA species occurs at a urea concentration of about 2 M. These results indicate that FnbA receptor forms a compact structure and that introduction of Gln → Ala and Lys → Ala substitutions did not affect the structural integrity of the mutants.

Binding to Fibrinogen and Fibrin. To evaluate fibrin(ogen)-binding activities of the FnbA species, we performed a comparative analysis of their binding properties using an enzyme-linked immunosorbent assay. In ELISA experiments fibrinogen was first immobilized onto plastic wells, and then some of these wells were treated with thrombin to convert fibrinogen into fibrin. Increasing concentrations of FITC-labeled WT FnbA, 1QFnbA, and 4Q4K FnbA were added to the wells, followed by incubation and subsequent detection of the bound FnbA species with monoclonal anti-FITC antibody alkaline phosphatase conjugate. A dose-dependent binding with similar affinities was observed for the wild type and mutated forms of FnbA (Figure 3). Binding of FnbA species to fibrinogen occurred with K_d = 1140 nM for the WT FnbA, 1550 nM for the 1Q FnbA, and 1170 nM for the 4Q4K FnbA (Figure 3A). WT FnbA, 1Q FnbA, and 4Q4K FnbA interacted with fibrin with K_d = 480, 670, and 470 nM, respectively. The affinities of both FnbA mutants toward immobilized fibrinogen and fibrin was found to be similar to that of the wild type FnbA.

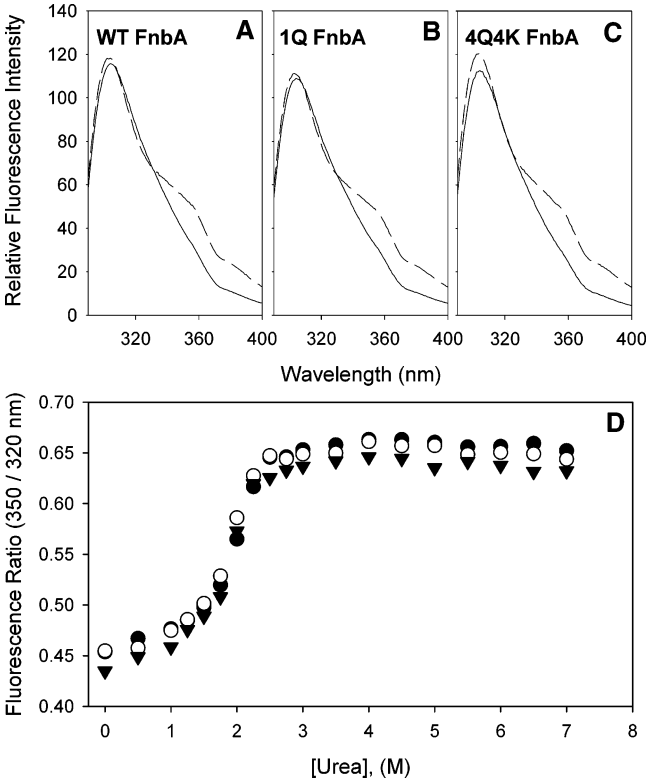

FIGURE 2: Fluorescence spectra and fluorescence-detected denaturation of the wild type and mutated forms of FnbA receptor. Top panels show fluorescence spectra of (A) WT FnbA, (B) 1Q FnbA, and (C) 4Q4K FnbA obtained in TBS, pH 7.4 (solid lines) and in TBS, pH7.4, containing 5 M urea (dashed lines). The bottom panel (D) shows urea-induced denaturation curves of the wild type FnbA (filled circles), 1Q FnbA (open circles), and 4Q4K FnbA (filled triangles) mutants detected by changes in intrinsic fluorescence.

To further characterize fibrin(ogen) binding properties of FnbA mutants, we employed a fibrin polymerization assay. The thrombin-catalyzed formation of polymeric fibrin from purified fibrinogen was examined using a turbidometric method, which allows monitoring the increase of light scattering due to the formation of the fibers. The effect of added FnbA on the polymerization process was investigated by a determination of turbidity after 1800 s of reaction during the plateau phase. Figure 4 shows the relationship between concentrations of various FnbA forms and turbidity, which is proportional to the average number of protofibrils per fiber (cross-sectional area of the fibers) (*28*, *29*). It was found that either form of FnbA incorporated into the reaction mixture effectively decreased the thickness of the fibers in a dose-dependent manner. The effect of the 1Q and 4Q4K FnbA mutants on the fiber formation was very similar to that of the WT FnbA (Figure 4). Therefore, the results of both fibrin polymerization assay and ELISA binding experiments revealed that fibrin(ogen)-binding properties of the 1Q FnbA and 4Q4K FnbA mutants are preserved.

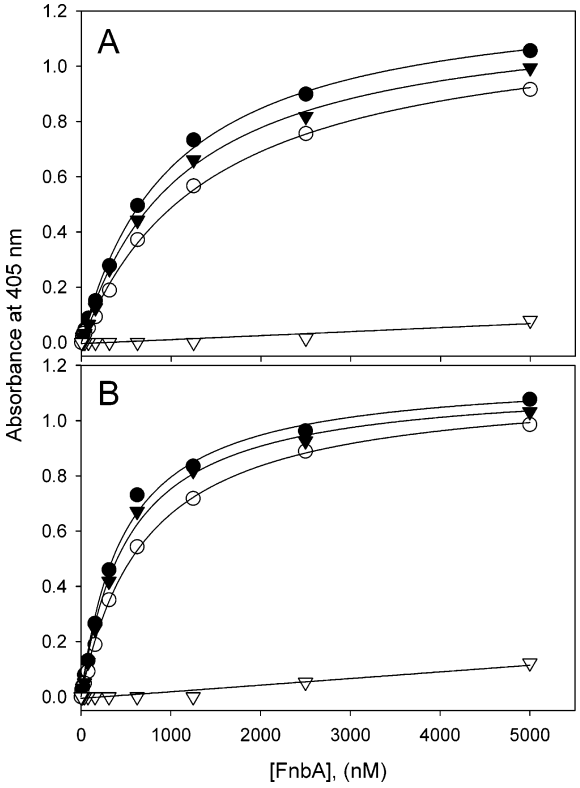

FIGURE 3: Binding of the wild type and mutated forms of FnbA to immobilized fibrinogen (A) and fibrin (B) as determined by ELISA. Increasing concentrations of FITC-WT FnbA (filled circles), FITC-1Q FnbA (open circles), and FITC-4Q4K FnbA (filled triangles) were incubated in microtiter wells coated with fibrinogen or fibrin. Bound FnbA species were detected with monoclonal anti-FITC antibody. FITC-labeled HSA (open triangles) was utilized as a control. The data are representative of three experiments, each performed in duplicate. Curves represent the best fit of the data to eq 1. The K_d values for interaction of FITC-labeled WT FnbA, 1Q FnbA, and 4Q4K FnbA with fibrinogen (or fibrin) were found to be 1140 ± 70 (480 ± 40) nM, 1550 ± 80 (670 ± 30) nM, and 1170 ± 90 (470 ± 30) nM, respectively.

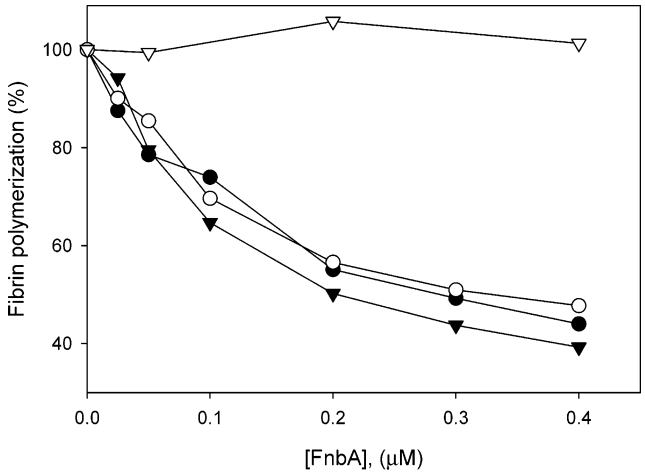

FIGURE 4: Effect of the wild type and mutated forms of FnbA on thrombin-catalyzed fibrin polymerization. Changes in turbidity after 30 min of reaction have been expressed as percent of fibrin polymerization and plotted as a function of different concentrations of WT FnbA (filled circles), 1Q FnbA (open circles), 4Q4K FnbA (filled triangles), and human plasma fibronectin (open triangles) as a control.

Incorporation of Dansylcadaverine and Dansyl-PGGQQIV Probes. To examine the effect of substitution of Gln and Lys reactive sites with Ala residues on reactivity of FnbA, we designed a series of experiments in which the wild type and mutated forms (1Q and 4Q4K) of FnbA were tested as substrates for factor XIIIa. Comparison of factor XIIIa reactivity of the wild type FnbA to that of the 1Q and 4Q4K FnbA mutants was initially performed using dansylcadaverine and dansyl-PGGQQIV probes (Figure 5). At various times, aliquots of reaction mixtures with dansylcadaverine or dansyl-PGGQQIV were collected, analyzed by SDS-PAGE, and examined under ultraviolet light prior to staining with Coomassie blue. In the presence of factor XIIIa and a molar excess of dansylcadaverine, the band corresponding to the wild type FnbA undergoes a continual increase of fluorescence, reflecting the enzymatic attachment of increasing molecules of the probe (Figure 5A). Under the same experimental conditions, incorporation of dansylcadaverine into 1Q FnbA mutant was drastically reduced, suggesting that Gln at position 103 indeed acts as a major reactive Gln site in FnbA. Further reduction in fluorescence intensity was observed with the 4Q4K FnbA mutant in which all four identified reactive Gln residues (Gln 103, Gln 105, Gln783, and Gln 830) were replaced with Ala (Figure 5A). Incubation of the 4Q4K FnbA mutant with dansylcadaverine and factor XIIIa for 60 min, however, resulted in a weak but detectable incorporation of the probe (Figure 5A). The residual reactivity of the 4Q4K FnbA mutant observed in the reaction with dansylcadaverine may indicate the presence in FnbA of an additional minor reactive Gln site(s).

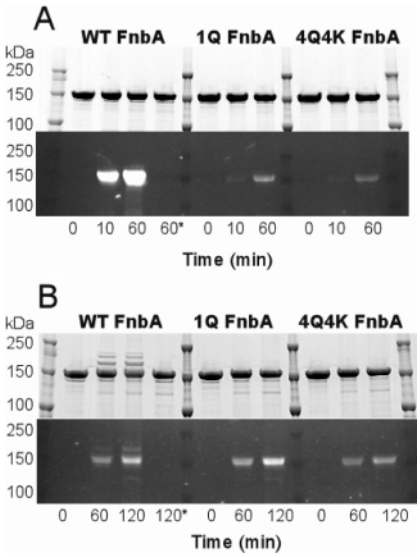

FIGURE 5: Factor XIIIa catalyzed incorporation of dansylcadaverine (A) and dansyl-PGGQQIV (B) probes into the wild type and mutated forms of FnbA receptor. Reactions were carried out at 37 °C in the presence of either 5 mM $CaCl_2$ or 2 mM EDTA (marked with an asterisk). Aliquots were removed at the indicated time points, mixed with SDS under reducing conditions, heated, and analyzed by SDS-PAGE on 4−20% gradient gels. After electrophoresis, the gels were photographed under ultraviolet light (A and B, bottom panels) and then stained with Coomassie Brilliant Blue (A and B, top panels). The outer lanes in the gels contain molecular mass standards as indicated.

Factor XIIIa catalyzed incorporation of the dansyl-PGGQQIV probe into the wild type FnbA is demonstrated in Figure 5B. When the 1Q FnbA mutant was assayed in the same reaction, its protein band exhibited fluorescence intensity that was slightly higher compared to that of the wild type FnbA (Figure 5B). This result suggests that

substitution of the major reactive Gln103 with Ala resulted in a more efficient dansyl-PGGQQIV labeling of the Lys sites within 1Q FnbA mutant. This effect is attributed to the high reactivity of the Gln103 site, which might effectively compete with the dansyl-PGGQQIV peptide probe by participating in intra- and/or intermolecular protein cross-linking. Occurrence of protein cross-linking upon incorporation of the dansyl-PGGQQIV probe in the wild type FnbA is supported by the presence of low-mobility bands detectable under ultraviolet light and upon staining with Coomassie blue (Figure 5B). Factor XIIIa incorporated the dansyl-PGGQQIV probe in the 4Q4K FnbA mutant at a reduced rate and with lower efficiency, suggesting that mutated Lys157, Lys503, Lys620, and Lys762 serve as amine donor sites. Consistent with incorporation of the peptide probe into the wild type and mutated forms of FnbA, the upward shift of the major FnbA bands was observed at 60 and 120 min of reaction (Figure 5B, top panel). The overall reduction of factor XIIIa reactivity observed with Q4K4 FnbA mutant toward the dansyl-PGGQQIV probe was not as prominent as it was toward dansylcadaverine. This indicates that additional reactive Lys site(s) remain available for enzymatic modification with the dansyl-PGGQQIV probe.

Cross-Linking to Fibrin. Reactivity of the 1Q and 4Q4K FnbA mutants was compared to that of the wild-type FnbA in the factor XIIIa catalyzed fibrin cross-linking reaction. For this purpose we analyzed reaction mixtures composed of fibrin, thrombin, factor XIIIa, and the wild type or mutated forms of FnbA. Products of FnbA-fibrin cross-linking were evaluated at different time points by SDS-PAGE and Western blot analysis. In the control reaction, cross-linking was accompanied by the depletion of the band corresponding to the wild type FnbA and by the appearance of several low-mobility bands designated as **a**−**d** (Figure 6). The products of the cross-linking reaction (bands **a**−**d**) reacted with both anti-FnbA polyclonal antibody and anti-fibrinogen α chain monoclonal antibody (Figure 6B,C), suggesting that these complexes are composed of covalently attached FnbA and fibrin α chain. The apparent molecular mass of covalently cross-linked complexes is consistent with the formation of FnbA-α chain heterodimers (prominent band **a**), as well as FnbA-α chain heteropolymers (bands **b**−**d**) in which one or more FnbA units are attached to α chain polymers. Under the same experimental conditions, both 1Q and 4Q4K FnbA mutants exhibited extremely low cross-linking reactivity. Only traces of mutated FnbA-α fibrin chain heterodimer (band **a**) or heteropolymers (bands **b**−**d**) were detected upon Coomassie Blue staining (Figure 6A) or upon immunostaining with anti-FnbA polyclonal antibody (Figure 6B) and anti-fibrinogen α chain monoclonal antibody (Figure 6C). The rate of incorporation of the wild type and mutated FnbA species into the fibrin clot was also evaluated using densitometric analysis. As shown in Figure 7, the wild type FnbA reacted at a much higher rate than 1Q or 4Q4K FnbA mutants. After 120 min of incubation, the amount of free (un-cross-linked) 1Q or 4Q4K FnbA mutant remaining was about 85% more compared to that of the wild type FnbA. These data suggest that the Gln site at position 103 is mostly responsible for factor XIIIa catalyzed attachment of FnbA to fibrin α chains. Under the tested conditions, the factor XIIIa reactivities of the 1Q and 4Q4K FnbA mutants toward fibrin were indistinguishable.

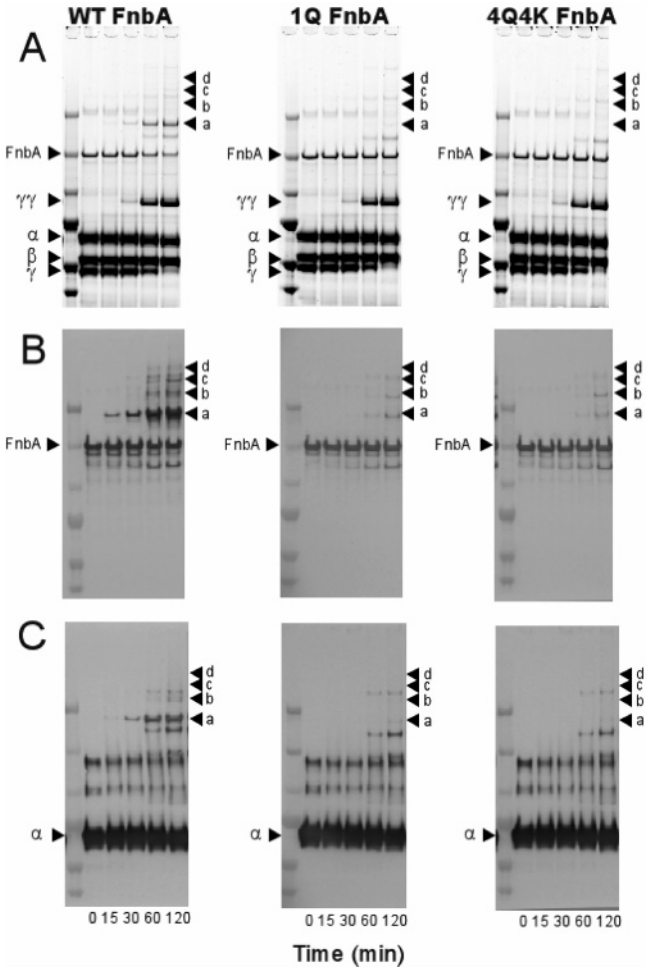

FIGURE 6: Factor XIIIa catalyzed cross-linking of the wild type and mutated forms of FnbA to fibrin. Cross-linking was initiated by the addition of thrombin (0.5 U/mL) at time zero to 5 μM fibrinogen and 15 μg/mL factor XIII in the presence of 2 μM of the wild type FnbA, 1Q FnbA mutant, and 4Q4K FnbA mutant. At the indicated time points, cross-linking reactions were terminated and analyzed by SDS-PAGE on 3−8% gradient gels under reducing conditions. After electrophoresis, the gels were either stained with Coomassie Brilliant Blue (A) or subjected to transfer to nitrocellulose filters followed by immunostaining with anti-FnbA (B) and anti-fibrinogen α chain (C) antibodies. Arrows show the positions of FnbA and the α, β, and γ chains of fibrin. The γ chain product of fibrin cross-linking is indicated as the $\gamma\gamma$ dimer, while the products of cross-linking between FnbA and fibrin are depicted as a spectrum of low-mobility bands **a**−**d**. The left-hand lane in each panel contains molecular mass standards having, from top to bottom, the following M_r values: 250, 150, 100, 75, 50, and 37 kDa.

The ability of 1Q and 4Q4K FnbA mutants to serve as factor XIIIa substrates upon cross-linking to fibrin was further investigated using ELISA experiments. When the wild type or mutated FnbA species were incubated with immobilized fibrin and factor XIIIa in the presence of 2 mM EDTA, the absorbance values observed for all three forms of FnbA were essentially the same (Figure 8). Since in the presence of EDTA the activity of factor XIIIa is completely blocked, the observed signals reflect noncovalent interaction between FnbA and fibrin and provide additional evidence that affinity of the 1Q and 4Q4K FnbA mutants toward fibrin was not affected. When the same reactions were carried out in the presence of 2 mM $CaCl_2$, the absorbance signals observed for each FnbA form were higher compared to that

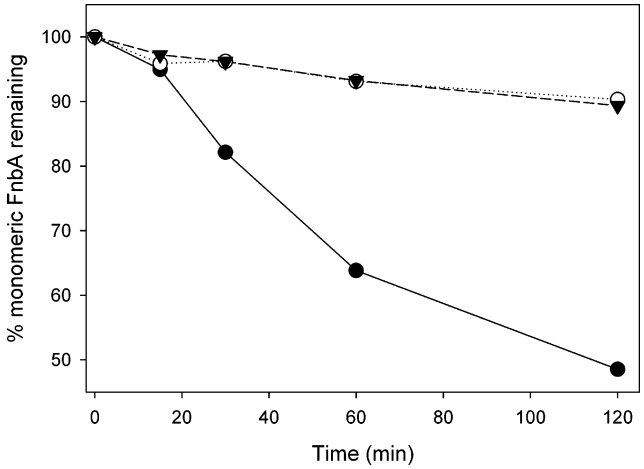

FIGURE 7: Rate of covalent incorporation of the WT FnbA (filled circles), 1Q FnbA mutant (open circles), and 4Q4K FnbA mutant (filled triangles) into a fibrin clot. For experimental details, see Materials and Methods.

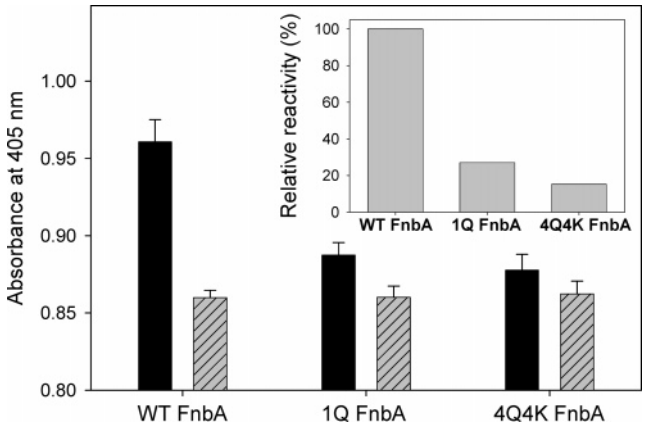

FIGURE 8: Factor XIIIa catalyzed incorporation of the wild type and mutated forms of FnbA into immobilized fibrin in the presence of 2 mM $CaCl_2$ (solid bars) or 2 mM EDTA (hatched bars) as determined by ELISA. The error bars reflect the standard deviation of eight determinations from a representative experiment. The inset shows the factor XIIIa reactivity of the WT FnbA, 1Q FnbA, and 4Q4K FnbA toward immobilized fibrin.

in the presence of EDTA. The difference in absorbance value for each form of FnbA detected in the presence of EDTA and $CaCl_2$ is equivalent to the amount of FnbA covalently incorporated to fibrin and, therefore, serves as an indicator of factor XIIIa reactivity. The inset in Figure 8 shows the relative reactivities of the wild type and mutated forms of FnbA upon factor XIIIa catalyzed cross-linking to immobilized fibrin. The 1Q FnbA mutant exhibited about 70% lower reactivity compared to that of the wild type FnbA. The 4Q4K FnbA mutant showed a further decrease of α chain cross-linking reactivity, which accounted for only 15% of the reactivity displayed by the wild type FnbA (Figure 8, inset). This indicates that some or all of the substituted Q105, Q783, Q830, K157, K503, K620, and K762 residues in 4Q4K FnbA mutant contribute to the overall fibrin cross-linking reactivity of FnbA. Since the reactivity of 4Q4K FnbA mutant toward low-molecular-mass probes (Figure 5) and fibrin (Figures 5, 7, and 8) was not completely eliminated, it is apparent that additional unidentified sites in FnbA are likely to participate in cross-linking reactions. Collectively SDS-PAGE, Western blot, and ELISA data strongly suggest that Gln103 is a major amine acceptor site of FnbA responsible for factor XIIIa catalyzed cross-linking to the α chain of fibrin. The Gln 103 substrate site provides from 70 to 85% of total fibrin cross-linking reactivity of FnbA. Several minor sites, including residues replaced in 4Q4K FnbA mutant, contributed to an additional 15−30% of the total fibrin cross-linking reactivity of FnbA.

DISCUSSION

It is well recognized that cell wall associated MSCRAMM receptors expressed by pathogenic Gram-positive bacteria contribute to microbial virulence (*2*). The important role of fibronectin-binding proteins (FnbA and FnbB) in the pathogenesis of staphylococcal infections was demonstrated in vivo using Fnb protein-defective *S. aureus* strains in an experimental endocarditis model (*30*). Heterologous expression of FnbA gene from *S. aureus* in a nonpathogenic strain of *Lactococcus lactis* provided another line of evidence suggesting the critical role of FnbA in staphylococcal virulence (*31*). Understanding of *S.* aureus adherence to host tissues and soluble molecules via its multifunctional FnbA receptor also requires in vitro information on interactions with fibrin(ogen) (*5*, *9*) and fibronectin (*6*, *9*, *10*). Such information provides a mechanistic explanation of the binding interactions between FnbA and human host proteins. In this study we investigate the role of reactive Gln and Lys sites within the staphylococcal FnbA receptor in factor XIIIa catalyzed cross-linking reactions with fibrin α chains.

Fluorescence spectroscopic analysis of the wild type and mutated forms (1Q and 4Q4K) of FnbA receptor confirmed that each protein forms a compact structure that underwent an unfolding transition when titrated with increasing concentrations of urea. Intrinsic fluorescence properties of FnbA utilized for monitoring of urea-induced denaturation depend on the microenvironment of the single Trp459 residue. The unfolding process observed upon monitoring changes in the fluorescence ratio, therefore, reflects the loss of a compact structure formed by the A region in which the reporting Trp459 is located (Figure 1). Analysis of the sequence homology of the NH_2-terminal A region of FnbA revealed about 25% identity with the A region of ClfA (*5*). The A regions of both FnbA and ClfA are responsible for binding to the same COOH-terminal portion of fibrin(ogen) γ chain (*5*, *32*, *33*) and apparently share a similar structural organization (*34*). The X-ray structural analysis of the ClfA fibrinogen-binding fragment revealed that its fibrinogen-binding region A is composed of the three DEv-IgG domains N1−N3. It was suggested that three N1−N3 IgG-like domains also form the fibrinogen-binding region A of the FnbA receptor (*34*). Therefore, four (Q103A, Q105A, K157A, and K503A) out of eight introduced mutations are situated within the fibrinogen-binding A region of FnbA formed by three N1−N3 domains. The remaining amino acid substitutions (K620A, K762A, Q783A, and Q830A) located in Du, D2, and D4 repeat, which do not form a compact structure and exist in an unfolded state (*35*). Consequently, it is unlikely that replacement of these residues would interfere with the overall folding of the FnbA receptor.

Recently we reported the results of ELISA binding experiments in which detection of FnbA bound to immobilized fibrin(ogen) was performed using anti-FnbA polyclonal antibodies (*9*). The K_d value estimated for the

FnbA-fibrin(ogen) interaction (29−30 nM) (*9*), however, was much lower than that initially reported by Wann et al. (2.4−11 μM) (*5*). These results prompted us to utilize FITC-labeled FnbA and to detect fibrin(ogen)-bound FnbA with anti-FITC monoclonal antibody. We found that detection of FITC-labeled FnbA bound to immobilized fibrin(ogen) with anti-FnbA antibody produced K_d, which was about 20−50-fold lower compared to that estimated by detection with anti-tag (FITC) antibody. Interestingly, when similar ELISA binding experiments were performed with another staphylococcal fibrinogen-binding protein ClfA, no difference in K_d value of FITC labeled ClfA was observed upon detection by either anti-ClfA or anti-FITC antibody (not shown). It appears that antibodies raised against FnbA promote binding of FnbA to fibrin(ogen). Similar properties were reported for monoclonal and polyclonal anti-FnbA antibodies when they were employed for evaluation of the FnbA−fibronectin interaction ((*36*) and references therein). It was suggested that immunization with FnbA may result in quick complex formation with plasma fibronectin before the immune system has time to recognize the free form of FnbA. Consequently, produced antibodies would recognize epitopes formed by FnbA-fibronectin complex rather than unoccupied FnbA (*36*). This model implies that FnbA and fibronectin remain predominantly in an associated state during the endocytic processing pathway. It seems unlikely, however, that even a high affinity complex such as FnbA-fibronectin would be preserved within an acidic environment upon degradation into small peptides. In contrast, the complex formed by FnbA and fibronectin or FnbA and fibrinogen may be processed as a single entity if it was covalently stabilized prior to entering into antigen-presenting cells. The results of a recent study reported by Siebenlist et al. (*37*) indicate that thrombin-uncleaved factor XIII in plasma provides a potent source of readily available cross-linking activity. Therefore, constitutive enzymatic activity displayed by plasma protransglutaminase ultimately may be responsible for the production of antibodies with such unusual properties.

Binding of FnbA to fibrino(gen) had a drastic impact on the different stages of thrombin-induced fibrin polymerization reactions. In fibrin formation, FnbA increased the lag period and reduced the maximum turbidity, suggesting that both protofibril formation and lateral association of protofibrils into fiber stages were affected, respectively (*9*). The prolonged time of fibrin polymerization and formation of altered (thinner) fibers caused by FnbA may be critical for *S. aureus* penetration through developing a hemostatic plug and for initiation of infection at the site of the vascular injury.

Comparison of the degree of factor XIIIa directed incorporation of dansylcadaverine in WT FnbA and 1Q FnbA allowed us to confirm that Gln103 indeed acts as a major reactive amine acceptor site in FnbA (Figure 5A). This observation is consistent with our earlier report, in which Gln103 was identified as a major Gln substrate site for factor XIIIa (*25*). The total reactivity of the Gln residues at positions 105, 783, and 830 represents only a small fraction compared to that of Gln103. The results of experiments with the dansyl-PGGQQIV probe demonstrated an overall reduction in the factor XIIIa reactivity of 4Q4K FnbA mutant that lacks four identified Lys sites at positions 157, 503, 620, and 762. It is apparent, however, that substitution of these Lys sites with Ala residues did not eliminate the ability of FnbA to incorporate a dansyl-PGGQQIV probe (Figure 5B). Factor XIIIa mediated labeling of FnbA species with dansyl-PGGQQIV probe and subsequent analysis of the reaction mixtures by SDS-PAGE revealed several interesting features associated with this reaction. First, replacement of the major Gln103 site with Ala resulted in a noticeable increase of dansyl-PGGQQIV peptide incorporation into the 1Q FnbA mutant, compared to that of the wild type FnbA. Second, incorporation of dansyl-PGGQQIV peptide into the 1Q FnbA mutant was not accompanied by the appearance of low-mobility bands that were observed upon treatment of the wild type FnbA (Figure 5B). These data suggest that the highly reactive Gln103 site interferes with incorporation of dansyl-PGGQQIV to reactive Lys sites by competing with the reactive Gln site of the probe. The formation of intra- and intermolecular isopeptide bonds between Gln103 and reactive Lys site(s) within FnbA impedes incorporation of the dansyl-PGGQQIV probe into the wild-type FnbA and leads to a restricted labeling of the Lys sites.

The results of fibrin cross-linking experiments clearly demonstrate that Gln103 of FnbA acts as a primary substrate site for factor XIIIa in cross-linking reactions with fibrin α chains. Interestingly, Gln103 is an extremely conserved residue and is present in all analyzed FnbA sequences from different *S. aureus* strains (*25*). This indicates that the covalent attachment of FnbA to the α chain of fibrin catalyzed by factor XIIIa is an important reaction for staphylococcal adhesion and colonization of the human host. The presence of additional minor sites participating in cross-linking of FnbA to fibrin is evident from the data obtained with the 1Q FnbA and 4Q4K FnbA mutants. Both 1Q FnbA and 4Q4K FnbA mutants exhibited the ability to participate in cross-link formation with fibrin, but at much lower efficiency than the WT FnbA. Cross-linking of FnbA mutants to fibrin analyzed by SDS-PAGE showed no detectable difference in reactivity between 1Q FnbA and 4Q4K FnbA mutants (Figures 6 and 7). In contrast, the results of ELISA-based cross-linking experiments revealed that reactivity of 4Q4K FnbA mutant is about 50% lower than that of the 1Q FnbA mutant and about 85% lower than that of the WT FnbA (Figure 8). Such a difference in estimation of factor XIIIa reactivity can be attributed to the various sensitivities of SDS-PAGE/densitometric assay and ELISA. Thus, the results of fibrin cross-linking experiments performed with the 4Q4K FnbA mutant indicate that Gln105, Gln783, Gln830, Lys157, Lys503, Lys620, and Lys762 act as minor reactive sites for factor XIIIa. From our data we were unable to assess if all of these sites or only some of them contribute to the cross-linking with fibrin. It is also not clear if the minor reactivity of FnbA toward fibrin is due to only Gln sites, only Lys sites, or a combination of both. It is well established that plasma fibronectin (*38, 39*) and α2-antiplasmin (*21, 40*) undergo cross-linking to the α chain of fibrin by contributing one major and several minor Gln sites. In contrast to FnbA, both fibronectin and α2-antiplasmin serve for factor XIIIa as monofunctional substrates that contain only reactive Gln sites. Therefore, the possibility that Lys sites of FnbA participate in cross-link formation with the α chain of fibrin cannot be excluded.

Covalent incorporation of *S. aureus* at the site of vascular injury through its FnbA receptor, and transglutaminase activity of factor XIIIa is likely to serve as an efficient

Table 1: Comparison of the Amino Acid Sequences Following Specific Factor XIIIa Reactive Gln Sites[a]

	*	1	2	3	4	5	6	7	
- S G D	Q_{103}	R	Q	V	D	L	I	P	- *S. aureus* FnbA (*25*)
N	Q_2	E	Q	V	S	P	L	T	- α2AP (*21*)
Q A	Q_3	Q	I	V	Q	P	P	S	- Fibronectin (*38*)
- **Q** Q I	Q_{86}	K	G	S	Y	P	D	A	- PAI-2 (*47*)
- N P E	Q_{93}	T	P	V	L	P	E	E	- Vitronectin (*48*)
- S Y S	Q_{292}	Q	I	L	F	P	Y	S	- TAFI (*49*)
- S L S	Q_{167}	S	K	V	L	P	V	P	- β casein (*43*)
- E G **Q**	Q_{399}	H	H	L	G	G	A	K	- fibrin(ogen) γ chain (*14*)
- F K S	Q_{221}	L	Q	K	V	P	P	E	- fibrin(ogen) α chain (*50*)
- D M P	Q_{237}	M	R	M	E	L	E	R	- fibrin(ogen) α chain (*50*)
- T G N	Q_{328}	N	P	G	S	P	R	P	- fibrin(ogen) α chain (*15*)
- S T G	Q_{366}	W	H	S	E	S	G	S	- fibrin(ogen) α chain (*15*)

[a] The asterisk shows the position of the reactive Gln site, while amino acid residues following reactive Gln are designated as numbers 1−7. Additional reactive Gln sites in PAI-2 (Gln83) and the fibrinogen γ chain (Gln398) are shown in boldface. Abbreviations: α2AP, α2-antiplasmin; PAI-2, plasminogen activator inhibitor 2; TAFI, thrombin-activatable fibrinolysis inhibitor.

mechanism for bacterial colonization and establishment of infection. It should be noted that although the site of injury represents a primary area for transglutaminase-mediated attachment of FnbA to fibrin or fibronectin, staphylococci have the capacity to utilize transglutaminase activity of factor XIIIa outside of the boundaries of activated coagulation cascade. Factor XIIIa mediated incorporation of FnbA to fibrin may depend on the activity of secreted by *S. aureus* coagulase. It was demonstrated that coagulase acts as a potent nonproteolytic activator of prothrombin (*41*) that specifically converts fibrinogen into fibrin and factor XIII into factor XIIIa (*42*). Prothrombin activation performed by staphylocoagulase may promote anchoring of *S. aureus* to the sites of fibrin(ogen) deposition, resulting in formation of vegetations consisting of bacteria, platelets, and fibrin.

Earlier studies demonstrated that specificity for factor XIIIa catalyzed cross-linking between proteins is directed by the primary structure in the vicinity of substrate glutamine residues (*43, 44*). However attempts to find homology within the primary sequence of known FXIIIa protein substrates surrounding the Gln cross-linking site revealed no apparent sequence pattern (*15, 27, 45*). In this study we performed yet another attempt to identify common features in the sequence adjacent to the reactive Gln site. The well-documented FXIIIa reactivity demonstrated for NH_2-terminally located Gln2 in α_2-antiplasmin (*21, 40*) and Gln3 in fibronectin (*38*) indicated that substrate specificity determinants must be present within the sequence following the amine acceptor site. This prompted us to focus our analysis on the portion of the polypeptide chain following the reactive Gln site. Careful examination of the amino acid sequences from different factor XIIIa substrates revealed the presence of a distinctive common pattern (Table 1). It appears that factor XIIIa displays a preference for the glutamine residue in an x**Q**AxBxPx sequence, where **Q** represents the reactive glutamine site, x is any amino acid residue, A is a polar residue, B is either valine or leucine, and P is proline. Amino acid residues other than valine or leucine (position 3) and proline (position 5) are accepted by factor XIIIa in case of PAI-2, FnbA, and fibrin(ogen) γ chain. It is apparent, however, that the capacity to serve as a Gln substrate for factor XIIIa is not exclusive for the x**Q**AxBxPx sequence and a number of Gln-containing polypeptides lacking this pattern are still recognized by factor XIIIa. Fibrin(ogen) α chain serves as the main physiological substrate for factor XIIIa but shows little or no homology in the sequences following the amine acceptor sites (Table 1). This indicates that additional mechanism(s) may also participate in the determination of factor XIIIa substrate specificity. The exo site responsible for the binding of factor XIIIa to fibrin (*46*) may contribute to the selection of Gln amine acceptor sites within α chains. The higher order structure (tertiary and quaternary) of the protein substrate may also play an important role in determining which Gln residue might act as an amine acceptor (*27*). Further studies including kinetic analysis are needed to define the role of the x**Q**AxBxPx linear sequence in determination of the substrate specificity of factor XIIIa.

ACKNOWLEDGMENT

We thank Dr. Stephen Olmsted for critical reading of the manuscript and Ms. Marjolaine Carriere for assistance with protein sequence analysis.

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BI0521240