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Formation of stable microspikes containing actin and the 55 kDa actin bundling protein, fascin, is a consequence of cell adhesion to thrombospondin-1: implications for the anti-adhesive activities of thrombospondin-1

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SUMMARY

The organisation of the actin cytoskeleton was examined in H9c2 and human intestinal smooth muscle cells adherent on fibronectin or thrombospondin-1. Whereas cells adherent on fibronectin adopted a polygonal shape and rapidly assembled prominent stress fibres and focal contacts, cells adherent on thrombospondin-1 assumed a more irregular morphology with large lamellae containing radial actin microspikes. Focal contacts were not detected in cells adherent on thrombospondin-1, as determined by indirect immunofluorescence staining for vinculin and other focal contact components. Instead, the radial microspikes stained positively for the actin-bundling protein, 55 kDa/fascin, and myosins. In cells adherent on fibronectin, 55 kDa/fascin immunoreactivity was diffuse and tended to be concentrated in the perinuclear region. In long-term adherent cells cultured in serum-containing medium, 55 kDa/fascin was detected in membrane ruffles, in stress fibres and in the perinuclear region. The microspikes formed within 40 minutes of plating cells on thrombospondin-1 and remained present when cells were treated with sodium orthovandate and hydrogen peroxide to increase intracellular phosphotyrosine levels. Indeed, although vanadate-treated cells tended to retract, the microspikes became more prominent and showed an increased intensity of staining for fascin. Under these conditions, a proportion of the microspikes did not appear to be in contact with the substratum: these spikes stained weakly for focal adhesion kinase, talin and vinculin. Cells treated with genistein also spread and formed fascin-containing microspikes which tended to be more slender than those of control cells. In contrast, cells adherent on fibronectin displayed a complex rearrangement of the actin cytoskeleton and a transient enrichment of 55 kDa/fascin-containing structures at the cell surface when treated with sodium orthovanadate and hydrogen peroxide. These observations indicate that cell interactions with fibronectin or thrombospondin-1 send distinct organisational signals to the actin cytoskeleton and may offer a mechanistic framework for further investigations of the anti-adhesive properties of thrombospondin-1.

Key words: thrombospondin-1, fibronectin, actin microfilament, fascin, vinculin

INTRODUCTION

Interactions between cells and their extracellular matrix play an important role in tissue organisation and also influence many aspects of cell behaviour. The extracellular matrix (ECM) is structurally very diverse, and so exposure of cells to different types of matrix macromolecules may offer one mechanism by which particular types of cell behaviour can be coordinated (reviewed by Hynes and Lander, 1992; Adams and Watt, 1993). Amongst the many glycoprotein components of the ECM, the thrombospondins form a gene family of structurally unique multidomain proteins (reviewed by Bornstein, 1992; Adams and Lawler, 1993a) which display restricted patterns of expression in embryonic and adult tissues (Laherty et al., 1992; Iruela-Arispe et al., 1993, Lawler et al., 1993; Tucker, 1993; Qabar et al., 1994). Currently, thrombospondin-1 (TSP-1) has been most extensively studied in terms of function. In addition to its ability to bind both calcium ions and other ECM glycoproteins, TSP-1 interacts with cell surfaces and, in substratum-bound form, promotes cell attachment, spreading or migration in appropriate cell types. Soluble TSP-1 modulates cell proliferation and can disrupt focal contacts in stably adherent cells (Murphy-Ullrich and Hook, 1989; Murphy-Ullvich et al., 1993; reviewed by Frazier, 1991; Sage and Bornstein, 1991; Lahav, 1993). TSP-1 thus exhibits both adhesive and antiadhesive properties in vitro and so it has been suggested that it plays a unique role in modulating cell behaviour in vivo.

To date, the molecular basis for these activities has not been well defined. However, the mechanisms by which TSP-1

interacts with cell surfaces appear to differ from those used by cells to attach to other adhesive glycoproteins, such as fibronectin and laminin. In the case of fibronectin, it is well established that the primary attachment interaction involves recognition of specific peptide sequences within the fibronectin molecule by integrins on the cell surface (reviewed by Hynes, 1992). In contrast, cell attachment to TSP-1 generally involves concurrent interactions with multiple cell binding sites, and the overall conformation of the TSP-1 molecule affects its adhesive activity (Asch et al., 1991; Sun et al., 1992; Adams and Lawler, 1993b). Although the $\alpha_v\beta_3$ integrin on vascular smooth muscle cells and endothelial cells binds to TSP-1 (Lawler et al., 1988), integrins have not been found to function as primary TSP-1 receptors in all cell types; instead, several types of cell surface molecules including proteoglycans, sulphatides and CD36 appear to be involved (see, for example, Frazier, 1991; Asch et al., 1991, Adams and Lawler, 1993b, 1994).

On a fibronectin substratum integrin-mediated attachment is typically followed by cell spreading and stabilisation of adhesive interactions through assembly of focal contacts and organisation of actin microfilaments into stress fibre bundles (reviewed by Hynes, 1989; Turner and Burridge, 1991a). In contrast, cell attachment to TSP-1 does not always lead to cell spreading (Lawler et al., 1988; Asch et al., 1991; Stomski et al., 1992; Adams and Lawler, 1993b, 1994). Although the adhesive activity of TSP-1 can be affected by the exact method of purification used and the number of disulphide bonds present within each molecule (Sun et al., 1992), cellular factors must also play a part, since individual cell types display different types of adhesive behaviour when exposed to the same preparations of TSP-1 (Adams and Lawler, 1993b, 1994). Since the ability of cells to spread upon a TSP-1 substratum does not appear to correlate with the recognition of a particular set of cell-binding sites (Adams and Lawler, 1993b), this behaviour may relate to the expression of intracellular mediators.

We have previously shown that rodent skeletal myoblast cell lines attach and spread on substrata coated with fibronectin or TSP-1 with similar concentration dependencies and kinetics. Whereas myoblast adhesion to fibronectin is an RGD-dependent process, probably mediated by the $\alpha_5\beta_1$ integrin (Menko and Boettiger, 1987; Enomoto et al., 1993; Adams, unpublished observation), myoblast adhesion to TSP-1 is not RGD-dependent and does not appear to be integrin-mediated. Instead, interactions with the cell suface involve the thrombospondin type 1 repeats and the carboxy-terminal domain, and are mediated by cell-associated chondroitin sulphate proteoglycans. Although attachment to either fibronectin or TSP-1 results in cell spreading, the spread cells exhibit different states of organisation of the actin-based cytoskeleton. Myoblasts adherent on fibronectin adopt a polygonal shape and organise actin filaments into stress fibre bundles. Myoblasts adherent on TSP-1 have an irregular shape, form large lamellae containing radial actin microspikes and contain few stress fibre bundles (Adams and Lawler, 1994). Since the actin-based cytoskeleton plays an important role in post-attachment intracellular signalling events (reviewed by Turner and Burridge, 1991; Juliano and Haskill, 1993; Adams and Watt, 1993; Ingber, 1993), these results raised the possibility that cell spreading on TSP-1 could involve a different set of intracellular events from cell spreading on fibronectin. Insight into the nature of these events may be of use in understanding the anti-adhesive properties of TSP-1.

Here, I describe experiments in which the organisation of actin microfilaments and various actin-associated proteins have been examined in myoblasts adherent on fibronectin or TSP-1. The data obtained indicate that actin microfilaments are functionally involved in cell spreading on both fibronectin and TSP-1. Adhesion to TSP-1 does not result in the formation of focal contacts, but rather in the formation of lamellae in which the actin-bundling protein, 55 kDa/fascin, and myosins localise to radial microspikes. Once formed, the microspike structures appear relatively stable: thus, when treated with vanadate and hydrogen peroxide, cells adherent on fibronectin undergo a complex reorganisation of the actin-based cytoskeleton, whereas cells adherent on TSP-1 retain their microspikes. However, in vanadate-treated cells, the staining intensity for fascin is increased and the distribution of the microspikes appears altered. Under these conditions, a proportion of the microspikes no longer appear to be in contact with the substratum, and these spikes display weak staining for focal adhesion kinase, vinculin and talin. The potential significance of these results is discussed with respect to the adhesive and antiadhesive roles of TSP-1 in the ECM.

MATERIALS AND METHODS

Cell culture

H9c2 (Kimes and Brandt, 1976) rat myoblasts were obtained from the ATCC and maintained in DMEM containing 20% fetal calf serum. Human intestinal smooth muscle cells (HISM; Graham et al., 1984), also obtained from the ATCC, were maintained in DMEM containing 10% fetal calf serum. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Adhesion assays

Plasma fibronectin was obtained from Telios Pharmaceuticals (San Diego, CA) and TSP-1 was prepared from human platelets as previously described (Adams and Lawler, 1994). For cell adhesion assays, both molecules were diluted to a concentration of 50 nM and allowed to adsorb to glass coverslips at 4°C overnight. The coverslips were blocked with 1 mg/ml heat-denatured BSA for 1 hour at room temperature. Cell adhesion assays were carried out at 37°C as previously described (Adams and Lawler, 1994), for periods from 20 minutes to 4 hours. Nonadherent cells were removed by washing in TBS containing 2 mM $CaCl_2$ and adherent cells were processed for immunofluorescence as described below. In some experiments, cells were pretreated for 2 hours with 10 μg/ml cytochalasin D or 3 μg/ml nocadazole (Sigma Chem. Co) before being processed for adhesion assays. In a separate series of experiments, cells were allowed to attach, and then the medium was replaced with DMEM containing 1 mM sodium orthovanadate and 2 mM hydrogen peroxide, to inhibit the action of intracellular phosphotyrosine phosphatases (Heffetz et al., 1990). Cells were exposed to these reagents for periods of 1 minute to 15 minutes, before being processed for immunofluorescence. In some experiments, cells were exposed to 250 μM genistein (ICN Immunobiologicals) for 2 hours prior to use in adhesion assays.

Immunofluorescence experiments

For staining with Texas Red isothiocyanate-phalloidin (TRITC-phalloidin, Sigma Chemical Co.), or with mouse monoclonal antibodies directed against vinculin (VIN 11.5, ICN ImmunoBiologicals); talin

(clone 8d4; Sigma Chem Co); paxillin (a gift from Dr C. Turner, Department of Anatomy and Cell Biology, SUNY Health Science Center, Syracuse, NY; Turner and Burridge, 1991b); phosphotyrosine (PY20; ICN Immunobiologicals), and pp125 focal adhesion kinase ($pp125^{FAK}$; antibody 2A7, a gift from Dr T. Parsons, Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA; Kanner et al., 1990; Schaller et al., 1992), or a rabbit polyclonal serum against $pp125^{FAK}$ (antiserum 331, a gift from Dr S. Hanks, Department of Cell Biology, Vanderbilt University, Nashville, TN; Hanks et al., 1992), cells were fixed in 3.7% formaldehyde for 10 minutes, then permeabilised for 10 minutes in 50 mM MES, pH 6.1, 5 mM $MgCl_2$, 3 mM EGTA, 100 mM KCl and 0.2% Triton X-100 (O'Neill et al., 1990). For staining with mouse monoclonal antibody 55K2 to human 55 kDa actin-bundling protein/fascin (a generous gift from Dr G. Mosialos, Department of Medicine, Harvard Medical School, Boston, MA and Dr F. Matsumura, Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ; Yamashiro-Matsumura and Matsumura, 1986), or a rabbit polyclonal antiserum to bovine uterine myosins (Sigma Chemical Co.), cells were fixed and permeabilised in absolute methanol for 10 minutes. Staining was carried out for 1 hour at room temperature: cells stained with TRITC-phalloidin were then washed and mounted in Vectastead mounting medium (Vector Laboratories, Loughborough, UK), whereas cells stained with primary antibodies were washed and stained for 45 minutes with appropriate FITC-conjugated secondary antibodies (ICN Biomedicals), then washed again and mounted. Samples were examined under epifluorescence using a Zeiss Axioplan microscope and photographs were taken using Kodak T-MAX 400 film.

RESULTS

Organisation of actin microfilaments as a consequence of cell adhesion to fibronectin or TSP-1

It has been demonstrated that integrin-mediated cell attachment is separable from the process of cell spreading, which depends upon polymerisation of actin into microfilaments (Orlando and Cheresh, 1991). To demonstrate that assembly of actin microfilaments is also functionally important for cell spreading on TSP-1, growing cultures of H9c2 cells were treated with 10 μg/ml of cytochalasin D for 2 hours, then prepared for adhesion assays and plated on coverslips coated with 50 nM TSP-1 or 50 nM fibronectin for 2 hours. The cytochalasin D-treated cells remained competent to attach to either substratum, but were not able to spread. In contrast, cultures of H9c2 cells pretreated with 3 μg/ml nocadazole, a microtubule-destabilising agent, retained the ability to spread on either substratum (data not shown). Thus, even though myoblast attachment to TSP-1 or to fibronectin involve different binding interactions at the cell surface, cell spreading on either substratum depends on the ability of cells to organise actin into microfilaments. Further experiments therefore were focussed on actin microfilament organisation in adherent cells.

We have previously observed that actin microfilament organisation differs markedly in skeletal myoblasts plated for 2 hours on substrata coated with TSP-1 or fibronectin (Adams and Lawler, 1994). To investigate this process in more detail, TRITC-phalloidin was used to visualise the distribution of polymerised actin in H9c2 cells which had attached to substrata coated with 50 nM TSP-1 or fibronectin for times between 20 minutes and 240 minutes.

After 20 minutes, H9c2 myoblasts had attached to both substrata but had not undergone spreading. On both substrata, the strongest staining for filamentous actin was at the periphery of the cells, where long, fingerlike protrusions or filopodia could be observed. A small number of the cells attached to fibronectin had begun to flatten and displayed a circumferential band of actin, but this type of actin organisation was not apparent in any of the cells attached to TSP-1 (Fig. 1a, cells on fibronectin; Fig. 1e, cells on TSP-1). After 40 minutes, cells attached to either substratum had begun to spread and a clear distinction in actin microfilament organisation had become apparent. Cells adherent to fibronectin had assumed a polygonal shape and had organised actin stress fibres, which ran both longitudinally across the cell body and also circumferentially (Fig. 1b). Cell adherent to TSP-1 had assumed a much more irregular shape, with large areas of lamellae, which contained radial actin microspikes. Circumferential actin bundles were observed behind these lamellae (Fig. 1f). These differences in microfilament organisation were maintained at 120 minutes, by which time further cell spreading had occurred on both substrata (Fig. 1c, cells on fibronectin; Fig. 1g, cells on TSP-1).

After 240 minutes, cells on TSP-1 had lost their irregular outline and lamellae, and assumed a polygonal shape similar to that observed in cells attached to fibronectin. At this time, cells adherent to either substratum displayed filamentous actin predominantly organised into longitudinal stress fibres (Fig. 1d, cells on fibronectin; Fig. 1h, cells on TSP-1, see also Adams and Lawler, 1994). Since myoblasts secrete fibronectin and laminin, both of which can bind to TSP-1 (Lawler et al., 1986), this change in morphology is likely to be caused by secretion of endogenous matrix. Thus, for at least 120 minutes after plating, actin microfilament organisation in cells spread on TSP-1 differed from that displayed by cells which had spread on fibronectin.

Cells adherent on TSP-1 do not assemble focal contacts

Cell spreading on fibronectin typically correlates with the organisation of focal contacts at the plasma membrane, in which an array of actin-associated proteins and signal-transducing molecules colocalise with the termini of actin filament bundles and clustered, ligand-occupied integrin receptors (reviewed by Burridge et al., 1988; Turner and Burridge, 1991a). To examine whether cells which had undergone spreading on TSP-1-organised focal contacts, H9c2 cells were allowed to attach and spread on fibronectin or TSP-1 for 90 minutes, and then stained for vinculin, an abundant component of focal contacts (Geiger, 1979). Whereas cells which had spread on fibronectin displayed a typical arrowhead staining pattern, indicative of the formation of a large number of focal contacts (Fig. 2a), vinculin staining in cells adherent on TSP-1 was diffuse and no organisation of focal contacts was apparent. Some concentration of staining above background was apparent in the perinuclear region (Fig. 2b). However, cells plated on TSP-1 were not rendered incapable of forming focal contacts at later times, as demonstrated by the many focal contacts present in cells incubated overnight in serum-containing medium (Fig. 2c). Cells allowed to adhere to fibronectin for 90 minutes also stained positively for paxillin (Turner and Burridge, 1991b), pp125 focal adhesion kinase ($pp125^{FAK}$; Schaller et al., 1992) and phosphotyrosine-containing proteins (Maher et al., 1985; Guan et al., 1991) in focal

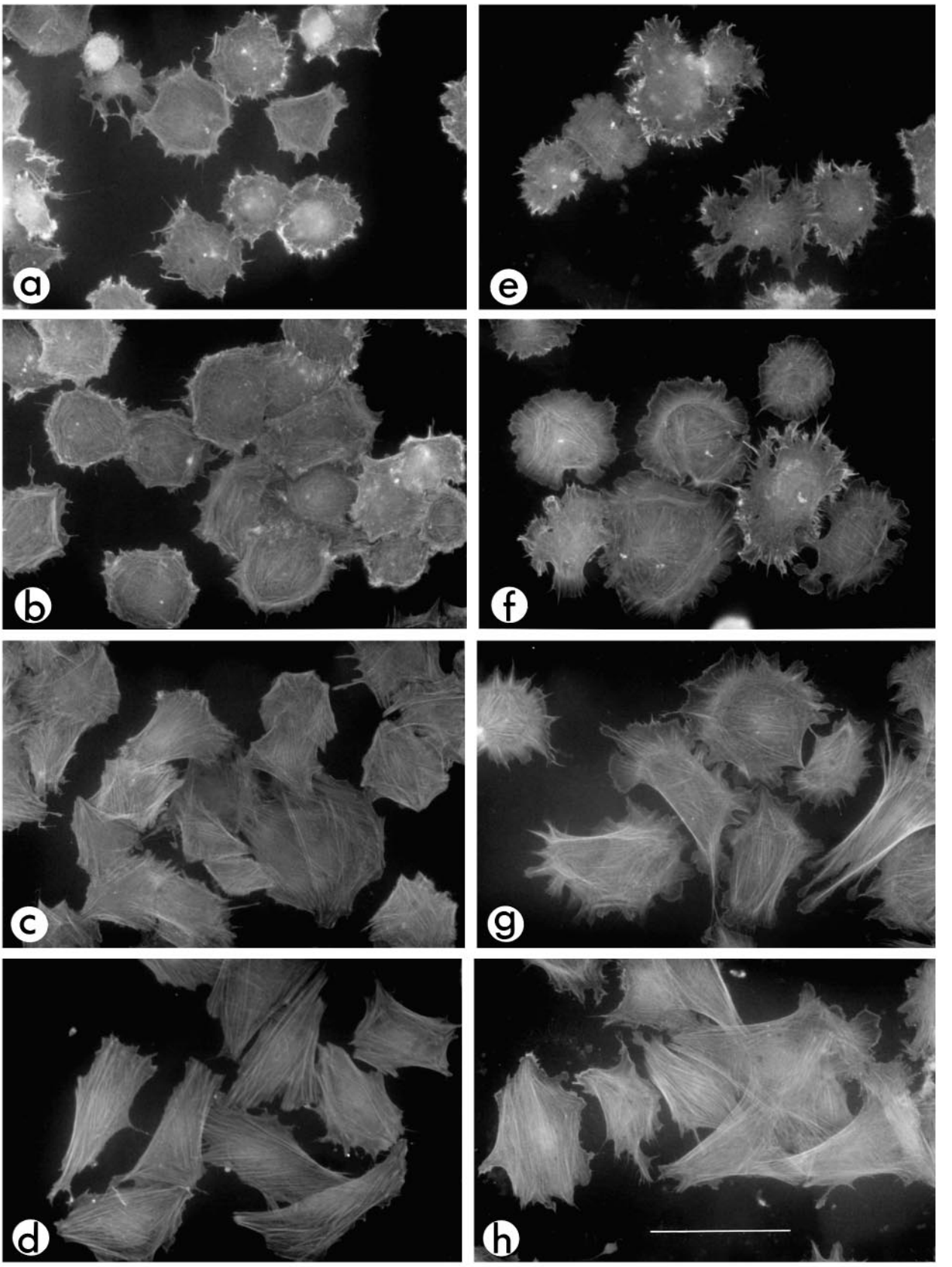


Fig. 1. Organisation of actin microfilaments in cells adherent on fibronectin or TSP-1. H9c2 cells were plated onto substrata coated with 50 nM fibronectin (a,b,c,d) or 50 nM TSP-1 (e,f,g,h) for 20 minutes (a,e); 40 minutes (b,f); 120 minutes (c,g); or 240 minutes (d,h); then fixed and stained with TRITC-phalloidin. Bar, 30 μm.

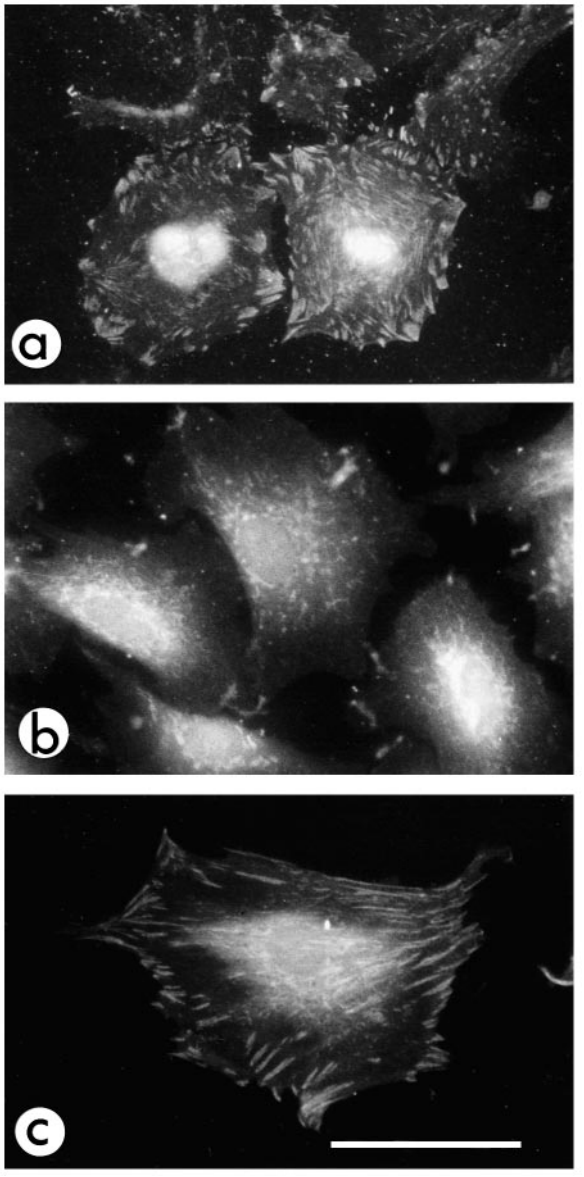


Fig. 2. Localisation of vinculin in adherent cells. H9c2 cells were plated on substrata coated with 50 nM fibronectin (a), or 50 nM TSP-1 (b,c), then fixed and stained for vinculin. In (a and b), adhesion was for 90 minutes in serum-free medium; in (c), cells were cultured overnight in DMEM containing 10% FCS. Bar, 30 μm.

contacts, whereas cells adherent on TSP-1 did not display distinctive staining patterns for these molecules (data not shown). Thus it appeared that cell adhesion to fibronectin, as expected, resulted in the formation of focal contacts, whereas cell adhesion to TSP-1 did not.

Adhesion to TSP-1 results in the organisation of radial microspike structures containing 55 kDa actin-bundling protein/fascin

Since cell adhesion to TSP-1 did not lead to focal contact formation, yet depended on actin polymerisation, the next question was to examine the spatial organisation of actin-associated proteins which are not typical components of focal contacts. A 55 kDa actin-bundling protein has been described which localises to stress fibres and membrane ruffles in stably adherent cells grown in serum-containing medium (Yamashiro-Matsumura and Matsumara, 1985, 1986). Molecular cloning has now shown this protein to be a human homologue of sea urchin fascin (Bryan et al., 1993; Mosialos et al., 1994; Otto, 1994), and so the protein will be referred to here as 55 kDa/fascin. In agreement with previously published data from a number of cell types (Yamashiro-Matsumura and Matsumura, 1986), the antibody to 55 kDa/fascin reacted with a single protein with an apparent molecular mass of 55 kDa on Western blots of H9c2 cells (unpublished observation). The antibody was then used to examine the distribution of 55 kDa/fascin in cells adherent on fibronectin or TSP-1 with respect to time after plating.

After 20 minutes, cells attached to fibronectin stained intensely but diffusely for 55 kDa/fascin. After 40 minutes, cells spread on fibronectin displayed diffuse staining throughout the cytoplasm, with small areas of more intense staining being present in ruffled membranes at the margins of the cells. After 120 minutes, the cytoplasmic staining remained diffuse but tended to be more prominent in the perinuclear region. Some cells displayed more intense staining in areas of membrane ruffles (Fig. 3a,c,e, cells plated on fibronectin). At no time did the 55 kDa/fascin staining coincide with stress fibres or focal contacts, even though these structures were clearly formed by 100 minutes post-plating on fibronectin (see Fig. 1c and Fig. 2a).

At 20 minutes post-plating, cells attached to TSP-1 also displayed intense, uniform staining for 55 kDa/fascin. However, as cell spreading proceeded, a restricted and striking distribution of 55 kDa/fascin became apparent. After 40 minutes, intense staining of abundant arrays of radial microspikes within lamellae was observed: indeed more of these structures could be detected by using this antibody than by TRITC-phalloidin staining. Although microspikes were present in all the adherent cells, the distribution of the spikes was quite variable. In some cells the microspikes had a polarised distribution, which suggested the existence of a leading edge, but many cells exhibited near circumferential arrays of microspikes. Intense, diffuse staining in the perinuclear region was also observed in the majority of cells. This probably represents a true perinuclear concentration of the protein, since a similar concentration of staining has been observed in various mammalian cell types grown in serum-containing medium (Yamashiro-Matsumura and Matsumura, 1986). A perinuclear cytoskeletal ‘cage’ structure which contains fascin has also been described in echinoderm coelomocytes (Otto et al., 1979). In H9c2 cells adherent on TSP-1, 55 kDa/fascin staining was absent in the zone of cytoplasm between the perinuclear region and the lamellae. This staining pattern was maintained at 120 minutes after plating cells on TSP-1 (Fig. 3b,d,f, cells plated on TSP-1). At 240 minutes or later, the overall change in cell morphology correlated with loss of the 55 kDa/fascin-containing microspikes (unpublished observation).

To further examine the structure of the microspikes, cells adherent to fibronectin or TSP-1 for 120 minutes were stained with a polyclonal antiserum to myosins, reactive with both conventional and unconventional myosins (Titus, 1993). In cells adherent on fibronectin, diffuse staining was observed throughout the cytoplasm and most cells also contained a con-

centration of more intensely staining spots within the perinuclear region (Fig. 3g). Cells adherent on TSP-1 also displayed this staining pattern within the cell body but, in addition, staining was present within the microspike structures (Fig. 3h). Thus, in addition to actin and 55 kDa/fascin, the microspikes also appear to contain myosins.

It has previously been reported that 55 kDa/fascin protein is found in association with both stress fibres and ruffled

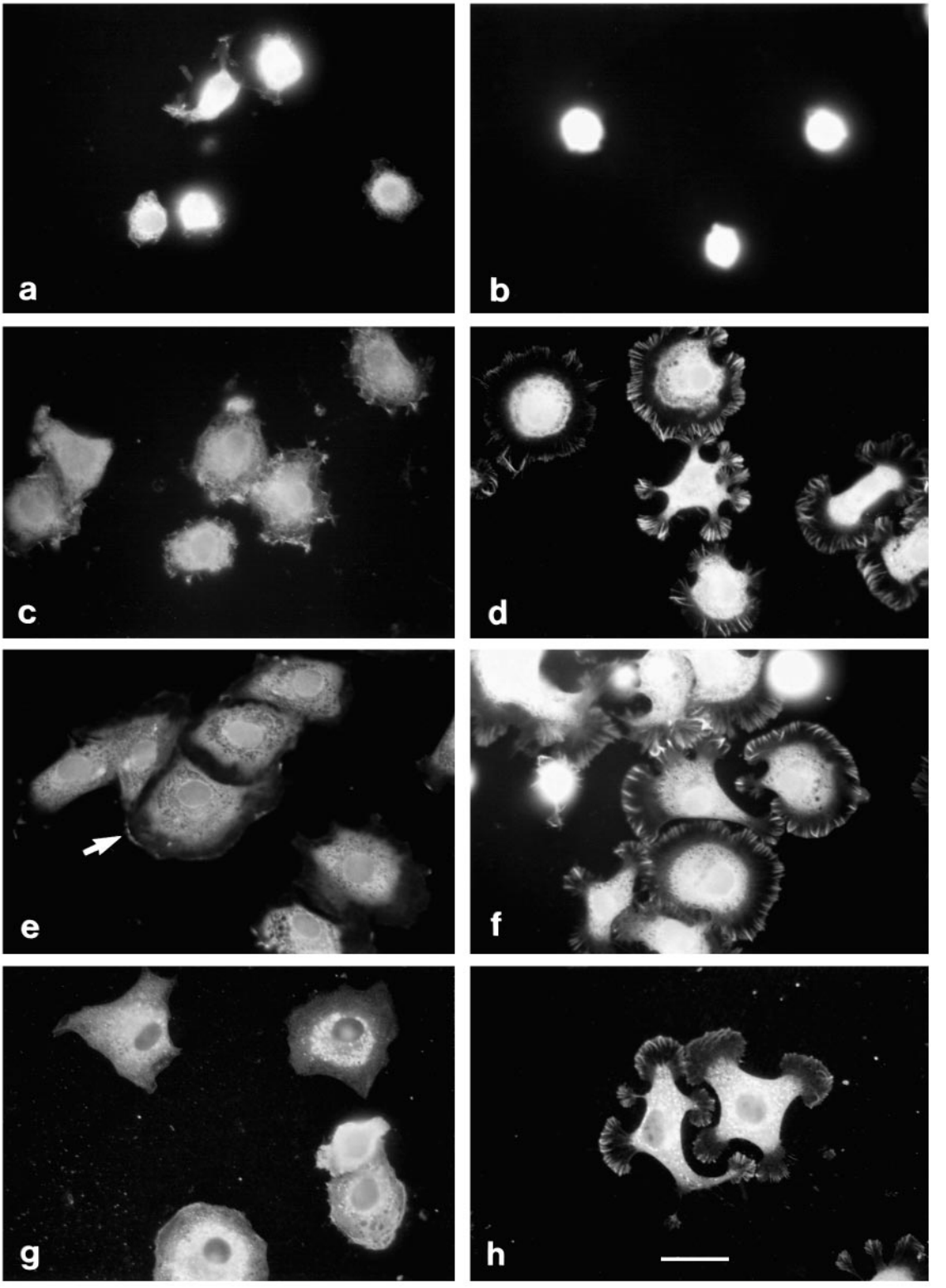


Fig. 3. Localisation of 55 kDa/fascin amd myosins in cells adherent on fibronectin or TSP-1. H9c2 cells were plated on substrata coated with 50 nM fibronectin (a,c,e,g) or 50 nM TSP-1 (b,d,f,h) for 20 minutes (a,b); 40 minutes (c,d) or 120 minutes (e,f,g,h); then fixed and stained for 55 kDa/fascin (a-f), or for myosins (g,h). Arrow in (e) shows an area at the cell margin which stained positively for 55 kDa fascin. Bar, 10 μm.

membranes in stably adherent cells (Yamashiro-Matsumura and Matsumara, 1985, 1986). Within the period of time over which the adhesion assay experiments were carried out, colocalisation of 55 kDa/fascin with stress fibres was not apparent in cells adherent on TSP-1 or fibronectin. To examine whether H9c2 cells were capable of organising stress fibres which contained 55 kDa/fascin, H9c2 cells were grown overnight in serum-containing medium. Under these conditions, the cells organised large actin stress fibres (Fig. 4a), and 55 kDa/fascin was detected in several locations including areas of ruffled membranes and the perinuclear region, and also in association with stress fibres (Fig. 4b). Although focal contacts were present (see Fig. 2c), 55 kDa/fascin was not detectable as part of these structures. Thus, 55 kDa/fascin can adopt the previously reported distribution within H9c2 cells, given the appropriate environmental conditions.

To confirm that these observations made using H9c2 cells had general applicability, the organisation of filamentous actin and 55kD/fascin was examined in a human smooth muscle cell line, HISM, which also undergoes spreading on both fibronectin and TSP-1 (Adams and Lawler, 1993b; Adams, unpublished observation). For these assays an adhesion time of 100 minutes was used. HISM cells adherent to TSP-1 displayed intense staining for polymerised actin within the cell body, but organisation of actin into stress fibre bundles was not apparent. However, like the H9c2 cells, HISM cells adherent on TSP-1 assumed an irregular morphology and formed large lamellae which contained many radial actin microspikes (Fig. 5a). HISM cells attached to fibronectin were also strongly stained by TRITC-phalloidin, indicating that filamentous actin was present within the spread cells. However, little organisation of actin stress fibres was apparent; instead most cells exhibited a diffuse, somewhat punctate staining pattern, and a concentration of actin microfilaments in areas of ruffled membranes. A minority of cells did contain slender actin stress fibres (Fig. 5b).

When stained for 55 kDa/fascin, the distribution of the protein in HISM cells adherent on TSP-1 was similar to that observed in H9c2 cells. Thus, 55 kDa/fascin localised to large arrays of radial microspikes and also appeared to be concentrated in the perinuclear region (Fig. 5c). In HISM cells adherent on fibronectin, 55 kDa/fascin protein appeared concentrated around the nucleus, and in small areas of ruffled membrane (Fig. 5d). These differences again correlated with the presence of focal contacts in cells adherent on fibronectin but not on TSP-1, as determined by vinculin staining (data not shown). Thus, the correlation between cell adhesion to TSP-1 and the formation of microspikes containing the 55 kDa/fascin actin bundling protein was observed in two cell lines derived from different tissues and different species.

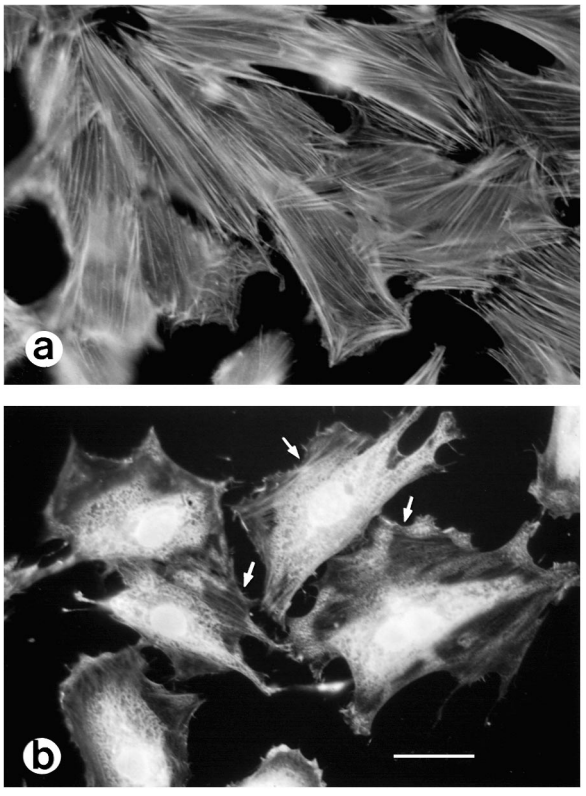


Fig. 4. Localisation of 55 kDa/fascin in long-term adherent cells. H9c2 cells were plated overnight in serum-containing medium, then fixed and stained with TRITC-phalloidin (a), or 55 kDa antibody (b). Small arrows in (b) indicate areas where 55 kDa was associated with stress fibres. Bar, 10 μm.

The microspike structures are stable under conditions which increase intracellular phosphotyrosine levels

Integrin-activation signals such as cell adhesion to fibronectin result in the activation and tyrosine phosphorylation of $pp125^{FAK}$ and other focal contact components during focal contact formation (reviewed by Hynes, 1992; Juliano and Haskill, 1993). Experimental elevation of phosphotyrosine levels in adherent cells leads to changes in the organisation of various actin-containing structures including focal contacts (Burridge et al., 1992) and adherens junctions (Volberg et al., 1992). Since it had not been possible to detect phosphotyrosine or $pp125^{FAK}$ in the TSP-1-induced microspikes, it was of interest to examine the stability of these structures under conditions of increased intracellular phosphotyrosine levels (Heffetz et al., 1990).

In preliminary experiments, it was found that treatment of cells with sodium orthovanadate and hydrogen peroxide for 10 minutes prior to the attachment assay completely inhibited cell attachment to either fibronectin or TSP-1. This was not a toxic effect, since the treated cells still excluded trypan blue. If vanadate-treated cells were plated onto tissue culture plastic in serum-containing medium, attachment was delayed for over 10 hours compared to control cells. Cells then attached, spread and proliferated similarly to untreated cells (unpublished observations). In contrast, cells plated onto fibronectin or TSP-1 for 120 minutes showed little change in microfilament organisation in response to vanadate treatment (Burridge et al., 1992).

Therefore, cells were allowed to adhere to TSP-1 or fibronectin for 60 minutes, treated with sodium orthovanadate and hydrogen peroxide for times between 1 minute and 15 minutes, and then processed for immunofluorescence. In the case of cells adherent upon fibronectin, vanadate treatment induced a complex series of changes in the organisation of filamentous actin and 55 kDa/fascin. After one minute of treatment with sodium orthovanadate, areas of marginal

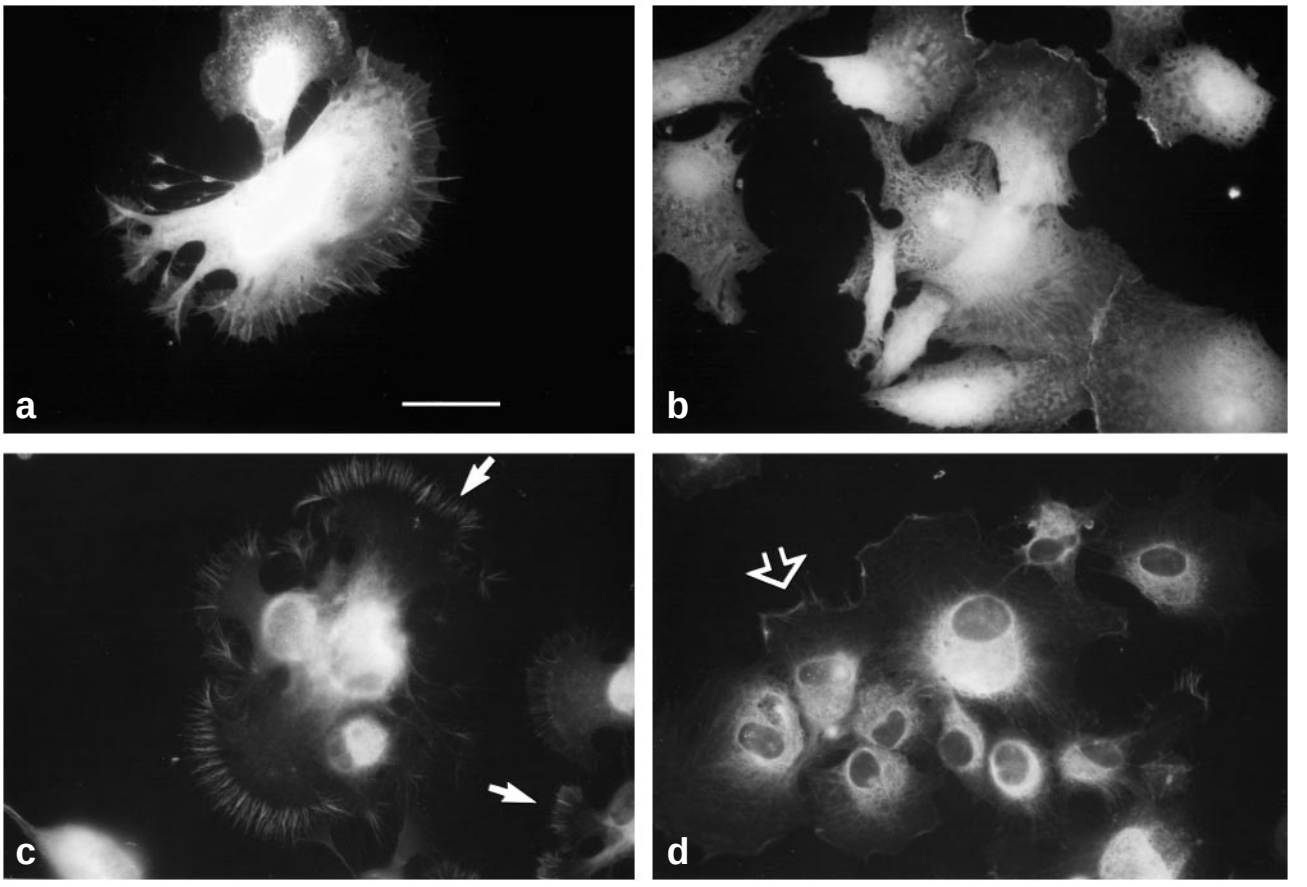


Fig. 5. Localisation of actin microfilaments and 55 kDa/fascin in HISM cells adherent on TSP-1 or fibronectin. Cells were plated on substrata coated with 50 nM TSP-1 (a,c) or 50 nM fibronectin (b,d), for 100 minutes, then stained with TRITC-phalloidin (a,b) or antibody to 55 kDa/fascin (c,d). Filled arrows in c indicate regions of microspikes. The open arrow in d indicates an area of ruffled membrane that stained positively for 55 kDa/fascin. Bar, 10 μm.

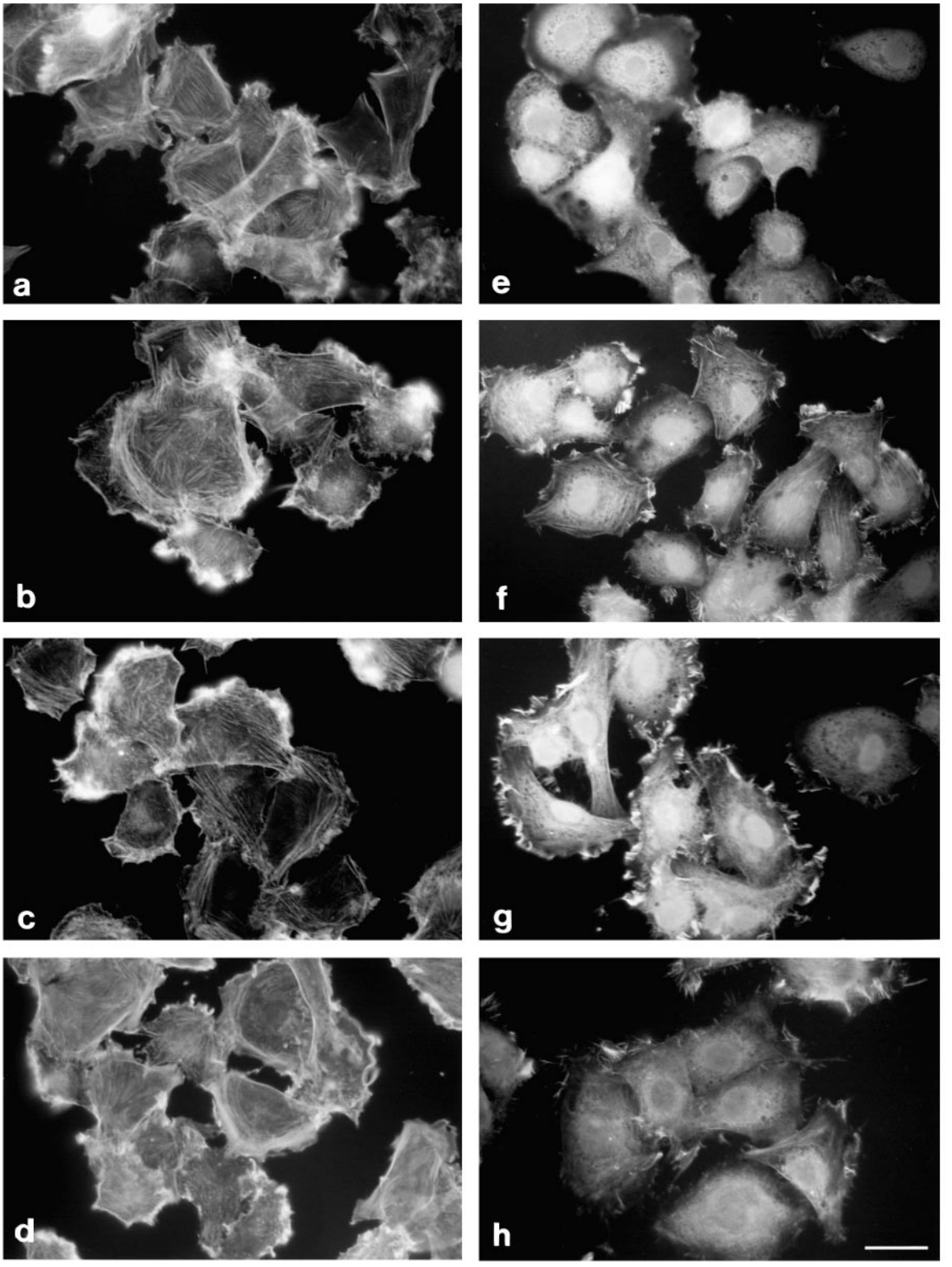
ruffling membrane had appeared, and this correlated with increased areas of staining for 55 kDa/fascin at the cell margins. In some cells, colocalisation of 55 kDa/fascin with stress fibres became apparent, although much of the 55 kDa/fascin staining remained diffuse within the cytoplasm (Fig. 6b,f, cells plated on fibronectin). After 5 minutes of treatment with sodium orthovanadate, actin stress fibre organisation within the cell body appeared altered and membrane ruffling had intensified, in that it tended to involve larger areas of the cell margins. These changes correlated with the appearance of large arrays of ruffles and spikes at the cell surface which stained positively for 55 kDa/fascin (Fig. 6c,g, cells plated on fibronectin). During these changes in the staining pattern of 55 kDa/fascin at the cell margins, the overall intracellular staining remained diffuse. After 15 minutes of treatment with vanadate, longitudinal and circumferential arrays of stress fibres were again prominent within the cells, and membrane ruffling tended to be restricted to smaller areas of the cell surface. Most of the 55 kDa/fascin staining was diffuse within the cytoplasm, but staining also remained more prominent in ruffles and cell surface projections than in untreated cells (Fig. 6d,h; compare Fig. 6e with h; cells plated on fibronectin).

In cells adherent on thrombospondin for 60 minutes, treatment with vanadate for 1 minute caused an intensification of TRITC-phalloidin staining of microspikes within the lamellae (Fig. 7a,b; cells plated on TSP-1). This correlated with increased staining intensity for 55 kDa/fascin within the microspikes (Fig. 7e,f). After 5 minutes of vanadate treatment, some cells were less well spread; however, all cells still displayed large arrays of radial microspikes which stained strongly for 55 kDa/fascin (Fig. 7c,g). Although the microspikes remained distributed at the cell margins, rather than over the apical surfaces of the cells, the morphology of the lamellae appeared changed and some of the microspikes formed fingerlike projections which did not appear to be in contact with the substratum (arrowed in Fig. 7c and h). After 15 minutes of vanadate treatment, most cells had retracted and few stress fibres could be observed, although filamentous actin was still present within the cells. However, the cells were still attached to the TSP-1 substratum and displayed very

Fig. 6. Changes in organisation of filamentous actin and 55 kDa/fascin caused by vanadate treatment in cells adherent on fibronectin. H9c2 cells were plated on substrata coated with 50 nM fibronectin for 60 minutes, then washed and fixed (a,e), or treated with sodium orthovanadate and hydrogen peroxide for 1 minute (b,f); 5 minutes (c,g); or 15 minutes (d,h) before fixation. Cells were stained with TRITC-phalloidin (a,b,c,d) or 55 kDa antibody (e,f,g,h). Bar, 10 μm.

prominent, circumferential arrays of microspikes. In general, the spikes now appeared longer and more slender than those of control, untreated cells and the staining intensity for 55 kDa/fascin was somewhat diminished (Fig. 7d,h). Thus, these experiments showed that the microspikes were not disrupted by increasing intracellular phophotyrosine levels, whereas cells adherent on fibronectin underwent complex changes in actin cytoskeletal organisation in response to this stimulus.

Since the numbers of spikes and the staining intensity for fascin appeared altered in vanadate-treated cells, additional experiments were carried out to investigate these changes in more detail. To investigate the role of tyrosine kinases in



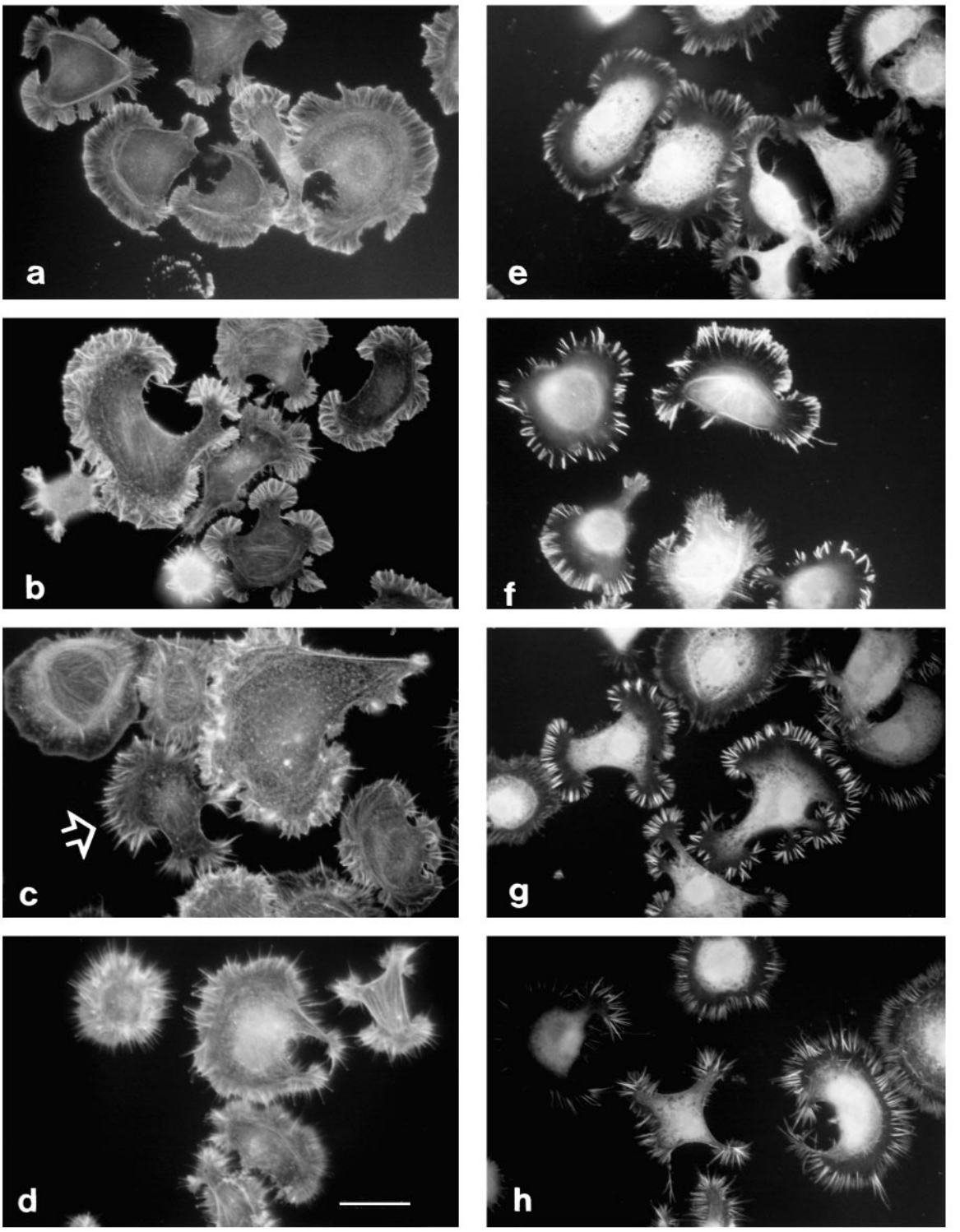


Fig. 7. Changes in the organisation of filamentous actin or 55 kDa/fascin caused by vanadate treatment of cells adherent on TSP-1. H9c2 cells were plated on substrata coated with 50 nM TSP-1 for 60 minutes, then washed and fixed (a,e), or treated with sodium orthovanadate and hydrogen peroxide for 1 minute (b,f), 5 minutes (c,g) or 15 minutes (d,h) before fixation. Cells were stained with TRITC-phalloidin (a,b,c,d) or 55 kDa antibody (e,f,g,h). Arrow in (c) indicates microspikes which appear to be raised above the substratum. Bar, 10 μm.

microspike formation, cells were treated with genistein, an inhibitor of tyrosine kinases (Akiyama et al., 1987). Genistein-treated cells spread on TSP-1 substrata and, in terms of actin organisation appeared similar to control cells (unpublished observation). When stained for 55 kDa/fascin it was apparent that the cells had indeed assembled large arrays of radial microspikes. However, in general the spikes appeared somewhat more slender than those of control cells (Fig. 8a; see

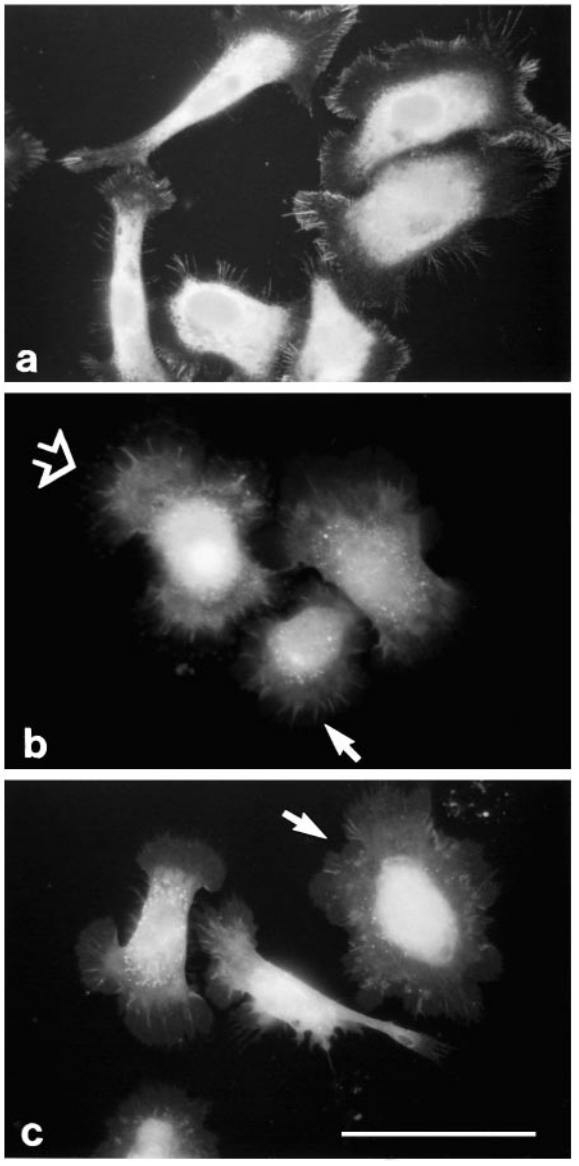


Fig. 8. Localisation of actin-associated proteins in cells adherent on TSP-1 after alteration of cellular phosphotyrosine levels. H9c2 cells were plated on substrata coated with 50 nM TSP-1 for 60 minutes after treatment with 250 μM genistein (a), or were treated with sodium orthovanadate and hydrogen peroxide for 10 minutes after the 60 minute adhesion period (b, c). Cells were stained for 55 kDa/fascin (a), vinculin (b), or talin (c). Arrowhead in (b) indicates regions where spots of staining were visible at the edges of the lamellae. Arrows in (b) and (c) indicate upraised microspikes which stained positively. Bar, 30 μm.

also Fig. 3f and Fig. 7e). Thus, inhibition of tyrosine kinases does not prevent microspike formation, but may affect certain aspects of microspike structure.

The distribution of the focal contact components pp125^{FAK}, vinculin and talin were then examined in cells which had been treated with orthovanadate for 10 minutes after adhesion to TSP-1 for 60 minutes. In these cells, discrete localisation of the FAK protein was apparent, in that some of the microspikes which were not in contact with the substratum displayed a low level of staining with antibodies to FAK (unpublished observation). Since the intensity of staining was very low, the distribution of the more abundant focal contact components, vinculin and talin, was also examined. Both talin and vinculin antibodies displayed diffuse staining above background levels in the perinuclear region and also in the fingerlike, projecting microspikes which appeared raised above the substratum (Fig. 8b and c). The distribution of staining in the microspikes was similar to that obtained with the FAK antibodies, but the intensity of staining was greater. A small number of cells also displayed 'spots' of staining at the margins of the lamellae (shown for vinculin, open arrowhead in Fig. 8b). The microspikes which were in contact with the substratum were not stained by these reagents.

DISCUSSION

In this paper I have compared the distribution of actin microfilaments, myosins, focal contact components, and the actin-bundling protein, 55 kDa/fascin in cells adherent on fibronectin or TSP-1 substrata. The consequences of cell attachment and spreading on a fibronectin substratum have been well characterised (reviewed by Hynes, 1989), and so by examining cells which are also capable of spreading on TSP-1, it has become possible to identify substratum-specific responses to TSP-1. The central conclusion of these experiments is that cells adherent on TSP-1 do not assemble focal contacts, but instead form radial microspike structures which contain actin, 55 kDa/fascin and myosin. These results are novel and may offer a mechanistic framework for clarifying the proposed anti-adhesive activities of TSP-1 in the extracellular matrix.

Whereas vinculin is widely used as a molecular marker for focal contact formation (reviewed by Burridge et al., 1988), I have found that 55 kDa/fascin is a sensitive marker for the TSP-1-induced microspikes. Indeed, the staining patterns obtained with vinculin and 55 kDa/fascin antibodies show a reciprocal distribution on the two substrata. Thus, on a fibronectin substratum, 55 kDa/fascin remains largely diffuse, whereas on TSP-1 the protein is rapidly incorporated into microspike structures. In contrast, vinculin becomes localised to focal contacts in fibronectin-adherent cells, but remains diffuse in cells attached to TSP-1. These results indicate that the interactions of TSP-1 or fibronectin with the cell surface may send quite distinct organisational signals to the actin-based cytoskeleton. Such mechanistic differences could account for the observed differences in functional properties of these two matrix glycoproteins.

55 kDa/fascin is a structurally unique actin-bundling protein (reviewed by Matsudaira, 1991) which appears to be well-conserved in evolution (reviewed by Otto, 1994) and which has been localised to a variety of actin-containing structures in diverse organisms. These include the membrane ruffles and lamellae of adherent mammalian cells (Yamashiro-Matsumura and Matsumura, 1985, 1986); the filopodia formed by echinoderm coelomocytes which have undergone osmotic shock (Otto et al., 1979; Otto and Bryan, 1981); the microspikes formed by starfish oocytes undergoing meiotic maturation (Otto and Schroder, 1984), and the acrosomal processes of starfish sperm (Maekawa et al., 1982). 55 kDa/fascin is also found as a component of more stable structures include stress fibre bundles in cultured mammalian cells (Yamashiro-

Matsumura and Matsumura, 1986) and the perinuclear cytoskeletal cage detected in sea urchin coelomocytes (Otto et al., 1979). The *Drosophila melanogaster* homologue of echinoderm fascin is the *singed* gene product (Paterson and O'Hare, 1991; Bryan et al., 1993). *singed* mutants exhibit bristle malformations and, in extreme alleles, are also female sterile due to defects in oogenesis involving aberrant nurse cell function (Paterson and O'Hare, 1991; Cant et al., 1994). Although actin and fascin form the major components of some of these structures (Otto and Bryan, 1981; Otto and Schroder, 1984), others such as stress fibres contain multiple actin-associated proteins. The TSP-1-induced microspikes contain myosin as well as 55 kDa/fascin and in future it will be of interest to examine their composition in more detail.

The morphology of cells adherent on TSP-1 is reminiscent of that of motile cells, which typically contain few focal contacts, lack well-developed actin stress fibres and display areas of lamellae at the leading edge (Abercrombie et al., 1971; Couchman and Rees, 1979; Izzard and Lochner, 1976, 1980; Kolega et al., 1982; reviewed by Stossel, 1993). Indeed, several types of cells undergo chemotatic or haptotactic movements in response to TSP-1 in Boyden chamber assays (Mansfield et al., 1990; Taraboletti et al., 1987, 1990). However, in cells moving over a substratum, the leading and trailing edges are morphologically distinct and a small number of focal contacts are present within the leading lamellae (Abercrombie et al., 1971; Izzard and Lochner, 1976; Heath and Dunn, 1978). Since the cells adherent on the pure TSP-1 substratum display near circumferential arrays of microspikes and lack focal contacts, it is not clear that all the structures required for movement are assembled. Within the extracellular matrix, TSP-1 is typically incorporated as small patches in association with cell surfaces (for example, Raugi et al., 1982; Murphy-Ullrich et al., 1988), and thus could stimulate cell movement in the context of other adhesive glycoproteins. In this respect, it will be of interest to examine the formation of microspikes and focal contacts by cells plated on a mixed fibronectin/TSP-1 substratum.

Interference reflection microscopy has been used to determine the separation between a cell and its substratum (Curtis, 1964). Three types of apposition have been defined by this method: focal contacts, which are small areas where the plasma membrane is within 10 nm to 15 nm of the substratum; close contacts, which are larger areas where the separation is about 30 nm, and regions where the separation is of the order of 100 nm to 140 nm (Izzard and Lochner, 1976, 1980; Heath and Dunn, 1978). Close contacts predominate in the leading lamallae of motile cells and are therefore thought to form weak, or low affinity, contacts which permit the forward protrusion of the lamellae (Izzard and Lochner, 1976, 1980; Couchman and Rees, 1979). Although, as indicated above, the cells spread on TSP-1 resemble motile cells in terms of morphology and actin organisation, it will be necessary to examine them by interference reflection microscopy to determine whether the fascin-containing contacts also share the characteristics of close contacts.

Adhesive strength does not show a simple correlation with cell motility (Calof and Lander, 1991) and so it will be informative to compare the strength of cell adhesion to fibronectin or TSP-1 more quantitatively. Such comparisons have been made for fibronectin and tenascin, an ECM glycoprotein which also has adhesion-modulating properties (reviewed by Chiquet-Ehrismann, 1991). In this study, cells were found to have similar initial affinities of interaction with fibronectin and tenascin, but whereas the strength of adhesion to fibronectin then increased over time at 37°C, no strengthening of adhesion was observed in cells attached to tenascin. However, the cell types used did not spread when exposed to tenascin (Lotz et al., 1989).

Although it seems plausible that the TSP-1-induced microspikes may form a weaker adhesive contact than that provided by a focal contact, several lines of evidence suggest that the microspikes themselves are relatively stable structures. First, they are present for at least two hours after plating cells on TSP-1, whereas membrane ruffling in response to growth factors such as PDGF persists for about 30 minutes (Mellström et al., 1988). Secondly, increased intracellular phosphotyrosine levels caused a substantial reorganisation of the actin cytoskeleton in cells adherent on fibronectin, yet cells adherent on TSP-1 retained their microspikes even as cell retraction proceeded. Similarly, although treatment of cells with tyrosine kinase inhibitors prevents focal contact formation (Burridge et al., 1992), cells treated with genistein still formed microspikes when plated on TSP-1. In vitro assays, using purified echinoderm fascin or mammalian 55 kDa actin bundling protein, have shown that actin/fascin bundles are stable structures, in that actin bundling activity is not affected by changes in the concentration of ATP, magnesium or calcium ions, or pH (Bryan and Kane, 1978; Maekawa et al., 1982; Yamashiro-Matsumura and Matsumura, 1985).

However, the increased staining intensity for 55 kDa/fascin and the alterations in structure of the lamellae and microspikes observed in vanadate-treated cells do suggest that some changes in the composition or structure of the microspikes may have occurred when cellular phosphotyrosine levels were raised. In support of this observation, the fingerlike microspikes which appeared free of the substratum and which stained positively for fascin were also found to contain focal contact components. Since genistein-treated cells tended to display more-slender microspikes than control cells, it appears possible that tyrosine kinase substrates are present within the microspikes under conditions of normal cellular phosphotyrosine levels, although proteins such as paxillin and focal adhesion kinase were not detected.

Soluble TSP-1 has previously been shown to prevent cell adhesion to glass or fibronectin substrata (reviewed by Lahav, 1993) and to destabilise cell-matrix contacts by inhibiting focal contact formation and assembly. This activity has been mapped to a peptide sequence within the amino-terminal heparin-binding domain (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1993). These experiments utilised a cell type which spread on fibronectin but not on TSP-1 substrata. The data indicated one mechanism by which TSP-1 can regulate cell-substratum adhesion and so promote cell migration and mitosis (Murphy-Ullrich and Hook, 1989). By using the myoblasts, a cell type which can spread on both TSP-1 and fibronectin substrata, I have found that substratum-bound TSP-1 stimulates the formation of a type of adhesive contact which is different from the focal contact. These data indicate a mechanism by which matrix-bound TSP-1 may be capable of supporting cell adhesion, yet readily permit the changes in cell shape which are necessary for cell movement or cell proliferation. The formation of microspike structures rather than focal

contacts may indicate either that TSP-1 triggers a separate series of intracellular events to fibronectin, or that signals which are required for stabilisation of adhesion and focal contact formation are lacking. For example, focal contact formation in cells adherent on fibronectin requires both integrin-mediated attachment and interactions with the heparin-binding domain of fibronectin and involves protein kinase C (Woods et al., 1986; Woods and Couchman, 1992). A more detailed examination of the formation and structure of the microspikes may serve to resolve these possibilities.

An alternate, but not exclusive, mechanism of action for TSP-1 may be that the microspikes have a role separable from that of substratum adhesion. The lamellae and microspikes provide an enlarged area of cell surface which could offer a high local concentration of membrane proteins such as receptors, transporters or ion channels. This function of cell-surface projections is exemplified by the brush border microvilli of absorptive epithelia (reviewed by Louvard, 1989). In this respect, it is interesting that the fascin-containing microspikes described in invertebrates are formed in response to soluble mediators, rather than adhesion to a substratum. By stimulating the formation of microspikes, TSP-1 may increase the sensitivity of cells to extracellular growth or motility factors and thereby bring about changes in cell behaviour by indirect mechanisms.

In terms of biochemical composition, the fascin-containing structures of invertebrates represent a different type of cell-surface projection to the villin-containing microvilli of absorptive epithelia (Bryan et al., 1993). Organisation of cortical actin microfilaments into radial microspikes has been observed in many cell types under various experimental conditions but, as discussed above, these structures tend to be formed only on a portion of the cell surface. Because of this structural complexity, and also because of the transient nature of the cell surface projections, it has proved difficult to analyse cortical actin-containing structures other than brush border microvilli at the biochemical level (reviewed by Bretscher, 1991). As described in this paper, a pure TSP-1 substratum clearly provides a strong signal for the formation of 55 kDa/fascin-containing microspikes. Thus, a more detailed analysis of the formation and molecular composition of the TSP-1-induced microspikes may not only further an understanding of the mechanisms by which TSP-1 regulates cell behaviour, but may also provide information on a distinct type of cortical actin structure.

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