A cryptic fragment from fibronectin’s III1 module localizes to lipid rafts and stimulates cell growth and contractility

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The interaction of cells with the extracellular matrix (ECM) form of fibronectin (FN) triggers changes in growth, migration, and cytoskeletal organization that differ from those generated by soluble FN. As cells deposit and remodel their FN matrix, the exposure of new epitopes may serve to initiate responses unique to matrix FN. To determine whether a matricryptic site within the III1 module of FN modulates cell growth or cytoskeletal organization, a recombinant FN with properties of matrix FN was constructed by directly linking the cryptic, heparin-binding COOH-terminal fragment of III1 (III1H) to the integrin-binding III8–10 modules (glutathione-S-transferase [GST]–III1H,8–10). GST–III1H,8–10 specifically stimulated increases in cell growth and contractility; integrin ligation alone was ineffective. A construct lacking the integrin-binding domain (GST–III1H,2–4) retained the ability to stimulate cell contraction, but was unable to stimulate cell growth. Both GST–III1H,2–4 and matrix FN colocalized with caveolin and fractionated with low-density membrane complexes by a mechanism that required heparan sulfate proteoglycans. Disruption of caveolae inhibited the FN- and III1H-mediated increases in cell contraction and growth. These data suggest that a portion of ECM FN partitions into lipid rafts and differentially regulates cytoskeletal organization and growth, in part, through the exposure of a neoepitope within the conformationally labile III1 module.

Introduction

Fibronectins are a family of multidomain glycoproteins that are deposited in the extracellular matrix (ECM)* where they promote adhesive and migratory events (Hynes, 1990). The polymerization of soluble fibronectin (FN) into insoluble fibrils within the ECM is a dynamic, cell-dependent process that is mediated by a series of events involving the actin cytoskeleton and integrin receptors (Magnusson and Mosher, 1998). Studies indicate that the interaction of cells with the ECM form of FN triggers changes in cell cycle progression, cell migration, and cytoskeletal organization that differ from those generated by the interaction of cells with nonpolymerized FN (Morla et al., 1994; Sottile et al., 1998; Sechler and Schwarzbauer, 1999; Hocking et al., 2000). Other studies suggest that FN polymers formed in vitro have antitumor metastatic (Pasqualini et al., 1996) and antiangiogenic properties (Yi and Ruoslahti, 2001). The mechanism by which the ECM form of FN gives rise to cellular phenotypes distinct from soluble FN is not known.

Current models of FN matrix assembly propose that binding of soluble FN to the cell surface triggers a series of conformational changes that result in the exposure of homophilic binding sites and the subsequent elongation of FN fibrils (Magnusson and Mosher, 1998). The first type III module of FN (III1) contains a binding site for the NH2 terminus of FN that is not exposed within soluble FN (Hocking et al., 1994), but may become exposed on cell surfaces during FN polymerization (Zhong et al., 1998; Tellier et al., 2000). Moreover, proteolytic cleavage of III1 at Ile597 exposes a cryptic heparin-binding activity in III1 (Litvinovich et al., 1992). These data suggest the possibility that during FN polymerization, a “matricryptic” (Davis et al., 2000), heparin-binding activity is exposed in III1, which serves to trigger cellular responses that are unique to ECM FN.

Previous work has shown that FN stimulates cell growth (Sottile et al., 1998) and contractility (Hocking et al., 2000) by a mechanism that requires the polymerization of FN into the ECM. To construct a recombinant FN with properties of matrix FN, we engineered a glutathione-S-transferase (GST)–tagged fusion protein in which the cryptic, heparin-binding III1 fragment (III1H) was linked directly to the inte-
grin-binding III8–10 modules (GST–III1H,8–10). FN-null (FN$^{-/-}$) cells, which do not produce FN and are grown under serum-free conditions (Sottile et al., 1998), were used in conjunction with recombinant FN fragments to eliminate any effects of endogenous FN or FN fragments. Our data indicate that treatment of FN$^{-/-}$ cells with GST–III1H,8–10 stimulates a specific increase in cell growth and contractility. A construct in which the II2–4 modules were substituted for the integrin-binding III8–10 modules (GST–III1H,2–4) retained the ability to stimulate cell contraction, but did not stimulate cell growth. Both GST–III1H,2–4 and polymerized FN colocalized with caveolin and fractionated with low-density membrane complexes by a mechanism that required heparan sulfate proteoglycans (HSPGs). Treatment of cells with sterol-binding compounds to disrupt caveolae blocked the localization of GST–III1H,2–4 and FN to lipid rafts and inhibited the increase in cell contraction and growth induced by either FN or the III1H-containing constructs. These data suggest that a portion of ECM FN partitions into lipid rafts and differentially regulates cytoskeletal organization and cell growth, in part, through the exposure of a neoepitope within the conformationally labile III1 module.

Results

A cryptic fragment of III1 stimulates cell growth

In the absence of FN, FN$^{-/-}$ cells that are adherent to type I collagen exhibit a typical cell doubling rate of 20 h (Sottile et al., 1998). Addition of GST–III1H,8–10 to collagen-adherent FN$^{-/-}$ cells stimulated a dose-dependent increase in cell growth compared with untreated cells (Fig. 1 A). Substitution of the heparin-binding III13 module (Bloom et al., 1999) for III1H (GST–III8–10,13) did not stimulate cell growth (Fig. 1 A). Treatment of cells with an FN construct in which the II2–4 modules were substituted for the III8–10 modules also did not increase cell growth (Fig. 1 A), suggesting that integrin ligation is necessary for the growth response to GST–III1H,8–10. However, treatment of cells with integrin-binding constructs in which the III1H fragment was absent (GST–III8–10 and GST–III9–10), or in which the corresponding COOH-terminal III2 fragment (III2H) was substituted for III1H (GST–III2H,8–10) failed to increase cell growth (Fig. 1 B), suggesting that III1H is also required. Cell growth was not increased when individually expressed GST–III1H was added to cells either alone or in combination with GST–III8–10 (GST–III1H + GST–III8–10; Fig. 1 B), suggesting that clustering of cell surface–bound III1H by binding of III8–10 to integrin receptors (Hynes, 1990) may be important for its activity.

To further define the role of III1H, FN$^{-/-}$ cells were treated with GST–III1H,8–10 in the absence and presence of increasing concentrations of GST–III1H. As shown in Fig. 2 A, GST–III1H,8–10-stimulated growth was inhibited by co-incubation of cells with the III1H fragment. In contrast, GST–III1H,8–10-stimulated growth was not inhibited when cells were co-incubated with the fragment of II2 corresponding to the III1H truncation (GST–II2H; Fig. 2 A). GST–III1H,8–10-stimulated growth was also inhibited by addition of the integrin-binding constructs GST–III8–10 and GST–III9–10 (Fig. 2 B). Moreover, mutation of the integrin syn-

Figure 1. Effect of GST–III1H,8–10 on cell growth. FN$^{-/-}$ cells were seeded on collagen-coated wells. 4 h after seeding, cells were treated with increasing concentrations of either intact FN (+Fn, ○), GST–III1H,8–10 (●), GST–III1H,2–4 (□), or GST–III8–10,13 (▲). In B, cells were treated with 250 nM of the various recombinant proteins. 4 d after seeding, cell number was determined as described in the Materials and methods. Data are presented as fold increase in cell number over control (growth in the absence of additional protein). *Significantly different from control, $P < 0.001$.

Heparin-binding activity is required for increased growth

The ability of GST–III1H,8–10 to bind to heparin was assessed in solid phase binding assays. As shown in Fig. 3 A, GST–III1H,8–10 bound biotin-labeled heparin, whereas GST–III8–10 did not. Heparin-binding activity was also observed with GST–III8–10,13 and was greater than that observed with GST–III1H,8–10 (Fig. 3 A). The heparin-binding activity of GST–III8–10,13 was similar to that observed with the FN construct that contains both the III12 and III13 modules (GST–III12–13) (Fig. 3 A). Control studies indicated that an equal amount of fusion protein bound in each well (unpublished data).

To determine whether the heparin-binding activity of III1H plays a role in GST–III1H,8–10-stimulated growth, cells were incubated with GST–III1H,8–10 in the absence and presence of either sulfated or nonsulfated heparin. Coincubation with sulfated heparin, but not nonsulfated heparin, reduced GST–III1H,8–10 growth to control levels (Fig. 3 B). Similarly, treatment with heparinase I inhibited the growth response to GST–III1H,8–10 by $\sim50\%$, suggesting a role for HSPGs (Fig. 3 C). Heparinase treatment had no effect on control cell growth (Fig. 3 C). To determine whether other known heparin-bind-
either with 100 nM GST–III1H,8–10 in the absence (control) and presence of GST–III1H or 1

in the absence (0) and presence of GST–III1H or 1 μM GST–III2H. * + GST–III1H (1 μM) differs significantly from + GST–III2H, P < 0.01. In B, cells were treated with 100 nM GST–III1H,8–10 in the absence (control) and presence of either 1 μM GST–III8–10, GST–III9–10, or GST–III9–10R1374,1379A. Cells were processed as indicated in the legend to Fig. 1. * + GST–III9–10R1374,1379A differs significantly from + GST–III9–10, P < 0.01.

In contrast, addition of the NH2-terminal 70-kD fragment to control cells had no effect on GST–III1H,8–10-stimulated growth. Addition of the heparin-binding domains to control cells had little effect on GST–III1H,8–10-stimulated growth. Addition of the heparin-binding domains to control cells had no effect on basal cell growth (Fig. 3 D). Taken together, these data suggest that the heparin-binding activity of III1 is necessary for the stimulation of cell growth.

Figure 2. Role of III1H and III9–10 in GST–III1H,8–10-stimulated growth. FN−/− cells were seeded as in the legend to Fig. 1. In A, cells were treated with 250 nM GST–III1H,8–10 in the absence (0) and presence of GST–III1H or 1 μM GST–III2H. * + GST–III1H (1 μM) differs significantly from + GST–III2H, P < 0.01. In B, cells were treated with 100 nM GST–III1H,8–10 in the absence (control) and presence of either 1 μM GST–III8–10, GST–III9–10, or GST–III9–10R1374,1379A. Cells were processed as indicated in the legend to Fig. 1. * + GST–III9–10R1374,1379A differs significantly from + GST–III9–10, P < 0.01.

Effect of III1H on cell contractility

FN polymerization has also been shown to trigger cytoskeletal tension generation (Hocking et al., 2000). To determine the role of III1H in cell contractility, FN−/− cells were imbedded in floating collagen gels in the absence and presence of increasing concentrations of either intact FN or the various fusion proteins. GST–III1H,8–10 stimulated an increase in cell contractility to a similar extent as that observed with intact FN (Fig. 4 A). Furthermore, in contrast to the negative results obtained in the growth studies, treatment of cells with GST–III1H,2–4 also promoted a significant increase in collagen gel contraction (Fig. 4 A). As with the growth assays, substitution of the heparin-binding III13 module for III1H (GST–III1H–10,13) failed to stimulate collagen gel contraction (Fig. 4 A). Addition of the III1H fragment resulted in a dose-dependent inhibition of GST–III1H,8–10-induced contraction; intact III1 had no effect (Fig. 4 B). Moreover, treatment of cells with GST–III1H–4,

in which III1H was replaced with the intact III1 module, did not stimulate contraction (Fig. 4 C), suggesting that exposure of cryptic residues within III1 is required to induce cytoskeletal tension generation. Constructs in which one or more of the COOH-terminal type III modules were deleted (GST–III1H,2–3, GST–III1H,2, and GST–III1H) did not stimulate cell contractility (Fig. 4 C), suggesting that secondary sequences contribute to the ability of III1H to stimulate contraction. These data suggest that a cryptic, heparin-binding region within the III1 module of FN plays a unique role in modulating both cell contractility and growth.

Figure 3. Role of heparin-binding activity in GST–III1H,8–10 growth. (A) Glutathione-coated plates were incubated with saturating concentrations of the various fusion proteins. Binding of biotin–heparin was assayed by EIA. Data represent the mean absorbance of triplicate wells ± SD. In B–D, FN−/− cells were seeded as indicated in the legend to Fig. 1. After 4 h, cells were treated with 100 nM GST–III1H,8–10 or an equal volume of PBS. In B, cells were coincubated with 50 μM sulfated (heparin I-S) or nonsulfated (heparin IV-A) heparin. *Significantly different from + GST–III1H,8–10, P < 0.001. In C, cells were pretreated with heparinase I or an equal volume of DME. *Significantly different from + GST–III1H,8–10, P < 0.05. In D, cells were coincubated with 1 μM of the various heparin-binding fragments. *Significantly different from GST–III1H,8–10 alone, P < 0.001.

GST–III1H,8–10 does not localize to focal contacts

To explore the mechanism by which the III1H constructs stimulate growth and contractility, studies were conducted to determine whether GST–III1H,2–4 and GST–III1H,8–10 localize to specific cell surface structures. Collagen-adherent FN−/− cells were treated with the various fusion proteins and then examined by immunofluorescence microscopy for GST to visualize the fusion proteins, and for vinculin, a marker of focal adhesions (Jockusch et al., 1995). As shown in Fig. 5, staining of GST–III1H,2–4 (Fig. 5 A, arrow) and GST–III1H,8–10 (Fig. 5 B, arrow) was detected in discrete clusters that did not colocalize with vinculin (Fig. 5, E and F). In contrast, GST–III8–10,13 staining strongly colocalized with vinculin (Fig. 5, C, G, and K, arrows) in a pattern similar to that observed with GST–III8–10 (unpublished...
data). No staining was observed when cells were treated with GST alone (Fig. 5 D).

**GST–III1H,2–4 colocalizes with caveolin by an HSPG-dependent mechanism**

To further identify the structures in which the III1H constructs localized, cells were incubated with either GST–III1H,2–4 or GST and examined by immunofluorescence microscopy for GST and caveolin, a marker of cell surface membrane invaginations, termed caveolae (Anderson, 1998).

As shown in Fig. 6, GST–III1H,2–4 staining (Fig. 6 A, arrows) colocalized with caveolin staining (Fig. 6, B and C, arrows). When cells were treated with GST alone, GST staining was not observed (Fig. 6 D), whereas caveolin staining was still detected (Fig. 6 E, arrow). Pretreatment of cells with filipin III, which binds cholesterol and disrupts caveolae (Anderson, 1998), caused a concomitant decrease in both GST–III1H,2–4 and caveolin staining (Fig. 6, G and H). Filipin III did not disrupt cell adhesion, as cells treated with filipin III remained well spread (Fig. 6 I).

To determine whether the cell surface localization of GST–III1H,2–4 was dependent upon HSPGs, collagen-adherent FN−/− cells were pretreated with heparinase before the addition of GST–III1H,2–4. As shown in Fig. 7 A, clustering of GST–III1H,2–4 on the cell surface was abolished by pretreatment with heparinase. In contrast, pretreatment of cells with either chondroitinase ABC (Fig. 7 a, C) or neuraminidase (Fig. 7 a, D) had no effect on the cell surface staining of GST–III1H,2–4. Heparinase treatment did not affect cell adhesion, as demonstrated by vinculin staining of focal adhesions (Fig. 7 a, F).

Proteins associated with caveolae are resistant to solubilization by either Triton X-100 or high pH carbonate buffer and can be purified by flotation on sucrose gradients (Schwab et al., 2000). To determine whether GST–III1H,2–4 associates with low-density membrane complexes by a mechanism that is dependent upon HSPGs, collagen-adherent FN−/− cells
were pretreated with heparinase I or an equivalent volume of DME, before the addition of GST–III1H,2–4. Cells were extracted with 1% Triton X-100 and fractionated by high-speed centrifugation. Under these conditions, the majority of cellular protein remains associated with the high-density sucrose (Fig. 7 b, fractions 9–12); proteins associated with low-density lipids are buoyant and rise to the interface of the 5 and 30% sucrose layers (Schwab et al., 2000). As previously demonstrated, both caveolin and β-actin, but not LAMP-1, associate with low-density membrane domains and thus migrate to the interface of the 5 and 30% sucrose layers (Anderson, 1998). GST–III1H,2–4 also comigrated to these low-density membrane fractions (Fig. 7 b, fractions 5 and 6). Moreover, pretreatment of cells with heparinase significantly reduced the amount of GST–III1H,2–4 associated with the low-density lipid complexes (Fig. 7 b). In contrast, heparinase treatment had no effect on the association of either caveolin or β-actin with low-density lipid complexes (Fig. 7 b). These data suggest that GST–III1H,2–4 localizes to caveolin-enriched membrane domains by an HSPG-dependent mechanism. GST–III1H,8–10 was also detected in the caveolin-enriched fractions, whereas GST–III8–10,13 was not (Fig. 7 c), providing further evidence that III1H mediates the localization of these FN fragments to lipid rafts.

**Partitioning of FN to lipid rafts requires FN polymerization and HSPGs**

To determine whether intact FN also colocalizes with caveolin, confluent FN−/− cells were incubated with FN for 4 h. Cells were costained for FN and caveolin. As demonstrated in Fig. 8 a, areas of FN (Fig. 8 a, A, arrow) and caveolin (Fig. 8 a, A, arrow) were colocalized, whereas areas of FN (Fig. 8 a, A, arrow) and caveolin (Fig. 8 a, A, arrow) were not. These results suggest that FN polymerization is required for its colocalization with caveolin.

**Figure 6.** Colocalization of GST–III1H,2–4 with caveolin. Collagen-adherent FN−/− cells were incubated with 250 nM of GST (D, E, and F) or GST–III1H,2–4 in the absence (A, B, and C) and presence of 2.5 μg/ml filipin III (G, H, and I). Cells were then processed for immunofluorescence. Fusion proteins were visualized using an anti-GST mAb followed by an FITC-labeled goat anti-mouse antibody (A, D, and G). Caveolin was visualized using a polyclonal anticaveolin antibody followed by a Texas red–labeled goat anti-rabbit antibody (B, E, and H). In the merged images shown in C and F, areas of colocalization appear yellow. The phase contrast image of cells treated with GST–II1H,2–4 and filipin III is shown in I. Bar, 10 μM.

**Figure 7.** Effect of heparinase on the localization of GST–III1H2–4 to caveolin-enriched lipid rafts. (a) Collagen-adherent FN−/− cells were pretreated with either heparinase I (B and F), chondroitinase ABC (C and G), neuraminidase (D and H), or an equal volume of DME (A and E) before the addition of 250 nM GST–III1H,2–4. Staining for GST (A–D) and vinculin (E–H) was conducted as in the legend to Fig. 5. Bar, 10 μM. (b) Confluent Fn−/− cells were treated with either heparinase I or DME, before the addition of 250 nM GST–III1H,2–4. (c) Cells were incubated with 250 nM GST–III1H,2–4 or GST–III8–10,13. Cell lysates were fractionated by sucrose density gradient centrifugation, as described in the Materials and methods. Aliquots (120 μl) of each fraction were analyzed by PAGE and immunoblotting. GST–III1H,2–4 was identified using an anti-FN mAb clone CCBD that recognizes an epitope in III4. The blot was then stripped and reprobed for LAMP-1. A separate immunoblot containing 5 μl of each fraction was analyzed for caveolin followed by β-actin. GST–III1H,8–10 and GST–III8–10,13 were identified using an anti-GST monoclonal antibody. Molecular mass markers are indicated to the left.
enriched fractions (fractions 5 and 6). Pretreatment of cells with heparinase significantly reduced the amount of FN associated with these fractions (Fig. 8 b, fractions 5 and 6).

To determine whether the localization of FN to caveolin-enriched lipid rafts requires FN polymerization, FN-null cells were pretreated with FN in the presence of either control mouse IgG or the anti-FN mAb, 9D2. The 9D2 mAb recognizes an epitope within the III1 module of FN and inhibits FN polymerization, but does not block the initial association of soluble FN with cell surfaces (Chernousov et al., 1991). After an 18-h incubation, cells were extracted using a high pH carbonate buffer and fractionated. As shown in Fig. 8 c, when FN polymerization was inhibited by the 9D2 mAb, FN staining was not detected in the low-density fractions (Fig. 8 c, fractions 5–8).

β-Cyclodextrin removes cholesterol from cell membranes and alters the association of proteins with caveolae (Keller and Simons, 1998). To determine whether disruption of caveolae would disrupt the association of FN with low-density lipid rafts, cells were allowed to polymerize an FN matrix and then were treated with cyclodextrin for 2 h. As shown in Fig. 8 d, cyclodextrin preferentially removed the FN associated with the low-density lipid fractions (fractions 5 and 6); cyclodextrin had no effect on the interaction of nonlipid-associated FN with cells (Fig. 8 d, fraction 12). Taken together, these data indicate that a portion of matrix FN partitions to lipid rafts by a mechanism that requires HSPGs.

### III1-mediated growth and contractility are inhibited by sterol-binding agents

To determine whether the fraction of GST–III1H,2–4 and FN that associates with caveolae is biologically active, Fn−/− cells were pretreated with the cholesterol-binding compounds nystatin and filipin III. Cells were then embedded in collagen gels in the presence of either FN or GST–III1H,2–4 and collagen gel contraction was assessed. As demonstrated in Fig. 9 A, pretreatment of cells with nystatin inhibited the contraction induced by GST–III1H,2–4 and partially inhibited the contraction induced by FN. In contrast, basal contraction was not altered by nystatin treatment (Fig. 9 A). A similar inhibition profile was obtained when cells were pretreated with filipin III (Fig. 9 B).

Studies were also conducted to determine whether disruption of caveolae inhibits GST–III1H,8–10- and FN-induced cell growth. Pretreatment of cells with nystatin inhibited the increase in cell growth induced by GST–III1H,8–10 and partially inhibited the increase in cell growth induced by intact FN (Fig. 9 C). Nystatin did not significantly affect basal cell growth (Fig. 9 C, +PBS). Pretreatment of cells with cyclodextrin also inhibited GST–III1H,8–10 and FN-stimulated growth while having no effect on basal growth (Fig. 9 D). Taken together, these data suggest that the ability of the heparin-binding fragment of III1 to stimulate cell contractility and growth is dependent upon its localization to caveolin-containing lipid rafts.

### Discussion

Although the ECM form of FN is thought to be the primary functional form in vivo (Hynes, 1990), studies to date have focused primarily on the effects of soluble FN on cell func-
A cryptic fibronectin fragment stimulates growth

Recent studies indicate that the interaction of cells with matrix FN triggers changes in cell behavior that are distinct from those generated by the interaction of cells with soluble FN (Morla et al., 1994; Sottile et al., 1998; Sechler and Schwarzbaucer, 1999; Hocking et al., 2000). The ability of FN constructs containing the cryptic heparin-binding region of III1 to stimulate cell growth and contractility suggests that one mechanism by which matrix FN gives rise to distinct cellular phenotypes is through the exposure of a neoepitope within III1 during FN matrix deposition or remodeling. Both the III1H-containing constructs, as well as intact FN, colocalized with caveolin and fractionated with caveolin-enriched membrane domains by a mechanism that required HSPGs. Moreover, localization of FN to caveolin-enriched fractions was blocked by an inhibitor of FN polymerization, suggesting that the matrix form of FN specifically translocates to lipid rafts. In addition, disruption of caveolae inhibited both the localization and the cellular responses to the III1H constructs and intact FN. Taken together, these data suggest that during ECM remodeling, a portion of matrix FN partitions into lipid rafts and differentially regulates cytoskeletal organization and cell growth, in part, through the exposure of a matricryptic, heparin-binding site within the III1 module.

The loss of lipid-associated FN upon treatment of cells with the inhibitor of FN polymerization, 9D2 mAb (Cherneousov et al., 1991), suggests that the association of FN with lipid rafts occurs as FN is either assembled or remodeled within the ECM. Furthermore, the cholesterol-binding compound nystatin does not inhibit FN matrix deposition (unpublished data). These data suggest that the formation of an FN matrix does not depend on FN localization to lipid rafts. Rather, these data indicate that as a consequence of FN matrix deposition, a portion of the matrix FN translocates to lipid rafts where it modulates cell function. In support of this, previous work has shown that cell cycle progression is altered in cells that have assembled a matrix consisting of an FN construct in which modules III1–7 were deleted (Sechler and Schwarzbaucer, 1998).

Previous studies have demonstrated that treatment of cells with an isolated fragment of III1, termed III1C (NH2 terminus → Asn600), inhibits lysophosphatidic acid–mediated actin organization and tyrosine phosphorylation (Bourdoulous et al., 1998), decreases cell migration (Morla et al., 1994), and inhibits tumor cell angiogenesis (Yi and Ruoslahti, 2001). The ability of III1C to affect cytoskeletal function has been attributed to its ability to either disrupt preexisting FN matrices (Bourdoulous et al., 1998) or to stimulate increased FN matrix deposition (Yi and Ruoslahti, 2001), depending upon its concentration. In the present study, the III1H-mediated increases in cell growth and contraction occurred in the absence of any endogenous FN. These results indicate that, in addition to its ability to affect cellular events by altering matrix composition, the III1 module of FN can also directly affect cell function.

Sottile et al. (2000) recently demonstrated that HSPGs play a role in the stimulation of cell growth induced by FN polymerization. Similarly, in the present study, the stimulation of cell growth by GST–III1H,8–10 was inhibited by either heparin or pretreatment of cells with heparinase. In addition, hepar-
arinase inhibited the localization of both GST–III1H,2–4 and intact FN to caveolae. These data suggest that HSPGs function as the cell surface receptor for the heparin-binding region of III1 and further, that a similar HSPG-dependent mechanism is involved in the partitioning of III1H and matrix FN into caveolae. Previous studies have localized the stress fiber-promoting activity of the COOH-terminal heparin-binding domain of FN to III13 (Bloom et al., 1999). In the present study, GST–III8–10,13 was unable to increase cell growth or contraction and did not localize to caveolae. However, addition of excess GST–III12–13 to cells inhibited GST–III1H,8–10-stimulated growth, suggesting that III13 can compete with III1H for binding to the glycosaminoglycan chains of the HSPGs. Additional interactions, possibly involving the core protein of the HSPG (Woods, 2001), likely contribute to the subsequent generation of distinct intracellular signals that modulate cell growth and contraction.

The molecular processing of information received by cells from their extracellular environment occurs in specific membrane domains, including caveolae, focal adhesions, and areas of cell–cell contact. Caveolae exist as cholesterol- and sphingolipid-enriched microdomains within the cell membrane and play a key role in signal transduction events (Brown and London, 1998). Several growth factor receptor tyrosine kinases as well as nonreceptor signaling proteins, including Src family kinases, MAPK, and PKCα, have been shown to localize to caveolae (Brown and London, 1998). The ability of heparin-binding III1 FN fragments, as well as matrix FN, to partition to caveolin-enriched microdomains represents a novel mechanism by which signals derived from the ECM may be transmitted to the interior of the cell to modulate growth and contractility.

During wound healing, migrating epithelial cells and fibroblasts deposit and remodel the ECM in order to support further cell ingrowth and neovascularization (Clark, 1995). Under normal circumstances, the synthesis of ECM proteins gradually decreases during tissue repair (Clark, 1995). However, in the absence of appropriate inhibitory signals, the synthetic phenotype of wound fibroblasts persists and can lead to the development of fibrotic changes within tissues, including excess deposition of FN, stimulation of fibroblast proliferation, and III1H,8–10-stimulated growth. Thus, the core protein of the HSPG (Woods, 2001), likely contributes to the subsequent generation of distinct intracellular signals that modulate cell growth and contraction.

Materials and methods

**Reagents**

Human plasma FN was isolated from Cohn’s fraction I and II, as previously described (Miekkä et al., 1982). The NH₂-terminal 70-kD FN fragment was generated as previously described (Hocking et al., 1994). Vitronectin was purified from fibrinogen-depleted human plasma (Hocking et al., 1999). Unless otherwise indicated, chemical reagents were from Sigma-Aldrich.

**Cell culture**

Mouse embryo cells, derived from FN⁻⁻ embryos and adapted to grow under serum-free conditions (Sottile et al., 1998), were provided by Jane Sottile (University of Rochester, Rochester, NY). FN⁻⁻ cells were cultured on collagen-coated dishes in a 1:1 mixture of Cellgro (Mediatech) and Am V (Life Technologies).

**Recombinant FNs**

Fig. 10 illustrates the FN constructs used in this study. PCR was used to amplify human FN cDNA (a gift of Jean Thiery, Institut Curie, Paris, France) encoding the following amino acids (Petersen et al., 1989): a COOH-terminal fragment of the first type III module (III1H; bases 1802–2032); III1H plus the II2 module (III1H;2; bases 1802–2347); III1H plus the II2 and III3 modules (III1H;2–3; bases 1802–2615; III1H plus the II3, III4, and III modules (III1H,2–4; bases 1802–2905; Ile 597–Thr 964), the full-length first through fourth type III modules (III1–4; bases 1745–2905; Ser 578–Thr 1600), and the II1H module (II1H; bases 2132–2347; Val 707–Thr 778), the 8–10th type III modules (II8–10; bases 3716–4565; Ala 1326–Thr 1600), and the III1 module (III13; bases 5357–5623; Asn 1873–Thr 1961). The sense primer for III1H,1H,2, III1H,2–3, and III1H,2–4 (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) contains an BamHI site (shown in bold) at the 5′ end. The sense primer for III8–10 and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) includes a BglII site. The sense primer for III9–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) was synthesized with a Cla site. The antisense primers for III1H (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) were designed using the same as those for III1H and III2H. The antisense primers for III1H (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) contain an EcoRI site. The antisense primers for III8–10 (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) contain a Smal site. III1H,8–10 and III8–10,13 were made by PCR amplification of either III1H or III2H with BamHI sites at both the 5′ and 3′ ends. The sense primers used were the same as those for III1H and III2H. The antisense primers for III1H (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) were synthesized with a Cla site, which generates two additional amino acids (Ile and Asp) between III1H and III2H. The sense primer for III1H,1H,2, III1H,2–3, and III1H,2–4 (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) contains an BamHI site (shown in bold) at the 5′ end. The sense primer for III8–10 and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) includes a BglII site. The sense primer for III9–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) was synthesized with a Cla site. The antisense primers for III1H (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) were designed using the same as those for III1H and III2H. The antisense primers for III1H (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) contain an EcoRI site. The antisense primers for III8–10 (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) contain a Smal site. III1H,8–10 and III8–10,13 were made by PCR amplification of either III1H or III2H with BamHI sites at both the 5′ and 3′ ends. These restriction enzyme sites generate two additional amino acids (Ile and Asp) between III1H and III2H. The sense primer for III1H (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) were synthesized with a Cla site, which generates two additional amino acids (Ile and Asp) between III1H and III2H. The sense primer for III1H,1H,2, III1H,2–3, and III1H,2–4 (5’-CCCGGATCCATCGTGGAAATGCACACAG3′) contains an BamHI site (shown in bold) at the 5′ end. The sense primer for III8–10 and III8–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) includes a BglII site. The sense primer for III9–10,13 (5’-CCCGGAACGCCCGCTCGCTCCCTCACCTAGACAG3′) was synthesized with a Cla site.
CATCTTCTCG–3′ (antisense). The outer primers were the same as those used to amplify nonmutant III9–10 (Hocking et al., 1999). The amplification of III1, III9–10, III12–13, and the heparin-binding domain of vitronectin have been previously described (Hocking et al., 1999). The PCR products were cloned into pGEX-2T (Amersham Biosciences) and translocated into DH5α bacteria (Sambrook et al., 1989). Proteins were isolated on glutathione–agarose (Amersham Biosciences) (Hocking et al., 1994). GST–III1 and GST–III1H were digested with TPCK–trypsin (Hocking et al., 1994). III1 was separated from GST by passing the digested material over SP-Sephadex C–25 (Amersham Biosciences). III1H was separated from GST by retention on heparin–Sepharose (Amersham Biosciences). GST–III8–10, 13 bound to heparin-Sepharose and eluted at a concentration of 0.628 ± 0.009 M NaCl. By comparison, the 30–40 COOH-terminal heparin-binding FN fragment elutes at 0.63 M NaCl (Ingham et al., 1990).

**Antibodies**

Antibodies and their sources are as follows: anti-FN III IgG (9D2; Chernousov et al., 1991; a gift from Deane Mosher, University of Wisconsin, Madison, WI); mouse IgG (Cappel); anticaenovirus polyclonal and LAMP-1 mAbs (BD Transduction Laboratories); anti-GST monoclonal and polyclonal IgG (Upstate Biotechnology); anti-vinuclear mAb, anti-β-actin mAb, and anti-FN polyclonal IgGs (Sigma–Aldrich).

**Growth assays**

Growth assays were conducted as previously described (Sottile et al., 1998). For some experiments, recombinant FN constructs, nystatin, cycloheximide, or heparinase I (5 μU/ml; Seikagaku Corp.) were added to collagen–agarose FN gels 30 min before the addition of proteins. Heparinase treatment was repeated every 24 h. After a 3- or 4-d incubation, cells were fixed, stained with crystal violet, and the absorbance at 590 nm was determined (Sottile et al., 1998).

**Collagen gel contraction assays**

Floating type I collagen gels were prepared as previously described (Hocking et al., 2000). Collagen gels imbedded with cells were incubated for 20 h and then removed from the wells and weighed. Collagen gel contraction was measured as a decrease in gel weight (Hocking et al., 2000). Data are reported as percent contraction: (1 − weight of the test gels/weight of gels not containing cells) × 100.

**Solid-state heparin binding assay**

Glutathione-coated 96-well plates (Pierce Chemical Co.) were incubated with saturating concentrations of proteins (400 nM; 25 μg/ml GST–III8–10,13) in TBS with 0.5% Tween. Plates were then washed and incubated with heparin–albumin–biotin followed by HRP–NeutrAvidin (Pierce Chemical Co.). Parallel plates were incubated with a polyclonal anti-GST antibody followed by an HRP–conjugated goat anti–rabbit IgG (Bio-Rad Laboratories). The assay was developed using 2,2′-azino–bis (3-ethylbenzthiazolium sulfonyl) acid and the absorbance at 405 nm was measured.

**Statistical analysis**

Data are expressed as mean ± SEM and represent one of at least three separate experiments performed in triplicate or quadruplicate. Significance was determined using one-way analysis of variance (ANOVA) or the t test for unpaired samples. Differences <0.05 were considered significant.

**Immunofluorescence microscopy**

Collagen-adherent FN−/− cells were incubated for 5 h with 250 nM of the GST fusion proteins. For inhibition studies, cells were pretreated for 0.5 h with 2.5 μg/ml filipin III or an equal volume of ethanol, 5 μg/ml heparinase I (Seikagaku Corp.), 25 U/ml neuraminidase (Calbiochem–Novabiochem), or an equal volume of MBS. Samples were placed in a centrifuge tube and overlaid with 4 ml of each of 30 Triton extraction or 15% (carbonate extraction) sucrose followed by 5% sucrose in MBS. Samples were centrifuged at 200,000 g at 4°C for 15 h. Sequential 1-ml fractions were collected and aliquots were analyzed by PAGE and immunoblotting.

**Immunoblotting**

PAGE and immunoblotting were performed as previously described (Hocking et al., 2000). 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% BSA followed by a goat anti–rabbit, –mouse, or –rat HRP–linked secondary antibody. Blots were developed using ECL (Amersham Biosciences). After detection, blots were stripped by incubation with 0.2 M glycine, 0.1% SDS, 0.1% Tween, pH 4.0 (Miller et al., 1997). Blots were then washed, reblocked, and reprobed.

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