A Shared Mechanism of Adhesion Modulation for Tenascin-C and Fibulin-1

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Adhesion modulatory proteins are important effectors of cell–matrix interactions during tissue remodeling and regeneration. They comprise a diverse group of matricellular proteins that confer antiadhesive properties to the extracellular matrix (ECM). We compared the inhibitory effects of two adhesion modulatory proteins, fibulin-1 and tenascin-C, both of which bind to the C-terminal heparin-binding (HepII) domain of fibronectin (FN) but are structurally distinct. Here, we report that, like tenascin-C, fibulin-1 inhibits fibroblast spreading and cell-mediated contraction of a fibrin–FN matrix. These proteins act by modulation of focal adhesion kinase and extracellular signal-regulated kinase signaling. The inhibitory effects were bypassed by lysophosphatidic acid, an activator of RhoA GTPase. Fibroblast response to fibulin-1, similar to tenascin-C, was dependent on expression of the heparan sulfate proteoglycan syndecan-4, which also binds to the HepII domain. Therefore, blockade of HepII-mediated signaling by competitive binding of fibulin-1 or tenascin-C represents a shared mechanism of adhesion modulation among disparate modulatory proteins.

INTRODUCTION

Tissue architecture is determined by positioning of cells within the fibrillar assemblage of proteins, proteoglycans, and other components that comprise the extracellular matrix (ECM). Organization and composition of the ECM have significant impacts on many cellular functions. By binding to transmembrane receptors, individual ECM components affect cell arrangements and initiate signaling events that alter gene expression and regulate cell communication. Fibronectin (FN) is a ubiquitously expressed, multifunctional ECM glycoprotein that promotes cell adhesion and plays important roles in tissue development, repair, and remodeling (Hynes, 1990). Cell interactions with FN are largely dependent on integrin receptors with subsequent engagement of intracellular signaling and cytoskeletal components (Hynes, 2002). Tissue development and remodeling require changes in cell adhesion to allow cell proliferation, cell motility, and ECM reorganization (Singer and Clark, 1999). Modulation of cell adhesion can be achieved by modifications in FN matrix organization or composition (Sechler *et al.*, 1998; Midwood *et al.*, 2006) or by the deposition of adhesion modulatory or matricellular proteins (Bornstein and Sage, 2002), including tenascin-C, fibulin-1, thrombospondin-1 (TSP-1), and many others (Fassler *et al.*, 1996; Bornstein, 2001; Chiquet-Ehrismann and Chiquet, 2003; Midwood *et al.*, 2004b).

Expression of proteins that modulate cell adhesion causes reduced cell interactions with adhesive ECM, thus allowing cell movement, shape changes, proliferation, and other related processes (Chiquet-Ehrismann, 1991; Orend and Chi-

quet-Ehrismann, 2000). The suppression of adhesion-related signal transduction produces dramatic changes in cytoskeletal organization and in the kinetics of cell contact with the ECM. Adhesion modulatory proteins comprise an increasingly diverse group, suggesting that their mechanisms for control of cell–ECM responses may also be varied. In fact, evidence supports several different mechanisms to explain the modulatory effects. For example, TSP-1 induces focal adhesion dissociation through calreticulin and phosphatidylinositol 3-kinase signaling (Goicoechea *et al.*, 2000; Orr *et al.*, 2002). Blockade of integrin interactions with ECM ligands has been reported previously (Hadari *et al.*, 2000; Hughes, 2001; Wu *et al.*, 2007) as well as indirect effects on adhesion through modulation of growth factor signaling (Bornstein and Sage, 2002; Hollier *et al.*, 2005, 2008). Finally, the FN-binding properties of tenascin-C reduce cell adhesion and modify intracellular signaling by preventing syndecan-4 receptor binding to the C-terminal heparin-binding (HepII) domain of FN (Huang *et al.*, 2001; Orend *et al.*, 2003; Midwood *et al.*, 2004a).

Tenascin-C and other adhesion modulatory proteins, including fibulin-1 and vitronectin, are present at sites of tissue injury in contact with the wound provisional matrix and available to interact with FN, syndecans, and other binding partners (Midwood *et al.*, 2004b). Interestingly, like tenascin-C, fibulin-1 binds to the HepII domain (Balbona *et al.*, 1992) and has been shown to have cell adhesion- and motility-suppressive effects on FN-coated substrates (Twal *et al.*, 2001). Vitronectin, in contrast, binds to syndecans (Wilkins-Port and Mckeown-Longo, 1996; Wilkins-Port *et al.*, 2003) but not to FN. This raises the question whether these proteins modulate adhesion by FN- or syndecan-dependent interactions. To address this question, we compared the effects of fibulin-1, vitronectin, and tenascin-C on cell interactions with a covalently cross-linked fibrin–FN matrix model of the provisional matrix. Our results show that the proteins with HepII domain binding activity modulate cell spreading and contractility on FN matrix.

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MATERIALS AND METHODS

Protein Production

Rat plasma FN was purified by gelatin-Sepharose (Pharmacia Biotech, Arlington Heights, IL) affinity chromatography from freshly drawn plasma (Wilson and Schwarzbauer, 1992). The construction and production of the recombinant protein 70Ten have been described previously and have been shown to have identical effects on cells as full-length tenascin-C (Wenk *et al.*, 2000; Midwood and Schwarzbauer, 2002). Fibulin-1 purified from human placental extracts was the kind gift of Drs. Waleed O. Twa and W. S. Argraves (Medical University of South Carolina). Recombinant human vitronectin was commercially available from Millipore Bioscience Research Reagents (Temecula, CA). To generate the HV120-glutathione transferase (GST) construct, cDNA was prepared by polymerase chain reaction (PCR) by using a V120-containing human cDNA as template and primers to the 5' end of FN repeat III₁₂ and the 3' end of III₁₅. This cDNA was inserted into a pGEX vector (GE Healthcare) in-frame with the GST coding region. The fusion protein was purified from bacterial lysates as recommended by the manufacturer.

Cell Culture

NIH3T3 fibroblasts were cultured in DMEM and 10% calf serum (HyClone Laboratories, Logan, UT). Syndecan-4-deficient mouse dermal fibroblasts (Echtermeyer *et al.*, 2001) were donated by Dr. Paul Goetinck (Massachusetts General Hospital). The cells were maintained in DMEM plus 10% fetal bovine serum and were used only up to 10 passages.

Fibrin-FN Matrix Preparation and Immunofluorescence

Fibrin-FN matrices were prepared as described previously by Midwood and Schwarzbauer (2002). We mixed 600 $\mu\text{g}/\text{ml}$ human fibrinogen (America Diagnostica, Greenwich, CT), 30 $\mu\text{g}/\text{ml}$ FN, and 15 $\mu\text{g}/\text{ml}$ coagulation factor XIIIa (Calbiochem-Novabiochem, San Diego, CA) with thrombin at 2 U/ml, and the mixture was pipetted onto a glass coverslip (Thermo Fisher Scientific, Waltham, MA). After overnight incubation at 4°C, the clots were gently aspirated leaving a fibrillar network attached to the surface of the coverslip. A solution of 11 $\mu\text{g}/\text{ml}$ fibulin-1, 120 $\mu\text{g}/\text{ml}$ 70Ten, or 15 $\mu\text{g}/\text{ml}$ vitronectin in 2% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) was added and allowed to bind for 30 min at room temperature. Fibrin-FN matrices without fibulin-1, 70Ten, or vitronectin were treated with 2% BSA in PBS under identical conditions. For fibrin-FN matrices containing HV120-GST, 11 $\mu\text{g}/\text{ml}$ fibulin-1, and 120 $\mu\text{g}/\text{ml}$ 70Ten were incubated for 1 h at room temperature with the equivalent mass concentrations of HV120-GST before addition to the clots. HV120-GST at 30 $\mu\text{g}/\text{ml}$ in 2% BSA/PBS was added to the fibrin-FN matrix without fibulin-1 or 70Ten for 30 min at room temperature as a control.

Cells were plated at 1×10^5 per well on substrate-coated glass coverslips for 1 h, after which time the cells were washed, fixed, and permeabilized (Midwood and Schwarzbauer, 2002). Cells were incubated with primary or secondary antibody or phalloidin in 2% ovalbumin (Sigma-Aldrich, St. Louis, MO) in PBS at 37°C for 1 h. Focal adhesions were detected with an anti-vinculin monoclonal antibody (mAb) (Sigma-Aldrich) diluted 1:300 and fluorescein-conjugated goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) at 1:600. For staining actin filaments, rhodamine-conjugated phalloidin (Invitrogen) was used at 1:1000. FN matrix was detected with the culture supernatant from hybridoma cells producing anti-human FN 7.1 at 1:10 (Brenner *et al.*, 2000). A polyclonal anti-fibulin-1 antibody, rb2954 (Godyna *et al.*, 1996), a kind gift of Dr. W. S. Argraves, and rhodamine-conjugated goat anti-rabbit secondary antibody (Invitrogen) were used for the immunofluorescence staining of fibulin-1 at dilutions of 1:100 and 1:400, respectively. Coverslips were mounted with SlowFade Light Antifade kit (Invitrogen). Staining was visualized with a Nikon Eclipse TE2000-U microscope with epifluorescence. Images were captured using a SensiCam digital camera (Cooke, Auburn Hills, MI) and analyzed with IPLab (Madison, WI) and Adobe Photoshop software (Adobe Systems, Mountain View, CA). For cell attachment assays, fibrin-FN matrices were incubated for 1 h at room temperature with anti-human HFN 7.1 ascites diluted 1:100. Vitronectin was then added to the antibody solution and incubated with the matrix followed by cell plating and immunofluorescence as described above.

Quantification of Cell Morphology

NIH3T3 fibroblasts plated on fibrin-FN matrices were immunostained for actin and vinculin to visualize cell shape, cytoskeletal organization, and focal adhesion formation. Three categories of cell morphology were used: spread, intermediate, and rounded. Morphological and molecular features that were scored included membrane protrusions, focal adhesions, actin stress fibers, and cortical actin filaments. Cells possessing actin-filled membrane protrusions (containing actin filaments as determined by rhodamine-phalloidin staining), actin stress fibers, and focal adhesions were categorized as spread. Cells lacking all three of these structural features and containing mainly cortical actin filaments were scored as rounded. Intermediate morphologies were assigned to cells that possessed actin-positive protrusions but lacked stress fibers and focal adhesions, or to cells containing protrusions and focal

adhesions without the presence of stress fibers. We counted 200 cells for each experimental condition, and the number of cells scored under each category was expressed as a percentage of the total.

Immunoblotting

Matrices were prepared as for immunofluorescence studies. For dosage-dependence assays, fibulin-1 was added to fibrin-FN matrices in the following concentrations: 1, 2, 5, 10, and 20 $\mu\text{g}/\text{ml}$. Cells were plated at a density of 2×10^5 per well and allowed to spread on matrices for 1 h. Immediately after incubation, cells were washed with cold PBS and then lysed on ice with 250 μl of radioimmunoprecipitation assay lysis buffer (Wierzbicka-Patynowski and Schwarzbauer, 2002), and relative protein concentrations of the lysates were determined using the β -N-acetylglucosaminidase assay kit (Sigma-Aldrich). Lysate samples containing equal amounts of total protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide-SDS gel and electrophoretically transferred to nitrocellulose. Phosphorylated proteins were detected using an anti-FAK-397 polyclonal immunoglobulin G (IgG) (BioSource International, Camarillo, CA) at 1:5000 and a monoclonal anti-mitogen-activated protein (MAP) kinase/diphosphorylated extracellular signal-regulated kinase (ERK) 1 and 2 (Sigma-Aldrich) at 1:10,000. Total protein levels were detected using an anti-focal adhesion kinase (FAK) mAb (BD Biosciences Transduction Laboratories, Lexington, KY) and an anti-ERK (pan-ERK) mouse IgG (BD Biosciences Transduction Laboratories) at dilutions of 1:100 and 1:10,000, respectively. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Pierce Chemical, Rockford, IL) at 1:30,000 dilution and SuperSignal chemiluminescent detection reagent (Pierce Chemical). Quantitative enhanced chemiluminescence (ECL) was conducted using ECL Plus western blotting detection reagents (GE Healthcare) according to the manufacturer's protocols. Immunoblotted proteins were detected and analyzed using Storm 860 Molecular Imager and ImageQuant software analysis package (GE Healthcare). Quantitative ECL (qECL) values were determined for pFAK and pERK1/2 in equal amounts of lysate from cells plated on fibrin-FN, fibrin-FN + fibulin-1, or fibrin-FN + 70Ten matrices. The qECL values for each phosphorylated protein on fibrin-FN matrix were assigned relative values of 1, and then values from + fibulin-1 and + 70Ten matrices were normalized to the fibrin-FN controls. Data from two independent experiments were averaged. The amounts of pERK1/2 detected by qECL approached the limits of detection of the Storm Imager; therefore, the actual proportions of phospho ERK could be even less than the reported 0.25 ± 0.02 .

Contraction Assays

Contraction assays were performed as described previously (Midwood and Schwarzbauer, 2002; Midwood *et al.*, 2004a). Matrices were prepared as for immunofluorescence studies except cells were added to the matrix components. For culture in three-dimensional matrices, cells were resuspended in 0.025 M HEPES, pH 7.4, 0.13M NaCl at $1 \times 10^6/\text{ml}$. FN at 30 $\mu\text{g}/\text{ml}$ in 3-(cyclohexylamino)propanesulfonic acid buffer was neutralized with an equal volume of 1 M Tris-HCl, pH 7.4, and mixed with either 11 $\mu\text{g}/\text{ml}$ fibulin-1 or 120 $\mu\text{g}/\text{ml}$ 70Ten for 30 min at room temperature, before polymerization. For dosage-dependence assays, the following quantities of fibulin-1 were mixed with FN before inclusion into clots: 1.25, 5, and 10 $\mu\text{g}/\text{ml}$. Immediately after the addition of thrombin, the mixture was pipetted into 48-well plates which had been coated with 1% BSA overnight at 4°C. The cell-matrix mixture was allowed to polymerize for 30 min at 37°C. The clots were then carefully detached from the walls of the wells, and matrix contraction was visualized. The area of the matrix was measured over time, subtracted from the starting area (predetachment), and expressed as a percentage of the starting area.

RhoA Activation Assay

Detection of levels of active RhoA protein in NIH3T3 lysates was conducted using a RhoA G-LISA activation assay kit (Cytoskeleton, Denver, CO). Matrices were prepared as per immunoblotting studies. Lysate preparation, total protein quantification, and active RhoA detection were performed according to manufacturer's protocols.

Pretreatments

Before treatment with activators or inhibitors, cells were serum starved for 24 h. After release with EDTA, cells were incubated with 5 μM lysophosphatidic acid (LPA) for 30 min at 37°C in suspension before addition to matrix proteins. Suspended cells were treated with 50 μM PD98059 or 40 μM SP600125 or an equivalent volume of dimethyl sulfoxide (DMSO) for 30 min at 37°C. For the combined PD98059 and LPA assays, cells were pretreated with either 50 μM PD98059 or an equivalent volume of DMSO and then added to a clot mixture containing LPA (Sigma-Aldrich) at concentrations of 5, 10, and 50 μM .

RESULTS

Fibulin-1 Alters Fibroblast Morphology on a Fibrin–FN Matrix

Fibulin-1 is present in blood plasma, is incorporated into fibrin matrices during coagulation, and mediates platelet adhesion to fibrinogen (Balbona *et al.*, 1992; Tran *et al.*, 1995; Godyna *et al.*, 1996). We took advantage of these properties to test the capacity of fibulin-1 to alter cell morphology within the context of a fibrin–FN matrix. The effects of fibulin-1 on cell adhesion and spreading were compared with the modulatory influences of a recombinant version of tenascin-C (70Ten), whose antiadhesive properties within the provisional matrix have been well characterized (Wenk *et al.*, 2000; Midwood and Schwarzbauer, 2002; Midwood *et al.*, 2004a). Fibulin-1 was incubated with polymerized fibrin–FN matrix to allow binding to matrix components. Colocalization with FN was demonstrated by immunofluorescence, which revealed a fibrillar meshwork containing FN (Figure 1, A and C) and associated fibulin-1 (Figure 1, B and C).

To test whether fibulin-1 affects cell–ECM interactions, NIH3T3 cells were compared after plating on fibrin–FN matrices with or without addition of fibulin-1 or 70Ten. Fibroblast cells exhibited notable changes in morphology when exposed to fibulin-1 or 70Ten (Figure 2). With inclusion of either protein, adherent cells failed to spread fully and exhibited significant changes in the organization and distribution of actin filaments when compared with cells plated on fibrin–FN matrix. Rhodamine-phalloidin staining revealed that the majority of cells on fibulin-1- or 70Ten-containing matrix did not organize actin into stress fibers but instead showed cortical localization of actin with membrane ruffles and few vinculin-positive focal adhesions (Figure 2A). Fibroblasts adherent on fibrin–FN matrix, however, displayed extensive actin stress fibers terminating in vinculin-rich focal adhesions (Figure 2A).

These effects on cell shape and cytoskeletal organization were quantified by scoring fibroblasts as spread, rounded, or intermediate based on the presence or absence of salient morphological features (Figure 2B). Representative examples of each type of morphology are illustrated in Supplemental Figure S1. After 1 h of adhesion, the majority of cells plated on the fibrin–FN matrix were spread, whereas only ~20% of cells exhibited a spread morphology in the presence of fibulin-1 or 70Ten. Instead the majority of fibroblasts on these matrices assumed intermediate morphologies, and higher numbers of rounded cells were observed on fibulin-1- and 70Ten-containing matrices compared with fibrin–FN matrix. These results demonstrate that the inclusion of fibulin-1 or 70Ten in a fibrin–FN matrix alters fibroblast morphology toward a less adhesive phenotype.

Cell Contractility Is Inhibited by Fibulin-1

Cell–ECM interactions after the establishment of the provisional matrix include the generation of contractile force. Tenascin-C is known to modulate the capacity of fibroblasts to contract fibrin–FN matrices in vitro (Midwood and Schwarzbauer, 2002). Because fibulin-1 and 70Ten both alter cell morphology in a similar manner, we tested whether the incorporation of fibulin-1 into the fibrin–FN matrix can also induce inhibitory effects on cell contractility by using a well-characterized matrix contraction assay (Corbett and Schwarzbauer, 1999; Midwood and Schwarzbauer, 2002). NIH3T3 fibroblasts embedded in a fibrin–FN network rapidly contracted the matrix to <60% of original area (Figure 3A). In contrast, the inclusion of fibulin-1 in the fibrin–FN

Figure 1. Fibulin-1 associates with FN in the provisional matrix. A fibrin–FN matrix incubated with fibulin-1 was immunostained with anti-FN (A) and anti-fibulin-1 (B) antibodies. Colocalization of fibulin-1 and FN is shown in the merged image (C). Bar, 20 μ m.

matrix impaired the cells' capacity to contract the matrix. We measured a 2.5-fold reduction in matrix contraction capacity of fibroblasts seeded within fibulin-1-containing matrices versus fibrin–FN matrix controls (Figure 3A). This suppression of cell contractility induced by fibulin-1 was comparable to the inhibitory effect of 70Ten on matrix contraction.

Figure 2. Fibulin-1 alters fibroblast morphology. NIH3T3 fibroblasts were plated for 1 h on fibrin-FN, fibrin-FN + fibulin-1, and fibrin-FN + 70Ten matrices before staining for actin with rhodamine-phalloidin (A, top) and with an anti-vinculin mAb (A, bottom). Bar, 10 μ m. (B) Cell morphologies were assessed as rounded, intermediate, or spread based on the presence or absence of actin-filled membrane protrusions, stress fibers, and focal adhesions. For each matrix condition, 200 cells were counted and categorized per experiment. The data are expressed as the mean $\pm$ the range of values from two independent experiments.

Fibrin-FN contraction assays with varying amounts of fibulin-1 showed a concentration-dependent inhibition of matrix contraction (Figure 3B). Therefore, fibulin-1 can specifically suppress cell contractile capacity to a degree that is similar to the inhibitory effect of 70Ten.

Fibulin-1 Alters ECM-mediated Cell Signaling

The modulation of cell morphology and contractility by tenascin-C is known to occur by regulating the activity of key signaling components. RhoA GTPase and FAK activation are suppressed by the presence of tenascin-C on FN substrates (Wenk *et al.*, 2000; Midwood and Schwarzbauer, 2002; Orend *et al.*, 2003). Given the similarities of their inhibitory effects, we investigated whether fibulin-1 and tenascin-C regulate the activation of common signaling pathways. Immunoblot analyses of total cell lysates showed that although equivalent levels of total cellular FAK were present under all conditions, fibulin-1- and 70Ten-containing matrices both reduced levels of phosphorylated (active) FAK (Figure 4A). Fibulin-1 had a concentration-dependent effect with an obvious reduction in phospho-FAK at 10 μ g/ml (Figure 4B). This concentration is equivalent to the level at which contraction was maximally inhibited (Figure 3).

Fibrin-FN matrices containing fibulin-1 and 70Ten also had suppressive effects on RhoA GTPase activation (Figure 4C). Enzyme-linked immunosorbent assay (ELISA)-based detection of guanosine triphosphate (GTP)-bound RhoA from the lysates of cells plated on + fibulin-1 and + 70Ten matrices yielded quantities of active RhoA that were signif-

icantly lower than the fibrin-FN control. Compared with cells on fibrin-FN, the inclusion of fibulin-1 or 70Ten reduced GTP-bound RhoA two- to threefold (68.7 and 46.4%, respectively).

Fibulin-1 has been established previously as a modulator of FN-stimulated ERK signaling (Twal *et al.*, 2001). To determine whether the regulation of ERK by fibulin-1 occurs within the context of the fibrin-FN matrix and whether this modulatory effect is shared by tenascin-C, NIH3T3 lysates were probed with anti-phospho-ERK antibodies. The results revealed that both fibulin-1 and 70Ten suppressed activation of ERK (Figure 4D), whereas total cellular ERK remained equivalent under all conditions.

Quantitative detection of phosphorylated forms of FAK and ERK corroborated qualitative immunoblot analyses. Compared with levels on fibrin-FN matrix, relative levels of phospho-FAK were reduced to 0.25 ± 0.03 on fibrin-FN + 70Ten and 0.24 ± 0.01 on fibrin-FN + fibulin-1 matrix. Similar reductions were observed for phospho-ERK1/2 with a relative level of 0.25 ± 0.02 on both fibrin-FN + fibulin-1 and fibrin-FN + 70Ten matrices. Together, these results represent approximately a fourfold reduction in levels of pFAK and pERK1/2 and demonstrate that fibulin-1 and tenascin-C exert comparable suppressive effects on these signaling components.

ERK Activation Is Required for Matrix Contraction

We have shown previously that sustained activation of FAK is required for cell contractility (Midwood and Schwar-

Figure 3. Cell contractility is inhibited by fibulin-1. (A) NIH3T3 fibroblasts were seeded within fibrin-FN, fibrin-FN + fibulin-1, or fibrin-FN + 70Ten matrices. After the matrix was detached from the dish, the area of the matrix was measured in duplicate samples at the indicated times and expressed as the mean $\pm$ SD for three independent experiments. (B) Rates of matrix contraction were assayed from clots containing varying amounts fibulin-1. The mean $\pm$ SD of the percentage contraction for each fibulin-1 concentration was calculated from duplicate samples in two independent experiments.

zbauer, 2002). To investigate whether ERK activity is also required, we examined matrix contraction by cells treated with PD98059, a pharmacological inhibitor of ERK (Alessi *et al.*, 1995; Cheresh *et al.*, 1999), compared with cells treated with SP600125, an inhibitor of the MAP kinase c-Jun NH₂-terminal kinase (Bennett *et al.*, 2001), and DMSO, the drug solvent. Cells treated with PD98059 exhibited a reduced capacity to contract the matrix (Figure 5A). SP600125-treated and DMSO-treated fibroblasts showed no inhibition of cell contractility. Furthermore, matrix contraction by PD98059-treated cells was inhibited to levels equivalent to those induced by fibulin-1- and 70Ten-containing matrices. Thus, it seems that activation of ERK is necessary for contractile behavior.

LPA Stimulation Reverses Inhibition of Cell Contractility

Stimulation of the activity of RhoA by the preincubation of cells with LPA has been demonstrated to supersede the inhibitory effects of tenascin-C on cell contractility (Midwood and Schwarzbauer, 2002). To test whether the same was true for fibulin-1, we assayed the contractile capacity of fibroblasts pretreated with LPA. Treatment of NIH3T3 cells with LPA restored contraction of fibrin-FN + fibulin-1 ma-

Figure 4. Fibulin-1 and tenascin-C alter ECM-mediated cell signaling. NIH3T3 fibroblasts were allowed to adhere to fibrin-FN matrices $\pm$ fibulin-1 and $\pm$ 70Ten for 1 h before lysis. Proteins were resolved by SDS-PAGE, and immunoblots were probed using the indicated antibodies. (A) Western blot of cell lysates probed with an anti-FAK mAb to detect total cellular FAK and a polyclonal antibody to detect FAK phosphorylated on tyrosine 397 (pFAK). (B) Lysates of cells plated on matrices containing different amounts of fibulin-1 were probed to detect levels of FAK by using the same antibodies described in A. (C) ELISA-based detection of active RhoA in lysates of NIH3T3 cells plated on fibrin-FN matrices plus and minus fibulin-1 or 70Ten as indicated. * $p < 0.05$ compared with fibrin-FN matrix as determined by Student's *t* test. (D) Immunoblot probed with phospho-specific anti-ERK1/2 mAb to detect phosphorylated forms of ERK1 (pERK1) and ERK2 (pERK2). A monoclonal pan-ERK antibody was used to detect total cellular ERK. The data shown in A–C are representative of two independent experiments.

trix to levels equivalent to the fibrin-FN control (Figure 5B). This restoration of fibroblast contractility was comparable with the level observed with LPA-treated cells in the fibrin-FN + 70Ten matrix. This result shows that RhoA acts downstream of fibulin-1 and tenascin-C, and stimulation of

Figure 5. Cell signaling and matrix contraction. (A) ERK activation is required for matrix contraction. NIH3T3 fibroblasts were pretreated with PD98059, SP600125, or DMSO for 30 min before incorporation into fibrin-FN matrix. Contraction was measured after 3 h, and data are expressed as described in Figure 3 for duplicate samples from two independent experiments. (B) Rho activation reverses

RhoA activity can completely reverse the inhibition of cell contractility induced by fibulin-1.

To gain insight into the roles of ERK and Rho GTPase in matrix contraction signaling, we tested whether RhoA stimulation by LPA reverses PD98059-induced inhibition of cell contractility. Cells were pretreated with PD98059 and then incorporated into fibrin-FN clots containing varied concentrations of LPA. The presence of LPA restored the capacity of PD98059-treated cells to contract the matrix (Figure 5C). The effect of LPA on cell contractility was concentration-dependent and at 50 μ M matrix contraction was restored to levels comparable with controls. This finding further corroborates the participation of ERK and RhoA in regulating contractility and suggests that RhoA is downstream of ERK in a pathway modulated by fibulin-1 and tenascin-C.

Syndecan-4 Contributes to Adhesion Modulation

Cell signaling and matrix contraction depend on syndecan-4 binding to FN (Couchman, 2003). Fibulin-1 directly binds to the HepII domain of FN (Balbona *et al.*, 1992), suggesting that, like tenascin-C, it modulates adhesion through blockade of syndecan-4 binding (Orend *et al.*, 2003; Midwood *et al.*, 2004a). Spreading of wild-type mouse embryo fibroblasts (MEFs), but not syndecan-4-null MEFs, is reduced by tenascin-C (Midwood *et al.*, 2004a) (Figure 6A). Syndecan-4-null MEFs on fibrin-FN matrices lacking or containing fibulin-1 or 70Ten exhibited comparable morphologies (Figure 6A). Therefore, in the absence of syndecan-4, fibroblasts are insensitive to the presence of these modulatory proteins, indicating that syndecan-4 function is necessary for both fibulin-1 and tenascin-C to exert their modulatory effects on the fibrin-FN matrix.

Although the results with fibulin-1 and tenascin-C strongly implicate interactions with the HepII domain in adhesion modulation, the possibility exists that interaction with syndecan-4 has modulatory activity. Therefore, we determined the effects on fibroblast spreading of vitronectin, a modulatory protein that binds syndecan-4 (Wilkins-Port and Mckeown-Longo, 1996; Wilkins-Port *et al.*, 2003). NIH3T3 cells failed to spread fully in the presence of fibulin-1 (Supplemental Figure S2A). However, cells plated on vitronectin-containing matrices assumed a spread morphology, similar to cells on fibrin-FN matrix. Cell adhesion in the presence of vitronectin was dependent on FN and could be completely inhibited by a function blocking anti-FN cell binding domain antibody (Supplemental Figure S2B), showing that the spreading was not due to cell-vitronectin interactions. These results indicate that the presence of an adhesion modulatory protein that does not bind to FN is not sufficient to modulate cell interactions with a fibrin-FN matrix.

To confirm that adhesion modulation by fibulin-1 and tenascin-C depends on interactions with the HepII domain

Figure 5 (cont). inhibition of cell contractility by fibulin-1. LPA-treated (LPA+) and untreated (LPA-) NIH3T3 fibroblasts were incorporated into clots containing fibrin-FN, fibrin-FN + fibulin-1, and fibrin-FN + 70Ten. Matrix contraction was measured after 3 h and expressed as a percentage. The means $\pm$ SD of duplicate samples from two independent experiments are shown. (C) Stimulation by LPA overrides the inhibition of cell contractility by PD98059 in a concentration-dependent manner. Cells were pretreated with 50 μ M PD98059 or an equivalent volume of DMSO (control) for 30 min before incorporation into clots containing indicated concentrations of LPA. Contraction was measured at the indicated time intervals, and the data points plotted represent the mean contraction of duplicate samples from two experiments.

Figure 6. Syndecan-4 expression is required for modulation of cell adhesion. (A) Syndecan-4-deficient mouse fibroblasts were plated on fibrin-FN, fibrin-FN + fibulin-1, or fibrin-FN + 70Ten matrices for 1 h and stained with rhodamine-phalloidin. Bar, 10 μ m. (B) Cells were plated on fibrin-FN matrices containing fibulin-1, 70Ten, or either of these proteins preincubated with the HepII fragment HV120-GST. Cell morphologies were categorized as described (Supplemental Figure S1). The data are expressed as the mean $\pm$ the range of values from two independent experiments.

of FN, we tested the effects of a recombinant HepII domain on cell adhesion. The recombinant protein HV120-GST contains the HepII domain (repeats III₁₂₋₁₅ + the V region) expressed as a GST fusion protein. Solid phase binding assays verified that both fibulin-1 and 70Ten bind to this domain as reported previously (Balbona *et al.*, 1992; Huang *et al.*, 2001; data not shown). Fibrin-FN matrices were incubated with fibulin-1 or 70Ten in the presence or absence of HV120-GST to competitively block their HepII-interacting sites. Cell morphology assays revealed that HV120-GST mitigated the inhibitory effects of these proteins on cell spreading (Figure 6B). The presence of HV120-GST with either fibulin-1 or 70Ten dramatically increased the numbers of spread cells to levels comparable with the fibrin-FN (+HV120) control. HV120-GST alone was unable to support adhesion and spreading (data not shown). Therefore, its antagonistic effects on the antiadhesive activities of fibulin-1 and 70Ten are due to interference with interactions between FN in the matrix and the adhesion modulatory proteins. This result shows that binding to the HepII domain by fibulin-1 and 70Ten defines an essential step in the process of adhesion modulation.

DISCUSSION

Adhesion modulatory proteins share the functional capacity to reduce cell interactions with adhesive ECM. Not only are the structures of these proteins varied, the mechanisms underlying adhesion modulation identified thus far have been distinct. The results presented here, however, show that the suppressive effects of tenascin-C and fibulin-1 share a common mechanism. Comparison of cell interactions with fibrin-FN matrix in the presence of these proteins shows that they have similar effects on cell shape, cytoskeletal organization, and intracellular signaling. Cell adhesion on fibrin-FN matrix stimulated FAK and ERK activation and cell contractility. These effects were inhibited by fibulin-1 and 70Ten, and contractility was restored with LPA treatment, indicating a role for Rho GTPases. Thus, these two modula-

tory proteins work through effects on a common set of intracellular signaling pathways. Also, like tenascin-C, we show that the effects of fibulin-1 on cell shape require expression of syndecan-4. Together, tenascin-C and fibulin-1 share a common pathway for adhesion modulation defined by syndecan-4 dependence and impacting FAK and ERK activation and cytoskeletal organization.

The HepII domain of FN participates in cytoskeletal rearrangements by interacting with heparan sulfate proteoglycans, including transmembrane syndecans (Woods, 2001; Couchman, 2003). Syndecan-4 localizes at focal adhesions (Woods *et al.*, 2000) and modulates FAK and Rho activation (Wilcox-Adelman *et al.*, 2002). The HepII domain is adjacent to the RGD cell binding domain, which engages integrins to activate FAK and downstream pathways (Hynes, 2002). Therefore, regulation of fibroblast morphology and function is accomplished by cytoskeletal reorganization and signal pathway activation through collaborations between receptors for cell binding and HepII domains. Tenascin-C modulates adhesion to FN by competing with syndecan-4 for binding to the HepII domain (Huang *et al.*, 2001; Orend *et al.*, 2003; Midwood *et al.*, 2004a). Modulation by fibulin-1 also requires FN and syndecan-4. In contrast, vitronectin, which binds syndecan-4 (Wilkins-Port and Mckeown-Longo, 1996; Wilkins-Port *et al.*, 2003) and fibrin (Podor *et al.*, 2001), did not affect fibroblast adhesion and spreading on a fibrin-FN matrix. This result indicates that fibrin and syndecan-4 interactions are not sufficient to modulate adhesion in this system. Mechanistic similarities between fibulin-1 and tenascin-C, and the differences from vitronectin, support the conclusion that fibulin-1 masks the HepII-syndecan-4 interactions required for adhesion and cell contractility.

We have shown previously that $\alpha 5 \beta 1$ binding to FN and downstream activation of FAK and Rho GTPase are essential for productive cell adhesion to and contractility of a fibrin-FN matrix (Corbett and Schwarzbauer, 1999; Wenk *et al.*, 2000; Midwood and Schwarzbauer, 2002). RhoA induces stress fiber formation and FAK activation plays a pivotal role in the remodeling of focal adhesions explaining how

reduced activation of these signals impairs cell spreading and matrix contraction. Alterations in cell contractility by tenascin-C result from the suppression of syndecan-4-mediated RhoA and FAK activation (Midwood and Schwarzbauer, 2002; Wilcox-Adelman *et al.*, 2002; Midwood *et al.*, 2004a). Inclusion of fibulin-1 had similar effects on FAK activation, and the reversal by LPA treatment implicates Rho GTPases, linking tenascin-C and fibulin-1 activities through common signaling pathways.

Fibulin-1 has been shown to suppress cell adhesion and motility on FN-coated substrates through down-regulation of ERK/MAP kinase signaling (Twal *et al.*, 2001). We have now identified ERK activity as a requisite for matrix contraction and showed that fibrin-FN matrix induction of phospho ERK was reduced in the presence of either 70Ten or fibulin-1. Activation of ERK1/2 promotes assembly of myosin filaments by regulating myosin light chain phosphorylation during cell migration and matrix contraction (Cheresh *et al.*, 1999). The down-regulation of ERK by fibulin-1 or tenascin-C can therefore diminish cell contractility by modulating the assembly of the actomyosin machinery. Together, the suppression of FAK, RhoA, and ERK signaling provides a mechanism that integrates the disruption of stress fibers, focal adhesions, and actomyosin complexes into a shared modulatory effect on cell contractility. Reversal of adhesion modulation by LPA suggests that Rho GTPase acts downstream of FAK and ERK in this process. Furthermore, the similarities between the effects of fibulin-1 and tenascin-C underscore the idea that a FN-dependent mechanism of adhesion modulation involves common lines of intracellular communication initiated by the competitive blockade of HepII.

Other adhesion modulatory proteins have been shown to exert their antiadhesive properties by altering signal transduction pathways. TSP-1 modulates FAK-dependent signaling and has differential effects on Rho, Rac, and Cdc42 GTPases (Adams and Schwartz, 2000; Wenk *et al.*, 2000; Orr *et al.*, 2004). Vitronectin induces Rac-mediated cytoskeletal rearrangements in fibroblasts (Kjoller and Hall, 2001). The counteradhesive effects of SPARC on adherent cells are mediated through a tyrosine kinase-dependent pathway and heterotrimeric G proteins (Motamed and Sage, 1998; Murphy-Ullrich, 2001). However, these mechanisms are not FN-specific, whereas fibulin-1 and tenascin-C affect FN-cell interactions.

The process of wound healing involves multiple sequential, integrated and overlapping cellular events such as adhesion, proliferation, migration, differentiation, and matrix contraction (Clark, 1996; Singer and Clark, 1999). Proteins that regulate cell-ECM interactions, including tenascin-C, fibulin-1, and vitronectin, are present at sites of tissue injury very early in the repair process (Tran *et al.*, 1995; Fassler *et al.*, 1996; Midwood *et al.*, 2004b). The ability of these proteins to affect the association of cells with the provisional matrix indicates that they act as important modulators of wound cell behaviors. For example, tenascin-C deposited at the edge of the wound bed is likely to promote a microenvironment conducive to cell migration into the wound bed and to limit cell contractility in this region (Midwood and Schwarzbauer, 2002; Midwood *et al.*, 2004a). We postulate that in vivo fibulin-1 acts in a similar manner to tenascin-C but with a spatially distinct role within the wound bed. Because fibulin-1 is localized to the provisional matrix, it may operate within that context to suppress excessive cell adhesion to the provisional matrix early in the healing process. Given the similarities of their modulatory properties, it is possible that fibulin-1 and tenascin-C also assume both complemen-

tary and compensatory roles in the regulation of cell-ECM interactions. The data from our investigation offer further insight into the regulatory functions performed by fibulin-1 during wound healing and, given the striking similarity of its activity to tenascin-C, provide evidence of a shared mechanism of adhesion modulation.

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