Fibulin-1 suppression of fibronectin-regulated cell adhesion and motility

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Accepted 16 August 2001

SUMMARY

Fibulin-1 is an extracellular matrix protein often associated with fibronectin (FN) in vivo. In this study, the ability of fibulin-1 to modulate adhesion, spreading and motility-promoting activities of FN was investigated. Fibulin-1 was found to have pronounced inhibitory effects on the cell attachment and spreading promoted by FN. Fibulin-1 was also found to inhibit the motility of a variety of cell types on FN substrata. For example, the FN-dependent haptotactic motility of breast carcinoma (MDA MB231) cells, epidermal carcinoma (A431), melanoma (A375 SM), rat pulmonary aortic smooth muscle cells (PAC1) and Chinese hamster ovary (CHO) cells was inhibited by the presence of fibulin-1 bound to FN-coated Boyden chamber membranes. Cells transfected to overproduce fibulin-1 displayed reduced velocity, distance of movement and persistence time on FN substrata. Similarly, the incorporation of fibulin-1 into FN-containing type I collagen gels inhibited the invasion of endocardial cushion mesenchymal cells migrating from cultured embryonic heart explants. By contrast, incorporation of fibulin-1 into collagen gels lacking FN had no effect on the migration of endocardial cushion cells.

These results suggest that the motility-suppressive effects of fibulin-1 might be FN specific. Furthermore, such effects are cell-type specific, in that the migration of gingival fibroblasts and endothelial cells on FN substrata is not responsive to fibulin-1. Additional studies found that the mechanism for the motility-suppressive effects of fibulin-1 does not involve perturbations of interactions between α5β1 or α4 integrins, or heparan sulfate proteoglycans with FN. However, fibulin-1 was found to inhibit extracellular signal regulated kinase (ERK) activation and to suppress phosphorylation of myosin heavy chain. This ability to influence signal transduction cascades that modulate the actin-myosin motor complex might be the basis for the effects of fibulin-1 on adhesion and motility.

Key words: Fibulin, Fibronectin, Adhesion, Migration, Actinomyosin

INTRODUCTION

Cell movement is a fundamental feature of many normal and pathological processes, including developmental morphogenesis, inflammation, wound healing and metastasis. The movement of cells in these contexts is largely dependent on cell interactions with specific components of the extracellular matrix (ECM) (Ruoslahti, 1992; Sheetz et al., 1998). Cell-ECM interactions promote, sustain, orient and inhibit cell movement (Lukashev and Werb, 1998). The literature is replete with examples of ECM proteins that promote cell motility, such as fibronectin (FN) (Duband et al., 1990), laminin (Davis et al., 1989), thrombospondin-1 (Taraboletti et al., 1987) and collagens (Olvera and Furcht, 1993; Perris et al., 1991). A growing number of ECM proteins that negatively regulate cell motility have also been described, including tenascin, osteonectin/SPARC, aggrecan and versican (Hasselea and Sage, 1992; Landolt et al., 1995; Perris et al., 1996; Spring et al., 1989).

Fibulin-1, a calcium-binding glycoprotein, is found in association with ECM structures such as microfibrils, basement membranes, elastic fibres and fibrin (Roark et al., 1995; Tran et al., 1995). The association of fibulin-1 with these ECM structures is probably based on its ability to bind ECM proteins such as FN, laminin, nidogen, endostatin (C-terminal domain NC1 of collagen XVIII), tropoelastin and fibrinogen (Balbona et al., 1992; Pan et al., 1993; Sasaki et al., 1998; Sasaki et al., 1999; Tran et al., 1995). Several studies suggest that the interaction between fibulin-1 and FN might be of particular importance. For example, fibulin-1 can be detected with FN in focal adhesion sites an hour after seeding fibroblastic cells on FN-coated surfaces (Argraves et al., 1989; Argraves et al., 1990). Within 12-24 hours after seeding of such cells, fibulin-1 can be found decorating FN-containing microfibrils (Argraves et al., 1990). Treatment of fibroblastic cells with antagonists of FN matrix assembly, such as integrin antibodies, blocks the incorporation of fibulin-1 into ECM fibrils (Godyna et al., 1994; Roman and McDonald, 1993). Furthermore, cells that fail to assemble a FN matrix do not incorporate fibulin-1 into ECM fibrils (Godyna et al., 1994). The association of fibulin-1 and FN has been demonstrated in vivo in embryonic tissues such as the cardiac cushions (Bouchey et al., 1996) and in tissues of the adult such as bone marrow stroma (Gu et al., 2000). Given the close relationship between FN and fibulin-1, we explored the possibility that fibulin-1 functions to regulate important...
biological activities of FN including promotion of cell adhesion and motility.

MATERIALS AND METHODS

Proteins

Fibulin-1 was purified from extracts of human placenta by immunoaffinity chromatography using mouse monoclonal 3A11 anti-fibulin-1 IgG-Sepharose (Argraves et al., 1990; Godyna et al., 1994). FN was purified from human plasma as described by Brew and Ingham (Brew and Ingham, 1994). Tissue type collagenogen (rat tail) was purchased from Becton Dickinson (Collaborative Biomedical, Bradford, MA). Pepsin digestion of type I collagen was done according to Leibovich and Weiss (Leibovich and Weiss, 1970). Bovine serum albumin (BSA) was purchased from USB (Cleveland, OH). Integrin αβ1 was purified from detergent extracts of human placenta according to Argraves and Tran (Argraves and Tran, 1994).

Antibodies

Mouse monoclonal anti-fibulin-1 antibody 3A11 has been described previously (Argraves et al., 1990). Mouse monoclonal anti-integrin-β1 antibody 442 was provided by E. Ruoslahti (Burnham Institute, La Jolla, CA). Rabbit polyclonal anti-β1-integrin cytoplasmic domain antibody 363 was provided by R. Hynes (Massachusetts Institute of Technology, Cambridge, MA). Rabbit polyclonal anti-integrin-α4 antibody was provided by M. Hemler (Dana-Farber Cancer Institute, Boston, MA). Rabbit antibodies to human non-muscle myosin heavy chain were purchased from Biomedical Technologies (Stoughton, MA). Polyclonal antibodies to unphosphorylated ERK (a MAP kinase) and monoclonal antibody to phosphorylated ERK1/2 were purchased from Cell Signaling (Beverly, MA).

Cells

Human breast adenocarcinoma MDA MB231 cells (ATCC; HTB-26) were cultured in Leibovitz L 15 medium with 10% foetal bovine serum (FBS) (Mediatech, Herdon, VA), 100 units ml\(^{-1}\) penicillin and 100 μg ml\(^{-1}\) streptomycin (Mediatech). Human malignant melanoma A375 SM cells were obtained from I. J. Fiddler (University of Texas, MD Anderson Cancer Center) and were cultured in Minimum Essential Medium (MEM) containing 10% FBS, MEM vitamins, L-glutamine, sodium pyruvate and non-essential amino acids (Mediatech). Human epidermoid carcinoma A-431 cells were grown in Dulbecco's Essential Medium (MEM) containing 10% FBS, MEM vitamins, L-glutamine, sodium pyruvate and non-essential amino acids (Mediatech). Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologics and grown in Medium 200 containing low serum growth supplement (Cascade Biologics, Portland, OR). Bovine aortic endothelial cells (BAECs) were released by scraping from the inside surface of an adult bovine aorta and cultured in MEM. 10% FBS, basic fibroblast growth factor (bFGF; 10 ng ml\(^{-1}\)) and 4.5 g l\(^{-1}\) glucose. Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologics and grown in Medium 200 containing low serum growth supplement (Cascade Biologics, Portland, OR). Bovine aortic endothelial cells (BAECs) were released by scraping from the inside surface of an adult bovine aorta and cultured in MEM and used for all experiments (Mediatech).

Transfection of fibulin-1 expression constructs

A cDNA comprising the complete coding sequence of human fibulin-1D (Tran et al., 1997) was ligated into vector pcDNA3Neo (Invitrogen, San Diego, CA). The pcDNANeo-fibulin-1D plasmid and the empty vector were separately introduced into MDA MB231 cells using Lipofectamine reagent (Gibco BRL, Rockville, MD). The cells were then grown in DMEM, 20% BCS for 18 hours and then in DMEM containing 10% BCS for 24 hours. The cells were then replated and grown in DMEM, 10% BCS and 1 mg ml\(^{-1}\) G418. 18 days after transfection, individual colonies were picked using a sterile cotton swab and transferred to 24-well plates. To assay for fibulin-1 production, the transfected cells were grown in serum-free DMEM (sDMEM) containing ITS (5 μg ml\(^{-1}\) insulin, 5 μg ml\(^{-1}\) transferrin, 5 ng ml\(^{-1}\) selenous acid (Beckton Dickinson, Franklin Lakes, NJ). Enzyme-linked immunosorbent assay (ELISA) was used to measure fibulin-1 in conditioned culture medium. Colonies that tested positive for high level fibulin-1D production were cloned by the limited dilution method.

Cell adhesion assay

Non-tissue-culture polystyrene (Dynatech, Chantilly, VA) and tissue-culture polystyrene (Corning, Corning, NY) microtitre wells were coated with fibulin-1, FN or BSA (over a range of concentrations 0.012-50 μg ml\(^{-1}\)) in 150 mM NaCl, 50 mM Tris (as TBS, pH 8.0) for 18 hours at 4°C. Unoccupied sites were then blocked with 1 mg ml\(^{-1}\) BSA at room temperature for 1 hour. Cells were released with 0.05% trypsin, 0.53 mM EDTA (trypsin-EDTA), washed once with DMEM containing 0.5 mg ml\(^{-1}\) soybean trypsin inhibitor (Sigma, St Louis, MO) and once with DMEM, and then suspended in DMEM at 3.5×10\(^5\) cells ml\(^{-1}\). Cells were added to the coated wells (3.5×10\(^5\) cells ml\(^{-1}\)) and allowed to attach for 1 hour at 37°C, 5% CO\(_2\). After gentle rinsing with TBS pH 7.4, attached cells were fixed for 30 minutes with 10% formaldehyde in Dulbecco's PBS (dPBS; Sigma) and then stained with 0.25% crystal violet for 4 hours. The cells were rinsed with deionized water and the stain released using 1% SDS and quantified by spectrophotometry (560 nm) using a Molecular Devices plate reader.

Video microscopy analysis of cell spreading

To evaluate the effect of fibulin-1 on FN-stimulated cell spreading, tissue-culture polystyrene microtitre wells and non-tissue-culture polystyrene microtitre wells were first coated with FN (8 μg ml\(^{-1}\)) and unoccupied sites blocked as above. Fibulin-1 or BSA (50 μg ml\(^{-1}\) in dPBS) were then incubated with the FN-coated surfaces for 4 hours at 37°C. Cells (2×10\(^4\) in DMEM) were added to each well and the plates placed on the heated stage of a microscope equipped with a video camera interfaced with a computer operating DIAS software (Solliteek, Oakdale, IA). Images of the cells were captured every 2.5 minutes for 60 minutes. Spread cells were counted manually in each frame and the percentage of spread cells plotted as a function of time. Spreading rates of fibulin-1-transfected MDA MB231 cells and empty-vector-transfected cells were determined on microtitre wells coated with FN and blocked with BSA as above.

Haptotactic Transwell migration assays

The undersurfaces of Transwell 0.64 cm\(^2\) filter inserts (8 μm pores, Becton Dickenson, Franklin Lakes, NJ) were coated with FN (100 μg ml\(^{-1}\)) in TBS pH 8.0 overnight at 4°C. The FN-coated surfaces were rinsed with sterile dPBS, dried by vacuum aspiration and incubated with 1 mg ml\(^{-1}\) BSA for 1 hour at room temperature. The filters were then rinsed in dPBS and incubated with either fibulin-1 or BSA (100 μg ml\(^{-1}\) in dPBS, 1 mM CaCl\(_2\)) for 4 hours at 37°C in a humidified chamber. The filters were rinsed, dried and placed in wells of 24-well plates containing 0.5 ml sDMEM supplemented with the serum supplemented with 100 U ml\(^{-1}\) penicillin and 100 μg ml\(^{-1}\) streptomycin (Mediatech).

4588 JOURNAL OF CELL SCIENCE 114 (24)
 Videomicroscopy analysis of two-dimensional cell migration

A computer-controlled microscopy system was developed to capture time-lapse images of cells migrating on tissue culture dishes. The system uses a Pentium II computer to control motorized movement of the stage of an Olympus CK-2 inverted phase microscope and image capture from a video camera connected to a frame grabber card. The microscope is contained within a 37°C incubator. The instrument control software was designed to direct an autofocus operation involving incrementing the stage through a series of optical planes (in the z direction) and capturing a digital image from the camera at each focal plane. An algorithm selects from the series of images the one with the maximal standard deviation of the histogram of grey scale intensity. This process can be repeated such that images are collected over varying periods of time, typically 24 hours or longer. Furthermore, the computer software also controls motorized x- and y-axis movement of the stage, which permits the collection of images of cells cultured in multiple wells of a 24-well dish. The time-lapsed images were analysed using another custom program that allows manual placement of a symbol over the cell centroid. The position of the centroids were updated in consecutive images so that the trajectory and migration rate of each cell during the observation period could be derived. In this study, time-lapse photography was initiated after cells had been allowed to attach for 1 hour and velocity calculations were based on cell movements measured during 5-hour windows of time incremented every 20 minutes. Average cell displacements were calculated for a series of time windows ranging from 20 minutes up to 20 hours. For example, the average displacement over individual 20-minute intervals was calculated at multiple times during a 20 hour period of culture and the mean of these values plotted at 20 minutes. The process was repeated for successively longer intervals (e.g. 40 min, 60 min, 80 min…20 hour) over a 20 hour period of culture.

Three-dimensional gel migration assay

The migration behaviour of cardiac cushion tissue cells in a collagen-lattice culture system was performed essentially as described by Bernanke and Markwald (Bernanke and Markwald, 1982). Briefly, chick embryos (stage 18) were collected and placed into sterile Earle’s balanced salt solution. The atrioventricular region of the hearts was dissected from the embryos and cut longitudinally to expose the lumen. The explants were placed on an ~0.4 mm thick gel of 1.0 mg ml⁻¹ pepsin-digested rat type I collagen (Becton Dickinson/ Collaborative Biomedical Products), 20 μg ml⁻¹ bovine FN (Sigma) with or without fibulin-1 (100 μg ml⁻¹) in M199, 1% chicken serum, ITS, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. The explants were incubated at 37°C, 5% CO₂. After varying periods of incubation, the number of cells at different depths from the surface of the gel (0 μm, 40 μm, 80 μm, 120 μm, 200 μm, 240 μm, 280 μm and 320 μm) was counted using an inverted microscope (Olympus, IMT-2). Only cells in the plane of focus were counted at each optical plane.

Assessment of the role of proteoglycans in fibulin-1 suppression of cell motility

MDA MB231 cells were grown for 18 hours in the presence or absence of chlorate (20 mM) in low-sulfate Ham’s F12 medium (Gibco BRL), 2 mM glutamine, 1 mM pyruvate and 10% dialysed BCS. The cells were then released using non-enzymatic dissociation solution (Sigma) and used in migration assays as described above. To quantify the magnitude of the chlorate effect on proteoglycan sulfation, the cells were cultured in the aforementioned medium containing carrier-free [³⁵S]-Na₂SO₄ (50 μCi ml⁻¹; NEN, Boston, MA). The medium was removed and the cell layer washed with ice-cold TBS and then extracted with extraction buffer (1% Triton X-100, 0.5 M NaCl, 0.5% Tween 20, 50 mM Hapes, pH 7.5) containing a

![Fig. 1. Fibulin-1 as an adhesion protein. The indicated cells (3.5x10⁴ per well) were incubated in microtitre wells coated with varying concentrations of FN, fibulin-1 or BSA for 1 hour at 37°C, 5% CO₂. After fixation the bound cells were stained with 0.25% crystal violet and the stain quantified by spectrophotometry after release with 1% SDS.](image)
proteinase inhibitor cocktail (Complete Mini EDTA-free, Boehringer Mannheim). The extract was passed through a 21 gauge needle several times and centrifuged at 100,000 g for 30 minutes. The supernatant was collected, the ionic strength adjusted to 0.3 M NaCl and insoluble material removed by centrifugation at 12,000 g for 30 minutes. Equal amounts of protein (50 μg) from chlorate-treated and untreated cells were mixed with 130 μl of DEAE Fast Flow Sepharose (Amersham Pharmacia, Piscataway, NJ) and incubated at room temperature for 30 minutes by nutational motion. The DEAE Sepharose was centrifuged at 1000 g for 2 minutes, the supernatant removed and the resin washed five times with TBS containing 0.3 M NaCl. Bound proteins were eluted from the DEAE-Sepharose by adding 1 ml of 1.5 M NaCl to the resin followed by vortex mixing and centrifugation to pellet the resin. The amount of radioactivity in the eluates was measured using a scintillation counter.

**RT-PCR analysis of integrin α4 subunit expression**

Total RNA was extracted from various cultured human cell lines using RNA Stat 60 (Tel-Test, Friendswood, TX) and cDNA was made with random hexamer oligodeoxynucleotide primers using Superscript reverse transcriptase (Gibco) as per the manufacturer’s instructions. Primers for human α4 subunit were designed based on the human α4 subunit sequence (X16983) and human GAPDH (NM_002046). Primers for human α4 subunit were CTGAAACGTGCAATGGTGAG (residues 2432-2451) and CATCGCAGAATTCTCATCC (residues 2907-2926). Primers for human GAPDH were CCGAGTCAACGG-ATTGGTCG (residues 93-113) and GCCCTCTCATGTTGGTG-AAG (residues 378-398). PCR was performed with these primer pairs and Taq polymerase (Qiagen) using 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes, with a final extension of 7 minutes.

**α4 immunoblotting**

Cells were extracted using extraction buffer as above. Equal amounts of protein (20 μg) from each cell extract were electrophoresed on 4-12% polyacrylamide gels (Invitrogen). The gel was then used either for autoradiography or immunoblot analysis.

**Receptor selection using magnetic bead-ligand conjugates**

Tosyl activated M-450 Dynabeads (Dynal Oslo, Norway) were conjugated to FN, fibulin-1 or BSA (5 μg per 10² beads) according to the manufacturer instructions. The FN beads were incubated with either BSA or fibulin-1 (each at 100 μg ml⁻¹) for 36 hours at 4°C. FN, FN-fibulin-1, fibulin-1 or BSA beads were incubated with cell surface labelled MDA MB231 cells (3.5x10⁵ per 10² beads) for 35 minutes in sDMEM containing either 50 μg ml⁻¹ fibulin-1 or 50 μg ml⁻¹ BSA. The cells had been surface radioiodinated using a lactoperoxidase/glucose oxidase procedure (Hammad et al., 1999). In a manner similar to that described by Plopper and Ingber (Plopper and Ingber, 1993), the beads were rinsed with CSK buffer (50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM Pipes pH 6.8 and enzyme inhibitors) and bound cells were extracted by sonication on ice in CSK buffer containing 0.5% Triton X-100. The beads were rinsed five times with CSK buffer and then mixed with SDS-PAGE sample buffer (non-reducing) and subjected to electrophoresis on 4-20% polyacrylamide gels (Invitrogen). The gel was then used either for autoradiography or immunoblot analysis.

**Solid phase binding assays**

Analysis of the effect of fibulin-1 on α5β1 binding to FN was done using an ELISA similar to the procedure of Hautanan et al. (Hautanan et al., 1989). Briefly, non-tissue-culture polystyrene and tissue-culture-treated polystyrene microtitre plate wells were coated with FN (3 μg ml⁻¹ in TBS, pH 8.0) and unoccupied binding sites were blocked using 3% non-fat dry milk in TBS pH 7.4. The wells were then incubated with either fibulin-1 or BSA at 50 μg ml⁻¹ in PBS, pH 7.4, for 4 hours at 37°C. The wells were rinsed and incubated with α5β1 (0.013-10 nM) in incubation buffer (TBS pH 7.4 containing 25 mM n-octyl-β-D-glucopyranoside, 1 mM MnSO₄). The incubation was performed at 4°C for 20 hours, after which the wells were rinsed with incubation buffer and bound α5β1 was quantified using monoclonal anti-β1 IgG, HRP-conjugated anti-mouse IgG and the chromogenic substrate o-phenylenediamine (Sigma). Binding affinity was estimated by fitting the data to a form of the binding isotherm as described by Balbana et al. (Balbana et al., 1992) using Sigmaplot (Jandel Scientific, San Rafael, CA).

**MHC phosphorylation analysis**

Immunoprecipitation analysis was performed to evaluate the effect of fibulin-1 on the phosphorylation of MHC. Cultured MDA MB231 cells were serum starved for 24 hours, released with trypsin-EDTA and metabolically labelled in suspension with [32P]-orthophosphate (0.25 mCi ml⁻¹) in phosphate-free DMEM for 1 hour. The cells were plated into dishes either coated with FN (100 μg ml⁻¹) and blocked with 0.2% BSA or coated with FN (100 μg ml⁻¹), blocked with 0.2% BSA and incubated with fibulin-1 (50 μg ml⁻¹) (FN-fibulin-1). After a 30 minute incubation period, the cells were washed with dPBS and lysed in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium vanadate, 10 mM Na pyrophosphate, 100 mM NaF, 10 μg ml⁻¹ leupeptin, 10 μg ml⁻¹ aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 12,000 g and the supernatants absorbed with protein-G-Sepharose. The protein concentration of extracts was quantified using the BCA method (Pierce, Rockford, IL). Equal amounts of protein (100 μg) from each extract were mixed with MHC antibody and incubated for 2 hours at 4°C, and the immune complexes were precipitated.
using protein-G-Sepharose. The immunoprecipitated material was electrophoresed on a 10% polyacrylamide gel and subjected to autoradiography.

**RESULTS**

**Most cells tested do not adhere to fibulin-1 substrata**

To address the question of whether fibulin-1 might have a role in cellular motility, we initially evaluated the possibility that fibulin-1 was an adhesive protein. As shown in Fig. 1, microtitre well adhesion analysis indicated that fibulin-1 (over a range of coating concentrations) was not adhesive for MDA MB231 breast carcinoma cells, A375 melanoma cells, gingival fibroblasts, BAECs or CEM lymphoblastic leukaemia cells. Similar results were obtained using Molt-4 lymphoblastic leukaemia cells, HUVECs, WI-38 lung fibroblasts and HT1080 fibrosarcoma cells (data not shown). However, A431 epidermal carcinoma cells displayed a moderate degree of adhesion to fibulin-1-coated surfaces compared with FN-coated surfaces (Fig. 1).

**ERK analysis**

MDA MB231 cells were cultured in 0.5% serum for 16 hours. The cells were then removed using trypsin-EDTA and washed with sfDMEM and resuspended in 0.2% BSA-containing DMEM. The cells were incubated in suspension for 4 hours and then allowed to attach to substrata of FN (30 µg ml⁻¹) blocked with 0.2% lipid-free BSA (Sigma) or to FN (30 µg ml⁻¹) blocked with 0.2% BSA and incubated with fibulin-1 (60 µg ml⁻¹). After various periods of attachment (5 minutes, 10 minutes and 20 minutes), unattached cells were collected and centrifuged for 5 minutes at 1000 g and at 4°C. These cell and attached cells extracted in combination using RIPA. Equal amounts of protein (12.5 µg) from each extract were run on 4-12% polyacrylamide gels, transferred to PVDF and probed with anti-phosphorylated-ERK IgG and HRP-conjugated anti-mouse antibody with ECL reagent. The blots were stripped by incubation with 2% SDS, 100 mM mercaptoethanol, 62.5 mM Tris, pH 6.7, and re-probed with anti-unphosphorylated-ERK IgG.

**Fig. 3.** Effect of fibulin-1 on FN-stimulated cell spreading. (A,B) The proportion of MDA MB231 cells that spread on tissue-culture polystyrene (A) or non-tissue-culture polystyrene (B) coated with FN or FN-fibulin-1 as a function of time. (C) Spreading of MDA MB231 cells transfected to express fibulin-1 and vector-transfected controls on FN substrata on tissue-culture polystyrene. (A-C) Unoccupied sites were blocked with BSA. Digital images of fields of cells containing 60-100 cells were collected at 2.5 minute intervals for 1 hour after seeding, and the number of cells spread in each successive image was quantified. (D,E) MDA MB231 cells transfected with empty vector (D) or fibulin-1 expression construct (E) were seeded onto FN coatings and allowed to attach for 40 minutes. The cells were then fixed, stained and photographed. Notice the spread and migratory morphology of the empty-vector-transfected cells as opposed to the rounded morphology of fibulin-1-transfected cells.

**Fig. 4.** Cell type-specific inhibition of FN-stimulated haptotactic migration by fibulin-1. The undersurfaces of Transwell membranes (8 µm pores) were coated with FN (100 µg ml⁻¹), unoccupied sites were blocked with BSA and were then incubated with fibulin-1 or BSA (100 µg ml⁻¹ each). Cells (1×10⁵) in sfDMEM were added to the upper chambers of inserts placed in wells containing sfDMEM plus fibulin-1 or BSA (50 µg ml⁻¹ each). After 18 hours of incubation at 37°C, 5% CO₂, the number of cells that had migrated to the undersurface of the membranes was determined. The plotted values are means ± the standard deviations of cell counts from five independent optical fields from triplicate experiments. The results shown were obtained using fibulin-1 isolated by monoclonal antibody 3A11 IgG-Sepharose. Similar findings were obtained using fibulin-1 isolated with a monoclonal antibody 5D12 which binds to an epitope distinct from that of 3A11 (data not shown).
Fibulin-1 inhibits cell adhesion and spreading on FN

Considering that fibulin-1 interacts with FN, a major goal of this study was to evaluate the ability of fibulin-1 to modulate FN-mediated cellular adhesion, spreading and migration. We first evaluated the capacity of fibulin-1 to modulate FN-mediated cellular adhesion and spreading. As shown in Fig. 2, fibulin-1 had inhibitory effects on the attachment of cells to FN-coated surfaces. The magnitude of the inhibitory effects of fibulin-1 were greatest when the FN was coated on tissue-culture polystyrene (Fig. 2A) as opposed to non-tissue-culture polystyrene (Fig. 2B). In addition, the rate of cell spreading on FN-fibulin-1 substrate was lower than that on FN substrate (Fig. 3A). As was the case for adhesion, the magnitude of the anti-spreading effect of fibulin-1 was higher on tissue-culture polystyrene coated with FN (Fig. 3A) than on non-tissue-culture polystyrene (Fig. 3B). Control ELISA experiments showed that the type of plastic to which FN was coated did not affect the binding of fibulin-1 to FN (estimated \( K_d \) of 210 nM for fibulin-1 binding to FN immobilized on tissue-culture polystyrene versus 214 nM on non-tissue-culture polystyrene, assuming a 150 kDa fibulin-1 dimer).

Consistent with these observations, MDA MB231 cells that were transfected to overexpress fibulin-1 showed impaired ability to spread on FN substrate compared with vector-transfected cells (Fig. 3C). Microscopic examination of the MDA MB231 cells transfected to overexpress fibulin-1 that were allowed to attach to FN-coated dishes for 1 hour showed that they displayed a rounded morphology and generally lacked lamellipodia (Fig. 3E). By contrast, vector-transfected cells spread well and had pronounced lamellipodia, consistent with migratory activity (Fig. 3D). Taken together, the findings indicate that both exogenously added fibulin-1 and fibulin-1 produced endogenously through recombinant means inhibit spreading of cells on FN.

Fig. 5. Fibulin-1 inhibits the invasion of endocardial cushion mesenchymal cells into gels of type I collagen and fibronectin. Stage 18 chick endocardial cushion explants were placed onto the surface of gels of type I collagen (1 mg ml\(^{-1}\)) containing FN (20 \( \mu \)g ml\(^{-1}\)) plus or minus fibulin-1 (100 \( \mu \)g ml\(^{-1}\)). The number of cells that had invaded into the gels (A) and the depth of invasion (B) were quantified after 50 hours. The plotted values in A are means ± the standard deviations from measurements made on four cushion explants. (A,B) The number of cells in optical planes extending through a depth of 400 \( \mu \)m of the gel in four areas underlying each explant was counted and summed. Asterisk (*) indicates that the value is significantly different from control (\( P<0.05 \)).

Fig. 6. Two-dimensional cell migration analysis. (A) Average velocity of MDA MB231 cells on FN or FN-fibulin-1 substrata. Each value that is plotted represents an average velocity calculated from the displacement of ~20 cell centroids during a 5 hour period. Shown are data from two and three independent experiments of cell migration on FN and FN-fibulin-1 substrata, respectively. Lines represent linear fits of all points in all experiments for each substratum. (B) Average distance that a cell travelled during a given time on FN versus FN-fibulin-1 substratum. Each plotted value represents an average of the distance travelled for ~20 cells for various times beginning 1 hour after seeding onto FN or FN-fibulin-1. Shown are data from two and three independent experiments of cell migration on FN and FN-fibulin-1 substrata, respectively. (C) Average distance that MDA MB231 cells transfected with either empty vector or fibulin-1 expression construct travel as a function of time on FN substratum. Each plotted value represents an average of the distance travelled by ~20 cells for various times after seeding onto FN. (D) Quantification by ELISA of the amount of fibulin-1 secreted into the medium by MDA MB231 cells transfected with fibulin-1 expression construct.
Fibulin-1 suppresses FN-dependent haptotactic motility

Quantitative analyses of the effect of fibulin-1 on FN-stimulated cell motility were performed using a haptotactic Boyden chamber-type assay, a collagen gel invasion assay and a computerized video microscopy assay of two-dimensional migration. Fibulin-1 inhibited the haptotactic migration stimulated by FN of a number of cell lines, including human (highly metastatic) melanoma (A375 SM), epidermoid carcinoma (A431), rat pulmonary aortic smooth muscle cells (PAC1), MDA MB231 cells, CHO cells and SV40-transformed WI-38 cells (WI-38 V A13) (Fig. 4). The magnitude of the inhibition varied according to the cell type. For example, we measured 73.7 ± 17% inhibition (n = 29) of migration of MDA MB231 cells, 68.33 ± 24% inhibition (n = 9) for A375 SM and 38.5 ± 19% inhibition (n = 4) for PAC1 cells. By contrast, the FN-stimulated migration of BAECs, gingival fibroblasts, HUVECs and lung fibroblasts (WI-38) was not inhibited by fibulin-1 (Fig. 4). These findings indicate that the motility-suppressing activity of fibulin-1 is cell-type specific. Through the course of these studies, we also noted that MDA MB231 cells became refractory to the motility suppressive effects of fibulin-1 after many passages (>43) (data not shown).

Fibulin-1 suppresses the invasiveness of mesenchymal cells when FN is present

Using a collagen gel invasion assay (Bernanke and Markwald, 1982; Sugi and Markwald, 1996), the effect of fibulin-1 on the invasive behaviour of mesenchymal cells leaving explanted endocardial cushions (stage 18) was measured. In these experiments, cushion explants were placed onto gels of type I collagen, type I collagen-FN or type I collagen-FN-fibulin-1 and cultured for 50 hours. The number of cushion mesenchymal cells that migrated into the gels and the depth that the cells penetrated into the gels were then measured. The presence of fibulin-1 (100 µg ml⁻¹) in type I collagen-FN gels resulted in ~50% inhibition (P < 0.001) in both the number of cells that entered the gel (Fig. 5A) and the distance that mesenchymal cells penetrated into the gel (Fig. 5B). By contrast, fibulin-1 had little or no effect on the invasion of mesenchymal cells into type I collagen gels that lacked FN (Fig. 5A,B). Interestingly, the presence of FN in the collagen gel did not effect the magnitude of mesenchymal cell invasion as compared to collagen alone suggesting that collagen was the principle motility promoting factor. The fact that mesenchymal cell invasiveness was reduced when both fibulin-1 and FN were present in the gel suggests that the two proteins generate a motility suppressing signal.

Fibulin-1 reduces migration velocity

Using a computerized system to analyse two-dimensional cell migration, rates of cell migration on culture dishes coated with FN or FN plus fibulin-1 were measured. The data shown in Fig. 6 indicate that fibulin-1 inhibits the rate (Fig. 6A) and distance (Fig. 6B) of migration of MDA MB231 cells by as much as 50% that of controls. Interestingly, the migration velocity of cells on a FN-fibulin-1 substratum remains uniform over at least 35 hours. These findings indicate that the

Fig. 7. α4-Integrins are not required for fibulin-1 inhibition of FN-stimulated haptotactic migration. (A) Evaluation of the effect of fibulin-1 on haptotactic migration of CHO cells engineered to express α4β1. Analysis of expression of the integrin α4 subunit in various cultured lines by RT-PCR (B) and immunoblotting (C). The arrowheads in C indicate the intact ~150 kDa and ~180 kDa forms of the α4 polypeptide typically observed under non-reducing conditions.

Fig. 8. Fibulin-1 does not bind to cell surface proteins and does not effect cell surface β1 integrin binding to FN. Radiolabelled MDA MB231 cell surface proteins were bound to magnetic beads conjugated with fibulin-1, FN or BSA. Before incubation with cells, FN-beads were incubated with either fibulin-1 (FN + fibulin-1) or BSA (FN + BSA). (A) Autoradiograph and (B) immunoblot analysis using anti-β1-integrin cytoplasmic domain antibody.
the role of binding to either of the integrin binding sites. To investigate The fibulin-1 binding site in FN is located in type III module13, which binds integrins such as α5β1, and the variable region (V-region or IIICS region), which binds α4 integrins (e.g. α4β1 and α4β7) (Guan and Hynes, 1990; Mould and Humphries, 1991; Mould et al., 1990). It was therefore possible that fibulin-1 binding to type III module13 might sterically or allosterically modulate integrin binding to either of the integrin binding sites. To investigate the role of α4 integrins in the process of fibulin-1 suppression of FN-stimulated cell motility, α4-deficient cells were tested for their responsiveness to fibulin-1. It was found that the migration of α4-deficient CHO cells on FN substratum could be inhibited by fibulin-1 to the same extent as observed with CHO cells transfected to express α4 and β1 subunits (Fig. 7A). In addition, analysis of α4 expression in the fibulin-1-responsive cell lines A431 and MDA MB231 showed that the cells had no detectable α4 mRNA or protein (Fig. 7B,C). These findings indicate that the inhibitory effects of fibulin-1 do not necessarily involve perturbations of the interaction of α4 integrins with FN or the signals that these interactions elicit.

**Fibulin-1 does not perturb α5β1 binding to FN**

The previous conclusion left open the possibility that fibulin-1 might modulate (sterically or allosterically) integrin (e.g. α5β1) interaction with the RGD site within type III module10 of FN. To address this possibility, we evaluated the effect of fibulin-1 on the ability of β1 integrin expressed on the surface of cells to bind FN-conjugated magnetic beads. Surface radiolabelled MDA MB231 cells were mixed with FN beads and the bead-associated cells extracted and bead-bound protein evaluated by autoradiography after SDS-PAGE. Several radiolabelled cell surface proteins bound to FN-conjugated magnetic beads (Fig. 8A). Immunoblot analysis of the FN-bead-bound protein fraction revealed the presence of a polypeptide that reacts with anti-integrin-β1-subunit antibody (Fig. 8B). This polypeptide had an electrophoretic mobility corresponding to a prominent radiolabelled polypeptide in the FN-bead-bound fraction. Preincubation of the beads with fibulin-1 and inclusion of fibulin-1 in the medium during incubation of the beads with cells did not affect the level of integrin β1 subunit that bound to the beads (Fig. 8B). It was also observed that no cell-surface protein bound to magnetic beads conjugated to fibulin-1, suggesting that fibulin-1 does not interact directly with a cell surface receptor.

We next used an ELISA to evaluate the binding of purified integrin α5β1 to FN in the presence and absence of fibulin-1. The estimated binding affinities (i.e. half-maximal saturation values) measured for α5β1 binding to FN on tissue culture polystyrene were 2.21 nM (n=2) in the absence of fibulin-1 and 2.11 nM (n=2) in the presence of fibulin-1 bound to FN (Fig. 9A). When the assays were performed using FN immobilized on non-tissue-culture polystyrene, an estimated binding affinity of 0.75 nM (n=2) was obtained in the absence of fibulin-1, and 0.57 nM (n=2) was obtained in the presence of fibulin-1 (Fig. 9B). The findings indicate that fibulin-1 does not perturb α5β1 interaction with FN. The aforementioned assays were performed in the presence of manganese to augment α5β1 interaction with FN. This suggests that fibulin-1 does not perturb α5β1 interaction with FN.
binding affinity (Hautanen et al., 1989), but similar findings were obtained when calcium was substituted for manganese (data not shown).

**Fibulin-1 motility-suppressive activity is independent of glycosaminoglycan sulfation**

Type III module13 is the major heparin-binding site in FN and mediates binding to heparan sulfate moieties of cell surface proteoglycans such as syndecan-4 (Saoncella et al., 1999). We therefore hypothesized that fibulin-1 binding to FN module type III13 might stericly or allosterically inhibit cell surface proteoglycan interactions either by interfering with glycosaminoglycan (GAG) or proteoglycan core protein binding to type III module13. To test this hypothesis, cells were treated with chlorate, a drug that inhibits GAG sulfation, and evaluated for their responsiveness to fibulin-1. The chlorate treatment was effective at reducing GAG sulfation (Fig. 10A) but it did not alter the ability of fibulin-1 to suppress motility (Fig. 10B). These findings suggest that sulfate moieties on GAG chains are not required in the process of fibulin-1 suppression of FN-stimulated cell adhesion and motility.

**Fibulin-1 modulates signalling associated with regulation of actin-myosin complex assembly**

We were interested in characterizing the signal transduction pathways that fibulin-1 modulates, particularly those that regulate cellular locomotion machinery. Conventional myosins (myosin-IIIIs) generate forces for cell shape change and cell motility (Sinard and Pollard, 1989) and phosphorylation of myosin heavy chain (MHC) regulates myosin function (van Leeuwen et al., 1999). The level of [35S]-phosphorylation of MHC was reduced in MDA MB231 cells seeded for 30 minutes onto FN-fibulin-1 compared with cells seeded onto FN alone (Fig. 11A).

ERK is known to be activated by cell adhesion to FN and to modulate actin-myosin motor assembly (Zhu and Assoian, 1995; Stupack, 2000). We observed that fibulin-1 could inhibit FN-mediated activation of ERK (Fig. 11B). The inhibitory effect of fibulin-1 was apparent as early as 5 minutes after incubation of cells with surfaces coated with FN, and the effect persisted for at least 20 minutes. During the 20 minute period after incubation of cells with FN substratum, the level of ERK2 phosphorylation was observed to oscillate, and this oscillation was suppressed by fibulin-1.

**DISCUSSION**

The findings presented here indicate that fibulin-1 is an inhibitor of in vitro cell adhesion and motility. The in vivo significance of this activity remains to be determined. However, a motility-suppressing role for fibulin-1 is consistent with observations showing that, during development, fibulin-1 expression is found in association with certain migratory populations of cells (Spence et al., 1992). For example, endocardial cushion mesenchymal cells have fibulin-1 on their surfaces and deposited in adjacent ECM (Bouchez et al., 1996; Miosge et al., 1996; Spence et al., 1992). Similarly, fibulin-1 is expressed by epicardial cells that differentiate into cells that
migrate into the myocardium and endocardial cushions (Miosge et al., 1996; Zhang et al., 1996). In addition, fibulin-1 expression has been observed in and around cells migrating from the neural crest (Spence et al., 1992).

An in vivo motility-suppressing role for fibulin-1 would be expected to extend beyond the phase of embryonic development, because fibulin-1 is also a component of the ECM of many adult tissues (Gu et al., 2000; Roark et al., 1995). For example, fibulin-1 is prominently associated with the ECM that surrounds vascular smooth muscle cells (Roark et al., 1995), perhaps acting to suppress movement of quiescent smooth muscle cells or leukocytes. Likewise, fibulin-1 has been shown to be deposited into fibrin-containing thrombi (Tran et al., 1995) and might modulate the motility of cells that infiltrate clots and thus participate in remodelling of provisional matrices of wounds. Additionally, fibulin-1 has been found in peritumour stroma of human ovarian cancer (Clinton et al., 1996) and shown to suppress the motility of ovarian carcinoma cells in vitro (Hayashido et al., 1998). Furthermore, fibulin-1 has been shown to also suppress the growth of fibrosarcoma tumours in nude mice, presumably through its ability to suppress fibrosarcoma cell invasiveness (Qing et al., 1997). Data presented here highlights the fact that fibulin-1 suppresses the motility of a wide array of cancer cell lines. Therefore, fibulin-1, which is normally present in basement membranes and loose connective tissues, might suppress tumour cell invasion.

We show that fibulin-1 specifically suppresses the motility-promoting activity of FN. The underlying mechanism for this activity remains uncertain. In this regard, it is important to point out that fibulin-1 binds to FN within type III repeat module13 (Balbona et al., 1992). This repeat module contains the major cell surface heparan sulfate proteoglycan (HSPG)-binding domain of FN (Bloom et al., 1999; Saoncella et al., 1999; Woods et al., 1986), and an α5β1 integrin binding site (McCarthy et al., 1986; Mould and Humphries, 1991). The type III13 module is also near to the other integrin-binding sites in FN, the RGDb site contained within the type III10 module (Rossi et al., 1988) and the alternatively spliced V (IIICS) region (Humphries et al., 1986).

Given the location of the fibulin-1-binding site in the vicinity of binding sites for integrins and cell surface HSPGs, we evaluated the possibility that fibulin-1 might modulate the interaction of integrins and proteoglycans with FN, which are important for cellular adhesion and motility. Our results show that fibulin-1 does not significantly affect the binding of α5β1 to the RGDb site of FN. Furthermore, the motility suppressive activity of fibulin-1 is independent of α4 integrin interactions with FN and also of sulfation of GAG moieties on cell surface proteoglycans. In light of these findings, other less obvious mechanisms must now be considered to account for the motility suppressive activity of fibulin-1. For example, it is plausible that fibulin-1 binding to FN acts to expose the cryptic anti-adhesive site in FN that has recently been located in the Hep-2 region of FN, the same region that contains the fibulin-1-binding site (Watanabe et al., 2000). This cryptic anti-adhesive site is exposed following conformational changes in FN induced by heparin binding or urea denaturation (Watanabe et al., 2000). Absorption of FN onto different types of polystyrene induces distinct conformational changes in FN that result in differential cell spreading (Garcia et al., 1999). Indeed, we found that fibulin-1 had different effects on cell spreading depending on the type of polystyrene to which FN was absorbed. One interpretation for this effect is that the absorption of FN to certain plastics limits the ability of fibulin-1 to induce alteration in the conformation of FN required to expose the cryptic anti-adhesive site. Other mechanisms are certainly possible; one that we are presently investigating involves FN-promoted proteolytic degradation of fibulin-1, leading to the production of a biologically active fragment.

MHC phosphorylation has been postulated to initiate spreading by releasing F-actin from actinomyosin complexes, allowing it to reassemble within lamellipodial protrusions (van Leeuwen et al., 1999). This results in a loss of actinomyosin-based contractility, leading to cell spreading. Our findings indicate that fibulin-1 suppresses cell spreading and MHC phosphorylation. Cells attached to surfaces of FN plus fibulin-1 have a rounded morphology, generally lack lamellipodia and display a lower rate of spreading than cells on FN substrata. Fibulin-1 was also found to inhibit FN-mediated activation of ERK. It is not known whether there is a relationship between ERK and MHC phosphorylation. MHC phosphorylation has been shown to involve an influx of extracellular calcium and activation of a calmodulin-dependent kinase (CaM kinase) (van Leeuwen et al., 1999). Franklin et al. (Franklin et al., 2000) have shown that increases in intracellular calcium induce activation of ERK1/2 in human T cells via activation of CaM kinase. Inhibition of CaM kinase has been shown to prevent ERK1/2 activation (Rosengart et al., 2000). Fibulin-1 might reduce intracellular calcium levels, thus reducing the activation of both ERK and MHC. It might accomplish this by modulating α5β1-FN signalling, which has been shown to regulate a tyrosine phosphorylation cascade that controls the function of the L-type calcium channel (Wu et al., 2001).

Although fibulin-1 was shown to suppress the motility-promoting activity of FN, it had little or no effect on the motility-promoting activity of type I collagen (gelatin). Whether fibulin-1 is capable of modulating the motility-promoting activity of ECM proteins other than FN remains to be established. However, there is indirect evidence in support of such a possibility. We have reported previously that fibrosarcoma cells transfected to express fibulin-1 exhibited a greatly reduced ability to migrate through a reconstituted basement membrane compared with vector-transfected control fibrosarcoma cells (Qing et al., 1997). Because basement membranes contain little or no FN (Grant et al., 1985), the results suggest that fibulin-1 can also inhibit the migration-promoting activity of basement membrane components that include laminin, nidogen and type IV collagen. Interestingly, fibulin-1 has been shown to interact with each of these constituents (Pan et al., 1993).

This work was supported by National Institutes of Health Grants GM42912 and HL52813 (W.S.A.). W.O.T. is a recipient of a fellowship from the American Heart Association-South Carolina Affiliate. We also acknowledge support provided to W.O.T. by the W. M. Keck Dynamic Image Analysis Facility for use of instrumentation to analyse cell adhesion and motility. In this regard, we thank D. R. Soil (University of Iowa, Iowa City, IO) for providing technical training.
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