A 49-Residue Peptide from Adhesin F1 of *Streptococcus pyogenes* Inhibits Fibronectin Matrix Assembly*

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F1 is an adhesin of *Streptococcus pyogenes* which binds the N-terminal 70-kDa region of fibronectin with high affinity. The fibronectin binding region of F1 is comprised of a 43-residue upstream domain and a repeat domain comprised of five tandem 37-residue sequences. We investigated the effects of these domains on the assembly of fibronectin matrix by human dermal fibroblasts, MG63 osteosarcoma cells, or fibroblasts derived from fibronectin-null stem cells. Subequimolar or equimolar concentrations of recombinant proteins containing both the upstream and repeat domains or just the repeat domain enhanced binding of fibronectin or its N-terminal 70-kDa fragment to cell layers; higher concentrations of these recombinant proteins inhibited binding. The enhanced binding did not result in greater matrix assembly and was caused by increased ligand binding to substratum. In contrast, recombinant or synthetic protein containing the 43 residues of the upstream domain and the first 6 residues from the repeat domain exhibited monophasic inhibition with an IC\textsubscript{50} of ~10 nM. Truncation of the 49-residue sequence at its N or C terminus caused loss of inhibitory activity. The 49-residue upstream sequence blocked incorporation of both endogenous cellular fibronectin and exogenous plasma fibronectin into extracellular matrix and inhibited binding of 70-kDa fragment to fibronectin-null cells in a fibronectin-free system. Inhibition of matrix assembly by the 49-mer had no effect on cell adhesion to substratum, cell growth, formation of focal contacts, or formation of stress fibers. These results indicate that the 49-residue upstream sequence of F1 binds in an inhibitory mode to N-terminal parts of exogenous and endogenous fibronectin which are critical for fibronectin fibrillogenesis.

Fibronectin extracellular matrix is a substratum for cell adhesion, migration, and proliferation during physiological and pathological processes such as embryogenesis, wound healing, inflammation, and tumorigenesis (1). Fibronectin extracellular matrix is also important for adhesion and invasion of certain pathogenic bacteria. Bacteria express surface molecules known as adhesins or microbial surface components recognizing adhesin-specific matrix molecules (MSCRAMMs), which mediate binding to extracellular matrix molecules (2). A number of adhesins have been identified, cloned, and analyzed for their binding to fibronectin, and some have been determined to be virulence factors and potential targets for the development of vaccines (3).

*Streptococcus pyogenes* is an extracellular pathogen responsible for diseases of varying severity, from nonsuppurative infections of the pharynx and skin to necrotizing fasciitis and toxic shock syndrome (4). *S. pyogenes* expresses several fibronectin-binding molecules that have been implicated in the invasion of epithelial cells (5–8). Parasitization of epithelial cells is believed to allow bacteria to escape from immune surveillance or antibiotic treatments and therefore to favor persistent or recurrent infections. F1 of *S. pyogenes* (9), also called Sfb1 (10), is a typical adhesin in its organization with its C terminus anchored on the bacterial cell wall, followed outwardly (C to N terminus) by a series of five 37-residue sequences repeated in tandem (RD5) and the so-called upstream nonrepetitive domain (UD) (Fig. 1). The binding of F1 to fibronectin has been localized to UD and RD5, but the binding regions do not match the homology units just described (11, 12). As depicted in Fig. 1, the first fibronectin binding motif, referred to as the functional upstream domain (FUD), encompasses the 43 residues of UD plus the first 6 residues of the first 37-residue repeat of the RD5 region. The second fibronectin binding motif, referred to as functional repeat domain (FRD), consists of 44 amino acids and includes the C-terminal part of one 37-residue repeat and the N-terminal part of the next; it begins and ends with the MGQQSES sequence in the middle of each repeat. Binding of FRD has been localized to the most N-terminal 27-kDa fragment of fibronectin, whereas binding of the FUD was localized to a larger region of fibronectin that includes the 27-kDa fragment and adjacent gelatin binding domain (12). The fibronectin-binding adhesin of *Staphylococcus aureus* also recognizes the N-terminal 27-kDa fragment (13, 14).

The mechanism of fibronectin matrix assembly requires that fibronectin bind at or near the cell surface to polymerize into fibrils (1, 15). One region of fibronectin which is critical for

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1 The abbreviations and nomenclature used are: MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; F1, functional F1 (region of F1 which binds fibronectin); FITC, fluorescein isothiocyanate; FRD, functional repeat domain (region represented by a construct starting, approximately from the middle of one repeat to the middle of the next repeat, including at both ends the sequence MGQQSES; there are four equivalent FRDs in RD5); FUD, functional upstream domain (region represented by a construct consisting of the 43-residue nonrepeated upstream domain (UD) and 6 amino acids from the first 37-residue repeat; 49-mer is the peptide equivalent to FUD); LPA, lysophosphatidic acid; RD5, repeat domain 5 (consists of five tandem 37-residue repeats downstream of FUD).
assembly lies in the N-terminal 70-kDa cathaptic D fragment, which binds reversibly to cells with the same affinity as intact fibronectin (16). Although the 70-kDa fragment does not become incorporated into insoluble matrix, it competes for the insolubilization of intact fibronectin, a process that takes place subsequent to reversible binding of fibronectin to cells (16). The binding activity of the 70-kDa fragment is largely in the N-terminal 27-kDa subfragment (16–18), and each of the five type I repeats of the 27-kDa fragment is required for strong binding (19). The 70-kDa fragment also binds to cryptic sites elsewhere in fibronectin which are important for assembly (20–26).

Internalization of fibronectin-coated S. pyogenes by cells is mediated by the integrins α5β1 or α5β2 (7), cell surface receptors that recognize the cell adhesion sequence RGD in fibronectin. Cell adhesive activity of fibronectin is cryptic in soluble fibronectin (27–29). The mechanism by which F1 mediates bacterial invasion of epithelial cells, therefore, is hypothesized to involve binding of F1 to the N-terminal part of fibronectin with consequent exposure of the integrin binding RGD-containing domain of fibronectin. This proposed process is in some ways similar to what is hypothesized (1) to occur during fibronectin matrix assembly, i.e. the N-terminal part of fibronectin binds surface sites on attached cells resulting in exposure of integrin binding and self-association sites on fibronectin required for its insolubilization (30, 31).

We have investigated if the fibronectin binding motifs of F1 influence fibronectin matrix assembly, e.g. increase fibronectin polymerization by exposing cryptic sites involved in self-assembly or decrease polymerization by blocking binding sites for cell surface molecules or for fibronectin itself. We found the effects of F1 motifs to be complex but mostly inhibitory. The 49-residue sequence of FUD, in particular, has potent inhibitory activity, most probably resulting from binding to the N-terminal sites of fibronectin responsible for binding to molecules at cell surfaces.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (BSA), fatty acid-free BSA, and monoclonal mouse antibody to human vinculin were from Sigma. Rheumatoid arthritis serum from the mouse adjuvant arthritis line 8.1 was a gift of Dr. R. M. Zvaifler (Division of Rheumatology, University of California, Los Angeles). Rho-damino-phalloidin and fluorescent isothiocyanate (FITC) was from Molecular Probes (Eugene, OR). Monoclonal antibody IST-9, which recognizes the EDa type III module present in cellular but not plasma fibronectin, was from Harlan Sera-Lab (Loughborough, U. K.). Polyclonal antibodies to fibronectin were raised in rabbits. Lysoosphatidic acid (LPA) was from Avanti Polar Lipids (Birmingam, AL). Soluble rat tail type I collagen was from Upstate Biotechnology (Lake Placid, NY). Human plasma fibronectin and the 70-kDa fragment were isolated and radiolabeled with 125I as described previously (16, 32). Vimentin was purified from human plasma (33). The proteins were stored in aliquots at −70 °C.

The His-tagged constructs corresponding to various regions of the S. pyogenes adhesin F1 have been described previously (12). The nomenclature utilized in this paper, depicted in Fig. 1, is as follows. FUD corresponds to the functional upstream domain (clone pUR4) which encompasses the nonrepeate domain and 6 amino acids from the first repeat region. FRD corresponds to the functional repeat domain (clone RD2–8) encompassing the region from the middle of one repeat to the middle of the next, beginning and ending with the sequence MG-GQSES. RD5 corresponds to the five repeats in tandem (clone RD217) and thus contains four FRDs. FF1 encompasses the entire functional F1 region (clone UR19) from UD to the end of RD5. Constructs were transfected into Escherichia coli. Colonies were monitored for protein expression by SDS-polyacrylamide gel electrophoresis after induction with isopropyl-1-thio-galactopyranoside. His-tagged proteins were purified on nickel nitrilotriacetic acid-Sepharose as described by the manufacturer (Qiagen, Chatsworth, CA). Fusion proteins were analyzed for purity by polyacrylamide gel electrophoresis in the presence of SDS. Concentrations of FRD and RD5 were estimated using the BCA Protein Assay Reagent Kit (Pierce) with soybean trypsin inhibitor as a standard. Concentrations of FUD and FF1 were estimated by absorbance at 280 nm. Estimates of concentration were validated by amino acid analyses (Protein/Nucleic Acid Shared Facility at the Medical College of Wisconsin, Milwaukee).

Peptides corresponding to FUD as well as the N- and C-terminal truncations indicated in Fig. 1 were synthesized and purified by high performance liquid chromatography at the Macromolecular Chemistry facility in the Department of Clinical Chemistry, Malmo General Hospital and Lund University, Malmo, Sweden. Concentrations were based on weights of dried peptides.

Preparation of FITC-labeled fibronectin or 70-kDa fragment was carried out as described previously (32, 34). FITC-labeled FUD, FRD, RD5, and FF1 were prepared using a similar technique.

Cells—Human foreskin dermal fibroblasts (AH1F) were a gift from Dr. M. L. Gospodarowicz (University of Wisconsin-Madison). MGC6, a human osteosarcoma cell line, was purchased from the American Type Culture Collection (Rockville, MD). Fibronectin−/− mouse fibroblasts had been derived from the fibronectin−/− embryonic stem cells as described previously (35). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) with 10% fetal bovine serum (Intergen, Purchase, NY).

Binding Assay—Tissue culture plastic 1.5-cm² wells (24-well plates) were coated with fibronectin, vitronectin, or collagen type I at 3–10 μg/ml in phosphate-buffered saline overnight at 4 °C. Cells (3 × 10⁵ in 0.5 ml/well) were plated in DMEM and 0.2% BSA and studied 4–16 h after seeding. Binding of fibronectin to cells was assayed using 2–4 nM FITC-labeled fibronectin (1–2 μg/ml or 3–5 nM 125I-labeled 70-kDa fragment (0.2–0.5 μg/ml) in 0.5 ml of DMEM and 0.2% BSA as described previously (36). 125I-Labeled 70-kDa fragment was also used at 50 nM when binding to fibronectin-null cells to compare quantitative binding to the binding of FITC-labeled 70-kDa observed by immunofluorescence. Fusion protein or peptide was added at the concentration indicated in the text. Fatty-acid free BSA was used when 400 nM LPA was included. Monolayers were incubated with the labeling mixture for 60 min followed by three washes with Tris-buffered saline and recovery of bound label by the addition of 1 M NaOH. Unlabeled 715 nM 70-kDa fragment or 1,000 nM fibronectin (molecular weight assumed to be 450,000) was included in some wells to assess nonspecific binding. Specific binding was determined by subtracting the nonspecific binding (always < 40% of specific binding for conditions in which no inhibitor was present) from the total binding.

Fluorescence Microscopy—For visualization of bound exogenously added FITC-labeled fibronectin, 1 × 10⁵ cells/well in 0.5 ml were plated in wells containing adhesive protein-coated coverslips and incubated in DMEM and 0.2% BSA for 2–18 h, as indicated in the text. Fusion protein or peptide to be tested as potential modifier was added immediately prior to the addition of 40 nM FITC-labeled fibronectin. After being incubated for 60 min at 37 °C, cells were washed twice, fixed with

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peptide at concentrations subequimolar to equimolar to the ligand but are inhibitory at higher concentrations. These results indicate that constructs containing both FUD and RD5 (Fig. 1). To assess possible effects on fibronectin matrix assembly, the four fusion proteins were included at increasing concentrations in culture when plating human dermal fibroblasts on fibronectin-coated coverslips. After overnight incubation, cells were fixed and analyzed by immunofluorescence utilizing IST-9, which recognizes cellular fibronectin. In the presence of 50 nM FUD, fibronectin matrix was largely absent in the monolayer after overnight culture, whereas in the presence of 50 nM FRD or RD5, fibronectin matrix was similar or only slightly inhibited compared with control (Fig. 2). The effect of FF1 was similar to that of RD5 (not shown).

To assess quantitatively their effects on matrix assembly, fusion proteins were included in assays measuring amounts of exogenously added 125I-labeled fibronectin or 125I-labeled 70-kDa fragment bound to fibroblast monolayers. At concentrations of 100–500 nM, FUD inhibited almost 100% of the binding of 4 nM fibronectin or 70-kDa fragment to cell monolayers (Fig. 3, A and B). The IC50 values for inhibition by FUD were ~10 nM. RD5 and FF1 were also inhibitory in the concentration range of 70–500 nM. RD5 and FF1 were more inhibitory for 70-kDa fragment than for intact fibronectin. At concentrations of 1–5 nM, RD5 or FF1 enhanced binding of fibronectin or 70-kDa fragment. FRD, like FUD, was solely inhibitory but 100-fold less active. These results indicate that constructs with the FUD sequence or a single FRD sequence inhibit binding, whereas constructs with repeat sequences in tandem stimulate binding of fibronectin or 70-kDa fragment to cell monolayers at concentrations subequimolar to equimolar to the ligand but are inhibitory at higher concentrations.

The Entire FUD Is Required for Inhibition of Fibronectin Matrix Assembly—FUD was studied further because of its strong, monophasic capacity to inhibit matrix assembly. FUD, like each of the recombinant fusion proteins, contains a free 4% paraformaldehyde for 10 min, and washed again. Coverslips were mounted using Vectashield (Vector, Burlingame, CA). Cells were viewed on an Olympus epifluorescence microscope with a microphotography attachment. For visualization of endogenous fibronectin, cells were plated on adhesive protein-coated coverslips in the presence of test agents and incubated for 24 h. After washing and fixation, cellular fibronectin was detected using 10 μg/ml IST-9 followed by incubation with FITC-conjugated or rhodamine-conjugated anti-mouse IgG. Coverslips were mounted for microscopy as described above. Visualization of FITC-labeled fibronectin or 70-kDa fragment on fibronectin-null cells was carried out as above except for the following modifications. 3 × 105 cells were plated on collagen-coated coverslips for 2–4 h followed by incubation with 20 nM FITC-labeled fibronectin or 50 nM FITC-labeled 70-kDa fragment for 1 h at 37 °C in the presence or absence of various concentrations of FUD or RD5. Coverslips were then treated as above and photographed by fluorescence and phase-contrast microscopy.

For visualization of FITC-labeled FUD, FRD, FF1, or RD5 together with previously deposited exogenously added plasma fibronectin, a similar procedure was followed as described above for visualization of FITC-FN. Fibronectin-null fibroblasts were plated and incubated on collagen-coated coverslips for 2–4 h. Plasma fibronectin at 130 nM was added to half of the coverslips and incubated for 16–24 h. Medium was removed, and cells were incubated with 40 nM FITC-labeled FUD or 120 nM FITC-labeled FF1, FRD, or FRD. Coverslips were washed, fixed, and incubated with polyclonal antibody to fibronectin followed by anti-rabbit antibody conjugated to rhodamine. For detection of focal contacts and stress fibers by double fluorescence, 5 × 104 cells were plated on fibronectin-coated coverslips and allowed to adhere and spread for 2 h in the presence or absence of test peptide. Cells were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline, washed, permeabilized with 1% Triton X-100 in phosphate-buffered saline for 5 min, washed, and blocked with 5% BSA for 2 h. Coverslips were incubated with rhodamine-phalloidin simultaneously with 20 μg/ml mouse anti-human vinculin IgG for 2 h at room temperature followed by FITC-conjugated rabbit anti-mouse IgG for 1 h. Coverslips were then mounted with Vectashield as described above.

Cell Adhesion and Growth Assays—Cell adhesion assays were carried out on fibronectin-, vitronectin-, or BSA-coated wells in 96-well plates, essentially as described previously (37), except that cells were plated in the presence or absence of test peptide. Cell growth analyses were performed by plating 5 × 103 cells/well in 96-well plates in DMEM containing 10% fetal calf serum in the presence or absence of 500 nM recombinant FUD or synthetic peptide. Replicate plates were monitored for cell number on days 1, 3, and 5 by metabolic activity as assessed by the MTS assay performed according to the manufacturer’s instructions (Promega, Madison, WI). Relative cell number was determined by comparison with a standard curve obtained with a known number of cells plated 1 h prior to the assay on each day. Coverslip cultures treated in the same way were stained for examination of fibronectin matrix by immunofluorescence at the end of the 5-day assay. Additional experiments were carried out on 24-well plates in which the cells were incubated for 5 days in the presence and absence of synthetic or recombinant FUD, and cell number was measured by trypsin treatment and counting on a hemocytometer.

**RESULTS**

Fibronectin Binding Regions of F1 Both Enhance and Inhibit Assembly of Fibronectin.—We studied the effects of His-tagged fusion proteins corresponding to FUD, FRD, RD5, and FF1 containing both FUD and RD5 (Fig. 1). To assess possible effects on fibronectin matrix assembly, the four fusion proteins were included at increasing concentrations in culture when plating human dermal fibroblasts on fibronectin-coated coverslips. After overnight incubation, cells were fixed and analyzed by immunofluorescence utilizing IST-9, which recognizes cellular fibronectin. In the presence of 50 nM FUD, fibronectin matrix was largely absent in the monolayer after overnight culture, whereas in the presence of 50 nM FRD or RD5, fibronectin matrix was similar or only slightly inhibited compared with control (Fig. 2). The effect of FF1 was similar to that of RD5 (not shown).

**FIG. 2.** FUD inhibits assembly of endogenous fibronectin by fibroblasts. Human foreskin fibroblasts in DMEM and 0.5% BSA were plated on coverslips coated with plasma fibronectin in the absence (NA) or presence of 50 nM FUD, FRD, or RD5 fusion proteins derived from F1. Cells were incubated for 24 h in the presence of 400 nM LPA. Cells were fixed and analyzed by immunofluorescence for cellular fibronectin with antibody IST-9. Bar = 30 μm.
cysteine residue in the sequence immediately after the N-terminal His tag (12) and forms dimers as assessed by SDS-polyacrylamide gel electrophoresis in the absence of reducing agent (not shown). To test whether the activity of FUD is influenced by the additional sequence, we studied FUD as a synthetic 49-residue peptide and a series of related peptides truncated at the N-terminal or C-terminal ends (Fig. 1). As shown in Fig. 4, A and B, the 49-mer blocked binding of 125I-labeled fibronectin or 125I-labeled 70-kDa fragment to cell monolayers with an IC50 of −10 nM, similar to the IC50 of the FUD fusion protein. The absence of the N-terminal KDQSPA resulted in the loss of inhibitory activity by at least 100-fold. Peptides lacking N-terminal 20 or 37 residues, resulting in peptides of 29 and 12 amino acids, completely lacked activity. Peptides corresponding to the first 7 (7-mer) and 20 (20-mer) amino acids were also synthesized and tested. The 7-mer did not have inhibitory activity. The inhibitory activity of the 20-mer, like the 42-mer, was at least 100-fold less than activity of the 49-mer (Fig. 4C). Inhibitory activity was not increased when 20-mer and 42-mer were added simultaneously (data not shown).

FIG. 3. Fibronectin binding motifs from F1 alter binding of radiolabeled fibronectin or 70-kDa fragment to fibroblasts. Human foreskin fibroblasts were plated on fibronectin-coated 2.5-cm diameter wells in DMEM and 0.2% BSA and studied upon reaching confluence. 4 nM 125I-labeled fibronectin (panel A) or 4 nM 125I-labeled 70-kDa fragment (panel B) was added in the absence or presence of increasing amounts of F1 fusion proteins for 1 h at 37 °C in the presence of 400 nM LPA and assayed in duplicate for radiolabel bound specifically to the cell monolayer as indicated under “Experimental Procedures.” Results are expressed as percent of control (in the absence of F1-derived proteins; 100% = 4.3 ng of fibronectin or 2.2 ng of 70-kDa fragment bound/well). Each panel shows one experiment representative of the three experiments performed for each ligand. Bars indicate the range of duplicate values in the experiment. Note the monophasic inhibitory effects of FUD and FRD compared with increased ligand binding to cell monolayers at low concentrations of the RD5 and FF1 constructs and inhibitory effects at higher concentrations. Arrows indicate the points at which recombinant proteins and ligand were equimolar.

FIG. 4. The entire FUD sequence is required for inhibitory activity on binding of radiolabeled fibronectin or 70-kDa fragment to cells. Human MG63 osteosarcoma cells were plated on vitronectin-coated wells and were analyzed at confluence for binding of 2 nM 125I-labeled fibronectin (panel A) or 3 nM 125I-labeled 70-kDa fragment (panels B and C) in the absence or presence of increasing amounts of FUD fusion protein or synthetic peptides. This incubation was carried out for 1 h at 37 °C in the presence of 400 nM LPA, after which the radiolabeled ligand bound specifically to the cell monolayers was quantitated as indicated under “Experimental Procedures.” Panels A and B show the effects of FUD-derived 49-mer, 42-mer, 29-mer, and 12-mer, and panel C shows the effects of 49-mer, 20-mer, and 7-mer. Results are shown as percent of control; 100% = 3.3 ng of fibronectin (panel A) and 0.48 ng of 70-kDa fragment (panels B and C) bound/well. Each panel shows the average and S.D. of duplicates from two experiments performed for each ligand. Arrows indicate the point at which peptides and ligand were equimolar. Experiments performed with human dermal fibroblasts plated on fibronectin showed similar results (data not shown).
shown). Thus, most or all of the entire sequence and/or length of the 49-mer is required for optimal inhibition of fibronectin or 70-kDa fragment binding to cell monolayers. Similar results were obtained whether cells were plated on fibronectin, vitronectin, or collagen and in the absence or presence of LPA, a stimulator of fibronectin matrix assembly.

The peptides were also tested for their ability to affect assembly of endogenous fibronectin, as ascertained by immunofluorescence using antibody IST-9, and exogenous FITC-fibronectin. As shown in Fig. 5, 500 nM 49-mer attenuated deposition of both FITC-fibronectin and endogenous fibronectin by cell monolayers. Loss of exogenous or endogenous fibronectin was nearly complete; only short strands of fibronectin remained. The 42-mer (Fig. 5) or 29-mer or 12-mer (not shown) did not visibly affect either exogenous or endogenous fibronectin assembly.

**FIG. 6.** The 49-mer does not affect cell adhesion, cell growth, or focal contact or stress fiber formation. Panel A, cell adhesion. Human foreskin fibroblasts were plated on 96-well plates coated with 10 μg/ml fibronectin, 10 μg/ml vitronectin, or 0.2% BSA. Cells were incubated for 1 h in DMEM containing 0.2% BSA in the absence (NA) or presence of increasing concentrations of 49-mer. Duplicate wells were washed, fixed, and stained with 0.1% crystal violet. The relative absorbance was measured on a microplate reader as a measure of cells adhered/well. Panel B, focal contacts and stress fibers. Human dermal fibroblasts were plated on fibronectin-coated coverslips and incubated for 2 h in the absence (NA) or presence of 500 nM 49-mer in DMEM and 0.2% BSA. Cells were washed, fixed, and permeabilized as described under “Experimental Procedures.” Double fluorescence was carried out by incubation with rhodamine-phalloidin (actin) and mouse anti-human vinculin, followed by incubation with FITC-rabbit anti-mouse IgG. Bar = 30 μm. Panel C, cell proliferation. Human dermal fibroblasts or MG63 cells were plated in triplicate in 96-well plates in the absence or presence of 500 nM FUD in DMEM and 10% fetal calf serum. Cells were allowed to grow for 1, 3, or 5 days and reacted with MTS reagent. Absorbance was measured at 490 nm, and cell number was estimated by comparison with a standard curve established on the same day of absorbance versus known cell number. Brackets indicate mean ± S.D. of triplicate samples.
primary smooth muscle cells (40). We therefore tested the 49-mer for possible effects on cell adhesion and growth. As shown in Fig. 6A, 500 nM 49-mer, a concentration that nearly fully blocks fibronectin matrix assembly, did not affect adhesion to fibronectin or vitronectin. Focal adhesions (determined by staining for vinculin) or stress fibers (determined by staining with rhodamine-phalloidin) were not disrupted by the treatment with 49-mer (Fig. 6B). To assess possible effects on proliferation, FUD was added at the time of plating of dermal fibroblasts or MG63 cells in medium containing 10% fetal calf serum. As assessed by cytosolic NADPH activity measured with the MTS reagent, there were no significant differences in growth curves over 5 days in the presence or absence of FUD (Fig. 6C). Similar results were obtained with the 49-mer when cells were suspended by trypsin treatment at various times and counted on a hemocytometer (not shown). To address the possibility that FUD was inactivated or that cells overcome the inhibitory effect of the peptide during prolonged culture, coverslip cultures of dermal fibroblasts or MG63 cells grown for 5 days in 10% fetal calf serum in the presence or absence of FUD
were processed for immunofluorescence with IST-9. A profound loss of fibronectin matrix similar to that shown in Fig. 5 was detected when FUD was present (not shown). Cell morphology after 5 days in culture, as visualized by phase-contrast microscopy, was typical for a cell monolayer and similar whether in the presence or absence of FUD. Cells in the presence or absence of FUD (not shown).

**FUD Binds to Fibronectin Fibrils but Inhibits Fibronectin Assembly in the Absence of Cellular or Preassembled Plasma Fibronectin—**

To understand the role of endogenous fibronectin in the mechanism by which the fibronectin-binding domains of F1 exert their effects on fibronectin fibrillogenesis, we utilized mouse fibroblasts derived from stem cells in which the fibronectin gene was knocked out (34). Cells were studied on a collagen substrate in the absence of serum, allowing us to control the presence or absence of fibronectin. Fig. 7 shows that fibronectin-null cells incubated with 20 nM FITC-fibronectin assembled a fibronectin matrix within 1 h. FUD at 20 nM inhibited fibril formation with only punctate staining remaining. At 100 nM, FUD completely inhibited deposition of FITC-fibronectin (not shown). Thus, endogenous fibronectin is not required for FUD inhibition of FITC-fibronectin assembly.

FITC-labeled FUD, FRD, or FF1, was incubated for 1 h with fibronectin-null cells possessing or lacking a fibronectin matrix; that is, the fibronectin-null cells were incubated in the absence or with plasma fibronectin overnight, resulting in the formation of an extensive matrix detected by immunofluorescence with a rhodamine conjugate (Fig. 8, pFN) which was totally lacking in cell layers not incubated with plasma fibronectin (not shown). As seen in the center and right panels of Fig. 8, FITC-FUD, -FRD, or -FF1 co-localized with predeposited fibronectin but did not bind to monolayers incubated in the absence of fibronectin (left panels). The same result was obtained with FITC-RD5 (not shown). These findings indicate that the inhibitory effects of FUD, FRD, or FF1 are not caused by binding molecules other than fibronectin on cell surfaces or in the matrix.

**FUD Inhibits Binding of 70-kDa Fragment Binding to Cells in the Absence of Fibronectin—** The results described above indicate that FUD could inhibit fibronectin fibrillogenesis by binding soluble fibronectin at the site with which it interacts with cell surface assembly sites, by binding to sites on preexisting or incipient fibronectin fibrils involved in fibronectin-fibronectin interactions, or by a combination of both mechanisms. To sort through these mechanisms, we studied the 70-kDa fragment and fibronectin-null cells. Radiolabeled 3 nM 70-kDa fragment (not shown) or 50 nM (Fig. 9A) bound specifically to fibronectin-null cells plated on collagen in the absence of serum. FUD monopa-
Equimolar concentrations of RD5 enhance the binding of 70-kDa fragment to substratum. Fibronectin-null cells were plated on collagen-coated coverslips for 2 h and incubated with 50 nM 125I-labeled 70-kDa fragment (panel A) or FITC-70-kDa fragment (panel B) for 1 h at 37 °C in the presence or absence of various concentrations of RD5. Shown as fold change is the ratio of the amount of ligand bound in the presence of RD5 over the amount bound in the absence of RD5; the arrow points to the concentration of RD5 equimolar with ligand. Cells in panel B were washed, fixed, mounted, and photographed for fluorescence and corresponding phase using the same settings as in Fig. 9B. Shown are the cells treated with 50 nM RD5. Bar = 10 μm.

Enhanced Binding of 70-kDa Fragment or Fibronectin by Equimolar RD5 Does Not Result in Increased Fibrillogenesis but Is Instead Caused by Increased Binding to Substratum—The fibronectin-null cells also allowed us to investigate the mechanism whereby equimolar concentrations of tandem repeat containing F1 domains (RD5 and FF1) promote enhancement of fibronectin or 70-kDa fragment binding to cell layers. One possibility was that RD5 or FF1, because of multivalence with respect to fibronectin, bridges soluble ligand to preassembled fibronectin. An equimolar concentration of RD5, however, promoted a 3-fold increase in binding of 125I-labeled 70-kDa fragment to fibronectin-null cell monolayers, even in the absence of a fibronectin matrix (Fig. 10A). As seen in Fig. 10B, the increase in the binding of FITC-labeled 70-kDa fragment promoted by an equimolar concentration of RD5 was caused by binding to collagen substrate not covered by adherent cells. Similar enhanced binding was found when the substratum included vitronectin, or the fluorescent ligand was fibronectin (not shown).

**DISCUSSION**

We have investigated the effects of parts of the fibronectin-binding protein F1 from *S. pyogenes* on fibronectin matrix assembly. FUD, a His-tagged construct consisting of the 43-residue domain upstream of the RD5 region in F1 plus 6 residues from the first 37-residue repeat (12), was a potent inhibitor of assembly of either exogenous or endogenous fibronectin. FRD, a His-tagged construct that begins and ends with MG-GQSES sequence present in each repeat of RD5, also inhibited fibronectin assembly but was at least 100-fold less potent than FUD. Binding of FUD has been localized to the N-terminal 70-kDa region of fibronectin, whereas FRD was shown to bind primarily to the N-terminal 27-kDa subfragment of the 70-kDa fragment (12). Previous studies have shown that most of the cell surface binding activity of the 70-kDa fragment important for fibronectin assembly resides within the 27-kDa region (16–19). FUD and FRD, therefore, likely inhibit fibronectin binding and assembly by binding to the five N-terminal type I modules that comprise the 27-kDa region.

RD5 and the FF1 construct containing both FUD and RD5 increased binding of radiolabeled fibronectin and 70-kDa fragment at concentrations subequimolar to equimolar to those of the ligands and inhibited binding at higher concentrations. These constructs contain multiple binding sites for fibronectin or 70-kDa fragment, similar to the triplicated D repeats of the FnbpA fibronectin-binding adhesin of *S. aureus* (41) and have the potential to increase binding by several different mechanisms. Fluorescence microscopy indicated that the increased binding did not result in increased deposition of fibrillar fibronectin and instead was associated with enhanced binding of fibronectin or 70-kDa fragment to the substratum. The enhanced binding is likely caused by the ability of the tandem FRD sequences in RD5 and FF1 to bind multiple fibronectins or 70-kDa fragments that by virtue of multivalence or altered conformation have increased avidity for collagen or vitronectin on the substratum. At higher concentrations of FF1 or RD5, fibronectin or 70-kDa fragment would be expected to form 1:1 complexes with the fusion protein and thereby be inhibited in binding.

Among the F1 domains, FUD was characterized further because of its high monophasic potency in inhibition of fibronectin binding and assembly. A synthetic peptide encompassing the entire FUD (49-mer) had the same level of activity as
FUD is the first described specific inhibitor of fibronectin matrix assembly which is not based on fibronectin itself. We speculate that FUD mimics the cell surface binding site for the five N-terminal type I modules of fibronectin. Dissection of the determinants of the interaction between FUD and fibronectin, therefore, may help elucidate the steps of fibronectin matrix assembly. Because FUD does not seem to affect cell morphology or growth, it may be a lead compound to develop agents that disrupt unwanted fibronectin assembly in inflammatory and fibrotic conditions.

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**REFERENCES**

1. Magnusson, M. K., and Mosher, D. F. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1383–1370
2. Joh, D., Wann, E. R., Kreikemeyer, B., Speziale, P., and Hook, M. (1999) Matrix Biol. 18, 211–223
3. Guzmán, C. A., Talay, S. R., Molinari, G., Medina, E., and Chhatwal, G. S. (1986) J. Infect. Dis. 155, 59–66
4. Bismar, A. G., and Stevens, D. L. (1996) N. Engl. J. Med. 334, 340–345
5. Molinari, G., Talay, S. R., Valentín-Weigand, P., Rohde, M., and Chhatwal, G. S. (1997) Infect. Immun. 65, 1357–1363
6. Cederquist, D., Dombeck, P. E., Lam, H., and Cleary, P. P. (1998) Infect. Immun. 66, 4535–4601
7. Ozeri, V., Rosenhaim, I., Mosher, D. F., Fassler, R., and Hanski, E. (1998) Mol. Microbiol. 30, 625–637
8. Jadouin, J., Ozeri, V., Burstein, E., Skutelsky, E., Hanski, E., and Sela, S. (1998) J. Infect. Dis. 178, 147–158
9. Hanski, E., and Caparon, M. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 6172–6176
10. Talay, S. R., Valentín-Weigand, P., Timmins, K. N., and Chhatwal, G. S. (1994) Mol. Microbiol. 13, 531–539
11. Sela, S., Ariv, A., Tov, I., Burstein, I., Caparon, M. G., and Hanski, E. (1995) Mol. Microbiol. 10, 1049–1055
12. Ozeri, V., Tovi, A., Burstein, I., Natsanson-Yaron, S., Caparon, M. G., Yamada, K. M., Akiyama, S. K., Vlodavsky, I., and Hanski, E. (1996) EMBO J. 15, 989–998
13. Mosher, D. F., and Proctor, R. A. (1980) Science 209, 927–929
14. Joh, D., Speziale, P., Gurussiddappa, S., Manor, J., and Hook, M. (1998) Eur. J. Biochem. 258, 897–905
15. Schwarzwaular, J. E., and Sechler, J. L. (1999) Curr. Opin. Cell. Biol. 11, 622–627
16. McKeown-Longo, P. J., and Mosher, D. F. (1985) J. Cell Biol. 100, 364–374
17. McDonald, J. A., Quade, B. J., Breckelmann, T. J., LaChance, R., Forsman, K., Hasegawa, E., and Akiyama, S. (1987) J. Biol. Chem. 262, 2957–2967
18. Schwarzwaular, J. E. (1991) J. Cell. Biol. 113, 1463–1473
19. Sottile, J., Schwarzwaular, J., Selegue, J., and Mosher, D. F. (1991) J. Biol. Chem. 266, 12440–12443
20. Chernousov, M. A., Fogerty, F. J., Koteliansky, V. E., and Mosher, D. F. (1991) J. Biol. Chem. 266, 10851–10858
21. Morita, A., and Russelahi, E. (1992) J. Cell Biol. 111, 421–429
22. Hocking, D. C., Sottile, J., and McKeown-Longo, P. J. (1994) J. Biol. Chem. 269, 19183–19191
23. Hocking, D. C., Smith, R. K., and McKeown-Longo, P. J. (1996) J. Cell Biol. 133, 431–444
24. Sechler, J. L., Corbett, S. A., and Schwarzwaular, J. E. (1997) Mol. Cell. Biol. 17, 2563–2573
25. Bittorf, S. V., Williams, E. C., and Mosher, D. F. (1993) J. Biol. Chem. 268, 2573–2575
26. Sottile, J., Hocking, D. C., and Swiatek, P. J. (1998) J. Cell Sci. 111, 2933–2943
27. Ugarova, T. P., Zamarron, C., Veklich, Y., Bowditch, R. D., Ginsberg, M. H., Hasegawa, E., and Akiyama, S. (1987) J. Biol. Chem. 262, 2590–2597
28. Grinnell, F., Lang, B. R., and Phan, T. V. (1982) Exp. Cell Res. 142, 499–504
29. Pearlstein, E. (1978) Int. J. Cancer 20, 386–392
30. Mosher, D. F., and Proctor, R. A. (1980) Science 209, 927–929
31. Hynes, R. O. (1999) J. Cell Biol. 141, 539–541
32. Bieter, S. V., Williams, E. C., and Mosher, D. F. (1993) J. Biol. Chem. 268, 24838–24846
33. Peters, D. M., Portz, L. M., Fullenwider, J., and Mosher, D. F. (1990) J. Cell Biol. 111, 249–256
35. Saoncella, S., Echtermeyer, F., Denhez, F., Nowlen, J. K., Mosher, D. F., Robinson, S. D., Hynes, R. O., and Goetinck, P. F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2805–2810
36. Zhang, Q., Checovich, W. J., Peters, D. M., Albrecht, R. M., and Mosher, D. F. (1994) J. Cell Biol. 127, 1447–1459
37. Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996) J. Cell Biol. 132, 227–238
38. Sechler, J. L., and Schwarzauer, J. E. (1998) J. Biol. Chem. 273, 25533–25536
39. Bourdoulous, S., Orend, G., MacKenna, D. A., Pasqualini, R., and Ruoslahti, E. (1998) J. Cell Biol. 143, 267–276
40. Mercurius, K. O., and Morla, A. O. (1998) Circ. Res. 82, 548–556
41. Morla, A., Zhang, Z., and Ruoslahti, E. (1994) Nature 367, 193–196
42. Chernousov, M. A., Metsis, M. L., and Koteliansky, V. E. (1985) FEBS Lett. 183, 365–369
43. Sottile, J., and Wiley, S. (1994) J. Biol. Chem. 269, 17192–17198
44. Zhang, Q., and Mosher, D. F. (1996) J. Biol. Chem. 271, 33284–33292