Specific Interactions between F1 Adhesin of \textit{Streptococcus pyogenes} and N-terminal Modules of Fibronectin*

Received for publication, June 12, 2001, and in revised form, July 23, 2001
Published, JBC Papers in Press, July 23, 2001, DOI 10.1074/jbc.M105417200

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Protein F1 is a surface protein of \textit{Streptococcus pyogenes} that mediates high affinity binding to fibronectin (Fn) and facilitates \textit{S. pyogenes} adherence and penetration into cells. The smallest portion of F1 known to retain the full binding potential of the intact protein is a stretch of 49 amino acids known as the functional upstream domain (UFBD). Synthetic and recombinant versions of FUD were labeled with fluorescein isothiocyanate and used in fluorescence anisotropy experiments. These probes bound to Fn or the 70-kDa fragment of Fn with dissociation constants of 8–30 nM. Removal of the N-terminal seven residues of FUD did not cause a change in binding affinity. Further N- or C-terminal truncations resulted in complete loss of binding activity. Analysis of recombinant versions of the 70-kDa fragment that lacked one or several type I modules indicates that residues 1–7 of the 49-mer bind to type I modules I1 and I2 of the 27-kDa subfragment and the C-terminal residues bind to modules I4 and I5. Fluorescein isothiocyanate-labeled 49-mer also bound with lower affinity to large Fn fragments that lack the five type I modules of the 27-kDa fragment but contain the other seven type I modules of Fn. These results indicate that, although FUD has a general affinity for type I modules, high affinity binding of FUD to Fn is mediated by specific interactions with N-terminal type I modules.

Fibronectin (Fn)\(^1\) is a 500-kDa dimer of 250-kDa subunits that are composed of three types of repeating homologous sequences, known as type I, II, and III modules (Fig. 1A) (1–3). Dimerization occurs via disulfide bonds at the extreme C terminus of each monomer. There are 12 type I finger modules, each of which contains about 45 residues and two conserved disulfide bonds (2). Type I modules are located near the N and C termini of each monomer. The N-terminal type I modules are interrupted by a protease sensitive connecting sequence and two type II modules. Type II modules also have a pair of disulfides (2). The majority of Fn consists of type III modules that contain about 90 residues and no disulfide bonds (1, 2). Four examples of alternative mRNA splicing have been identified. Two occur with the splicing in or out of additional type III modules known as extra domains A and B. Another involves RNA encoding 120 residues termed the variable segment, which can be included completely, partially, or left out (2). The final example involves exclusion of RNA encoding modules III-15 and I-10 known as the (V+C)\(^-\) splice variant (4). Fn is readily cleaved into functional domains by controlled proteolysis (5). These functional domains retain binding activity to substrates such as cells, extracellular matrix proteins, and bacteria (6).

Many bacterial pathogens have exploited Fn as a means to adhere, colonize, and invade host tissue (7). Group A streptococcus has traditionally been viewed as an extracellular pathogen responsible for human diseases of varying severity, ranging from nonsuppurative infections of the pharynx and skin to toxic shock syndrome, necrotizing fasciitis, and sepsis (8). Recent studies showed that Group A streptococcus is capable of inducing its own entry into several eukaryotic cell types \textit{in vitro} (9–12). Entry of Group A streptococcus into cells also occurs \textit{in vivo} and may contribute to bacterial persistence despite antibiotic therapy (13, 14).

Protein F1 of \textit{Streptococcus pyogenes} is a major adhesin and invasin of Group A streptococcus that binds soluble and extracellular matrix-associated Fn with a high affinity by a mechanism involving two domains of the molecule (15, 16). Fn bound to F1 acts as a bridge that connects F1 to integrin receptors. This brings the bacterium to a close contact with the host cell, consequently leading to a very efficient bacterial uptake (17, 18).

F1 is one of a number of microbial adhesins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that bind Fn (19). The external portion of F1 contains two types of repeats known as repeat domain 1 (RD1) and repeat domain 2 (RD2) (15). RD2 consists of 37 amino acid residues repeated fully four times and partially a fifth time (15). RD2 is similar to the Fn-binding D repeat region in the Fn-binding protein of \textit{Staphylococcus aureus} (20). Immediately N-terminal to the RD2 repeat is a 43-residue upstream fibronectin binding domain (UFBD) that is unique to F1 compared with other MSCRAMMs (15). Two known functional binding domains of F1 cross the homology boundaries of UFBD and RD2. A recombinant protein consisting of an RD2 and the adjacent UFBD completely blocks adherence of \textit{S. pyogenes} to Fn (15). Further analysis defined a functional upstream do-

\(^{*}\) These studies were supported in part by National Institutes of Health Grants HL21644 (to D. F. M.) and HL50549 (to J. S.) and by the Center for the Study of Emerging Diseases (to E. H.). The costs of this study were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^{\dagger}\) The abbreviations used are: Fn, fibronectin; FITC, fluorescein isothiocyanate; FRD, functional, repeated domain; FUD, functional upstream domain; HPLC, high-performance liquid chromatography; MALDI-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MSCRAMM, microbial surface component recognizing adhesive matrix molecules; RD, repeat domain; UFBD, upstream fibronectin binding domain.
main (FUD) as a 49-residue sequence that consists of the 43 residues of the UFBD and the N-terminal six residues of the first RD2 (15). FUD is the smallest sequence that retains the affinity of F1 for Fn (15). The second functional binding domain, known as the functional repeated domain (FRD), includes 44 residues of two contiguous RD2 repeats beginning and ending with the sequence MGGQSES (15). Recombinant FUD or a protein encompassing UFBD and RD5 blocks binding of iodinated Fn to F1-bearing S. pyogenes with ID_{50} concentration a 50% inhibitory of 0.5 nm, whereas RD5, a protein with four FRDs, blocks with an ID_{50} of 50 nm (21).

We recently found that recombinant FUD and FUD-derived peptides are potent inhibitors of assembly of Fn by fibroblastic cells, a process that is initiated by binding of the N-terminal part of Fn to cell surfaces of fibroblastic cells (22). FUD is known to bind to the N-terminal region of Fn (15). Huff et al. (23) used fluorescence anisotropy to define interactions between soluble Fn or the N-terminal modules of Fn and the D repeats of S. aureus Fn-binding protein. We describe a similar approach to quantify interactions between the binding sequences of F1 and Fn. Specifically, we measured interactions between a variety of proteolytic and recombinant pieces of Fn and a series of recombinant and synthetic peptides representing full-length and truncated versions of FUD. These experiments demonstrated that subsites within the 49 residues of FUD bind to specific N-terminal type I modules of Fn.

EXPERIMENTAL PROCEDURES

Preparation of Bacterial Peptides—Synthetic peptides were synthesized by the Macromolecular Chemistry Unit of the Department of Clinical Chemistry, Malmö General Hospital, Malmö, Sweden. Recombinant His-tagged FUD (pUR-4) or FRD (RD2-4) were expressed in E. coli and purified using the expression vector pQE30 (Qiagen, Valencia, CA) according to manufacturer’s instructions and as reported previously (15). The recombinant proteins included an N-terminal polyhistidine tag and linker sequence with a cysteine residue. Gel electrophoresis in SDS with and without inclusion of reducing agent indicated that >50% of each purified fusion protein was dimerized via a disulfide bond.

Production of Labeled Peptides—Peptides or recombinant FUD or FRD were dissolved in 0.1 M bicarbonate buffers (pH 9.5) and labeled with fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. The samples were resolved with a Vydac C18 (Vydac, Hesperia, CA) column (4.6 × 250 nm, 5 μm particle size, 300 Å pore size). A gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid was used over 135 min at a flow rate of 1 ml/min. Absorbance at 215 and 280 nm was monitored. Fractions were collected at 2-min intervals and manually as needed. Each fraction was then exhaustively dialyzed against H_{2}O prior to lyophilization.

To localize which amino group is modified by FITC, we took advantage of the fact that susceptibility to trypsin cleavage would be lost upon modification of the e-amino group with FITC (Fig. 2). The 49-mer has no arginines and three lysines, one at the N terminus and two adjacent to one another at residues 31 and 32 (Fig. 1B). Each peptide was dissolved in a 0.1 M ammonium bicarbonate (pH 9.5). Sequencing grade-modified trypsin (Promega, Madison, WI) was used according to the manufacturer’s suggestions. Following the addition of a 1:20 dilution of trypsin, each sample was incubated at 37 °C for 4.5 h. Reactions were quenched with the addition of 0.1% trifluoroacetic acid. Each sample was washed with a ZipTip C_{18} (Millipore, Bedford, MA) according to the manufacturer’s protocol. Samples were then subjected to MALDI-MS at the Mass Spectrometry/Bioanalytical facility located in University of Wisconsin Biotechnology Center. The results were compared with predicted masses for trypptic products of peptide labeled at individual sites.

Anisotropy Measurements—Fluorescence polarization measurements were made in Tris-buffered saline containing 0.2% bovine albumin at 25 °C with constant stirring. Polarization (P) is related to anisotropy (r) by the following equation:

\[ r = \frac{P(3 - P)}{Y} \]  

Excitation and emission wavelengths were 493 and 520 nm, respectively. Data were generated on an SLM 8000C photon counting spectrofluorometer operating with an SLM 8100-hardware/software upgrade (Thermo Spectronic, Rochester, NY). Fn fragments were titrated into a 25 nm solution of FITC-labeled peptide. For competition studies, a 25 nm concentration of the 70-kDa fragment of Fn was preincubated for 10 min with 500 nm concentrations of competitor followed by the addition of 25 nm FITC-labeled 49-mer.

Determination of Dissociation Constants K_{d}—Anisotropy data were analyzed using Sigma Plot (SPSS Science, Chicago, IL) by fitting the data from replicate experiments done on two occasions to a nonlinear reciprocal equation. The analysis assumed 1 binding site, allowing K_{d} values to be generated. The equation used for this fit was as follows,

\[ Y = \frac{Q_{max}}{K_{d} + [\text{fragment}]} + K_{d} \]  

where Y is the change in anisotropy, Q_{max} is the maximal change in anisotropy, K_{d} is the association constant of the interaction, [fragment] is the concentration of the titrant added, and K_{d} is 1/K. The standard error was calculated based on the fit of the curve to the individual points.

RESULTS

All Populations of Peptide with Single Labels Interact with the 70-kDa Fragment of Fn—A synthetic 49-residue peptide (49-mer) based on the FUD region of F1 was synthesized and labeled with FITC. The peptide has four potential sites of labeling, the α- and ε-amino groups of Lys-1 and the ε-amino groups of Lys-31 and Lys-32 (Fig. 1B). HPLC analysis of labeled 49-mer separated the mixture into six peaks (Fig. 2A). MALDI-MS revealed that the major peak contained unlabeled peptide. Four lower peaks contained peptides labeled at one site. The minor broad peak contained peptides with multiple FITC labels. The four singly labeled peptides were analyzed by trypsin digestion followed by MALDI-MS (Fig. 2B). The masses of fragments from peaks 1 and 2 indicated that these peptides are labeled at Lys-1. The two peaks are presumably due to derivation of either the α- or ε-group. The digestion patterns of
peaks 3 and 4 indicated that these peptides are labeled at Lys-31 and Lys-32, respectively.

In order to test whether labeling at specific sites alters binding and the validity of using the mixture of labeled and unlabeled peptides, we analyzed the fractionated FITC-labeled peptides in titrations with the 70-kDa fragment of Fn. Baseline anisotropy (not shown) and maximal change in anisotropy (Fig. 3) were smallest for the peptides labeled at Lys-1 and largest for the peptides labeled at Lys-31 and Lys-32. Calculated $K_D$ values also fell into two groups (Table I). $K_D$ values for peaks 1 and 2 (Lys-1) were 30 and 26 nM, respectively, whereas $K_D$ values for peaks 3 (Lys-31) and 4 (Lys-32) were 10 and 8 nM, respectively. The binding curve for labeled unfractinated 49-mer was intermediate between the curves of HPLC-purified singly labeled peptides (Fig. 3). These results indicate that derivation of any one group does not abolish binding, and in most subsequent studies, a labeled mixture was used.

**Type I Modules of Fn Are Necessary for an Interaction with the 49-mer**—In order to determine which parts of Fn contribute to binding, titrations of Fn fragments encompassing the whole protein were performed while the anisotropy of the unfractinated labeled 49-mer was measured. As shown in Fig. 4, a change in anisotropy was recorded with the addition of Fn or 70-kDa, 27-kDa, and 160/180-kDa fragments. The absolute change in anisotropy was smallest for the 27-kDa fragment and largest for intact-Fn and the 70-kDa fragment. Apparent affinities ($K_D$) of binding of labeled 49-mer were as follows: 27-kDa fragment, 12 nM; Fn, 21 nM; 70-kDa fragment, 26 nM; and 160/180-kDa fragments, 53 nM (Table I). No anisotropy change occurred following the addition of 40-kDa gelatin-binding fragment, 31-kDa C-terminal fragment, or fingerless fragment lacking type I modules (Table I). The 27-kDa, 40-kDa, 70-kDa, 160/180-kDa, and 31-kDa fragments contain five, four, nine, seven, and three type I modules, respectively. The binding studies indicate that multiple type I modules are important for binding and that the type I modules in the N-terminal 27-kDa fragment are particularly important.

To localize further binding activity within the 27-kDa region of Fn, recombinant derivatives of the 70-kDa fragment (Gaps) lacking one or more of the first five type I modules that make up the 27-kDa region (Fig. 1A) were used in the titration assay. Titrations showed that the 49-mer failed to bind Gap 1-3 but bound to Gap 4-5 and Gap 3 (Fig. 5). The apparent affinities of binding were lower than for binding to 70-kDa fragment, 50 nM for Gap 3, and 149 nM for Gap 4-5 (Table I). This result shows major binding activity within the first two type I modules.

Recombinant FUD Peptide Binds Slightly Differently Than the Synthetic 49-mer—His-tagged recombinant FUD encoded...
by pUR-4 was also labeled with FITC followed by titrations with Fn fragments in the anisotropy assay. Fig. 6 shows that FITC-FUD interacted with 40-kDa fragment of Fn as well as with the 27-kDa and 70-kDa fragments. The apparent affinity of FITC-labeled FUD for the 70-kDa fragment was 22 nM, comparable to the affinity of unfractuated labeled 49-mer (Table I). The affinity of FITC-labeled FUD for the 27-kDa fragment was 63 nM, less than the affinity of labeled 49-mer (Table I). The affinity of FITC-labeled FUD for the 40-kDa fragment was 144 nM (Table I). These results suggest that the 27- and 40-kDa fragments contain lower affinity binding sites that, when linked together, result in high affinity binding. This conclusion agrees with previous evidence of binding of FUD to 27-, 40-, and 70-kDa fragments (15).

We also tested recombinant FRD encoded by RD2-8 in our assay. FRD has two internal lysines that are available for binding with Fn fragments in the anisotropy assay. Fig. 6 shows that FITC-FUD interacted with 40-kDa fragment of Fn as well as with the 27-kDa and 70-kDa fragments. The apparent affinity of FITC-labeled FUD for the 70-kDa fragment was 22 nM, comparable to the affinity of unfractuated labeled 49-mer (Table I). The affinity of FITC-labeled FUD for the 27-kDa fragment was 63 nM, less than the affinity of labeled 49-mer
labeling (Fig. 1B). We found that FITC-FRD interacted with the 70-kDa and 27-kDa fragments of Fn but not the 40-kDa fragment (Fig. 7). This result is also in agreement with evidence that this recombinant protein interacts with the 70-kDa fragment but not the 40-kDa fragment (15). Apparent affinities of the interaction were 18 and 63 nM for the 70- and 27-kDa fragments, respectively (Table I). Thus, FRD binds to the 70-kDa fragment with a similar affinity to FUD but has a lower affinity for the 27-kDa fragment.

Identification of Critical Residues within the 49-mer—To determine which portions of 49-mer are important for Fn binding, we labeled the truncated versions of the 49-mer shown in Fig. 1B with FITC. The truncated peptides were designed to include or omit the four clusters of charged residues (shown above the sequence of the 49-mer in Fig. 1B). The FITC-labeled synthetic peptide representing the C-terminal 42 residues of the FUD (42-mer) bound the same Fn fragments as the 49-mer (Fig. 8A). Affinities of the 42-mer peptide were 18 and 63 nM for the 70- and 27-kDa fragments, respectively (Table I), similar to the affinities of FITC-labeled 49-mer. The 42-mer, however, failed to bind any of the Gap proteins (Fig. 8B). Labeled 29-mer, 20-mer, or 7-mer (Fig. 1B) showed no change in anisotropy upon addition of Fn fragments (data not shown).

49-mer and pUR-4 Cross-compete for Fn Binding, Whereas Heparin and the CB-7 Fragment of Collagen Do Not—The subtle differences in the specificities of binding of 49-mer and FUD to various Fn-derived proteins raise the question of whether the two peptides bind to overlapping or entirely different sites in Fn. Therefore, cross-competition experiments were performed. Fig. 9 shows that preincubation of 25 nM 70-kDa fragment with 500 nM unlabeled recombinant 49-mer or FUD blocked the change in anisotropy when 25 nM FITC-labeled FUD was added. Conversely, unlabeled 49-mer or FUD blocked binding of FITC-labeled 49-mer to the 70-kDa fragment. When the 70-kDa fragment was preincubated with heparin, which binds to the 27-kDa region (6), or the CB-7 fragment of collagen, which binds to the 40-kDa region (6), the change in anisotropy upon the introduction of FITC-labeled FUD was the same as seen with the 70-kDa fragment alone. These findings suggest that 49-mer or FUD bind to the same or overlapping sites on the 70-kDa fragment that are distinct from sites of interactions with heparin or collagen.
We characterized the interaction between Fn and the 49-residue FUD and 44-residue FRD sequences of Fl from S. pyogenes using a solution-phase fluorescence polarization assay and synthetic 49-mer, synthetic 49-mer derivatives, and recombinant dimeric fusion proteins as probes. Several important conclusions emerged. 1) Both the 49-mer and FUD bound tightly ($K_D$ of $<20$ nM) to the N-terminal fragment of Fn. 2) Optimal binding of the 49-mer to the N-terminal portion of Fn required modules I1 and/or I2. 3) Both probes also bound with lower affinity to parts of Fn that do not include I1 or I2 but are composed of other type I modules, viz. I6–I9 (recombinant FUD) or I6–I9 plus I10–I12 (synthetic 49-mer and recombinant FUD). 4) Although the 49-mer bound to the N-terminal portion of Fn lacking I4 and I5, a 42-mer lacking the N-terminal seven residues of the 49-mer did not. 5) Further truncation of the 49-mer abolished all binding.

**FIG. 7.** Interaction of labeled recombinant FRD with Fn N-terminal fragments. FITC-labeled FRD encoded by RD2-8 (25 nM) was titrated with the 70-kDa fragment, 40-kDa fragment, or 27-kDa fragment of Fn while the change in anisotropy was monitored. The arrows indicate the concentration of labeled FRD in the assay.

**FIG. 8.** Interaction of labeled recombinant FRD with Fn N-terminal fragments. A, FITC-labeled 42-mer (25 nM) was titrated with the 70-kDa fragment, 40-kDa fragment, or 27-kDa fragment of Fn while the change in anisotropy was monitored. B, FITC-labeled 42-mer (25 nM) was titrated with Gap 4-5 or Gap 1-3 while the change in anisotropy was monitored. The arrows indicate the concentration of labeled 42-mer in each assay.

**FIG. 9.** Competition for the 70-kDa fragment of Fn. The 70-kDa fragment (25 nM) was premixed with 500 nM CB7, heparin (Hep), unlabeled 49-mer, or recombinant FUD and allowed to incubate for a period of 10 min before 25 nM FITC-labeled recombinant FUD or FITC-labeled 49-mer was added while the anisotropy was monitored. Results are shown as a percentage of the difference in anisotropy found between FITC-labeled FUD or 49-mer incubated with 70-kDa fragment and FITC-labeled FUD or 49-mer alone. Error bars represent the range from two experiments.

**DISCUSSION**

We characterized the interaction between Fn and the 49-residue FUD and 44-residue FRD sequences of Fl from *S. pyogenes* using a solution-phase fluorescence polarization assay and synthetic 49-mer, synthetic 49-mer derivatives, and recombinant dimeric fusion proteins as probes. Several important conclusions emerged. 1) Both the 49-mer and FUD bound tightly ($K_D$ of $<20$ nM) to the N-terminal fragment of Fn. 2) Optimal binding of the 49-mer to the N-terminal portion of Fn required modules I1 and/or I2. 3) Both probes also bound with lower affinity to parts of Fn that do not include I1 or I2 but are composed of other type I modules, viz. I6–I9 (recombinant FUD) or I6–I9 plus I10–I12 (synthetic 49-mer and recombinant FUD). 4) Although the 49-mer bound to the N-terminal portion of Fn lacking I4 and I5, a 42-mer lacking the N-terminal seven residues of the 49-mer did not. 5) Further truncation of the 49-mer abolished all binding.
The increase in anisotropy upon binding to 70-kDa fragment of FITC-49-mer labeled at the N-terminal lysine was less than the increase upon binding of 49-mer labeled at the internal lysines. These findings indicate that FITC tethered at either the N terminus or the middle of the 49-mer is immobilized upon binding of the 49-mer to the 70-kDa N-terminal fragment, although to different degrees. The affinities of binding of purified singly labeled derivatives were similar within a factor of three. We do not know whether the differences are due to loss of binding affinity upon derivation of Lys-1 or gain of binding affinity upon derivation of Lys-31 or Lys-32. The increase in anisotropy also varied depending on the target in order: 70-kDa fragment = Fn > 27-kDa fragment. Such variation in change in anisotropy is consistent with the 27-kDa fragment being smaller than the 70-kDa fragment and with the 70-kDa fragment having segmental flexibility within Fn (30). The 49-mer bound to recombinant Gap mutants of the 70-kDa construct lacking I4 and I5 but with less avidity than to intact 70-kDa fragment. A peptide lacking the N-terminal seven residues of the 49-residue sequence did not bind to the Fn construct having I1 and I2 but lacking I4 and I5. The six C-terminal residues derived from the first RD2 sequence have previously been demonstrated to be required for binding of FUD to Fn (15). The findings, taken together, suggest that the entire lengths of the 49-mer and of modules I1–I5 contribute to binding. Proteins and peptides based on sequences from the Fn-binding MSCRALLM of S. aureus are in a random conformation in solution (31). Far-UV circular dichroism of recombinant FUD indicates that it is in a random conformation also. A 49-residue peptide in a random conformation would have a maximum length of 16 nm. This length approximates the length of an array of five type I modules (3, 32, 33). Therefore, we conclude that the 49-mer binds in a single mode to modules I1–I5 of Fn such that specific subsites in the 49-mer interact with specific Fn modules.

The specificity of binding of the 49-mer contrasts with the specificity of the three 38-residue D motifs found in the Fn-binding protein of S. aureus. The third D motif binds best to modules I4 and I5 of the 27-kDa fragment of Fn, but with micromolar rather than nanomolar affinities (23). When multiple D motifs, which each contain a sequence similar to the essential VETEDT sequence found at the C terminal of FUD (15), are expressed together, however, low nM affinities are achieved (23). NMR studies indicate that the sequence PNYQF-GGHNSVDFEEDT at C-terminal part of the D3 repeat, which is similar to the PNETGFSGN MVETEDT sequence at the C terminus of the 49-mer, converts from a disordered to a more ordered extended conformation on binding to recombinant I4–I5 (34). The comparisons of binding of 49-mer and 42-mer to Gap mutants indicate that the N-terminal seven residues of the 49-mer bind to I1 and/or I2. Thus, we speculate that binding of the 49-mer to I1–I5 is driven by an interaction of the N-terminal residues with I1 and/or I2 that is specific for the 49-mer and an interaction of the C-terminal residues with I4 and I5 that is similar to the interaction of D3 with I4 and I5. Peptides representing the N-terminal half of D3 bind with low affinity to recombinants I1–I2 (35). However, there is no obvious similarity between the N-terminal sequences of the 49-mer and D3.

The type I module folds as two β-sheets stabilized by two disulfides and a hydrophobic core (3). One sheet has two strands (A and B); the second has three strands (C, D, and E). Neither the CB-7 fragment of collagen nor heparin competed for binding of the probes to the 70-kDa fragment of Fn. These findings indicate that the sites of interaction of FUD with the 70-kDa fragment are distinct from sites where collagen or heparin bind. In vitro mutagenesis studies indicate that important residues for the interaction of the 27-kDa fragment with heparin are within the D–E, or Ω, loops of type I modules (36). Lack of competition by heparin, therefore, suggests that the interaction between the FUD of F1 and Fn involve other parts of type I modules than the D–E loops. NMR studies of the binding of staphylococcal D3 to recombinant I4–I5 indicate that binding occurs on one face of the I4–I5 structure that includes both loops and strands (34). NMR studies also indicate that the I4–I5 module pair is fixed due to a well defined intermodule interface (32), whereas the I1–12 module pair is joined by a flexible linker without a discernible interface (33). It will be interesting to learn how the 49-mer interacts with I1–I2 versus I4–I5.

Our solution phase binding results were, in general, compatible with previous studies of binding using competitive assays and recombinant FUD (15, 21). In competitive assays, recombinant FUD was found to compete for binding interactions of both the 27-kDa fibrin-binding fragment and the 40-kDa gelatin-binding fragment, but FUD competed best when the fragments were linked together as the 70-kDa N-terminal fragment (15). In our fluorescence polarization assay, recombinant FUD bound better to the 70-kDa fragment than to its 40-kDa gelatin-binding or 27-kDa N-terminal subfragments. The 49-mer, in contrast, bound to the 70-kDa fragment and 27-kDa subfragment equally well but did not bind to the 40-kDa subfragment. The 49-mer also bound to gelatin-binding 160/180-kDa tryptic fragments containing three C-terminal type I modules plus the four type I modules of the gelatin-binding region. These results suggest that synthetic 49-mer and recombinant FUD recognize and bind a general feature of type I modules. This general binding likely is polyomodal in the sense that a given subsite in the 49-mer can interact with multiple type I modules and vice versa. The dimeric character of recombinant FUD in which the two 49-residue sequences are tethered near their N termini by a disulfide may constrain and alter the binding specificity of subsites within the recombinant protein for modules I1–I5 and thus account for the differences in binding activities of the recombinant FUD versus 49-mer.

Synthetic 49-mer and recombinant FUD are both inhibitors of assembly of Fn and binding of 70-kDa N-terminal fragment of Fn to sites on the surface of fibroblasts where assembly takes place (22). Studies of Fn-null fibroblasts lacking Fn matrix indicate that the effects of 49-mer and FUD are due to binding to soluble Fn or 70-kDa fragment (22). The ID₅₀, for inhibition of binding of 4 nM Fn to fibroblasts by 49-mer or FUD is ~10 nM (22), very similar to the Kᵢ values of binding estimated by the present fluorescence polarization assay. However, recombinant FRD and synthetic 42-mer, which bound to the 70-kDa fragment with affinities only slightly less than FUD or 49-mer as estimated by the fluorescence polarization assay, are poor inhibitors of the binding of 70-kDa fragment to fibroblasts, with ID₅₀ values of >500 nM (22). The discrepancies between the fluorescent polarization and fibroblast binding assays indicate that the specific mode of binding to Fn and not the affinity of binding determines whether a given sequence blocks binding of Fn to cells. In relation to bacterial adhesion and entrance into cells, the specificity of the FUD sequence for the five N-terminal modules of Fn may be important for the conformation of Fn bound to F1 on the surface of S. pyogenes. Rather than simply capturing Fn, the FUD sequence of F1 may bind the N-terminal region of one or both subunits of Fn with an exact polarity and thus determine whether other parts of Fn bind other parts of

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2 J. Spanbauer and D. F. Mosher, unpublished observation.
Interactions between F1 Adhesin and Fibronectin

35613

F1 and adopt conformations that would facilitate bacterial entry into epithelial cells.

Acknowledgment—Special thanks go to Oleg Tsodikov for help with data analysis.

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