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Cell adhesion and signaling on the fibronectin 1st type III repeat; requisite roles for cell surface proteoglycans and integrins

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Abstract

Background: The first type III repeat of fibronectin is known to be involved in fibronectin matrix assembly, and recombinant proteins from this type III repeat can inhibit cell proliferation, tumor metastasis and angiogenesis. We have analyzed the way rat aortic smooth muscle cells (RASMCs) interact with a recombinant protein encompassing a C-terminal portion of the first type III repeat of fibronectin (protein III1-C).

Results: Cells are able to adhere to and spread on III1-C coated on a dish. Both β1 integrins and cell surface heparan sulfate proteoglycans serve as receptors for III1-C. For example, cell attachment to III1-C is partially inhibited by agents that block β1 integrins or by heparin. Complete inhibition of cell attachment is seen only when integrin blocking agents are combined with heparin. Affinity chromatography revealed the binding of proteins that likely represent the integrin β1 and α5 submits to a III1-C column. Cell adhesion to III1-C results in robust ERK1/2 activation that is blocked by integrin-blocking agents. In addition, cell adhesion to III1-C and ERK1/2 activation by III1-C are both inhibited by heparan sulfate but not by chondroitin sulfate. Moreover, heparitinase treatment, but not chondroitinase treatment of RASMCs results in reduced cell adhesion and ERK1/2 activation. Affinity chromatography experiments demonstrated that $^{35}SO_4$-labeled cell surface heparan sulfate proteoglycans bound specifically to III1-C.

Conclusions: The results suggest that the 1st type III repeat of fibronectin contains a previously unrecognized cell adhesion domain that stimulates robust ERK1/2 activation in RASMCs. Cells interact with this domain through cell surface heparan sulfate proteoglycans and integrins, and both classes of receptors are required for optimal cell adhesion and ERK1/2 activation.

Background

Fibronectin can control many aspects of cell behavior, including cell growth, migration and differentiation [1]. Fibronectin exists in the blood as a dimer, but in tissues it is in the form of an insoluble fibrillar matrix. The fibrillar form of fibronectin is thought to be the most relevant form in vivo because this is the form with which most cells interact [1]. Various lines of evidence suggest that the fibrillar matrix form of fibronectin exerts effects on cells that are not duplicated by the dimeric form of fibronectin. For example, high concentrations of dimeric fibronectin enhance cell migration whereas high concentrations of fibrillar fibronectin reduce cell migration [2]. Also, inhibition of fibronectin matrix assembly has been

shown to inhibit cell growth in various systems [3–5]. These findings suggest that fibrillar fibronectin may stimulate signal transduction pathways in cells that are either not stimulated, or only marginally stimulated, by dimeric fibronectin.

Fibronectin matrix assembly is a cell-mediated process that requires the activity of integrins [1,6–8]. The integrin that is primarily responsible for assembling fibronectin into the matrix is $\alpha5\beta1$, although $\alpha v\beta3$, $\alpha IIb\beta3$ and $\alpha4\beta1$ can also function in this capacity [9–14]. In addition to integrins, fibronectin matrix assembly also depends on self-association sites within fibronectin. For example, the N-terminal 70 kDa region, the 1st type III repeat and the 10th type III repeat are thought to be important for the proper alignment of fibronectin molecules during matrix assembly [15–22]. We and others have shown that a recombinant protein representing a portion of the first type III repeat (protein III1-C) can affect fibronectin matrix assembly and cell growth [2–4,16]. Moreover, a mutant recombinant fibronectin molecule that is lacking the first seven type III repeats has been shown to lead to defective fibronectin matrix assembly in cell culture and in vivo, and to inhibition of cell proliferation [23,24]. Although the matrix produced with fibronectin lacking the first seven type III repeats was somewhat aberrant, this study does show that some matrix assembly can occur without the first type III repeat. Therefore, several approaches reveal that the fibronectin type III repeats, and especially the first type III repeat, play important roles in the regulation of fibronectin matrix assembly and cell growth.

III1-C is known to inhibit cell proliferation [3,4]. Recently, III1-C (also known as anastellin) has been shown to inhibit angiogenesis, and tumor growth and metastasis [25]. In the present study we examine how cells interact with and respond to III1-C, with a particular focus on signaling through the Ras/ERK pathway. We find that cells can adhere and spread on III1-C and that two classes of receptors function in cell adhesion to III1-C; integrins and cell-surface proteoglycans.

Results

Cell attachment and spreading on the first type III repeat of fibronectin

III1-C is a recombinant fibronectin fragment that encompasses most of the first type III repeat, but is missing the A and B β-strands of the repeat [2]. We had previously found that III1-C inhibited the growth of normal diploid smooth muscle cells (SMCs) in culture [4]. During these experiments we noticed that the presence of III1-C in the culture medium affected the morphology of cells at early times after plating. As shown in Figure 1, cells plated onto fibronectin coated dishes attached and spread well, typically containing numerous lamellipodia per cell (Fig. 1A, panel FN). Cells seeded onto fibronectin coated dishes with III1-C in the culture medium spread more extensively and in a more circular pattern at early times after plating than cells plated on fibronectin in the absence of III1-C (Fig. 1A, panels FN and FN+C, and Fig. 1B). Cells cultured on fibronectin for 4 hours show approximately the same extent of spreading as cells on fibronectin plus III1-C (Fig. 1B), indicating that III1-C accelerated the rate of spreading of the cells but did not affect the eventual extent of spreading. The effect on cell morphology was specific for III1-C because the negative control protein III 11-C (a recombinant protein derived from the 11th type III repeat in fibronectin, which, similar to III1-C, is missing the A and B β-strands of this repeat) had no effect on the morphology of the cells (Fig. 1A, panel FN+11C, and Fig. 1B). Interestingly, the effect of III1-C on cell morphology was seen even when the plates had *not* been coated with fibronectin (Fig. 1A, panel C, and Fig. 1B), indicating that intact fibronectin was not required for cell attachment and spreading when III1-C was in the culture medium. Again, the negative control protein III 11-C had no effect on cell morphology; cells seeded onto uncoated plates with III 11-C in the culture medium remained rounded and did not appreciably spread over several hours (Fig. 1A, panel 11C, and Fig. 1B). These results indicated that III1-C had an effect on the morphology of freshly plated cells. Moreover, the plates did not need to be coated with any adhesive protein prior to seeding the cells with III1-C in the culture medium, indicating that III1-C may have adsorbed onto the tissue culture plastic and that cells then attached and spread on the adsorbed III1-C.

To directly test whether cells were able to bind to III1-C coated on a dish, various concentrations of either fibronectin, III1-C or III 11-C were coated onto dishes and cell attachment to these substrates was measured. As shown in Figure 2, cells attach well to fibronectin and to III1-C, but not to III 11-C coated on a dish. On a molar basis, III1-C was less effective at supporting cell attachment than fibronectin, however, it was clear that RASMCs do attach and spread well on III1-C coated on a dish. The morphology of the cells seeded onto the III1-C–coated wells was indistinguishable from the morphology shown in Fig. 1A, panel C.

Role of integrins in cell attachment to III1-C

The attachment of RASMCs to III1-C was analyzed further to determine the role played by integrins as potential adhesive receptors for III1-C. Various integrin-blocking reagents were tested for their ability to block RASMC attachment to III1-C and to fibronectin. The adhesion of SMCs to fibronectin is mediated through β1 integrins [1,26]. We confirmed these previous findings for

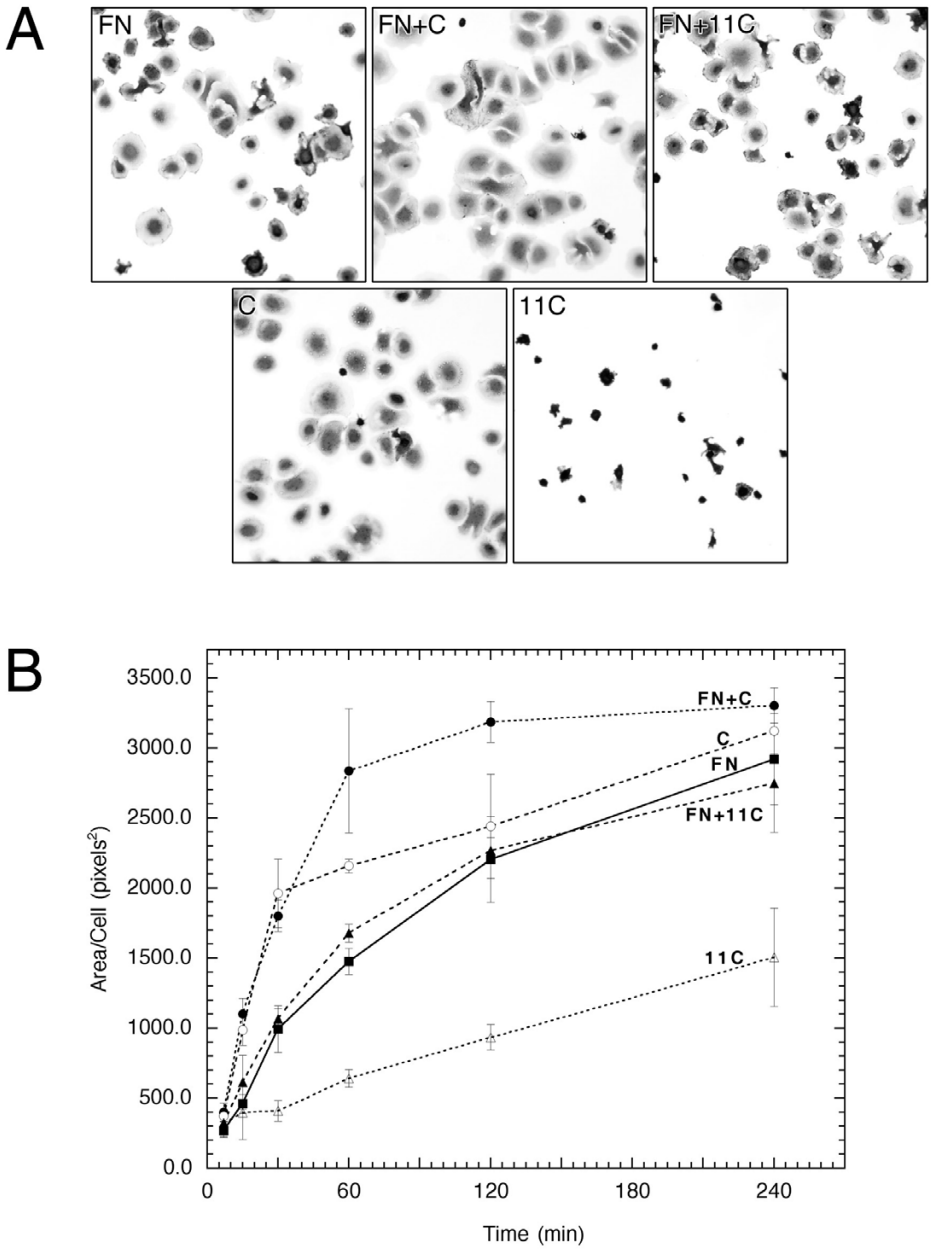

Figure 1
Effect of III1-C on cell spreading. Panel A. RASMCs were plated onto either uncoated dishes (C and 11C panels) or dishes coated with 20 μg/ml fibronectin (FN, FN+C and FN+11C panels) in the presence of either no recombinant protein in the media (FN panel), or 25 μM III1-C (FN+C and C panels), or 25 μM III 11-C in the media (FN+11C and 11C panels). After 30 min at 37°C the cells were fixed, stained with coomassie blue and photographed under a light microscope. Bar equals 100 μm. **Panel B.** Quantitation of cell spreading over time in the culture conditions described for Panel A. Each data point is the average of four samples; similar results were obtained from four independent experiments.

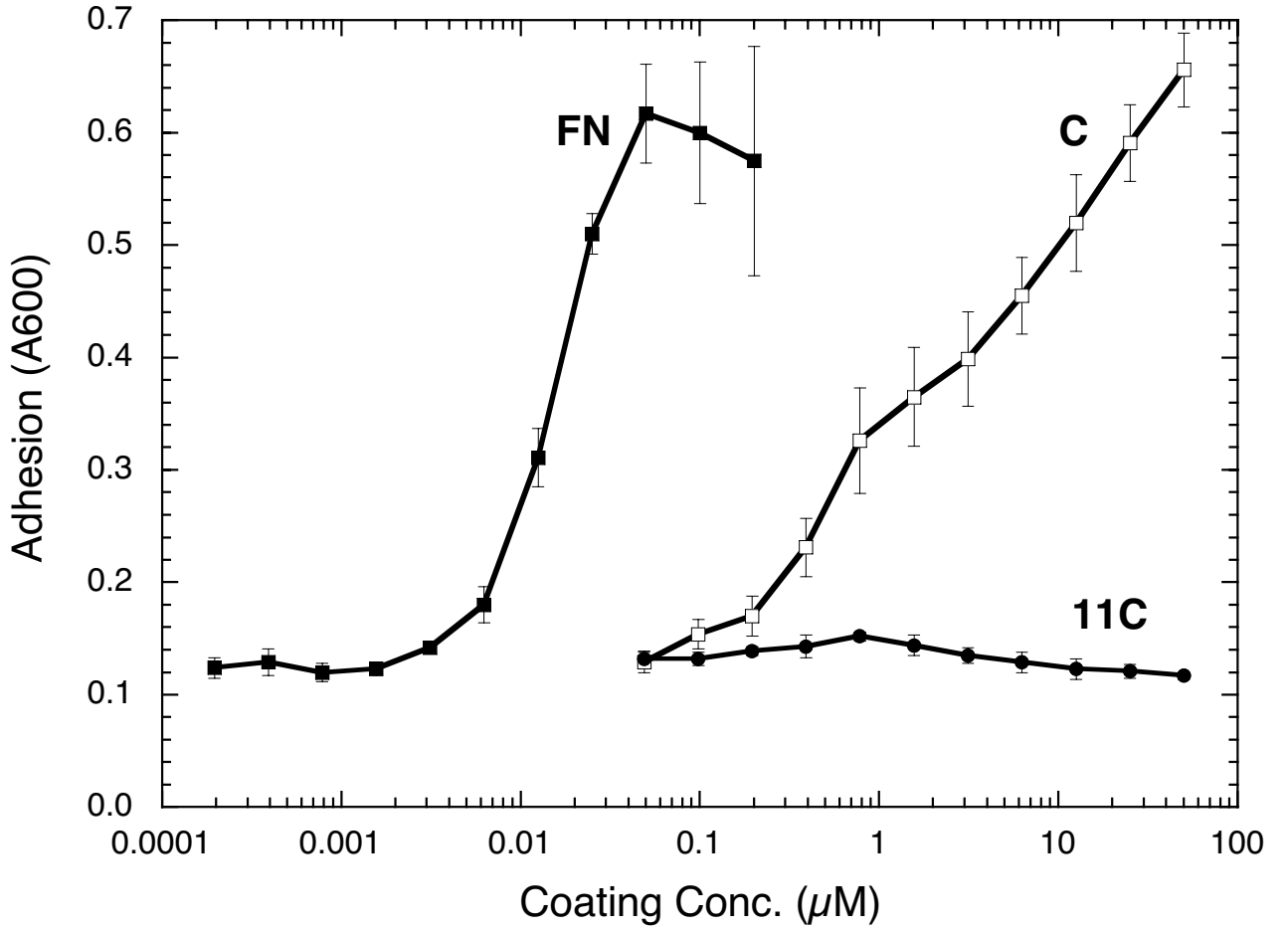

Figure 2
Cell attachment to III1-C. Fibronectin (■), III1-C (□) or III 11-C (●) at various concentrations were coated onto plastic wells. RASMCs were seeded onto the coated wells and the cells were allowed to adhere for 30 min at 37°C. The cells were fixed, stained and the amount of cell attachment was measured. Each sample was performed in triplicate and the experiment was done three times with comparable results each time.

our RASMCs, by showing a complete block of cell attachment to fibronectin by EDTA (an agent that blocks all integrins), anti-β1 blocking mAb, and RGD peptide, but not by the negative control RAD peptide (Fig. 3A). Anti-fibronectin antibodies also effectively blocked RASMC attachment to fibronectin (Fig. 3A). Heparin did not inhibit cell attachment to fibronectin (Fig. 3A), however, it did modestly reduce cell spreading on fibronectin (15% inhibition of cell spreading, data not shown). These results indicate that RASMC adhesion to fibronectin is mediated by β1 integrins.

Blocking studies indicated that cell attachment to III1-C was partially mediated by β1 integrins. For example, RASMC attachment was partially blocked by EDTA, anti-β1 mAb, and RGD peptide (Fig. 3B), indicating the involvement of integrins. However, cell attachment was also partially blocked by heparin, which did not inhibit cell attachment to fibronectin (Fig. 3A and 3B, Hep samples), indicating that a non-integrin receptor also mediated cell attachment to III1-C. A complete block of cell attachment to III1-C was only observed when heparin was combined with integrin blocking agents; e.g., heparin + anti-β1 mAb or heparin + RGD peptide (Fig. 3B). Anti-fibronectin antibodies modestly inhibited cell attachment, but anti-III 1-C antibodies effectively blocked cell attachment to III1-C (Fig. 3B). These results demonstrate that cell attachment to III1-C was mediated through both β1 integrins and another class of receptors on RASMCs.

One way in which integrins were originally shown to be fibronectin receptors was by affinity chromatography of radioiodinated cell surface proteins on a fibronectin col-

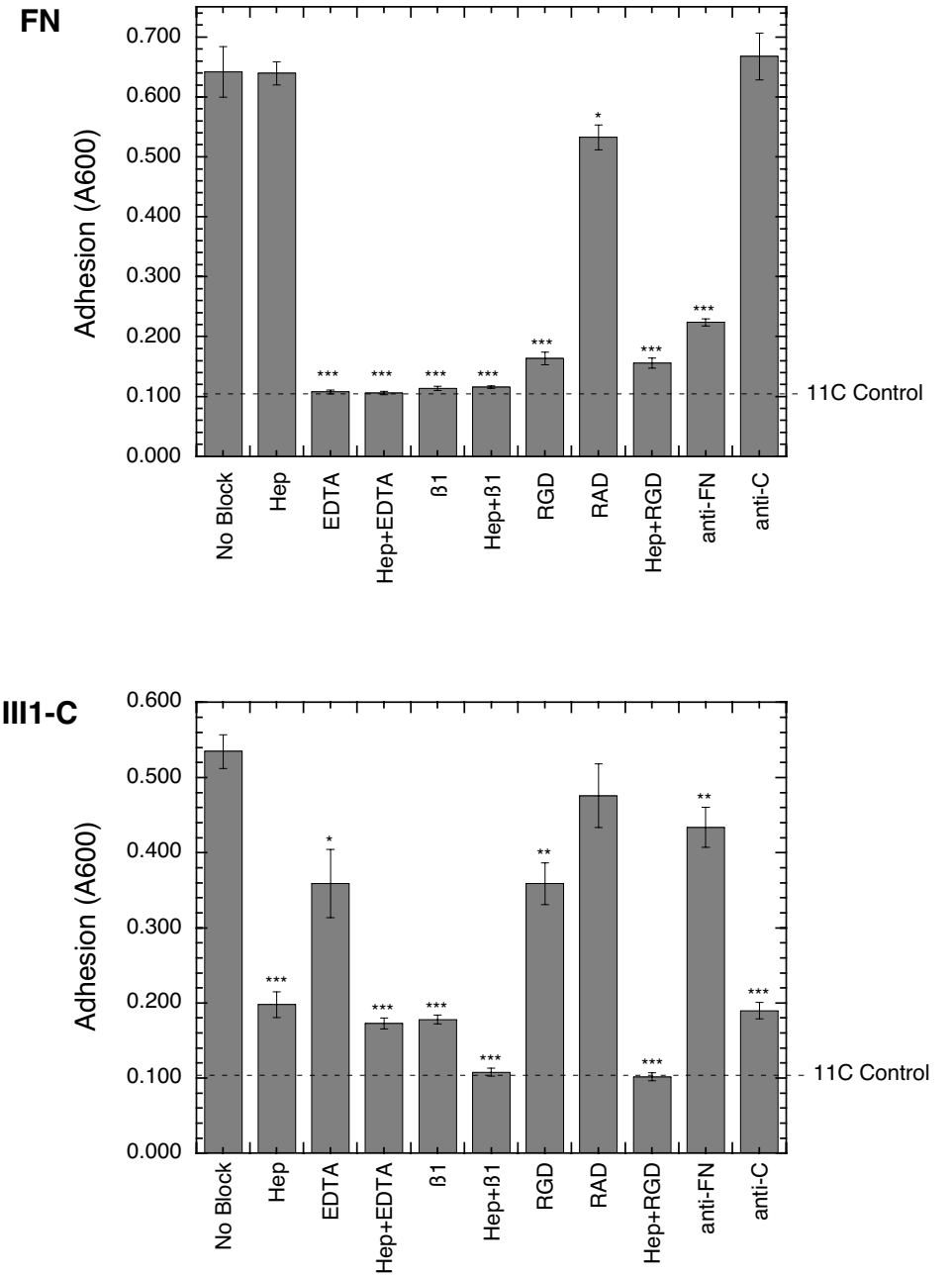

Figure 3
Blocking of cell attachment to III1-C RASMCs were seeded onto wells coated with either 20 μg/ml FN (top panel), or 25 μM III1-C (bottom panel) in the absence or presence of various inhibitors. The inhibitors were 100 μg/ml heparin (Hep), 10 mM EDTA (EDTA), 50 μg/ml anti-β1 blocking mAb Ha2/5 (pi), 0.5 mg/ml GRGDSP peptide (RGD), 0.5 mg/ml GRADSP peptide (RAD), 100 μg/ml anti-FN polyclonal antibody (anti-FN), and 60 μg/ml anti-IIII-C polyclonal antibody (anti-C). After 30 min at 37°C the cells were fixed, stained with crystal violet and the amount of cell attachment was measured. The dashed line indicates the amount of signal seen on the negative control protein III 11-C, and represents the background A600 reading in these experiments. Statistical analysis was done by Student's T-test (2-tailed); * $p < .05$, ** $p < .005$, *** $p < .0005$. Each sample was done in quadruplicate and the experiment was performed three times with similar results each time.

umn [27,28]. Consistent with these earlier studies, cell surface radioiodination of RASMCs followed by affinity chromatography on fibronectin Sepharose and elution with EDTA revealed the binding of two major bands at 120 kDa and 140 kDa (Fig. 4,E lane under FN). These sizes match those of the β1 and α5 integrin submits, respectively, that have been shown by affinity chromatography to bind to the cell binding domain (i.e., 10th type III repeat) of fibronectin [27–31]. Affinity chromatography on a III1-C Sepharose column revealed the binding of three major bands of sizes 120 kDa, 140 kDa and 150 kDa (Fig. 4,E lane under C). The 120 kDa and 140 kDa bands likely represent the β1 and α5 subunits (as above), while the 150 kDa band may represent either the α2 or α3 integrin subunits, which are known to be expressed by VSMCs [26]. Affinity chromatography on the control III 11-C column yielded no radioiodinated proteins that were eluted from the column (Fig. 4,E lane under 11C). These results are consistent with the hypothesis that one or more pi integrins act as receptors for III1-C. Taken together with the integrin-blocking results described above, it is clear that RASMC adhesion to III1-C is partially mediated through β1 integrins.

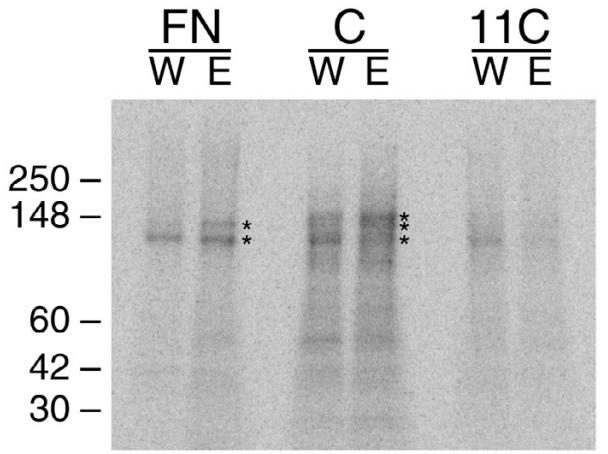

Figure 4
Affinity chromatography of ^{125}I surface labeled proteins on III1-C Sepharose RASMCs were surface labeled with ^{125} I, lysed in octylglucoside lysis buffer, and these cell lysates were passed over affinity columns containing either FN (FN), III1-C (C), or III 11-C (11C). The columns were washed and bound proteins were eluted with 10 mM EDTA in lysis buffer. Samples from the final wash before elution (W lanes), and from the elution (E lanes) fractions of each column were analyzed by SDS-PAGE and phosphorimager analysis. Numbers to the left of the panel indicate the migration of molecular mass markers (kDa). Asterisks mark the positions of bands that bound to and were eluted from the FN and III1-C columns.

Cell adhesion to III1-C activates ERK1/2

Integrins are known to activate signaling pathways in cells [32–35]. Among the most well-studied signaling pathways is the Ras/MAPK pathway leading to activation of ERK1 and ERK2 [36]. We therefore decided to test the effect of cell adhesion to III1-C on ERK activation. As expected, treatment of RASMCs with PDGF resulted in activation of ERK1/2 (Fig. 5, PDGF lanes). Plating RASMCs onto a fibronectin coated dish in the absence of growth factors yielded moderate activation of ERK1/2, which peaked at 30 min then decreased somewhat over the next 30 min of the experiment (Fig. 5, bottom panel). However, plating cells onto a III1-C coated dish gave strong activation of ERK1/2, which peaked at 30 min and remained relatively constant for the subsequent 30 min (Fig. 5, top panel). The control protein III 11-C did not appreciably stimulate ERK1/2 phosphorylation (Fig. 5, 11C lanes, both panels). Thus, the adhesion of RASMCs to III1-C resulted in robust activation of ERK1/2 that was comparable to the activation obtained with PDGF.

ERK activation by III1-C is dependent on β1 integrins

As shown above, several agents that block β1 integrin function partially inhibit cell attachment to III1-C. We therefore tested the effects of these agents on ERK1/2 activation by III1-C. The results obtained with ERK1/2 activation closely paralleled the results obtained with cell attachment to III1-C, except that the individual blocking agents were relatively more efficient at inhibiting ERK1/2 activation. For example, heparin, EDTA, anti-β1 mAb and RGD peptide all significantly inhibited ERK1/2 activation by cell adhesion to III1-C (Fig. 6). Heparin was most effective and completely blocked ERK1/2 activation. (Fig. 6). In contrast, the negative control RAD peptide did not inhibit ERK1/2 activation by III1-C (Fig. 6, C+RAD lane). These results indicate that both integrins and the class of receptors inhibited by heparin were required for III1-C mediated activation of ERK1/2.

Role of heparan sulfate proteoglycans in cell adhesion to III1-C and ERK1/2 activation by III1-C

Because heparin was able to inhibit the attachment of cells to III1-C and the activation of ERK1/2 by III1-C, we tested the effect of other glycosaminoglycans on III1-C stimulated ERK1/2 activity. Heparin, heparan sulfate and dermatan sulfate blocked ERK1/2 activation by III1-C (Fig. 7), and cell adhesion to III1-C (not shown). In contrast, hyaluronic acid, chondroitin-4-sulfate and chondroitin-6-sulfate had no effect on the ability of III1-C to stimulate ERK1/2 activity (Fig. 7) or on the adhesion of cells to III1-C (not shown). It is possible that heparin, heparan sulfate and dermatan sulfate inhibit III1-C mediated cell adhesion and ERK1/2 activation due to common chemical features. For example, heparin, heparan

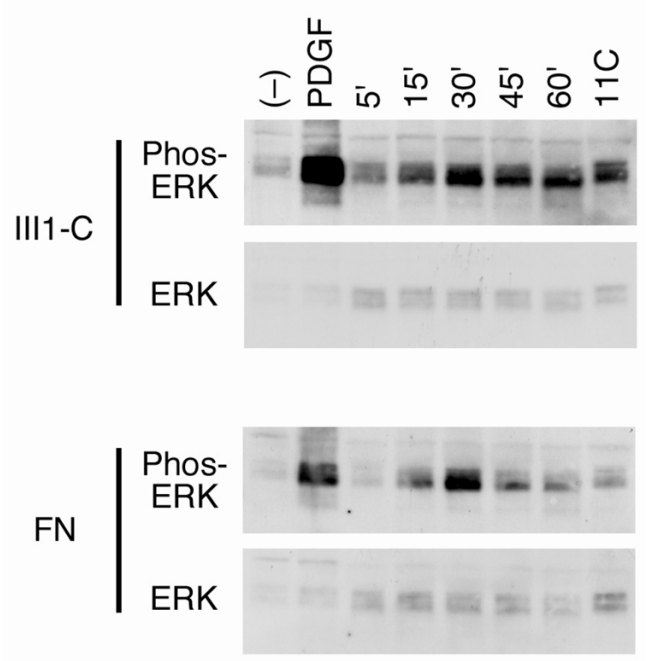

Figure 5
Activation of ERK by cell adhesion to III1-C. RASMCs were collected into DMEM + 0.5% BSA and either kept in suspension in the absence (-) or presence of 2 ng/ml PDGF (PDGF), or were seeded onto dishes precoated with either 20 μg/ml FN (FN), 25 μM III1-C (C), or 25 μM III 11-C (11C). Note that the only cells that received growth factor are the cells in the PDGF lane, which received the growth factor 10 min before lysis. After 5, 15, 30, 45 or 60 min at 37°C the cells were lysed in sample buffer and analyzed by inununoblotting with anti-phospho-p44/42 MAPK antibodies (Phos-ERK panel). The blot was then stripped and reprobed with anti-ERK antibodies (ERK panel) to analyze protein loading. This experiment was done more than 3 times with similar results each time.

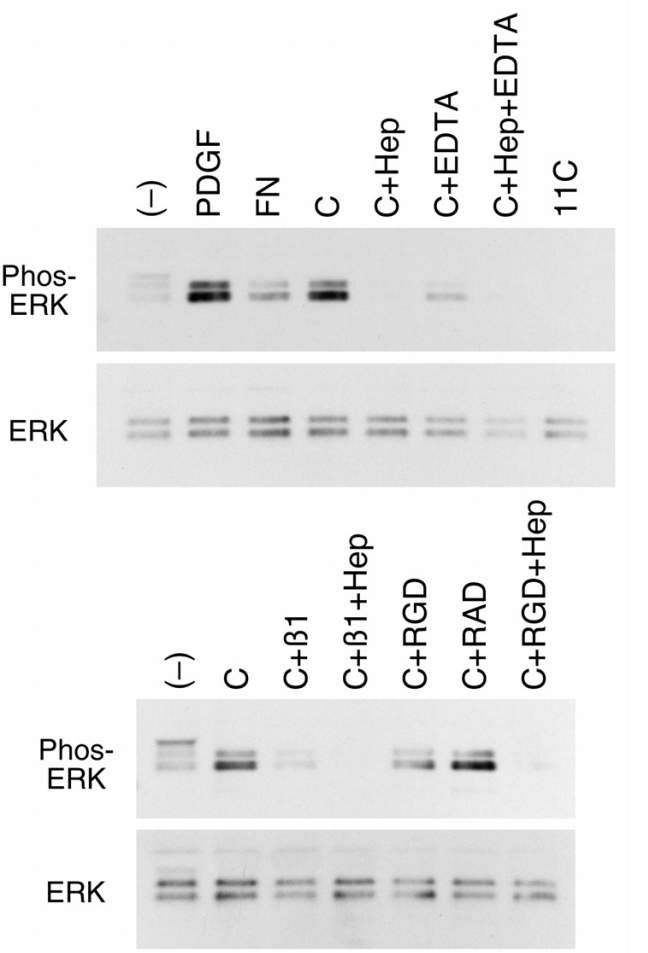

Figure 6
Inhibition of III1-C mediated ERK activation by various integrin inhibitors. RASMCs were collected in DMEM+0.5% BSA and were then either left in suspension in the absence (-) or presence of PDGF (PDGF), or plated onto dishes precoated with either fibronectin (FN), III1-C (C and C+... lanes), or III 11-C (11C), as described in the legend to Fig. 5. Some samples of cells plated onto III1-C also contained either 100 μg/ml heparin (C+Hep), 10 mM EDTA (C+EDTA), 50 μg/ml anti-β1 blocking mAb Ha2/5 (pi), 0.5 mg/ml GRGDSP peptide (RGD), 0.5 mg/ml GRADSP peptide (RAD), or combinations of heparin plus integrin inhibitors, as indicated. After 30 min at 37°C cells were lysed in SDS-PAGE sample buffer and samples were analyzed by immunoblotting with anti-phospho-p44/42 MAPK antibodies (Phos-ERK panels). Blots were then stripped and reprobed with anti-ERK antibodies (ERK panels) to analyze protein loading. The experiment was done 3 times with similar results each time.

sulfate, and dermatan sulfate all contain iduronic acid residues whereas chondroitin sulfate and hyaluronic acid do not. The results presented above indicate that a heparan sulfate proteoglycan (HSPG) or dermatan sulfate proteoglycan on the surface of RASMCs may serve as a receptor for III1-C.

To further explore the possibility that a cell surface proteoglycan serves as a receptor for III1-C, RASMCs were treated with the glycosaminoglycan-degrading enzymes seeded onto III1-C, and analyzed for the activation of ERK1/2. Heparitinase treatment, but not chondroitinase ABC treatment decreased the amount of ERK1/2 activation by III1-C (Fig. 8). Heparitinase treatment also inhibited cell adhesion by 25–40%, while chondroitinase ABC treatment did not inhibit cell adhesion to III1-C (not shown). This result is consistent with the idea that HSPGs but not chondroitin sulfate proteoglycans serve as signaling receptors for III1-C.

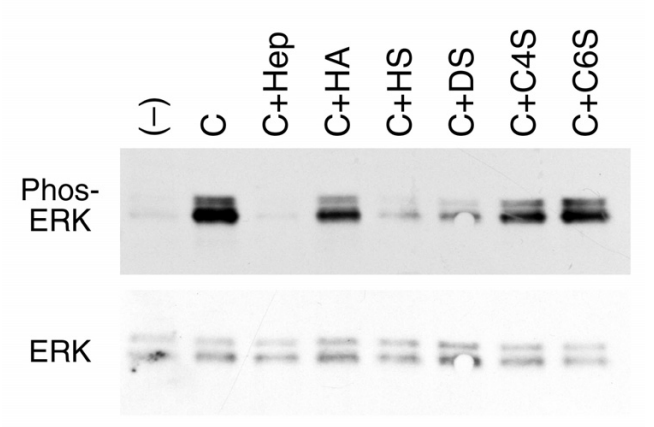

Figure 7
Inhibition of III1-C mediated ERK activation by glycosaminoglycans. RASMCs were collected into DMEM + 0.5% BSA and either kept in suspension (-) or cells were seeded onto dishes precoated with 25 μM III1-C (all other lanes). Cells seeded onto III1-C contained either no glycosaminoglycan in the medium (C) or 80 μg/ml heparin (C+Hep), hyaluronic acid (C+HA), heparan sulfate (C+HS), dermatan sulfate (C+DS), chondroitin-4-sulfate (C+C4S), or chondroitin-6-sulfate (C+C6S). After 30 min at 37°C cells were processed and analyzed by immunoblotting with anti-phospho-p44/42 MAPK antibodies (Phos-ERK panel). The blot was subsequently stripped and reprobed with anti-ERK antibodies (ERK panel) as described in the legend to Fig. 5. The experiment was done 3 times with similar results each time.

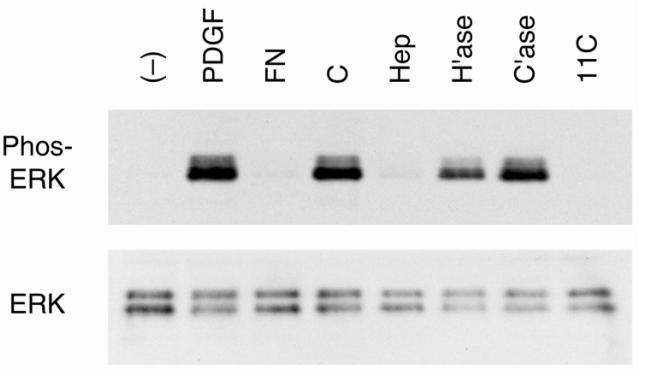

Figure 8
Inhibition of III1-C mediated ERK activation by heparitinase. RASMCs were collected in DMEM+0.5% BSA and were then treated in suspension with either no GAGase ((-), PDGF, FN, C, Hep, and 11C samples) or with 0.1 u/ml of heparitinase (H'ase), or 0.1 u/ml of chondroitinase ABC (C'ase) for 1 hr at 37°C. Cells were then either left in suspension in the absence (-) or presence of PDGF (PDGF) for 10 min, or cells were plated onto dishes precoated with either fibronectin (FN), III1-C (C, Hep, H'ase, and C'ase lanes), or III 11-C (11C). One sample of cells plated onto III1-C contained 100 μg/ml heparin (Hep) in the medium. Cell samples were analyzed by immunoblotting with anti-phospho-p44/42 MAPK antibodies (Phos-ERK panel) followed by anti-ERK antibodies (ERK panel) as described in the legend to Fig. 5. The experiment was performed four times with similar results each time.

To identify the cell surface proteoglycans that may serve as receptors for III1-C, RASMCs were labeled with $^{35}SO_4$ and cell lysates were applied to III1-C and III 11-C columns. The most highly labeled band, migrating in a smear at about 450 kDa, bound efficiently to the III1-C column (Fig. 9A, compare flow through and elution lanes). This proteoglycan resisted elution by 8 M urea but was eluted by boiling the Sepharose beads in SDS (Fig. 9A). This same proteoglycan did not bind appreciably to the III 11-C column (Fig. 9A). Heparitinase and chondroitinase ABC treatment of the $^{35}SO_4$ labeled samples revealed that the 450 kDa proteoglycan was composed mostly of HSPGs (Fig. 9B). These results indicate that most of the cell surface HSPGs of RASMCs can bind to III1-C in affinity chromatography.

RASMC HSPGs were analyzed further by immunoblotting with the monoclonal antibody 3G10 which specifically recognizes desaturated uronates on the heparan sulfate stubs that remain associated with core proteins after heparitinase digestion [37]. Complete digestion of RASMC lysates with heparitinase reveals major bands at 46 kDa and 70 kDa, indicating that these are the sizes of the major HSPG core proteins (not shown). RASMC lysates were subjected to partial heparitinase treatment (full digestion was not performed in order to allow some GAGs to remain associated with the core proteins) then applied to either III1-C or III 11-C Sepharose. As shown in Fig. 10, most of the 46 kDa and 70 kDa HSPGs bound to III1-C Sepharose but not to III 11-C Sepharose. In addition, the presence of heparin in the lysate inhibited the 46 kDa and 70 kDa HSPGs from binding to the III1-C column (Fig. 10, compare C and C•H lanes). The inhibition of 46 kDa and 70 kDa binding to III1-C by heparin suggests that these HSPGs may serve as receptors for III1-C in cell adhesion and ERK1/2 signaling.

Discussion

We have found that cells can interact with a portion of the fibronectin first type III repeat, leading to cell adhesion and activation of ERK, and that this signaling requires both cell surface HSPGs and integrins. RASMCs are able to adhere to and spread on III1-C. Adhesion to III1-C results in robust activation of ERK; comparable to that seen with PDGF stimulation. Several independent lines of evidence indicate that cell surface HSPGs and β1 integrins act as receptors for III1-C and are required for

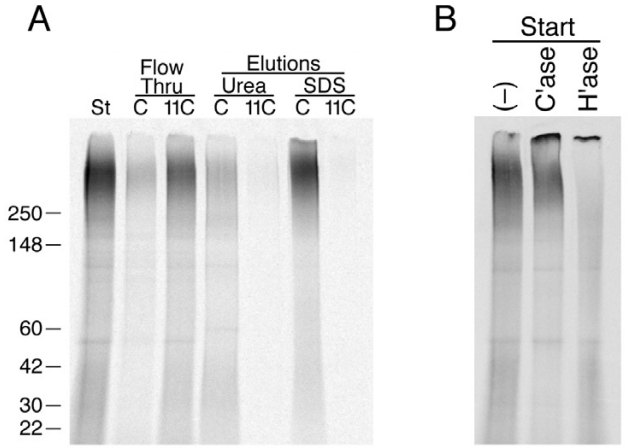

Figure 9
Affinity chromatography of $^{35}SO_4$-labeled proteoglycans on III1-C Sepharose. Panel A. RASMCs were labeled with $^{35}SO_4$, which preferentially labels the glycosaminoglycan chains of proteoglycans. Cells were lysed in NP40 buffer and lysates were applied to either III1-C Sepharose (C lanes) or III 11-C Sepharose (11C lanes) columns. The flow through fractions were collected (Flow Thru lanes), the columns were washed, and the bound material was eluted by first applying 8 M urea elution buffer (Urea lanes), washing the columns with PBS, then collecting the Sepharose beads and boiling them in SDS-PAGE sample buffer (SDS lanes). The lane labeled St shows a sample of the starting material. All samples were separated on SDS-PAGE gels then detected by phosphorimager analysis. Numbers to the left of the panel indicate the migration of molecular mass markers (kDa). **Panel B.** Samples of the starting material were treated with either no enzyme ((-)), or with 0.03 u/ml chondroitinase ABC (C'ase), or 0.03 u/ml heparitinase (H'ase), then analyzed by SDS-PAGE and phosphorimager. The experiment was performed twice with similar results both times.

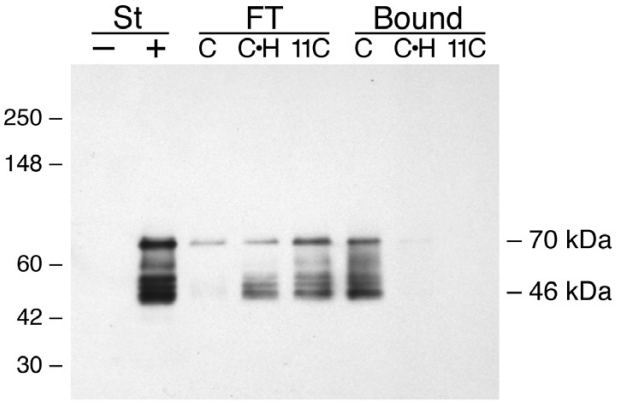

Figure 10
Affinity chromatography HSPGs on III1-C Sepharose. RASMCs were collected by trypsinization and lysed in NP40 buffer. The lysate was then treated either without (-) or with (+) 0.1 u/ml heparitinase for 1 hr at 37°C. Heparitinase treated samples were then applied to either III1-C Sepharose (lanes marked C) or III 11-C Sepharose (lanes marked 11C). One sample was applied to a III1-C Sepharose column in the presence of 0.5 mg/ml heparin (lanes marked C•H). The flow through fractions were collected (FT lanes), the columns were washed extensively, then the Sepharose beads were collected and boiled in SDS-PAGE sample buffer (Bound lanes). Samples were analyzed by immunoblotting with the 3G10 antibody. Antibody 3G10 recognizes the uronate stubs that remain associated with core proteins after heparitinase digestion. Thus, after heparitinase digestion 3G10 shows the sizes of HSPG core proteins. Note that the major HSPGs have core protein sizes of 46 kDa and 70 kDa, and both of these HSPGs bind to the III1-C column but not the III 11-C column. The experiment was performed twice with similar results both times.

cell adhesion and ERK activation by III1-C. For example, various β1 integrin blocking agents inhibit cell attachment to III1-C and ERK activation by III1-C. In addition, inhibition by heparin, heparan-sulfate, and heparitinase, but not by chondroitin sulfates or chondroitinase ABC all point to the importance of HSPGs as receptors. Affinity chromatography with ^{125}I surface labeled proteins resulted in the binding of proteins of the appropriate sizes of β1 integrins to both FN and III1-C columns. Moreover, affinity chromatography with $^{35}SO_4$-labeled cells suggests that HSPGs with core protein sizes of 46 kDa and 70 kDa serve as III1-C receptors. Taken together, these results indicate that the first type III repeat of fibronectin can serve as a cell binding domain and that both integrins and cell surface HSPGs function as receptors for this type III repeat.

Our results demonstrate that both integrins and cell surface HSPGs are required for robust ERK 1/2 activation when cells adhere to III1-C. These results are one of the first demonstrations that HSPGs can activate the ERK signaling pathway. Another example of this closely parallels our findings [38]. The adhesion of Jurkat T cells to thrombospondin-1 has been shown to require the cooperation of three classes of receptors; β1 integrins, CD47 and HSPGs [38]. Adhesion of the Jurkat T cells to thrombospondin-1 stimulates ERK1/2 activation and the binding of HSPGs to thrombospondin-1 is required for this signaling. The particular HSPGs that mediate ERK1/2 activation in Jurkat T cells have not been identified, however, the authors speculate that syndecans may participate in the adhesion and signaling in that system [38]. This may also be the case in our system, as described below.

One major family of cell surface HSPGs is the syndecan family. There are 4 members in the syndecan family (syndecan-1 through-4) [39,40]. Syndecan-1, -2 and -4 are known to bind to extracellular matrix proteins and syndecan-1 and -4 have been shown to support stress fiber formation in conjunction with integrins [41–45]. The HSPGs we have identified as III1-C receptors have core proteins that migrate at the approximate sizes of syndecan-1 (~69 kDa core protein) and syndecan-2 (~48 kDa core protein) [40]. It is therefore possible that our 46 kDa and 70 kDa HSPGs represent syndecan family members. As described above, the syndecans may cooperate with β1 integrins and CD47 to activate the ERK signaling pathway in T cells [38]. A similar mechanism may apply to the adhesion of RASMCs to III1-C. Future studies will determine the identity of the HSPGs that act as III1-C receptors.

The Ras/MAPK pathway is typically thought to be a part of a mitogenic pathway [36]. However, we and others have shown that prolonged treatment with III1-C can inhibit cell proliferation [3,4], and here we show that cell adhesion to III1-C can activate ERK1/2. One possible explanation for this apparent discrepancy is that although ERK1/2 are often involved in mitogenesis, in SMCs ERK activation can lead to inhibition of proliferation [46]. This occurs through ERK-mediated stimulation of PGE_2 production, which then inhibits the proliferation of SMCs [46]. Further experiments will determine whether RASMCs produce PGE_2 after treatment with III1-C. Another possibility is that III1-C stimulates other signaling pathways apart from the ERK pathway, and that one of the other signaling pathways inhibits proliferation of the cells. The relationship between ERK activation, stimulation of other signaling pathways and growth inhibition by III1-C will be elucidated by future experiments.

The III1-C protein has recently been shown to inhibit angiogenesis, tumor growth and metastasis [25]. Those findings, along with our present findings reveal some intriguing parallels between III1-C and the angiogenesis inhibitor endostatin [47]. For example, both III1-C and endostatin are fragments of matrix proteins and can inhibit angiogenesis and tumor growth. In addition, recent work has shown that cells interact with endostatin through two classes of receptors; integrins and glypicans (cell surface HSPGs) [48,49]. This parallels our findings in this present report, that cells interact with III1-C through integrins and cell surface HSPGs. Therefore, III1-C and endostatin may inhibit angiogenesis through related mechanisms involving both integrins and HSPGs. Future work will determine the relative contributions of these two classes of receptors in the biological effects of III1-C.

Conclusions

We have found that cells can interact with the fibronectin first type III repeat, leading to cell adhesion and activation of ERK, and that this signaling requires both cell surface HSPGs and integrins. Results from various independent types of experiments indicate that the first type III repeat of fibronectin can serve as a cell binding domain and that both integrins and cell surface HSPGs function as receptors for this type III repeat.

Materials and Methods

Materials

DMEM and glutamine Pen-Strep were obtained from Life Technologies, Inc. FBS was purchased from HyClone Laboratories, Inc. Plasma fibronectin was purified from human plasma by gelatin-agarose affinity chromatography [50]. 4–20% gradient SDS-PAGE gels were from Novex. Anti-phospho-p44/42 MAPK polyclonal antibody and anti-p44/42 ERK polyclonal antibody were purchased from New England Biolabs. Anti-β1 blocking antibody (Ha2/5) was from PharMingen. Peptides GRGDSP and GRADSP were purchased from Life Technologies, Inc. Texas red and FITC labeled secondary antibodies and $^{35}SO_4$ were from ICN. Heparitinase, chondroitinase ABC and mAb 3G10 were from Seikagaku. Anti-FN antibodies were a gift of Dr. Erkki Ruoslahti (Burnham Institute). ECL plus reagent and Hyperfilm were obtained from Amersham. Complete™ protease inhibitor cocktail tablets were purchased from Boehringer Mannheim Biochemicals. The expression vector pQE-12 was obtained from Qiagen. Heparin and all other chemicals were obtained from Sigma.

The purified glycosaminoglycans used in this study were a generous gift of Dr. Nancy Schwartz (Univ. of Chicago). The glycosaminoglycans were produced under a contract from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH) and serve as reference standards. The tissue sources of the glycosaminoglycans were as follows: hyaluronic acid, human umbilical cord; heparan sulfate, beef lung; dermatan sulfate, hog mucosa; chondroitin-4-sulfate, notochord from river sturgeon (S. platorhynchus), and chondroitin-6-sulfate, cranial cartilage of river sturgeon.

Cell culture

Primary rat aortic smooth muscle cells (RASMCs) were isolated from 12–16 week old Sprague-Dawley rats as described previously [51]. RASMCs were cultured in DMEM supplemented with 10% FBS and glutamine Pen-Strep. RASMCs were typically between passages 2 and 7 for experiments.

Recombinant protein production

Proteins III1-C and III 11-C (previously called III_{11}) were produced in bacteria and purified as described previously [2]. III1-C encompasses fibronectin amino acids 600–674 (Asn-Ala-Pro-Gln-...Thr-Ser-Thr-Pro), and III 11-C amino acids 1,532–1,599 (Leu-Pro-Ser-Ser-...Thr-Ala-Val-Thr), according to the previously published numbering method [52]. The recombinant proteins were expressed by the pQE-12 vector, which places a 6-His coding sequence at the C-terminus of each protein. The proteins were purified on Ni-agarose columns and were >98% pure as judged by SDS-PAGE and coomassie staining. Stock protein solutions were typically 700 μM for III1-C and 520 μM for III 11-C in PBS.

Anti-III1-C antibodies were produced by immunizing rabbits with III1-C. After one month the rabbits were boosted and serum samples were collected every two weeks. The rabbit serum was applied to an affinity column to purify the anti-III 1-C antibodies. In order to avoid purifying antibodies to the 6-His tag in III1-C, the affinity column was made with a GST fusion protein. The 1st type III repeat of fibronectin was expressed as a fusion protein coupled to GST, and this GST-3FN1 fusion protein was coupled to Sepharose and used in affinity purification. Bound antibodies were eluted with 100 mM triethylamine, pH 11.5 and the pH was neutralized with 1 M Tris, pH 8; (the antibody concentration was 0.4 mg/ml). BSA (0.5 mg/ml) was added to stabilize the antibody for subsequent handling. The antibody prep was dialyzed extensively against PBS before use.

Cell attachment and spreading assays

For the cell spreading assay shown in Fig. 1, fibronectin (20 μg/ml) was coated onto 24-well dishes for 1 hr at RT. The wells were washed 3 times with PBS before adding cells. Growing RASMCs were harvested by trypsinization and collected into DMEM + 0.5% BSA + soybean trypsin inhibitor. Cells were washed 3 times with DMEM + 0.5% BSA, then resuspended at 1×10^5 cells/ml in DMEM + 0.5% BSA, in the absence or presence of 25 μM III1-C or 25 μM III 11-C in the medium. Cells were seeded onto the wells (0.5 ml/well, ~30% confluence), placed in a 37°C incubator and allowed to adhere for either 7, 15, 30, 60, 120 or 240 min. Cells were then fixed with 3.7% paraformaldehyde, 60 mM sucrose in TBS, followed by staining with Coomassie Blue dye (1 mg/ml Coomassie Brilliant Blue, 45% MeOH, 10% acetic acid). Cells were destained with water, air-dried, then photographed with a Leica DM IRB phase microscope connected to a Polaroid CCD camera and an Apple Macintosh computer for capturing images. To quantitate the amount of spreading, each image was analyzed with the NIH Image 1.61 application to determine the total amount of area covered by cells and this number was divided by the total number of cells in the image to give an average area per cell (in pixels^2/cell).

Cell attachment assays shown in Fig. 2 and 3 were done essentially as described previously [2]. Briefly, fibronectin, III1-C or III 11-C at various concentrations were coated onto 96-well dishes for 1 hr at RT. The wells were then washed 3 times with PBS before adding cells. Growing RASMCs were harvested by trypsinization and collected into DMEM + 0.5% BSA + soybean trypsin inhibitor. Cells were washed 3 times with DMEM + 0.5% BSA, then resuspended at 4×10^5 cells/ml in DMEM + 0.5% BSA with or without inhibitors, as indicated in the figures. Cells were seeded onto the coated wells and allowed to adhere for 30 min at 37°C. The cells were then fixed with 3.7% paraformaldehyde, 60 mM sucrose in TBS, followed by 20% methanol, and staining in 0.5% crystal violet in 20% methanol. Stained cells were washed with water and the dye was solubilized with 0.1 M sodium citrate, pH 4.2, 50% ethanol. Attachment was quantitated by measuring the absorbance at 600 nm.

^{125}I cell surface labeling and affinity chromatography

The procedure was a modification of the procedure used to isolate the fibronectin receptor and other integrins [27,28]. The affinity resins were prepared by coupling fibronectin, III1-C or III 11-C to CNBr-activated Sepharose CL-4B according to the manufacturer's recommendations. The concentration of protein was typically 4–5 mg fibronectin/ml of resin, or 8–10 mg protein/ml of resin for III1-C and III 11-C. Ten 15-cm dishes of RASMCs were washed twice with PBS, then harvested by detachment of cells in PBS + 10 mM EDTA. The cells were washed twice more with PBS, then resuspended at 4×10^7 cells/ml in PBS. Cells were labeled with 1 mCi $Na^{125}I$ and lactoperoxidase-H_2O_2, as previously described [27]. After labeling the cell pellet was washed with ice-cold PBS + NaN_3, then lysed in 2 volumes of OG Lysis Buffer (200 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, TBS, PMSF) at 4°C. The lysate was spun at 14,000 × g for 15 min at 4°C, and the supernatant was used for affinity chromatography. Lysate was applied to the affinity columns (0.2 ml lysate was applied to 0.1 ml affinity matrix bed volume), flow through fractions were collected and passed over the columns twice more. Columns were washed with 10 column volumes of Wash Buffer (40 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, TBS), before elution of bound protein with 10 mM EDTA in Wash Buffer. Samples from the final wash and eluted samples were separated on 4–20% gradient Novex gels, the gels were dried and exposed to phosphorimager cassette and the radioactive signal was measured with a Storm phosphorimager instrument.

MAPK assay and immunoblotting

Wells of a 6-well plate were coated with either 20 μg/ml fibronectin, 25 μM III1-C or 25 μM III 11-C for 1 hr at RT. Wells were washed 3 times with PBS before adding cells. Growing RASMCs were harvested by trypsinization and collected into DMEM + 0.5% BSA + soybean trypsin inhibitor. Cells were washed 3 times with DMEM + 0.5% BSA, then resuspended at 3×10^5 cells/ml in DMEM + 0.5% BSA with or without integrin blocking agents or other additions, as described in the figure legends. Cells were either left in suspension (for suspended and PDGF stimulated samples) or were seeded onto the coated wells (1 ml per well) and allowed to adhere for 30 min at 37°C, or for the times indicated in the figure legend (Fig. 5). The cells stimulated with PDGF (2 ng/ml PDGF-BB) received the growth factor 10 min before collecting the cells for lysis, while the other samples received no growth factor or serum. After the adhesion period plates were placed on ice, unattached cells were collected and spun down, while cell monolayers were washed once with ice cold PBS then lysed with SDS-PAGE sample buffer (100 μl/well). Centrifuged pellets of unattached cells were combined with the appropriate monolayer lysate sample and the samples were heated to 100°C for 5 min. Samples were separated on 4–20% gradient Novex gels, transferred to Immobilon P membranes, blocked with 5% nonfat dry milk in TBS-Tween, then probed with anti-phospho-p44/42 MAPK antibody according to the manufacturer's recommendations. Blots were then stripped in Stripping Buffer (2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris, pH 6.7) at 50°C for 30 min, washed 4 times with TBS-Tween, blocked with 5% nonfat dry milk in TBS-Tween, then probed with anti-p44/42 ERK antibody according to the manufacturer's recommendations. All blots were developed with the ECL plus reagent and exposed to Hyperfilm. For both antibodies the typical exposure times were under 5 min.

Heparitinase treatment of cells and lysates

6 well plates were coated with either fibronectin, III1-C or III 11-C as described above. Growing RASMCs were harvested by trypsinization and washed in DMEM + BSA as described above. For GAGase treatment of intact cells, the cells remained in suspension and received either no enzyme or 0.1 u/ml heparitinase or 0.1 u/ml chondroitinase ABC for 1 hr at 37°C with constant gentle rotation. Cells were then plated onto precoated dishes and processed for immunoblotting with anti-phospho-p44/42 MAPK antibody as described above. Alternatively, after heparitinase digestion cells were lysed directly in SDS sample buffer and analyzed by immunoblotting with the 3G10 mAb (essentially as described above for anti-phospho-p44/42 MAPK immunoblotting).

For GAGase treatment of cell lysates, cells were lysed on ice in NP40 Lysis buffer (1% Nonidet P 40, 150 mM NaCl, 50 mM Tris, pH 8.0) plus protease inhibitors (Complete™ protease inhibitor cocktail) at 2×10^6 cells/ml. Insoluble material was removed by centrifugation at 14,000 × g for 15 min at 4°C. Lysate was prepared for heparitinase treatment by adding $CaCl_2$ to 10 μM final concentration. Heparitinase was added to 0.1 u/ml final concentration and the lysate was incubated at 37°C for 1 hr. The lysate was then applied to affinity chromatography columns as described below.

$^{35}SO_4$ labeling and affinity chromatography

Growing RASMCs labeled for 20 hr at 37°C with 200 μCi/ml 35S04 in DMEM + 10% dialyzed FCS + Pen-Strep. Cells were placed on ice, washed once with PBS, then lysed in NP40 Lysis buffer as described above. III1-C and III 11-C columns were produced as described above. Cell lysates were applied to III1-C or III 11-C columns (typically 500 μl lysate was applied to 250 μl affinity matrix bed volume), flow through fractions were collected and passed over the columns twice more. The final flow through fractions were then collected. Columns were washed with 10 column volumes of NP40 Lysis buffer, then bound material was removed by boiling the Sepharose beads in 2 column volumes of SDS sample buffer. Alternatively, the bound material was first eluted with 2 column volumes of 8 M urea buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris, pH 8.0), followed by washing with 10 column volumes of NP40 Lysis buffer, and then removing any remaining bound material by boiling the Sepharose beads in SDS sample buffer. Samples were separated on 4–20% Novex SDS-PAGE gels, the gels were fixed and dried and the radioactive material was detected by using a phosphorimager.

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