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Chimeric fibronectin matrix mimetic as a functional growth- and migration-promoting adhesive substrate

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Abstract

Therapeutic protein engineering combines genetic, biochemical, and functional information to improve existing proteins or invent new protein technologies. Using these principles, we developed an approach to deliver extracellular matrix (ECM) fibronectin-specific signals to cells. Fibronectin matrix assembly is a cell-dependent process that converts the inactive, soluble form of fibronectin into biologically-active ECM fibrils. ECM fibronectin stimulates cell functions required for normal tissue regeneration, including cell growth, spreading, migration, and collagen reorganization. We have developed recombinant fibronectin fragments that mimic the effects of ECM fibronectin on cell function by coupling the cryptic heparin-binding fragment of fibronectin's first type III repeat (FNIII1H) to the integrin-binding domain (FNIII8-10). GST/III1H,8-10 supports cell adhesion and spreading and stimulates cell proliferation to a greater extent than plasma fibronectin. Deletion and site-specific mutant constructs were generated to identify the active regions in GST/III1H,8-10 and reduce construct size. A chimeric construct in which the integrin-binding, RGDS loop was inserted into the analogous site in FNIII8 (GST/III1H,8^{RGD}), supported cell adhesion and migration, and enhanced cell proliferation and collagen gel contraction. GST/III1H,8^{RGD} was expressed in bacteria and purified from soluble lysate fractions by affinity chromatography. Fibronectin matrix assembly is normally up-regulated in response to tissue injury. Decreased levels of ECM fibronectin are associated with non-healing wounds. Engineering fibronectin matrix mimetics that bypass the need for cell-dependent fibronectin matrix assembly in chronic wounds is a novel approach to stimulating cellular activities critical for tissue repair.

Keywords

fibronectin; recombinant protein; extracellular matrix; cell proliferation; cell adhesion

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1. Introduction

Fibronectin is an abundant glycoprotein that is evolutionarily conserved and broadly distributed among vertebrates [1]. Soluble fibronectin is composed of two nearly identical subunits that are joined by disulfide bonds [2]. 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 are found in a number of ECM proteins and consist of two overlapping β sheets [3,4]. Molecular modeling and atomic force microscopy studies predict that reversible unfolding of the type III repeats contributes to the remarkable elasticity of fibronectin, which may be extended up to six times its initial length without denaturation [5,6]. In the ECM, fibronectin is organized as an extensive network of elongated, branching fibrils. The three-dimensional organization of ECM fibronectin likely arises from the ability of cells to repeatedly exert a mechanical force [7] on discrete regions of the protein [5] to facilitate the formation of fibronectin-fibronectin interactions [8]. As cells contact fibronectin fibrils, tractional forces induce additional conformational changes [9] that are necessary for both lateral growth and branching of the fibrils [10].

The polymerization of fibronectin into the ECM is a cell-dependent process that is mediated by coordinated events involving the actin cytoskeleton and integrin receptors [8,11]. Most adherent cells, including epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells, polymerize a fibrillar fibronectin matrix [1]. Recent studies have provided evidence that the interaction of cells with either the soluble or ECM form of fibronectin gives rise to distinct cellular phenotypes [12–18]. ECM fibronectin stimulates cell spreading [19], growth [13,20] and migration [21], as well as collagen deposition [22,23] and organization [18]. Others have shown a role for fibronectin matrix assembly in the deposition of fibrinogen [24], fibrillin [25], and tenascin C [26] into the ECM. Fibronectin matrix polymerization stimulates the formation of endothelial ‘neovessels’ in collagen lattices [27]. Moreover, blocking fibronectin matrix polymerization inhibits cell growth [13,16] and contractility [18], alters actin organization [28] and cell signaling [15], and inhibits cell migration [21]. Together, these studies indicate that fibronectin matrix polymerization plays a key role in establishing the biologically-active extracellular environment required for proper tissue function.

Fibronectin matrix assembly is rapidly up-regulated following tissue injury, while reduced fibronectin matrix deposition is associated with abnormal wound repair [29]. Altered fibronectin matrix deposition is also associated with a large number of chronic diseases including asthma, liver cirrhosis, and atherosclerosis [29–31]. Given the role of the fibronectin matrix in orchestrating ECM organization and in regulating cell and tissue responses critical for tissue repair, defective or diminished fibronectin matrix deposition by cells is likely to have profound effects on the ability of tissues to heal. Therapies that provide injured cells with synthetic fibronectin matrices have the potential to stimulate or correct defects in ECM deposition or organization and thus, have important applications to the treatment of chronic, degenerative illnesses.

In earlier studies, we localized the ECM-specific effects of fibronectin to a cryptic heparin-binding site in the first type III module of ECM fibronectin [19,21,32,33]. This conformation-dependent site is not exposed in soluble fibronectin [32,34,35] but becomes unmasked either during fibronectin matrix assembly or when cells and tissues exert tension on polymerized fibronectin fibrils [33,36]. In the present study, we have engineered several recombinant fibronectin constructs that incorporate the “open” conformation of FNIII1 and thus, mimic the ECM form of fibronectin. We compare the ability of ECM fibronectin mimetics to support cell adhesion, proliferation, migration, and collagen reorganization to that of full-length fibronectin and integrin-binding fibronectin fragments.

2.1. Reagents, antibodies, and cells

Human plasma fibronectin was isolated from Cohn's fraction I and II [37]. Type I collagen was extracted from rat tail tendons using acetic acid and precipitated with NaCl [38]. Human fibrinogen was a gift from Dr. Patricia Simpson-Haidaris (University of Rochester, Rochester, NY). Recombinant vitronectin was expressed and purified as described [39]. GRGDSP peptides were obtained from Sigma (St. Louis, MO). FN12-8 monoclonal antibody was obtained from Takara (Madison, WI); horseradish peroxidase-conjugated goat anti-mouse antibody was from Bio-Rad (Hercules, CA). Tissue culture supplies were from Corning/Costar (Cambridge, MA). Unless otherwise indicated, chemical reagents were from J.T. Baker (Phillipsburg, NJ) or Sigma.

Mouse embryonic fibronectin-null fibroblasts (FN-null MEFs; provided by Dr. Jane Sottile, University of Rochester, Rochester, NY) were cultured on collagen I-coated dishes under fibronectin- and serum-free conditions using a 1:1 mixture of Cellgro® (Mediatech, Herndon, VA) and Aim V (Invitrogen) [13].

A schematic of the recombinant proteins used in this study is shown in Figure 1A. FNIIIH is comprised of amino acids Ile⁵⁹⁷-Thr⁶⁷³ (bases 1802-2032). Recombinant GST/IIIH,8-10, GST/IIIH, GST/III8-10, GST/III8-10,13 and GST/IIIH were produced in *E. Coli* and purified as described [19, 32]. A fibronectin fragment in which a C-terminal fragment of Extra domain-B (ED_B-C) was coupled to the integrin-binding modules, III8-10 (GST/IIIED_B-C,8-10), was produced using the sense primer: 5'-CCCAGATCTCTGAGGTGGACCCCGCTAAAC-3' and antisense primer: 5'-CCCCCGGGCTATGTTTCGGTAATTAATGGAAATTG-3' (*Sma*I site in bold). The human fibronectin cDNA construct pCEF103 was used as a template [40]. GST/IIIH, 8-10ΔRRK (R⁶¹³T, R⁶¹⁵T, K⁶¹⁷A) was produced with the forward primer 5'-CCCGGTACCATCCAGTGAATGCACCACAGCCATCTCACATTTCOAAGTACATTCTCACGTGGACACCTGCAAATTCTGTAGGC-3'. The *Kpn*I site is shown in bold and the mutant codons are underlined. The reverse primer was 5'-CCCGAATTCCTATGTGCTGGTGCTGGTGGTG-3' with the *Eco*R1 site in bold. GST/IIIH,8-10ΔEGQ (E⁶⁴⁷D,Q⁶⁴⁹N) was produced using the following mutant sense primer: 5'-GGTATACGACGGCAACCTGATCAGCATCCAGCACTACG-3'. Mutations are underlined; a *Bst*Z17I site is shown in bold. The antisense primer for GST/IIIH,8-10ΔEGQ was the same as that used for GST/IIIH,8-10 (5'-CCCCCGGGCTATGTTTCGGTAATTAATGGAAATTG-3'), which contains a *Sma*I site (bold). Human full-length fibronectin cDNA pFH100 was used as a template.

Recombinant fibronectins that contained deletions to type III modules within the cell binding domain were made using GST/IIIH,8-10 plasmid DNA as the template. The sense primer, beginning in FNIII1, for these constructs was: 5'-**CCCGGATCCATCCAGTGAATGCACCACAG**-3'. The BamHI site is shown in bold. GST/IIIH,8 was made with the antisense primer: 5'-GTCACGATCAATT**CCCGGG**CTATGTTTTCTGTCTTCCTCTA-3', which contains a SmaI site shown in bold. GST/IIIH,8,10 was made with the following antisense primer that includes the 5' end of FNIII10 and the 3' end of FNIII8: 5'-GGTCCCTCGGAACAT**CAGAA**CTGTTTTCTGTCTTCCTCTAAGAGG-3'. An AlwNI site is shown in bold. GST/IIIH,10 was made using the antisense primer: 5'-**CCAGGTCCCTCGGAACATCAGAA**CTGTGCTGGTGGTGG-3', which runs from the PpuMI site in FNIII10 (bold) into the 3' end of FNIII1-H. GST/IIIH,9-10 was made by

first engineering a ClaI site (bold) into FNIII1H of pGEX-2T/III1H,8-10 with the sense primer: 5'-GGTATACGAGGGCCAGCTCATATCGATCCAGCAGTACGG-3'. The BstZ17I site in FNIII1 is underlined. FNIII1H was then coupled directly to FNIII9 by deleting FNIII8 from pGEX2T/III1H,8-10 with the sense primer: 5'-CATATCGATCCAGCAGTACGGCCACCAAGAAGTGACTCGCTTTGACTTCACCAC CACCAGC ACCAGCACAGGTCTTGATTCCCCAACTGG-3'. The ClaI site, shown in bold, was used to move this fragment into pGEX-2T/III1H,8-10.

The RGD chimera, GST/III1H,8^{RGD}, was produced using the following mutant sense primer: 5'-GTGAGTGTCTCCAGTGTCTACGGCCGTGGAGACTCGCCGGCAAGCAGCACACCT CTTAGAG GAAGACAGAAAACATAGGAATTC-3'. The insert from FNIII10 (bases 4487–4510 in pFH100; underlined), which encodes for the GRGDSPAS sequence, was inserted between base 3946 and base 3959 (Y¹⁴⁰² and S¹⁴⁰⁷) of FNIII8, leading to the addition of four amino acids (RGDS) to FNIII8. The EcoRI site is shown in bold. The FNIII10 insert contains an engineered NgoMIV site as a marker. The antisense primer for GST/III1H,8^{RGD} (5'-GGTATACGAGGGCCAGCTCATATCGATTTCAGCAGTACGGCC-3') contains a BstZ17I site shown in bold. GST/III8^{RGD} was engineered using pGEX-2T/III1H,8^{RGD} as a template. The sense primer used was: 5'-CGGATCCGCTGTTCTCCTCCCCTGACCTGCG-3', with the BamHI site shown in bold. The antisense primer for GST/III8^{RGD} (5'-CGAATTCCTATGTTTTCTGTCTTCC-3') contains an EcoRI site shown in bold. For all protein constructs, PCR-amplified DNA was cloned into pGEX-2T (Amersham Biosciences) and transfected into DH5 α bacteria [32]. DNA was sequenced to confirm the presence of the mutations. GST-tagged fibronectin constructs were isolated on glutathione-Sepharose (Amersham Biosciences) and dialyzed extensively against PBS [34]. Proteins were filter-sterilized and purity was assessed by SDS-polyacrylamide gel electrophoresis [32] (Fig. 1B). Purified proteins were stored in aliquots at -80°C .

2.3. Cell adhesion and proliferation assays

Tissue culture plates (48- or 96-well) were coated with recombinant fibronectin proteins or full-length ECM proteins diluted in PBS at the indicated concentrations for 90 min at 37°C . Unbound protein was removed and wells were washed with PBS. For cell proliferation assays, FN-null MEFs were seeded at 2.5×10^3 cells/cm² in Aim V/Cellgro and incubated for 4 days at 37°C , 8% CO₂. For cell adhesion assays, FN-null MEFs were seeded at 1.5×10^5 cells/cm² in Aim V/Cellgro and cells were allowed to adhere for 30 min at 37°C , 8% CO₂. After incubation, cells were washed with PBS and fixed with 1% paraformaldehyde. Cells were stained with 0.5% crystal violet, solubilized in 1% sodium dodecyl sulfate (SDS), and the absorbance at 590 nm was measured with a microplate spectrophotometer (Bio-Rad) [41].

2.4. Solid-phase enzyme linked immunosorbent assay

Tissue culture plates (96-well) were incubated with recombinant fibronectin proteins or plasma fibronectin diluted in PBS at the indicated concentrations for 90 min at 37°C . Wells were washed with PBS to remove unbound protein, then blocked with 1% BSA in PBS. Bound proteins were detected using an anti-FNIII10 monoclonal antibody (FN12-8, 1 $\mu\text{g}/\text{ml}$) followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibodies. Wells were washed with PBS and the assay was developed using 2,2-azino-bis(3-ethylbenzthiazolinesulfonic acid) and the absorbance at 405 nm was measured.

2.5. Cell migration assay

An in vitro wound repair assay was used to measure cell migration [21]. Tissue culture plates (35-mm) were coated with recombinant fibronectin constructs or full-length fibronectin at 200 nM in PBS for 90 min at 37°C. FN-null MEFs were seeded at 7.4×10^4 cells/cm² in Aim V/Cellgro. Cells were allowed to adhere and spread for either 4 or 16 h to establish a confluent monolayer. A thin section of the monolayer was carefully removed with a sterile, plastic microspatula. Cells were washed twice with Aim V/Cellgro. Five non-overlapping regions of the wound were viewed immediately after wounding with an Olympus inverted microscope using a 4x objective (time = 0 h). Phase contrast images of each region were obtained using a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were obtained of the five non-overlapping regions at various times after wounding. A numbered grid was marked on the bottom of the tissue culture plates prior to cell seeding to ensure that the same regions were being photographed at each time point. The average width of the initial wound was ~500 μ m. Wound area measurements were obtained using ImagePro-Plus software (Media Cybernetics, Silver Spring, MD). Cell migration was calculated as original cleared area (time = 0 h) – cleared area (time = 2, 4, 6, or 8 h).

2.6. Collagen gel contraction assay

Collagen gel contraction assays were performed as described previously [18]. Neutralized collagen gels were prepared by mixing collagen type I with 1x Dulbecco's modified Eagle's medium (DMEM; Life Technologies), 2X concentrated DMEM, and 0.1 NaOH on ice for final concentrations of 0.8 mg/ml collagen and 1X DMEM, pH 7.2. FN-null MEFs were added to the collagen mixture to a final concentration of 2×10^5 cells/ml. An equal volume of Aim V/Cellgro was added to aliquots of the collagen mixture for 'no cell' controls. Recombinant fibronectin proteins or plasma fibronectin were added to the collagen/cell mixtures at various concentrations. Aliquots (0.1 ml) of the collagen/cell mixture were added to wells of tissue culture plates (96-well) precoated with 2% BSA in PBS. Gels were allowed to polymerize for 1 h at 37°C, 8% CO₂. To form floating gels, DMEM (0.1 ml/well) was added and wells were scored with a pipette tip. Following a 20 h incubation at 37°C, 8% CO₂, gels were removed from their wells and weighed (Model AE260, Mettler, Toledo, OH). Volumetric collagen gel contraction was calculated as the decrease in gel weight relative to the control, no-cell gel weight.

2.7. Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM) and represent one of at least three experiments performed in either triplicate or quadruplicate. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-test, with GraphPad Prism Version 4 software (LaJolla, CA). Differences were considered significant for *p* values less than 0.05.

3. Results

3.1. Recombinant fibronectin fragments as adhesive substrates

We previously developed a recombinant fibronectin construct (GST/III1H,8-10) that mimics many of the cellular effects of ECM fibronectin, by directly coupling the cryptic, heparin-binding fragment of the first type III repeat of fibronectin to the integrin-binding domain [32]. Addition of soluble GST/III1H,8-10 to the culture media of adherent FN-null MEFs enhances cell spreading, growth, and contractility [32], increases the migration rate of small airway epithelial cells [21], and induces local arteriolar vasodilation in vivo [33]. To develop fibronectin matrix mimetics as functional ligands for adhesive biomaterials, studies were

conducted to characterize the proliferative response of FN-null MEFs cells to immobilized GST/III1H,8-10. The use of FN-null MEFs in the current study allows us to characterize cell behavior in response to the recombinant fibronectin proteins in the absence of endogenous full-length fibronectin and to identify specific effects of individual domains and amino acid sequences of fibronectin on cell function. Cells were seeded at low density onto tissue culture plates coated with various recombinant fibronectin fragments and cell number was determined after 4 days. The extent of cell proliferation on GST/III1H,8-10-coated substrates was compared to that observed in response to substrates coated with the following ligands: (i) an integrin-binding fragment lacking FNIII1H (GST/III8-10), (ii) a construct in which the carboxyl-terminal heparin-binding domain of fibronectin was coupled to FNIII8-10 (GST/III8-10,13), and (iii) plasma fibronectin. At 24 hours after seeding, cells adherent to the fusion protein substrates were well-spread and displayed morphologies similar to that observed with cells adherent to plasma fibronectin (Fig. 2A). Cells proliferated over the 4 day period on all substrates tested and formed a near-confluent monolayer on substrates coated with GST/III1H,8-10 (Fig. 2A). Relative cell number on Day 4 was quantified as a function of protein coating concentration. Cell number on surfaces pre-coated with either 200 nM or 400 nM GST/III1H,8-10 was significantly greater than that observed on surfaces pre-coated with either plasma fibronectin or the integrin-binding fragment, GST/III8-10 (Fig. 2B). Average cell numbers obtained from surfaces pre-coated with GST/III1H,8-10 at 200 nM were 1.32 ± 0.08 ($n = 5$) and 1.49 ± 0.05 ($n = 15$) -fold greater than those obtained on surfaces coated with the same molar concentration of plasma fibronectin or GST/III8-10, respectively. The increase in cell number observed on GST/III1H,8-10-coated surfaces required FNIII1H, as cell number on GST/III8-10,13-coated surfaces was similar to GST/III8-10-coated surfaces (Fig. 2B).

To compare ligand densities of the substrates, ELISAs were performed on tissue culture plates coated with increasing concentrations of protein. At protein coating concentrations less than 200 nM, differences in the relative amount of protein bound to the tissue culture plastic were observed, with GST/III8-10 and plasma fibronectin binding more efficiently than either GST/III1H,8-10 or GST/III8-10,13 (Fig. 3A). For all fusion proteins tested, ligand surface density reached saturation at a concentration of 200 nM. No differences in ligand density were observed among the various recombinant fusion proteins at coating concentrations of 200 nM and 400 nM (Fig. 3A). The ligand density of plates coated with plasma fibronectin at 400 nM was slightly greater than that observed with the recombinant proteins (Fig. 3A). Together, these data indicate that the increase in cell number observed on substrates coated with GST/III1H,8-10 (Fig. 2B) was not due to differences in the coating density of the proteins.

To quantify cell attachment to the various proteins, adhesion assays were performed 30 min after seeding cells at high density onto tissue culture plates pre-coated with increasing concentrations of protein. No differences in cell number were detected among the various groups at coating concentrations greater than 50 nM (Fig. 3B), indicating that the differences in cell number observed after 96 h were not due to differences in initial cell adhesion. As expected, cells did not adhere to wells coated with GST alone (Fig. 3B). Taken together, these data indicate that surfaces coated with GST/III1H,8-10 support cell adhesion and spreading and promote cell proliferation to a greater extent than surfaces coated with either plasma fibronectin or fragments with only integrin-binding activity.

3.2. Role of FNIII1H in proliferation

To further evaluate the role of FNIII1H in the proliferative response to GST/III1H,8-10, a fusion protein was produced in which the analogous C-terminal region of the ED-B module of fibronectin was substituted for FNIII1H (GST/IIIED_B-C,8-10). FN-null MEFs were seeded onto surfaces coated with 200 nM GST/III1H,8-10, GST/IIIED_B-C,8-10, GST/

III8-10, or GST/III1H and cell number was determined after 96 h. Similar to results shown in Fig. 2B, surfaces coated with GST/III1H,8-10 produced a significant increase in cell number versus surfaces coated with either GST/III8-10 or GST/IIIED_B-C,8-10 (Fig. 4A). No difference in initial cell adhesion to surfaces coated with GST/III1H,8-10, GST/III8-10, or GST/IIIED_B-C,8-10 were observed (Fig. 4B). Surfaces coated with GST/III1H alone did not support either cell adhesion (Fig. 4B) or proliferation (Fig. 4A).

We previously localized the growth-promoting site in soluble GST/III1H,8-10 to the heparin-binding sequence, Arg⁶¹³-Trp⁶¹⁴-Arg⁶¹⁵-Pro⁶¹⁶-Lys⁶¹⁷, in FNIII1 [19]. To further localize the growth-stimulating site in FNIII1H, a construct was produced in which only the charged amino acid sequences within the R⁶¹³WRPK sequence were mutated to non-charged amino acids (R⁶¹³T, R⁶¹⁵T, K⁶¹⁷A; GST/III1H,8-10ΔRRK). An additional control fusion protein was produced in which a separate, highly conserved sequence in FNIII1H, E⁶⁴⁷G⁶⁴⁸Q⁶⁴⁹, was mutated (E⁶⁴⁷D, Q⁶⁴⁹N; GST/III1H,8-10ΔEGQ). FN-null MEFs were seeded onto surfaces coated with 200 nM of the various fusion proteins and cell number was determined after 96 h. Cell number on surfaces coated with GST/III1H,8-10ΔRRK was less than that observed on surfaces coated with GST/III1H,8-10, and was similar to that observed on GST/III8-10,13-coated surfaces (Fig. 4C). Cell number on GST/III1H,8-10ΔEGQ was not different than that observed on GST/III1H,8-10 (Fig. 4C), providing additional evidence that the growth-promoting activity of FNIII1 is specific to Arg⁶¹³, Arg⁶¹⁵, Lys⁶¹⁷ and that differences in cell number were not due to non-specific structural changes in the mutated protein. No differences in initial cell adhesion were observed among the groups (Fig. 4D). Taken together, these results indicate that the Arg⁶¹³-Arg⁶¹⁵-Lys⁶¹⁷ sequence in FNIII1H mediates the enhanced growth response to GST/III1H,8-10, but that in the absence of FNIII8-10, FNIII1H is unable to support cell adhesion.

3.3. Role of integrin-binding modules in proliferation

FNIII8, FNIII9, and FNIII10 form the primary integrin-binding region of fibronectin, regulating cell adhesion and cell surface receptor specificity [11]. FNIII10 contains the integrin-binding, RGD sequence [42], whereas FNIII9 contains a sequence, termed the synergy site, which promotes binding to α5β1 integrins [43]. Thus far, our data indicate that the integrin-binding domain, FNIII8-10, is necessary for adhesion and growth in response to GST/III1H,8-10 (Fig. 4). To determine whether all three integrin-binding modules are necessary for the proliferative response to GST/III1H,8-10, a series of fusion proteins were produced in which one or two modules within the integrin-binding region were deleted. The modules deleted and the constructs produced were: (i) FNIII9 (GST/III1H,8,10), (ii) FNIII8 (GST/III1H,9-10), (iii) FNIII8 and FNIII9 (GST/III1H,10), and (iv) FNIII9 and FNIII10 (GST/III1H,8). FN-null MEFs were seeded onto surfaces coated with the various fusion proteins and cell number was determined on Day 4. Deleting FNIII9 from GST/III1H,8-10 did not lead to a difference in cell number compared to the original matrix mimetic (GST/III1H,8,10 versus GST/III1H,8-10; Fig. 5A). In contrast, deleting FNIII8 from GST/III1H,8-10 resulted in a reduction in cell number on Day 4 (GST/III1H,9-10 and GST/III1H,10 versus GST/III1H,8-10; Fig. 5A). Cell adhesion to surfaces coated with GST/III1H,8,10, GST/III1H,9-10 or GST/III1H,10 was similar to that observed on GST/III1H,8-10-coated surfaces (Fig. 5B), indicating that the differences in cell number observed on Day 4 were not due to differences in initial cell attachment. Cells did not adhere to the construct lacking FNIII10 (GST/III1H,8; Fig. 5B), indicating that FNIII8 cannot support cell adhesion in the absence of FNIII10. Taken together, these data indicate that GST/III1H,8,10 supports cell adhesion and promotes cell growth to a similar extent as the original construct, GST/III1H,8-10. In addition, FNIII8 and FNIII10, but not FNIII9, are required for the full proliferative response to the fibronectin matrix mimetics.

3.4. Development of RGD-chimeras

The cell attachment activity of fibronectin can be duplicated by peptides containing the highly conserved, integrin-binding Arg-Gly-Asp (RGD) sequence [42]. To further refine GST/III1H,8,10 as a fibronectin matrix mimetic, we engineered fusion proteins in which FNIII10 was deleted and instead, the adhesive RGDS sequence was inserted into FNIII8. To do so, we took advantage of the fact that the RGDS sequence in FNIII10 is located in a unique loop connecting β -strands F and G [44], and inserted the FNIII10 sequence, Arg-Gly-Asp-Ser-Pro-Ala-Ser (GRGDSPAS) into the analogous site in FNIII8. Two fusion proteins were produced: (i) GST/III1H,8^{RGD} and (ii) GST/III8^{RGD}. Cell adhesion assays were conducted to compare cell attachment activities of the RGD chimeras to the original construct, GST/III1H,8-10. Cell adhesion to surfaces coated with GST/III1H,8^{RGD} was similar to that observed on GST/III1H,8-10-coated surfaces at all protein coating concentrations (Fig. 6A). Cells did not adhere to GST/III1H lacking the RGD sequence (Fig. 6A). These data indicate that the adhesive activity of FNIII10 can be duplicated by inserting the RGDS-loop into FNIII8.

To assess the RGD-containing chimeras as growth-promoting adhesive substrates, FN-null MEFs were seeded onto wells coated with 200 nM of the various fusion proteins and cell number was determined after 96 h. Surfaces coated with GST/III1H,8^{RGD} supported a similar level of cell growth as did surfaces coated with the original mimetic, GST/III1H,8-10 (Fig. 6B), and was greater than that observed with cells adherent to surfaces coated with RGD peptides alone (Fig. 6B). Of note, after 4 days, cells were no longer adherent to wells coated with GRGDSP peptides (Fig. 6B).

Dose-response studies indicate that the growth-promoting activity of GST/III1H,8^{RGD} was nearly identical to that of GST/III1H,8-10 at various protein coating concentrations (Fig. 7A). Removal of FNIII1H from GST/III1H,8^{RGD} (GST/III8^{RGD}) resulted in significantly lower cell number on Day 4 (Fig. 7A), providing further evidence that the heparin-binding fragment of FNIII1 is required for the enhanced cell growth response. No differences in initial cell adhesion were observed on surfaces coated with GST/III8^{RGD}, GST/III1H,8-10 and GST/III1H,8^{RGD} (Fig. 7B). Furthermore, a comparison of the proliferative activity of the fibronectin matrix mimetics to that of other intact ECM ligands revealed that surfaces coated with GST/III1H,8^{RGD} promoted cell growth to a similar extent as vitronectin-coated surfaces, and to a greater extent than that observed for fibrinogen- or gelatin-coated surfaces (Fig. 8). Taken together, these data indicate that by inserting the RGD loop into FNIII8, both FNIII9 and FNIII10 can be removed from the initial matrix mimetic without loss of cell adhesion and growth-promoting activity.

3.5. Matrix mimetics and cell migration

We next assessed the ability of the recombinant fibronectin constructs to support cell migration using an in vitro assay of wound repair. FN-null MEFs were seeded at high densities onto surfaces coated with 200 nM of the various fusion proteins and allowed to form confluent monolayers. A region of the monolayer was then removed and migration of cells into the denuded space was quantified. Surfaces coated with either the original matrix mimetic (GST/III1H,8-10) or the integrin-binding fragment of fibronectin (GST/III8-10) promoted cell migration into the denuded area to a greater extent than surfaces coated with intact, plasma fibronectin (Fig. 9A). Substituting the heparin-binding module, FNIII13, for FNIII1H decreased the rate of cell migration (GST/III1H,8-10 versus GST/III8-10,13; Fig. 9A), resulting in a rate of migration similar to that of plasma fibronectin-coated surfaces (GST/III8-10,13 versus FN; Fig. 9A).

Similar to results obtained in the growth assays (Fig. 5A), removal of FNIII9 from the original matrix mimetic did not reduce the rate of cell migration, which was similar to that of the integrin-binding fragment, GST/III8-10 (GST/III1H,8,10 versus GST/III8-10, Fig. 9B). The rate of cell migration on surfaces coated with the growth-promoting, chimeric construct, GST/III1H,8^{RGD}, was similar to that of plasma fibronectin (Fig. 9B). Removing FNIII8 from GST/III1H,8,10 decreased the rate of cell migration (GST/III1H,10 versus GST/III1H,8,10; Fig. 9C), indicating a possible role for FNIII8 in promoting higher rates of cell migration. These data indicate that as adhesive substrates, the recombinant fibronectin matrix mimetics equal or exceed the ability of plasma fibronectin to promote cell migration.

3.6. Matrix mimetics and collagen reorganization

Collagen gel contraction assays are utilized as in vitro models of collagen matrix reorganization during wound repair [45]. Our previous studies showed that fibronectin matrix polymerization stimulates collagen gel contraction [18] and that this activity is mimicked by the original matrix mimetic, GST/III1H,8-10 [32]. To assess the ability of the new fibronectin matrix mimetics to stimulate collagen gel contraction, FN-null MEFs were embedded in collagen gels and the extent of contraction after 20 h was determined. As shown previously [32], addition of plasma fibronectin or the original matrix mimetic (GST/III1H,8-10) to FN-null MEFs embedded in floating collagen gels stimulated collagen gel contraction (Fig. 10). Addition of either GST/III1H,8,10 or GST/III1H,8^{RGD} to cell-embedded collagen gels stimulated collagen gel contraction to a similar extent as that observed in response to the original matrix mimetic, GST/III1H,8-10 (Fig. 10). The extent of collagen gel contraction in response to GST/III1H,8,10 and GST/III1H,8^{RGD} was significantly greater than that observed in response to the control protein, GST (Fig. 10).

4. Discussion

We have developed several recombinant fibronectin proteins that couple the “open” conformation of FNIII1 with integrin-binding sequences in order to mimic the effects of the ECM form of fibronectin on cell growth and migration. We compared the bioactivity of the fibronectin matrix mimetics to full-length, plasma fibronectin and to integrin-binding fragments and peptides. Our studies show that a chimeric fibronectin fragment composed of FNIII1H and FNIII8, with the RGDS loop inserted into FNIII8, supports cell adhesion and migration, stimulates collagen gel reorganization, and displays enhanced proliferative activity over integrin-binding fibronectin fragments and other full-length ECM proteins. We localized the growth-promoting activity in FNIII1H to amino acids Arg⁶¹³, Arg⁶¹⁵, and Lys⁶¹⁷, and present new evidence that FNIII8 has cell growth- and migration-promoting properties.

Surfaces presenting the RGD-loop in FNIII8 exhibited identical adhesive activities compared to the original matrix mimetic (GST/III1H,8-10; Fig. 6A), which was comparable to that observed with integrin-binding fragments (Fig. 3B). Deleting FNIII9 from the original matrix mimetic did not decrease the proliferation response of cells (Fig. 5A) and was not required for cell migration (Fig. 9B), indicating that binding of the PHSRN sequence in FNIII9 to $\alpha 5\beta 1$ integrins [43,46] does not contribute to the observed activities. Surfaces coated with the chimeric RGD-construct produced higher levels of cell growth over surfaces coated with linear GRGDSP peptides (Fig. 6B), suggesting that the structural framework of the FNIII module provides support for the RGD loop to allow for higher affinity interactions with integrin receptors [47]. The growth-promoting activity of the chimeric fibronectin matrix mimetic, GST/III1H,8^{RGD}, was comparable to that of the ECM protein, vitronectin, and was greater than that observed for other full-length ECM substrates including fibrinogen and gelatin (Fig. 8). Thus, tethering or incorporating chimeric

fibronectin matrix mimetics into biomaterials could provide robust proliferative activity to tissue-engineered scaffolds and medical devices.

The bioactivities of the fibronectin fragments tested were not identical. Surfaces coated with either GST/III1H,8-10 or GST/III1H,8,10 induced high-growth and high-migration responses; GST/III1H,8^{RGD}-coated surfaces provided high-growth and moderate-migration activities; GST/III8-10 induced low-growth but high-migration activities. Hence, engineering interfaces with different fibronectin matrix mimetics and fragments may represent a strategy to spatially pattern different cellular responses. In contrast to results obtained in the growth studies, a large decrease in cell migration rate occurred when FNIII10 was removed and replaced by the RGD loop (compare Fig. 6B with Fig. 9B), suggesting that an auxiliary site in FNIII10 [47] may contribute to enhanced cell migration, but not to enhanced cell growth. On the other hand, deletion of FNIII8 from GST/III1H,8-10 reduced both growth (Fig. 5A) and migration (Fig. 9C). Previous studies have identified a region within the amino-terminus of FNIII8 that is involved in cell adhesion [48]. Taken together, the results suggest that at least two additional sites within the cell-binding domain of fibronectin, one in FNIII10 and one in FNIII8, contribute to integrin-mediated cellular responses.

Proteolysis of the first type III repeat of fibronectin at residue Ile⁵⁹⁷ removes both the A and B β strands and results in a carboxyl-terminal fragment that binds to heparin [49]. Our previous studies localized the growth-promoting activity of GST/III1H,8-10 to the heparin-binding sequence, K⁶¹¹R⁶¹⁵WR_K⁶¹⁹. Here, we demonstrate that FNIII1H is also required for the enhanced growth response to surfaces coated with GST/III1H,8-10 and further, we pinpoint the growth-enhancing sequence in FNIII1 to R⁶¹³, R⁶¹⁵, and K⁶¹⁷. The cell surface receptor that binds to FNIII1H is currently unknown. Our previous work suggests that FNIII1 interacts with cells via heparan sulfate proteoglycans [32]. Aortic smooth muscle cells adhere to surfaces coated with micromolar concentrations of a similar FNIII1 fragment, III1-C, via a heparin-dependent mechanism that involves $\alpha 5\beta 1$ integrins [50]. Together, these studies suggest that FNIII1H may bind either directly or indirectly to $\alpha 5\beta 1$ integrins via heparan sulfate proteoglycans to elicit cellular responses. In support of this, we have shown that FNIII1H must be directly coupled to FNIII8-10 to stimulate cell growth, as individual fragments added simultaneously to cells do not have growth-promoting effects [32]. Similarly, the proliferative response to surfaces coated with a mixture of FNIII1H and FNIII8-10 fragments was not different from that observed with GST/III8-10-coated surfaces (data not shown).

Fibronectin type III repeats are found in ~2% of all human proteins and show a high degree of structural similarity [51]. 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 that overlap to form a hydrophobic core [3,4], imparting a high degree of stability to the protein's structure [52]. The six loops formed at the poles between β strands (termed AB, BC, etc.) are highly variable and can withstand extensive modification without loss of stability [53,54]. This inherent stability likely facilitated the insertion of the RGDS sequence into the FG loop of FNIII8. The stability of fibronectin type III repeats has also allowed for the development of FNIII-domains as scaffolds for the display of binding elements [55], while an engineered FNIII scaffold is currently in Phase II trials as an anti-angiogenesis agent [51]. Both FNIII1 and FNIII8 lack cysteine residues and post-translation modifications [2], which facilitates production in bacteria. For our studies, GST/III1H,8^{RGD} was generated with a cleavable GST-tag that allows for rapid purification via glutathione chromatography; we obtained yields of ~ 4 mg of purified GST/III1H,8^{RGD} per liter of bacterial culture. The molecular mass of the chimeric matrix mimetic, excluding the GST-tag, is ~19 kDa. In contrast, the molecular mass of a single, soluble fibronectin molecule is ~500 kDa. The small size of the

fibronectin matrix mimetic decreases potential immunogenicity, provides increased structure and stability over peptides, and reduces the number of binding sites for other receptors.

5. Conclusion

We have developed a small, chimeric fibronectin matrix mimetic by inserting the integrin-binding RGDS sequence into the FG loop of FNIII8 and then coupling FNIII8^{RGD} to the heparin-binding fragment of FNIII1. Surfaces passively coated with GST/III1H₈^{RGD} support cell adhesion and migration, induce collagen matrix contraction, and display enhanced proliferative activity over either integrin-binding fibronectin fragments or full-length fibronectin. These results provide proof-of-principle for the development of chimeric fibronectin type III repeats as novel adhesive ligands for synthetic biomaterials that support cell migration and stimulate high rates of cell proliferation.

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The abbreviations used are

ECM	extracellular matrix
FNIII	fibronectin type III repeat
FN-null MEFs	fibronectin-null mouse embryonic fibroblasts
GST	glutathione-S-transferase

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Figure 1. Fibronectin fusion proteins

(A) Schematic representation of a fibronectin subunit and recombinant fibronectin fusion proteins. Relevant type III modules are numbered. FNIII1H is the heparin-binding, C-terminal fragment of FNIII1; ED_B-C is the corresponding C-terminal fragment of Extra domain-B. In (B), aliquots (5 μ g) of recombinant proteins were electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Numbered lanes correspond to fusion protein identification numbers in (A). Molecular mass markers are shown on left.

Figure 2. GST/III1H,8-10 exhibits greater growth-promoting activity than fibronectin
 FN-null MEFs were seeded on tissue culture plates (2.5×10^3 cells/cm²) precoated with either (A) 200 nM or (B) increasing concentrations of the various proteins and incubated for 4 days (96 h). (A) Phase contrast images were obtained at 24 h and 96 h. Bar = 100 μ m. (B) Cell number was quantified at 96 h as described in “Methods”. Data are presented as mean absorbance of quadruplicate wells $\pm$ SEM and represent 1 of 3 experiments. *Significantly greater than FN, $p < 0.05$ (ANOVA).

Figure 3. Analysis of protein surface adsorption and cell adhesion

Tissue culture plates were coated with increasing concentrations of proteins. (A) The relative amount of protein bound to wells was determined by ELISA. (B) FN-null MEFs were seeded onto protein-coated wells (1.5×10^5 cells/cm²) and allowed to attach for 30 min. The number of adherent cells was determined as described in “Methods”. Data are presented as mean absorbance of triplicate wells $\pm$ SEM and represent 1 of 3 experiments.

*Significantly different from GST/III1H,8-10, $p < 0.05$ (ANOVA).

Figure 4. FNIII1H is essential for the enhanced growth response

FN-null MEFs were seeded (A and C, 2.5×10^3 cells/cm²; B and D, 1.5×10^5 cells/cm²) on tissue culture plates coated with 200 nM recombinant fibronectin constructs. (A and C) Relative cell number was determined after 4 days. (B and D) Cells were allowed to adhere for 30 min and attached cell number was determined. Data are expressed as mean absorbance of quadruplicate wells $\pm$ SEM and represent 1 of 3 experiments. *Significantly different from GST/III1H,8-10, $p < 0.05$ (ANOVA).

Figure 5. FNIII8 and FNIII10 are required for cell growth responses

FN-null MEFs were seeded (A, 2.5×10^3 cells/cm²; B, 1.5×10^5 cells/cm²) onto tissue culture plates precoated with 200 nM of the recombinant fibronectin constructs. (A) Relative cell number was determined after 4 days. (B) Cells were allowed to adhere for 30 min and the number of attached cells was determined. Data are expressed as mean absorbance of quadruplicate wells $\pm$ SEM and represent 1 of 3 experiments. *Significantly different from GST/IIIH,8-10, $p < 0.05$ (ANOVA).

Figure 6. Chimeric matrix mimetics support cell adhesion and growth

FN-null MEFs were seeded (A, 1.5×10^5 cells/cm²; B, 2.5×10^3 cells/cm²) on tissue culture plates coated with protein at (A) increasing concentrations or (B) 200 nM. GRGDSP peptides were coated at 20 μ g/ml (34 μ M). (A) Cells were allowed to adhere for 30 min and attached cell number was determined. (B) Relative cell number was determined after 4 days. Data are expressed as mean absorbance of (A) triplicate wells or (B) quadruplicate wells $\pm$ SEM and represent 1 of 3 experiments. *Significantly different from GST/III1H,8-10, $p < 0.05$ (ANOVA).

Figure 7. Role of FNIII1H in the proliferative response to GST/III1H,8^{RGD}

FN-null MEFs were seeded (A, 2.5×10^3 cells/cm²; B, 1.5×10^5 cells/cm²) on tissue culture plates coated with recombinant fibronectin constructs at (A) increasing concentrations or (B) 400 nM. (A) Relative cell number was determined after 4 days. (B) Cells were allowed to adhere for 30 min and attached cell number was determined. Data are expressed as mean absorbance of quadruplicate wells $\pm$ SEM and represent 1 of 3 experiments. *Significantly different from GST/III1H,8-10, $p < 0.05$ (ANOVA).

Figure 8. Proliferative response to fibronectin matrix mimetics versus other ECM proteins
FN-null MEFs were seeded (2.5×10^3 cells/cm²) on tissue culture plates coated with various proteins (200 nM). Relative cell number was determined after 4 days. Data are expressed as mean absorbance of quadruplicate wells $\pm$ SEM and represent 1 of 3 experiments.

*Significantly different from GST/III1H,8-10, $p < 0.05$ (ANOVA).

Figure 9. Fibronectin matrix mimetics support cell migration

FN-null MEFs were seeded (7.4×10^4 cells/cm²) on tissue culture plates coated with 200 nM plasma fibronectin (FN) or various recombinant fibronectin constructs. Cells were allowed to spread to confluence for 16 h (A,B) or 4 h (C). Cell monolayers were wounded and images of five non-overlapping regions were obtained at 0, 2, 4, 6, and 8 hours post-wounding. Data are presented as mean area migrated at each time point $\pm$ SEM and represent 1 of 3 experiments. (A,B) *Significantly different from FN, $p < 0.05$. (C) *Significantly different from GST/III1H,8,10, $p < 0.05$ (ANOVA).

Figure 10. Fibronectin matrix mimetics stimulate collagen gel contraction

FN-null MEFs were imbedded (2×10^5 cells/ml) in neutralized collagen gels in the presence of recombinant fibronectin constructs, the control protein, GST (400 nM) or plasma fibronectin (20 μ g/ml; 40 nM). Collagen gel contraction was determined after 20 h as described in “Methods”. Data are presented as average percent contraction versus ‘no-cell’ controls from 3 experiments performed in quadruplicate $\pm$ SEM. *Significantly different from GST, $p < 0.05$. #Significantly different from GST/III1H,8-10, $p < 0.05$ (ANOVA).