Fibronectin polymerization stimulates cell growth by RGD-dependent and -independent mechanisms

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SUMMARY

Many aspects of cell behavior are regulated by cell-extracellular matrix interactions, including cell migration and cell growth. We previously showed that the addition of soluble fibronectin to collagen-adherent fibronectin-null cells enhances cell growth. This growth-promoting effect of fibronectin depended upon the deposition of fibronectin into the extracellular matrix; occupancy and clustering of fibronectin-binding integrins was not sufficient to trigger enhanced cell growth. To determine whether the binding of integrins to fibronectin’s RGD site is required for fibronectin-enhanced cell growth, the ability of fibronectin lacking the integrin-binding RGD site (FN\textsubscript{D}RGD) to promote cell growth was tested. FN\textsubscript{D}RGD promoted cell growth when used as an adhesive substrate or when added in solution to collagen-adherent fibronectin-null cells. Addition of FN\textsubscript{D}RGD to collagen-adherent fibronectin-null cells resulted in a 1.6-1.8× increase in cell growth in comparison with cells grown in the absence of fibronectin. The growth-promoting effects of FN\textsubscript{D}RGD and wild-type fibronectin were blocked by inhibitors of fibronectin polymerization, including the anti-fibronectin antibody, L8. In addition, FN\textsubscript{D}RGD-induced cell growth was completely inhibited by the addition of heparin, and was partially blocked by either heparitinase-treatment or by addition of recombinant fibronectin heparin-binding domain. Heparin and heparitinase-treatment also partially blocked the growth-promoting effects of wild-type fibronectin, as well as the deposition of wild-type fibronectin into the extracellular matrix. These data suggest that cell surface heparan-sulfate proteoglycans contribute to the growth-promoting effects of FN\textsubscript{D}RGD and wild-type fibronectin. Addition of heparin, treatment with heparitinase, or incubation with monoclonal antibody L8 all inhibited the formation of short linear FN\textsubscript{D}RGD fibrils on the cell surface. Inhibitory β1 integrin antibodies had no effect on FN\textsubscript{D}RGD fibril formation, FN\textsubscript{D}RGD-induced cell growth, or cell adhesion on FN\textsubscript{D}RGD-coated substrates. These data suggest that fibronectin fibril formation can promote cell growth by a novel mechanism that is independent of RGD-integrin binding, and that involves cell surface proteoglycans.

Key words: Fibronectin, Extracellular matrix, Cell growth

INTRODUCTION

The interaction of cells with the extracellular matrix is important in regulating cell growth, differentiation, migration and survival (Hynes, 1990). Integrins are a major class of transmembrane receptors that mediate cell adhesion to fibronectin (Hynes, 1992; Pytela et al., 1985) as well as to other extracellular matrix proteins (Hynes, 1992; Ruoslahti, 1988; Yamada, 1989). Binding of extracellular matrix proteins to integrins leads to the generation of intracellular signals, many of which are similar to intracellular signals generated by growth factor stimulation (Assoian, 1997; Juliano, 1996; Schwartz, 1997). Integrin-extracellular matrix interactions are also important for cell survival, as disruption of integrin-mediated attachment to the extracellular matrix induces apoptosis in epithelial and endothelial cells (Frisch and Francis, 1994; Meredith et al., 1993; Ruoslahti and Reed, 1994). Integrins and integrin-dependent signalling events also regulate the deposition of fibronectin into the extracellular matrix (Giancotti and Ruoslahti, 1990; Wu et al., 1993; Wu et al., 1998).

Fibronectin also binds to cell surface proteoglycans, including syndecans (Carey, 1997; Saunders and Bernfield, 1988) and CD44 (Naor et al., 1997). Binding of fibronectin to syndecan involves the interaction of fibronectin’s carboxy-terminal heparin-binding domain with heparan sulfate glycosaminoglycan chains on syndecan (Saunders and Bernfield, 1988). Much data have suggested that the heparin-binding domain of fibronectin contributes to cell spreading and stress fiber formation in a variety of cell types (Bloom et al., 1999; Izzard et al., 1986; Woods et al., 1986). The addition of heparin-binding fragments of fibronectin in solution or
adsorbed to the substrate can restore stress fiber formation in cells adherent to the cell-binding fragment of fibronectin (Bloom et al., 1999; Woods et al., 1986). In some systems, the requirement for the heparin-binding domain of fibronectin can be bypassed by treatment of cells with activators of protein kinase C (PKC) (Woods and Couchman, 1992) or Rho (Saoncella et al., 1999). These data suggest that cell surface proteoglycans cooperate with integrins in mediating maximal cell adhesion and spreading through PKC- and/or Rho-dependent mechanisms.

Our recent data demonstrate that deposition of fibronectin into the extracellular matrix stimulates adhesion-dependent cell growth, and that ligation and clustering of fibronectin-binding integrins are not sufficient to promote enhanced cell growth (Sottile et al., 1998). These data are consistent with other studies showing that inhibition of fibronectin deposition, or disruption of a preformed fibronectin matrix, decreases cell proliferation (Bourdoulous et al., 1998; Clark et al., 1997; Mercurius and Morla, 1998). Others have shown that cell growth in response to fibronectin can also be regulated by the three-dimensional organization of fibronectin fibrils within the matrix (Sechler and Schwarzbauer, 1998). Taken together, these data indicate that extracellular matrix fibronectin plays an important role in regulating adhesion-dependent cell growth.

We previously demonstrated that integrin ligation is not sufficient to promote fibronectin-dependent cell growth (Sottile et al., 1998). To determine whether RGD-integrin binding is required for fibronectin-induced cell growth, we examined the effects of fibronectin lacking the RGD sequence (FNαRGD) on the growth of fibronectin-null cells. Our data demonstrate that cells adhere, spread and grow on substrates coated with FNαRGD, and that addition of FNαRGD to substrate-adherent fibronectin-null cells results in a 1.6-1.8× increase in cell growth in comparison with cells grown in the absence of fibronectin. FNαRGD stimulated cell growth to approximately 50-60% of the levels induced by wild-type fibronectin, indicating that both RGD-dependent and -independent mechanisms are involved in the cell growth response to fibronectin. The ability of FNαRGD to enhance growth of collagen-adherent cells was completely inhibited by the addition of heparin, and was partially blocked by heparitinase treatment, or by addition of recombinant fibronectin heparin-binding domain. These treatments also blocked the formation of short linear FNαRGD fibrils on the cell surface. Interestingly, our data also demonstrate that α5 and β3 integrin antibodies inhibit cell adhesion to FNαRGD. However, α5 and β3 integrin antibodies did not inhibit either FNαRGD fibril formation or the ability of FNαRGD to induce growth of collagen-adherent cells. These data indicate that fibronectin fibril formation and fibronectin-induced cell growth can occur in the absence of RGD, and suggest a role for proteoglycans in the cell growth response to fibronectin polymerization.

MATERIALS AND METHODS

Immunological reagents

Monoclonal antibody L8, which recognizes an epitope in fibronectin’s 1-9 and III-1 modules, was a generous gift of Dr Michael Chernousov (Penn State College of Medicine, Danville, PA, USA) (Chernousov et al., 1991). Rabbit polyclonal antibody to α5 integrin cytoplasmic domain (a gift of Dr Susan LaFlamme, Albany Medical College, Albany, NY, USA) and polyclonal anti-fibronectin antibody were previously described (LaFlamme et al., 1992; Sottile and Mosher, 1993). Polyclonal anti-vinculin antibody was from Sigma (St Louis, MO, USA). FITC-phalloidin was purchased from Molecular Probes (Eugene, OR, USA). Monoclonal anti-integrin antibodies to α5, αv, β1 and β3 subunits, and control hamster IgG and IgM were purchased from Pharmingen (San Diego, CA, USA).

Proteins

Human fibronectin was purified from Cohn’s fractions I and 2 (a generous gift from Dr Ken Ingham, American Red Cross, Bethesda, MD, USA) as previously described (Miekka et al., 1982). Rat plasma fibronectin was purified on columns of gelatin-Sepharose. Full-length recombinant rat fibronectin, and fibronectin lacking the RGD site (FNαRGD) were expressed in insect cells and purified from insect cell conditioned medium as described (Hocking et al., 2000). Production and purification of recombinant rat 70 kDa and 40 kDa fibronectin fragments have been previously described (Sottile and Mosher, 1997). Recombinant III12-13 was produced in bacteria and purified as described (Hocking et al., 1999).

Cell culture

Mouse embryo cells were derived from fibronectin-null embryos and adapted to grow under serum-free conditions in defined medium (a 1:1 mixture of Cellgro (Mediatech, Herndon, VA, USA) and Aim V (Life Technologies, Gaithersburg, MD, USA) as described (Sottile et al., 1998). These media do not require serum supplementation. Thus, the cells are cultured under conditions where no exogenous source of fibronectin or other extracellular matrix proteins is present. The insect cell line IPLB-SF-21, adapted to grow in the serum-free medium SF900-II, was obtained from Life Technologies (Gaithersburg, MD, USA). SF21 cells do not produce any detectable endogenous fibronectin. Thus, recombinant proteins produced by these cells do not contain any contaminating fibronectin.

Immunofluorescence

Fibronectin-null cells were plated onto 18 mm glass coverslips precoated with vitronectin (5 µg/ml), recombinant fibronectin (10 µg/ml) or FNαRGD (10 µg/ml). Cells were seeded in defined medium, and incubated at 37°C for various lengths of time. Defined medium contains 1.5 mg/ml albumin, which would effectively block any remaining protein binding sites on the dishes. After allowing cells to attach and spread for 6 hours, some wells were supplemented with fibronectin, FNαRGD or heparin. Cells were then fixed with paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with the indicated primary antibodies for 60 minutes. After washing, cells were incubated with fluorescein isothiocyanate (FITC-) or Texas Red-conjugated antibodies for 30 minutes. Following washing, cells were mounted in glycerol gel (Sigma) and examined using an Olympus BX60 microscope equipped with epifluorescence.

Cycloheximide treatment

Fibronectin-null cells were preincubated for 2 hours with 20 µg/ml cycloheximide (Sigma). Cells were then trypsinized, washed twice with medium containing soybean trypsin inhibitor (Sigma) and cycloheximide, then replated onto vitronectin-coated 18mm glass coverslips in defined medium in the presence of cycloheximide (20 µg/ml). Control cells were cultured in the absence of cycloheximide. Cells were allowed to attach and spread for 1-3 hours before being processed for immunofluorescence.

Cell adhesion assays

Tissue culture dishes (96-well) were coated with wild-type fibronectin or FNαRGD at 10 µg/ml in PBS at 4°C overnight. Collagen was coated onto dishes in 0.02 N acetic acid at 50 µg/ml at 4°C overnight.
Wells were washed with phosphate-buffered saline (PBS), then blocked with 1% bovine serum albumin (BSA) in PBS for 60 minutes at 37°C. Cells were preincubated with anti-integrin or control antibodies at 25-50 μg/ml, or with various fibronectin fragments for 30 minutes at room temperature prior to plating at 5×10^5 cell/ml in 0.1 ml of defined medium. The cells were allowed to attach for 30-60 minutes at 37°C. Cells were washed with PBS, then fixed with 1% paraformaldehyde for 30 minutes at room temperature. Cells were stained with 0.5% Crystal Violet, then air dried. The dye was solubilized with 1% sodium dodecyl sulfate, and the absorbance at 592 nm was determined using a Wallac Victor2 (Gaithersburg, MD, USA) plate reader. Background absorbance of protein-coated wells in the absence of cells was subtracted from each data point.

Cell growth assays on fibronectin- and FNΔRGD-coated dishes

Tissue culture dishes (24 well; Corning, Cambridge, MA, USA) were coated with recombinant fibronectin or recombinant FNΔRGD in PBS at 2.5-20 μg/ml at 37°C overnight. Wells were washed with PBS before seeding cells at 0.5×10^5 cell/cm^2 in 1 ml of defined medium. The cells were allowed to grow for various lengths of time at 37°C. Cells were washed with PBS, then fixed with 1% paraformaldehyde for 30 minutes at room temperature. Cells were stained with 0.5% Crystal Violet, and the absorbance determined on a spectrophotometer as described (Hocking et al., 1998; Sottile et al., 1998).

Cell growth assays on collagen-coated dishes

Collagen was coated onto dishes in 0.02 N acetic acid at 50 μg/ml at 4°C overnight. Fibronectin-null cells cultured on collagen-coated dishes have a doubling time of approximately 20 hours (Sottile et al., 1998). Collagen-coated wells were washed with PBS before seeding cells at 0.5×10^5 cell/cm^2 in 1 ml of defined medium. 4-6 hours after seeding, plasma fibronectin, recombinant wild-type fibronectin or recombinant FNΔRGD (20 nM) were added to some of the wells. For inhibition experiments, heparin, recombinant fibronectin fragments, purified L8 IgG or integrin antibodies were added at the time of addition of soluble fibronectin. Control wells received PBS, nonimmune mouse IgG (Cappel), hamster IgG or IgM (Phar côngen) or recombinant 70 kDa amino-terminal or 40 kDa gelatin-binding fragments of fibronectin. Cells were allowed to grow for the indicated times, then assayed for cell number using Crystal Violet, as described above.

Heparitinase and chondroitinase treatment

Fibronectin-null cells were seeded in defined medium onto collagen-coated dishes. Following a 4 hour incubation, wells were either supplemented with 0.005 U/ml heparitinase or 0.6 U/ml chondroitinase ABC (Seikagaku America, Falmouth, MA, USA) (Chen et al., 1996a; Minden et al., 1995; Shishido et al., 1995). Following a 2 hour incubation, some wells were supplemented with 20 nM wild-type (WT) fibronectin or 20 nM FNΔRGD. Fresh heparitinase or chondroitinase were added daily. Cells were allowed to grow for 3 days, then processed for immunofluorescence microscopy or assayed for cell number using Crystal Violet, as described above.

RESULTS

Cell spreading and focal contact formation on FNΔRGD

Identifying the role of fibronectin and fibronectin polymerization in regulating adhesion-dependent cell growth has been complicated by the presence of fibronectin in the serum used to culture cells, and by the ability of most adherent cells to constitutively synthesize and deposit fibronectin into the extracellular matrix. To circumvent these problems, we established fibronectin-null cell lines that do not make fibronectin, but are capable of polymerizing exogenously added fibronectin (Sottile et al., 1998).

In many cell types, the α5β1 integrin plays a major role in mediating cell attachment to fibronectin. We previously

Fig. 1. Cell spreading on wild-type and ΔRGD fibronectin. Tissue culture dishes were precoated with 10 μg/ml of wild-type (WT) or ΔRGD fibronectin. Fibronectin-null cells were seeded onto coated wells in defined medium. 1 hour (A) or 3 hours (B) after seeding, the cells were fixed with 2.5% paraformaldehyde, permeabilized with 0.5% Triton X-100, then incubated with FITC-phalloidin (to detect actin) and a mouse monoclonal antibody to vinculin. After washing, cells were incubated with Texas Red-anti mouse antibody to detect vinculin. Actin (Panels 1,3,5,7) and vinculin (Panels 2,4,6,8) staining was detected using an Olympus microscope equipped with epifluorescence. Photographs were taken with a Spot digital camera. Bar, 10 μm.
showed that fibronectin-null cells have α5- and αv-containing integrins on their cell surface, but no detectable cell surface α4 integrins (Sottile et al., 1998). The major binding site in fibronectin for α5β1 is the Arg-Gly-Asp (RGD) sequence that is contained within the III-10 module (Pierschbacher and Ruoslahti, 1984a; Pierschbacher and Ruoslahti, 1984b). Cell adhesion to fibronectin can be disrupted by addition of RGD peptides, demonstrating the importance of the RGD sequence in mediating cell adhesive events (D’Souza et al., 1991; Pierschbacher and Ruoslahti, 1984a). To determine whether regions of fibronectin other than the RGD sequence are able to promote cell attachment and growth, recombinant fibronectin that lacks the RGD sequence (FNΔRGD) was coated onto tissue culture dishes, and the ability of fibronectin-null cells to attach, spread and grow was monitored. As shown in Fig. 1, cells attached, spread and formed vinculin-containing focal contacts (top panels) on FNΔRGD-coated substrates. Cell spreading and stress fiber formation on FNΔRGD was delayed in comparison with spreading on wild-type fibronectin (compare Fig. 1A,B). However, by 3 hours, cells seeded on FNΔRGD were well spread and contained prominent stress fibers (Fig. 1, Panel 5).

Others have shown that the presence of α5β1 in focal contacts depends upon ligand occupancy (LaFlamme et al., 1992). The α5β1 integrin is not present in focal contacts of fibroblasts (LaFlamme et al., 1992) or fibronectin-null cells (Sottile et al., 1998) adherent to surfaces coated with extracellular matrix proteins other than fibronectin, but redistributes to focal contacts following addition of RGD-containing fibronectin fragments or peptides (LaFlamme et al., 1992; Sottile et al., 1998). Although RGD is the major binding site in fibronectin for α5β1 (Pierschbacher and Ruoslahti, 1984a; Pierschbacher and Ruoslahti, 1984b), α5β1 has also been shown to interact with the amino-terminal 70 kDa portion of fibronectin (Hocking et al., 1998). Therefore, we asked whether adhesion to FNΔRGD resulted in the clustering of α5β1 in focal contacts in an RGD-independent manner. As shown in Fig. 2, α5-integrin was not detected in focal contacts of cells seeded on FNΔRGD (Fig. 2F,H). As expected, cells adherent to wild-type fibronectin contained α5 integrin in their focal contacts (Fig. 2B,D).

Since cell spreading was delayed on FNΔRGD in comparison with spreading on wild-type fibronectin (Fig. 1), we next tested whether cell spreading on FNΔRGD was mediated by production of cell-derived adhesive molecules other than fibronectin. Fibronectin-null cells cultured in the presence of the protein synthesis inhibitor, cycloheximide, spread (Fig. 2C,D,G,H) and formed vinculin-containing focal contacts (Fig. 2C,G) when seeded onto FNΔRGD (Fig. 2G,H) or wild-type (Fig. 2C,D) fibronectin. Cells did not spread on uncoated tissue culture wells in the absence (Fig. 2I) or presence (not shown) of cycloheximide. Taken together, these data indicate that cell spreading on FNΔRGD does not require the production of cell-derived adhesive factors, and that fibronectin sequences other than the integrin-binding RGD site are able to mediate cell spreading and focal contact formation.

Cell adhesion to FNΔRGD is αv-integrin dependent

The α5β1 integrin has been shown to bind to the 70 kDa amino terminal region of fibronectin (Hocking et al., 1998).

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Fig. 2. Ability of FNΔRGD to promote cell spreading and focal contact formation. Fibronectin-null cells were seeded in defined medium onto uncoated coverslips (I,J) or onto coverslips coated with wild-type fibronectin (WT) or FNΔRGD in the absence (~CHX: A,B,E,F,I,J) or presence (+CHX: C,D,G,H) of cycloheximide. 3 hours after seeding, cells were fixed then incubated with a polyclonal antibody to α5 integrin and a monoclonal antibody to vinculin, followed by a Texas Red-conjugated anti-rabbit antibody to visualize α5 (B,D,F,H, J) and a FITC-anti-mouse antibody to visualize vinculin (A,C,E,G,I). Few cells attached to uncoated coverslips; to visualize cells that did attach, the cells shown in I and J were not washed prior to fixation. Cells were examined using an Olympus microscope equipped with epifluorescence, and photographed with a Spot digital camera. Bar, 10 μm.
Therefore, to determine whether adhesion of fibronectin-null cells to FNΔRGD is mediated by 70 kDa-integrin interactions, we tested whether addition of the 70 kDa fragment inhibited cell adhesion to FNΔRGD. As shown in Fig. 3, the 70 kDa amino-terminal fragment and the control 40 kDa gelatin-binding fragment of fibronectin did not inhibit adhesion to FNΔRGD or wild-type fibronectin. To determine whether cell adhesion to FNΔRGD is integrin mediated, we tested the ability of integrin antibodies to block adhesion to FNΔRGD. As shown in Fig. 3A, inhibitory α5 and β1 integrin antibodies did not block adhesion to FNΔRGD. In contrast, adhesion to collagen I-coated dishes was blocked by β1 antibodies (not shown). Surprisingly, β3 integrin antibodies partially inhibited (40%) cell adhesion to FNΔRGD (Fig. 3A), but had little effect on adhesion to wild-type fibronectin (Fig. 3B). When antibodies to αv and β3 integrins were added together, >70% of adhesion to FNΔRGD was inhibited (Fig. 3A). Control antibodies had no effect on cell adhesion to wild-type fibronectin or FNΔRGD. These data indicate that in the absence of RGD-integrin binding, αv- and β3-containing integrins mediate cell adhesion to fibronectin.

Cell growth in response to FNΔRGD

Cell spreading is critical for cell cycle progression (Chen et al., 1997; Hansen et al., 1994; Huang et al., 1998). However, spreading by itself is not sufficient to promote cell growth (Davey et al., 1999). Therefore, to determine whether FNΔRGD was able to promote cell growth in addition to cell spreading, fibronectin-null cells were seeded onto tissue culture plates coated with increasing concentrations of FNΔRGD or wild-type fibronectin. As shown in Fig. 4, cells grown on dishes coated with FNΔRGD achieved cell densities approximately 60% of those observed with cells seeded on wild-type fibronectin. This cell density is similar to those achieved by fibronectin-null cells adherent to type I collagen (Sottie et al., 1998). Maximal cell growth on both fibronectin and FNΔRGD occurred at a coating concentration of approximately 10 μg/ml.

Adhesion and growth of cells on FNΔRGD-coated substrates was not blocked by function-blocking anti-β1 integrin antibodies (Fig. 5), indicating that cell growth in response to FNΔRGD is β1 integrin-independent. In contrast, cell growth on collagen I-coated substrates was drastically reduced by β1 antibodies. The effect of β3 antibodies on cell growth could not be assessed, since β3 antibodies inhibit cell adhesion to FNΔRGD (Fig. 3A).

We previously showed that addition of soluble fibronectin to collagen, fibronectin, or laminin-adherent fibronectin-null cells

![Fig. 3. Effect of integrin antibodies on cell adhesion to fibronectin and ΔRGD fibronectin. Fibro...](image-url)

![Fig. 4. Comparison of cell growth on wild-type and ΔRGD fibronectin-coated dishes. Tissue culture dishes were precoated with various amounts of wild-type (WT, ○) or ΔRGD (●) fibronectin. Fibro...](image-url)
resulted in a 2-5× increase in cell growth (Sottile et al., 1998). This fibronectin-enhanced cell growth depended upon deposition of fibronectin into the extracellular matrix (Sottile et al., 1998). Fibronectin deposition is a cell-mediated process (McDonald, 1988; McKeown-Longo and Mosher, 1983) in which binding of fibronectin to cell surface receptors triggers homophilic binding interactions between fibronectin molecules (Aguirre et al., 1994; Chernousov et al., 1991; Hocking et al., 1994; Morla and Ruoslahti, 1992). To determine whether fibronectin can enhance cell growth of collagen-adherent cells in the absence of RGD-integrin interactions, collagen-adherent

![Graph](image1)

**Fig. 5.** Effect of β1 integrin antibodies on cell growth. Fibronectin-null cells in defined medium were seeded onto dishes precoated with collagen type I (white bars) or FNΔRGD (black bars). After allowing cells to attach and spread for 2 (FNΔRGD) or 6 (collagen) hours, the cell culture medium was supplemented with β1 integrin or control IgM antibodies at 25 μg/ml. Cells were allowed to grow for 4 days and were then processed as described in Fig. 4. Data are presented as percentage growth of cells incubated in the absence of antibodies. Values are means of duplicate determinations, and error bars the range.

![Graph](image2)

**Fig. 6.** Effect of FNΔRGD on cell growth. Fibronectin-null cells were seeded on collagen-coated wells in defined medium. 6 hours after seeding, cells were supplemented with 20 nM human plasma fibronectin (pFN, ●), wild-type recombinant fibronectin (WTFN, ○), recombinant FNΔRGD (□) or were given an equivalent volume of PBS (−FN, ■). Various times after seeding, cells were processed as described in Fig. 4. Values are means of duplicate determinations, and error bars the range.

![Images](image3)

**Fig. 7.** Deposition of wild-type and FNΔRGD into the extracellular matrix. Fibronectin-null cells were seeded in defined medium onto vitronectin-coated dishes. Following a 6 hour incubation, wells were either supplemented with 20 nM fibronectin (FN; A-D) or FNΔRGD (E-H). At the time of fibronectin addition, some wells were supplemented with the 350 nM 70 kDa amino-terminal fragment of fibronectin (70K) or the 40 kDa gelatin-binding fragment of fibronectin (40K). Following a 3 day incubation, cells were fixed, then permeabilized with 0.5% Triton-X-100. Cells were incubated with a polyclonal antibody to fibronectin followed by incubation with fluorescein-conjugated goat anti-rabbit IgG (A,C,E,G,H). Cells were examined using an Olympus microscope equipped with epifluorescence. (B,F) The corresponding phase pictures to A and E, respectively. Bar, 10 μm.
fibronectin-null cells were incubated in the presence of FNΔRGD, and cell growth was monitored over the course of 4 days. Fig. 6 demonstrates that addition of FNΔRGD to fibronectin-null cells resulted in a 1.8-fold increase in cell growth over that observed in cells adherent to collagen alone. Wild-type recombinant fibronectin and plasma fibronectin increased cell growth 3.5 and 4-fold, respectively, over that observed in cells adherent to collagen alone (Fig. 6). These data indicate that fibronectin can stimulate the growth of collagen-adherent cells in the absence of RGD-integrin interactions.

**Monoclonal antibody L8 inhibits cell surface staining of FNΔRGD and inhibits FNΔRGD-induced growth**

Previous studies have shown that fibronectin lacking the RGD site is impaired in its ability to form fibronectin fibrils; in CHO cells, short linear fibrils formed at the periphery of cells, mostly in areas of cell-cell contact (Sechler et al., 1996). Addition of FNΔRGD to fibronectin-null cells results in the formation of short stitch-like fibrils on the cell surface (Fig. 7E). In contrast, cells incubated with wild-type fibronectin (Fig. 7A) elaborate an extensive fibronectin matrix. Formation of FNΔRGD fibrils was blocked by agents known to inhibit formation of wild-type fibronectin matrix, including the 70 kDa amino-terminal fragment of fibronectin (Fig. 7G) and the anti-fibronectin antibody, L8 (see below).

The III-1 module of fibronectin is thought to be involved in fibronectin-fibronectin interactions that are important during fibronectin fibrillogenesis (Chernousov et al., 1991; Hocking et al., 1994; Morla and Ruoslahti, 1992; Sechler et al., 1996). Previous studies have shown that antibodies that bind to III-1, such as 9D2 and L8, inhibit fibronectin polymerization (Chernousov et al., 1987; Chernousov et al., 1991). The inhibition of wild-type fibronectin polymerization by 9D2 blocks the ability of fibronectin to promote cell growth (Sottile et al., 1998). To determine whether III-1 plays a role in the formation of FNΔRGD fibrils and in the ability of FNΔRGD to enhance cell growth, we asked whether these events could be blocked by monoclonal antibody L8. As shown in Fig. 8, addition of L8 to fibronectin-null cells blocked the deposition of FNΔRGD (Fig. 8E) and wild-type fibronectin (Fig. 8B) on the cell surface, as detected by indirect immunofluorescence microscopy; control IgG had no effect (Fig. 8C,F). Addition of L8 did not result in any change in cell morphology (not shown). To determine whether L8 could block the ability of FNΔRGD to promote cell growth, collagen-adherent fibronectin-null cells were incubated with FNΔRGD in the presence or absence of L8. As shown in Fig. 9, addition of L8 blocked >80% of FNΔRGD-induced cell growth. Addition of L8 resulted in a similar inhibition of wild-type fibronectin-induced cell growth (Fig. 9). Taken together, these data indicate that the organization of FNΔRGD into short linear fibrils depends upon the 70 kDa amino-terminal domain, as well as III-1-fibronectin interactions, and thus occurs by a mechanism similar to that used to polymerize wild-type fibronectin. These data also indicate that III-1-fibronectin interactions are critical for the cell growth response to FNΔRGD.

Integrins are known to participate in fibronectin fibril formation (Akiyama et al., 1989; Fogerty et al., 1990; Giancotti

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**Fig. 8.** Effect of L8 on fibronectin and FNΔRGD deposition. Fibronectin-null cells were seeded in defined medium onto vitronectin-coated dishes. Following a 6 hour incubation, wells were either supplemented with 20 nM wild-type fibronectin (WT; A-C) or FNΔRGD (D-F). At the time of fibronectin addition, some wells were also supplemented with 50 μg/ml monoclonal antibody L8 (B,E) or mouse IgG (C,F). Control wells received an equal volume of PBS (A,D). Following a 3 day incubation, cells were fixed, then permeabilized with 0.5% Triton-X-100. Cells were incubated with a polyclonal antibody to fibronectin followed by incubation with fluorescein-conjugated goat anti-rabbit IgG. Cells were examined using an Olympus microscope equipped with epifluorescence to visualize fibronectin and FNΔRGD. Bar, 10 μm.
and Ruoslahti, 1990; Zhang et al., 1993), and antibodies to α5 and β1 integrins partially inhibit fibronectin polymerization (Akiyama et al., 1989; Fogerty et al., 1990). To compare the effects of anti-integrin antibodies on fibronectin and FNΔRGD fibril formation, and fibronectin- and FNΔRGD-induced growth, function-blocking β1 and β3 antibodies were added to fibronectin-null cells that were incubated with either FNΔRGD or with non-mutant fibronectin. β1 and β3 antibodies did not prevent the deposition of FNΔRGD on the cell surface (Fig. 10B). Similarly, addition of αv and β3 antibodies did not inhibit FNΔRGD fibril formation (not shown). In contrast, fibril formation of non-mutant fibronectin was partially blocked by the presence of β1 and β3 antibodies, resulting in the formation of shorter, less extensive fibrils (Fig. 10E). To determine whether β3 integrin antibodies inhibit cell growth, we asked whether FNΔRGD-induced growth on collagen-coated dishes was blocked by addition of β3 antibodies. As shown in Fig. 11, β3 integrin antibodies partially blocked cell growth both in the absence (29%) and presence (22%) of FNΔRGD. However, the relative increase in cell growth induced by FNΔRGD was unchanged by β3 integrin antibody treatment (without anti-β3=1.7×; with anti-β3=1.8×). No further decrease in cell growth was observed when αv and β3 integrin antibodies were added together (not shown).

**Heparin and heparitinase-treatment inhibit FNΔRGD induced cell growth**

Antibodies to β3 integrins did not prevent enhanced cell growth induced by FNΔRGD (Fig. 11). These data suggest that binding of FNΔRGD to a non-integrin receptor may contribute to the cell growth response. Since fibronectin is known to bind to proteoglycans, we asked whether heparin could block the

![Fig. 9. Effect of L8 on fibronectin- and FNΔRGD-induced growth. Fibronectin-null cells were seeded in defined medium onto collagen-coated dishes. Following a 6 hour incubation, wells were supplemented with either 20 nM wild-type fibronectin (WT) or FNΔRGD. At the time of fibronectin addition, some wells were also supplemented with 50 μg/ml monoclonal antibody L8 or mouse IgG. Following a 5 day incubation, cells were fixed with 1% paraformaldehyde, then stained with 0.5% Crystal Violet and the absorbance at 540 nm determined. Data is expressed as fold increase in growth; growth of control wells (cells incubated in the absence of fibronectin) was set equal to 1. Values are means of duplicate determinations, and error bars the range.](image)

![Fig. 10. Effect of integrin antibodies on fibronectin and FNΔRGD deposition. Fibronectin-null cells were seeded in defined medium onto vitronectin-coated dishes. The day after seeding, wells were supplemented with 20 nM nonmutant fibronectin (D-F) or FNΔRGD (A-C). At the time of fibronectin addition, some wells were also supplemented with β1 and β3 (25 μg/ml each) antibodies (B,E) or with control IgG and IgM antibodies (25 μg/ml each; C,F). Control wells were supplemented with an equal volume of PBS (A,D). Following a 4 day incubation, cells were fixed, then permeabilized with 0.5% Triton-X-100. Cells were incubated with a polyclonal antibody to fibronectin followed by incubation with fluorescein-conjugated goat anti-rabbit IgG. Cells were examined using an Olympus microscope equipped with epifluorescence to visualize fibronectin or FNΔRGD. Bar, 10 μm.](image)
Cell growth regulation by fibronectin

ability of FNΔRGD to promote growth. As shown in Fig. 12A, the growth-promoting effects of FNΔRGD were completely abolished by the addition of 1 mg/ml heparin (H1), and were partially (45%) inhibited by addition of the recombinant fibronectin heparin-binding domain containing modules III12,13 (HBD). The addition of heparin (1 mg/ml) to fibronectin-null cells also decreased growth induced by wild-type fibronectin by 45% (Fig. 12A). Lower doses of heparin (10 µg/ml) partially inhibited growth induced by FNΔRGD (70%) and wild-type fibronectin (30%). Heparin (1 mg/ml) had little effect (<15% decrease) on cell growth in the absence of added fibronectin (data not shown). The ability of heparin and heparin-binding fragments of fibronectin to inhibit cell growth suggests that fibronectin-proteoglycan binding may be a critical component of fibronectin- and FNΔRGD-induced growth.

To further explore the possibility that cell surface proteoglycans may be involved in fibronectin-induced cell growth, we examined the effect of heparitinase and chondroitinase treatment on fibronectin-induced cell growth. As shown in Fig. 12B, heparitinase treatment (+H) of fibronectin-null cells attenuated the growth-promoting effects of both wild-type fibronectin and FNΔRGD, causing a 43% reduction in the growth-promoting effects of FNΔRGD and a 34% decrease in the growth-promoting effects of wild-type fibronectin. Chondroitinase treatment (+C) had no effect on FNΔRGD or fibronectin induced cell growth (Fig. 12B). Taken together, these data support a role for cell surface heparan sulfate proteoglycans in mediating the growth-promoting effects of fibronectin.

Cell surface staining of FNΔRGD is attenuated by heparin and heparitinase treatment

Many studies have shown that clustering of cell surface receptors, including growth factor receptors and integrins, is necessary for transducing downstream signalling events (Akiyama et al., 1994; Clark and Brugge, 1995; Heldin, 1995; Miyamoto et al., 1995; Weiss and Schlessinger, 1998). To determine whether the heparin-binding activity of fibronectin is important for the clustering of FNΔRGD into fibrils on the cell surface, we asked whether FNΔRGD fibril formation could...
be blocked by either the addition of heparin or by heparitinase treatment (Fig. 13). FNΔRGD staining was eliminated when cells were incubated in the presence of heparin (Fig. 13G), or when cells were treated with heparitinase (Fig. 13I). Both treatments also attenuated fibril formation in cells incubated with wild-type fibronectin (Fig. 13B,D). Addition of heparin or treatment with heparitinase did not cause any noticeable decrease in cell attachment or spreading (not shown). In addition, chondroitinase treatment did not affect the deposition of either wild-type fibronectin (Fig. 13E) or FNΔRGD (Fig. 13J). These data suggest that heparan sulfate proteoglycans may participate in FNΔRGD fibril formation on the cell surface.

DISCUSSION

We previously demonstrated that fibronectin polymerization into the extracellular matrix promotes adhesion-dependent growth, and that integrin-ligation and clustering are not sufficient to promote enhanced cell growth in response to fibronectin (Sottile et al., 1998). In this study, we have extended these findings by demonstrating that fibronectin can stimulate cell growth in the absence of RGD-integrin ligation by a mechanism that remains dependent on fibronectin polymerization. FNΔRGD promoted cell growth to approximately 50-60% of the levels induced by wild-type fibronectin (Figs 4, 6). Thus, it is likely that both RGD-dependent and -independent mechanisms are involved in the cell growth response to wild-type fibronectin.

We previously showed that fibronectin-null cells do not express α4 integrins on their cell surface (Sottile et al., 1998). In addition, all of the remaining fibronectin-binding integrins, including those that contain αv (αvβ1, αvβ3, αvβ5, αvβ6) have been shown to interact with fibronectin via the RGD site in III-10 (Chen et al., 1996b; Plow et al., 2000). Therefore, it was surprising to find that cell adhesion to FNΔRGD could be blocked with antibodies to αv and β3 integrins (Fig. 3). To our knowledge, this is the first demonstration that binding of fibronectin to αv-containing integrins can occur independently of the RGD sequence. αvβ3 binds to a surprisingly large number of molecules, by both RGD-dependent and independent mechanisms (Plow et al., 2000). In addition, binding of fibronectin to the platelet receptor, αIIbβ3, can occur by RGD-dependent and -independent mechanisms (Bowditch et al., 1991). The site in fibronectin that mediates cell attachment to αv-containing integrins in the absence of RGD is not currently known.

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Our previous data indicate that the ability of fibronectin to enhance cell growth depends upon its ability to become deposited into the extracellular matrix (Sottile et al., 1998). FNΔRGD does not polymerize into robust fibrils in the extracellular matrix of fibronectin-null cells, but forms short stitch-like fibrils on the cell surface (Fig. 7). Others have shown that FNΔRGD can be induced to form fibrils by an α4β1-dependent mechanism following integrin activation (Sechler et al., 2000). The FNΔRGD fibrils reported here are generated by a distinct mechanism, since fibronectin-null cells do not have cell surface α4 integrins (Sottile et al., 1998). The short linear FNΔRGD fibrils are abolished by the exogenous addition of heparin, and by heparitinase-treatment, which also inhibited the growth-promoting effects of both FNΔRGD and wild-type fibronectin (Figs 12, 13). These data suggest that cell surface heparan sulfate proteoglycans may play an important role in...
fibronectin fibril formation, as well as in mediating fibronectin’s growth-promoting effects.

Interestingly, both the formation of FNΔRGD fibrils on the cell surface, and the ability of FNΔRGD to induce cell growth were blocked by the presence of monoclonal antibody L8. L8 recognizes an epitope in fibronectin’s I0 and III1 modules (Chernousov et al., 1991), and inhibits fibronectin deposition into the extracellular matrix, presumably by blocking fibronectin-fibronectin interactions (Chernousov et al., 1987; Chernousov et al., 1991). It is possible that binding of FNΔRGD to the cell surface exposes fibronectin-interactive sites within III-1, as has previously been proposed for wild-type fibronectin (Hocking et al., 1994; Morla and Ruoslahti, 1992; Sechler et al., 1996). The ability of L8 to block both FNΔRGD fibril formation and the growth-promoting effects of FNΔRGD suggests that exposure of self-interactive sites that lead to clustering of fibronectin into fibrils is a critical event during fibronectin-stimulated cell growth and can occur in the absence of RGD-integrin interactions. The loss of FNΔRGD fibrils with heparitinase treatment suggests the possibility that heparan sulfate proteoglycans may play a role in exposing fibronectin self-interactive sites on the cell surface.

Our data are consistent with a model in which fibronectin can trigger cell growth by two mechanisms: (1) by serving as an adhesion substrate for cells and thus allowing for cell spreading and adhesion-dependent cell cycle progression; and (2) as a consequence of its polymerization into fibrils in the extracellular matrix. αv-containing integrins may promote cell growth via effects on cell adhesion and spreading, since cell adhesion to FNΔRGD is dependent upon αv and β3 integrins (Fig. 3). β3 integrin antibodies partially inhibited the growth of collagen-adherent cells in the presence and absence of FNΔRGD. However, the relative increase in growth induced by FNΔRGD was not affected by antibody treatment (Fig. 11). The effect of β3 integrin antibodies on cell growth could be due to the ability of these antibodies to partially block adhesion (not shown) and growth (Fig. 11) on collagen I in the absence of FNΔRGD. In contrast, our data suggest that heparan sulfate proteoglycans participate in the cell growth response to fibronectin by a mechanism that depends upon fibronectin deposition into the extracellular matrix. The ability of heparitinase to inhibit FNΔRGD cell surface staining and to attenuate FNΔRGD-induced growth suggests that binding of fibronectin to heparan sulfate proteoglycans contributes to the growth-promoting effects of FNΔRGD. It is likely that proteoglycans also contribute to the growth-promoting effects of wild-type fibronectin, as treatment of cells with heparitinase, or addition of soluble heparin decreased deposition of wild-type fibronectin into the extracellular matrix (Fig. 13) and partially inhibited fibronectin-induced cell growth (Fig. 12).

Several heparin-binding domains have been identified within the fibronectin molecule. Heparin-binding activity has been localized to modules I1-5 (the 27 kDa amino-terminal region), III1, and the carboxyl-terminal heparin-binding domain containing III12-14 (Hynes, 1990; Litvinovich et al., 1992). Previous studies have demonstrated a role for fibronectin heparin-binding domains in fibronectin deposition into the extracellular matrix. The amino-terminal 27-kDa fragment of fibronectin is important for binding of soluble fibronectin to the surface of substrate-attached cells (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988), while the carboxyl-terminal heparin-binding domain has been shown to partially inhibit fibronectin binding and deposition into the extracellular matrix (Bultmann et al., 1998). Our data demonstrate that recombinant III12,13 partially inhibited FNΔRGD-induced cell growth (Fig. 12); the 70 kDa amino-terminal fragment (which contains modules I1-5) also partially blocks FNΔRGD-induced growth (data not shown). The ability of III12,13 and the 70 kDa fragment to inhibit FNΔRGD-induced growth could be due to the direct involvement of these domains in fibronectin-proteoglycan interactions.

Others have proposed that heparan sulfate proteoglycans act as coreceptors with integrins to promote maximal cell spreading on fibronectin (Saoncella et al., 1999; Woods et al., 1986). Our data demonstrate that fibronectin can promote adhesion and focal contact formation in the absence of fibronectin-RGD interactions, and that this adhesion depends upon binding to αv- and β3-containing integrins. Our data also suggest an important role for fibronectin-proteoglycan interactions in mediating FNΔRGD’s growth-promoting effects, and in promoting the formation of FNΔRGD fibrils on the cell surface. Although FNΔRGD can form short stitch-like fibrils on the cell surface, its inability to form extensive fibrils suggests that exposure of additional fibronectin-fibronectin interactive sites critical for fibril growth may depend upon integrin interactions with fibronectin’s RGD site. Our data also suggest that proteoglycans are important regulators of wild-type fibronectin function, since heparin and heparitinase treatment attenuated both fibronectin deposition into the extracellular matrix and the growth-promoting effects of wild-type fibronectin. Fibronectin is known to interact with a number of cell surface and cell-associated proteoglycans that have been shown to regulate cell migration and cell growth, including syndecan, perlecan and CD44 (Carey, 1997; Mathiak et al., 1997; Naor et al., 1997). Our data suggest that one mechanism whereby proteoglycans affect cell growth may be by regulating fibronectin deposition into the extracellular matrix. This hypothesis is supported by a recent study showing that transfection of a mutant syndecan 2 into CHO cells can inhibit fibronectin deposition into the extracellular matrix (Klass et al., 2000). Together, these data suggest that fibronectin fibril formation can promote cell growth by a novel mechanism that is independent of RGD-integrin binding, and that involves cell surface proteoglycans.

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