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Tenascin-C Suppresses Rho Activation

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Abstract. Cell binding to extracellular matrix (ECM) components changes cytoskeletal organization by the activation of Rho family GTPases. Tenascin-C, a developmentally regulated matrix protein, modulates cellular responses to other matrix proteins, such as fibronectin (FN). Here, we report that tenascin-C markedly altered cell phenotype on a three-dimensional fibrin matrix containing FN, resulting in suppression of actin stress fibers and induction of actin-rich filopodia. This distinct morphology was associated with complete suppression of the activation of RhoA, a small GTPase that induces actin stress fiber formation. Enforced activation of RhoA circumvented the effects of tenascin. Effects of active Rho were reversed by a Rho inhibitor C3 transferase. Suppression of GTPase activation allows tenascin-C expression to act as a regulatory switch to reverse the effects of adhesive proteins on Rho function. This represents a novel paradigm for the regulation of cytoskeletal organization by ECM.

Key words: tenascin-C • provisional matrix • fibronectin • Rho GTPase • filopodia

Introduction

Changes in tissue organization that govern development, disease, aging, and injury require defined alterations in extracellular matrix (ECM)[1] composition and architecture (Adams and Watt, 1993; Fleischmajer et al., 1998). Expression levels of fibronectin (FN), an adhesive protein, and tenascin-C, an ECM protein that modulates cell–FN interactions, vary during wound repair, tumor formation, and embryonic development. This provides a mechanism for modulating cell functions through temporal and spatial variations in proportions of adhesive and anti-adhesive ECM proteins. FN mediates cell adhesion primarily through heterodimeric integrin receptors binding to the arg-gly-asp (RGD) and adjacent sequences in the central cell binding domain (Hynes, 1992). Cell-FN interactions direct cytoskeletal organization and intracellular signaling and connect cells to other matrix components such as collagen, fibrin, and glycosaminoglycans (Mosher, 1989; Hynes, 1990).

Contrary to the adhesive role of FN, tenascin-C induces loss of focal adhesions and prevents cell adhesion and spreading on FN (Spring et al., 1989; Murphy-Ullrich et al., 1991). Tenascin-C signals can be mediated directly by interactions with cell surface proteins such as annexin II (Chung and Erickson, 1994) or with several different integrin receptors (Joshi et al., 1993; Prieto et al., 1993; Sriramarao et al., 1993; Yokosaki et al., 1994). Alternatively, control of cell responses may be indirect through binding to other matrix components including heparin and FN (Chiquet-Ehrismann et al., 1991; Aukhil et al., 1993; Chung et al., 1995). These complex effects and multiple binding partners suggest that tenascin-C functions in a context-dependent manner to modulate cell–matrix interactions. Indeed, the protein is expressed in areas of reduced cell adhesion during development, wound healing, and tumorigenesis (Mackie et al., 1988; Erickson and Bourdon, 1989; Chiquet-Ehrismann, 1993; Zagzag et al., 1995).

Signals from the matrix are communicated through integrins to intracellular pathways including the Rho family of small GTPases (Rho, Rac, and Cdc42; Hall, 1998; Schoenwaelder and Burridge, 1999). Rho activation promotes actin stress fiber assembly and focal adhesion formation which are common cell responses to FN (Ridley and Hall, 1992; Hall, 1998). Active Rac and Cdc42 induce extension of lamellipodia and filopodia, respectively (Hall, 1998), and appear to contribute to cell spreading on FN (Price et

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[1]*Abbreviations used in this paper:* ECM, extracellular matrix; 70Ten, recombinant chimeric protein containing FN and tenascin-C sequences; FN, fibronectin; RGD, arginine-glycine-aspartic acid cell-binding sequence.

al., 1998). Soluble extracellular factors including LPA, PDGF, and bradykinin stimulate activation of individual members of this family. However, direct regulation of Rho GTPases by specific components of the ECM has not been fully defined.

To address the opposing stimuli of FN and tenascin-C in a physiologically relevant matrix model, we used a covalently cross-linked fibrin-FN matrix as a three-dimensional substrate. This matrix resembles the fibrin-FN provisional matrix that forms at sites of tissue injury. Compared with FN alone, this matrix has distinct effects on cell behavior (Corbett et al., 1996; Corbett and Schwarzbauer, 1997). Here we show that inclusion of tenascin-C in the fibrin-FN matrix resulted in an altered fibroblast morphology with actin-rich filopodial projections. This unusual cell phenotype differs from the cortical actin arrangement that develops on fibrin-FN matrix. We find that the change in actin organization occurs because RhoA activation is completely suppressed by tenascin-C. These results show that signals from tenascin-C, and possibly other ECM proteins, modulate cell responses to FN through control of GTPase activities.

Materials and Methods

Protein Production

Rat plasma FN was purified by gelatin-Sepharose (Pharmacia Biotech) affinity chromatography from freshly drawn plasma (Wilson and Schwarzbauer, 1992). Recombinant amino-terminal 70-kD fragment of rat FN and mouse tenascin-C cDNA have been previously described (Schwarzbauer, 1991; Luczak et al., 1998). Native human tenascin-C from U251 glioma cells and consisting of >90% large splice variant was generously provided by Dr. Harold Erickson (Duke University Medical Center) (Aukhil et al., 1990).

Recombinant 70Ten cDNA was constructed in the baculovirus vector pVL1393 by ligating a 1,810-bp BamHI-PstI fragment encoding FN 70 kD with two tenascin-C fragments spanning the 3′ two thirds of the coding sequence, a 342-bp PstI-XbaI fragment and a terminal 3,891-bp XbaI-XhoI fragment. The junction between FN and tenascin-C sequences is a natural PstI site at position 1810 in FN and an engineered site at position 1987 in tenascin-C. A primer (5′-GGCTGCAGTCTGAGGTGTCCCC-3′) with a PstI site in frame (denoted in bold) was used with a downstream primer to amplify a 342-bp PstI-XbaI fragment of tenascin-C. PCR amplification was carried out for 30 cycles under the following conditions: 94°C, 30 s; 47°C, 1 min; 72°C, 30 s. The DNA sequence of the PCR product was verified. 70Ten was produced using the baculovirus insect cell expression system and protein was purified from cell culture medium by gelatin-Sepharose chromatography (Sechler et al., 1996).

Substrate Preparation

Fibrin-FN matrices were prepared using a mass ratio of 20:1 fibrinogen/FN and give identical adhesion results to matrices prepared at a physiologic ratio of 10:1. The ratio of FN/tenascin-C was 1:3, a 1:1 molar ratio of dimeric FN to hexameric tenascin-C. Ratios lower than 1:2.5 did not induce filopodia. 0.6 mg/ml fibrinogen (American Diagnostica, Inc.), 30 μg/ml FN, 120 μg/ml tenascin-C and/or 72 μg/ml 70Ten, and 10 μg/ml coagulation factor XIII (Calbiochem-Novabiochem) were mixed in 150 mM NaCl, 20 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, as described (Corbett and Schwarzbauer, 1999). Immediately after addition of thrombin (Sigma Chemical Co.) at 1.5 U/ml, the mixture was pipetted onto a glass coverslip (Fisher Scientific). Fibrinogen and thrombin were reconstituted and contaminating FN was removed from fibrinogen as previously described (Corbett et al., 1996; Wilson and Schwarzbauer, 1992). Human coagulation factor XIII was diluted to 0.5 mg/ml with 50% glycerol, 0.5 mM EDTA before use. After overnight incubation at 4°C, the clots were carefully aspirated from the coverslip leaving a matrix attached to the surface (Corbett et al., 1996) and the substrate was blocked with 1% BSA in PBS. Covalent cross-linking was monitored by SDS-PAGE (Wilson and Schwarzbauer, 1992).

Cell Attachment

Mouse NIH 3T3 fibroblasts were cultured in DME (GIBCO BRL) containing 10% calf serum (Hyclone Laboratories). Rat1 fibroblasts stably transfected with activated RhoA-V14 (Qiu et al., 1995) or Cdc42-V12 (Qiu et al., 1997) cDNA (gifts from Dr. Marc Symons, Picower Institute) were maintained in DME containing 10% fetal calf serum, 2 mM glutamine, 400 μg/ml G418 (GIBCO BRL). Because RhoA-V14 is driven by a tetracycline-repressible promoter, medium also contained 2.5 μg/ml puromycin and 2 μg/ml tetracycline. Tetracycline was withdrawn from the medium 2 d before the start of each experiment (Qiu et al., 1995). Cells were grown to confluence and prepared for cell attachment and spreading assays as described (Corbett et al., 1996).

Cells spread on substrate-coated glass coverslips for varying times were fixed, permeabilized, and stained with rhodamine-phalloidin as described (Corbett et al., 1996). Coverslips were mounted with SlowFade Light Antifade Kit (Molecular Probes Inc.). Cells were visualized with a Nikon Optiphot-2 microscope and images were captured using an Optronics three sensor cooled CCD camera.

Preparation of Recombinant C3 Transferase

Recombinant C3 transferase cDNA in the pGEX-2T vector (a gift from Dr. Larry Feig, Tufts University) was expressed in *E. coli* DH12S as a glutathione S-transferase (GST) fusion protein and purified as described (Ridley et al., 1992) with the following modifications. Cells were lysed with Bacterial Protein Extraction Reagent (B-PER; Pierce Chemical Co.) and GST-C3 transferase in the pellet was released by incubation for 10 min in 200 μg/ml lysozyme at room temperature, dialyzed into 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and isolated by binding to glutathione agarose beads. The GST fusion protein bound to beads was cleaved with thrombin, thrombin was removed, and purified C3 transferase was dialyzed into DME. Purity was checked by SDS-PAGE. C3 transferase protein was added to the culture medium at 25 μg/ml for 24 h (Zhong et al., 1998).

GTPase Activity Assay

GTP-bound RhoA and Cdc42 were affinity isolated from cell lysates using the Rho-binding domain of murine Rhotekin (GST-RBD; Ren et al., 1999) or the Cdc42-binding domain of murine $p65^{PAK}$ (GST-PBD; Bagrodia et al., 1995) (gifts from Dr. Keith Burridge, University of North Carolina). Fusion proteins expressed in *E. coli* strain BL21 were induced with 0.3 mM IPTG, cells were lysed in B-PER and solubilized proteins were incubated with glutathione-agarose beads. Bound protein concentrations were determined using the BCA Protein Assay (Pierce Chemical Co.).

Serum-starved NIH 3T3 fibroblasts spread on matrices for 1 h were lysed in cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM $NaVO_4$, 1 mM EGTA, 50 μg/ml leupeptin, and 0.5% aprotinin), and spun at 4°C for 10 min. Lysates with equal amounts of protein were added to glutathione-agarose beads with 20 μg bound GST-RBD or GST-PBD. After a 30-min incubation at 4°C, beads were washed and protein was eluted by boiling in electrophoresis sample buffer (Waterman-Storer et al., 1999). Bound proteins and whole cell lysates were separated on 13% polyacrylamide–SDS gels, transferred to nitrocellulose and detected with anti-RhoA or anti-Cdc42 monoclonal antibodies diluted 1:250 (Transduction Laboratories). Primary antibodies were detected with horseradish peroxidase–conjugated secondary antibody diluted 1:50,000 (Pierce Chemical Co.) and ECL Plus detection reagent (Amersham Pharmacia Biotech).

Results

Tenascin-C Alters Actin Organization on Fibrin-FN Matrix

In wounds, the fibrin-FN provisional matrix supports fibroblast movements and interactions vital for tissue repair (Clark, 1996; Corbett and Schwarzbauer, 1999). Tenascin-C expression is upregulated during tissue injury (Fors-

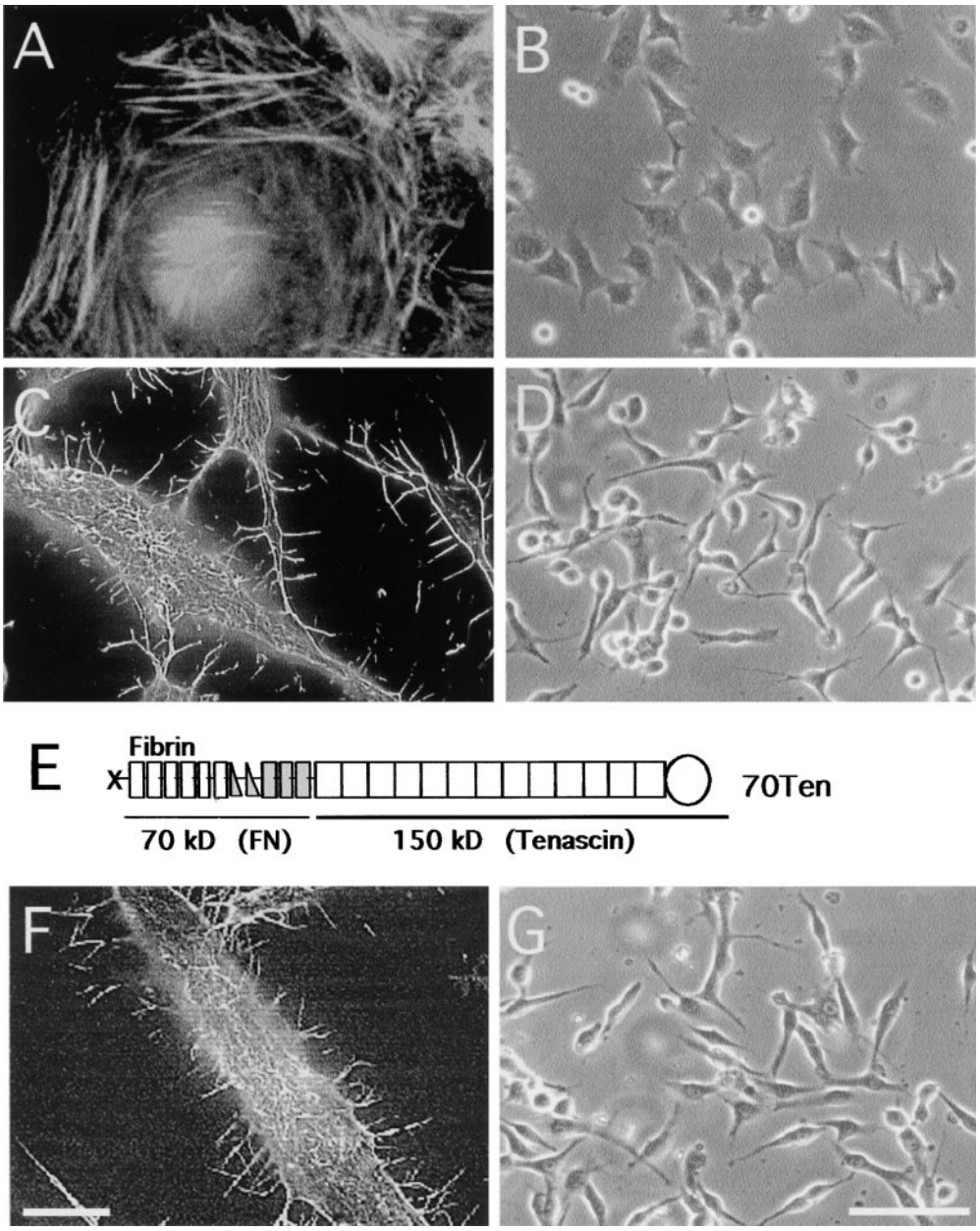

Figure 1. Filopodia form in response to a fibrin-FN+tenascin-C matrix. Fibrin-FN (A and B), fibrin-FN+tenascin-C (C and D), and fibrin-FN+70Ten (F and G) matrices were formed on glass coverslips and cells were allowed to spread for 1 h. Cells were analyzed by phase microscopy or washed, fixed, permeabilized, and incubated with rhodamine-phalloidin to stain filamentous actin. (E) 70Ten contains the amino-terminal 70-kD region of FN including the fibrin cross-linking site (X) connected to all type III repeats and the terminal knob of tenascin-C containing adhesive and anti-adhesive domains. Bars: (A, C, and F) 10 μm; (B, D, and G) 100 μm.

berg et al., 1996; Mackie et al., 1988) and is known to affect ECM–dependent cell functions (Chiquet-Ehrismann, 1993; Erickson, 1993; Crossin, 1996). Tenascin-C's modulatory effects are also apparent in cells on a three-dimensional fibrin-FN matrix. NIH3T3 fibroblasts showed circumferential spreading with a cortical arrangement of actin stress fibers on fibrin-FN matrix (Fig. 1, A and B). In contrast, inclusion of tenascin-C during formation of the matrix gave a fibrin-FN+tenascin-C substrate that induced a distinct cell morphology with a dramatically different actin organization that lacked stress fibers (Fig. 1, C and D). Instead, actin was organized into short filaments throughout the cytoplasm with numerous filopodia extending out from the cell bodies and processes. Thus, native tenascin-C markedly altered cellular responses to the fibrin-FN matrix.

To eliminate the possibility that proteins other than tenascin-C contributed to the cell response, we used a recombinant tenascin-C polypeptide expressed in insect cells under serum-free conditions. This recombinant, 70Ten, is a chimeric molecule consisting of the amino-terminal 70-kD region of FN joined to the carboxy-terminal 150-kD of tenascin-C (Fig. 1 E). The 70-kD FN segment promotes specific, efficient covalent cross-linking to the fibrin matrix. The 150-kD region includes the 13 type III repeats and terminal knob from tenascin-C and contains multiple sites for interacting with cells (Crossin, 1996). Highly purified recombinant 70Ten had an effect on cells identical to native tenascin-C (Fig. 1, F and G). Therefore, this recombinant protein provides a reliable source of pure tenascin-C sequences for use in characterizing the cytoskeletal and morphological responses to the fibrin-FN+tenascin-C provisional matrix.

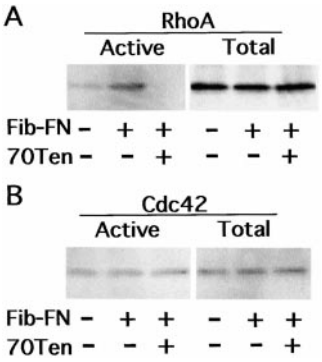

Figure 2. Tenascin-C inhibits Rho activation. Relative amounts of activated (GTP-bound) Rho and Cdc42 were determined using an affinity assay. (A and B) NIH3T3 fibroblasts were serum starved, then plated on tissue culture plastic (−) or on matrices with the indicated components. Active GTPases were isolated and analyzed along side total cell lysates by immunoblotting with anti-Rho or anti-Cdc42 antibodies.

Tenascin-C Suppresses Activation of Rho

Rho and Cdc42 are small GTPases that regulate the organization of the actin cytoskeleton into stress fibers and filopodia, respectively (Hall, 1998). To determine the effects of tenascin-C sequences on RhoA and Cdc42 activities, active GTPases were isolated from spread cell lysates by binding to GST fusion proteins containing the binding domain of rhotekin for Rho or of $p65^{PAK}$ for Cdc42 (Bagrodia et al., 1995; Benard et al., 1999; Ren et al., 1999). Under all conditions, equivalent levels of total RhoA or Cdc42 protein were found in whole cell lysates (Fig. 2, A and B). A low level of active RhoA was present in serum-starved NIH3T3 cells plated on tissue culture plastic in the absence of a FN matrix (Fig. 2 A, lane 1). Active RhoA levels were elevated when cells were plated on a fibrin-FN matrix (Fig. 2 A, lane 2). Therefore, adhesion and spreading on this matrix stimulates Rho activation. Surprisingly, with the inclusion of 70Ten in the fibrin-FN matrix, absolutely no active RhoA could be detected (Fig. 2 A, lane 3). Levels were far below those seen in control cells, indicating that tenascin-C sequences in the matrix suppress RhoA activation.

This was not the case, however, with Cdc42. Fibroblasts plated with or without fibrin-FN matrix showed similar levels of active protein (Fig. 2 B, lanes 1 and 2) which did not increase with inclusion of 70Ten (Fig. 2 B, lane 3). Clearly, tenascin-C does not act by increasing the levels of active Cdc42. Instead, the presence of tenascin-C sequences in the fibrin-FN matrix caused complete suppression of RhoA activation while maintaining the level of active Cdc42 and allowing induction of filopodia.

Filopodium Formation Is Abrogated by Active Rho

The matrix-dependent reduction in levels of active Rho suggested that Rho activity regulates cell responses to this provisional matrix. Activation of Rho by treatment of 3T3 cells with lysophosphatidic acid (LPA), a component of serum (Ridley and Hall, 1992), induced cell spreading and development of prominent actin stress fibers but no filopodia on a fibrin-FN+70Ten matrix (Fig. 3, A and B). Inhibition of active Rho by coincubation with LPA and the specific inhibitor C3 transferase abolished assembly of stress fibers (Fig. 3, C and D). C3 transferase alone did not alter cell responses to fibrin-FN+70Ten matrix (not shown).

The C3 transferase sensitivity of stress fiber formation induced by LPA indicates that inhibition of Rho activation is a key step in cell interactions with this matrix. To test directly the effects of active Rho, we used Rat1 fibroblasts carrying a constitutively active form of RhoA cDNA

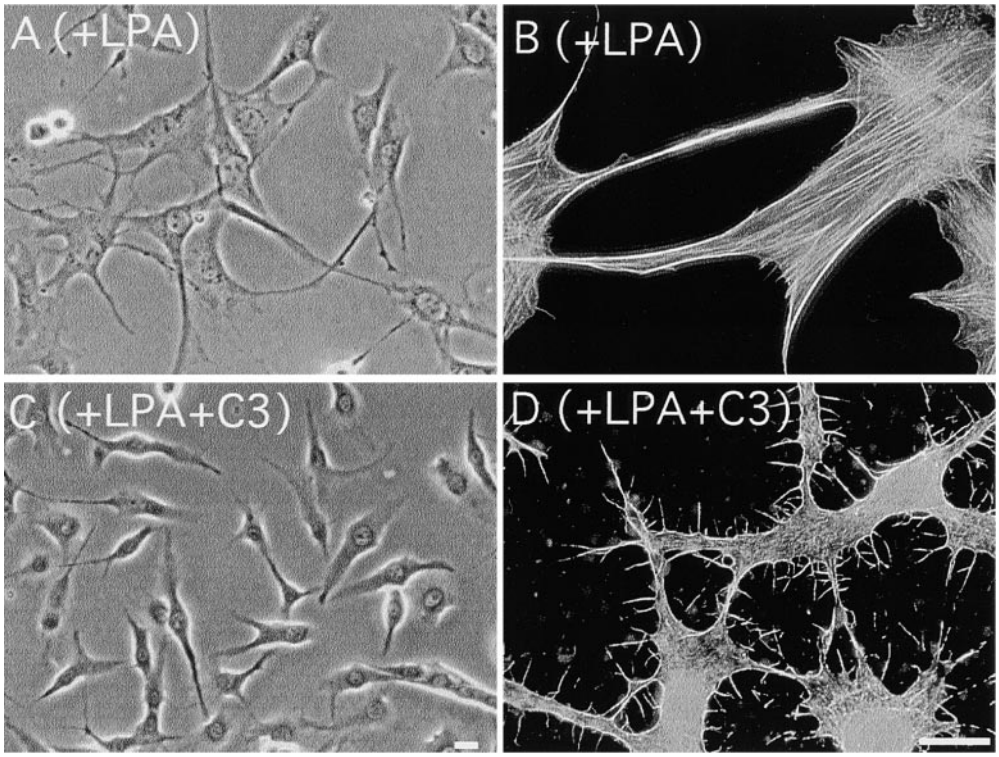

Figure 3. Rho inactivation causes restoration of filopodia. NIH3T3 cells were serum starved for 24 h either without (A and B) or with (C and D) 25 μg/ml C3 transferase added to the medium. Before plating on fibrin-FN+70Ten matrices, all cells were pretreated with 200 ng/ml LPA while in suspension for 30 min. Cells were allowed to spread for 1 h before examination by phase optics (A and C) or visualization of the actin cytoskeleton (B and D). Bars, 20 μm.

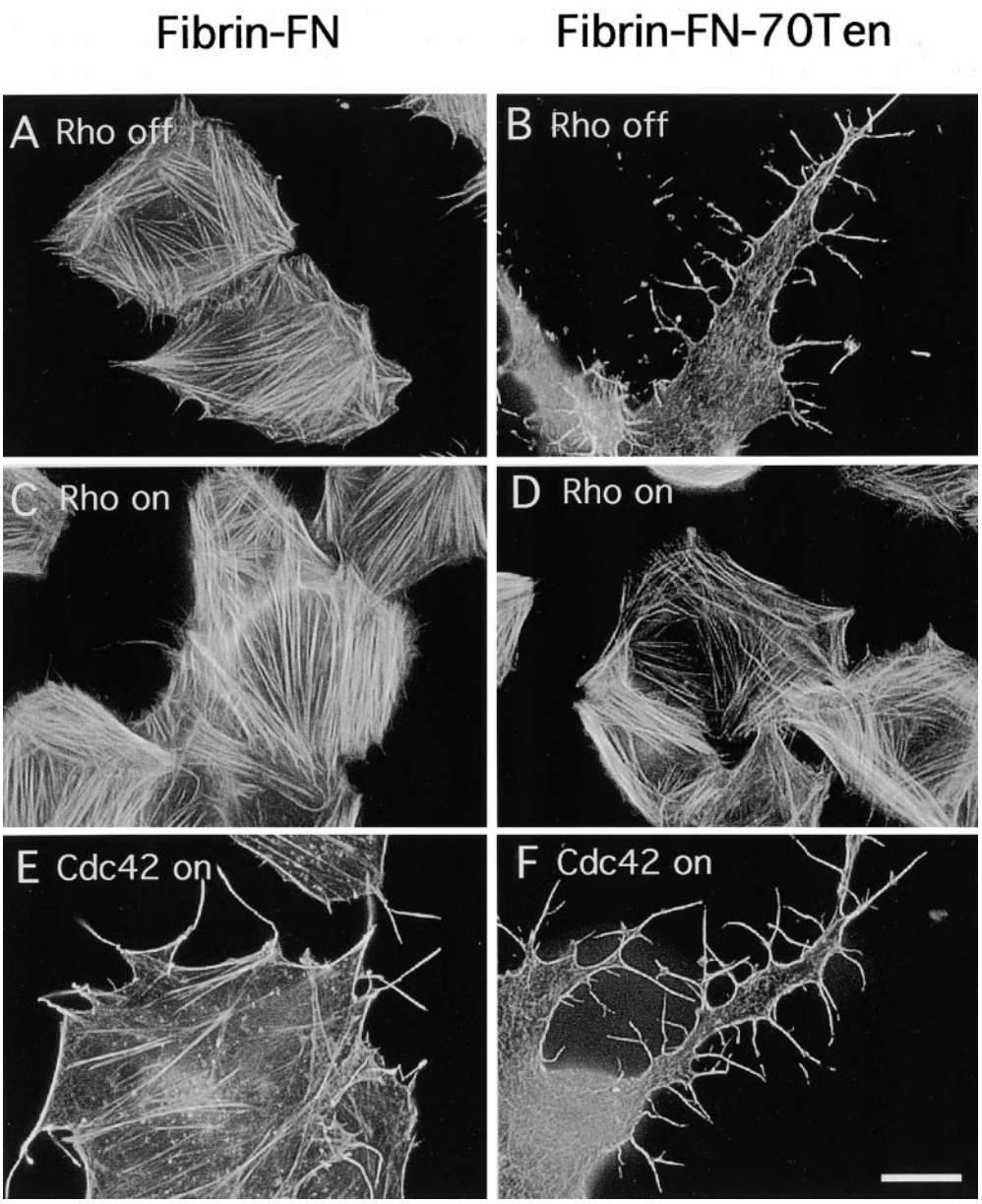

Figure 4. RhoA activity ablates effect of tenascin-C. Rat1 fibroblasts carrying active RhoA-V14 under the control of a tetracycline repressible promoter were allowed to spread on fibrin-FN (A and C) or fibrin-FN+70Ten matrix (B and D). Cells under repressed conditions (no active RhoA-V14 protein; A and B) or after induction to express active RhoA-V14 (C and D) were stained with rhodamine-phalloidin. Rat1 cells constitutively expressing active Cdc42-V12 were plated on fibrin-FN (E) or fibrin-FN+70Ten (F) matrix for 1 h. Bar, 20 μm.

(RhoA-V14) under the control of a tetracycline-repressible promoter (Qiu et al., 1995). Rat1 cells also express endogenous RhoA and, when RhoA-V14 expression was repressed by tetracycline, these cells reacted to fibrin-FN or fibrin-FN+70Ten matrices in much the same way as NIH3T3 fibroblasts (Fig. 4, A and B). Constitutive activation of RhoA by expression of RhoA-V14 gave identical actin organization into stress fibers in cells on fibrin-FN and on fibrin-FN+70Ten matrix (Fig. 4, C and D). Conversely, cells overexpressing constitutively active Cdc42-V12 (Qiu et al., 1997) extended filopodia and showed reduced actin rearrangement into stress fibers on fibrin-FN matrix (Fig. 4 E). However, Cdc42-V12 expression did not enhance filopodial projections on fibrin-FN+70Ten matrix (Fig. 4, compare F with B). Thus, enforced RhoA activation completely reverses the cytoskeletal effects of the tenascin-C sequences. Together, these results show that suppression of Rho activation is an important step in matrix induction of filopodia.

Provisional Matrix Components Collaborate to Organize the Cytoskeleton

Distinct cytoskeletal organizations were induced by cell interactions with these matrices. Circumferential spreading with development of stress fibers on fibrin-FN matrix (Fig. 5, left) was contrasted by extension of actin-rich filopodial processes detectable at the earliest time point on fibrin-FN+70Ten matrix (Fig. 5, 12 min, right). These results show that cells actively reorganized their cytoskeleton in response to tenascin-C sequences within a fibrin-FN matrix.

FN is essential for cell interactions with this matrix. Cells adhered poorly and did not spread on fibrin-70Ten

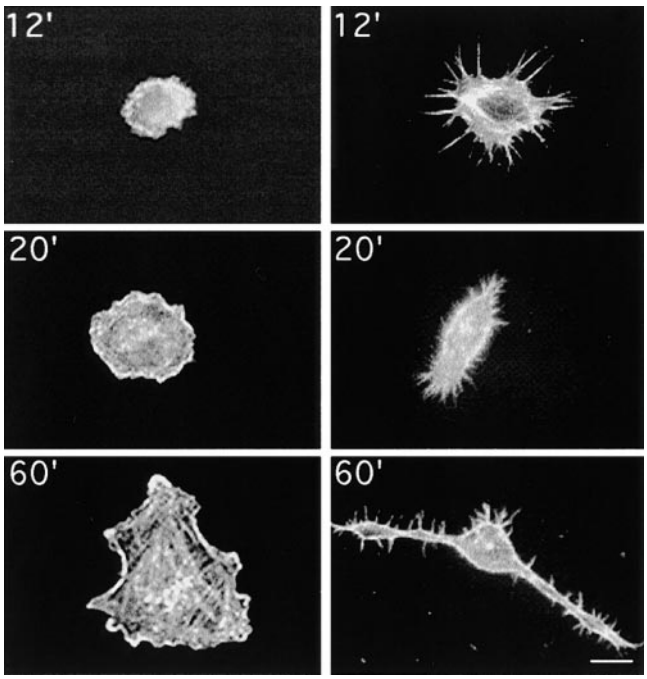

Figure 5. Matrix induction of filopodia formation. Cells were fixed and stained with rhodamine-phalloidin at the indicated times after plating on fibrin-FN (left) or fibrin-FN+70Ten matrix (right). Bar, 20 μm.

matrix lacking FN and cell attachment was ablated with an RGD peptide that blocks integrin-FN interactions (not shown). In addition, matrix presentation of FN and tenascin-C sequences was required. A planar substrate coated with FN and 70Ten supported circumferential fibroblast spreading. Furthermore, soluble tenascin-C added to the medium after cells had spread on a fibrin-FN matrix did not induce actin reorganization (not shown). Taken together, these results show that FN and tenascin-C collaborate within the context of a fibrin provisional matrix to induce a cytoskeletal organization distinct from either protein alone. This cell regulation relies on adhesive signals from FN through integrins and suppressive signals from tenascin-C to Rho family GTPases.

Discussion

We have identified a novel mechanism for tenascin-C regulation of cell phenotype through suppression of Rho GTPases. Inhibition of Rho activation by tenascin-C sequences prevented stress fiber formation and allowed projection of numerous actin-rich filopodia. Apparently, Cdc42 was functionally masked by active Rho and, by blocking Rho activation, filopodia replaced stress fibers. Surprisingly, these dramatic changes were stimulated by a relatively modest change in matrix composition, in this case by addition of a single anti-adhesive protein to an otherwise adhesive network. Thus, increased tenascin-C expression can function as a regulatory switch to counteract matrix-derived signals that activate Rho.

The link between tenascin-C suppression of Rho activation and extension of filopodia was confirmed by induction of stress fibers through enforced activation of RhoA and by filopodial extension when active Rho was inhibited by C3 transferase. Analyses of integrin clustering and focal adhesion formation in response to FN substrates have shown that ECM contributes to Rho-induced cytoskeletal changes (Hotchin and Hall, 1995; Machesky and Hall, 1997) and that adhesion on FN stimulates Rho activation in Swiss 3T3 cells (Ren et al., 1999) and NIH3T3 cells (this report). Thus, it is clear that integrin interactions with FN upregulate Rho activity. However, it has not been previously reported that specific ECM components can prevent this activation. While the dynamics of GTPase activation remain to be elucidated during the course of spreading on these matrices, it is clear that at 1 h, levels of active RhoA but not active Cdc42 were altered in response to variations in ECM. It appears that the suppression of RhoA activation by tenascin-C is a key element in allowing expression of Cdc42 function.

Reduced focal adhesions, rounding of adherent cells, formation of fascin microspikes, and increased cell migration and proliferation are documented cellular responses to tenascin-C (Spring et al., 1989; Murphy-Ullrich et al., 1991; Wehrle-Haller and Chiquet, 1993; Fischer et al., 1997) and could result from tenascin-C blockade of Rho activation. Tenascin-C may downregulate cell adhesion strength through direct cell receptor binding and signaling. Suppression of Rho by tenascin-C receptor ligation could either be a direct downstream response or could result from transdominant inhibition of signals initiated by FN receptors (Blystone et al., 1994). The alternatively spliced region is probably not responsible for the suppression since the effects of both small and large tenascin-C splice variants were similar (unpublished observations). Tenascin-C binding to FN (Chiquet-Ehrismann et al., 1991; Chung et al., 1995) presents an alternative regulatory mechanism whereby cell-FN interactions are modulated, leading to changes in FN signaling. It is of interest that fibroblast spreading on recombinant FN fragments indicates a role for FN's heparin domain in filopodium extension (Bloom et al., 1999). Perhaps access to this domain is enhanced in the presence of fibrin and tenascin-C.

In the wound bed, the combination of fibrin, FN, and tenascin-C may collaborate to induce appropriate shape changes and migratory behavior needed to properly position cells for tissue remodeling. The more adhesive fibrin-FN–rich regions of the provisional matrix would promote stable cell-matrix contacts needed for new matrix deposition and wound contraction. In a dynamic situation such as wounds or developing tissues, the deposition of tenascin-C may be designed to provide an important regulatory switch that signals cells to modulate their responses to an adhesive environment. Although tenascin-C has a significant role in the fibrin-FN provisional matrix, tenascin-C–null animals do not show overt defects in wound repair (Forsberg et al., 1996). It seems likely that the presence of functionally related proteins such as thrombospondin or SPARC at injury sites could provide related or additional signals that would similarly modify cell behavior (Sage and Bornstein, 1991). This suggests the interesting hypothesis that other antiadhesive matrix proteins contribute to cytoskeletal patterning and cell shape through differential activation of Rho family GTPases.

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