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Characterization of Cell–Matrix Adhesion Requirements for the Formation of Fascin Microspikes

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Cell adhesion to thrombospondin-1 (TSP-1) correlates with assembly of cell–substratum contact structures that contain fascin microspikes. In this analysis, cell-matrix requirements for assembly of fascin microspikes were examined in detail. In six cell lines, cell spreading on a TSP-1 substratum correlated with expression of fascin protein and formation of fascin microspikes. Microspikes were not formed by H9c2 cells adherent on fibronectin, vitronectin, collagen IV, or platelet factor 4. However, both fascin microspikes and focal contacts were assembled by cells adherent on laminin-1. Using mixed substrata containing different proportions of TSP-1, and fibronectin, fascin microspike formation by H9c2 and C2C12 cells was found to be reduced on substrata containing 25% fibronectin and abolished on substrata containing 75% fibronectin. Adhesion to intermediate mixtures of TSP-1 and fibronectin resulted in coassembly of fascin microspikes and focal contacts, colocalization of fascin with actin stress fiber bundles and altered distributions of β1 integrins, cortical α-actinin, and tropomyosin. In cells adherent on 50% TSP-1:50% fibronectin, GRGDSP peptide treatment decreased focal contact assembly and altered cytoskeletal organization but did not inhibit microspike assembly. Treatment with chondroitin sulfate A or *p*-nitrophenol β-D-xylopyranoside decreased microspike formation and modified cytoskeletal organization but did not inhibit focal contact formation. In polarized migratory and postmitotic C2C12 cells, fascin microspikes and ruffles were localized at leading edges and TSP matrix deposition was also concentrated in this region. Depletion of matrix TSP by heparin treatment correlated with decreased microspike formation and cell motility. Thus, the balance of adhesive receptors ligated at the cell surface during initial cell–matrix attachment serves to regulate the type of substratum adhesion contact assembled and subsequent cytoskeletal organization. A role for fascin microspikes in cell motile behavior is indicated.

INTRODUCTION

It has long been appreciated that the interactions of cells with extracellular matrix (ECM) macromolecules play a role in regulating cell adhesion, shape, and movement: processes that are central to tissue organization in metazoan organisms and that contribute to many pathological conditions if disregulated (reviewed by Hynes and Lander, 1992; Gumbiner, 1996).

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To date, the cell–substratum contact structure most thoroughly studied in vitro has been the focal contact, or focal adhesion, that corresponds morphologically to a site of very close apposition between the ventral plasma membrane of a cell and its substratum (Abercrombie *et al.*, 1971; Izzard and Lochner, 1976; Heath and Dunn, 1978). In molecular terms, focal contacts are distinguished by the colocalization of ligand-occupied clustered integrins on the cell surface with the termini of actin microfilament bundles and a characteristic assembly of intracellular proteins including talin, vinculin, paxillin, and focal adhesion kinase (FAK) beneath the the plasma membrane. Focal con-

tacts not only link integrins to the actin cytoskeleton but also generate intracellular signals (reviewed by Burridge *et al.*, 1988; Turner and Burridge, 1991; Juliano and Haskill, 1993; Jockusch *et al.*, 1995; Schwartz *et al.*, 1995). The abundance of focal contacts in fibroblastic cells correlates inversely with their speed of movement on a planar substratum (Izzard and Lochner, 1976; Couchman and Rees, 1979; Kolega *et al.*, 1982).

Various other actin-containing structures implicated in cell–substratum interactions have been described, including close contacts (Izzard and Lochner, 1976), point or dot contacts (Streeter and Rees, 1987; Tawil *et al.*, 1993), invadopodia (Mueller and Chen, 1991), podosomes (Tarone *et al.*, 1985), and filopodia (reviewed by Grinnell, 1978). These structures tend to be transient localized features of the cell surface or cell-type–specific structures and remain less well characterized than focal contacts. However, the available information indicates that the molecular composition of these structures has similarities with that of focal contacts. Point contacts contain certain β_1 integrins and vinculin but lack talin or FAK (Tawil *et al.*, 1993; Arregui *et al.*, 1994); invadopodia contain talin, phosphotyrosine-containing proteins, and β_1 integrins, predominently $\alpha_3\beta_1$ (Mueller *et al.*, 1989, 1992; Coopman *et al.*., 1996); and podosomes contain vinculin (Tarone *et al.*, 1985). Filopodia that attach to substratum display small adhesion plaques containing integrins and vinculin, and filopodial formation and dynamics are altered in the absence of vinculin (Samuels *et al.*, 1993; Varnum-Finney and Reichardt, 1994; Goldman *et al.*, 1995). Acute laser inactivation of vinculin or talin affects filopodial bending or motility, respectively (Sydor *et al.*, 1996).

Analysis of the structure and regulation of focal contacts has been aided by the use of cell adhesion assays in serum-containing medium or on matrix glycoproteins such as fibronectin. These conditions stimulate cell spreading and the rapid assembly of many focal contacts, which are readily visualized by staining for abundant structural components such as vinculin or talin (see, for example, Geiger, 1979; Singer *et al.*, 1988; reviewed by Hynes, 1990). In contrast, cells that spread on thrombospondin-1 (TSP-1), a matrix glycoprotein to which cells adhere through multiple cell surface binding molecules including proteoglycans, glycolipids, CD36, CD47, several less well-characterized molecules, and in some cell types certain integrins, predominantly $\alpha_v\beta_3$ (Lawler *et al.*, 1988; reviewed by Frazier, 1991; Lahav, 1993; Adams *et al.*, 1995; Bornstein, 1995), do not assemble focal contacts but display a distinctive organization of actin microfilaments in the cell cortex, characterized by radial actin microspikes (Adams and Lawler, 1994). These microspikes contain the actin-bundling protein fascin. Whereas fascin is diffusely distributed in cells adherent on fibronectin, vinculin remains diffuse in cells adherent on TSP-1 and is not present in microspikes. Thus, cell spreading on fibronectin or on TSP-1 results in the formation of biochemically distinct structures involved in cell–substratum adhesion (Adams, 1995). It is possible that these two types of contact structure could play functionally distinct roles in the coordination of cell adhesive and motile behavior.

The aim of this analysis was to examine the phenomenon of fascin microspike formation in detail, with reference to ECM substratum requirements. To this end, the correlation among cell spreading on TSP-1, fascin expression, and microspike formation was examined, also the ability of cells to form microspikes or focal contacts when adherent on different adhesive substrata or on mixed TSP-1/fibronectin substrata. Because the cortical microspikes and lamellae formed by cells adherent on platelet TSP-1 resemble structures at the leading edge of motile cells (Abercrombie *et al.*, 1971; Couchman and Rees, 1979; Heath and Holifield, 1991), the distributions of fascin, TSP, and fibronectin and the role of matrix TSP in microspike formation were examined in polarized migratory C2C12 myoblasts. The results of these experiments demonstrate that the formation of fascin microspikes or focal contacts and microfilament organization is regulated according to the ECM ligand provided and provide evidence for a role of matrix TSP and fascin microspikes in cell migratory behavior.

MATERIALS AND METHODS

Cell Lines

The cell lines used included A10 rat aortic smooth muscle cells (Kimes and Brandt, 1976a), A549 human lung carcinoma cells, C2C12 mouse skeletal myoblasts (Blau *et al.*, 1985), C32 human melanoma cells, COS-7 green monkey kidney cells (Gluzman, 1981), G361 human melanoma cells, G8 mouse skeletal myoblasts (Christian *et al.*, 1977), H9c2 rat myoblasts (Kimes and Brandt, 1976b), HT1010 human fibrosarcoma (Rasheed *et al.*, 1974), MDCK canine kidney cells, MG-63 human osteosarcoma cells, RT4 human bladder carcinoma cells, and SK-N-SH human neuroblastoma cells (Spengler *et al.*, 1973). Most cell lines were cultured in DMEM containing 10% fetal calf serum; however, the myoblast cell lines were cultured in DMEM containing 20% fetal calf serum. All cells were maintained in a humidified 10% CO_2 atmosphere at 37°C.

Gel Electrophoresis and Western Blotting

To examine fascin expression in a panel of cell lines, growing cultures containing 5×10^5 cells per dish were lysed directly in SDS-PAGE sample buffer containing 100 mM dithiothreitol. SDS-PAGE was carried out according to the method of Laemmli (1970) on 12.5% polyacrylamide gels. Proteins were transferred to nitrocellulose (0.22 μm, pore size, Bio-Rad, Watford, UK) at 60 V for 18 h, using a Transblot apparatus (Bio-Rad) and transfer buffer composed of 25 mM Tris(hydroxymethyl)aminomethane base, 92 mM glycine, and 20% methanol (Towbin *et al.*, 1979). Nonspecific binding sites were blocked by overnight incubation at 4°C in TBS containing 2% bovine serum albumin and 0.05% Tween 20. Blots were incubated with a 1:750 dilution of fascin antibody in TBS containing 0.05% Tween 20 (TBS-Tween) for 2 h at room temperature with vigorous

shaking, washed three times over 45 min in TBS-Tween, shaken with a 1:750 dilution of peroxidase-conjugated rabbit anti-mouse IgG (ICN Biomedicals Inc.) for 2 h and washed 3 times in TBS-Tween. Bound antibody was visualized by incubating the blots in TBS containing chloronaphthol (Sigma, St. Louis, MO; 1 μg/ml) and 30% (v/v) hydrogen peroxide solution (2 μl/ml).

Adhesion Assays

Platelet TSP-1 was prepared as previously described (Adams and Lawler, 1994). Rat plasma fibronectin was obtained from Telios (San Diego, CA); human placental collagen IV, rat plasma vitronectin, mouse EHS laminin (laminin-1; Wewer and Engvall, 1994), platelet factor 4, chondroitin sulfate A, heparin, and β-xylosides were obtained from Sigma. The 40-kDa chymotryptic heparin-binding fragment of plasma fibronectin and GRGDSP peptide were obtained from Life Technologies (Paisley, Scotland). Cell adhesion assays were carried out in serum-free medium on coated glass coverslips as previously described (Adams and Lawler, 1994; Adams, 1995), typically for periods of 1 h at 37°C. In experiments using a mixed substratum, glycoproteins were diluted to equimolar coating concentrations, mixed in different ratios by volume, and used for coating. Soluble inhibitors were added at the time of plating the cells. Nonadherent cells were removed by gentle washing in TBS containing 2 mM $CaCl_2$, and adherent cells were fixed and processed for immunofluorescence.

Immunofluorescence Microscopy

For immunofluorescent staining using rhodamine-phalloidin (Sigma), mouse monoclonal antibody against vinculin (VIN 11.5, ICN Immunobiologicals), mouse monoclonal antibody against tropomyosin (TM311, Sigma), mouse monoclonal antibody to phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) or mouse monoclonal antibody to paxillin (antibody 349, Transduction Laboratories, Lexington, KY), cells were fixed in 3.7% formaldehyde for 10 min, then permeabilized for 10 min in a buffer composed of 50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.1, 5 mM $MgCl_2$, 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 100 mM KCl, and 0.2% Triton X-100 (O'Neill *et al.*, 1990). For staining with mouse monoclonal antibody to fascin (Yamashiro-Matsumura and Matsumura, 1986, the generous gift from George Mosialos, Harvard Medical School) or mouse monoclonal antibody to α-actinin (BM-75.2, Sigma), cells were fixed and permeabilized in absolute methanol for 10 min. For staining with rabbit polyclonal antisera to platelet TSP (R1, as used in Adams and Lawler [1994], and R3, raised against a glutathione *S*-transferase fusion protein encoding the type 1 repeats of human TSP-1; a gift from Jack Lawler, Harvard Medical School), fibronectin (Sigma), or rabbit antiserum to human fibronectin receptor (Life Technologies), cells were fixed with 2% paraformaldehyde in PBS. Staining with primary antibodies or phalloidin was carried out for 90 min at room temperature, and then, if necessary, cells were washed and stained for 45 min with appropriate fluorescein isothiocyanate-conjugated secondary antibodies (ICN Immunobiologicals), washed, and mounted in Vectastead mounting medium (Vector Laboratories, Loughborough, UK). Samples were examined by epifluorescence using a Zeiss Axioplan microscope and photographs were taken on Kodak T-Max 400 film.

Confocal Microscopy

Cells were stained as described above and examined by confocal microscopy, using a Leica DM-RBE microscope and laser. Images were acquired in the Leica TCS NT programme. Thirty-two or 45 serial optical sections were made throughout the entire depth of a cell using a picture size of 512 × 512 pixels. Three-dimensional (3D) image reconstruction was carried out using the NIH Image programme.

Time-Lapse Videomicroscopy

C2C12 cells plated at 3×10^4 cells/flask in Nunc Slide Flasks were monitored over 4-h periods in a 37°C environmental chamber using a Zeiss Axiovert 100 microscope fitted with a Sony SS-M37OCE change-coupled devise camera linked to a video recorder and driven by an EOS BAC900 animation controller. Cultures treated with 500 μg/ml heparin 2 h after plating were monitored between 4 h and 8 h after the start of the treatment. Migration was recorded at 4 frames/min, and the movement distances and velocities of individual cells were calculated from traces. At least 50 cells were traced for each experimental condition. Statistical significance was determined using a two-tailed *t*-test. Parallel cultures were stained for fascin.

RESULTS

Correlation of Fascin Expression, Cell Spreading, and Fascin Microspike Formation on TSP-1 Substrata

The ability of cells to spread on TSP-1 does not correlate with the recognition of particular cell-binding domains of TSP-1, raising the possibility that intracellular factors may determine this behavior (Adams and Lawler, 1993; reviewed by Adams *et al.*, 1995). Because fascin localizes to cortical microspikes in two cell types that spread on TSP-1 (Adams, 1995), it was of interest to examine the relationship between fascin expression and cell spreading on TSP-1 in detail. A panel of cell lines were screened for their ability to attach or spread when plated on a platelet TSP-1 substratum (Table 1). As expected, a spectrum of morphologies from completely round through to well spread were observed. Some cell lines, such as SK-N-SH or MG-63, assumed spiky irregular shapes (see Figure 2 below; see also Adams and Lawler, 1993).

Next, expression of fascin protein was examined by Western blot analysis. All cell lines that underwent irregular or extensive spreading contained fascin (Figure 1, lanes 1–7). Of the cell lines that attached but remained rounded, four of six lines tested lacked fascin; however, HT1080 cells and C32 cell lines did contain fascin (Figure 1, lanes 8–13). Thus, although expression of fascin shows a positive correlation with the ability of cells to spread on a TSP-1 substratum, fascin expression per se is not restricted to cell types that spread on TSP-1.

H9c2 and HISM cells have previously been shown to form fascin microspikes when adherent on TSP-1 (Adams, 1995). To examine microspike formation in cell lines that spread to different extents on TSP-1, the three fascin-positive cell lines HT1080, SK-N-SH, and A10 were stained for fascin 1 h after plating on substrata coated with 50 nM TSP-1 or 50 nM fibronectin in serum-free medium. In HT1080 cells that attach but remain round on TSP-1, fascin was diffusely distributed throughout the cell and no microspike-type structures were observed (Figure 2a). In HT1080 cells spread on fibronectin, fascin was diffusely distributed

Table 1. Adhesive behavior of cell lines in response to a platelet TSP-1 substratum

Cell line	Attachment to TSP-1	Spreading	Reference
A549	+	−	Tuszynski *et al.* (1992); this study
C32	+	−	Roberts *et al.* (1987); Asch *et al.* (1991); this study
HT1080	+	−	Asch *et al.* (1991); Adams and Lawler (1994)
G361	+	−	Prater *et al.* (1991); Adams and Lawler (1993); see also Roberts *et al.* (1987); Asch *et al.* (1991)
MDCK	+	−	This study
RT4	+	−	This study
COS-7	+	(+)	This study
MG-63	+	(+)	Adams and Lawler (1993); Chen *et al.* (1994); see Clezardin *et al.* (1989)
SK-N-SH	+	(+)	This study
A10	+	+	This study
C2C12	+	+	Adams and Lawler (1994)
HISM	+	+	Adams and Lawler (1993); Adams (1995)
H9c2	+	+	Adams and Lawler (1994); Adams (1995)
G8	+	+	This study

The morphology of cells adherent on substrata coated with 50 nM TSP-1 was examined after adhesion for 1 h at 37°C in serum-free medium. With respect to attachment, + indicates qualitatively that cells could attach. With respect to spreading, − indicates no spreading and rounded cell morphology, (+) indicates partial spreading and irregular cell morphology, and + indicates well-spread. Examples of these morphologies can be found in Figure 2 and also in Adams and Lawler (1993).

throughout the cytoplasm (Figure 2b). SK-N-SH cells that spread partially on TSP-1 showed diffuse fascin staining within the cell body and in fingerlike peripheral protrusions and arrays of microspikes (Figure 2c). SK-N-SH cells adherent on fibronectin adopted a smooth-edged polygonal shape and fascin staining appeared diffuse (Figure 2d). In A10 cells, which undergo extensive spreading on TSP-1, circumferential arrays of fascin microspikes were observed at the cell margins and diffuse perinuclear fascin staining was also present (Figure 2e). This staining pattern is similar to that previously described in other cells that spread extensively on TSP-1 (Adams, 1995). In A10 cells spread on fibronectin, fascin staining was diffuse throughout the cytoplasm (Figure 2f). Thus, cell types that undergo spreading on TSP-1 show localization of fascin to cortical microspikes. Depending on the extent of cell spreading, the microspikes are apparent as individual protrusions from the cell margin or as "ribs" within a lamellar structure. Cell adhesion on fibronectin does not stimulate the formation of these fascin-containing structures.

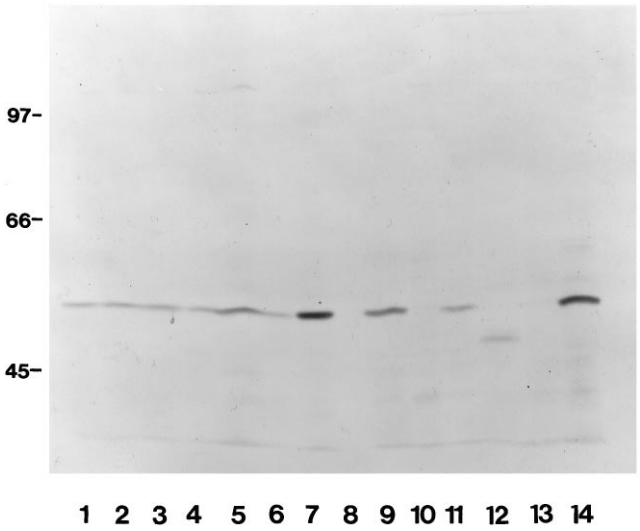

Figure 1. Expression of fascin by cell lines used in attachment assays. Equivalent numbers of cells were lysed in SDS-PAGE sample buffer, the extracts were resolved on a 12.5% polyacrylamide gel under reducing conditions, transferred to nitrocellulose, and probed with antibody 55K2 to fascin. Lanes: 1, A10 cells; 2, HISM cells; 3, MG-63 cells; 4, SK-N-SH cells; 5, H9c2 cells; 6, G8 cells; 7 and 14, C2C12 cells; 8, A549 cells; 9, C32 cells; 10, RT4 cells; 11, HT1080 cells; 12, G361 cells; 13, MDCK cells. Molecular mass markers are indicated in kDa.

Ability of Other Adhesive Substrata to Stimulate Fascin Microspike Formation

To obtain further insight into the ECM signals that stimulate fascin microspike formation, the distributions of fascin and vinculin were examined in H9c2 cells adherent for 60 min on substrata coated with 50 nM TSP-1, vitronectin, collagen IV, or laminin-1. The cells spread on all four substrata and as expected, cells adherent on platelet TSP-1 formed abundant fascin microspikes, whereas vinculin remained diffuse and focal contacts were not detected (Figure 3, a and b). In

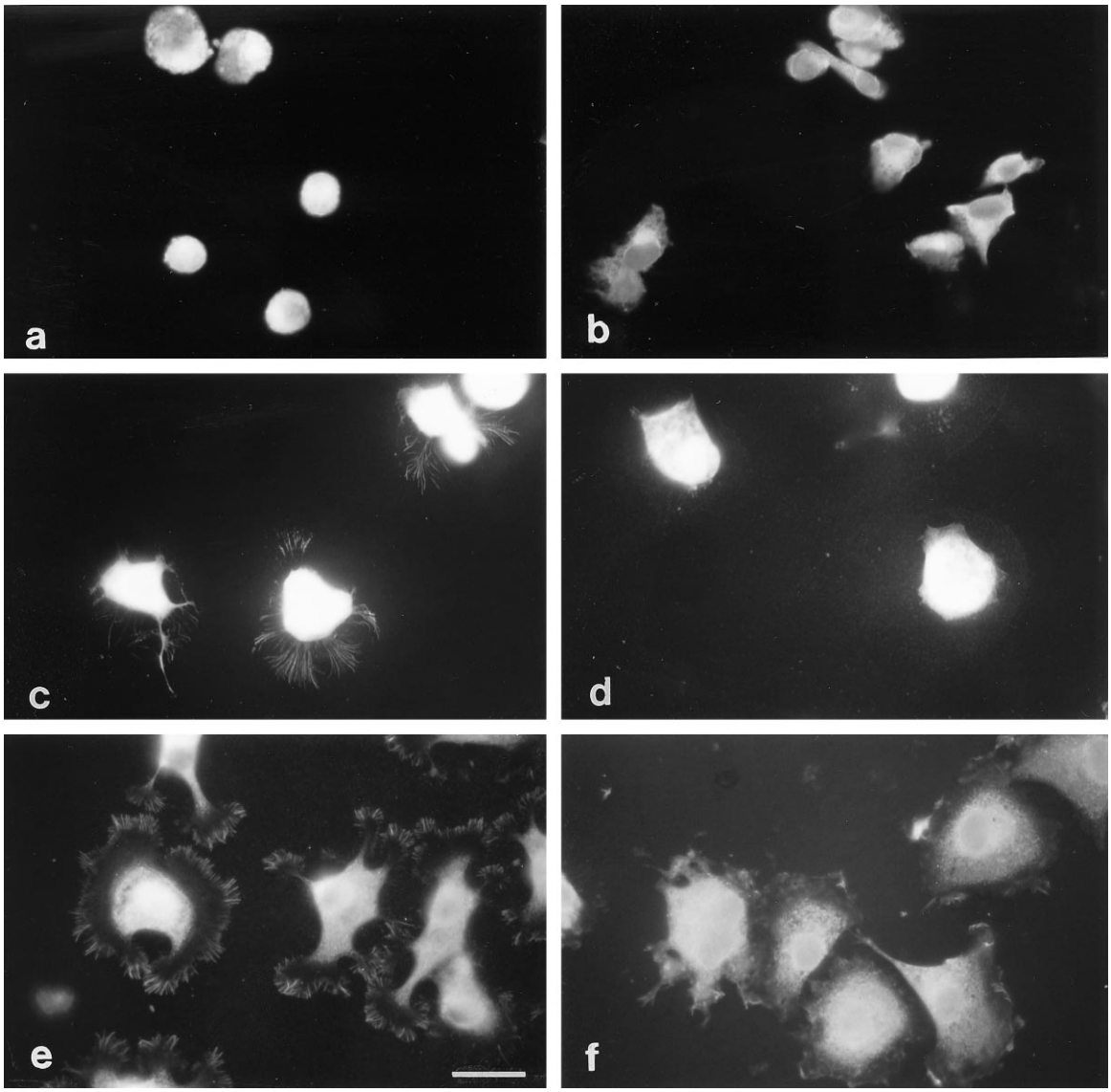

Figure 2. Distribution of fascin in cells adherent on TSP-1 or fibronectin substrata. HT1080 cells (a and b), SK-N-SH cells (c and d), or A10 cells (e and f) were stained for fascin 1 h after plating on substrata coated with 50 nM TSP-1 (a, c, and e) or 50 nM fibronectin (b, d, and f) under serum-free conditions. Bar, 12 μm.

contrast, H9c2 cells adherent on vitronectin exhibited diffuse cytoplasmic fascin staining (Figure 3c). Vinculin localized to many focal contacts distributed over the ventral surfaces of the cells (Figure 3d). The distribution of these two proteins in response to adhesion to vitronectin thus resembles that observed upon cell adhesion to fibronectin (Adams, 1995; see Figure 2, b, d, and f). Cells adherent on a collagen IV substratum displayed a different distribution of fascin, in that in addition to the diffuse intracellular staining, staining of upraised cortical structures was apparent in some cells and colocalization of fascin protein with actin microfilament bundles was detectable in some cells (Figure 3e). All cells adherent on collagen IV displayed prominent vinculin staining in focal contacts (Figure 3f).

Cells adherent on laminin-1 displayed a distinctive distribution of fascin. In the majority of cells, colocalization of fascin with actin microfilament bundles was readily apparent. Fascin localized to prominent but irregular upraised microspikes and ruffles of various sizes at the cell margins (Figure 3g). The morphological heterogeneity and upraised position of these structures distinguished them from the arrays of radial fascin microspikes assembled by H9c2 cells adherent on TSP-1 (Figure 3, compare a and g). Cells

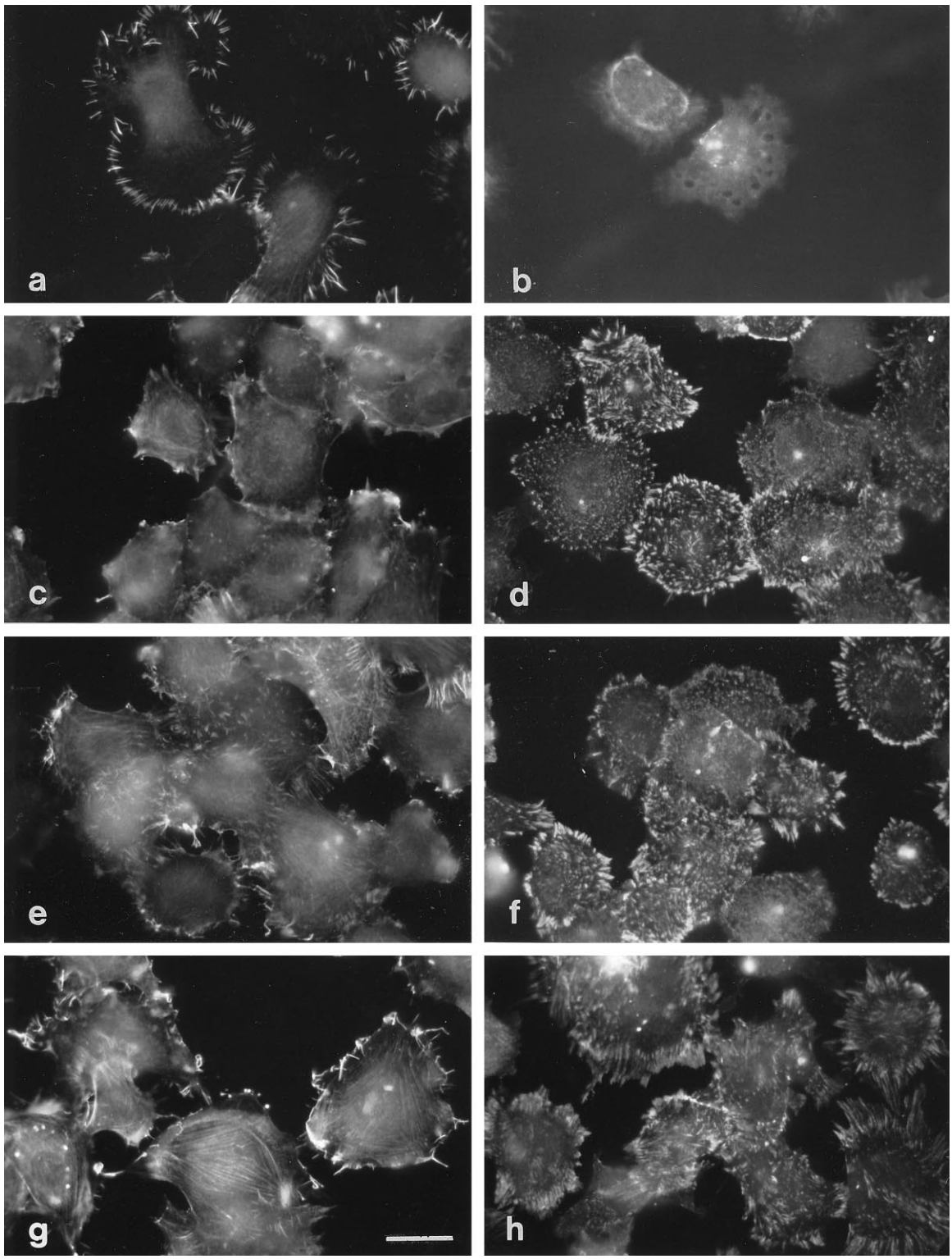

Figure 3. Formation of fascin microspikes and focal contacts by H9c2 cells plated on various ECM glycoproteins. H9c2 cells were stained for fascin (a, c, e, and g) or vinculin (b, d, f, and h) 1 h after plating on substrata coated with 50 nM TSP-1 (a and b), vitronectin (c and d), collagen IV (e and f), or EHS laminin (g and h). Bar, 12 μm.

adherent on laminin-1 also assembled vinculin-positive focal contacts (Figure 3h). Thus, the assembly of large arrays of radial fascin microspikes appears to be a unique response to a TSP-1 substratum, yet cortical fascin microspikes form concurrently with focal contacts when H9c2 cells adhere to laminin-1.

To determine whether cell adhesion via heparan sulfate proteoglycan-mediated mechanisms results in microspike formation, adhesion assays were carried out using the HepII heparin-binding domain of fibronectin or platelet factor 4. These substrata support cell adhesion through heparan sulfate proteoglycan-mediated interactions (Laterra *et al.*, 1983; Lark *et al.*, 1985; Izzard *et al.*, 1986). The heparin-binding domain of fibronectin did not support H9c2 cell attachment. H9c2 cell attachment to platelet factor 4 did not lead to cell spreading or formation of focal contacts or fascin microspikes (my unpublished observations). Thus, ligation of cell surface heparan sulfate proteoglycans is not a sufficient stimulus to trigger fascin microspike formation in H9c2 cells.

Fascin Microspikes and Focal Contacts Are Coassembled in Cells Adherent on Mixed TSP-1/Fibronectin Substrata

To examine the assembly of fascin microspikes and focal contacts in cells simultaneously exposed to different matrix components, H9c2 cells were plated for 60 min on substrata cocoated with different proportions of equimolar platelet TSP-1 and fibronectin and then stained for fascin or for vinculin. Cells adherent on 100% TSP-1 formed large arrays of fascin microspikes but did not assemble focal contacts (Figure 4, a and b). Cells adherent on 90% TSP-1:10% fibronectin also formed fascin microspikes but not focal contacts (Figure 4, c and d). A few cells displayed weak colocalization of fascin with actin microfilament bundles (Figure 4c, arrow). In response to 75% TSP-1:25% fibronectin, cells spread more extensively (Figure 4, compare e with a), fewer fascin microspikes were present at cell margins and fascin was readily apparent in association with microfilament bundles (Figure 4e). Vinculin staining localized in an arrowhead pattern, indicative of the formation of focal contacts, although the staining intensity was weak (Figure 4f). Increased cell spreading, decreased formation of microspikes, association of fascin with microfilament bundles, and formation of vinculin-containing focal contacts were also all apparent in cells adherent on a 50% TSP-1:50% fibronectin substratum (Figure 4, g and h). Some cells exhibited diffuse perinuclear vinculin staining (Figure 4h).

Formation of fascin microspikes was almost undetectable in cells adherent on 25% TSP-1:75% fibronectin, although some cells bore a few fascin-positive ruffles and isolated microspikes. Fascin was also less prominently associated with actin microfilament bundles than in the cells adherent on the 50% TSP-1:50% fibronectin mixture (Figure 4, compare j to g). Cells also exhibited perinuclear vinculin staining and many focal contacts (Figure 4k). In H9c2 cells adherent on 100% fibronectin, fascin staining was uniformly diffuse (Figure 4l), the staining intensity for vinculin in focal contacts was greater than that observed in cells adherent on any of the TSP-1/fibronectin mixed substrata (Figure 4, compare m with f, h, or l) and perinuclear vinculin staining was reduced (Figure 4, compare m with k and h).

Because the colocalization of fascin with microfilament bundles on mixed substrata was surprising, the same experiment was carried out with C2C12 cells, which also assemble actin/fascin microspikes when adherent on TSP-1 (Adams and Lawler, 1994; my unpublished observation). A similar shift from microspike-based to focal contact-based substratum adhesion was observed in C2C12 cells adherent on substrata containing at least 25% fibronectin. In addition, colocalization of fascin with microfilament bundles was apparent in cells adherent on substrata containing 25%, 50%, or 75% fibronectin (my unpublished observations). These results demonstrate that cells adherent on a mixed TSP-1/fibronectin substratum assemble fascin microspikes and focal contacts concomitantly and that adhesion to substrata containing between 25% and 50% fibronectin also stimulates association of fascin with microfilament bundles. The presence of either 25% fibronectin or 25% TSP-1 is sufficient to affect formation of the cell–substratum contact structure assembled in response to the other glycoprotein.

Cell Adhesion to Mixed TSP-1/Fibronectin Substrata Modulates Cytoskeletal Organization

To examine the effects of mixed substrata on the development of cytoskeletal organization in more depth, the distribution of a series of focal contact and cytoskeletal components was compared in H9c2 cells adherent on 100% fibronectin or on 50% TSP-1:50% fibronectin. In cells adherent on 100% fibronectin, β1 integrins were distributed in focal contacts and diffusely over the apical surfaces of cells (Figure 5a). β1 integrins were also present on long fine processes of varying thickness that extended from the margins of some cells (Figure 5a, arrow). On the basis of their apparently flexible and in some cases branching morphology, these processes appear to correspond to retraction fibers (Taylor and Robbins, 1963). In H9c2 cells adherent on the 50:50 mixed TSP-1/fibronectin substratum, punctate staining for β1 integrins was present over cell surfaces, was detectable on branched processes present at the margins of some cells, and was barely detectable in focal contacts (Figure 5b).

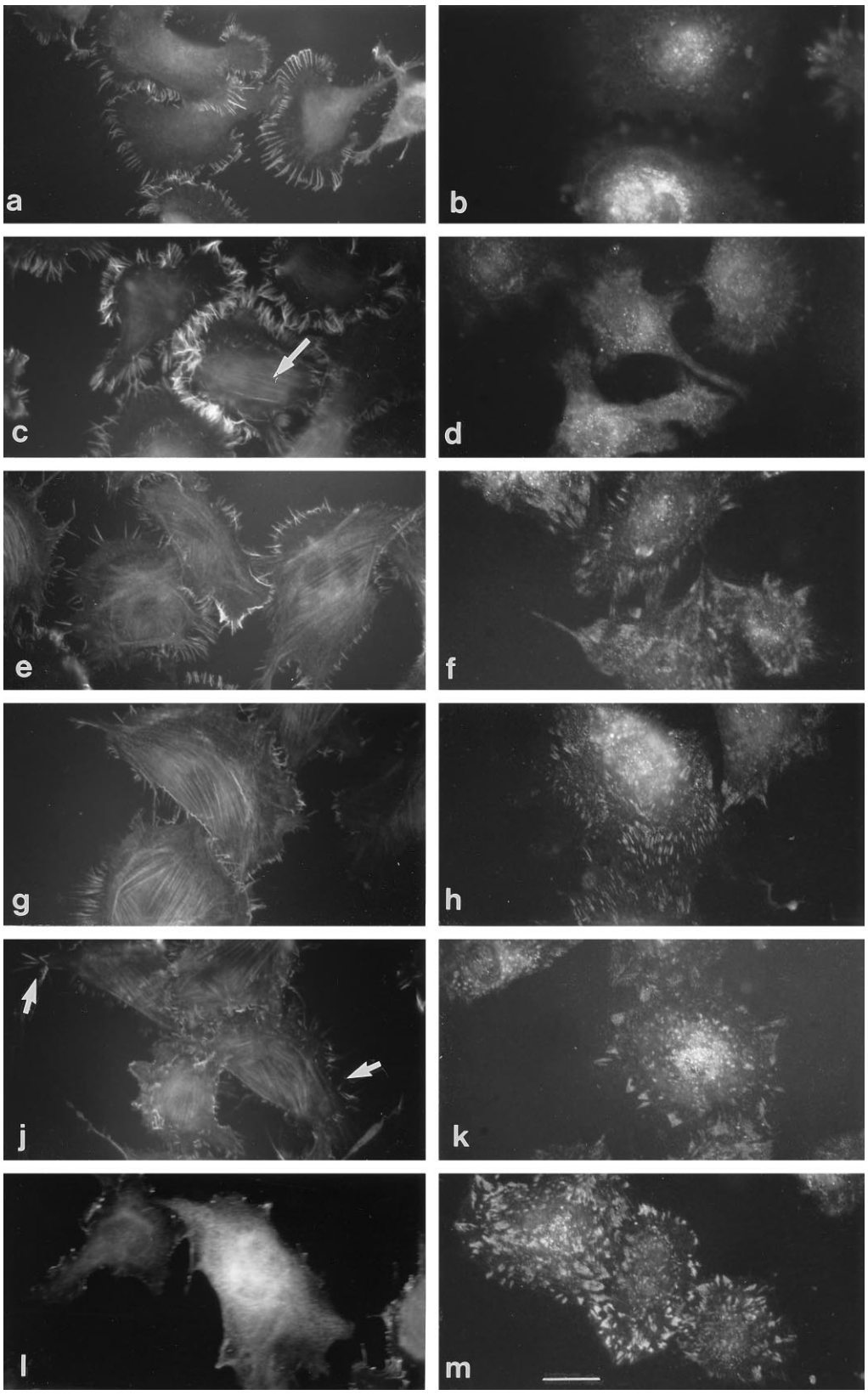

Figure 4.

Thus, one of the effects of the mixed substratum is to alter β1 integrin distribution.

Assembly of focal contacts was also examined by staining for paxillin and phosphotyrosine. Focal contacts assembled by cells adherent on 100% fibronectin stained strongly for paxillin (Figure 5c), whereas the staining intensity was much lower in the focal contacts of cells adherent on 50:50 TSP-1/fibronectin (Figure 5d). Intense phosphotyrosine staining is typically associated with focal contacts at cell peripheries or nascent contacts in which FAK is activated (Burridge *et al.*, 1992). The focal contacts of cells adherent on 100% fibronectin all stained for phosphotyrosine and as expected, the greatest intensity of staining was apparent in focal contacts at cell margins (Figure 5e). In contrast, focal contacts within the central regions of cells adherent on 50:50 TSP-1/fibronectin stained very weakly for phosphotyrosine, and phosphotyrosine-containing proteins were strongly concentrated in peripheral focal contacts (Figure 5f). Thus, exposure of cells to the mixed substratum affects the incorporation of both structural and regulatory components into focal contacts.

To analyze actin microfilament bundle organization, cells were stained for the actin-binding proteins tropomyosin and α-actinin, which are involved in actin microfilament organization within muscle sarcomeres and which characteristically associate with stress fiber bundles in stably adherent cultured cells (Lazarides, 1975; Lazarides and Burridge, 1975; reviewed by Pittenger *et al.*, 1994; Jockusch *et al.*, 1995). In H9c2 cells adherent on 100% fibronectin, tropomyosin localized to longitudinal and circumferential actin microfilament bundles (Figure 5g), whereas in H9c2 cells adherent on the 50:50 mixed substratum, tropomyosin staining of longitudinal stress fibers was of lower intensity (Figure 5, compare g and h) and tropomyosin in the cortical regions of these cells had a diffuse distribution. The zone of diffuse tropomyosin staining did not extend to the peripheral region of microspike formation (Figure 5h). α-Actinin distribution was punctate in cells adherent on 100% fibronectin and this pattern could be resolved as periodic staining along the length of microfilament bundles (Figure 5j, example shown with arrow). The central regions of cells adherent on the 50:50 mixed substratum also displayed a punctate distribution of α-actinin, but in the cortical regions α-actinin localized in elongated radial streaks that extended to the margins of the cells (Figure 5k). Thus, with the association of fascin with microfilament bundles (see Figure 4g), these results demonstrate that exposure to the mixed TSP-1/fibronectin substratum affects both the spatial organization and the biochemical composition of microfilament bundles.

Figure 4 (facing page). Formation of fascin microspikes and focal contacts by H9c2 cells adherent on mixed TSP-1/fibronectin substrata. H9c2 cells were stained for fascin (a, c, e, g, j, and l) or vinculin (b, d, f, h, k, and m) 1 h after plating on substrata consisting of 100% TSP-1 (a and b), 90% TSP-1:10% fibronectin (c and d), 75% TSP-1:25% fibronectin (e and f), 50% TSP-1:50% fibronectin (g and h), 25% TSP-1:75% fibronectin (j and k), or 100% fibronectin (l and m). A cell displaying weak colocalization of fascin with microfilaments is shown with an arrow in c. Residual isolated microspikes are shown with arrows in j. Bar, 10 μm.

The Roles of Integrins and Proteoglycans in Development of Cytoskeletal Organization on Mixed TSP-1/Fibronectin Substrata

Previous experiments have demonstrated that myoblast attachment to fibronectin is an RGD-dependent, α5β1 integrin-mediated process, whereas attachment to TSP-1 does not involve the RGD site of TSP-1 and is inhibitable by chondroitin sulfate A or β-D-xylopyranoside, suggestive of the involvement of proteoglycans (Enomoto *et al.*, 1993; Adams and Lawler, 1994). It was therefore of interest to test the contributions of β1 integrins or proteoglycans to cytoskeletal organization on mixed substrata. As expected, 1 mM GRGDSP peptide inhibited cell attachment to the 100% fibronectin substratum and did not affect cell adhesion to 100% TSP-1. GRGDSP peptide at 1 mM also had little effect on cell adhesion to a 50% TSP-1: 50% fibronectin mixed substratum, as determined by phase-contrast microscopy. Chondroitin sulfate A at 100 μg/ml prevented H9c2 cell attachment to TSP-1, did not affect cell adhesion to fibronectin, and caused cell rounding on the mixed substratum, although the number of attached cells was not significantly decreased (my unpublished observations; see also Figure 7).

Next, cells were treated for 20 h with 5 nM *p*-nitrophenol β-D-xylopyranoside, a competitive inhibitor of glycosaminoglycan side chain addition to protein cores, or 5 nM *o*-nitrophenol β-D-xylopyranoside, an inactive isomer (Schwartz, 1977), and tested for their ability to assemble focal contacts or microspikes. The active isomer decreases quantitative cell attachment to TSP-1 by 60% (Adams and Lawler, 1994), but residual adherent cells are still spread and so can be examined with respect to cytoskeletal organization. Treatment of cells with the inactive isomer did not affect formation of focal contacts or microspikes. In contrast, cells treated with *p*-nitrophenol β-D-xylopyranoside showed no alteration in focal contact assembly on fibronectin but exhibited a 94% reduction in microspike formation on TSP-1 (Figure 6). Focal contact formation by cells adherent on 50:50 mixed TSP-1/fibronectin was unaltered, yet microspike formation appeared decreased. However, microspike formation on the mixed substratum is much more irregular than on 100% TSP-1 (see Figure 4) and so this distinction

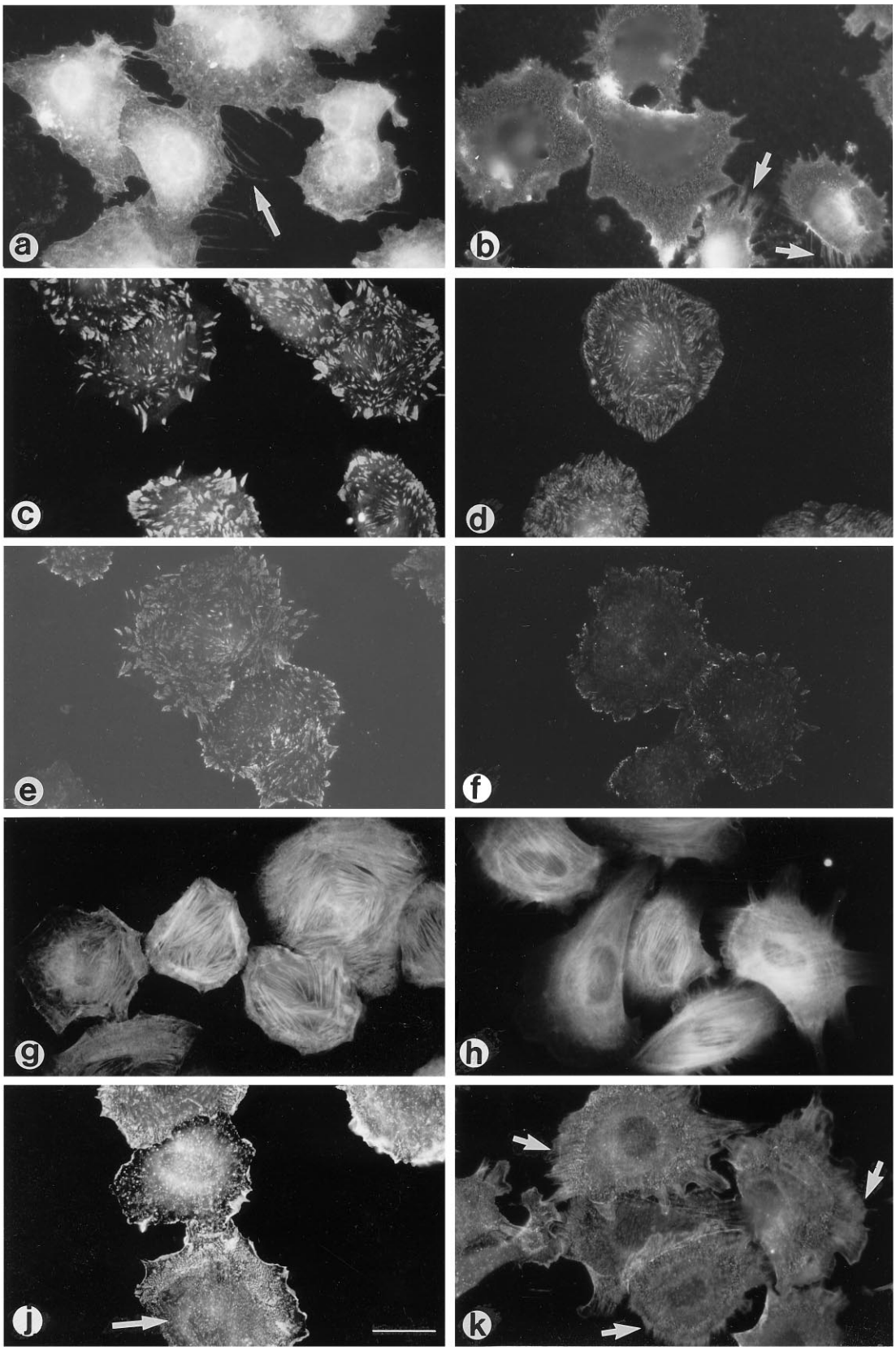

Figure 5.

was not amenable to quantification and was not pursued further.

To further examine the linkage between cell surface adhesion receptors and cytoskeletal organization, the effects of soluble GRGDSP peptide or chondroitin sulfate A on cytoskeletal organization in cells adherent on 50:50 TSP-1/fibronectin substrata were examined. Cells treated with 1 mM GRGDSP peptide spread and assembled irregular arrays of fascin microspikes. However, localization of fascin to microfilament bundles was markedly decreased (Figure 7, compare a and d). As expected, GRGDSP-treated cells displayed less organization of vinculin to focal contacts and increased diffuse cytoplasmic vinculin staining (Figure 7, compare b and e). Although fascin did not colocalize with microfilament bundles, association of tropomyosin with microfilament bundles in the central regions of cells was apparent. In comparison to control cells, increased diffuse tropomyosin staining was present along cell margins (Figure 7, compare c and f). Thus, perturbation of integrin-mediated adhesion by GRGDSP peptide affects both focal contact assembly and cytoskeletal organization on the mixed substratum.

Cells treated with 100 μg/ml chondroitin sulfate A during the attachment period spread poorly (Figure 7, compare a–c with g–j). Fascin appeared diffuse and did not colocalize with microfilament bundles. Fascin microspike formation was diminished but not abolished and the residual microspikes tended to be upraised rather than substratum adherent (Figure 7, compare a and g). Focal contact assembly was not reduced relative to untreated control cells (Figure 7, compare b and h). Localization of tropomyosin to stress fibers was not apparent and instead tropomyosin staining appeared uniformly diffuse throughout the cells (Figure 7, compare c and j). Thus, chondroitin sulfate A treatment affects both fascin microspike formation and cytoskeletal organization on the mixed substratum. Thus, these data implicate a combination of cell surface interactions involving both $\alpha5\beta1$ integrin and proteoglycans in the development of cytoskeletal organization in H9c2 cells adherent on a mixed TSP-1/fibronectin substratum.

Figure 5 (facing page). Effect of 50:50 mixed TSP-1/fibronectin substratum on cytoskeletal organization in H9c2 cells. H9c2 cells were stained for $\beta1$ integrins (a and b; apparent retraction fibers are shown with arrows), paxillin (c and d), phosphotyrosine (e and f), tropomyosin (g and h), or α-actinin (j and k) 1 h after plating on 100% fibronectin (a, c, e, g, and j) or 50% TSP-1:50% fibronectin (b, d, f, h, and k). The arrow in j indicates zone where periodic distribution of α-actinin along stress fibers is apparent; short arrows in k indicate cortical regions that show a different distribution of α-actinin on the mixed substratum. Bar, 10 μm.

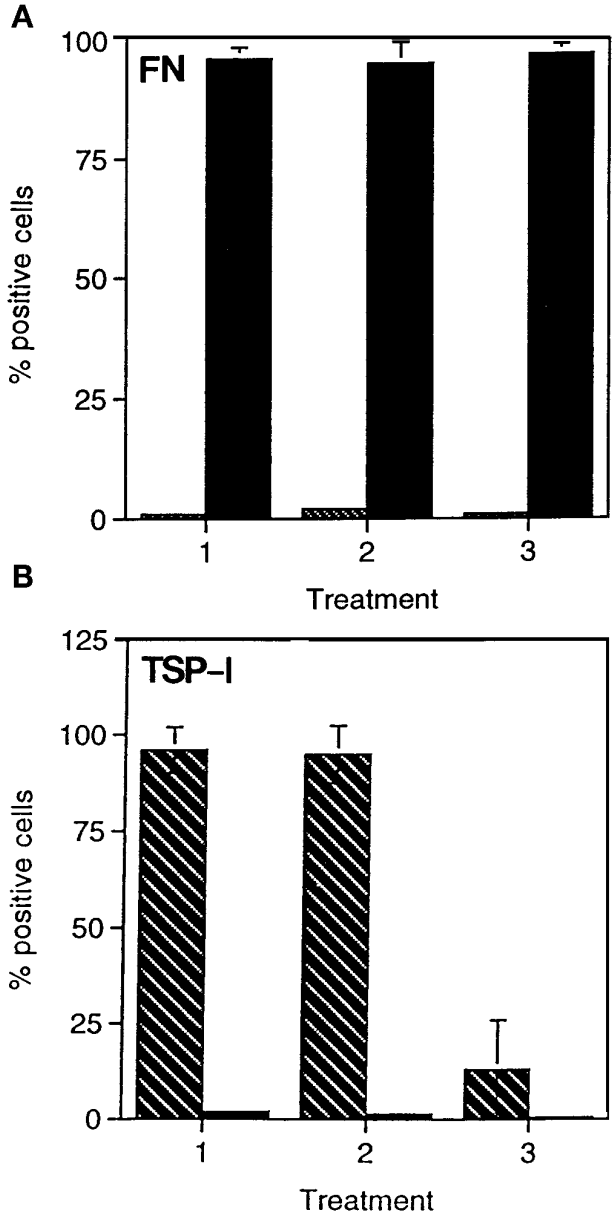

Figure 6. Effects of xylosides on assembly of focal contacts or microspikes. Control H9c2 cells (bar 1) or cells treated for 20 h with *o*-nitrophenol β-D-xylopyranoside (bar 2) or *p*-nitrophenol β-D-xylopyranoside (bar 3) were allowed to adhere for 1 h to substrata coated with 50 nM fibronectin (A) or 50nM TSP-1 (B) and then scored for formation of microspikes (hatched columns) or focal contacts (solid columns) by staining for fascin or vinculin, respectively. Each column is the mean of duplicate experiments. Error bars, SD.

Fascin Microspikes Are Present at the Protrusive Margins of Migratory and Postmitotic C2C12 Cells

The organization of filamentous actin (F-actin) into microspikes in the cortex of H9c2, C2C12, HISM, or A10 cells spread on a platelet TSP-1 substratum has

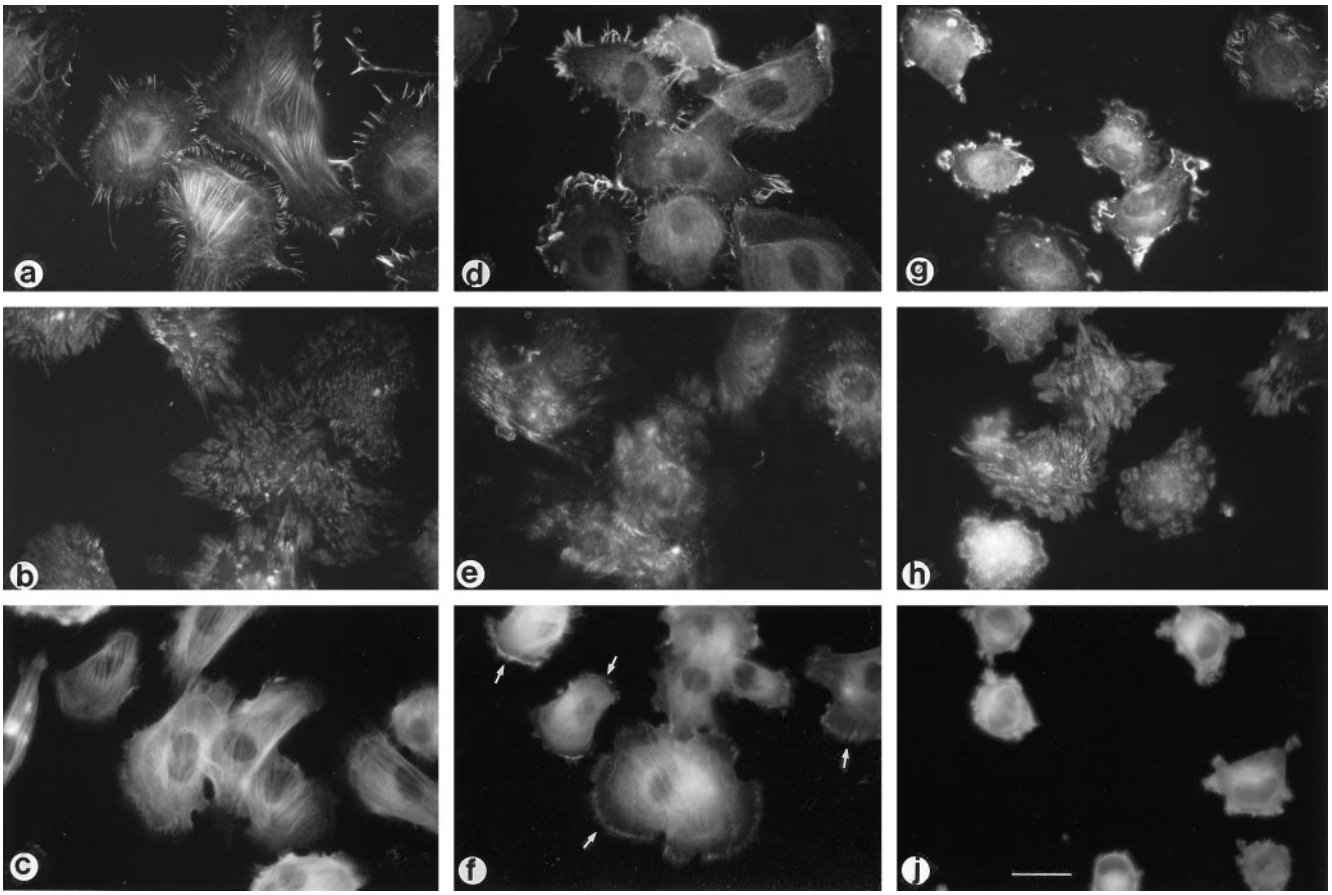

Figure 7. Role of integrins and proteoglycans in cytoskeletal organization of H9c2 cells adherent on 50% TSP-1:50% fibronectin substrata. H9c2 cells were allowed to adhere for 1 h to 50:50 TSP-1/fibronectin under control conditions (a–c) or in the presence of 1 mM GRGDSP peptide (d–f) or 100 μg/ml chondroitin sulfate A (g, h, j) and then stained for fascin (a, d, and g), vinculin (b, e, and h), or tropomyosin (c, f, and j). Arrows in f indicate tropomyosin localization at cell margins. Bar, 10 μm.

similarities with actin organization in the microspike-containing lamellipodium, which characterizes the leading edge of crawling cells (Abercrombie *et al.*, 1970a,b, 1971; Couchman and Rees, 1979; Izzard and Lochner, 1980; reviewed by Stossel, 1993). Thus it was of interest to examine the distribution of fascin microspikes and TSP in polarized cells. C2C12 skeletal myoblasts were chosen because they display the same mechanisms of attachment to TSP-1 as H9c2 cells (Adams and Lawler, 1994), undergo similar cytoskeletal responses (my unpublished observations), and are migratory in sparse culture. Polarized cells were examined within a random cell population (i.e., random with respect to their motile behavior and lack of synchronicity in cell cycle progression) that displayed a variety of morphologies, including polygonal well-spread cells, single isolated fan-shaped cells that typify polarized cells moving over a substratum (Couchman and Rees, 1979; Stossel, 1993), and rounded postmitotic pairs of cells.

Staining for F-actin and vinculin was carried out to provide context for the fascin staining data. Small isolated fan-shaped cells were characterized by a leading edge and well-spread rear part behind the nucleus. These cells stained diffusely with phalloidin within the cell body, exhibited an intensely staining peripheral zone of ruffles and microspikes at the margin of the lamellipodium, and occasionally displayed actin stress fiber bundles aligned with the rear margin of the cell (Figure 8, a and b). Typical vinculin-containing focal contacts were not apparent; instead, vinculin staining appeared diffuse or in small cytoplasmic spots (Figure 8c). In these cells, intensely staining fascin microspikes were present along the leading edges (Figure 8d, arrow). More elongated cells displayed diffuse fascin staining throughout the cytoplasm and intense staining of ruffles and microspikes at their anterior margin (Figure 8d).

A second type of fan-shaped cell had a larger spread area and displayed more pronounced polarity, with a

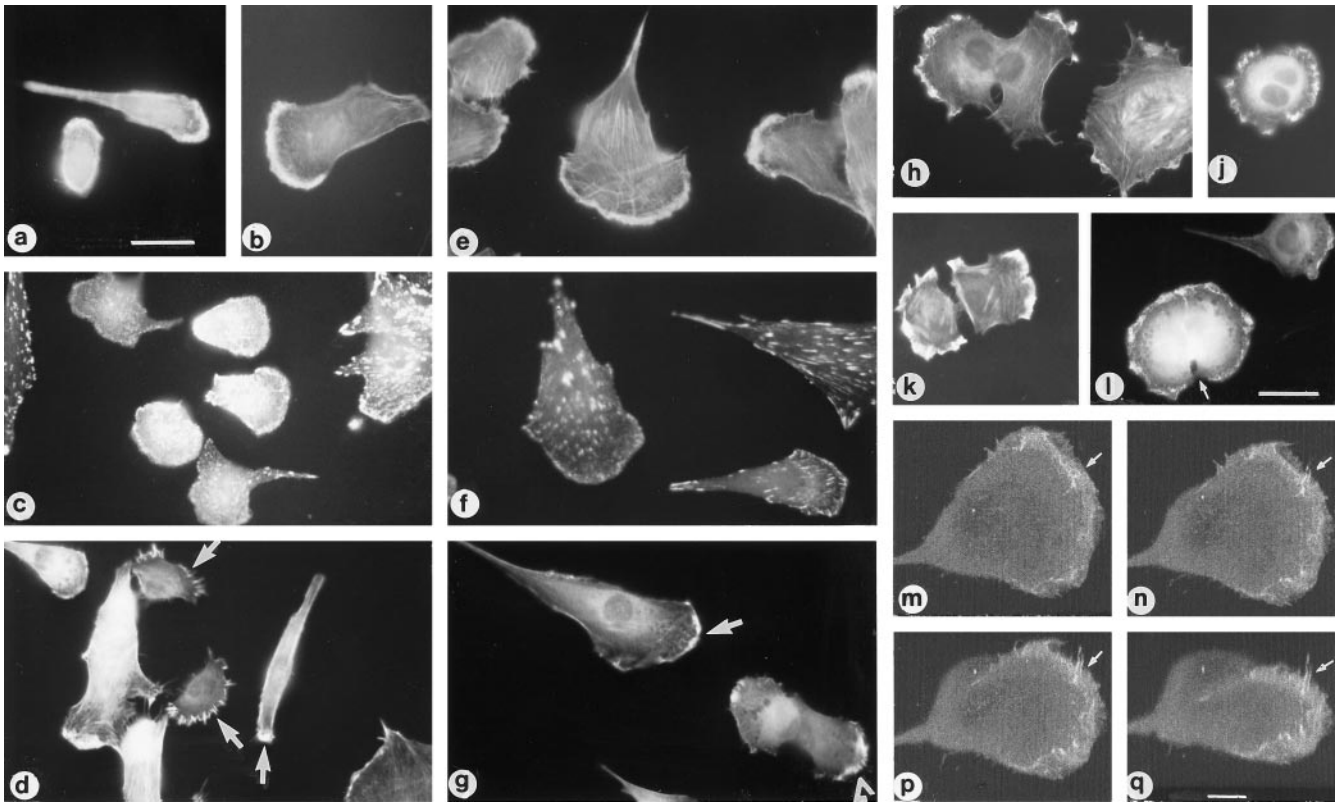

Figure 8. Presence of fascin-positive microspikes and ruffles at the protrusive margins of migratory and postmitotic C2C12 cells. Sparse cultures of C2C12 myoblasts were stained with rhodamine-phalloidin (a, b, and e), antibody to vinculin (c and f), or antibody to fascin (d, g, and h–q) and examples of small polarized cells (a, b, c, and d), larger fan-shaped cells (e, f, and g), or postmitotic pairs of cells (h–l) photographed under epifluorescence. Arrows in d and g indicate fascin microspikes and ruffles at leading edges; arrowhead in g indicates postmitotic pair of cells. (h) Asymmetric pair of cells nearing completion of cytokinesis which bear fascin microspikes and ruffles at their protrusive margins. (j and l) Examples of cells at the onset of cytokinesis: arrow in l indicates cleavage furrow. (k) Example of postmitotic pair stained 2 h after release from nocadazole block. Bars: a and l, 10um. (m–q) Series of projections from 3D reconstruction of confocal serial optical sections through one cell of a postmitotic pair stained for fascin. Projections were made at 180° (m), 210° (n), 230° (p), and 250° (q) to the original plane of section. Arrows indicate an upraised group of fascin microspikes displayed by the projections. Bar, 2.5 μm.

highly contracted trailing tail. In these cells, actin microfilament organization was complex and included microfilament bundles within the cell body and trailing tail and an elaborate mesh work of filaments within the leading lamellae, behind the F-actin-rich lamellipodium (Figure 8e). Large focal contacts were present at the rear end of the retracted trailing tail and the central region of such cells. As reported for chick heart fibroblasts (Couchman and Rees, 1979), spot-like focal contacts were present within the lamellar region and at the margins of lamellipodia (Figure 8f). In these cells fascin was diffusely distributed throughout the cell body and was also concentrated in ruffle or microspike structures at the anterior margin of the lamellipodium (Figure 8g).

Postmitotic cell pairs undergoing cytokinesis also exhibited regions of fascin-positive structures on their spreading margins. These took the form of smooth-edged fascin-positive ruffles (Figure 8g) or complex arrays of upraised fascin microspikes and ruffles (Figure 8h). Cells that had undergone nuclear division but that were in the early stages of cytokinesis exhibited near-circumferential arrays of fascin microspikes and ruffles (Figure 8, j and l). Although cells transiting mitosis are covered with apical villi (Erickson and Trinkhaus, 1976), no apical fascin-positive structures were observed. To enable more examples of postmitotic pairs to be examined, C2C12 cell cultures were treated with 0.1 μg/ml nocadazole for 6 h to arrest cells at an early phase of mitosis and then grown on in nocadazole-free medium for 2 h. In these cultures, postmitotic pairs of cells exhibited very large arrays of fascin-positive microspikes and ruffles on their outer spreading edges (Figure 8k).

Confocal microscopy was used to analyze the fascin microspikes in detail by taking serial optical sections through the outer margins of individual postmitotic cells and assembling 3D reconstructions that could be examined at different angles to the original plane of section. The projections revealed some microspikes in

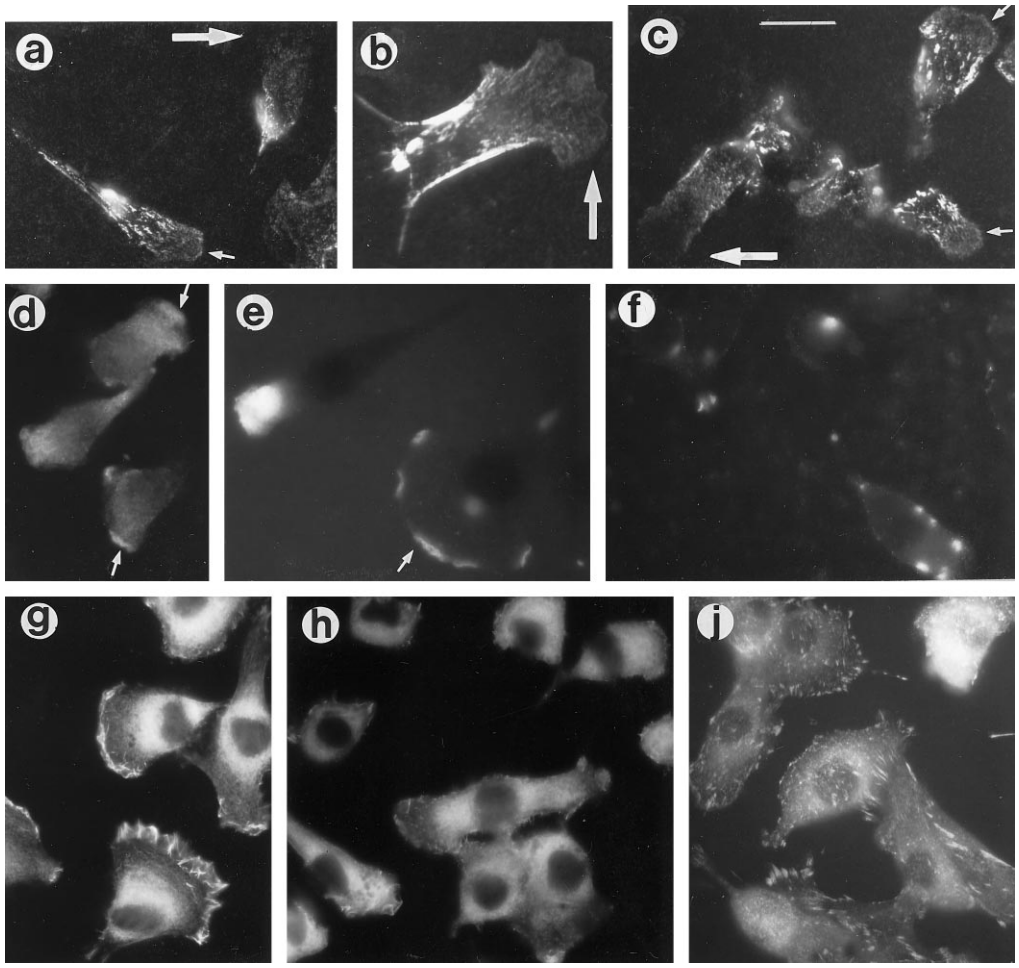

Figure 9. Deposition of extracellular TSP and development of the motile phenotype in sparse cultures of C2C12 cells. Cells from control cultures (a–e and g) or cultures treated with 500 μg/ml heparin for 6 h (f, h, and j) were stained for fibronectin (a–c), TSP (d–f), fascin (g and h), or vinculin (j). Large arrows in a–c indicate cells with no detectable fibronectin at the leading edge; small arrows in a and c indicate examples of cells with some deposition of fibronectin in the lamellar region. Arrows in d and e indicate concentrations of TSP staining at leading edges. Bar, 9 μm.

contact with the substratum and also complex arrays of upraised fascin microspikes and elaborate ruffle-like structures (Figure 8, m–q). Thus, fascin microspikes and ruffles are located at sites of membrane protrusion in polarized C2C12 cells.

Matrix TSP Is Present around the Lamellar Region of Migratory C2C12 Cells and Plays a Role in Microspike Formation and Cell Motility

Given that fascin microspikes are induced in the experimental situation of cell adhesion to a platelet TSP-1 substratum, are not induced upon cell adhesion to fibronectin, vitronectin, or collagen IV, and are found at the protrusive leading edge of polarized cells, an obvious question was to determine whether sites of matrix deposition of TSP correlate with sites of microspike formation in polarized cells. Therefore, the low-density random cultures of C2C12 cells were stained for fibronectin or TSP-1 and examined by epifluorescence or confocal microscopy.

As previously reported for other cell types (reviewed by Hynes, 1990), fibrillar fibronectin was concentrated at the central and rear portions of polarized cells, particularly around the trailing tail (Figure 9, a–c). In general, fibronectin was absent from the protrusive, lamellar region (Figure 9, a and b, examples shown by arrows), although small deposits were present at the tip of the lamellipodium in some cells (Figure 9, a and c, examples indicated with small arrows). From a total of 300 polarized cells scored in three separate preparations, 82% exhibited fibronectin deposition in association with the central and rear

portions of the cells and 18% displayed small fibronectin deposits at their anterior margin. Confocal microscopy was used to confirm that fibronectin was indeed absent from the anterior region of most polarized cells (my unpublished observation).

Cell-associated TSP localized in granular patches. These patches were concentrated around the lamellar region and appeared diminished or absent from the posterior regions of polarized cells (Figure 9, d and e). This staining pattern was observed with two antisera to TSP-1 and was abolished if the antisera were preincubated with platelet TSP-1 (my unpublished observation). Of a total of 300 polarized cells examined in three separate preparations, 68% displayed such concentrations of TSP around the anterior lamellar region: the remaining 32% displayed TSP staining around the entire length of the cells. In the epifluorescence images, matrix TSP appeared to be present below and around zones of microspikes and ruffle formation at the leading edge: this distribution was confirmed by use of confocal microscopy (my unpublished observation).

To determine whether polarized deposition of matrix TSP is required in the development of fascin microspikes and the motile phenotype, low-density C2C12 cultures were treated with soluble heparin to inhibit the incorporation of newly synthesized TSP-1 into matrix (McKeown-Longo *et al.*, 1984; Majack *et al.*, 1985). After 6 h, the cultures were scored for the percentage of polarized fan-shaped cells using phase-contrast microscopy. Of a total of 1300 cells scored in triplicate assays, 25% (±4.4%, SD) of cells in control cultures displayed a polarized morphology, whereas only 13% (±3.1%, SD) of cells appeared polarized in the heparin-treated cultures, indicating a 52% decrease in the number of polarized cells (difference significant at $p < 0.001$). As expected, the heparin-treated cells displayed very little cell-associated TSP (Figure 9f), although fibronectin fibrils were still assembled (McKeown-Longo *et al.*, 1984; Majack *et al.*, 1985; my unpublished observation). In the heparin-treated cultures, fascin microspikes and ruffles were reduced compared with polarized cells in untreated cultures and fascin appeared diffuse (Figure 9, g and h). Cells from the heparin-treated cultures also tended to display nonpolarized distributions of vinculin-containing focal contacts (Figure 9j compared with Figure 8e). Thus, deposition of matrix thombospondin is requisite in the formation of fascin microspikes.

To test the effects of inhibition of TSP deposition and fascin microspike formation on cell migratory behavior, control and heparin-treated cultures of C2C12 cells were analyzed by time-lapse videomicroscopy. The mean velocity of cell movement in control cells was 48.4 ± 9.4 μm/h. In heparin-treated cultures, the mean velocity was 27 ± 10.9 μm/h, a statistically significant difference at $p < 0.001$. In addition, whereas control cells maintained the same leading edge throughout, 28% of the cells traced after heparin treatment were not morphologically polarized and moved sequentially in more than one direction, by virtue of membrane protrusions arising at different points on the cell surface. Thus, inhibition of matrix TSP deposition correlates with alterations in C2C12 cell morphology, decreased microspike formation, and reduced cell motility.

DISCUSSION

In this article, I describe experiments that analyze factors required in the formation of fascin microspikes by 1) investigating the correlation of fascin expression, cell spreading, and microspike formation on a platelet TSP-1 substratum; 2) testing the ability of various adhesive substrata to stimulate formation of fascin microspikes; 3) investigating the interrelationship of microspike and focal contact formation and cytoskeletal organization on mixed substrata and the roles of integrins and proteoglycans in these processes; 4) examining the distributions of fascin, TSP, and fibronectin in polarized cells and the role of TSP in cell motile behavior. The novel results obtained establish that assembly of fascin microspikes or focal contacts can be experimentally manipulated by exposure of cells to different adhesive substrata and that adhesion to mixed substrata affects actin cytoskeletal organization and also demonstrate TSP-dependent aspects of the motile phenotype.

Fascin is a structurally unique actin-bundling protein (reviewed by Matsudaira, 1994; Edwards and Bryan, 1995) that is expressed in a limited set of adult human tissues and in many tissues of adult *Xenopus* or mice (Holthuis *et al.*, 1994; Mosialos *et al.*, 1994; Edwards *et al.*, 1995). Its expression has previously been demonstrated in epithelial, mesenchymal, and Epstein-Barr virus-transformed lymphoblastoid cell lines (Yamashiro-Matsumura and Matsumura, 1986; Mosialos *et al.*, 1994). In this study, fascin was detected in a variety of normal and transformed cell types of several different tissue origins. Because all cell types capable of spreading on platelet TSP-1 were found to express fascin and to form microspikes, the data obtained support the speculation that fascin may be required for cell spreading and microspike formation on a TSP-1 substratum. However, because the two nonspreader cell lines C32 and HT1080 also expressed fascin, fascin cannot suffice as the sole intracellular determinant of spreading ability. Cell speading on TSP-1 likely requires the coexpression of additional structural proteins or appropriate regulatory proteins. Indeed, the complex distribution pattern of fascin in cells cultured in serum-containing medium (Yamashiro-Matsumura and Matsumura, 1986), its lack of

colocalization with the actin-based cytoskeleton in cells adherent on fibronectin (Adams, 1995) or vitronectin, and its localization to multiple actin-containing structures in cells adherent on laminin-1 or a mixed TSP-1/fibronectin substratum (this study) all suggest that fascin is under dynamic intracellular regulation.

Whereas cells respond to TSP-1 by forming large arrays of fascin microspikes, the β1- or αv-subclass integrin ligands fibronectin, collagen IV, and vitronectin trigger assembly of focal contacts. Cell adhesion to laminin-1, also mediated by β1 integrins (Hynes, 1992), results in a third type of response in which both focal contacts and individual upraised cortical fascin microspikes are formed and colocalization of fascin with microfilaments is also observed. These three types of responses presumably arise as a consequence of ligation of different adhesive receptors during initial cell attachment, leading to the recruitment of different cytoskeletal and regulatory components at the plasma membrane. The intracellular signals activated by cell attachment and spreading on TSP-1 substrata are not well characterized. Tyrosine kinases or phosphatases may be involved, because fascin microspike formation is modulated by alterations in intracellular phosphotyrosine levels (Adams, 1995). Heterotrimeric G proteins have been implicated in the chemotactic or haptotactic activities of TSP-1 (Mansfield and Suchard, 1994; Gao *et al.*, 1996; Suchard and Mansfield, 1996). Intracellular events of general importance in regulating actin microfilament organization and focal contact assembly include the activation of protein kinase C, phosphatidylinositol 3-kinase, or small guanine nucleotide binding proteins such as Rac, Rho, and CDC42 (reviewed by Hall, 1994; Chant and Stowers, 1995; Burridge and Chrzanowska-Wodnicka, 1996).

The different morphologies of the fascin microspikes formed upon adhesion to TSP-1 or to laminin-1 might result from either biophysical or biochemical factors. It is possible that the microspikes formed by H9c2 cells adherent on laminin-1 cannot contact the substratum because the predominance of focal contacts physically limits the formation of other types of apposition. On a biochemical level, the simultaneous formation of fascin microspikes and focal contacts might involve competition for limiting amounts of a common structural or regulatory component, so that assembly of many focal contacts would limit the number of microspikes formed. Alternatively or additionally, the microspikes formed in response to TSP-1 or to laminin-1 may have different physical properties because of a difference in biochemical composition. To date, components in common with focal contacts have not been identified in fascin microspikes assembled in response to TSP-1 under standard adhesion assay conditions (Adams, 1995; my unpublished observations); however, the possibility that such components exist cannot be excluded.

The experiments using mixed TSP-1/fibronectin substrata demonstrated that increasing ligation of β_1 integrins correlated with decreased microspike assembly and increased formation of focal contacts. In addition, modulation of either integrin or proteoglycan/glycosaminoglycan-based adhesions on the mixed substratum had effects on stress fiber assembly, suggestive of the existence of a complex interplay of intracellular processes governing the development of these supramolecular structures. A particularly interesting effect of the mixed substratum was the localization of fascin to actin microfilament bundles in cells adherent on substrata containing 25% or 50% fibronectin. Thus, as also suggested by the experiments using individual ECM components, there appear to be three compartments for fascin in cells: microspikes, microfilament-associated, and a diffuse pool. Alterations in cell–matrix adhesive interactions change the distribution of fascin between these compartments, again implicating signal inception at the plasma membrane in this process. Relocation of fascin has been observed in cells growing in serum-containing medium upon treatment with phorbol 12-myristate 13-acetate and correlated with changes in the phosphorylation status of fascin (Yamakita *et al.*, 1996). It will be interesting to examine whether cell adhesion to different ECM components also alters the phosphorylation state of fascin.

Stress fibers are required in the generation of tractional forces between a cell and its substratum. There is much evidence that the development of isometric tension plays a role in stress fiber assembly (reviewed by Burridge and Chrzanowska-Wodnicka, 1996) and the intrinsic contractility of different cell types correlates inversely with their migratory behavior (see for example, Tucker *et al.*, 1985). Thus, the formation of stress fibers by cells adherent on mixed TSP-1/fibronectin substrata can be taken to indicate the development of contractility. However, the stress fibers assembled on 50% TSP-1:50% fibronectin differed biochemically from those assembled in response to 100% fibronectin in that they contained both fascin and tropomyosin. They thus resemble the stress fibers of cells cultured in serum-containing medium (Yamashiro-Matsumura and Matsumura, 1986). In vitro, fascin and tropomyosin display cross-inhibition of actin-binding activity and certain tropomyosin isoforms also inhibit actin bundling by fascin (Yamashiro-Matsumura and Matsumura, 1985; Matsumura and Yamashiro-Matsumura, 1986). The effect of fascin association on the generation of mechanical tension by stress fibers has not been examined, but the differences in microfilament bundle organization demonstrated herein raise the possibility that cell adhesion to different matrix components affects on the functional properties of the actin cytoskeleton. This would be of interest in the context of mechanical coordination of

cellular function and behavior, as proposed by the tensegrity model (Ingber, 1993a,b).

The presence of microspikes at the protrusive edges of cultured cells and the possible duel role of such structures in sensory exploration and selective substratum adhesion has been appreciated since these structures were first studied morphologically by phase-contrast and electron microscopy (Taylor and Robbins, 1963). Regions of fascin microspikes and ruffles are present on nonpolarized cells (Yamashiro-Matsumura and Matsumura, 1986) and when this article was in preparation, localization of fascin at the leading edge of A431 cells and endothelial cells was reported (Tao *et al.*, 1996) and also the presence of fascin microspikes at the spreading margins of B28NG2.6 glioma cells (Lin *et al.*, 1996).

In this analysis, I have demonstrated that the microspikes and ruffles present at the protrusive leading edges of polarized migratory and postmitotic C2C12 cells also include fascin-containing structures. Disruption of TSP matrix deposition reduces both microspike formation and cell motility. Current models of actin-based cell movement invoke membrane protrusion and substratum adhesion as the first steps in a cycle of events that result in net forward migration (reviewed by Huttenlocher *et al.*, 1995; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). The experimental findings described herein offer a basis for further analyses of the molecular processes by which ECM components regulate assembly of fascin microspikes, the biochemical interplay of fascin microspikes, focal contacts, and cytoskeletal organization, and the role of microspikes in cell motile behavior.

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REFERENCES

Abercrombie, M., Heaysman, J.E.M., and Pegrum, S.M. (1970a). The locomotion of fibroblasts in culture I. Movements of the leading edge. Exp. Cell Res. *59*, 393–398.

Abercrombie, M., Heaysman, J.E.M., and Pegrum, S.M. (1970b). The locomotion of fibroblasts in culture II. "Ruffling." Exp. Cell Res. *60*, 437–444.

Abercrombie, M., Heaysman, J.E.M., and Pegrum, S.M. (1971). The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. Exp. Cell Res. *67*, 359- 367.

Adams, J.C. (1995). Formation of stable microspikes containing actin and the 55kDa actin-bundling protein, fascin, is a consequence of cell adhesion to thrombospondin-1: implications for the anti-adhesive activities of thrombospondin-1. J. Cell Sci. *108*, 1977–1990.

Adams, J.C., and Lawler, J. (1993). Diverse mechanisms for cell attachment to platelet thrombospondin. J. Cell Sci. *104*, 1061–1071.

Adams, J.C., and Lawler, J. (1994). Cell-type specific adhesive interactions of skeletal myoblasts with thrombospondin-1. Mol. Biol. Cell *5*, 423–437.

Adams, J.C., Tucker, R.P., and Lawler, J. (1995). The Thrombospondin Gene Family, Austin, TX: R.G. Landes.

Arregui, D.O., Carbonetto, S., and L. McKerracher, L. (1994). Characterization of neural cell adhesion sites: point contacts are the sites of interaction between integrins and the cytoskeleton in PC12 cells. J. Neurosci. *14*, 6967–6977.

Asch, A.S., Tepler, J., Silbiger, S., and Nachman, R.L. (1991). Cellular attachment to thrombospondin: cooperative interactions between receptor systems. J. Biol. Chem. *266*, 1740–1745.

Blau, H.M., Pavlath, G.K., Hardeman, E.C., Chiu, C-P., Silberstein, L., Webster, S.G., Miller, S.C., and Webster, C. (1985). Plasticity of the differentiated state. Science *230*, 758–766.

Bornstein, P. (1995). Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin-1. J. Cell Biol. *130*, 503–506.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility and signaling. Annu. Rev. Cell Dev. Biol. *12*, 463–519.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. *4*, 487–525.

Burridge, K., Turner, C.E., and Romer, L.H. (1992). Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J. Cell Biol. *119*, 893–903.

Chant, J., and Stowers, L. (1995). GTPase cascades choreographing cellular behavior: movement, morphogenesis and more. Cell *81*, 1–4.

Chen, H., Sottile, J., O'Rourke, K., Dixit, V., and Mosher, D.F. (1994). Properties of recombinant mouse thombospondin-2 expressed in *Spodoptera* cells. J. Biol. Chem. *269*, 32226–32232.

Christian, C.N., Nelson, P.G., Peacock, J., and Nirenberg, M. (1977). Synapse formation between two clonal cell lines. Science *191*, 995–998.

Clezardin, P., Jouishomme, H., Chavassieux, P., and Marie, P.J. (1989). Thrombospondin is secreted and synthesised by human osteoblasts and osteosarcoma cells. Eur. J. Biochem. *181*, 721–726.

Coopman, P.J., Thomas, D.M., Gehlsen, K.R., and Mueller, S.C. (1996). Integrin $\alpha 3\beta 1$ participates in the phagocytosis of extracellular matrix molecules by human breast cancer cells. Mol. Biol. Cell *7*, 1789–1804.

Couchman, J.R., and Rees, D.A. (1979). The behavior of fibroblasts migrating from chick heart explants: changes in adhesion, locomotion and growth, and in the distribution of actomysin and fibronectin. J. Cell Sci. *39*, 149–165.

Edwards, R.A., and Bryan, J. (1995). Fascins, a family of actin bundling proteins. Cell Motil. Cytoskeleton *32*, 1–9.

Edwards, R.A., Herrera-Sosa, H., Otto, J., and Bryan, J. (1995). Cloning and expression of a murine fascin homolog from mouse brain. J. Biol. Chem. *270*, 10764–10770.

Enomoto, M.I., Boettiger, D., and Menko, A.S. (1993). $\alpha 5$ integrin is a critical component of adhesion plaques in myogenesis. Dev. Biol. *155*, 180–197.

Erickson, C.A., and Trinkhaus, J.P. (1976). Microvilli and blebs as source of reserve surface membrane during cell spreading. Exp. Cell Res. *99*, 375–384.

Frazier, W.A. (1991). Thrombospondins. Curr. Opin. Cell Biol. *3*, 792–799.

Gao, A-G., Lindberg, F.P., Dimitry, J.M., Brown, E.J., and Frazier, W.A. (1996). Thrombospondin modulates $\alpha_v\beta_3$ function through integrin-associated protein. J. Cell Biol. *135*, 533–544.

Geiger, B. (1979). A 130K protein from chick gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. Cell *18*, 193–205.

Gluzman, Y. (1981). SV-40 transformed simian cells support the replication of early SV40 mutants. Cell *23*, 175–182.

Goldman, W.H., Schindl, M., Cardozo, T.J., and Ezzell, R.M. (1995). Motility of vinculin-deficient F9 embryonic carcinoma cells analyzed by video, lasar confocal, and reflection interference contrast microscopy. Exp. Cell Res. *221*, 311–319.

Grinnell, F. (1978). Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol. *53*, 65–144.

Gumbiner, B.M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell *84*, 345–357.

Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu. Rev. Cell Biol. *10*, 31–54.

Heath, J.P., and Dunn, G.A. (1978). Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high voltage electron-microscope study. J. Cell Sci. *29*, 197–212.

Heath, J.P., and Holifield, B. (1991). Cell locomotion: new research tests old ideas on membrane and cytoskeletal flow. Cell Motil. Cytoskeleton *18*, 245–257.

Holthuis, J.C.M., Schoonderwoert, V.T.G., and Martens, G.J.M (1994). A vertebrate homolog of the actin-bundling protein fascin. Biochim. Biophys. Acta *1219*, 184–188.

Huttenlocher, A., Sandborg, R.R., and Horwitz, A.F. (1995). Adhesion in cell migration. Curr. Opin. Cell Biol. *7*, 697–706.

Hynes, R.O. (1990). Fibronectins, Berlin: Springer-Verlag.

Hynes, R.O. (1992). Integrins: versatility, modulation and signalling in cell adhesion. Cell *69*, 11–25.

Hynes, R.O., and Lander, A.D. (1992). Contact and adhesive specificities in the associations, migrations and targeting of cells and axons. Cell *68*, 303–322.

Ingber, D.E. (1993a). Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. J. Cell Sci. *104*, 613–627.

Ingber, D.E. (1993b). The riddle of morphogenesis: a question of solution chemistry or molecular cell engineering? Cell *75*, 1249–1252.

Izzard, C.S., and Lochner, L.R. (1976). Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. J. Cell Sci. *21*, 129–159.

Izzard, C.S., and Lochner, L.R. (1980). Formation of cell-to-substrate contacts during fibroblast motility: an interference reflection study. J. Cell Sci. *42*, 81–116.

Izzard, C.S., Radinsky, R., and Culp, L.C. (1986). Substratum contacts and cytoskeletal reorganization of BALB/c 3T3 cells on a cell-binding fragment and heparin-binding fragments of plasma fibronectin. Exp. Cell Res. *165*, 320–336.

Jockusch, B.M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegal, M., Ridiger, M., Schluter, K., Stanke G., and Winkler, J. (1995). The molecular architecture of focal adhesions. Annu. Rev. Cell Dev. Biol. *11*, 379–416.

Juliano, R.L., and Haskill, S. (1993). Signal transduction from the extracellular matrix. J. Cell Biol. *120*, 577–585.

Kimes, B.W., and Brandt, B.L. (1976a). Characterization of two putative smooth muscle cell lines from rat thoracic aorta. Exp. Cell Res. *98*, 349–366.

Kimes, B.W., and Brandt, B.L. (1976b). Properties of a clonal muscle cell line from rat heart. Exp. Cell Res. *98*, 367–381.

Kolega, J., Shure, M.S., Chen, W., and Young, M.D. (1982). Rapid cellular translocation is related to close contacts formed between various cultured cells and their substrata. J. Cell Sci. *54*, 23–34.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lahav, J. (1993). Thrombospondin, Boca Raton, FL: CRC Press.

Lark, M.W., Laterra, J., and Culp, L.A. (1985). Close and focal contact adhesions of fibroblasts to a fibronectin-containing matrix. Fed. Proc. *44*, 394–403.

Laterra, J., Silbert, J.E., and Culp, L.A. (1983). Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices including fibronectin. J. Cell Biol. *96*, 112 -123.

Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. Cell *84*, 359–369.

Lawler, J., Weinstein, R., and Hynes, R.O. (1988). Cell attachment to thrombospondin: the role of Arg-Gly-Asp, calcium and integrin receptors. J. Cell Biol. *107*, 2351–2361.

Lazarides, E. (1975). Tropomyosin antibody: the specific localization of tropomyosin in nonmuscle cells. J. Cell Biol. *65*, 549–561.

Lazarides, E., and Burridge, K. (1975). α-Actinin: immunofluorescent localization of a muscle structural protein in non-muscle cells. Cell *6*, 289–298.

Lin, X.-H., Grako, K.A., Burg, M.A., and Stallcup, W.B. (1996). NG2 proteoglycan and the actin-binding protein fascin define separate populations of actin-containing filopodia and lammellipodia during cell spreading and migration. Mol. Biol. Cell *7*, 1977–1993.

Majack, R.A., Coates Cook, S., and Bornstein, P. (1985). Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. J. Cell Biol. *101*, 1059–1070.

Mansfield, P.J., and Suchard, S.J. (1994). Thrombospondin promotes chemotaxis and haptotaxis of peripheral blood monocytes. J. Immunol. *153*, 4219–4229.

Matsudaira, P. (1994). Actin crosslinking proteins at the leading edge. Semin. Cell Biol. *5*, 165–174.

Matsumura, F., and Yamashiro-Matsumura, S. (1986). Modulation of actin-bundling activity of 55kDa protein by multiple isoforms of tropomyosin. J. Biol. Chem. *261*, 4655–4659.

McKeown-Longo, P.J., Hanning, R., and Mosher, D.F. (1984). Binding and degradation of platelet thrombospondin by cultured fibroblasts. J. Cell Biol. *98*, 22–28.

Mitchison, T.J., and Cramer, L.P. (1996). Actin-based cell motility and cell locomotion. Cell *84*, 371–379.

Mosialos, G., Yamashiro, S., Baughman, R.W., Matsudaira, P., Vara, L., Matsumura, F., Kieff, E., and Birkenbach, M. (1994). Epstein-Barr virus infection induces expression in B lymphocytes of a novel gene encoding an evolutionarily conserved 55kDa actin-bundling protein. J. Virol. *68*, 7320–7328.

Mueller, S.C., and Chen, W-T. (1991). Cellular invasion into matrix beads: localization of β_1 integrins and fibronectin to the invapodia. J. Cell Sci. *99*, 213–225.

Mueller, S.C., Kelly, T., Dai, M., Dai, H., and Chen, W.T. (1989). Dynamic cytoskeleton-integrin associations induced by cell binding to immobilized fibronectin. J. Cell Biol. *109*, 3455–3464.

Mueller, S.C., Yeh, Y., and Chen, W-T. (1992). Tyrosine phosphorylation of membrane proteins mediates cellular invasion by transformed cells. J. Cell Biol. *119*, 1309–1325.

O'Neill, C., Jordan, P., Riddle P., and Ireland, G. (1990). Narrow linear strips of adhesive substratum are powerful inducers of both growth and total focal contact area. J. Cell Sci. *95*, 577–586.

Pittenger, M.F., Kazzaz, J.A., and Helfman, D.M. (1994). Functional properties of non-muscle tropomyosin isoforms. Curr. Opin. Cell Biol. *6*, 96–104.

Prater, C.A., Plotkin, J., Jaye, D., and Frazier, W.A. (1991). The properdin-like type 1 repeats of human thrombospondin contain a cell attachment site. J. Cell Biol. *112*, 1031–1040.

Rasheed, S., Nellson-Rees, W.A., Toth, E.M., Arnstein, P., and Garcher, M.B. (1974). Characterization of a newly derived human sarcoma cell line. Cancer *33*, 1027–1033.

Roberts, D.D., Sherwood, J.A., and Ginsberg, V. (1987). Platelet thrombospondin mediates attachment and spreading of human melanoma cells. J. Cell Biol. *104*, 131–139.

Samuels, M., Ezzell, R.M., Cardozo, T.J., Critchley, D.R., Coll, J.-L., and Adams, E.D. (1993). Expression of chick vinculin complements the adhesive-defective phenotype of a mutant mouse F9 embryonal carcinoma cell line. J. Cell Biol. *121*, 909–921.

Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. (1995). Integrins: emerging paradigms of signal transduction. Annu. Rev. Cell Dev. Biol. *11*, 549–599.

Schwartz, N.B. (1977). Regulation of chondroitin sulfate synthesis: effect of β-xylosides on synthesis of chondroitin sulfate proteoglycan, chondroitin sulfate chains, and core protein. J. Biol. Chem. *252*, 6316–6321.

Singer, I.I., Scott, S., Kawka, D.W., Kazazis, D.M., Gailit, J., and Ruoslahti, E. (1988). Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. J. Cell Biol. *106*, 2171–2182.

Spengler, B.A., Biedler, J.L., Helson, L., and Freedman, L.S. (1973). Morphology and growth, tumorigenicity and cytogenetics of human neuroblastoma cell lines established in vitro. In Vitro *8*, 410.

Stossel, T.P. (1993). On the crawling of animal cells. Science *260*, 1086–1093.

Streeter, H.B., and Rees, D.A. (1987). Fibroblast adhesion to RGDS shows novel features compared with fibronectin. J. Cell Biol. *105*, 507–515.

Suchard, S.J., and Mansfield, P.J. (1996). Neutrophil thrombospondin receptors are linked to GTP-binding proteins. J. Cell. Physiol. *168*, 217–227.

Sydor, A.M., Su, A.L., Wang, F-S., Xu, A., and Jay, D.G. (1996). Talin and vinculin play distinct roles in filopodial motility in the neuronal growth cone. J. Cell Biol. *134*, 1197–1207.

Tao, Y.S., Edwards, R.A., Tubb, B., Wang, S., Bryan, J., and McCrea, P.D. (1996). β-Catenin associates with the actin-bundling protein fascin in a non-cadherin complex. J. Cell Biol. *134*, 1271–1281.

Tarone, G., Cirillo, D., Giancotti, F.G., Comoglio, P.M., and Marchisio, P.C. (1985). Rous sarcoma virus-transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. Exp. Cell Res. *159*, 141–157.

Tawil, N., Wilson, P., and Carbonetto, S. (1993). Integrins in point contacts mediate cell spreading: factors that regulate integrin accumulation in point contacts vs. focal contacts. J. Cell Biol. *120*, 261–271.

Taylor, A.C., and Robbins, E. (1963). Observations on microextensions from the surface of isolated vertebrate cells. Dev. Biol. *7*, 660–673.

Towbin, H., Steahelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Tucker, R.P., Edwards, B.F., and Erickson, C.A. (1985). Tension in the culture dish: microfilament organization and migratory behavior of quail neural crest cells. Cell Motil. *5*, 225–237.

Turner, C.E., and Burridge, K. (1991). Transmembrane molecular assemblies in cell-extracellular matrix interactions. Curr. Opin. Cell Biol. *3*, 849–853.

Tuszynski, G.P., Rothman, V.L., Deutch, A.H., Hamiliton, B.K., and Eyal, J. (1992). Biological activities of peptides and peptide analogues derived from common sequences present in thrombospondin, properdin, and malarial proteins. J. Cell Biol. *116*, 209–217.

Varnum-Finney, B., and Reichardt, L.F. (1994). Vinculin-deficient PC12 cell lines extend unstable lamellipodia and filopodia and have a reduced rate of neurite outgrowth. J. Cell Biol. *127*, 1071–1084.

Wewer, U.M., and Engvall, E. (1994). Laminins. Methods Enzymol. *245*, 85–105.

Yamakita, Y., Ono, S., Matsumura, F., and Yamashiro, S. (1996). Phosphorylation of human fascin inhibits its actin binding and bundling activities. J. Biol. Chem. *271*, 12632–12638.

Yamashiro-Matsumura, S., and Matsumura, F. (1985). Purification and characterization of an F-actin-bundling 55-kilodalton protein from HeLa cells. J. Biol. Chem. *260*, 5087–5097.

Yamashiro-Matsumura, S., and Matsumura, F. (1986). Intracellular localization of the 55kD actin-bundling protein in cultured cells: spatial relationships with actin, alpha-actinin, tropomyosin, and fimbrin. J. Cell Biol. *103*, 631–640.