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Identification of the Heparin-binding Determinants within Fibronectin Repeat III₁

ROLE IN CELL SPREADING AND GROWTH*

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Fibronectins are high molecular mass glycoproteins that circulate as soluble molecules in the blood, and are also found in an insoluble, multimeric form in extracellular matrices throughout the body. Soluble fibronectins are polymerized into insoluble extracellular matrix (ECM) fibrils via a cell-dependent process. Recent studies indicate that the interaction of cells with the ECM form of fibronectin promotes actin organization and cell contractility, increases cell growth and migration, and enhances the tensile strength of artificial tissue constructs; ligation of integrins alone is insufficient to trigger these responses. Evidence suggests that the effect of ECM fibronectin on cell function is mediated in part by a matricryptic heparin-binding site within the first III₁ repeat (FNIII₁). In this study, we localized the heparin-binding activity of FNIII₁ to a cluster of basic amino acids, Arg⁶¹³, Trp⁶¹⁴, Arg⁶¹⁵, and Lys⁶¹⁷. Site-directed mutagenesis of a recombinant fibronectin construct engineered to mimic the ECM form of fibronectin demonstrates that these residues are also critical for stimulating cell spreading and increasing cell proliferation. Cell proliferation has been tightly correlated with cell area. Using integrin- and heparin-binding fibronectin mutants, we found a positive correlation between cell spreading and growth when cells were submaximally spread on ECM protein-coated surfaces at the time of treatment. However, cells maximally spread on vitronectin or fibronectin still responded to the fibronectin matrix mimetic with an increase in growth, indicating that an absolute change in cell area is not required for the increase in cell proliferation induced by the matricryptic site of FNIII₁.

Fibronectin is an abundant adhesive glycoprotein that is evolutionarily conserved and broadly distributed among vertebrates (1). The polymerization of soluble fibronectin into insoluble fibrils within the ECM³ is a dynamic, cell-dependent

process that is mediated by coordinate events involving the actin cytoskeleton and integrin receptors (2). Previous studies have shown that active fibronectin matrix polymerization promotes cellular functions critical for tissue repair, including cell growth (3–6), cell migration (7), and collagen matrix contraction (8). Fibronectin matrix polymerization also promotes collagen I deposition (9, 10) and enhances the tensile strength of collagen-based tissue constructs (11); integrin ligation alone is not sufficient to trigger these responses (3, 7–9, 11). The mechanism by which ECM fibronectin exerts its unique effect on cell function is only partially understood.

In the ECM, fibronectin is organized as an extensive network of elongated, branching fibrils. Time lapse microscopy of cells expressing green fluorescent protein-labeled fibronectin demonstrate that cells routinely stretch ECM fibronectin into long fibrillar strands that recoil when released (12). The three-dimensional organization of ECM fibronectin likely arises from the ability of cells to repeatedly exert a mechanical force (13) on discrete regions of the protein (14) to facilitate the formation of fibronectin-fibronectin interactions (15–19). As cells contact fibronectin fibrils, tractional forces induce additional conformational changes (20) that are necessary for both lateral growth and branching of the fibrils (17).

Fibronectin, like many other ECM molecules, is a mosaic protein composed of modular subunits (21). The primary structure of each subunit is organized into three types of repeating homologous units, termed types I, II, and III. Fibronectin type III repeats (FNIII) are found in a number of ECM proteins and consist of seven β -strands that overlap to form two β -sheets (22, 23). Molecular modeling and atomic force microscopy studies predict that reversible unfolding of the type III repeats contributes to the elasticity of fibronectin, which may be extended up to six times its initial length (14, 24, 25). Fibronectin modules contain multiple binding sites, including those for glycosaminoglycans, collagen or gelatin, fibrin, and integrin receptors; additional binding sites may become available as fibronectin modules are elongated and internal residues are exposed (18, 25, 26).

The anti-parallel β -sheets of FNIII repeats are composed of three (A, B, and E) and four (C, D, F, and G) β -strands. Proteolysis of the first type III repeat of fibronectin (FNIII₁) at residue Ile⁵⁹⁷ removes both the A and B β -strands and results in a C-terminal fragment that binds to heparin (27). FNIII₁ also exhibits cryptic homophilic binding activity (18, 28) that mediates fibronectin fibril formation (16, 29). These sites are not

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³ The abbreviations used are: ECM, extracellular matrix; GST, glutathione S-transferase; FNIII, fibronectin type III repeat; FN-null MF, fibronectin-null myofibroblast; HSPGs, heparan sulfate proteoglycans; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

exposed in soluble fibronectin (18, 27), but may be exposed either during fibronectin matrix polymerization or as cells exert tension on the insoluble matrix (30, 31). We previously hypothesized that the cryptic heparin-binding activity of FNIII₁ functions as a conformation-dependent site for cell surface heparan sulfate proteoglycans (HSPGs) and thus, serves as a mechanism by which the ECM form of fibronectin exerts its unique effect on cell function. To test this hypothesis, we developed a GST-tagged fusion protein in which the C-terminal, heparin-binding fragment of FNIII₁, comprised of residues Ile⁵⁹⁷-Thr⁶⁷³, was directly linked to the integrin-binding FNIII₈₋₁₀ modules (GST/III1H,8-10). Treatment of fibronectin-null myofibroblasts (FN-null MFs) with GST/III1H,8-10 stimulated cell growth, contractility, and migration to a similar extent as ECM fibronectin (7, 32). As such, this fibronectin matrix mimetic effectively bypasses some of the requirements for intact fibronectin to undergo conformational changes to initiate ECM fibronectin-specific signals. Here, we used site-directed mutagenesis of the matrix mimetic to map the heparin-binding, cell spreading, and growth-promoting activities of FNIII₁. We localized these activities to a cluster of basic amino acids, Arg⁶¹³-Trp⁶¹⁴-Arg⁶¹⁵-Lys⁶¹⁷, that are contained within the C-strand of FNIII₁. Using integrin- and heparin-binding fibronectin mutants, we found a positive correlation between increased cell spreading and increased cell growth when cell area at the time of exposure was submaximal. However, cells maximally spread on vitronectin or fibronectin substrates still responded to the fibronectin matrix mimetic with an increase in growth, indicating that an absolute change in cell area is not required for the increased rate of cell proliferation induced by ECM fibronectin.

EXPERIMENTAL PROCEDURES

Reagents—Human plasma fibronectin was isolated from Cohn's fraction I and II, as described previously (33). Human plasma vitronectin was a gift from Dr. Scott Blystone (SUNY Upstate Medical University, Syracuse, NY). Human fibrinogen was a gift from Dr. Patricia Simpson-Haidaris (University of Rochester, Rochester, NY). Laminin was from BD Biosciences. Type I rat tail collagen was obtained from Upstate (Lake Placid, NY). Fibronectin III₁ peptides were from Sigma. Antibodies and their sources are as follows: anti-FNIII₁ IgG (9D2 (29)), a gift from Dr. Deane Mosher, University of Wisconsin, Madison, WI; mouse IgG Cappel, Aurora, OH; anti-GST polyclonal IgG, Upstate; horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies, Bio-Rad. Tissue culture supplies were obtained from Corning/Costar (Cambridge, MA). Unless otherwise indicated, chemical reagents were from Sigma.

Cell Culture—Mouse embryonic FN-null MFs (3) were generously provided by Dr. Jane Sottile (University of Rochester, Rochester, NY). FN-null MFs do not produce endogenous fibronectin but are able to polymerize exogenously added fibronectin into the ECM (3). FN-null MFs were cultured on collagen I-coated dishes under serum-free conditions using a 1:1 mixture of Cellgro® (Mediatech, Herndon, VA) and Aim V (Invitrogen). These media do not require serum supplementation. Thus, no exogenous source of fibronectin is present during routine culture.

Recombinant Fibronectins—Recombinant GST/III1H, GST/III1H,8-10, GST/III1H,2-4, GST/III8-10, and GST/III8-10,13 were produced in bacteria and purified as described previously (32). FNIII1H is comprised of amino acids Ile⁵⁹⁷-Thr⁶⁷³ (bases 1802-2032). The FNIII1H,8-10 heparin-binding mutant (GST/III1H,8-10ΔKRWR; K609G,R613T,W614T,R615T,K617A) was produced using the following mutant sense primer: 5'-**CCCGGTACC**ATCCAGTGGAAATGCACCACAGCCATC-TCACATTTCCGGGTACATTCTCACGACGACACCTG-CAAATTCTGTAGGC. Mutations are underlined; a Kpn site is shown in bold. The mutant sense primer for GST/III1H,8-10ΔRWR (R613T,W614T,R615T) (5'-**CCCGGTA-CCATCCAGTGGAAATGCACCACAGCCATCTCACATT-TCCAAGTACATTCTCACGACGACACCTAAAAATTC-TGTAGGC**) also contains a Kpn site. The antisense primer used for both III1H mutants was the same as that used to amplify GST/III8-10 (32). The sense primer for GST/III1H,8-10RGE (D1495E) (5'-**CCCGGTACC**ATCCAGTGGAAATGCACCACAG) contains a Kpn site; the antisense mutant primer (5'-**CCCCCGGGCTATGTTCCGGTAATT-AATGGAAATTGGCTTGCTGCTTGCGGGGCTTCTC-CACGGCCAGTG**) contains a SmaI site. GST/III1H,8-10ΔSyn (R1374A,R1379A) was produced using the same mutant inner sense and antisense primers as were used previously to generate GST/III9-10^{R1374A,R1379A} (32). The outer primers were the same as those used to amplify GST/III1H,8-10RGE (sense) and GST/III8-10 (antisense) (32). Purified GST/III1H,8-10 DNA (32) was used as the PCR template for all mutant GST/III1H,8-10 constructs except GST/III1H,8-10ΔSyn/RGE. The production of GST/III1H,8-10ΔSyn/RGE was similar to that of GST/III1H,8-10ΔSyn except that GST/III1H,8-10RGE DNA was used for amplification.

The truncated GST/III₁ constructs were produced using the III₁ sense primer (5'-**CCCGGATCC**AGTGGTCCTGTGCGAAGTATTAT) containing a BamHI site (bold) and the following antisense primers: GST/III1F666 (bases 1745-2011; Ser⁵⁷⁸-Phe⁶⁶⁶): 5'-**CCCGAATT**CCTAGAAGTCAAAGCGAGTCACTTC; GST/III1F664 (bases 1745-2005; Ser⁵⁷⁸-Phe⁶⁶⁴): 5'-**CCCGGATCC**CCTAAAGCGAGTCACCTCTTGGTG; GST/III1Y656 (bases 1745-1981; Ser⁵⁷⁸-Tyr⁶⁵⁶): 5'-**CCCGAATT**-CCTAGTACTGCTGGATGCTGATGAG; GST/III1Y646 (bases 1745-1951; Ser⁵⁷⁸-Tyr⁶⁴⁶): 5'-**CCCGAATT**CCTAGT-ATACCACACCAGGCTTC. EcoRI sites are shown in bold. GST/III-1^{K641T} was produced using the following mutant primers: 5'-TCAAAGGCCTGACGCCTGGTGTGGT (sense) and 5'-ACCACACCAGGCGTCAGGCCTTTGA (antisense). The outer primers were the same as those used to amplify non-mutant GST/III1 (bases 1745-2905; Ser⁵⁷⁸-Thr⁶⁷³) (18).

PCR-amplified DNA was cloned into pGEX-2T (Amersham Biosciences) and transfected into DH5α bacteria (32). DNA was sequenced to confirm the presence of the mutations. Fusion proteins were isolated on glutathione-Sepharose (Amersham Biosciences) and dialyzed extensively against PBS, as described previously (18).

Collagen Gel Contraction Assay—Floating type I collagen gels were prepared as described previously (8). Collagen gels imbedded with FN-null MFs and FNIII₁ peptides were incu-

bated for 20 h and then removed from the wells and weighed. Collagen gel contraction was measured as a decrease in gel weight (8). Data are reported as percent of contraction: $(1 - \text{weight of the test gels} / \text{weight of gels not containing cells}) \times 100$.

Solid-phase Enzyme-linked Immunosorbent Assay and Heparin Binding Assay—Glutathione-coated 96-well plates (Pierce) were incubated with saturating concentrations of GST fusion protein, as previously described (32). Plates were then washed and incubated with either primary antibodies (1 $\mu\text{g}/\text{ml}$) or 5 $\mu\text{g}/\text{ml}$ heparin-albumin-biotin followed by horseradish peroxidase-linked secondary antibodies or horseradish peroxidase-NeutrAvidin (Molecular Probes, Eugene, OR). The assays were developed using 2,2-azino-bis(3-ethylbenzthiazolinesulfonic acid) and the absorbance at 405 nm was measured.

Cell Spreading Assay—Tissue culture dishes (24-well) were coated with collagen I (50 $\mu\text{g}/\text{ml}$ in 0.02 N acetic acid (3)), fibronectin (10 $\mu\text{g}/\text{ml}$), vitronectin (1 or 10 $\mu\text{g}/\text{ml}$), fibrinogen (10 $\mu\text{g}/\text{ml}$), or laminin (10 $\mu\text{g}/\text{ml}$). Proteins other than collagen were diluted in PBS. FN-null MFs were seeded at 2.7×10^3 cells/ cm^2 (34) in defined medium and incubated at 37 °C for 4 h. Cells were then treated with 20 nM fibronectin, 250 nM GST fusion proteins, or an equal volume of PBS. At various times, cells were fixed with 2% paraformaldehyde. Cells were visualized with an Olympus inverted microscope (IX70) using a $\times 40$ objective. Phase-contrast images of fixed cells from triplicate wells were obtained using a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). The areas of at least 49 randomly chosen cells per condition were determined using ImagePro-Plus software (Media Cybernetics, Silver Spring, MD) calibrated with a stage micrometer.

Cell Growth Assay—FN-null MFs were seeded on tissue culture plates (48-well) coated with collagen, fibronectin, laminin, fibrinogen, or vitronectin at 3×10^3 cells/ cm^2 for 4 h (32). Cells were then incubated with 250 nM of the various recombinant fibronectin constructs at 37 °C for 3 or 4 days. Cells were fixed with 1% paraformaldehyde and stained with 0.5% crystal violet. Cells were solubilized with 1% SDS and the absorbance at 590 nm was determined.

Immunoblotting—PAGE and immunoblotting were performed as described previously (18). Gel samples were reduced with 2% β -mercaptoethanol. Immunoblots were incubated with primary antibody in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 1% bovine serum albumin followed by goat anti-rabbit or -mouse horseradish peroxidase-linked secondary antibody. Blots were developed using ECL (Pierce). After detection, blots were stripped by incubation with 0.2 M glycine, 0.1% SDS, 1% Tween, pH 4.0 (35). Blots were then washed, reblocked, and reprobed.

Statistical Analysis—Data are expressed as mean \pm S.E. and represent one of at least two independent experiments performed in triplicate (cell area) or in quadruplicate (cell growth and collagen gel contraction). Statistical significance was determined using either one-way analysis of variance with Turkey's post-test or Student's *t* test for unpaired samples using Prism software (GraphPad Software, San Diego, CA). Differences less than 0.05 were considered significant.

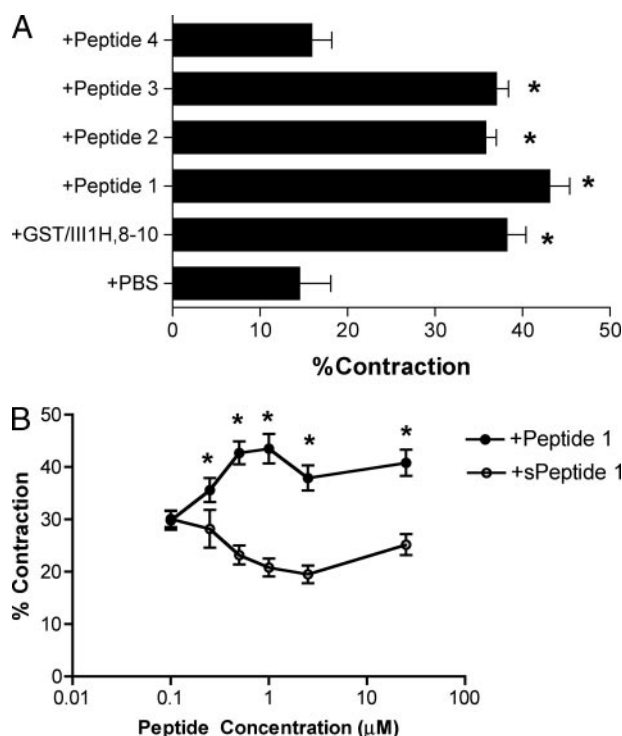


FIGURE 1. Effect of FNIII₁ peptides on collagen gel contraction. Collagen gel contraction assays were performed on FN-null MFs, as described under "Experimental Procedures." **A**, collagen gels were incubated in the absence and presence of 500 nM GST/III1H,8-10 or 100 μM of the various FNIII₁ peptides. *, significantly different from +PBS, $p < 0.01$. **B**, collagen gels were treated with increasing concentrations of Peptide 1 or the control, scrambled (s) Peptide 1. *, significantly different from corresponding dose of sPeptide 1, $p < 0.01$. Peptide sequences are shown in Fig. 3.

RESULTS

Identification of the Matricryptic Heparin-binding Site in FNIII₁—Our previous studies indicate that the effect of ECM fibronectin on cell function is mediated in part by a matricryptic, heparin-binding site within FNIII₁ (7, 32). To further define the role of FNIII₁ in promoting cell growth and contractility, studies were conducted to localize the functionally active site in FNIII₁. In cell growth and migration assays, the cellular response to the fibronectin matrix mimetic, GST/III1H,8-10, requires both FNIII_{1H} as well as $\alpha 5 \beta 1$ integrin ligation via the FNIII₈₋₁₀ modules. In contrast, cell-mediated collagen gel contraction is stimulated by a FNIII_{1H} construct that does not contain an integrin-binding site (32). Thus, overlapping 18-mer peptides encompassing a cluster of basic residues in FNIII₁ were used in collagen gel contraction assays to initially map the active site in FNIII_{1H}. As shown in Fig. 1A, Peptides 1, 2, and 3, containing the common sequence, ⁶¹³RWRPKNSVGR, stimulated collagen gel contraction; Peptide 4 had no effect. Concentrations of Peptide 1 ranging from 250 nM to 25 μM produced a significant increase in collagen gel contraction compared with the control scrambled Peptide 1 (sPeptide 1; Fig. 1B). The effects of Peptide 1 on contraction were saturable, reaching a maximum between 0.5 and 1 μM (Fig. 1B). These values are in agreement with previous studies demonstrating that dimeric FN, which contains two III₁ modules, stimulates maximum collagen gel contraction between 0.2 and 0.4 μM (32).

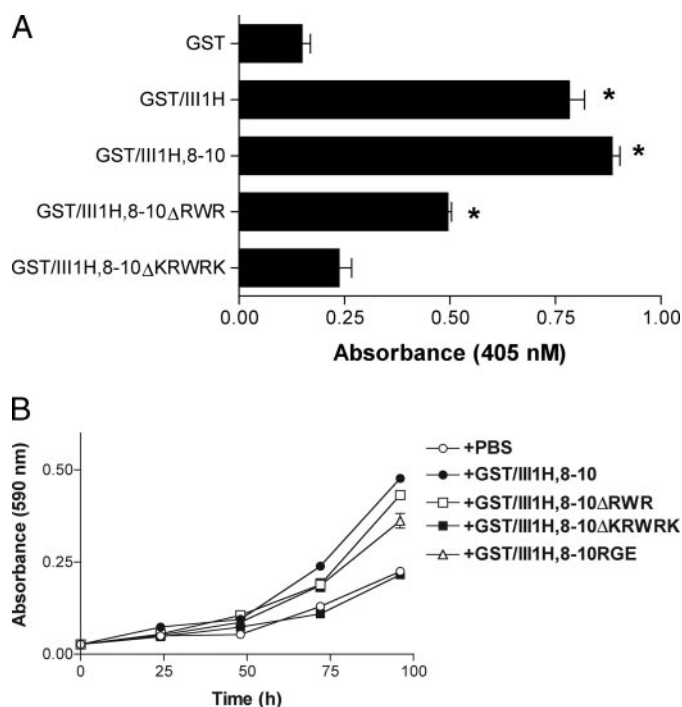


FIGURE 2. Localization of the heparin-binding and growth-promoting activities of FNIII₁. *A*, glutathione-coated plates were incubated with saturating concentrations of the various fusion proteins. Binding of biotin-heparin was assayed by enzyme-linked immunosorbent assay. Data represent the mean absorbance of triplicate wells \pm S.E. *, significantly different from +GST, $p < 0.01$. *B*, FN-null MFs were seeded on collagen-coated wells. Four hours after seeding, cells were treated with 400 nM of the various fusion proteins and cell number was determined at various times as described under "Experimental Procedures." Data are presented as the absorbance obtained at 590 nm and represent the mean \pm S.E. Maximal growth in response to GST/III1H,8-10 occurs with 100 nM protein (32). +GST/III1H,8-10, +GST/III1H,8-10ΔRWR, and +GST/III1H,8-10RGE are significantly different from +PBS on days 2, 3, and 4, $p < 0.01$.

Mutating Arg⁶¹³, Trp⁶¹⁴, and Arg⁶¹⁵ in GST/III1H,8-10 to the corresponding, non-charged amino acid sequence in FNIII₁ (Thr¹¹⁷⁸-Thr¹¹⁷⁹-Thr¹¹⁸⁰) resulted in a partial decrease in the heparin-binding activity of the construct (GST/III1H,8-10ΔRWR; Fig. 2*A*). Mutating two additional residues in GST/III1H,8-10ΔRWR (K609G and K617A; GST/III1H,8-10ΔKRWRK) abolished the heparin-binding activity (Fig. 2*A*), as well as the growth response to GST/III1H,8-10 (Fig. 2*B*). Mutating the integrin-binding RGD sequence in FNIII₁₀ to RGE only partially inhibited the growth response (Fig. 2*B*), underscoring the importance of FNIII_{1H} in the cellular response to the matrix mimetic.

Studies utilizing overlapping peptides extending from Lys⁶⁰⁹ to Thr⁶²⁷ were conducted next to confirm the importance of this region in fibronectin and GST/III1H,8-10-induced growth. Addition of FNIII₁ Peptides 5 or 6 to cells blocked the increase in growth induced by either GST/III1H,8-10 (Fig. 3*A*) or fibronectin (Fig. 3*B*). In contrast, Peptide 7 had no effect on fibronectin- or GST/III1H,8-10-induced growth (Fig. 3, *A* and *B*). Peptides 5 and 6 did not affect cell growth in the absence of fibronectin (not shown). A schematic of the FNIII₁ peptides used in this study is shown in Fig. 3*C*. Taken together, these data identify Arg⁶¹³, Trp⁶¹⁴, Arg⁶¹⁵, and Lys⁶¹⁷ as the novel heparin-binding and growth-promoting site in FNIII₁.

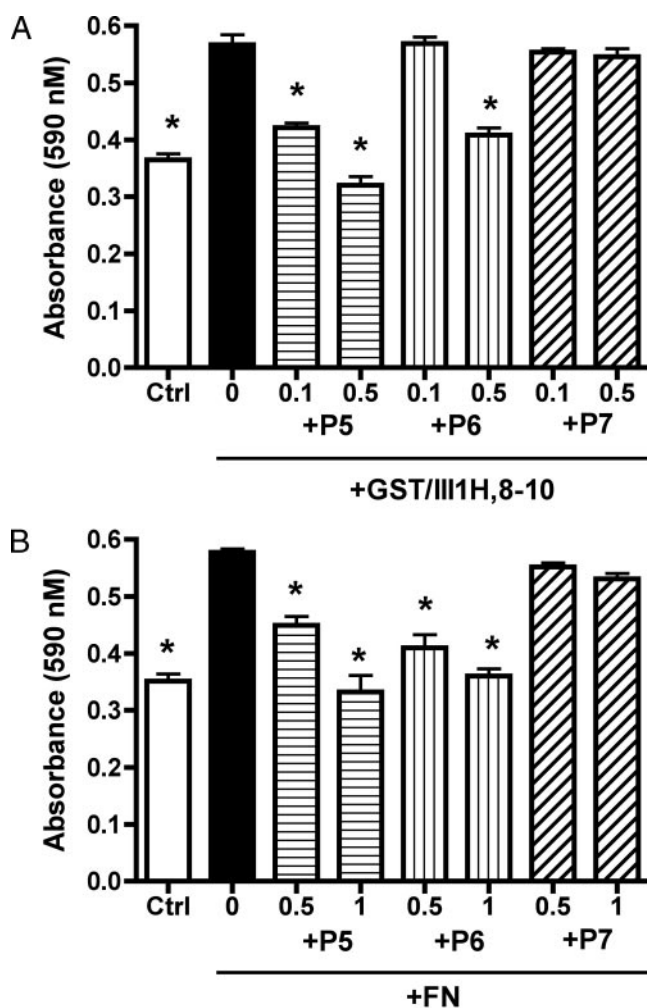


FIGURE 3. Effect of FNIII₁ peptides on fibronectin-induced cell growth. FN-null MFs were seeded on collagen-coated wells. Four hours after seeding, cells were treated with (A) GST/III1H,8-10 (100 nM), (B) fibronectin (FN; 40 nM), or an equal volume of PBS (Ctrl), in the absence ("no addition") and presence of FNIII₁ peptides (shown in mM). Following a 3-day incubation, cell number was determined. Data are presented as the absorbance obtained at 590 nm and represent the mean \pm S.E. *A*, *, significantly different from +GST/III1H,8-10, $p < 0.01$. *B*, *, significantly different from +FN, $p < 0.001$. Peptide sequences are shown in *C*.

Localization of the Epitopes for the Function-blocking Anti-FNIII₁ Antibody, 9D2—The 9D2 mAb binds to FNIII₁ and blocks fibronectin matrix assembly (29). As such, 9D2 has been used in several studies to distinguish the effects of soluble fibronectin from those of ECM fibronectin. 9D2 inhibits fibronectin-stimulated cell growth (3), contractility (8), and migration (7). 9D2 mAb does not block the initial association of fibronectin with cell surfaces (29), nor does it block cell adhesion to fibronectin (7). These studies suggest that the 9D2 epitope may be located at or near the matricryptic heparin-

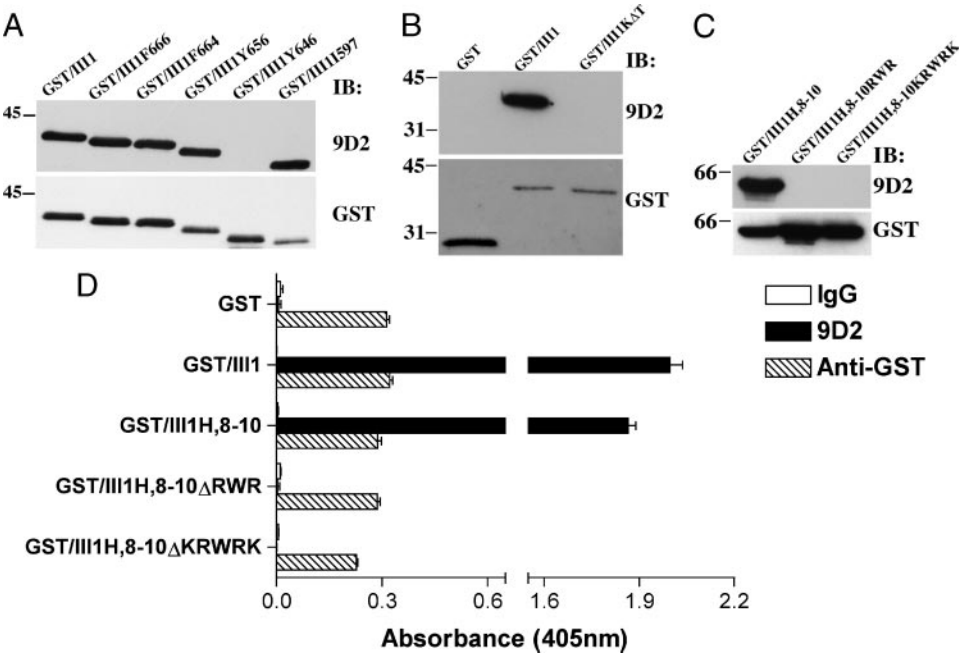


FIGURE 4. Localization of the 9D2 epitope in FNIII₁. In A–C, aliquots (0.2 μg) of GST/III₁ fusion proteins were electrophoresed on 10 or 12% polyacrylamide gels and analyzed by immunoblotting (IB) with the anti-FNIII₁ antibody, 9D2. Blots were then stripped and reprobed for GST. Molecular mass markers are indicated to the left. In D, glutathione-coated plates were incubated with saturating concentrations of the various fusion proteins. Binding of 9D2, mouse IgG, and anti-GST antibodies was assayed by enzyme-linked immunosorbent assay. Data represent the mean absorbance of quadruplicate wells ± S.E.

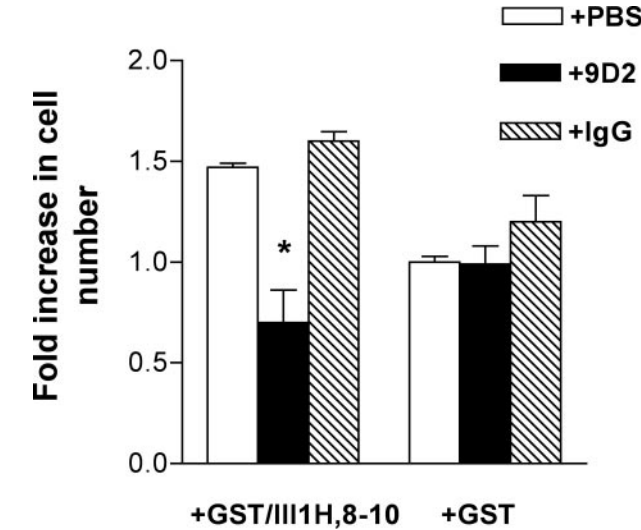


FIGURE 5. 9D2 blocks cell growth in response to GST/III1H,8-10. FN-null MFs were seeded on collagen-coated wells. Four hours after seeding, cells were treated with either 100 nM GST or GST/III1H,8-10 in the presence of 9D2 (500 nM) or non-immune IgG (500 nM), or an equal volume of PBS. Following a 3-day incubation, cell number was determined. Data are presented as the -fold increase in cell number over control (+GST) and represent the mean ± S.E. of 1 of 2 experiments performed in quadruplicate. *, significantly different from "GST/III1H,8-10+IgG," $p < 0.05$.

binding site. To localize the 9D2 epitope, a series of truncated FNIII₁ constructs were produced and analyzed by immunoblotting. As shown in Fig. 4A, sequential removal of C-terminal residues comprising either the connecting sequence to FNIII₂ (GST/III1F666), or the G β-strand of FNIII₁ (GST/III1F664 (36) and GST/III1Y656 (25)) had no effect on 9D2 recognition. Similarly, removal of both A and B β-strands from the N termi-

nus of FNIII₁ (GST/III1I597) did not alter 9D2 recognition (Fig. 4A). In contrast, removal of both F and G β-strands (GST/III1Y646) resulted in loss of 9D2 recognition (Fig. 4A), indicating that the 9D2 epitope is contained within the C-F β strands.

It was proposed previously that Lys⁶⁴¹ of human fibronectin is part of the 9D2 epitope, as rat fibronectin, which contains Thr at this site, is not recognized by 9D2 (29). Lys⁶⁴¹ is located within the loop connecting β strands E and F (25). Indeed, mutating Lys⁶⁴¹ to Thr in GST/III1 resulted in loss of 9D2 recognition (GST/III1K641T; Fig. 4B), demonstrating a role for Lys⁶⁴¹ in 9D2 binding to human fibronectin.

We next used the mutated GST/III1H,8-10 constructs to ask whether the cryptic heparin-binding site, located within the C β-strand of FNIII₁, is also part of the 9D2 epitope. As shown in Fig. 4C, GST/III1H,8-10 was recognized by 9D2 in immunoblots. In contrast,

9D2 did not immunoblot either GST/III1H,8-10ΔRWR or GST/III1H,8-10ΔKRWRK (Fig. 4C). Identical results were obtained when the constructs were analyzed by enzyme-linked immunosorbent assay (Fig. 4D). These results indicate that residues Arg⁶¹³, Trp⁶¹⁴, and Arg⁶¹⁵ are important components of the 9D2 epitope. Consistent with these findings, cell growth in response to GST/III1H,8-10 was inhibited by 9D2 IgG, but not non-immune IgG (Fig. 5).

ECM Fibronectin Increases Cell Area—Cell growth has been positively correlated with total cell area (37). Thus, one mechanism by which GST/III1H,8-10 may promote cell growth, as well as cell migration, is by increasing cell area. To analyze the effects of the matrix mimetic on cell area, FN-null MFs were seeded onto collagen I-coated dishes and incubated for 4 h to allow basal cell spreading to occur. FN-null MFs are grown under serum-free conditions, providing an ideal system for determining cell area in the complete absence of fibronectin, and for distinguishing the effects of soluble versus matrix fibronectin (3, 8, 9, 32, 38). In the absence of GST/III1H,8-10, the average area of collagen-adherent cells 4 h after seeding was ~900 μm², indicating that cells were well spread on the collagen substrate (39–41). Cells were then treated with GST/III1H,8-10, fibronectin, or control fusion proteins for an additional 2 h. As shown in Fig. 6A, addition of either GST/III1H,8-10 or intact fibronectin to collagen-adherent cells increased cell area. Cell spreading was not increased in response to a construct in which the heparin-binding III₁₃ module was substituted for III_{1H} (GST/III8-10,13; Fig. 6A), demonstrating the specificity of the III_{1H} fragment. Similarly, treatment of cells with a construct in which the III₂₋₄ modules were substituted for III₈₋₁₀ did not increase cell area (GST/

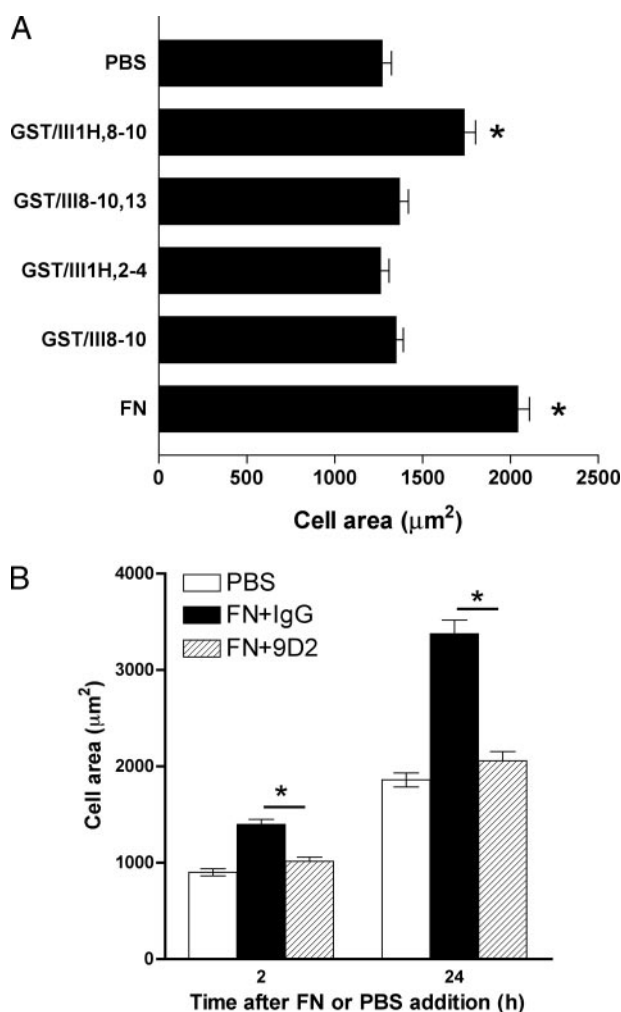


FIGURE 6. The ECM form of fibronectin increases cell area. FN-null MFs were allowed to adhere and spread on collagen I-coated wells for 4 h. *A*, cells were then treated for 2 h with 250 nM of the various fusion proteins. In *B*, cells were treated for 2 or 24 h with 20 nM FN in the presence of either 50 μg/ml non-immune mouse IgG or 50 μg/ml anti-FNIII₁, 9D2. Control cells were treated with PBS only. Cells were then fixed and phase-contrast images were obtained. Cell areas were determined as described under "Experimental Procedures." Data are presented as mean cell area (μm²) ± S.E. of at least 70 cells per condition and represent 1 of 2 experiments performed. *A*, *, significantly different from "+PBS," $p < 0.05$. *B*, *, "+FN+9D2" is significantly different from "+FN+IgG," $p < 0.05$.

III1H,2-4; Fig. 6A), suggesting that integrin ligation is necessary for the cell spreading response. However, treatment of cells with an integrin-binding fragment in which the III_{1H} fragment was absent (GST/III8-10; Fig. 6A) also failed to increase cell area, further demonstrating a role for III_{1H} in regulating cell area.

Our studies indicate that FNIII_{1H} contributes to cell spreading and that a portion of the 9D2 epitope lies within the functional heparin-binding domain of FNIII₁. Furthermore, 9D2 mAb blocks both cell growth (3) and migration (7) in response to fibronectin. Thus, to determine whether 9D2 inhibits cell spreading in response to fibronectin, FN-null MFs were allowed to spread on a collagen substrate and then treated with fibronectin in the presence of either 9D2 or non-immune IgG. As shown in Fig. 6B, fibronectin triggered a significant increase in projected cell area 2 and 24 h after its addition. Furthermore,

the increase in cell area in response to fibronectin was blocked by addition of 9D2 mAb, providing evidence that fibronectin matrix assembly and exposure of the matricryptic site in FNIII₁ are required for the increase in cell area.

Fibronectin Increases Cell Area by Transiently Increasing the Rate of Cell Spreading—To analyze the kinetics of fibronectin-induced cell spreading, collagen-adherent FN-null cells were treated with either fibronectin or an equal volume of PBS for various times and projected cell areas were determined. As shown in Fig. 7, fibronectin triggered a rapid increase in cell area that was evident within 10 min of its addition. This increase in cell spreading was followed by a plateau phase that began ~1 h after fibronectin addition and continued for ~4 h (Fig. 7A). Following the plateau phase, cell spreading resumed at a rate similar to that observed with control (PBS-treated) cells (Fig. 7, A and C). Linear regression analysis ($r^2 \geq 0.8$) was used to determine the rate of cell spreading immediately after fibronectin addition (<1 h; Fig. 7B) and following the plateau phase (4–24 h after fibronectin treatment; Fig. 7C). As shown in Fig. 7D, fibronectin transiently increased the rate of cell spreading from 103.7 (1.7 μm²/min) to 632.4 μm²/h (10.5 μm²/min). Following the plateau phase, the rate of cell spreading of fibronectin-treated cells returned to a rate similar to that observed in PBS-treated cells (66.8 versus 54.0 μm²/h, respectively; Fig. 7D). In contrast, the early and late phases of cell spreading of PBS-treated control cells were not significantly different (Fig. 7D). These data indicate that addition of fibronectin to collagen-adherent cells triggers a rapid yet transient increase in the rate of cell spreading.

Relationship between Cell Area and Growth—We next analyzed the effects of mutant heparin- and integrin-binding GST/III1H,8-10 constructs on cell spreading to (i) identify the amino acid residues responsible for increasing cell area and (ii) determine whether changes in cell area paralleled changes in cell growth. Addition of the FNIII₁ heparin-binding mutant, GST/III1H,8-10ΔKRWRK, to collagen-adherent FN-null MFs did not increase cell area (Fig. 8A) and likewise, did not enhance cell growth (Fig. 8B). Similarly, both the cell spreading response (Fig. 8A) as well as the cell growth response (Fig. 8B) to GST/III1H,8-10 was abolished by mutating the integrin-binding sequences in FNIII₈₋₁₀ (GST/III1H,8-10ΔSyn/RGE). Together, these data indicate that cell area and growth are co-regulated by the heparin-binding activity of FNIII₁ and the integrin-binding activity of FNIII₈₋₁₀. Treatment of cells with a construct in which only the integrin-binding synergy site in FNIII₉ was mutated (GST/III1H,8-10ΔSyn) stimulated cell spreading (Fig. 8A) and cell growth (Fig. 8B) to a similar extent as GST/III1H,8-10. These data suggest that for cells adherent to collagen I, increased cell spreading in response to the fibronectin matrix mimetic is correlated with enhanced cell growth.

To determine whether GST/III1H,8-10 can increase the area and growth of cells adherent to other ECM substrates, FN-null cells were seeded on various adhesive proteins and allowed to adhere and spread for 4 h. Vitronectin was used at a non-saturating concentration to avoid inducing maximal cell area prior to GST/III1H,8-10-treatment. In all experiments, wells were blocked with bovine serum albumin to eliminate

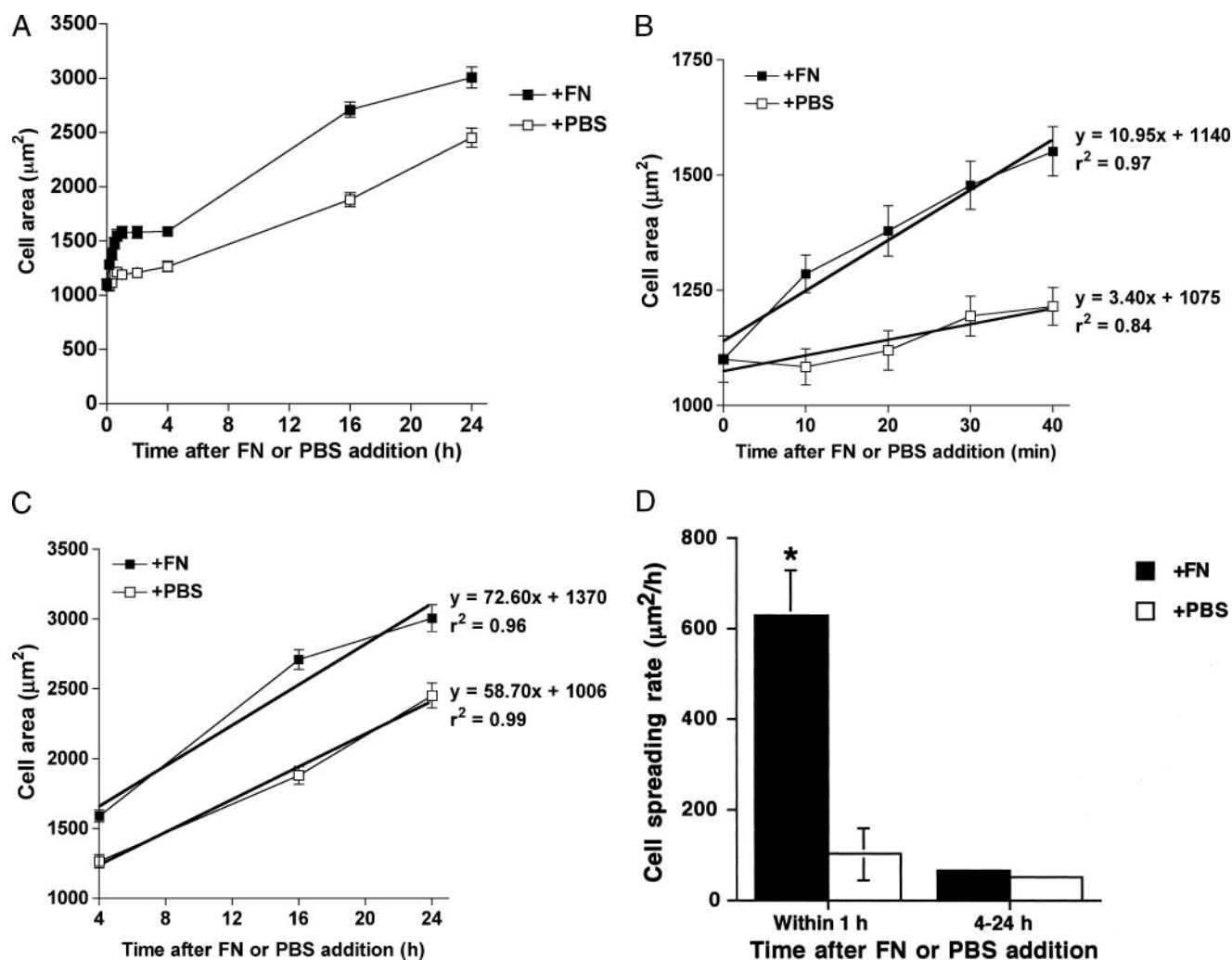


FIGURE 7. Fibronectin increases cell area by transiently increasing the rate of cell spreading. Fibronectin (20 nM) or an equal volume of PBS was added to collagen-adherent FN-null cells as described in the legend to Fig. 6. At various times, cells areas were determined. *A*, representative time course of cell area in response to fibronectin or PBS. Data are presented as mean cell area (μm^2) \pm S.E. of at least 49 cells per condition. The rate of cell spreading was determined from the slope (*B*, $\mu\text{m}^2/\text{min}$; *C*, $\mu\text{m}^2/\text{h}$) of the linear fit of the data. *D*, average cell spreading rates ($\mu\text{m}^2/\text{h}$) from four independent experiments. Data are presented as mean \pm S.E. *, $p < 0.05$ versus other groups.

adhesion of GST/III1H,8–10 to the tissue culture plastic. Cells seeded in bovine serum albumin-coated wells in the presence of soluble GST/III1H,8–10 are non-adherent (not shown). As shown in Fig. 9A, GST/III1H,8–10 stimulated an increase in cell area when cells were adherent to several different ECM proteins, including fibrinogen, vitronectin, and laminin. Furthermore, this increase in cell area was accompanied by a similar increase in cell growth (Fig. 9B).

Cell Area Expansion Is Not Required for GST/III1H,8–10-induced Growth—Cell spreading may physically trigger local changes in cytoskeletal conformation that in turn, allow for the recruitment and activation of signaling complexes involved in cell proliferation (42–44). As such, the relative change in cell area induced by ECM fibronectin may serve to mechanically stimulate the local activation of intracellular signals involved in cell growth control. To determine whether a change in cell area is required for enhanced cell growth in response to GST/III1H,8–10, cell area expansion in response to GST/III1H,8–10 was limited by seeding FN-null MFs on wells coated with saturating amounts of fibronectin or vitronec-

tin. These conditions were used to promote maximal cell spreading within the first 4 h of seeding. As shown in Fig. 10A, areas of cells adherent to either fibronectin or vitronectin were significantly greater than those of cells adherent to collagen I and as anticipated, the areas of fibronectin- and vitronectin-adherent cells did not increase in response to GST/III1H,8–10 (Fig. 10A). In contrast, the increase in cell growth was still observed in response to GST/III1H,8–10, even in the absence of an increase in cell area (Fig. 10B). These data indicate that an absolute increase in cell area is not required for enhanced cell growth in response to GST/III1H,8–10 and suggest that ECM FN-induced growth is not due to mechanical signals triggered in response to changes in cell shape.

DISCUSSION

Fibronectin matrix polymerization promotes cellular functions critical for tissue repair, including cell growth (3–6), cell migration (7), and collagen matrix contraction (8). Fibronectin matrix polymerization also promotes collagen I deposition (9,

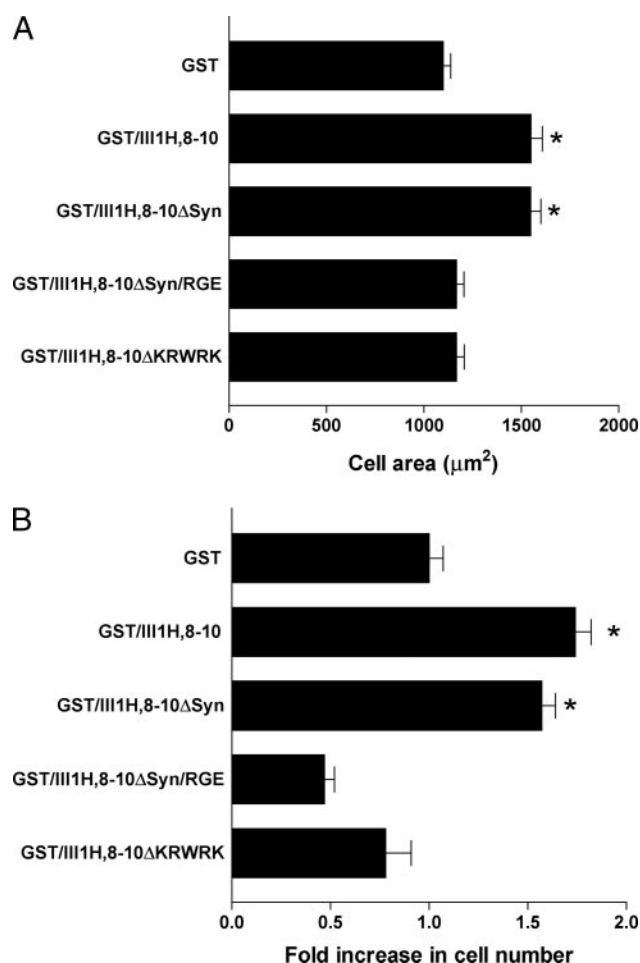


FIGURE 8. Cell area expansion and growth require both integrin- and heparin-binding activities. FN-null MFs were allowed to adhere and spread on collagen I-coated wells for 4 h. Cells were then treated with 250 nM of the various GST/III1H,8-10 constructs. **A**, 2 h after treatment, cells were fixed and areas were determined as described in the legend to Fig. 6. Data are presented as mean cell area (μm^2) \pm S.E. of at least 50 cells per condition. **B**, 4 days after addition of recombinant proteins, cells were fixed and cell number was determined. Data are presented as -fold increase in cell number over control (growth in the presence of GST). *, $p < 0.05$ versus GST.

10) and enhances the tensile strength of collagen-based tissue constructs (11). The mechanism by which ECM fibronectin exerts its unique effect on cell function is only partially understood. We previously hypothesized that conformational changes during fibronectin matrix remodeling expose a "matri-cryptic," heparin-binding activity in III₁ that serves to trigger cellular responses that are unique to ECM fibronectin. To test this hypothesis, we produced a recombinant fibronectin construct in which the heparin-binding fragment of FNIII₁ was directly linked to the integrin-binding FNIII₈₋₁₀ modules (GST/III1H,8-10). We found that treatment of cells with GST/III1H,8-10 stimulates cell growth, contractility, and migration to a similar extent as ECM fibronectin (7, 32). The interaction of GST/III1H,8-10 with cell surfaces appears to be mediated by HSPGs (32).

In the present study, we extended this work by localizing the functional heparin-binding site of FNIII_{1H} to the basic amino acids Arg⁶¹³, Trp⁶¹⁴, Arg⁶¹⁵, and Lys⁶¹⁷. The ⁶⁰⁹KYILRWPK sequence in FNIII₁ is unique among the type III repeats of

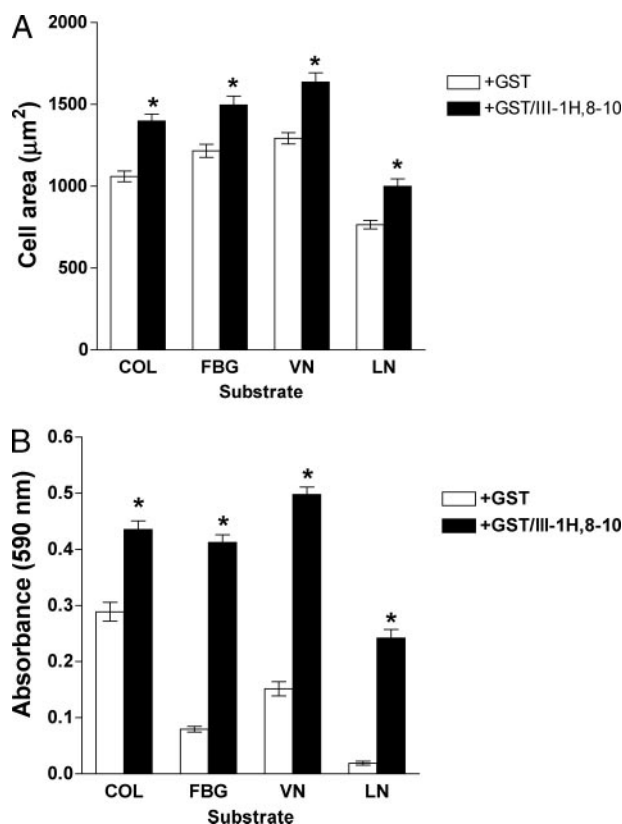


FIGURE 9. Effect of GST/III1H,8-10 on the area and growth of cells adherent to various ECM substrates. FN-null MFs were seeded on wells coated with collagen (COL), fibrinogen (FBG), vitronectin (VN; 1 $\mu\text{g}/\text{ml}$), or laminin (LN) and incubated for 4 h. Cells were then treated for an additional 2 h (**A**) or 4 days (**B**) with 250 nM GST/III1H,8-10 or GST. Cell area and growth were determined as described in the legend to Fig. 6. Data are presented as mean \pm S.E. of two independent experiments. *, $p < 0.05$ versus GST control.

fibronectin and is highly conserved among species (45). Human, rat, and mouse fibronectin sequences are identical in this region; Arg⁶¹³ is substituted with a lysine in *Xenopus* and bovine fibronectin. Cell spreading and growth in response to the fibronectin matrix mimetic required the Lys⁶⁰⁹-Arg⁶¹³-Trp⁶¹⁴-Arg⁶¹⁵-Lys⁶¹⁷ residues of FNIII₁ as well as integrin ligation. Constructs containing single integrin-binding domain mutations (GST/III1H,8-10RGE and GST/III1H,8-10 Δ Syn) retained the ability to promote cell growth, whereas the double integrin-binding mutant, GST/III1H,8-10 Δ Syn/RGE, did not. These findings suggest that ligation of $\alpha 5\beta 1$ integrins by either the RGD site in FNIII₁₀ or the synergy site in FNIII₉ is sufficient to promote cell growth when coupled with FNIII₁ binding to its receptor. These results indicate that an $\alpha 5\beta 1$ -dependent, but RGD-independent, response can be stimulated by ECM fibronectin. These results are consistent with previous observations that ECM fibronectin can stimulate cell growth by an RGD-independent, heparin-dependent mechanism (38). Interestingly, cell growth in response to GST/III1H,8-10 requires that the III1H module be co-expressed with III8-10 (32), suggesting that a physical interaction between the FNIII₁ HSPG receptor and $\alpha 5\beta 1$ integrins may be necessary for the integrin-mediated response.

Our data suggest that the heparin-binding site of FNIII₁ can enhance $\alpha 1\beta 1/\alpha 2\beta 1$ responses, as seen in collagen gel contrac-

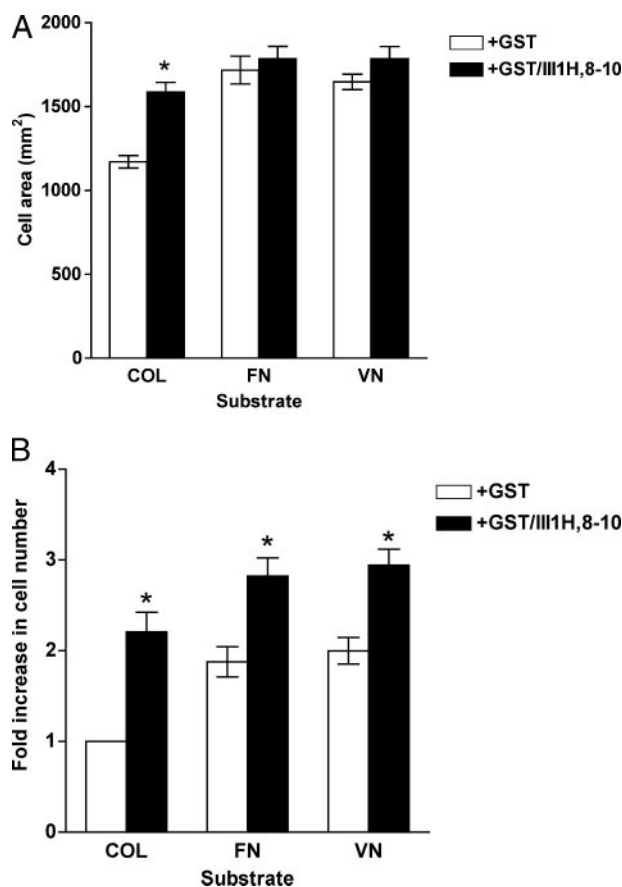


FIGURE 10. Cell area expansion is not required for GST/III1H,8-10-induced growth. FN-null MFs were seeded on wells coated with collagen (COL), fibronectin (FN), or vitronectin (VN; 10 μ g/ml) and incubated for 4 h. Cells were then treated for an additional 2 h (A) or 4 days (B) with 250 nM GST/III1H,8-10 or GST. Cell area and growth were determined as described in the legend to Fig. 6. Data are presented as mean \pm S.E. of four independent experiments. *, $p < 0.05$ versus GST control.

tion assays presented in Fig. 1A, as well as $\alpha 5\beta 1$ -mediated responses, as observed in the cell spreading and growth response to GST/III1H,8-10 (Fig. 8). The mechanism by which FNIII₁ modulates $\beta 1$ integrin function is currently unknown. HSPGs are known to function as co-receptors to modify integrin-mediated responses (46). A recombinant fibronectin construct containing both the C-terminal heparin-binding III₁₃ module and the integrin-binding III₇₋₁₁ domain (GST/III7-11,13) supports cell spreading and stress fiber formation when applied as an adhesive substrate (47). In the present study, we utilized a similar construct (GST/III8-10,13) to assess the specificity of the FNIII_{1H} module in enhancing cell area. When added in solution, GST/III8-10,13 did not increase the area of cells previously spread on a collagen substrate, consistent with the observation that GST/III8-10,13 is also unable to promote collagen gel contraction or enhance cell growth (32). The increase in cell area in response to fibronectin was associated with morphological changes indicative of a switch from isotropic to anisotropic cell spreading (results not shown). As such, distinct intracellular mechanisms may control cell area immediately following cell attachment to substrate-bound fibronectin monomers, where isotropic spreading and FNIII₁₃ signaling predominate (47), versus during fibronectin matrix formation

or remodeling, when anisotropic spreading and FNIII₁ signaling occur. The downstream effects of both FNIII₁₃ and FNIII_{1H} are mediated by HSPGs (32, 47, 48). As such, additional interactions, possibly involving the core protein of proteoglycans (46), may contribute to the subsequent generation of distinct intracellular signals that modulate cell spreading. The dissimilar effects of GST/III1H,8-10 and GST/III8-10,13 on cytoskeletal reorganization and cell spreading may also result from their different subcellular localization, as GST/III1H,8-10 co-localizes with HSPGs in lipid rafts, whereas GST/III8-10,13 localizes to focal contacts (32). A similar partitioning of the syndecan and glypican families of HSPGs has been observed (46, 49).

Fibronectin increased cell area by transiently increasing the rate of cell spreading from 103.7 to 632.4 μ m²/h. These results are consistent with previous data obtained from laminin-adherent hepatocytes that demonstrated an initial spreading rate of 740 μ m²/h (34). The rapid increase in cell area in response to fibronectin, combined with our data indicating a role for the ECM form of fibronectin in cell spreading, suggests that FNIII₁-mediated responses arise from early changes in fibronectin conformation that are initiated upon binding of fibronectin to the cell surface (20), and not from the formation of extensive fibronectin fibrils *per se*. The increase in cell area in response to fibronectin and GST/III1H,8-10 is accompanied by the formation of lamellipodia (not shown). Hence, it is likely that enhanced cell spreading and lamellipodia formation are key steps that mediate the increased rates of cell migration observed in response to ECM fibronectin and GST/III1H,8-10 (7).

Actin polymerization provides the driving force for cell spreading (50), whereas membrane tension generated by membrane-cytoskeletal adhesions (51) restricts spreading (42). Therefore, the appearance of a plateau following the rapid increase in cell area of fibronectin-treated cells may indicate that cells have reached a maximal area. Similarly, for cells spread on fibronectin or vitronectin, the absence of an increase in cell area in response to GST/III1H,8-10 may be due to the limitation of cell area. Approximately 4 h after the initiation of the plateau phase, spreading of fibronectin-treated cells resumed at a rate similar to control (PBS-treated) cells. The stimulus that reinitiated cell spreading is not known, but may be related to increased cell mass and/or renewed availability of adhesion receptors (41).

Several studies have provided evidence that changes in cell growth are tightly coupled to changes in cell area (37, 52). Others suggest that cell spreading may serve a permissive role for integrin-mediated proliferation (53). During cell spreading, changes in the conformation of the actin cytoskeleton may physically recruit and/or cluster signaling molecules that regulate cell growth (44). Our data indicate that whereas ECM FN can promote cell spreading, increased cell area is not the mechanism by which ECM fibronectin increases the rate of cell growth. Moreover, our data provide evidence that under certain circumstances, increased cell growth can be uncoupled from changes in cell area. These findings are important for understanding mechanisms that control ECM fibronectin-induced cell growth *in vivo*, as the inability to increase cell area

and hence, cytoskeletal tension (42), in intact tissue may normally serve to limit the cellular response to growth signals.

The amino acid residues involved in GST/IIIH₈-10-induced cell spreading and growth, Arg⁶¹³-Trp⁶¹⁴-Arg⁶¹⁵, contribute to the 9D2 epitope. Furthermore, 9D2 blocks GST/IIIH₈-10-stimulated cell growth as well as fibronectin-induced cell spreading. The 9D2 antibody has been shown to inhibit several other fibronectin-stimulated cell functions, including cell growth (3), cell migration (7), and collagen gel contraction (8). 9D2 mAb inhibits fibronectin matrix polymerization in a variety of cell types including dermal fibroblasts (29), aortic smooth muscle cells (9), microvascular endothelial cells (9), as well as the FN-null myofibroblasts (3). 9D2 mAb does not block fibronectin matrix polymerization in small airway epithelial cells, but does inhibit migration (7), suggesting that 9D2 affects migration in these cells by directly blocking the Arg⁶¹³-Trp⁶¹⁴-Arg⁶¹⁵ site in ECM fibronectin. Taken together, these data support the hypothesis that ECM fibronectin regulates cell behavior, in part, through the cell-dependent exposure of a neoepitope within the conformationally labile FNIII₁ module. Decreasing intracellular cytoskeletal tension preferentially reduces the appearance of an FNIII₁ epitope (30), suggesting that exposure of the matricryptic site in FNIII₁ may be dynamically regulated. If so, reduced cellular tension on ECM fibrils may impair tissue remodeling by "closing" matricryptic FNIII₁ sites that would normally stimulate cell growth, collagen fibril contraction, and re-epithelialization of injured tissues. Novel therapeutic approaches that provide injured cells with synthetic fibronectin matrix mimetics may circumvent either diminished fibronectin matrix assembly or decreased expression of matricryptic FNIII₁ sites and hence, accelerate wound repair by providing tissues with ECM fibronectin-specific signals.

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**Identification of the Heparin-binding Determinants within Fibronectin Repeat III₁
: ROLE IN CELL SPREADING AND GROWTH**

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