Identification of Factor XIIIa-Reactive Glutamine Acceptor and Lysine Donor Sites within Fibronectin-Binding Protein (FnbA) from *Staphylococcus aureus*

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ABSTRACT: Staphylococcal fibronectin-binding protein (FnbA) is a surface-associated receptor responsible for the reversible binding of bacteria to human fibronectin and fibrin(ogen). Recently we have shown that FnbA serves as a substrate for coagulation factor XIIIa and undergoes covalent cross-linking to its ligands, resulting in the formation of heteropolymers (Matsuka, Y. V., Anderson, E. T., Milner-Fish, T., Ooi, P., and Baker, S. (2003) *Staphylococcus aureus* fibronectin-binding protein serves as a substrate for coagulation factor XIIIa: Evidence for factor XIIIa-catalyzed covalent cross-linking to fibronectin and fibrin, *Biochemistry 42,* 14643−14652). Factor XIIIa also catalyzes the incorporation in FnbA of fluorescent probes dansylcadaverine and glutamine-containing synthetic peptide patterned on the NH_2-terminal segment of fibronectin. In this study, the above probes were utilized for site-specific labeling and identification of reactive Gln and Lys residues targeted by factor XIIIa in rFnbA. Probe-decorated rFnbA samples were subjected to trypsin or Glu-C digestion, followed by separation of labeled peptides using reversed phase HPLC. Sequencing and mass spectral analyses of isolated probe-modified peptides have been employed for the identification of factor XIIIa-reactive Gln and Lys residues. Analysis of dansylcadaverine-labeled peptides resulted in the identification of one major, Gln103, and three minor, Gln105, Gln783, and Gln830, amine acceptor sites. The labeling procedure with dansyl-PGGQQIV probe revealed that Lys157, Lys503, Lys620, and Lys762 serve as amine donor sites. The identified reactive glutamine acceptor and lysine donor sites of FnbA may participate in transglutaminase-mediated cross-linking reactions resulting in the covalent attachment of pathogenic *Staphylococcus aureus* to human host proteins.

Fibronectin-binding protein from *Staphylococcus aureus* is a surface-associated multifunctional receptor responsible for adhesion of the microorganism to fibronectin (*2*, *3*), fibrinogen (*4*), and fibrin (*1*). The interactions between host macromolecules and staphylococci represent a critical first step in the infection process. Attachment of *S. aureus* to host tissues is ensured by extremely high affinity of receptor−ligand interaction. Staphylococcal fibronectin-binding protein interacts with its ligands with a K_d in the low nanomolar range (*1*, *5*−*7*). Nevertheless reversible binding to human proteins may serve only as an initial phase in the process of staphylococcal adhesion to the host tissue. Recently we have reported that staphylococcal fibronectin-binding protein A (FnbA) from *S. aureus* strain ATCC49525 serves as a substrate for coagulation factor XIIIa (*1*). In this study we have demonstrated that FnbA can be covalently cross-linked to either fibronectin or fibrin by transglutaminase action of factor XIIIa, resulting in the formation of receptor−ligand heteropolymers. In the absence of fibronectin or fibrin, treatment of FnbA with factor XIIIa was accompanied by the formation of high molecular mass FnbA homopolymers. Coagulation factor XIIIa or plasma transglutaminase (EC 2.3.2.13) belongs to the transamidase class of enzymes that catalyze the covalent cross-linking of specific protein substrates through formation of intermolecular ϵ-(γ-glutamyl)-lysine isopeptide bonds. Cross-linking occurs via an acyl transfer reaction in which the γ carboxyamide group of glutamine serves as the acyl donor (amine acceptor) and the ϵ-amino group of lysine serves as the acyl acceptor (amine donor) (*8*, *9*). Factor XIII circulates in the blood as a nonactive tetramer precursor, A_2B_2, that is composed of two catalytic A subunits and two regulatory B subunits. Following exposure to thrombin, factor XIII zymogen undergoes a Ca^{2+}-dependent activation to factor XIIIa (factor XIII activated), which subsequently catalyzes the formation of covalent cross-links between γ chains and between α chains of a fibrin clot. This reaction represents the final event in the blood coagulation cascade and is essential for normal hemostasis. Factor XIIIa is also involved in the covalent incorporation of several different proteins into fibrin clots by the same mechanism. Among them are fibronectin and α_2-antiplasmin whose cross-linking to the clot plays an important role in wound healing and fibrinolysis. The protein−protein cross-linking reactions catalyzed by factor XIIIa represent a two-stage process. First, proteins specifically associate with each other to form a reversible (noncovalent) complex and then become covalently cross-linked by factor XIIIa. In general, protein cross-linking catalyzed by factor XIIIa produces a variety of fused homo- and heteropolymeric structures that play an important role in a number of physiological reactions (*10*). Our recent finding that *S. aureus* can utilize transglutaminase activity for

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adhesion to human ECM molecules suggests that protein cross-linking is involved in pathological reactions associated with bacterial infection.[1]

Most *S. aureus* strains express one (FnbA) or two (FnbA and FnbB) fibronectin-binding proteins encoded by two different genes (*11*, *12*). The physiological significance of the two copies of fibronectin-binding protein in staphylococci is not understood. The NH_2-terminal region of the mature fibronectin-binding protein is formed by about a 500-residue-long A region responsible for fibrinogen/fibrin binding activity of the receptor (*4*). The A region of FnbA contains the B1−B2 double copy of a 30-residue-long repeat of unknown function which is missing in the FnbB version of the protein. The COOH-terminal region of fibronectin-binding protein contains five conserved ~40-residue-long Du, D1, D2, D3 and D4 repeats that form the fibronectin-binding region of the receptor (*3*, *7*, *13*). The COOH-terminus of fibronectin-binding protein is covalently attached to the cell wall peptidoglycan by the transpeptidase activity of sortase (*14*). The reversible binding of FnbA to fibronectin or fibrin may serve as a prerequisite for efficient intermolecular covalent cross-linking. The association of FnbA with fibronectin or fibrin likely results in appropriately positioned donor lysine and acceptor glutamine residues that subsequently become cross-linked by factor XIIIa. Factor XIIIa also catalyzes the formation of an isopeptide bond between the γ-carboxamide group of peptide-bound reactive glutamine residues and the amino groups of a variety of primary amines, including those of putrecine, spermidine, and cadaverine (*15*, *16*). Incorporation of an alternative amine donor inhibits protein cross-linking and leads to an enzyme-directed, site-specific labeling of the participating glutamine residues in the acceptor protein (*9*). Similarly, by utilizing peptides patterned after the NH_2-terminal sequence of fibronectin or α_2-antiplasmin, containing reactive glutamine residues, specific labeling of the participating lysine residues in the donor protein can be achieved (*17*−*19*). The data presented in our previous study (*1*) indicated that, in the presence of factor XIIIa, staphylococcal rFnbA could be modified by the amine donor synthetic probe dansylcadaverine and dansylated peptide patterned after the NH_2-terminal sequence of fibronectin, which acts as amine acceptor probe. In the present paper we have identified the factor XIIIa-reactive Lys and Gln residues in rFnbA that participate as amine donor and acceptor sites in cross-linking reactions.

MATERIALS AND METHODS

Staphylococcal Fibronectin-Binding Protein. The recombinant fibronectin-binding protein A (rFnbA) comprising residues Ala1 through Pro839 from *S. aureus* strain ATCC49525 was produced in *Escherichia coli* and isolated from the soluble fraction of the cell lysate as described previously (*1*). The identity of isolated rFnbA was confirmed using SDS−PAGE, Western blot, and NH_2-terminal sequence analysis. Protein concentrations in all experiments were determined using bicinchoninic acid (BCA) assay according to instructions (Pierce Chemical Company).

Factor XIIIa Catalyzed Incorporation of Dansylcadaverine and Dansyl-PGGQQIV Probes into rFnbA. To incorporate dansylcadaverine (Sigma) or dansyl-PGGQQIV (custom synthesized by New England Peptide, Inc.) into rFnbA we have used preactivated factor XIII. For this purpose 500 μg/mL of factor XIII (Haematologic Technologies, Inc.) was activated by treatment with 0.25 unit/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 hirudin (Sigma) and this mixture was used as factor XIIIa (*20*). Factor XIIIa-catalyzed labeling of the reactive glutamine residues within rFnbA was carried out by incubating 1−2 mg of rFnbA for 4 or 18 h at 37 °C with 2.5 mM dansylcadaverine and factor XIIIa (30 μg/mL) in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM DTT, 5 mM $CaCl_2$ buffer. The labeling of factor XIIIa-reactive lysine residues was performed by incubating 1−2 mg of rFnbA for 4 or 18 h at 37 °C with 2 mM dansyl-PGGQQIV and factor XIIIa (30 μg/mL) in 20 mM Tris, pH 8.5, 15 mM NaCl, 5 mM DTT, 5 mM $CaCl_2$ buffer. The total volume of reaction mixture in both cases was 0.3 mL. At the end of the incubation period, the proteins were precipitated with 7% TCA and harvested by centrifugation (5 min at 14000*g*), and the pellets were extracted repeatedly (8 times) either with 1 mL of ethanol/ether (1:1 v/v) to remove unreacted dansylcadaverine or with 1 mL of *N*,*N*-dimethylformamide containing 1% *N*-methylmorpholine and 5% H_2O to remove unreacted dansyl-PGGQQIV probe (*21*).

Fragmentation of Dansylcadaverine- and Dansyl-PGGQQIV-Labeled rFnbA by Thrombin. Proteolytic fragmentation of dansylcadaverine- or dansyl-PGGQQIV-modified rFnbA was performed using thrombin (Sigma). After the removal of unreacted dansylcadaverine and dansyl-PGGQQIV probes, the modified rFnbA pellets were dissolved in 0.5 mL of TBS, pH 7.4 buffer containing 5 mM $CaCl_2$. Limited proteolysis was carried out by incubating the modified rFnbA with thrombin for 1 h at 25 °C at an enzyme/substrate ratio of 1:200 (w/w). The reaction was terminated by heating at 95 °C in the presence of 2% SDS, and the products of hydrolysis were analyzed by SDS−PAGE.

SDS−PAGE Analysis. Dansylcadaverine- or dansyl-PGGQQIV-modified rFnbA preparations and their thrombin-generated fragments were analyzed by SDS−PAGE using precast 4−20% (BioRad Laboratories) gradient gels. All SDS−polyacrylamide gels in this study were examined under 350 nm ultraviolet light and then stained with Coomassie Brilliant Blue R (BioRad Laboratories).

Digestion of Dansylcadaverine- and Dansyl-PGGQQIV-Labeled rFnbA. Enzymatic hydrolysis of the dansylcadaverine-modified rFnbA was achieved by treatment with Glu-C (V-8) protease (Worthington Biochemical Corp.) and TPCK-treated trypsin (Worthington Biochemical Corp.). Hydrolysis of the dansyl-PGGQQIV-modified rFnbA was performed using Glu-C protease only. Followed by TCA precipitation and extraction, the modified rFnbA pellets were dissolved in 0.3 mL of either TBS, pH 7.4 for cleavage with trypsin or PBS, pH 7.8 for cleavage with Glu-C protease. Enzymatic cleavage was carried out by incubating the modified rFnbA

[1] Abbreviations: HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; TBS, tris buffered saline (20 mM Tris buffer, pH 7.4, with 0.15 M NaCl); PBS, phosphate buffered saline (20 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.4, with 0.15 M NaCl); DTT, dithiothreitol; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TPCK, L-(tosylamido 2-phenyl) ethyl chloromethyl ketone; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PTH, phenylthiohydantoin; ECM, extracellular matrix proteins.

with trypsin or Glu-C protease for 16 h at 37 °C at an enzyme/substrate ratio of 1:20 (w/w). After 16 h of incubation an additional amount of trypsin or Glu-C protease was added to the reaction mixture, resulting in a final enzyme/substrate ratio of 1:10 (w/w), and the digestion was continued for another 8 h at 37 °C. The digestion mixture was diluted 1:1 (v/v) with 0.2% trifluoroacetic acid and centrifuged at 14000*g* for 5 min, and the supernatant was subjected to reversed phase HPLC.

Reversed Phase HPLC Separation of Dansylcadaverine- and Dansyl-PGGQQIV-Labeled Peptides. Dansylcadaverine- and dansyl-PGGQQIV-labeled peptides were separated on Aquapore RP-300 C_8 column (Brownlee Labs) by gradient elution with acetonitrile in 0.1% trifluoroacetic acid. Separation was carried out using a Dynamax HPLC station equipped with a ProStar fluorescence detector (Varian). Peptides were eluted with a 0−50% linear gradient of acetonitrile over a 90-min interval at a flow rate of 0.5 mL/min. Elution of peptides was detected by monitoring of absorbance at 210 nm and fluorescence at 550 nm with excitation at 350 nm. The fluorescent tracer peaks were collected and after concentration to smaller volumes (50−200 μL) were reinjected to the same column. The second round of elution was performed using a 10−20% or 20−35% linear gradient of acetonitrile over a 60-min interval at a flow rate of 0.5 mL/min. The isolated dansylcadaverine- or dansyl-PGGQQIV-labeled peptides were subjected to mass spectral and NH_2-terminal sequence analysis.

Sequence Analysis. The NH_2-terminal sequence analysis was performed with an Applied Biosystems model 490 sequenator. Selected samples were also submitted for service analysis to M-Scan Inc. These samples were analyzed using Applied Biosystems model 477A sequenator. The NH_2-termini of the isolated peptides were determined by sequencing for up to 18 cycles.

Theoretical Estimation of the Molecular Masses of Peptides. Calculation of the molecular masses of trypsin- and Glu-C proteinase-generated peptides was performed from the known primary sequence of staphylococcal rFnbA using Peptide Companion V1.25 software (CSPS Pharmaceuticals, Inc.). The effect of dansylcadaverine (335.17 Da) or dansyl-PGGQQIV (930.44 Da) modification on the mass of the peptide was calculated by considering the mass increase due to the probe. Since the formation of each ϵ-(γ-glutamyl)-lysine isopeptide bond is accompanied by the release of one ammonia (17.03 Da), the final molecular mass values were adjusted accordingly.

Mass Spectral Analysis. The determination of molecular masses of the isolated peptides was performed using MALDI-TOF mass spectrometer Voyager DE-STR (Perseptive Biosystems). Ions formed by laser desorption at 337 nm (N_2 laser) were recorded at an acceleration voltage of 20 kV in the reflector mode. In general, 200 single spectra were accumulated for improving the signal/noise ratio and analyzed by the use of the Data Explorer software. α-Cyano-4-hydroxycinnamic acid (Aldrich Chemical Co.) was used as the matrix. One microliter of a 10 mg/mL solution of the matrix compounds in 70% acetonitrile/0.1% trifluoroacetic acid was mixed with 1 μL of peptide solution (5−10 pmole/μL). For MALDI-TOF MS, 1 μL of this mixture was spotted on a stainless steel sample target and dried at room temperature. The mass spectra were externally calibrated using human Glu1-fibrinopeptide B, human angiotensin I, and synthetic des-Arg1-bradykinin.

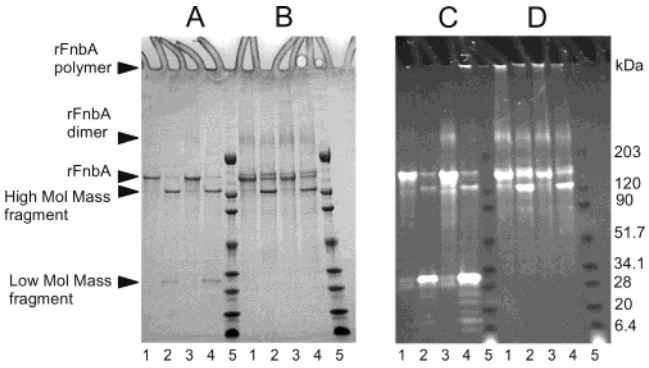


FIGURE 1: Factor XIIIa-catalyzed incorporation of dansylcadaverine (panels A and C) and dansyl-PGGQQIV (panels B and D) probes into rFnbA. Modification reactions were carried out for 4 and 18 h. After the removal of unreacted probes modified for 4 h (lane 1) and 18 h (lane 3) rFnbA samples were analyzed by SDS−PAGE. Alternatively, the rFnbA samples modified over 4 and 18 h were subjected to limited proteolysis by thrombin and then analyzed by SDS−PAGE (lanes 2 and 4). After electrophoresis the gels were photographed under 350 nm ultraviolet light (panels C and D) and then stained with Coomassie Brilliant Blue (panels A and B). Arrows show positions of probe-modified rFnbA and its thrombin-generated fragments. Lane 5 in each panel contains molecular mass standards as indicated.

The determination of the molecular mass of proteins (rFnbA) was performed using the same instrument. Ions were recorded at an acceleration voltage of 20 kV in the linear mode. Sinapinic acid (Aldrich Chemical Co.) was employed as the matrix. The mass spectra were calibrated using bovine serum albumin as an external standard.

RESULTS

Factor XIIIa-directed Incorporation of Dansylcadaverine and Dansyl-PGGQQIV into rFnbA and Fragmentation of Probe-Decorated Protein by Thrombin. In the previous study we have reported that fibronectin-binding protein A from *S. aureus* strain ATCC49525 serves as a bifunctional substrate for coagulation factor XIIIa and contains both reactive Gln and Lys residues (*1*). To assess the location of reactive Gln and Lys residues within FnbA, amine donor (dansylcadaverine) or amine acceptor (dansyl-PGGQQIV) fluorescent probes were incorporated into rFnbA by the catalytic action of factor XIIIa. Reactions were carried out for 4 or 18 h, followed by the removal of unreacted probes and the fragmentation of fluorescent-tracer-labeled rFnbA by thrombin. The existence of a single Arg202−Gly203 peptide bond within FnbA that is sensitive to thrombin attack allows the generation of two fragments representing the N- and C-terminal portions of rFnbA with theoretically estimated molecular masses of 22 and 70.7 kDa, respectively. The products of thrombin-mediated cleavage of rFnbA were evaluated by SDS−PAGE with subsequent examination of the gels under UV light prior to Coomassie Brilliant Blue staining (Figure 1). Incubation of dansylcadaverine-decorated rFnbA with thrombin resulted in the appearance of two discrete fragments, consistent with the hydrolysis of a single peptide bond. The mobility of the thrombin-generated low and high molecular mass fragments on SDS−PAGE was somewhat lower than that expected for 22 kDa and 70.7 kDa fragments. This observation, however, is coherent with the

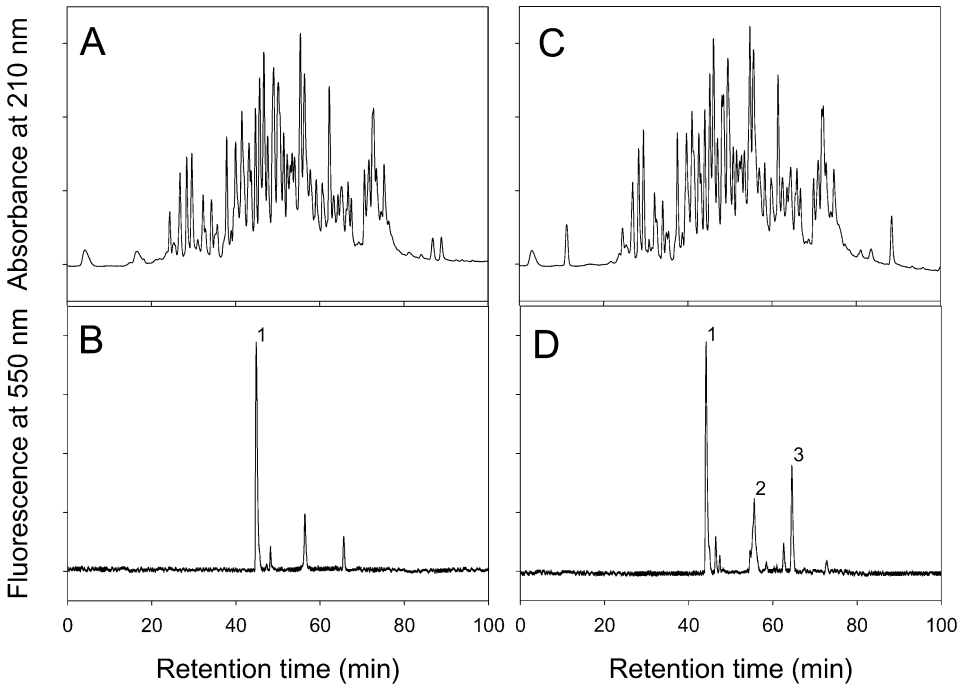


FIGURE 2: HPLC separation of dansylcadaverine-labeled peptides from the trypsin digest of factor XIIIa-modified rFnbA. Factor XIIIa-catalyzed incorporation of dansylcadaverine into rFnbA was carried out for 4 (panels A and B) and 18 h (panels C and D). The dansylcadaverine-labeled rFnbA preparations were digested by trypsin, and the peptides were separated on Aquapore RP-300 C_8 reversed phase column. The elution was monitored by absorbance at 210 nm as well as by fluorescence at 550 nm. Fluorescent peaks 1 (panels B and D), 2, and 3 (panel D) were collected, and after a second round of reversed phase chromatography the samples were subjected to NH_2-terminal sequence and mass spectral analysis.

overall low mobility on SDS−PAGE of the band corresponding to the parent rFnbA (both nonmodified and fluorescent probe-modified), which migrates between the 120 kDa and 203 kDa standards (*1*). The molecular mass obtained for rFnbA (93038 Da) using mass spectral analysis was close to the value estimated from the primary sequence (92656 Da), therefore, suggesting abnormally low migration of rFnbA and its fragments on SDS−PAGE.

The modification of rFnbA with dansylcadaverine catalyzed by factor XIIIa for 4 h resulted in the fluorescence of the band corresponding to monomeric rFnbA (Figure 1A,C, lane 1). The thrombin-generated cleavage of the dansylcadaverine-decorated rFnbA and subsequent analysis of the reaction mixture by SDS−PAGE revealed that fluorescence was mostly localized within the low molecular mass fragment. The band corresponding to the prominent high molecular mass fragment accommodated only a small fraction of the total dansylcadaverine fluorescence (Figure 1A,C, lane 2). Similar results were observed with rFnbA modified with dansylcadaverine over the extended, 18 h period of time (Figure 1A,C, lanes 3 and 4). Prolonged incubation of rFnbA with dansylcadaverine and factor XIIIa also resulted in the appearance of the minor high molecular mass band corresponding to rFnbA dimer (Figure 1A,C, lane 3). Incubation of rFnbA in the presence of factor XIIIa and dansyl-PGGQQIV peptide for 4 or 18 h resulted in the incorporation of the probe in the monomeric rFnbA as well as in the appearance of the bands corresponding to dimers and high molecular mass polymers (Figure 1B,D). Nevertheless, it is apparent that, under the experimental conditions employed, protein cross-linking was almost completely inhibited and the incorporation of dansylcadaverine and dansyl-PGGQQIV probes occur in the predominantly monomeric form of rFnbA. When the dansyl-PGGQQIV-modified rFnbA was subjected to thrombin-catalyzed cleavage, only the high molecular mass fragment exhibited fluorescence upon UV illumination (Figure 1D, lanes 2 and 4). The low molecular mass fragment was not detectable in UV light, nor was it visible upon staining of the gel with Coomassie Brilliant Blue (Figure 1B, lanes 2 and 4). Thus, limited proteolysis data indicated that the major glutamine acceptor and lysine donor sites are spatially separated within the polypeptide chain of the rFnbA molecule and presumably located in the N- and C-terminal regions, respectively.

Identification of the rFnbA Glutamine Acceptor Sites Involved in Factor XIIIa Cross-Linking Reactions. In order to identify the specific reactive Gln residue(s) within rFnbA, the latter was incubated for 4 and 18 h in the presence of factor XIIIa and a molar excess of the fluorescent probe dansylcadaverine. Following the dansylcadaverine labeling reaction, the modified rFnbA preparations were washed from the unreacted probe and then digested with trypsin. HPLC separation of the tryptic peptides produced after a 4 h factor XIIIa-catalyzed incorporation of dansylcadaverine into rFnbA revealed a complex profile at 210 nm (Figure 2A). In contrast, only one major peak with retention time of approximately 44 min was detected in the same sample upon monitoring of fluorescence at 550 nm (Figure 2B, peak 1). Extension of the incubation time of rFnbA in the presence of factor XIIIa and dansylcadaverine from 4 to 18 h and subsequent digestion with trypsin did not have an impact either on the elution profile at 210 nm (Figure 2 C) or on intensity or retention time of the major fluorescent peak (Figure 2D, peak 1). At the same time, extending the time of dansylcadaverine incorporation into rFnbA resulted in the increase of intensities of two minor fluorescent peaks depicted as 2 and 3 (Figure 2D). The fluorescent tracer-decorated peptides labeled as peak 1 (Figure 2B) and peaks

Table 1: Summary of the NH_2-Terminal Sequence and Mass Spectral Analysis of Dansylcadaverine-Modified Peptides Derived from rFnbA[a]

proteinase	peak	retention time (min)	amino acid sequence	peptide fragment	$[M + H]^+$ obsd	$[M + H]^+$ calcd
trypsin	1	43.84	ETTQSQDNSGD[**Q**]R	92−104	1783.83	1783.75
	3	64.52	[**Q**]VDLIPK	105−111	1130.68	1130.62
Glu-C	1	46.75	TTQSQDNSGD[**Q**]RQVD	93−107	1996.70	1996.86
	4	53.02	G[**Q**]QTIEE	829−835	1122.50	1122.51
	6	58.63	SVP[**Q**]IHGFNKHNE	780−792	1824.59	1824.88
	7	60.29	TTQSQDNSGD[**Q**]R[**Q**]VD	93−107	2314.99	2315.00

[a] [**Q**] indicates an Edman cycle without recovery of a known amino acid and assigned to be factor XIIIa-derivatized Gln. The calculated mass of tryptic peptides 1 and 3 and Glu-C proteinase peptides 1, 4, and 6 includes the mass of one incorporated dansylcadaverine molecule. The calculated mass of Glu-C proteinase peptide 7 includes the mass of two incorporated dansylcadaverine molecules (see Materials and Methods). Tryptic peptide from fluorescent peak 2 and Glu-C proteinase peptides from fluorescent peaks 2, 3, and 5 were not unambiguously identified.

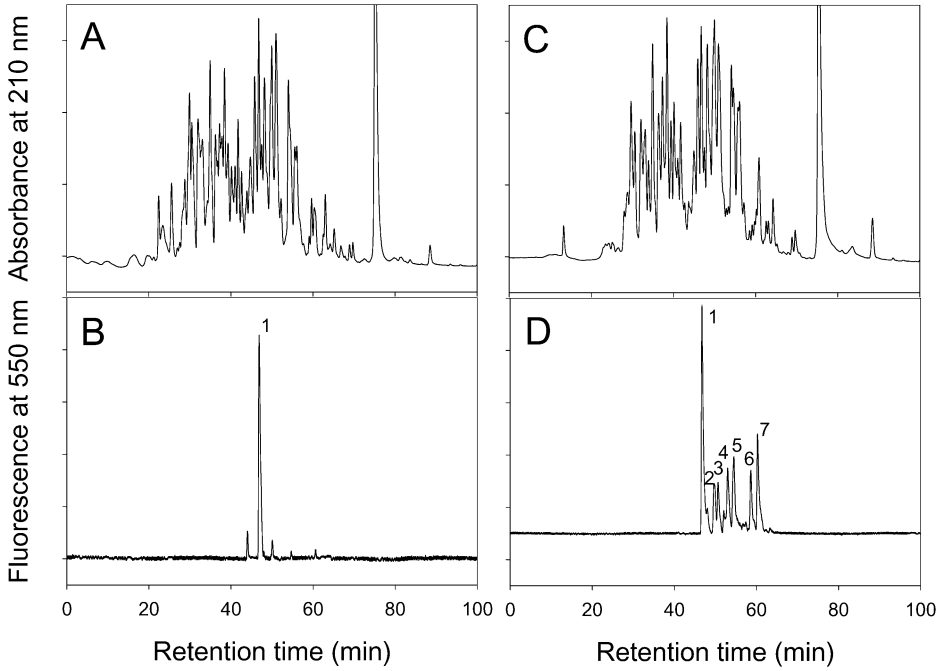


FIGURE 3: HPLC separation of dansylcadaverine-labeled peptides from the Glu-C protease digest of factor XIIIa-modified rFnbA. Factor XIIIa-catalyzed incorporation of dansylcadaverine into rFnbA was carried out for 4 (panels A and B) and 18 h (panels C and D). The dansylcadaverine-labeled rFnbA preparations were digested by Glu-C protease, and the peptides were separated on Aquapore RP-300 C_8 reversed phase column. The elution was monitored by absorbance at 210 nm as well as by fluorescence at 550 nm. Fluorescent peaks 1 (panels B and D), 2, 3, 4, 5, 6 and 7 (panel D) were collected, and after a second round of reversed phase chromatography the samples were subjected to NH_2-terminal sequence and mass spectral analysis.

1, 2, 3 (Figure 2D) were collected and after a second passage through a C_8 column were characterized by NH_2-terminal sequence and mass spectral analysis (Table 1). Sequence analysis of the peptide from the major fluorescent peak 1 revealed that it corresponds to a 13-mer fragment derived from the NH_2-terminal portion of the rFnbA. During Edman degradation, the residue in the 12th cycle was not detected, while proper sequencing was resumed in the next 13th cycle. The 12th residue, which did not yield a conventionally recognized amino acid in the Edman procedure, corresponds to Gln103 therefore suggesting that it was modified. Two other Gln residues (Gln95 and Gln97) located in this peptide were released as PTH (phenylthiohydantoin) derivatives in cycles 4 and 6, respectively. Sequencing data obtained for the tryptic 13-mer peptide (peak 1) are further supported by the results of mass spectral analysis. The mass spectrum of this peptide displayed an $[M + H]^+$ peak at m/z 1783.83, corresponding to the calculated mass of the peptide containing a single dansylcadaverine modification, 1783.75 (Table 1). Sequence and mass spectral analysis of the tryptic peptide from the minor fluorescent peak 3 in Figure 2D showed that this peptide was also derived from the NH_2-terminal region of the FnbA molecule. Upon sequencing, a single Gln residue (Gln105) was not detected in the first cycle, but the registration of PHT derivatives of the residues shown in Table 1 was resumed in the following cycles, suggesting that Gln105 can be identified as another acceptor site. The observed mass of this peptide corresponded to the theoretical value with a single dansylcadaverine modification (Table 1). The material from peak 2 was not sufficiently homogeneous for NH_2-terminal sequencing even after additional passage through a C_8 reversed phase column and, therefore, was not positively identified by Edman degradation.

Because some of the predicted tryptic peptides were rather large (particularly those originated from the COOH-terminal portion of the protein), digestion of dansylcadaverine-modified rFnbA was also performed using Glu-C proteinase, which generated smaller and more manageable peptides. The rFnbA and factor XIIIa were incubated in the presence of dansylcadaverine, as described in "Materials and Methods", and digested with Glu-C proteinase. A single fluorescent peak with a retention time of 46 min was

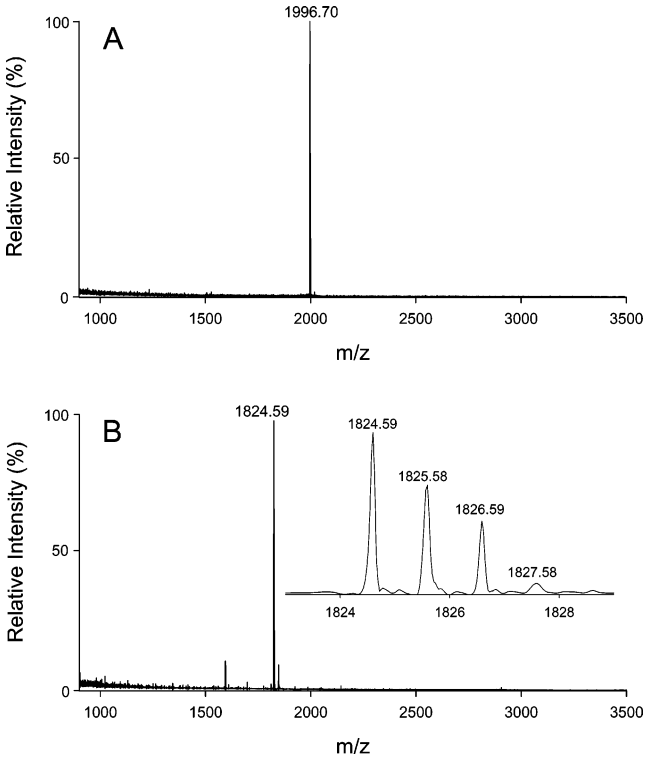


FIGURE 4: MALDI-TOF mass spectra of isolated dansylcadaverine-labeled peptides from the Glu-C protease digest of factor XIIIa-modified rFnbA. MALDI-TOF MS analysis of selected fluorescent peaks 1 (panel A) and 6 (panel B) is shown. The inset in panel B shows an example of the isotopic distribution obtained for dansylcadaverine-modified peptide from fluorescent peak 6 (Table 1).

repeatedly observed in the Glu-C proteinase digestion mixture after the modification of rFnbA with dansylcadaverine over a 4 h period (Figure 3B, peak 1). The extended, 18 h incorporation of dansylcadaverine into rFnbA followed by Glu-C digestion produced the same major peak 1 and multiple minor peaks designated as 2, 3, 4, 5, 6, and 7 (Figure 3D). As shown in Table 1, four peptides were recovered from the Glu-C proteinase digestion mixture and positively identified. The peptides from peaks 1 and 7 contained the same reactive Gln103 and Gln105 residues as those identified in the tryptic digests. Interestingly, both peptides correspond to the sequence 93−107 and differ only by the number of dansylcadaverine modifications. The peptide from the major fluorescent peak 1 contains one modified Gln103 residue, while both Gln103 and Gln105 are modified in the peptide from peak 7. The Glu-C proteinase digestion also produced two fluorescent peptides derived from the COOH-terminal portion of rFnbA. The peptides from peaks 4 and 6 contained modified Gln830 and Gln783 residues, respectively. Again, the presence of dansylcadaverine modifications in the isolated fluorescent peaks was confirmed by mass spectral analysis (Figure 4 and Table 1).

The analysis of the several separate dansylcadaverine labeling experiments performed over 4 and 18 h followed by either trypsin or Glu-C proteinase digestion suggested that Gln103 serves as a major amine acceptor site for factor XIIIa in rFnbA. The high reactivity of the Gln103 site is responsible for the origin of a single major fluorescent peak 1 corresponding to either the tryptic peptide ETTQSQDNSGD**Q**$_{103}$R (Figure 2B) or Glu-C proteinase-generated TTQSQDNSGD**Q**$_{103}$RQVD peptide (Figure 3B). Modification of the Gln103 was fully completed after 4 h of reaction (or earlier), since further incubation with factor XIIIa did not affect the intensity of peak 1 (Figures 2D and 3D). In contrast, recovery of additional fluorescent peptides from the trypsin (peaks 2, 3) or Glu-C proteinase (peaks 4, 6, and 7) digestion mixtures was achieved only upon extended treatment with factor XIIIa. Intensities of these fluorescent peaks were still significantly lower, compared to that of the major peak 1, indicating that only a fraction of reactive Gln residues at positions 105, 783, and 830 underwent modification. Thus, modification experiments with dansylcadaverine revealed that rFnbA contains one major (Gln103) and three minor (Gln105, Gln783, and Gln830) factor XIIIa-reactive amine acceptor sites.

Identification of the rFnbA Lysine Donor Sites Involved in Factor XIIIa Cross-Linking Reactions. The factor XIIIa-mediated titration of Lys side chains of rFnbA was performed using the dansyl-PGGQQIV peptide patterned on the NH_2-terminal sequence of fibronectin. The rFnbA was incubated for 4 h in the presence of factor XIIIa and dansyl-PGGQQIV probe and then digested with Glu-C proteinase. The HPLC separation of Glu-C proteinase-generated peptides again revealed multiple peaks detected at 210 nm and only a few fluorescent peaks, eluting in the range of approximately 60 min (Figure 5A,B). However, with the exception of the peak marked by an asterisk (Figure 5 B), the degree of labeling and consequently the level of purity of the probe modified peptides were not sufficient for required sequence analysis. To improve the recovery of dansyl-PGGQQIV-modified peptides, the rFnbA was incubated with factor XIIIa and dansyl-PGGQQIV probe for 18 h and digested with Glu-C proteinase as described in "Materials and Methods". The HPLC separation of this digestion mixture produced a total of seven fluorescent peaks (Figure 5D). The elution profile of fluorescent peaks 1−7 (Figure 5D) was similar to that of the digestion mixture generated after 4 h of rFnbA modification (Figure 5B), suggesting production of the same peptides with a higher degree of labeling. Each of these fluorescent peaks (asterisk-labeled peak from Figure 5B and peaks 1−7 from Figure 5D) was additionally purified on a reversed phase C_8 column, and then subjected to evaluation by mass spectral and sequencing analysis. The results of performed analysis are summarized in Table 2. A total of six modified peptides were positively identified using both mass spectral and sequencing analysis. High confidence sequences were obtained for peak 4, 5, and 7 fractions, identifying Lys157, Lys620, and Lys503 unambiguously as probe-modified residues (Table 2). During Edman degradation dansyl-PGGQQIV-modified Lys residues are not recognized as conventional PTH derivatives and, therefore, cannot be detected. Upon sequencing of peak 4 this result was observed in the 2nd cycle, while Val residue was released as PTH derivative in the 1st cycle. Sequencing resumed in the following 3rd cycle and continued without interruptions. The Lys residue yielded in the 9th cycle further reinforced the conclusion that the modified lysine (Lys157) was present in cycle 2. Analysis of peptides from peaks 5 and 7 revealed the interruption of sequencing in cycles 3 and 2, respectively. Again, these data suggest that Lys620 (cycle 3 in peptide 5) and Lys503 (cycle 2 in peptide 7) were modified by factor XIIIa. As can be seen from Table 2, the results of NH_2-terminal sequence analysis obtained for peaks 4, 5, and 7

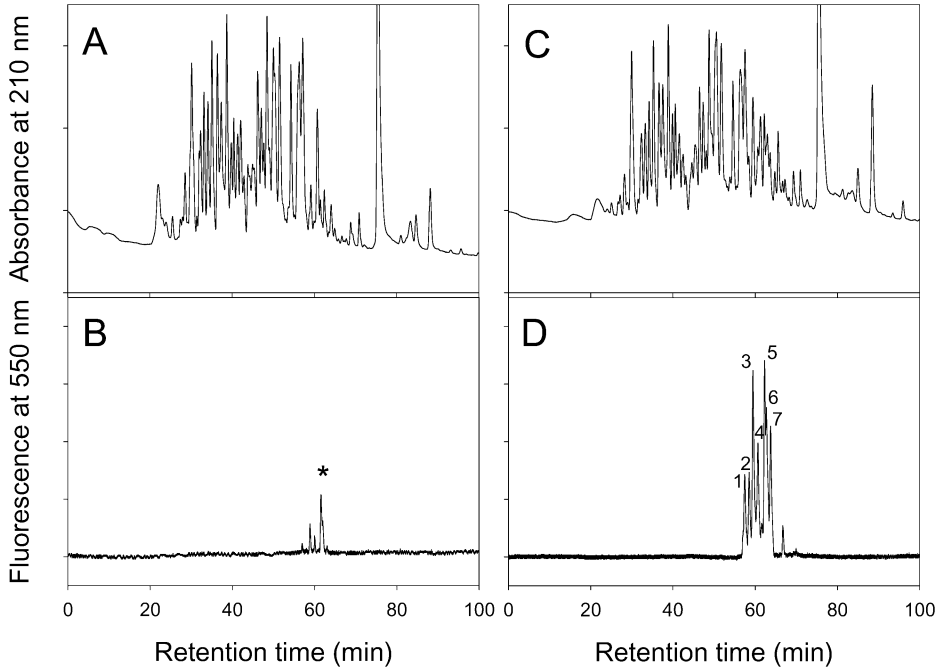


FIGURE 5: HPLC separation of dansyl-PGGQQIV-labeled peptides from the Glu-C protease digest of factor XIIIa-modified rFnbA. Factor XIIIa-catalyzed incorporation of dansyl-PGGQQIV into rFnbA was carried out for 4 (panels A and B) and 18 h (panels C and D). The dansyl-PGGQQIV-labeled rFnbA preparations were digested by Glu-C protease, and the peptides were separated on Aquapore RP-300 C_8 reversed phase column. The elution was monitored by absorbance at 210 nm as well as by fluorescence at 550 nm. The fluorescent peak depicted by an asterisk (panel B) and peaks 1, 2, 3, 4, 5, 6 and 7 (panel D) were collected, and after a second round of reversed phase chromatography the samples were subjected to NH_2-terminal sequence and mass spectral analysis.

Table 2: Summary of the NH_2-Terminal Sequence and Mass Spectral Analysis of Dansyl-PGGQQIV-Modified Peptides Derived from rFnbA[a]

proteinase	peak	retention time (min)	amino acid sequence	peptide fragment	$[M + H]^+$ obsd	$[M + H]^+$ calcd
Glu-C	1	57.18	V[**K**]GTD	156−160	1432.37	1432.68
	2	58.55	[**K**]DKPKYE	762−768	1820.77	1820.89
	4	60.24	V[**K**]GTDVTSKVTVE	156−168	2276.13	2276.15
	5 (*)	62.29	ST[**K**]GIVTGAVSD	618−629	2047.97	2048.00
	6	62.98	ST[**K**]GIVTGAVSDHTTVE	618−634	2614.95	2615.21
	7	63.77	A[**K**]QIIE	502−507	1614.50	1614.82

[a] [**K**] indicates an Edman cycle without recovery of a known amino acid and assigned to be factor XIIIa-derivatized Lys. The calculated mass includes the mass of one incorporated dansyl-PGGQQIV molecule (see Materials and Methods). Peptide from fluorescent peak 3 was not unambiguously identified.

are consistent with the observed $[M + H]^+$ values. Each of these probe-modified fractions exhibited an $[M + H]^+$ value that precisely matched the calculated mass of the respective peptide containing a single dansyl-PGGQQIV modification (Table 2). Each of the fluorescent peaks 1 and 2 represented a mixture of two labeled and unlabeled peptides, present in essentially equal amounts. Reliable reading of the double sequence was achieved by utilizing the known primary sequence of FnbA and the map of predicted cleavage sites. Similarly, reading of a low confidence sequence obtained for fluorescent peak 6 was also accomplished by knowing the amino acid sequence of FnbA and the location of predicted cleavage sites catalyzed by Glu-C proteinase. Analysis of the isolated fractions revealed that some dansyl-PGGQQIV-labeled peptides are derived from the same regions of the polypeptide chain. Partial hydrolysis of the Asp160−Val161 peptide bond by Glu-C proteinase resulted in the recovery of a shorter version of probe-modified peptide 1 (fragment 156−160) and a longer peptide 4 (fragment 156−168). Similarly, incomplete hydrolysis of the Asp629−His630 peptide bond resulted in the production of peptide 5 (fragment 618−629) and peptide 6 (fragment 618−634) (Table 2). Thus, Lys157 and Lys620 again were identified as probe-modified residues in fluorescent peaks 1 and 6, respectively. In addition to the probe-modified peptide V$\mathbf{K}_{157}$-GTD, fluorescent peak 1 also contained unlabeled peptide corresponding to residues 322−350 (Figure 7). Factor XIIIa-catalyzed modification of Lys762 was demonstrated by the sequencing of fraction corresponding to fluorescent peak 2 (Table 2). The secondary low confidence sequence detected in peak 2 was not positively identified. Presumably this sequence is originated from an unidentified peptide with observed mass $[M + H]^+$ of 2047.84 which is also present in peak 2 (Figure 6A). Overall, mass spectral analysis of fractions 1, 2, and 6 provided another line of evidence supporting the results of NH_2-terminal sequencing. The mass peaks corresponding to $[M + H]^+$ at *m*/*z* 1432.37, 1820.77, and 2614.95 were present in fractions 1, 2, and 6, respectively. The observed masses obtained for fluorescent peaks 1, 2, and 6 corresponded to the calculated values with a single dansyl-PGGQQIV modification each (Figure 6 and Table 2). The fluorescent peak depicted by an asterisk (Figure 5B) represented a mixture of two peptides. The reading of this fraction was again achieved by knowing the primary

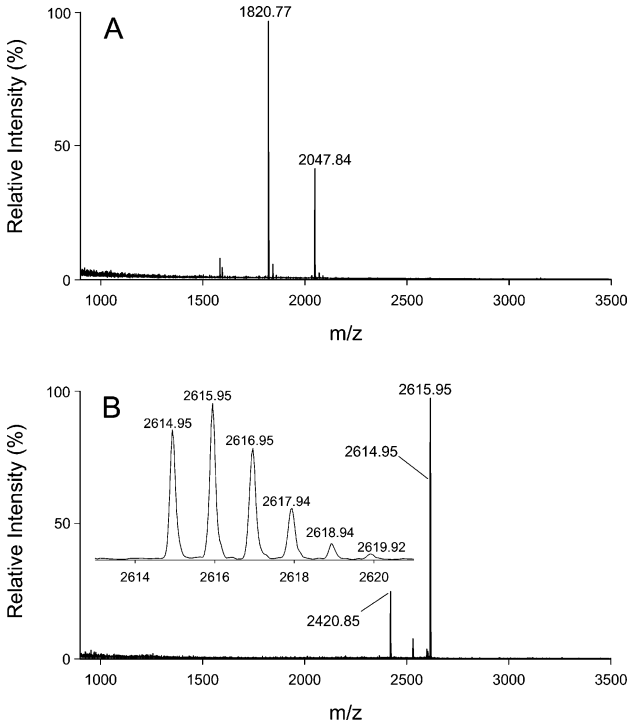
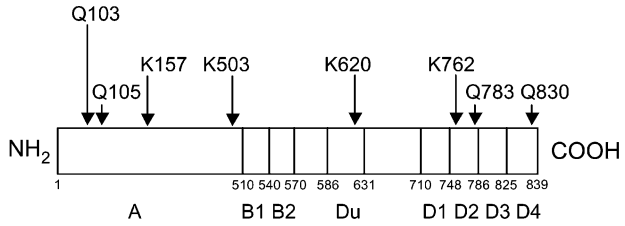


FIGURE 6: MALDI-TOF mass spectra of isolated dansyl-PGGQ-QIV-labeled peptides from the Glu-C protease digest of factor XIIIa-modified rFnbA. MALDI-TOF MS analysis of selected fluorescent peaks 2 (panel A) and 6 (panel B) is shown. The inset in panel B shows an example of the isotopic distribution obtained for dansyl-PGGQQIV-modified peptide from fluorescent peak 6 (Table 2).

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ASEQKTTTVEENGNSTTDNKVSETQTTTTNVNHIEETQSYNATVTEQPSN 50
ATQVTTEEAPKAVQAPQTAQPANVEKVKEEVVKEEAKPQVKETTQSQDNS 100
GD**Q**R**Q**VDLIPKKATQNQVAETQVEVAQPRTVSESKPRVTRSADVVEAKEG 150
MGVSEV**K**GTDVTSKVTVESGSIEAPQGNKVEPHAGQRVVLKYKLKFADGL 200
KRGDYFDFTLSNNVNTYGVSTARKVPEIKNGSVVMATGEILGNGNIRYTF 250
TNEIEHKVEVTANLEINLFIDPKTVQSDGEQKITSKLNGEETEKTIPVVY 300
NPGVSNSYTNVNGSIETFNKESNKFTHIAYIKPMNGNQSNTVSVTGTLTE 350
GSNLAGGQPTVKVYEYLGKKDELPQSVYANTSDTNKFKDVTNEMNGKLSV 400
QDNGSYSLNLDKLDKTYVIHYTGEYLQGSDQVNFRTELYGYPERAYKSYY 450
VYGGYRLTWDNGLVLYSNKADGNGKNGQIIQNNDFEYKEDTAKGTMSGQY 500
DA**K**QIIETEENQDNTPLDIDYHTAIDGEGGYVDGYIETIEETDSSAIDID 550
YHTAVDSEAGHVGGYTESSEESNPIDFEESTHENSKHHANVVEYEEDTNP 600
GGGQVTTESNLVEFDEEST**K**GIVTGAVSDHTTVEDTKEYTTESNLIELVD 650
ELPEEHGQAQGPIEEITENNHHISHSGLGTENGHGNYGVIEEIEENSHVD 700
IKSELGYEGGQNSGNQSFEEDTEEDKPKYEQGGNIVDIDFDSVPQIQGQN 750
NGNQSFEEDTE**K**DKPKYEQGGNIIDIDFDSVP**Q**IHGFNKHNEIIEEDTNK 800
DKPNYQFGGHNSVDFEEDTLPKVSGQNEG**Q**QTIEEDTTP 839
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FIGURE 7: Location of factor XIIIa-reactive Gln and Lys residues within FnbA. Schematic illustration of the structural organization of the rFnbA (residues Ala1−Pro839) from *S. aureus* strain ATCC49525 used in this study (top) and its amino acid sequence (bottom). The cartoon 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. Arrows show the positions of factor XIIIa-reactive Gln (103, 105, 783, and 830) and Lys (157, 503, 620, and 762) residues within A, Du, D2, and D4 regions of FnbA. Bold letters denote the same factor XIIIa-reactive Gln and Lys residues in the primary sequence of FnbA. Underlined letters depict the location of predicted thrombin cleavage site Arg202−Gly203.

sequence of FnbA. The unlabeled peptide was identified as fragment 266−280 (Figure 7) while the tracer-containing peptide corresponded to peak 5 on Figure 5D and contained the single probe-modified Lys620 (Table 2). The fraction corresponding to peak 3 (Figure 5D) appeared to be a mixture of several peptides, sequences of which could not be resolved by Edman degradation. Thus, the treatment of rFnbA with factor XIIIa in the presence of dansyl-PGGQQIV probe resulted in the specific modification of Lys157, Lys503, Lys620, and Lys762, suggesting that these residues serve as amine donor sites.

DISCUSSION

In the present study we have identified within the staphylococcal FnbA reactive Gln and Lys residues that are targeted by the human coagulation factor XIIIa. To our knowledge, this is the first report on the localization of factor XIIIa-reactive amine acceptor and donor sites in a bacterial protein. The site-specific labeling of the factor XIIIa-reactive glutamines had been performed using the fluorescent lysine analogue dansylcadaverine (*15*, *16*). We have shown that factor XIIIa reacted with a restricted number of Gln residues present in the rFnbA receptor. Residues Gln103, Gln105, Gln783, and Gln830 serve as amine acceptor sites in FnbA, when the latter is incubated with dansylcadaverine and coagulation factor XIIIa. The reactive residues Gln103 and Gln105 are located within the NH_2-terminal A region of FnbA receptor (Figure 7), while the Gln783 and Gln830 residues are situated in the COOH-terminal part of the molecule and belong to the D1 and D4 repeats, respectively. Identification of the Gln103 as a major amine acceptor site of the FnbA is consistent with the results of limited proteolysis experiments. The cleavage of dansylcadaverine-modified rFnbA by thrombin results in the release of a low molecular mass NH_2-terminal fragment, which exhibited a high intensity of fluorescence upon UV illumination, due to the presence of the major Gln103 acceptor site. The high molecular mass band corresponding to the COOH-terminal fragment and containing the minor Gln783 and Gln830 sites (Figure 7) produced a low emission signal (Figure 1A,C). Thus, limited proteolysis data obtained for the dansylcadaverine-modified rFnbA provided another line of evidence suggesting various degrees of reactivity of identified acceptor sites.

The dansyl-PGGQQIV peptide probe patterned on the NH_2-terminal sequence of fibronectin containing reactive glutamine residue has been utilized for the labeling of factor XIIIa-reactive lysine residues (*17*, *18*). The labeling procedure revealed that 4 of the 56 potential lysine donor residues within rFnbA had incorporated the peptide probe. These

residues include Lys157, Lys503, Lys620, and Lys762. The identified factor XIIIa-reactive lysine sites are distributed between the fibrin(ogen)-binding region A and the fibronectin-binding D repeats. Lys157 is located within the NH_2-terminal part of the A region, while Lys503 is located in its COOH-terminal segment adjacent to the B1B2 repeats. The fibronectin-binding Du and D2 repeats contain the factor XIIIa-reactive Lys620 and Lys762 sites, respectively (Figure 7). Interestingly, despite the NH_2-terminal location of the reactive Lys157, thrombin cleavage of the dansyl-PGGQ-QIV-decorated rFnbA did not produce a fluorescent low molecular mass fragment (Figure 1D, lanes 2 and 4). The low molecular mass fragment was neither detectable upon staining of the gel with Coomassie Brilliant Blue (Figure 1 B, lanes 2 and 4). These observations indicate that the modification of Lys157 with the dansyl-PGGQQIV probe might induce higher susceptibility of the NH_2-terminal region of FnbA to thrombin attack resulting in the generation of small peptides that are not detectable on SDS−PAGE. Alternatively, the loss of the low molecular mass fragment can be explained by the high reactivity of the Gln103 acceptor site located in the same NH_2-terminal region of the protein. Due to its high reactivity, Gln103 may participate in the formation of inter- and possibly intramolecular isopeptide bond(s) with available Lys residue(s) in the presence of a molar excess of dansyl-PGGQQIV probe. This is supported by the presence of rFnbA cross-linked species, which are visible on the gel upon Coomassie staining (Figure 1B) or under UV illumination (Figure 1D). Consequently, generation of cross-linked rFnbA species may result in hampered SDS−PAGE detection of low molecular mass fragment produced by thrombin.

Overall, all of the identified reactive Gln acceptor and Lys donor residues tend to cluster in the NH_2- and COOH-terminal areas of FnbA that form the fibrin(ogen)- and fibronectin-binding sites. The existence of an additional Gln acceptor and Lys donor residues within the staphylococcal FnbA receptor, however, cannot be excluded since the fluorescent tracer containing peptides corresponding to peak 2 (Figure 2D), peaks 2, 3, 5 (Figure 3D), and peak 3 (Figure 5D) were not positively identified. Nevertheless, site-specific labeling allowed the localization of the exact positions of reactive Gln and Lys residues participating in factor XIIIa-catalyzed cross-linking reactions of FnbA with fibronectin, fibrin, and, possibly, other human host proteins. Up to now, only a little more than a dozen protein substrates have been identified for coagulation factor XIIIa. Among the known glutamine-containing substrates for factor XIIIa exists little sequence homology, and reactivity is difficult if not impossible to predict. The identified reactive glutamines are usually located in the solvent-exposed surface regions or flexible extensions (*22*−*24*). It seems that both the primary structure and the conformation of a protein appear to determine whether a glutamine residue can be reactive. These observations are consistent with the data obtained in this study for the staphylococcal FnbA receptor. The reactive Gln783 and Gln830 acceptor sites are situated in the D2 and D4 fibronectin-binding repeats, which, according to several reports, do not have a compact structure and exist in a rather unfolded state (*25*−*27*). The reactive Gln103 and Gln105 sites are located in the NH_2-terminal region of FnbA that appears to be sensitive to proteolysis and, therefore, again may indicate the lack of an ordered structure. The selectivity of factor XIIIa toward the amine donor lysine residues in proteins is not sufficiently understood, either. Despite the common notion that factor XIIIa is less selective toward lysine residues than to glutamine residues, only a restricted number of amine donor sites can participate in a particular protein−protein cross-linking reaction and undergo modifications with a peptide probe. It was shown that factor XIIIa exhibits broad yet clearly differentiated tolerance with respect to the residue preceding the amine donor lysine in protein substrates. Analysis of protein substrates for factor XIIIa or tissue transglutaminase revealed that the residues directly preceding the amine donor site are preferentially uncharged and basic polar residues, as well as the small aliphatic ones (*28*). The data obtained in the present study for the staphylococcal FnbA receptor further support these observations. Among the four identified factor XIIIa-reactive lysines, Val precedes Lys157, Ala precedes Lys503, and Thr precedes Lys620. The only exception is Glu, which precedes Lys762 (Figure 7).

In order to investigate whether the identified factor XIIIa-reactive sites are conserved in other fibronectin-binding proteins from different *S. aureus* strains, their amino acid sequences were analyzed using a multiple sequence alignment. The amino acid sequence of FnbA from *S. aureus* strain ATCC49525 was compared to the FnbA and FnbB sequences from strains 8325-4 (*11*, *12*), MW2 (*29*), EMRSA-16, MSSA-476, COL, Mu50, and N315 (30). The amino acid sequences of FnbA and FnbB receptors from strains EMRSA-16 and MSSA-476 were obtained from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk). The *S. aureus* COL sequence was obtained from the Institute for Genomic Research (http://www.tigr.org). Multiple sequence alignment was performed using CLUSTAL W (1.81) program (*31*). Figure 8 shows the amino acid sequence alignment of the regions surrounding the identified reactive Gln and Lys residues in the FnbA receptor from *S. aureus* strain ATCC49525. A multiple alignment revealed that the reactive Gln103, Gln105, Gln830, and Lys620 residues are topologically conserved in all analyzed FnbA sequences. In the FnbA receptor from strains MW2, MSSA-476, COL, 8325-4, and EMRSA-16 the reactive Lys157 is replaced with a Thr residue. In strains COL and 8325-4, the reactive Lys503 is substituted to an Asn residue. The segment of polypeptide chain containing reactive Lys762 is missing in the FnbA sequence from EMRSA-16 strain, and the reactive Gln783 is substituted to His in COL and 8325-4 strains (Figure 8). This observation indicates that the reactivity of FnbA receptors from different *S. aureus* strains toward the transglutaminase action of factor XIIIa may vary. Also, it is apparent that the factor XIIIa-reactive acceptor and donor sites are less preserved within the FnbB family of receptors. None of the analyzed FnbB sequences possess the reactive Gln103, Lys157, and Lys503, while Lys762 is missing in Mu50 and N315 strains. The reactive Gln105 and Gln783 are not preserved in the FnbB sequence from strains MW2, MSSA-476, COL, and 8325-4. Among all of the identified factor XIIIa-reactive sites, only the Lys620 and Gln830 residues are highly conserved and present in all analyzed FnbA and FnbB sequences (Figure 8). Interestingly, upon treatment of FnbA with factor XIIIa, the conserved reactive Lys620 residue consistently exhibited the highest reactivity

	Strain	Q103 Q105	K157	K503	K620	K762	Q783	Q830
FnbA	ATCC49525	DNSGD**Q**R**Q**VDLIP	GVSEV**K**GTDVT	GQYDA**K**QIIET	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	Mu50	DNSGN**Q**R**Q**VDLTP	DVSEV**K**GTDVT	GQYDA**K**QIIET	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	N315	DNSGN**Q**R**Q**VDLTP	DVSEV**K**GTDVT	GQYDA**K**QIIET	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	MW2	DNSGD**Q**R**Q**VDLTP	DEKVETGTDVT	GQYDA**K**QIIET	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	MSSA-476	DNSGD**Q**R**Q**VDLTP	DEKVETGTDVT	GQYDA**K**QIIET	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	COL	DNSGD**Q**R**Q**VDLTP	NAKVETGTDVT	GQYDKNLVTTV	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVPHIHGFN	GQNEG**Q**QTIEE
	8325-4	DNSGD**Q**R**Q**VDLTP	NAKVETGTDVT	GQYDKNLVTTV	DEEST**K**GIVTG	EEDTE**K**DKPKY	FDSVPHIHGFN	GQNEG**Q**QTIEE
	EMRSA-16	DNSGD**Q**R**Q**VDLTP	DAKVETGTDVT	GQYDA**K**QIIET	DEEST**K**GIVTG	----------	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
FnbB	Mu50	-----KS**Q**EDLPS	EEAKATGTDVT	-----DPIIEK	DEEST**K**GIVTG	EEDTEEDKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	N315	-----KS**Q**EDLPS	EEAKATGTDVT	-----DPIIEK	DEEST**K**GIVTG	EEDTEEDKPKY	FDSVP**Q**IHGFN	GQNEG**Q**QTIEE
	MW2	------SRVDLPS	EEAKATGTDVT	-----DPIIEK	DEEST**K**GILTG	EEDTE**K**DKPKY	FDSVPHIHGFN	GHNEG**Q**QTIEE
	MSSA-476	------SRVDLPS	EEAKATGTDVT	-----DPIIEK	DEEST**K**GILTG	EEDTE**K**DKPKY	FDSVPHIHGFN	GHNEG**Q**QTIEE
	COL	------SRVDLPS	EETKATGTDVT	-----EPIIEH	DEDST**K**GIVTG	EEDTE**K**DKPKY	FDSVPHIHGFN	GHNEG**Q**QTIEE
	8325-4	------SRVDLPS	EETKATGTDVT	-----EPIIEH	DEDST**K**GIVTG	EEDTE**K**DKPKY	FDSVPHIHGFN	GHNEG**Q**QTIEE

FIGURE 8: Alignment of the amino acid sequences of FnbA and FnbB species from various *S. aureus* stains. Regions surrounding factor XIIIa-reactive Gln and Lys residues are shown. Positions of the identified reactive Gln and Lys residues at the top correspond to that of the FnbA receptor from *S. aureus* strain ATCC49525. The bold letters in the primary sequences of FnbA and FnbB species highlight topologically conserved factor XIIIa-reactive Gln acceptor and Lys donor sites. Multiple sequence alignment was performed using the CLUSTAL W (1.81) program.

toward the dansyl-PGGQQIV probe (Figure 5B peak marked by an asterisk, Figure 5D peak 5, Table 2). This further indicates physiological importance of the Lys donor site at position 620. The fact that the major Gln103 acceptor site along with the Lys157 and Lys503 donor sites are absent in all evaluated FnbB sequences suggests that the B form of fibronectin-binding receptor may play a less prominent role in factor XIIIa-catalyzed cross-linking reactions. These differences also indicate that A and B forms of fibronectin-binding protein may exhibit various selectivity toward their human host protein cross-linking partners.

Except the staphylococcal FnbA receptor, all currently known factor XIIIa protein substrates are involved in blood coagulation, fibrinolysis, extracellular matrix assembly, and wound healing reactions. Our finding that *S. aureus* FnbA serves as a bifunctional substrate for factor XIIIa and undergoes cross-linking to fibronectin or fibrin (*1*) suggests that coagulation factor XIIIa also plays an important role in molecular pathogenesis. The ability of pathogenic *S. aureus* to utilize the transglutaminase activity of factor XIIIa for covalent attachment to human host molecules might explain the extremely high efficiency of bacterial colonization upon tissue injury. Following injury, the formation of a blood clot serves both to restore vascular integrity and to provide a provisional matrix for the initiation of wound repair (*32*). The clot's major protein components, fibrin and plasma fibronectin, are essential for these functions. Both fibrin and fibronectin also serve as ligands for the surface-associated FnbA receptor of *S. aureus* and, therefore, are responsible for the binding of the bacteria to the wound site. As the clot matures, coagulation factor XIIIa initiates catalysis of intermolecular cross-linking between fibrin molecules and between fibrin and fibronectin. Covalent cross-linking between fibrin molecules increases the structural stability of the clot (*8*), while the cross-linking of fibronectin to fibrin is important for cell adhesion and migration events required for the wound healing process (*33*−*35*). The staphylococcal FnbA receptor that is reversibly associated with fibrin or fibronectin at this stage can be covalently cross-linked to its ligands by factor XIIIa. The covalent incorporation of FnbA to fibrin or fibronectin increases the probability of staphylococcal colonization and establishment of infection. It also competes with fibrin−fibrin and fibrin−fibronectin cross-linking reactions (*1*, *36*, *37*) and, therefore, may affect the structural integrity of the clot and inhibit wound-healing reaction. Factor XIIIa-catalyzed cross-linking reactions resulting in formation of FnbA homo- or heteropolymers may also contribute to biofilm development. It is believed that the formation of a well-developed biofilm is one of the mechanisms which protects *S. aureus* from host immune defenses and systemic antibiotics (*38*). Such implications of factor XIIIa-catalyzed cross-linking of staphylococcal FnbA to itself and to human extracellular matrix proteins most likely served as a driving force for the molecular evolution of FnbA receptor, resulting eventually in the acquiring of a new property. The evolved reactivity of FnbA toward factor XIIIa might have provided a significant advantage in the colonization of the host and subsequently had a positive impact on the survival of *S. aureus*.

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