Focal Adhesion Integrity Is Downregulated by the Alternatively Spliced Domain of Human Tenascin

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Abstract. Tenascin, together with thrombospondin and SPARC, form a family of matrix proteins that, when added to bovine aortic endothelial cells, caused a dose-dependent reduction in the number of focal adhesion-positive cells to ~50% of albumin-treated controls. For tenascin, a maximum response was obtained with 20–60 μg/ml of protein. The reduction in focal adhesions in tenascin-treated spread cells was observed 10 min after addition of the adhesion modulator, reached the maximum by 45 min, and persisted for at least 4 h in the continued presence of tenascin. This effect was fully reversible, was independent of de novo protein synthesis, and was neutralized by a polyclonal antibody to tenascin. Monoclonal antibodies to specific domains of tenascin (mAbs 81C6 and 127) were used to localize the active site to the alternatively spliced segment of tenascin. Furthermore, a recombinant protein corresponding to the alternatively spliced segment (fibronectin type III domains 6–12) was expressed in *Escherichia coli* and was active in causing loss of focal adhesions, whereas a recombinant form of a domain (domain 3) containing the RGD sequence had no activity. Chondroitin-6-sulfate effectively neutralized tenascin activity, whereas dermatan sulfate and chondroitin-4-sulfate were less active and heparan sulfate and heparin were essentially inactive. Studies suggest that galactosaminoglycans neutralize tenascin activity through interactions with cell surface molecules. Overall, our results demonstrate that tenascin, thrombospondin, and SPARC, acting as soluble ligands, are able to provoke the loss of focal adhesions in well-spread endothelial cells.

CELL substrate adhesion typically involves cellular recognition of an adhesive matrix protein (e.g., fibronectin, laminin, vitronectin, or fibrinogen) by an integrin. Integrins are a family of heterodimeric cell surface receptors that recognize specific domains in adhesive extracellular matrix proteins (reviewed in reference 21). Subsequent to attachment of cells to a substrate via integrins, additional cell surface, cytoskeletal, and extracellular components become engaged in the adhesion process, resulting in cell spreading and the formation of stable adhesion plaques.

The dynamics of a living organism require that cell substrate adhesion be regulated. During wound healing, embryogenesis, and metastasis, processes in which cells proliferate and migrate, cell–substrate adhesions disassemble and reform (2, 5, 44). Previous studies have shown that exposure of cells to tumor-promoting phorbol esters (17, 36) or to certain growth factors (18, 19) can cause loss of focal adhesions. More recently, studies demonstrate that certain extracellular matrix molecules may participate in destabilizing or modulating cell–substrate contacts. Murphy-Ullrich and Höök showed that thrombospondin (TSP)[1] caused a loss of focal adhesion plaques from spread bovine aortic endothelial (BAE) cells (30). Sage et al. reported that SPARC (osteonectin) causes rounding of BAE cells (26, 35).

Tenascin (also referred to as hexabrachion or cytotactin) is a large oligomeric glycoprotein that has been implicated as an adhesion modulator; however, its physiological role remains unclear. Tenascin is localized primarily in developing tissues and in tumors (reviewed in 8, 12, 13) and, like many matrix glycoproteins, it is composed of multiple domains, which may have discrete functions (12, 16, 22, 23, 37). Alternative splicing of mRNA results in multiple forms of the molecule with varying numbers of a 91 amino acid motif resembling fibronectin type III (FN-III) domains (16, 23, 43).

Tenascin is known to interact with other molecules of the extracellular matrix. There is a strong binding of tenascin to

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial; C-4-S, chondroitin-4-sulfate; C-6-S, chondroitin-6-sulfate; FN-III, fibronectin type III; IRM, interference reflection microscopy; TSP, thrombospondin.

chondroitin sulfate proteoglycans from cell culture, cartilage extracts, and embryonic brains (7, 20, 42). Tenascin also binds to heparin and DNA (29), and a reversible binding of tenascin to fibronectin in solution has been reported (26).

Attachment of cells to tenascin-coated plastic has been reported by several investigators (4, 10, 15); however, cells attached to tenascin remain rounded and do not spread, unlike cells attached to fibronectin substrates. Other workers have emphasized the weakness or absence of cell attachment to tenascin substrates (13, 14, 26–28). A weak and/or transient attachment would be expected whenever a cell expresses receptors for the substrate molecule, even if these receptors are not involved in promoting physiological cell adhesion (12).

Several recent studies suggest that tenascin may act to inhibit adhesion to substrates like fibronectin. There are at least two different anti-adhesion mechanisms. The adhesion modulator may bind to the substrate in a manner which for steric reasons interferes with the recognition of the adhesive site by the appropriate integrin. This mechanism may be operative when the large tenascin molecule is incorporated into a mixed substrate or is allowed to bind to an adhesive substrate (26). A steric blocking may explain many of the anti-adhesion activities reported for tenascin in vitro (10, 26, 28, 37, 39); it may also play a role in modulating adhesion in vivo. A second anti-adhesion mechanism may involve interaction of an adhesion modulator with specific receptors that trigger anti-adhesive responses through second messenger systems. Soluble tenascin inhibited the adhesion of human mammary carcinoma cells to fibronectin-coated plastic and to collagen gels (11). Riou et al. (33) found that soluble tenascin inhibited the spreading and migration of mesodermal cells on fibronectin substrates, although Lightner and Erickson (26) reported no effect of soluble tenascin on initial adhesion of fibroblasts to fibronectin. These effects may involve a binding of tenascin to distinct cell receptors, resulting in a modulation of cell adhesion through a process involving second messengers.

In this report, we characterize the ability of tenascin to stimulate the loss of focal adhesions in BAE cells that have been grown to confluency in the presence of serum and are fully spread with well-developed focal adhesions. Furthermore, we map this activity to the alternatively spliced region of the hexabrachion arm and show that tenascin activity is neutralized by some galactosaminoglycans acting at the cell surface. It should be noted that while previous studies have focused on the ability of tenascin to modulate the initial attachment and spreading of cells, in this study we are observing loss of focal adhesions in cells that have been fully spread and attached for many hours.

Materials and Methods

Materials

The following items were purchased: DME (Cell-Gro; Mediatech, Inc., Herndon, VA); FBS (Hyclone Laboratories Inc., Logan, UT); trypsin-EDTA (Gibco Laboratories, Grand Island, NY); BSA, cycloheximide, and glutaraldehyde (Sigma Chemical Co., St. Louis, MO); NBD-phallicidin (Molecular Probes, Inc., Eugene, OR); and chondroitinase ABC (Seikagaku America Inc., Rockville, MD).

Proteins and Glycosaminoglycans

TSP was purified from fresh human platelets purchased from the Birmingham American Red Cross (Birmingham, AL) using affinity chromatography on a column of heparin-Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) and gel permeation chromatography on a column of A0.5m resin (Bio-Rad Laboratories, Richmond, CA) (31). Tenascin was purified from culture supernatants of U-251MG human glioma cells by ammonium sulfate precipitation, followed by gel permeation chromatography on a column of Sephacryl S-500 HR and anion exchange chromatography on a MonoQ column (1). Some preparations were further purified by a second chromatography step on a Sephacryl S-500 HR column (1). Protein concentration was determined from the absorbance at 278 nm, using the extinction coefficient 0.97 (40). The purified tenascin preparations from the U-251MG cells consisted of at least 95% of the large form (320-kD subunit) of the hexabrachion molecule. Tenascin was purified from U87 cells using the same protocol as for U-251 cells; however, yields of protein from this cell line were significantly lower than from U-251MG cells. Human fibronectin and vitronectin were generous gifts of Dr. Deane Mosher, Department of Physiological Chemistry, University of Wisconsin (Madison, WI). Bovine vitronectin and rat laminin were purchased from Telios Pharmaceuticals, Inc. (San Diego, CA). Human fibrinogen from KABI-Vitrium AB (Stockholm, Sweden) depleted of contaminating fibronectin was a gift of Dr. Lech Switalski, Department of Microbiology, University of Alabama at Birmingham (Birmingham, AL). SPARC purified from the culture supernatants of mouse PYS cells was a generous gift of Dr. Helene Sage, Department of Biological Structure, University of Washington (Seattle, WA) (35). The purity of the proteins was assessed by SDS-PAGE.

Isomers of chondroitin sulfate glycosaminoglycans were provided by Dr. John Baker, Department of Biochemistry, University of Alabama at Birmingham (Birmingham, AL). Chondroitin-6-sulfate (C-6-S) glycosaminoglycans were purified from basking shark cartilage and chondroitin-4-sulfate (C-4-S) glycosaminoglycans were purified from a rat chondrosarcoma after alkaline borohydride treatment of the proteoglycan according to the method of Carlson (6). Heparan sulfate from bovine kidney and chondroitin sulfate B (dermatan sulfate), C (C-6-S), D, and E, all "super special grade" when available, were purchased from Seikagaku America Inc. Chondroitin sulfate (shark cartilage, mixed isomers) and heparin (porcine intestinal mucosa) were obtained from Sigma Chemical Co. Low molecular weight heparin (3,000 mol wt) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Uronic acid contents were determined according to the carbazole method of Bitter and Muir (3).

Cells

BAE cells were provided by Dr. Robert Auerbach, Department of Zoology, University of Wisconsin (Madison, WI). Cells were characterized in Dr. Auerbach's laboratory as homogeneous populations of endothelial cells by positive fluorescence for factor VIII antigen and uptake of acetylated low-density lipoproteins, and by being positive for angiotensin-converting enzyme. Cells were routinely cultured in DME supplemented with 4.5 g/liter glucose, 2 mM glutamine, and 20% FBS. Cultures were passaged when confluent by detaching the cells with trypsin-EDTA. Cells were used between passages 6 and 13.

Antibodies

Monoclonal anti-vinculin ascites fluid (clone VIN-11-5) was purchased from Sigma Chemical Co. Rhodamine-labeled goat anti-mouse IgG was obtained from Cappel Laboratories (Malvern, PA). Rabbit anti-human tenascin antiserum was purchased from Telios Pharmaceuticals, Inc. The IgG fraction was isolated by affinity chromatography on a column of Protein A–Sepharose. The specificity of the antibody was analyzed by Western blots. The isolated IgG was tested against rat laminin, human fibronectin, human TSP, and SDS extracts of BAE cells and normal human skin fibroblasts fractionated by SDS-PAGE. The antibody recognized the purified tenascin and bands of the same molecular weight as tenascin in conditioned medium of normal skin fibroblasts. The antibody did not react with any of the other purified proteins or the cell extracts.

The locations of the epitopes for several hexabrachion mAbs have been mapped by electron microscopy and Western blotting (unpublished results), and are shown schematically in Fig. 1. Electron microscopy showed that mAb 58 bound to the central nodule and mAb 2A6 bound to the terminal knob. mAbs 190, 127, and 81C6 bound to the thick segment of the hexabrachion arm, which comprises the FN-III domains, and have been more

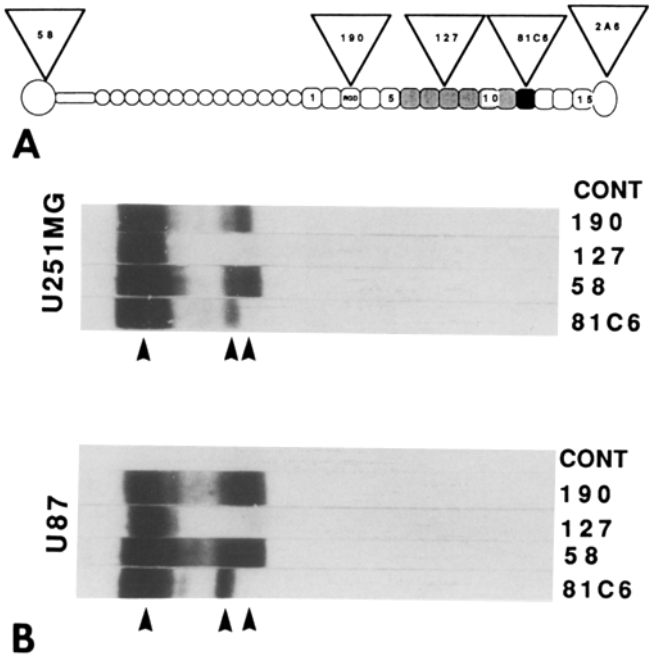

Figure 1. Mapping of mAb epitopes. (*A*) A diagram of one arm of the human hexabrachion showing the domain structure and likely positions of mAbs as determined by electron microscopy and Western blotting. The NH_2-terminus (at the central nodule) is on the left, and the COOH-terminus (the fibrinogen-like terminal knob) is on the right. EGF-like domains are round, and FN-III domains are rounded squares. The largest alternatively spliced domains are shaded. (*B*) Western blot of native tenascin with different mAbs. Electrophoresis on a 5% nonreducing gel was from left to right. The arrows, from left to right, mark the position of the 320-, 230-, and 220-kD splice variants. The upper panel shows tenascin purified from U-251MG cell culture, and the lower panel shows tenascin from the U87 glioma cell line, in which the two lower molecular weight splice variants are more abundant. The *CONT* lane was stained with a mAb not specific for tenascin. mAbs 190 and 58 stain all three splice variants, 81C6 stains the 320- and 230-, but not the 220-kD band, and 127 strains only the 320-kD band.

precisely mapped by Western blotting. mAb 190 recognized a recombinant form corresponding to FN-III domain 3 (the domain with the RGD sequence). mAbs 127 and 81C6 reacted with the recombinant protein HxB6-12, which is composed of the alternatively spliced segment (see below), and their epitopes are thus localized in this domain. mAb 81C6 can be mapped more precisely to FN-III domain 12 because it stains the 320- and 230-kD bands of native tenascin, but not the 220-kD bands (Fig. 1), which is probably missing domain 12 (12). Based on staining of proteolytic fragments, we tentatively map mAb 127 to one of the FN-III domains 7–9.

Focal Adhesion Assays

Focal adhesion assays were performed as described (30). Briefly, BAE cells were grown for 24–48 h on glass coverslips in DME with 20% FBS until just confluent. Cells were treated for 1 h with 10 μg/ml cycloheximide in serum-containing medium to minimize interference from proteins synthesized during the course of the experiments. Unless specifically indicated, cycloheximide was present during all stages of the experiment before fixation. Cells were then rinsed with warmed DME to remove serum components and incubated with tenascin or other agents diluted in DME for the indicated time. Cells were fixed with 3% warmed glutaraldehyde for 30 min, washed, and examined by interference reflection microscopy using a Nikon Optiphot microscope. A minimum of 200 cells per condition were routinely counted unless indicated otherwise. All experiments were performed at least twice, with most being performed three or four times. The central observations that tenascin and the HxB6-12 fragment cause a loss of focal adhesions were confirmed by a second person who performed the assays with blinded samples. Cells were prepared for immunofluorescence using methods and antibodies as previously described (30).

Cloning and Expression of Tenascin Segment HxB6-12

The bacterial expression system of Studier et al. (38), based on the T7 promoter and inducible T7 polymerase, was used to express specific segments of the tenascin molecule. The DNA corresponding to the entire alternatively spliced segment (FN-III repeats 6–12) was amplified by polymerase chain reaction, using cDNA prepared from the U-251MG cells as template. The forward and reverse primers corresponded to the amino acid sequences EQAPE and SAIAT (beginning of domain 6 and end of domain 12 (16). Restriction sites NdeI and BamHI were included in the primers to permit directional cloning of the amplified segment into the pET 11b expression vector. The resulting plasmid was used to transform the *Escherichia coli* strain BL21(DE3) (38). After the transformed *E. coli* was induced with 0.4 mM IPTG for 3 h, a prominent 67-kD protein was detected in the lysate of the cells and analyzed on SDS-PAGE (Fig. 2). This protein was only present in minute quantities before induction and was found to react with anti-tenascin antibodies. This recombinant protein segment of tenascin, named HxB6-12, was purified by precipitation with 25% ammonium sulfate, followed by chromatography on a Mono Q column. The Mono Q column gave a cleanly separated peak that eluted at 0.31 M NaCl, followed by several peaks that eluted at 0.37–0.41 M NaCl. All peaks showed predominantly or exclusively the 67-kD band on 10% SDS-PAGE (Fig. 2). The first peak was identified as a monomeric protein by glycerol gradient sedimentation and electron microscopy (see Fig. 2); this material was used in the focal adhesion assays. The later peaks were oligomers comprising 3–10 subunits. 32 mg of monomer and 120 mg of oligomer were obtained per liter of bacterial culture. On Western blot analysis (not shown) the 67-kD HxB6-12 stained with our polyclonal antibody and with mAbs 127 and 81C6, but not with mAb 190, consistent with our mapping of these antigenic sites (Fig. 1 *A*). A 10-kD recombinant protein segment of tenascin corresponding to FN-III domain 3, which contains the RGD sequence, was produced and purified by a similar protocol (I. Aukhil, P. Joshi, Y. Z. Yan, and H. P. Erickson, unpublished results).

Results

Modulation of Focal Adhesions Is a Property of Some, but Not All Matrix Molecules

TSP has previously been shown to stimulate the disappearance of focal adhesions from spread BAE cells (30). To determine whether this effect is a general property of extracellular matrix proteins, confluent BAE cells were incubated with 10–30 μg of fibronectin, fibrinogen, vitronectin, laminin, TSP, tenascin, or SPARC. Subsequently, cells were examined for focal adhesions. The results of this experiment showed that the matrix proteins can be divided into two groups based on their effects on preformed focal adhesions (Fig. 3). The first group consisted of fibronectin, fibrinogen, laminin, and vitronectin, all matrix molecules with known adhesive properties. These proteins did not affect the number of cells with focal adhesions or the distribution of adhesion plaques over the cell body. The second group of matrix proteins included TSP, tenascin, and SPARC, all of which caused the loss of focal adhesions from ~50% of the cells with preformed adhesion plaques. Cells with three or fewer adhesion plaques, which were generally located at the periphery of the cell, were scored as negative for focal adhesions. Immunohistochemical staining of tenascin-treated cells for vinculin and F-actin showed that the disappearance of focal adhesions as observed by interference reflection microscopy (Fig. 4) corresponded to the absence of vinculin plaques in the central region of the cells and a tendency for

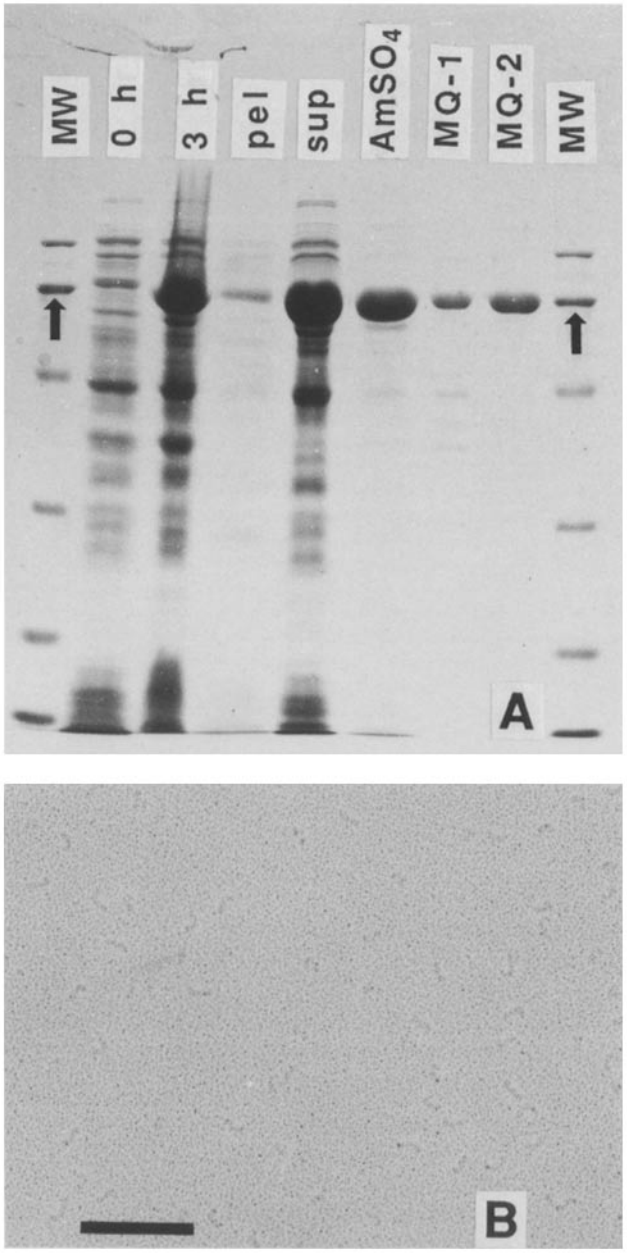

Figure 2. Bacterially expressed segment HxB-12. (*A*) SDS gel electrophoresis of expression and purification of HxB6-12. Lane *MW* shows molecular weight standards, with the arrow indicating BSA (67 kD); lane *0 h* is the bacterial pellet before induction; lane *3 h* is the bacterial pellet after 3 h of induction; lane *pel* is the pellet; and lane *sup* is the supernatant from the bacterial lysate. $AmSO_4$ is the 25% ammonium sulfate pellet. Lanes *MQ-1* and *MQ-2* are the monomeric and oligomeric peaks from the Mono Q column. (*B*) After sedimentation on glycerol gradients, rotary shadowed electron microscope specimens of peak MQ-1 show rod-shaped molecules measuring 20 nm in length. (Bar is 100 nm).

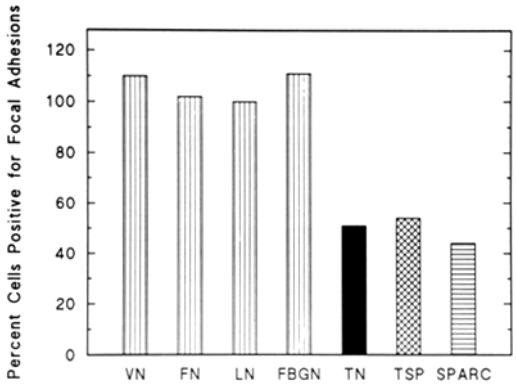

Figure 3. Effect of extracellular matrix proteins on focal adhesions in BAE cells. Cells grown on coverslips in the presence of serum were treated for 2 h at 37°C with the following proteins in a 500-μl volume: vitronectin (10 μg), fibronectin (30 μg), laminin (30 μg), fibrinogen (30 μg), tenascin (20 μg), TSP (30 μg), and SPARC (1:20). All cells were treated with 10 μg/ml cycloheximide for 1 h before and during the incubation with proteins. Cells were fixed after the 2-h incubation and examined by IRM for the presence of focal adhesions. Results are expressed as percent of cells positive for focal adhesions relative to BSA-treated controls (100% corresponds to 66% of total cells).

the F-actin to be distributed circumferentially (Fig. 5). Loss of vinculin staining from plaque-like structures was often observed in cells that had only a minimal alteration in F-actin distribution or thickness of the stress fibers. This is consistent with other observations which show that a loss of vinculin can precede dissolution of the focal adhesion structure (18).

Approximately 30% of cells in the tenascin-treated culture vs. 66% of cells in the BSA-treated cells retained focal adhesions. These cells usually had 10–15 adhesion plaques distributed in both the peripheral and central regions of the cells (Fig. 4). The cells that retained focal adhesions after treatment with tenascin seem to constitute a general refractory population, since the population of focal adhesion-positive cells was not further reduced by simultaneous treatment with tenascin and TSP. The refractory cells contained similar numbers and distributions of focal adhesions, as did positive untreated (control) cells, and the distribution of adhesion plaques in these two populations of cells was indistinguishable (Fig. 4).

In these assays, tenascin slightly reduced the extent of cell spreading, but it did not cause a general rounding of cells. There was a 15% decrease in the number of cells that were scored as spread when observed by phase contrast microscopy; however, there was no significant difference in the mean area of the spread cells after tenascin treatment (data not shown).

The effects of tenascin or TSP were reversible. If cells were washed free of adhesion-modulating protein, focal adhesions reassembled by ~1 h, even in the continued presence of cycloheximide (data not shown).

Characterization of Tenascin-mediated Loss of Focal Adhesions in Spread Cells

The reduction in focal adhesion positive cells was dependent on the amount of tenascin added to the cells (Fig. 6). Half-maximal tenascin activity (a decrease to 70% of control cells) was obtained with 2–6 μg/ml (1–3 nM hexabrachion or 6–18 nM monomer), and the maximum decrease in focal adhesion positive cells to nearly 40% of BSA-treated controls was seen in cells treated with 60–200 μg/ml tenascin.

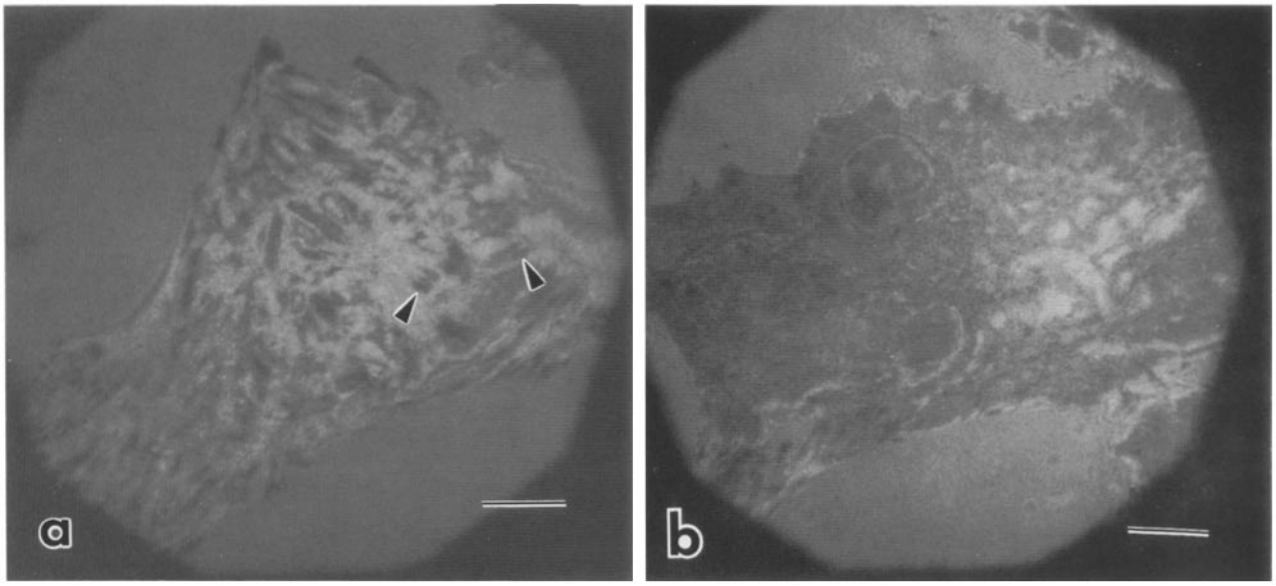

Figure 4. Interference reflection images of cells treated with tenascin. Cells grown in the presence of serum were washed and treated with 40 μg/ml tenascin or BSA in serum-free medium for 1 h at 37°C, fixed and examined by IRM. (*a*) BSA-treated cells have large focal adhesion plaques distributed over the entire cell body (*arrowheads*). (*b*) In contrast, tenascin-treated cells characteristically lack central focal adhesions and have a somewhat homogeneous gray appearance. Bars = 10 μm.

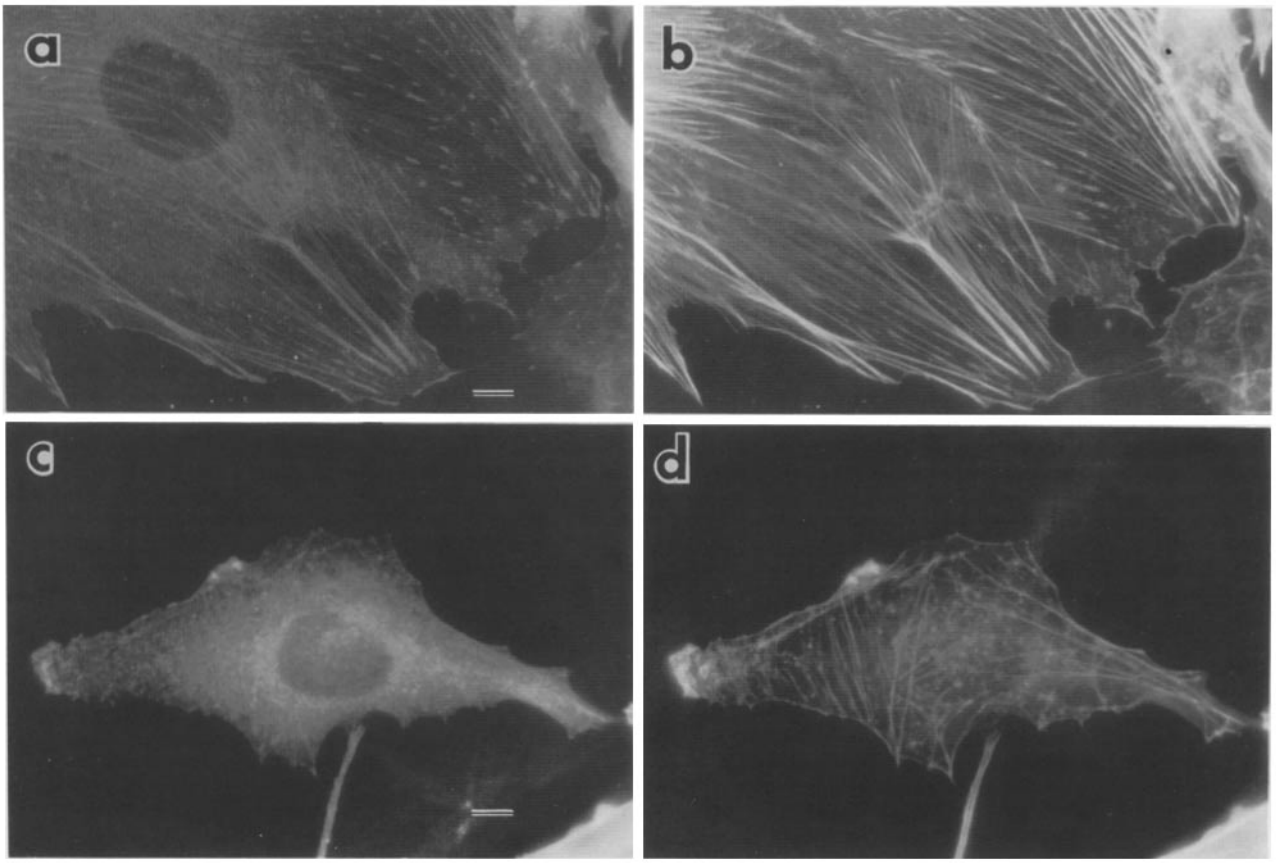

Figure 5. Vinculin and F-actin distribution in tenascin-treated cells is altered. Cells were treated with either 40 μg/ml BSA (*a*, *b*) or 40 μg/ml tenascin (*c*, *d*), fixed and permeabilized, and then stained with a mAb to vinculin (*a*, *c*) or with NBD-phallicidin (*b*, *d*). BSA-treated controls have an extensive network of actin-containing stress fibers (*b*) and vinculin-containing plaques located at the termini of these stress fibers (*a*). Tenascin-treated cells, in contrast, lack vinculin-containing plaques (*c*). Staining for F-actin in tenascin-treated cells is found primarily in a circumferential pattern (*d*). Bars = 10 μm.

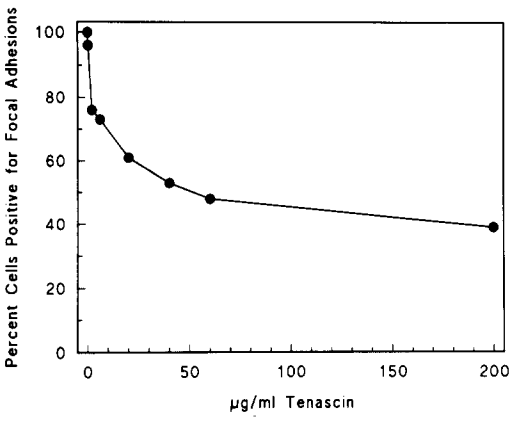

Figure 6. Tenascin-mediated loss of focal adhesions is dose dependent. BAE cells were grown for 24 h on coverslips in DME with 20% FBS and then treated with 10 μg/ml cycloheximide for 90 min before addition of increasing concentrations of tenascin in a 500-μl volume. Cells were incubated with proteins for 1 h at 37°C, fixed, and examined by IRM for the number of focal adhesion-positive cells. A minimum of 100 cells were counted per coverslip. Results are expressed as percent of spread cells positive for focal adhesions relative to BSA-treated controls: 71% of the BSA-treated cells were positive for focal adhesions.

In experiments performed over several months with different preparations of tenascin at 40 μg/ml, the percent of focal adhesion positive cells relative to the BSA-treated controls was 55 ± 10% (n = 34).

The kinetics of the tenascin-mediated effect were examined. A nearly 10% reduction in focal adhesion positive cells was observed as early as 10 min after the addition of tenascin. The maximal reduction was observed by 45 min and persisted over 4 h (Fig. 7). In contrast, cells treated with TSP did not show a reduction in focal adhesions until 20 min after addition of TSP, and a maximal effect required 1–2 h of incubation with TSP. Thus, BAE cells appear to be somewhat more responsive to tenascin as compared with TSP.

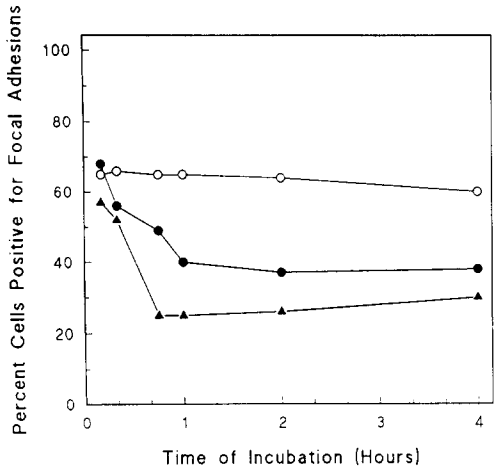

Figure 7. Kinetics of response to tenascin and TSP. Confluent BAE cells grown on coverslips in the presence of serum were pretreated with 10 μg/ml cycloheximide for 1 h, washed, and treated with either 60 μg/ml TSP (*closed circles*), 40 μg/ml tenascin (*closed triangles*), or 60 μg/ml BSA (*open circles*) with cycloheximide in serum-free DME. Cells were incubated with the proteins for various times (10, 20, 45, 60, 120, and 360 min) before fixation and examination by IRM. Results are expressed as the percent of cells positive for focal adhesions.

Effect of Tenascin Is Neutralized by Anti-Tenascin Antibodies

A rabbit polyclonal anti-tenascin antibody neutralized tenascin's effect on focal adhesions. When the antibody was added with tenascin (40 μg/ml) and incubated with the cells, 30 μg/ml IgG neutralized 50% of tenascin activity and 100 μg/ml antibody completely blocked tenascin activity (data not shown). Addition of antibody alone did not affect the number of cells positive for focal adhesions. Nonimmune rabbit IgG at 200 μg/ml did not inhibit tenascin activity and anti-tenascin antibody had no effect on TSP-mediated loss of focal adhesions (data not shown).

In attempts to identify the domain in the hexabrachion molecule responsible for its focal adhesion labilizing activity, mAbs reacting with different domains in the protein (Fig. 1 *A*) were tested for their ability to neutralize tenascin activity. Antibodies against the distal knob (mAb 2A6), the central knob (mAb 58), and mAb 190, which recognizes the third FN-III domain, had little or no effect on tenascin activity (Table I). However, antibodies 127 and 81C6 effectively neutralized the tenascin activity (Table I). mAb 127 was more effective at lower concentrations than 81C6. These two neutralizing antibodies bind to different FN-III domains in the alternatively spliced segment, suggesting that the anti-adhesion activity is in this segment. Antibodies incubated with cells in the absence of tenascin had no effect on focal adhesions (data not shown).

Recombinant Fragment HxB6-12 Has Tenascin-like Activity

To confirm the mapping of the focal adhesion modulating activity to the alternatively spliced domain of tenascin, a cDNA fragment corresponding to the entire alternatively spliced segment of human tenascin comprising FN-III domains 6–12 was expressed in *E. coli*. The recombinant pro-

Table I. Neutralization of Tenascin Activity by Domain-Specific Monoclonal Antibodies

	Percent of cells positive for focal adhesions Antibody concentration			
Antibody	0 μg/ml	10 μg/ml	20 μg/ml	60 μg/ml
Tenascin + mAb 58	–	n.t.	48 (25)*	n.t.
+ mAb 190	–	n.t.	40 (0)	n.t.
+ mAb 127	–	71 (122)	68 (108)	71 (122)
+ mAb 81C6	–	43 (3)	61 (78)	68 (108)
+ mAb 2A6	–	n.t.	51 (36)	n.t.
Tenascin	42			
BSA	66			

Cells grown on coverslips were pretreated for 1 h with 10 μg/ml cycloheximide, washed, and incubated for 1 h at 37°C with 40 μg/ml tenascin or BSA or with tenascin and antibodies. Cells were fixed and examined for focal adhesions by interference reflection microscopy (IRM). Antibodies alone did not affect focal adhesions (data not shown).

* Results in parentheses are expressed as percent neutralization of the reduction of focal adhesions in cells treated with antibody and tenascin as compared with cells incubated with tenascin alone.

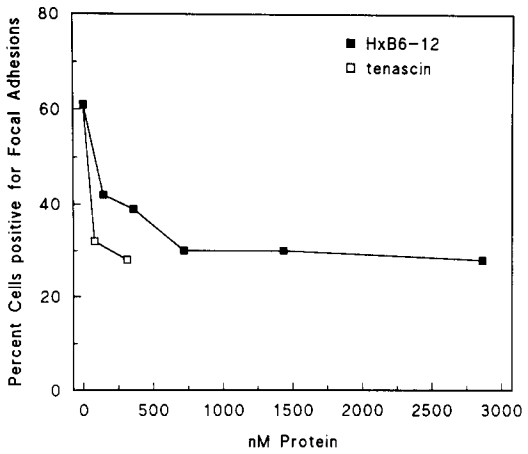

Figure 8. Recombinant protein HxB6-12 has focal adhesion labilizing activity. Increasing concentrations of monomeric HxB6-12 or native tenascin were tested in 1-h focal adhesion assays on BAE cells grown on coverslips. Cells treated with 100 μg/ml BSA served as positive controls. Results are expressed as percent of cells positive for focal adhesions.

Table II. Tenascin Activity Is Neutralized by C-6-S Glycosaminoglycan

Percent of cells positive for focal adhesions			
Experiment 1		Experiment 2	
BSA (40 μg/ml)	67 (100)	BSA	79 (100)
Tenascin (40 μg/ml)	40 (0)	Tenascin	55 (0)
Tenascin + C-6-S*	68 (104)	Tenascin + CS-D	57 (8)
+ C-6-S‡	62 (81)	+ CS-E	55 (0)
+ C-4-S	54 (52)	+ HS	54 (0)
+ CS-B	54 (52)	+ Heparin	57 (8)

Cells grown on coverslips were cycloheximide-treated before incubation for 1 h with tenascin and various glycosaminoglycans (used at 200 μg/ml). Cells were then fixed and examined for the presence of focal adhesions by IRM. Numbers in parentheses are data expressed as percent neutralization.
* This C-6-S is from Dr. John Baker, University of Alabama at Birmingham, and was prepared from basking shark cartilage.
‡ This C-6-S (CS-C) was purchased from Seikagaku America Inc. C-4-S was from Dr. John Baker and was purified from a rat chondrosarcoma. CS-B (dermatan sulfate), CS-D, and CS-E (oversulfated isomers of CS), and HS (bovine kidney) were obtained from Seikagaku America Inc. Heparin (3,000 D) was from Boehringer Mannheim Biochemicals.

tein, referred to as HxB6-12, was purified and shown to cause a loss of focal adhesions from spread BAE cells in a dose-dependent manner (Fig. 8). Although HxB6-12 reduced the number of focal adhesion positive cells to the same extent as native tenascin, it was significantly less effective on a molar basis. Oligomeric preparations of HxB6-12 were also tested and found to have activity at slightly lower concentrations than the monomeric form of HxB6-12. Another recombinant protein, a 10-kD polypeptide equivalent to FN-III domain 3, which contains the RGD sequence, had no focal adhesion labilizing activity.

Chondroitin Sulfate Glycosaminoglycans Neutralize Tenascin Activity

Since tenascin binds chondroitin sulfate proteoglycans (7, 20, 42) and TSP-induced loss of focal adhesions is neutralized by heparin (30), we examined various glycosaminoglycans for their ability to neutralize tenascin activity. Two preparations of C-6-S neutralized tenascin activity better than either chondroitin sulfate B (dermatan sulfate) or C-4-S (Table II). Over-sulfated isomers of chondroitin sulfate (chondroitin sulfate D and E), heparin, or heparan sulfate at 200 μg/ml had no neutralizing activity. None of the glycosaminoglycans alone affected the distribution or number of focal adhesions (data not shown). Treatment of C-6-S with chondroitinase ABC eliminated the ability of C-6-S to neutralize tenascin activity (Table III), suggesting that C-6-S activity was due to the glycosaminoglycan chains and not to a possible contaminant. Chondroitinase treatment of tenascin itself had no effect on tenascin activity.

C-6-S appears to neutralize tenascin activity through interactions with the cell surface and does not have to be present as a soluble molecule, since preincubation of cells with C-6-S, followed by removal of unbound glycosaminoglycan by washing before addition of tenascin, still resulted in neutralization of tenascin activity (Table IV).

These data suggest that C-6-S bound to the cell surface may either compete for binding to a tenascin receptor or, alternately, sterically interfere with tenascin interactions with this hypothetical receptor. In the latter case, longer glycosaminoglycan chains may be expected to block tenascin receptor accessibility more effectively than shorter chains. To determine if the tenascin-neutralizing activity of the galactosaminoglycans was related to the chain length of the polymer, C-6-S and C-4-S preparations were subfractionated according to size on a Sephacryl S-300 column. Fractions were analyzed for uronic acid content by the carbazole method and tested for neutralizing activity in focal adhesion assays at ~25 μg (C-6-S) or 15 μg (C-4-S) uronic acid. We observed a relationship between the size of the glycosaminoglycan chain and the ability to neutralize tenascin activity.

Table III. C-6-S Neutralization of Tenascin Activity Is Sensitive to Chondroitinase ABC Treatment

	Percent of cells positive for focal adhesions	
BSA	69 (100)	n = 100
Tenascin	46 (67)	n = 200
Tenascin + C-6-S	63 (91)	n = 300
Tenascin + [C-6-S + enzyme]*	40 (58)	n = 166
Tenascin + [C-6-S + inactive enzyme]*	61 (88)	n = 302
Tenascin + enzyme	43 (62)	n = 150
Tenascin + inactive enzyme	44 (64)	n = 200
Enzyme	62 (90)	n = 101
C-6-S + enzyme*	66 (96)	n = 100

500 μg of C-6-S was digested for 18 h at 37°C with 0.1 U chondroitinase ABC. 200 μg/ml of digested or untreated C-6-S and/or 0.02 U enzyme were incubated with cells in standard focal adhesion assays using 40 μg/ml tenascin. In some cases, enzyme was inactivated by boiling for 5 min before addition to C-6-S. In control experiments, tenascin incubated for 18 h at 37°C with enzyme showed no decrease in activity as compared with control tenascin incubated with buffer only. Results are expressed as the percentage of cells positive for focal adhesions. Numbers in parentheses are data expressed relative to BSA-treated controls.
n, number of cells counted.
* Enzyme was inactivated by boiling for 5 min before addition to cells.

Table IV. Neutralization of Tenascin Activity by Preincubation of Cells with Chondroitin Sulfate Glycosaminoglycan

	Tenascin	
	(−)	(+)
C-6-S	62 (102)	59 (97)
DME	61 (100)	39 (64)

Monolayers of BAE cells grown on coverslips were washed twice with DME and then incubated for 1 h at 37°C with 10 μg/ml cycloheximide and 60 μg/ml of chondroitin sulfate-C (Seikagaku America Inc.) or DME. Coverslips were then washed three times with warm DME and then incubated for 1 h with 40 μg/ml tenascin (+) or BSA (−), fixed, and examined for focal adhesions by IRM. Results are expressed as percent of cells positive for focal adhesions and numbers in parentheses are percent of cells positive for focal adhesions relative to the BSA-treated control.

Fractions of C-6-S of relatively longer chain length (glycosaminoglycans eluted at K_{av} 0.147 and 0.235) completely neutralized 20 μg tenascin activity; similarly, C-4-S chains eluted at K_{av} 0.235 neutralized activity to 95% of control. In contrast, glycosaminoglycan fractions of either C-6-S or C-4-S that eluted at greater K_{av}'s (0.31–0.5), i.e., having a shorter chain length, were decreasingly effective in neutralizing tenascin activity (Fig. 9).

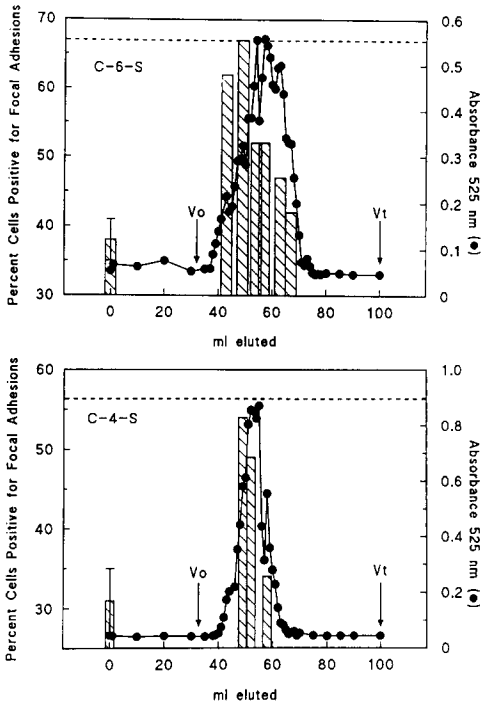

Figure 9. The ability of glycosaminoglycans to neutralize tenascin activity is related to the size of the chains. 50 mg C-6-S (Seikagaku America Inc.) (*top*) and 10 mg C-4-S (gift of Dr. John Baker) (*bottom*) were fractionated on a Sephacryl S-300 column equilibrated in PBS (v_o = 33 ml, v_t = 101 ml). Fractions were analyzed by the carbazole method for uronic acid content and the absorbance was read at 525 nm (*circles*). Various fractions were then analyzed for their ability to neutralize the activity of 20 μg tenascin in focal adhesion assays (*hatched bars*). C-6-S fractions were used at ~25 μg uronic acid and C-4-S fractions at ~15 μg uronic acid. The percent of cells positive in BSA controls is indicated by the dashed lines (63% for the C-6-S experiment and 57% for the C-4-S experiment). For both types of chondroitin sulfates, the larger size chains were more effective neutralizers of tenascin activity than the smaller size GAG chains.

Discussion

Previous observations, together with the data presented here, suggest the existence of two families of matrix glycoproteins that differ with respect to their effects on cell substrate adhesion. The family of adhesive matrix proteins, which include fibronectin, vitronectin, fibrinogen, laminin, and some collagens, promotes cell adhesion by facilitating cell attachment, spreading, and formation of focal adhesions. The second family of matrix proteins appears to negatively modulate cell–substrate adhesion by apparently stimulating the disassembly of focal adhesions in a subpopulation of spread cells. Members of this adhesion-modulating family, which include TSP, tenascin, and SPARC, are similar in that their expression is increased during neoplasia, embryogenesis, and tissue remodeling and repair, processes in which cells undergo mitosis and become motile (5). Cells change shape and lose their focal adhesions during mitosis. Furthermore, motile and transformed cells have fewer and smaller focal adhesions than nontransformed, stationary cells (2, 5, 44). The expression of adhesion modulators during tissue remodeling may provide the type of "loose" adhesion required by a motile or mitotic cell.

Like TSP, tenascin affects focal adhesions in only a subpopulation of cells in our BAE cultures. A combination of two adhesion modulators, TSP and tenascin, did not increase the percent of susceptible cells, suggesting that some cells are in a refractile phase in which they are unable to respond to adhesion modulators. In the population of susceptible cells, the modulation of focal adhesions is achieved without the need for de novo protein synthesis, since focal adhesion loss occurs in the presence of cycloheximide. Moreover, the effects are reversible: after tenascin or TSP is removed, focal adhesions are reassembled in the continued presence of cycloheximide. We also observed that cells adhering to fibronectin or vitronectin substrates are equally sensitive to tenascin or TSP (Murphy-Ullrich, J. E., unpublished results).

A panel of mAbs was used to locate the domain in the tenascin molecule responsible for its adhesion-modulating activity. Two neutralizing antibodies bound to the alternatively spliced segment, suggesting that this region contained the active site. A recombinant form of the alternatively spliced segment did, in fact, exhibit focal adhesion modulating activity, although it was less potent on a molar basis than native tenascin. This difference could reflect lack of glycosylation of this normally heavily glycosylated region (16), incorrect folding, or the monomeric nature of the recombinant protein. Nevertheless, these data strongly suggest that a major active site is located in the alternatively spliced segment. Mapping the adhesion-modulating activity to this segment also suggests that mechanisms regulating the splicing of tenascin mRNA may determine the activity of the protein. Recent evidence from Weller et al. (43), shows that, at least for mouse mesenchymal tissues, the larger form of tenascin predominates during embryogenesis. In other embryonic tissues the appearance of different splice forms of tenascin is more complex (32). Weakening of cell–substrate contacts and thereby "priming" cells for migration may be one of the

functions of the large form of tenascin in embryogenesis and tissue remodeling.

In our studies, we found that sulfated galactosaminoglycans neutralized the focal adhesion labilizing activity of tenascin. Several investigators reported that tenascin binds to chondroitin sulfate proteoglycan from muscle cell culture, cartilage, and chick brain (7, 20, 42), but this interaction may primarily involve the proteoglycan core protein (20). Tenascin does not appear to bind to the chondroitin sulfate glycosaminoglycan, since we failed to demonstrate a binding of biotin-labeled chondroitin sulfate to tenascin adsorbed to a microtiter well (Zhou, F., M. Höök, and J. E. Murphy-Ullrich, unpublished observations). Since chondroitin sulfate neutralized tenascin activity after unbound glycosaminoglycans had been removed from preincubated cells, the observed neutralization probably involves binding of the glycosaminoglycan to a cell surface component. This galactosaminoglycan binding component could be a hypothetical tenascin receptor (41) or a membrane component located in the vicinity of this receptor. As a result, the galactosaminoglycan could compete for or sterically hinder tenascin interactions with a receptor. The latter hypothesis is supported by data which show that longer glycosaminoglycan chains are more effective inhibitors of tenascin than are shorter chains. It is noteworthy that heparin-like polysaccharides, which neutralize the focal adhesion modulating activity of TSP, did not affect tenascin activity.

Focal adhesions involve a very close apposition of the membrane to the substrate (10–15 nm). It is unlikely that a molecule as large as a hexabrachion (~100–150 nm) would be able to intercalate under the cell and act directly at the extracellular face of the focal adhesion. It appears more likely that tenascin binds to a cell surface molecule distant from the focal adhesion site, perhaps on the dorsal surface.

Interaction of tenascin with a cell surface molecule might trigger a cytoplasmic signal that alters the cytoskeleton or disrupts interactions of cytoplasmic components of adhesion plaques with transmembrane receptors. A signaling mechanism has been proposed for the ability of tenascin to inhibit T cell activation, an immunomodulatory activity perhaps related to an inhibition of cell adhesion (34). It is possible that this hypothetical signaling involves a phosphorylation/dephosphorylation mechanism, although the role of phosphorylation of focal adhesion components in the maintenance of adhesion plaque integrity is presently unclear (reviewed in reference 5). Phorbol esters, which activate protein kinase C, cause a rapid loss of focal adhesions (36) and alterations in F-actin (17). Lamb et al. (24) demonstrated that activation or injection of protein kinase A into fibroblasts caused complete loss of actin-containing stress fibers and presumably, associated focal adhesions. On the other hand, Woods et al. recently showed that induction of focal adhesion formation by the heparin-binding fragment of fibronectin is blocked in the presence of agents which inhibit protein kinase C (45; Woods, A., M. R. Austria, and J. R. Couchman. 1990. *J. Cell Biol.* 111:19*a*. [Abstr.]). Physiologically, kinases are regulated by binding of ligands to specific receptors. We have shown that interaction of three extracellular matrix molecules, TSP, tenascin, and SPARC, with cells stimulates the loss of focal adhesions. Our future work will focus on determining the signal transduction mechanisms though which these matrix molecules might alter focal adhesion integrity.

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References

1. Aukhil, I., C. C. Slemp, V. A. Lightner, K. Nishimura, G. Briscoe, and H. P. Erickson. Purification of hexabrachion (tenascin) from cell culture conditioned medium and separation from a cell adhesion factor. *Matrix.* 10:98–111.
2. Bershadsky, A. D., I. S. Tint, A. A. Neyfakh, Jr., and J. M. Vasiliev. 1985. Focal contacts of normal and RSV-transformed quail cells: hypothesis of the transformation-induced deficient maturation of focal contacts. *Exp. Cell Res.* 158:433–444.
3. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330–334.
4. Bourdon, M. A., and E. Ruoslahti. 1989. Tenascin mediates cell attachment through an RGD-dependent receptor. *J. Cell Biol.* 108:1149–1155.
5. Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–526.
6. Carlson, D. M. 1968. Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. *J. Biol. Chem.* 243:616–626.
7. Chiquet, M., and D. M. Fambrough. 1984. Chick myotendinous antigen. II. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunit. *J. Cell Biol.* 98:1937–1946.
8. Chiquet-Ehrismann, R. 1990. What distinguishes tenascin from fibronectin? *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2598–2604.
9. Chiquet-Ehrismann, R., E. J. Mackie, C. A. Pearson, and T. Sakakura. 1986. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* 47:131–139.
10. Chiquet-Ehrismann, R., P. Kalla, C. A. Pearson, K. Beck, and M. Chiquet. 1988. Tenascin interferes with fibronectin action. *Cell.* 53:383–390.
11. Chiquet-Ehrismann, R., P. Kalla, and C. A. Pearson. 1989. Participation of tenascin and transforming growth factor-β in reciprocal epithelial-mesenchymal interactions of MCF7 cells and fibroblasts. *Cancer Res.* 49:4322–4325.
12. Erickson, H. P., and M. A. Bourdon. 1989. Tenascin: an extracellular matrix protein prominent in specialized embryonic tissues and tumors. *Annu. Rev. Cell Biol.* 5:71–92.
13. Erickson, H. P., and V. A. Lightner. 1988. Hexabrachion protein (tenascin, cytotactin, brachionectin) in connective tissues, embryonic brain, and tumors. *Adv. Cell Biol.* 2:55–90.
14. Erickson, H. P., and H. C. Taylor. 1987. Hexabrachion proteins in embryonic chicken and human tumors. *J. Cell Biol.* 105:1387–1394.
15. Friedlander, D. R., S. Hoffman, and G. M. Edelman. 1988. Functional mapping of cytotactin. Proteolytic fragments active in cell-substrate adhesion. *J. Cell Biol.* 107:2329–2340.
16. Gulcher, J. R., D. E. Nies, L. S. Marton, and K. Stefansson. 1989. An alternatively spliced region of the human hexabrachion contains a repeat of potential N-glycosylation sites. *Proc. Natl. Acad. Sci. USA.* 86:1588–1592.
17. Hedberg, K. K., G. B. Birrell, D. L. Habliston, and O. H. Griffith. 1990. Staurosporine induces dissolution of microfilament bundles by a protein kinase C-independent pathway. *Exp. Cell Res.* 188:199–208.
18. Herman, B., and W. J. Pledger. 1985. Platelet-derived growth factor-induced alterations in vinculin and actin distribution in BALB/c-3T3 cells. *J. Cell Biol.* 100:1031–1040.
19. Herman, B., M. A. Harrington, N. E. Olashaw, and W. J. Pledger. 1986. Identification of the cellular mechanisms responsible for platelet-derived growth factor induced alterations in cytoplasmic vinculin distribution. *J. Cell. Physiol.* 126:115–125.
20. Hoffman, S., K. L. Crossin, and G. M. Edelman. 1988. Molecular forms, binding functions, and developmental expression patterns of cytotactin and cytotactin-binding proteoglycan, an interactive pair of extracellular matrix molecules. *J. Cell Biol.* 106:519–532.
21. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.
22. Jones, F. S., M. P. Burgoon, S. Hoffman, K. L. Crossin, B. A. Cunningham, and G. M. Edelman. 1988. A cDNA clone for cytotactin contains sequences similar to epidermal growth factor-like repeats and segments

of fibronectin and fibrinogen. *Proc. Natl. Acad. Sci. USA.* 85:2186–2190.
23. Jones, F. S., S. Hoffman, B. A. Cunningham, and G. M. Edelman. 1989. A detailed structural model of cytotactin: protein homologies, alternative RNA splicing, and binding regions. *Proc. Natl. Acad. Sci. USA.* 86:1905–1909.
24. Lamb, N. J. C., A. Fernandez, M. A. Conti, R. Adelstein, D. B. Glass, W. J. Welch, and J. R. Feramisco. 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. *J. Cell Biol.* 106:1955–1971.
25. Lane, T. F., and E. H. Sage. 1990. Functional mapping of SPARC: peptides from two distinct Ca^{++}-binding sites modulate cell shape. *J. Cell Biol.* 111:3065–3076.
26. Lightner, V. A., and H. P. Erickson. 1990. Binding of hexabrachion (tenascin) to the extracellular matrix and substratum and its effect on cell adhesion. *J. Cell Science.* 95:263–277.
27. Lotz, M. M., C. A. Burdsal, H. P. Erickson, and D. R. McClay. 1989. Cell adhesion to fibronectin and tenascin. Quantitative measurements of initial binding and subsequent strengthening response. *J. Cell Biol.* 109:1795–1805.
28. Mackie, E. J., I. Thesleff, and R. Chiquet-Ehrismann. 1987. Tenascin is associated with chondrogenic and osteogenic differentiation in vivo and promotes chondrogenesis in vitro. *J. Cell Biol.* 105:2569–2579.
29. Marton, L. S., J. R. Gulcher, and K. Stefansson. 1989. Binding of hexabrachions to heparin and DNA. *J. Biol. Chem.* 264:13145–13149.
30. Murphy-Ullrich, J. E., and M. Höök. 1989. Thrombospondin modulates focal adhesions in endothelial cells. *J. Cell Biol.* 109:1309–1319.
31. Murphy-Ullrich, J. E., and D. F. Mosher. 1985. Localization of thrombospondin in clots formed *in situ*. *Blood.* 66:1098–1104.
32. Prieto, A. L., F. S. Jones, B. A. Cunningham, K. L. Crossin, and G. M. Edelman. 1990. Localization during development of alternatively spliced forms of cytotactin mRNA by in situ hybridization. *J. Cell Biol.* 111:685–698.
33. Riou, J. F., D. L. Shi, M. Chiquet, and J. C. Boucaut. 1990. Exogenous tenascin inhibits mesodermal cell migration during amphibian gastrulation. *Dev. Biol.* 137:305–317.
34. Rüegg, C. R., R. Chiquet-Ehrismann, and S. S. Alkan. 1989. Tenascin, an extracellular matrix protein, exerts immunomodulatory activities. *Proc. Natl. Acad. Sci. USA.* 86:7437–7441.
35. Sage, H., R. B. Vernon, S. E. Funk, E. A. Everitt, and J. Angello. 1989. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading in vitro and exhibits CA^{+2}-dependent binding to the extracellular matrix. *J. Cell Biol.* 109:341–356.
36. Schliwa, M., T. Nakamura, K. R. Porter, and U. Euteneuer. 1984. Tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J. Cell Biol.* 99:1045–1059.
37. Spring, J., K. Beck, and R. Chiquet-Ehrismann. 1989. Two contrary functions of tenascin: dissection of the active sites by recombinant tenascin fragments. *Cell.* 59:325–334.
38. Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89.
39. Tan, S. S., K. L. Crossin, S. Hoffman, and G. M. Edelman. 1987. Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. *Proc. Natl. Acad. Sci. USA.* 84:7977–7981.
40. Taylor, H. C., V. A. Lightner, W. F. Beyer, Jr., D. McCaslin, G. Briscoe, and H. P. Erickson. 1989. Biochemical and structural studies of tenascin/hexabrachion proteins. *J. Cell. Biochem.* 41:71–90.
41. Vaino, S., M. Jalkanen, and I. Thesleff. 1989. Syndecan and tenascin expression is induced by epithelial-mesenchymal interactions in embryonic tooth mesenchyme. *J. Cell Biol.* 108:1945–1954.
42. Vaughan, L., S. Huber, M. Chiquet, and K. H. Winterhalter 1987. A major six-armed glycoprotein from embryonic cartilage. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:349–353.
43. Weller, A., S. Beck, and P. Ekblom. 1991. Amino acid sequence of mouse tenascin and differential expression of two tenascin isoforms during embryogenesis. *J. Cell Biol.* 112:355–362.
44. Woods, A., and J. R. Couchman. 1988. Focal adhesions and cell-matrix interactions. *Collagen Relat. Res.* 8:155–182.
45. Woods, A., J. R. Couchman, S. Johansson, and M. Höök. 1986. Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:665–670.