Stimulation of Fascin Spikes by Thrombospondin-1 Is Mediated by the GTPases Rac and Cdc42

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Abstract. Cell adhesion to extracellular matrix is an important physiological stimulus for organization of the actin-based cytoskeleton. Adhesion to the matrix glycoprotein thrombospondin-1 (TSP-1) triggers the sustained formation of F-actin microspikes that contain the actin-bundling protein fascin. These structures are also implicated in cell migration, which may be an important function of TSP-1 in tissue remodelling and wound repair. To further understand the function of fascin microspikes, we examined whether their assembly is regulated by Rho family GTPases. We report that expression of constitutively active mutants of Rac or Cdc42 triggered localization of fascin to lamellipodia, filopodia, and cell edges in fibroblasts or myoblasts. Biochemical assays demonstrated prolonged activation of Rac and Cdc42 in C2C12 cells adherent to TSP-1 and activation of the downstream kinase p21-activated kinase (PAK). Expression of dominant-negative Rac or Cdc42 in C2C12 myoblasts blocked spreading and formation of fascin spikes on TSP-1. Spreading and spike assembly were also blocked by pharmacological inhibition of F-actin turnover. Shear-loading of monospecific anti-fascin immunoglobulins, which block the binding of fascin to actin into cytoplasm, strongly inhibited spreading, actin cytoskeletal organization and migration on TSP-1 and also affected the motility of cells on fibronectin. We conclude that fascin is a critical component downstream of Rac and Cdc42 that is needed for actin cytoskeletal organization and cell migration responses to thrombospondin-1.

Key words: cell adhesion • cell motility • cytoskeleton • fascin • small G proteins

Introduction

Dynamic structural rearrangement of the cortical actin cytoskeleton is fundamental to many cellular activities, including adhesion, migration, polarization, and mitosis. Within the tissues of metazoan organisms, regulatory extracellular cues in the form of extracellular matrix (ECM)[1] or soluble signaling molecules are transduced intracellularly by ligation of specific receptors to regulate the three-dimensional organization of cytoskeletal polymers (reviewed by Yamada and Miyamoto, 1995; Lauffenburger and Horwitz, 1996). A key question in molecular cell biology is to understand the mechanisms by which these extracellular cues are coupled to the integrated organization of cytoskeletal structure.

Thrombospondin-1 (TSP-1) is a multifunctional macromolecule that is expressed in organogenesis and during active remodelling of adult tissues, such as occurs in inflammatory response, wound healing, angiogenesis, or the establishment of tumors (Raugi et al., 1987; Watkins et al., 1990; Raivich et al., 1998, reviewed by Adams et al., 1995). At the cellular level, TSP-1 directly supports cell adhesion and migration and also has modulatory effects on adhesive and migratory behaviors in the context of a complex, organized ECM. These effects are mediated by the binding of TSP-1 to an array of cell-surface receptors. The adhesive receptors in different cell types include CD36, CD47/integrin-associated protein, integrin αvβ3 and proteoglycans (reviewed by Adams et al., 1995; Bornstein, 1995). For many cell types, attachment to TSP-1 is coupled to cell spreading, which correlates with a distinctive organization of the cortical actin cytoskeleton involving assembly of radial spikes which contain F-actin and the actin-bundling protein fascin (Adams, 1995, 1997). These structures are also present at the leading edge of polarized cells and growth cones and have been functionally implicated in cell

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[1]*Abbreviations used in this paper:* ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; PAK, p21-activated kinase; PBD, p21-binding domain; TSP-1, thrombospondin-1.

migration (Edwards and Bryan, 1995; Adams 1997). Thus, the molecular mechanisms that regulate spike formation are likely to be important for the physiological roles of TSP-1 in processes such as wound healing, which depend on coordinated cell adhesion, contact, and migration.

In consideration of factors that regulate F-actin polymerization and organization, it is well-established that members of the Rho family of small guanine nucleotide-binding proteins have major effects on actin-containing structures (reviewed by Hall, 1994, 1998). Rac, Cdc42 and Rho are each required in the assembly of morphologically distinct F-actin structures. Activation of Rac leads to formation of circumferential lamellipodia; activation of Cdc42 in subconfluent cells results in extension of mobile filopodia and activation of Rho protein is required in stress fibre formation and cell contractility (reviewed by Hall, 1994, 1998; Burridge and Chrzanowska-Wodnicka, 1996). Rac and Cdc42 also induce formation of small focal adhesions at cell margins termed focal complexes (Kozma et al., 1995; Nobes and Hall, 1995). Recent data indicate that the functional properties of each GTPase likely arise by the recruitment and activation of distinct sets of effector proteins (Dharmawardhane and Bokoch, 1997; Boettner and Van Aelst, 1999; Kaibuchi et al., 1999).

The fascin-containing structures formed by cells adherent to TSP-1 may appear as individual filopodial projections or as ribs within thin cytoplasmic sheets (Adams, 1995, 1997) and thus resemble both Rac- and Cdc42-dependent actin structures. Therefore, we tested the hypothesis that formation of fascin spikes involves Rho family GTPases. We report here that formation of fascin spikes in response to TSP-1 correlates temporally with activation of Rac and Cdc42 and requires their activation, but does not depend on the activation of Rho. The ability of cells to adhere, migrate and organize spikes in response to TSP-1 depends both on the activity of these GTPases and on the coherent organization of F-actin by fascin. This dependence on the interaction of fascin with F-actin in cells adherent to TSP-1 contrasts with a more limited requirement for fascin in cell adhesion to fibronectin. These novel results establish fascin as a functionally important target of Rac and Cdc42 and demonstrate that TSP-1 is a physiological extracellular activator of these cellular pathways.

Materials and Methods

Cell Microinjection

NIH 3T3 cells were plated overnight on glass coverslips in DME containing 2% calf serum, then serum starved in 0.5% calf serum for 24 h. Cells within a premarked area of the coverslip were microinjected with expression plasmid DNA at 0.05 μg/ul for activated constructs or 0.2 μg/ul for dominant-negative constructs, plus 1 mg/ml rabbit IgG as a marker. Cells expressing activated GTPases were incubated for 2–4 h and cells with dominant-negative GTPases were incubated for 4–6 h, before fixation in either 3.7% formaldehyde or in –20°C absolute methanol.

Immunofluorescence Microscopy

Expression of fascin by NIH and Swiss 3T3 fibroblasts was confirmed by Western blot using polyclonal antiserum FAS-C to fascin (Adams et al., 1999). Indirect immunofluorescent staining for fascin or vinculin was carried out as previously described (Adams, 1997) using FITC-conjugated anti–mouse secondary antibody. Rhodamine-conjugated anti–rabbit IgG (Sigma-Aldrich) was included during the second antibody step for identification of injected cells. Double labeling for focal contacts and F-actin used VIN 11.5 monoclonal antibody to vinculin (Sigma-Aldrich) with FITC-conjugated secondary antibody and rhodamine-phalloidin. To express a fascin-GFP fusion construct, human fascin cDNA in the pCDNA3 vector (prepared as described in Adams et al., 1999) was subcloned in frame into the EcoR1 site of pEGFP vector (CLONTECH Laboratories, Inc.). The polypeptide expressed from this construct contains enhanced green fluorescent protein (EGFP) fused NH_2-terminal to fascin by the linker peptide sequence YSDLELKLRIRGPRQPRSGLSSTAT. Double labeling for initial focal contacts used 4G10 monoclonal antibody to phosphotyrosine-containing proteins (Upstate Biotechnology) with Alexa 546-conjugated secondary antibody (Molecular Probes). Samples were viewed by epifluorescence using a Zeiss Axioplan microscope and photographs were taken on Kodak Tmax 100 film.

Transient Transfection and Adhesion Assays

Sparse cultures of C2C12 myoblasts were transfected with 1 ug of the expression plasmids pEGFP (CLONTECH Laboratories, Inc.), pEGFP-fascin or with a 1:4 mixture of pEGFP/fascin plus either pCMV6 expression plasmid encoding T19N Rho-myc, T17N Rac-myc, or T17N Cdc42-myc using Superfect according to the manufacturer's instructions (QIAGEN). 48 h after transfection the cell populations were used in TSP-1 adhesion assays as described previously (Adams, 1995). To quantitate the ability of transfected cells to attach to TSP-1, drops of the cell suspensions used for the adhesion assays were dried onto glass coverslips and scored for the percentage of EGFP-positive cells. In replicate experiments, the percentage of transfected cells was between 19 and 27%. To quantitate any effects of GTPase expression on cell attachment, this fraction was compared with the percentage of EGFP-positive cells in the TSP-1–adherent population. Adherent GTPase plasmid transfected cells were also identified by indirect immunofluorescence by use of mouse monoclonal antibody 9E10 against the myc epitope tag (Evan et al., 1985) and costained with TRITC-phalloidin (Sigma-Aldrich) for examination of F-actin organization. Replicate samples were scored in each experimental condition. Seven independent experiments were carried out for each transfection condition. For experiments in which cells were pretreated with 0.4 μM jasplakinolide for 60 min (Molecular Probes) or with 0.5 μM latrunculin B for 30 min (Calbiochem-Novabiochem), the compounds were first tested at a range of concentrations between 0.1 and 1 μM for their activity in affecting F-actin organization in the absence of cell toxicity as determined by trypan blue exclusion. The concentrations chosen for the adhesion assay experiments represented the minimal concentrations needed to achieve clear effects on F-actin organization.

Detection of Active Rac and Cdc42 by Affinity-binding Assay

C2C12 cells were used in adhesion assays on TSP-1 or fibronectin substrata, as described above. Pull down assays with the effector binding domain from PAK (GST-PBD) were performed essentially as described (Manser et al., 1994; Bagrodia et al., 1998). For identification of active Rac, cells were washed twice in ice-cold PBS and lysed in a buffer containing 0.5% NP-40, 50 mM Tris-HCl, 500 mM NaCl, 1 mM $MgCl_2$, and a protease inhibitor cocktail. For extraction of active Cdc42, cells were lysed in buffer containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 10 mM $MgCl_2$ and protease inhibitor cocktail. Extracts were clarified by centrifugation and mixed on ice for 30 min with PBD-GST in the presence of glutathione-agarose beads (Sigma-Aldrich). The beads were then pelleted by centrifugation, washed three times in the appropriate lysis buffer and the bound proteins eluted in SDS-PAGE sample buffer. Proteins were resolved on 13% polyacrylamide gels under reducing conditions, transferred to nitrocellulose and the blots probed sequentially with antibodies to Rac or Cdc42 (Transduction Labs or Santa Cruz Biotechnology, Inc.) using ECL detection. Amounts of Rac or Cdc42 which bound to PBD-GST were normalized relative to the total amounts of Rac or Cdc42 present in the cell extracts by scanning densitometry. Activity levels in adherent cells were compared with those in C2C12 cells suspended for 90 min at 37°C in BSA-blocked dishes.

PAK Immunoprecipitation and Kinase Assay

C2C12 cells were suspended in serum-free DMEM and plated on BSA-blocked bacteriological plastic dishes precoated with 30 μg/ml fibronectin

or TSP-1 for the indicated times. Cells were rinsed three times in ice-cold PBS, lysed on ice in 0.5% NP-40 buffer containing a cocktail of protease and phosphatase inhibitors, clarified by centrifugation, and snap-frozen in dry ice. Suspended cells were maintained in DMEM on uncoated, BSA-blocked dishes. Protein concentrations in the lysates were determined by the Bradford assay. The p65 p21-activated kinase (PAK) was immunoprecipitated and its kinase activity assayed as described in Price et al. (1998) using an in-gel kinase assay with myelin basic protein as the substrate.

Shear-Loading

Shear-loading of cells with immunoglobulin was carried out using an adaptation of the method of Clark and McNeil (1992). In brief, single cell suspensions in serum-free medium containing 2% wt/vol Pluronic-68 (Sigma-Aldrich) and immunoglobulin at concentrations between 0.2 and 1 mg/ml were passed through a 25-gauge needle by 4 strokes of a 1-ml syringe. Pilot experiments using FITC-conjugated IgG established that ~95% of cells became loaded and that cell viability was minimally affected as determined by trypan blue exclusion. The antibodies used were all purified IgG fractions of rabbit sera. Nonimmune IgG was from Sigma-Aldrich; the anti-fascin IgGs were FAS-C (Adams et al., 1999) and FAS-N, an antiserum raised against a synthetic peptide corresponding to residues 18–32 of human fascin (antiserum prepared at Zenaca-CRB). The FAS-C and FAS-N epitopes are 100% conserved between human and mouse (Edwards and Bryan, 1995). Preimmune and immune IgG fractions were prepared for both FAS-C and FAS-N, either on protein A–agarose or by use of an immunoglobulin purification kit (Amersham Pharmacia Biotech). In some experiments, the antibodies were preincubated with 15 μg/ml each of FAS-N and FAS-C peptides before shear-loading the mixture into cells. The nonimmune and preimmune IgGs did not react with proteins in C2C12 cells as determined by Western blot of cell extracts. Immune IgGs reacted specifically with fascin protein (Adams et al., 1999, and this study). Cells were used in adhesion assays and processed for immunofluorescence as described above. Time lapse videomicroscopy was carried out in a 37°C environmental chamber using a Zeiss Axiovert 100 microscope fitted with a Sony SS-M37OCE CCD camera linked to a video recorder and driven by a EOS BAC900 animation controller. Recordings were made at 5 frames/min. To calculate cell velocities, cell outlines were marked on traces made at 2-min intervals and the displacement of the cell centroid over time measured. Statistical significance was calculated using a two-tailed *t* test.

Metabolic Labeling and Immunoprecipitation

C2C12 cells were plated at 1.5×10^6 cells per 90-mm plate in serum-containing medium for 5 h. The medium was then changed to DME containing 5% dialysed fetal calf serum and 80 uCi/ml [^{35}S]methionine. After 18 h incubation, the cells were washed in ice-cold PBS and lysed for 20 min on ice in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM PMSF, and Complete protease inhibitor cocktail (Boehringer Mannheim). Lysates were clarified by centrifugation at 14,000 rpm for 5 min. Lysates corresponding to 5×10^5 cells were incubated with 2 μg of purified immunoglobulins for 2 h on ice and then mixed end-over-end with 40 μl of a 1:1 (vol/vol) suspension of protein A–Sepharose for 40 min at 4°C. Beads and immune complexes were washed once in PBS containing 0.5% Triton X-100, 0.1% SDS, once in PBS containing 0.5% Triton X-100 and 0.5 M NaCl, and twice more in the first buffer. The pellets were resuspended in SDS-PAGE sample buffer containing 100 mM dithiothreitol, boiled, and the precipitated protein resolved on 12.5% polyacrylamide gels under reducing conditions. The gels were treated with Amplify (Amersham Pharmacia Biotech), dried, and autoradiographed at –70°C.

Blot Overlay Assay for Fascin-Actin Binding

Samples of 5 ug/lane of rabbit skeletal muscle actin (Sigma-Aldrich) were resolved on 12% polyacrylamide gels and transferred to nitrocellulose. The protein was renatured on the membrane by sequential 5-min washes in 10 mM Hepes, pH 7.5, 60 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol containing stepwise decreasing concentrations of guanidinium.HCl from 6 M down to 0.1 M. After blocking for 1 h at 4°C in the above buffer containing 5% milk, the membrane strips were incubated overnight with radiolabeled fascin protein prepared by in vitro translation from the pCDNA3 expression vector (Promega TNT kit), in the presence of immunoglobulins plus or minus FAS-N and FAS-C peptides. After washing three times in buffer, binding of fascin to actin was detected by autoradiography using Kodak Biomax film.

Results

Activated Rac and Cdc42, but Not Rho, Stimulate Cortical Fascin Spike Formation

In pilot experiments designed to explore whether small GTPases might have a role in fascin spike formation, C2C12 myoblasts were treated for 18 h with lovastatin, an inhibitor of isoprenylation which prevents plasma membrane association and thereby functionality of small GTPases (Fenton et al., 1992). Cells were then tested for their ability to form spikes on TSP-1 substrata. Whereas control cells spread and formed arrays of spikes, lovastatin-treated cells remained round without detectable spike formation (not shown). This result suggested that an active GTPase is required for assembly of fascin spike structures. The roles of Rho family GTPases were therefore studied using more specific reagents.

To determine directly whether activation of a small GTPase can promote the organization of fascin spikes, we first assayed the effects of expressing constitutively active GTPases in fibroblasts. Serum-starved adherent NIH 3T3 cells on glass coverslips were microinjected with plasmids encoding constitutively active forms of Rho (Q63L RhoA), Cdc42 (Q61L Cdc42Hs), or Rac (Q61L Rac1) and examined for fascin organization after 2–4 h. Nonimmune rabbit IgG was included in the injection solution to facilitate subsequent identification of the injected cells. Since activation of Rac and Rho can occur as a consequence of Cdc42 activity (Hall, 1998), the Cdc42 plasmid was coinjected with a plasmid encoding dominant-negative Rac (T17N Rac1). In all cases, characteristic effects on cell morphology were detectable by phase contrast microscopy (data not shown).

Uninjected serum-starved NIH 3T3 cells typically contained moderate numbers of focal contacts. Weak but detectable localization of fascin to filamentous structures was noted under these conditions, which likely corresponded to association of fascin with microfilament bundles (see double labeling in Fig. 4 below and Adams et al., 1999). The staining patterns appeared largely unaltered in cells microinjected with rabbit IgG (Fig. 1, A–C, and data not shown). Cells microinjected with the Q63L Rho plasmid contracted to a smaller area and developed large numbers of arrowhead-shaped focal contacts as expected (Fig. 1 D). Fascin staining in these cells was more diffuse than in controls though some cells showed small specks of staining at cell margins which did not correspond to focal contacts (Fig. 1 E, and data not shown).

In cells injected with the Q61L Cdc42 and T17N Rac plasmids, vinculin localized to isolated focal contacts (Fig. 1 F). The density of these contacts distinguished them from the vinculin-containing contacts assembled in response to activated Rho (Fig. 1, compare F with D). These cells showed fascin projections of various lengths from short spikes to elongated, single filopodia, also prominent marginal staining in which fascin appeared as thin, uniform lines or bands (example arrowed in Fig. 1 G). In spatially isolated cells, (i.e., those lacking cell–cell contacts),

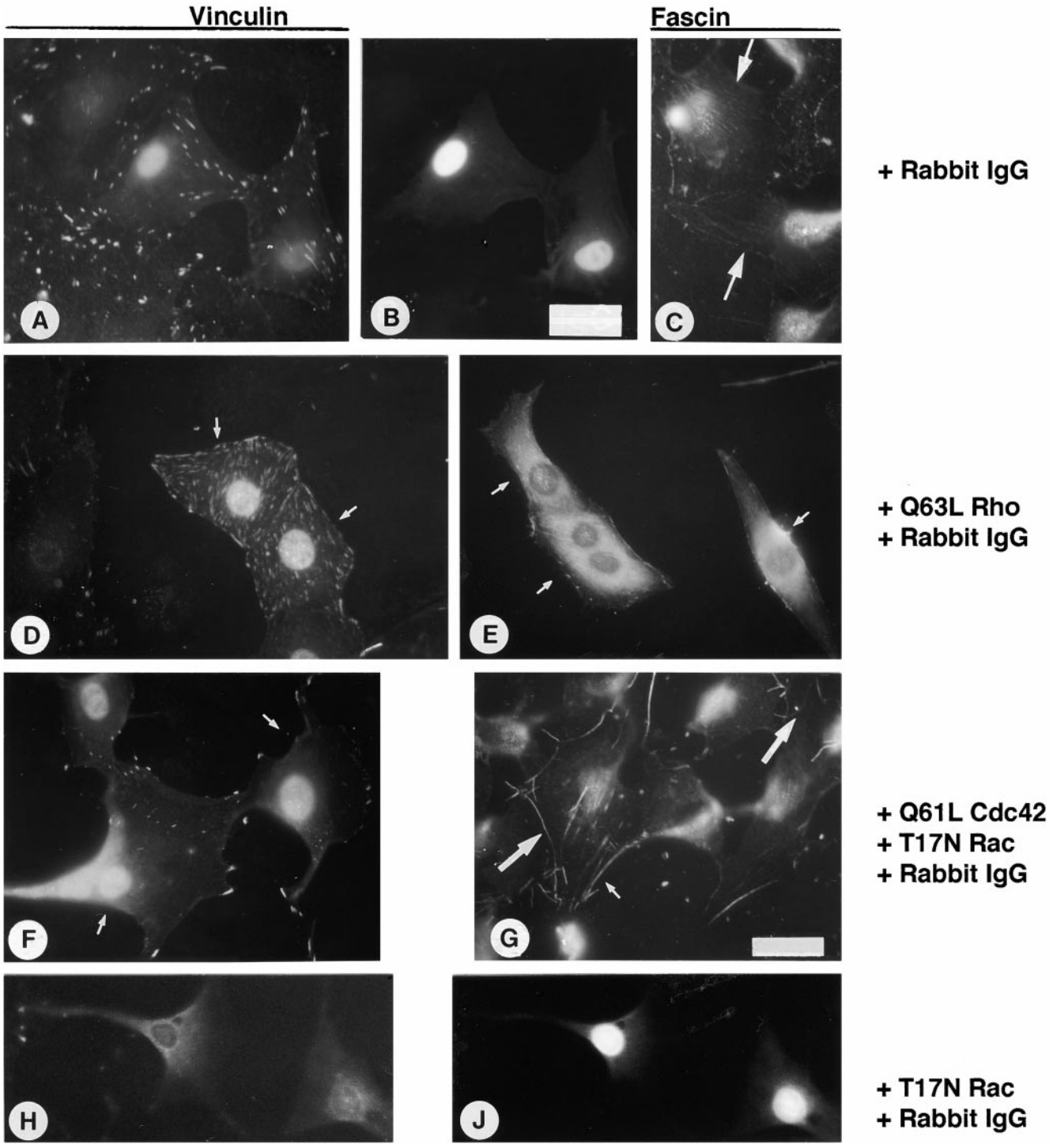


Figure 1. Effects of constitutively active Rho or Cdc42 on the localization of vinculin and fascin in quiescent fibroblasts. NIH 3T3 fibroblasts were stained with mouse monoclonal antibodies to vinculin (A, D, and F) or fascin (C, E, G, and H) 6 h after microinjection with rabbit IgG (A–C); IgG plus Q63L Rho plasmid (D and E); IgG plus Q61L Cdc42 and T17N Rac plasmids (F and G), or IgG plus T17N Rac plasmid alone (H and J). TRITC-anti–rabbit IgG staining was used to identify injected cells (exemplified by fields shown in A and B, H and J). Arrows in C–G indicate injected cells. Thin arrow in G indicates band of fascin at cell edge. Bars, 10 μm.

the fascin projections and lines were formed circumferentially. To establish that the assembly of these fascin-containing structures were due to the expression of active Cdc42, cells were also microinjected with the T17N Rac plasmid alone. In these cells, fascin remained diffuse and did not localize at cell margins (Fig. 1, H and J).

In cells expressing Q61L Rac, vinculin was concentrated in small focal contacts that presumably correspond to the previously described Rac-dependent focal complexes of Swiss 3T3 cells (Fig. 2 A). Cells that expressed Q61L Rac usually developed striking circumferential ribbons of fascin staining at the cell margins (Fig. 2 B). Closer inspection

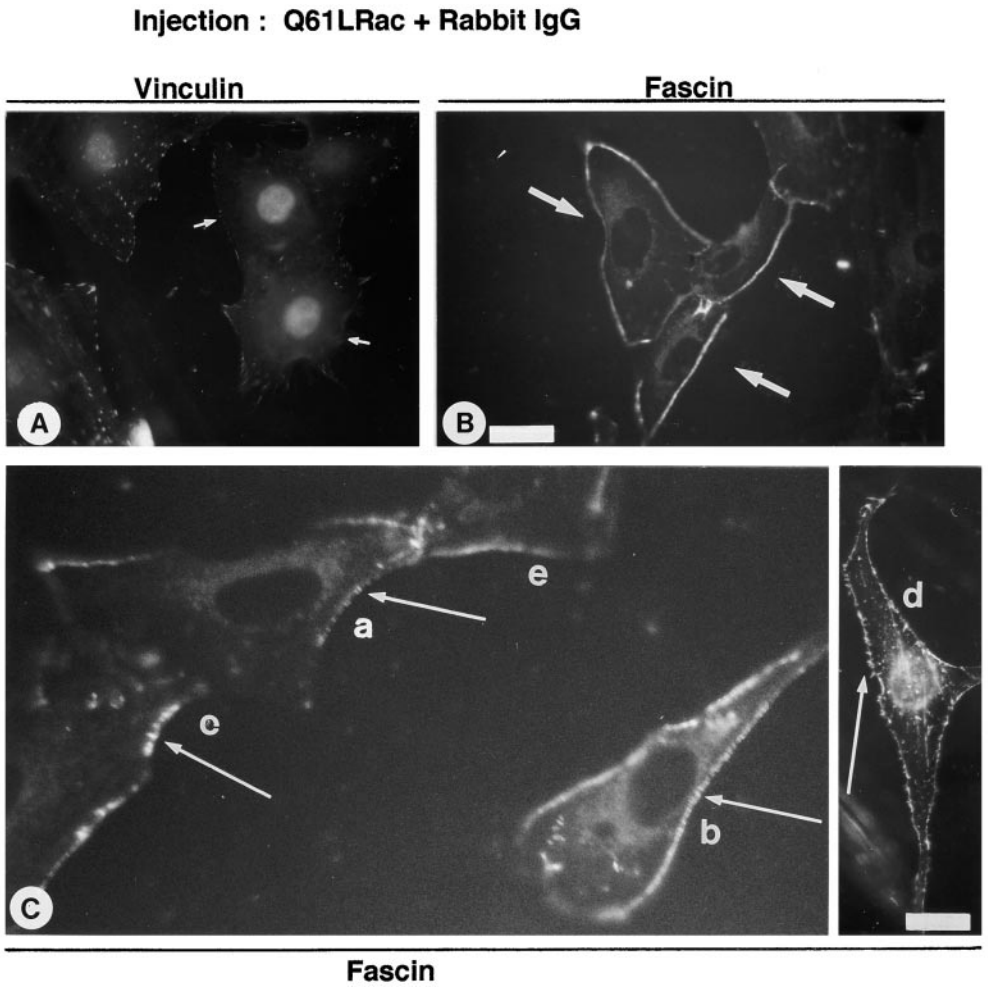


Figure 2. Effects of constitutively active Rac on the localization of vinculin and fascin in quiescent fibroblasts. NIH 3T3 fibroblasts were stained with mouse monoclonal antibodies to vinculin (A) or fascin (B and C) 6 h after injection with rabbit IgG plus Q61L Rac plasmid. The injected cells are indicated by arrows in each panel. Cells a and b in C illustrate different densities of the radial fascin staining intensities, cell c shows a cluster of short fascin projections and cell d illustrates the minority of cells which showed short projections rather than fascin ribbons as in B. Cell e in C is an injected cell not in the plane of focus. See text for further details. Bars: (A and B); 10 μm, (C) 5 μm.

at higher magnification revealed that the bands contained short, radial spoke-like intensities of staining (Fig. 2 C). Some cells showed a few disperse staining intensities (Fig. 2 C, cell a), in others the short spokes of fascin appeared more closely packed (Fig. 2 C, cell b). 18% of the injected cells did not show the ribbons of fascin staining but instead had short, individuated fascin projections at cell margins (Fig. 2 C, cells c and d). In some of these cells, the projections were present at cell margins and also on the apical cell surface (Fig. 2 C, cell d). These visual assessments of the activities of the three GTPases were substantiated by quantification of results pooled from five independent experiments (Table I). The results demonstrate that expression of constitutively active Cdc42 or Rac, but not Rho, leads to assembly of cortical fascin-containing structures. In this paper, we use spikes as a collective term for long and short fascin projections.

We wished to examine the spatial inter-relationships of fascin spikes and focal contacts, however, the fascin antibodies can only be used on methanol-fixed preparations which prevents use of available antibodies to focal adhesion components. We therefore undertook several types of colabeling to compare the localizations of actin projections, fascin and focal contacts. First, Q61L Cdc42/T17N

Table I. Effects of Constitutively Active GTPases on the Localization of Vinculin and Fascin in Matrix-adherent, Quiescent Fibroblasts

	Staining patterns relative to uninjected cells			
	Vinculin stain		Fascin stain	
Injection condition	% Injected cells with altered staining pattern	Predominant localization	% Injected cells with altered staining pattern	Predominant localization
IgG	12 (n = 24)	Small FC*	3 (n = 33)	On MF*
Q63L Rho + IgG	83 (n = 31)	Large FC	90 (n = 34)	Diffuse
Q61L Rac + Igg	73 (n = 31)	Sparse cortical FC	84 (n =55)	Cortical ribbons with short spikes
Q61L Cdc42 + T17N Rac + IgG	71 (n = 50)	Sparse FC	75 (n = 71)	Protrusions and marginal bands

*Localizations in IgG-injected cells principally resembled those of uninjected cells. 12% of IgG-injected cells had fewer FC, 3% displayed small cortical concentrations of fascin.

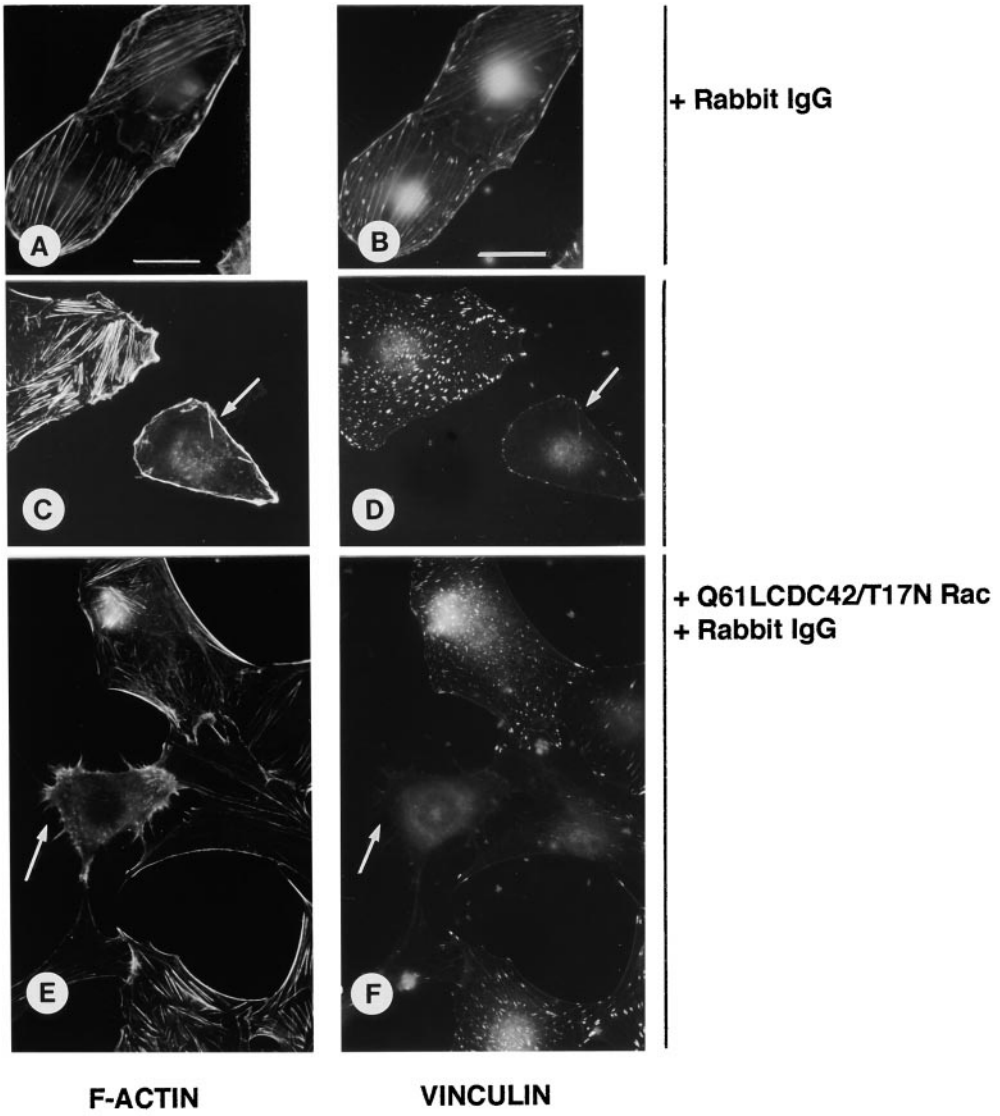


Figure 3. Spatial relationship of Q61L Cdc42-stimulated actin projections and focal contacts in fibroblasts. NIH 3T3 fibroblasts were injected with IgG (A and B) or IgG plus Q61L Cdc42 and T17N Rac plasmids (C–F) and double-stained 6 h later for F-actin (A, C, and E) and vinculin (B, D, and F). The large projection arrowed in C and D shows vinculin at the tip. Examples of actin projections which do not contain organized focal contacts are indicated by arrows in E and F. Bars, 10 μm.

Rac-injected cells fixed in formaldehyde were double stained for F-actin and vinculin. In comparison with uninjected or IgG-injected cells, microfilament organization in the central regions of the Q61L Cdc42/T17N Rac-injected cells was decreased and filopodial projections and spikes of various sizes were assembled (Fig. 3, A, C, and E). The Q61L Cdc42/T17N Rac–injected cells had low numbers of vinculin-containing focal contacts (Fig. 3, D and F, see also Fig. 1 F), a few of which colocalized with the F-actin in spikes (example arrowed in Fig. 3, C and D). However, the majority of projections did not show associated focal contacts (an example of a cell with many such projections is shown in Fig. 3, E and F). To examine the colocalization of fascin with actin projections, C2C12 cells coexpressing EGFP-fascin and Q61LCdc42 were plated on fibronectin and counterstained with phalloidin. These cells displayed filopodial projections of which all contained colocalized actin and fascin. Fascin also colocalized with microfilament bundles in the central regions of the cells (Fig. 4, A and B). Thus, the actin filament-containing projections regulated by Cdc42 also contain fascin but contain vinculin only occasionally.

To relate the distribution of fascin to the focal contact-type structures formed in response to constitutively activated Cdc42 and Rac, transiently transfected cells coexpressing EGFP-fascin with Q61LRac or Q61L Cdc42 were plated on fibronectin. Because fascin spikes and ruffles are normally formed transiently during cell spreading on fibronectin (Adams, 1995), cells were examined at early times of spreading, 30 min after plating. To maximize the detection of early focal contacts, antibodies to phosphotyrosine rather than to vinculin were used as a counterstain. At this time, Q61L Rac-expressing cells appeared more spread than control cells and showed small, dot-like focal contacts under the cell body and at scattered points along cell margins (Fig. 4 C). Fascin was located diffusely within the cell body and was also concentrated along the lamellipodial margin. Some points of coincidence with phosphotyrosine-containing dots were detectable, but in other areas fascin and phosphotyrosine localizations appeared distinct (Fig. 4 D). Cells expressing Q61L Cdc42 had irregular, protrusive morphologies and typically showed low numbers of focal contacts in comparison to control cells (Fig. 4, E and G). Phosphotyrosine was not detected in as-

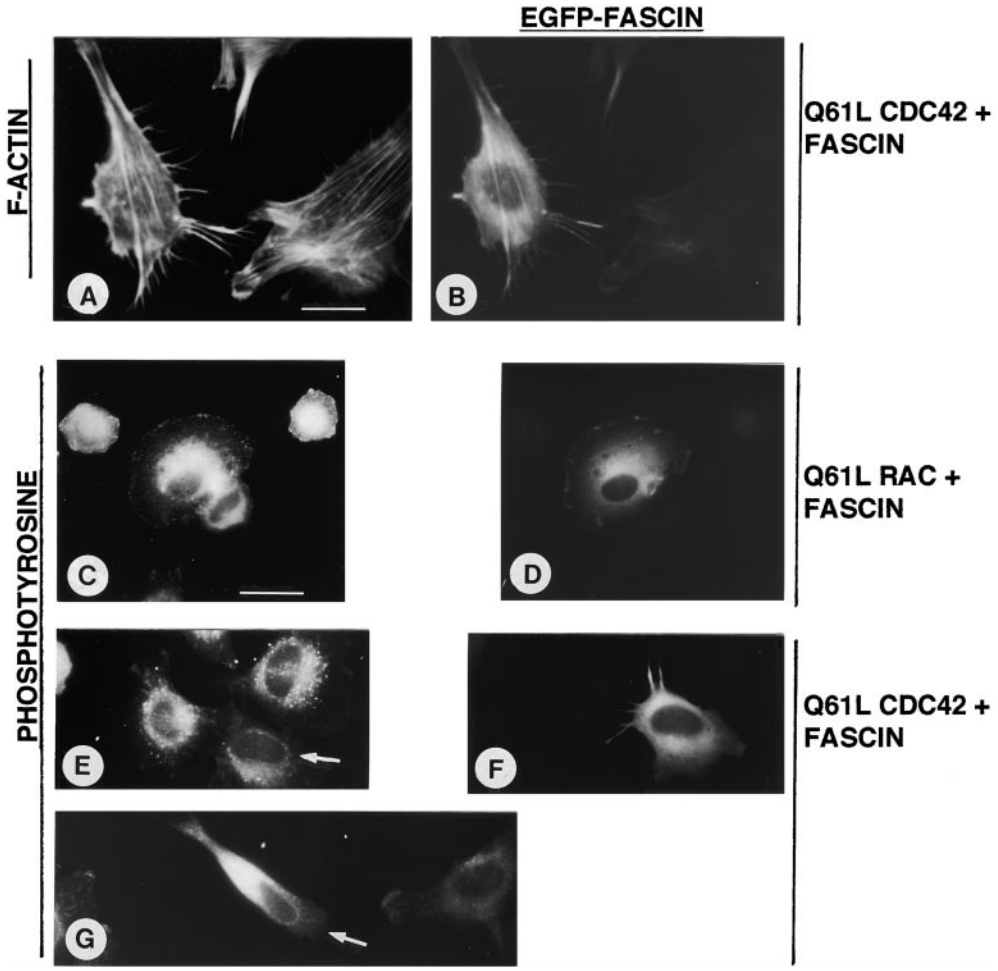


Figure 4. Distribution of fascin and phosphotyrosine-containing proteins in Cdc42 and Rac-stimulated cytoskeletal structures. C2C12 cells transiently cotransfected with Q61L Cdc42 and EGFP-fascin (A, B, E, F, and G) or Q61L Rac and EGFP-fascin (C and D) were plated on fibronectin for 60 min (A and B) or 30 min (C–G) and counterstained for F-actin (A) or phosphotyrosine (C, E, and G). Arrows in E and G indicate transfected cells. Bars: (A and B) 5 μm; (C–G) 10 μm.

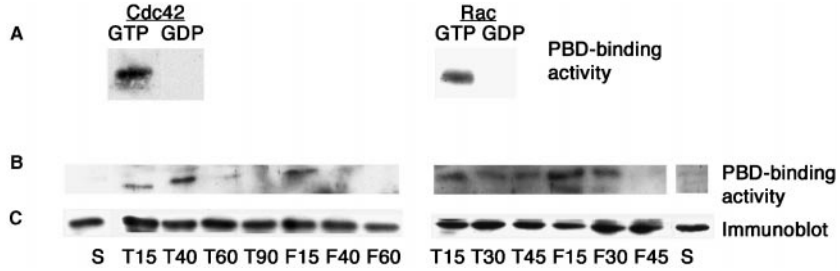
sociation with the protrusions, whereas fascin was readily observed (Fig. 4, E and F). We conclude that despite minor overlap in the distribution of fascin with initial focal contacts at the margins of Rac-dependent lamellipodia, fascin and focal adhesion components are largely distinct.

Adhesion to TSP-1 Activates Rac, Cdc42 and PAK

Cell adhesion to TSP-1 is a strong stimulus for fascin spike formation (Adams, 1995, 1997; Adams et al., 1999). Therefore, to further examine the roles of Rac and Cdc42 in formation of fascin spikes, we investigated whether Rac and Cdc42 are activated during cell adhesion on TSP-1. These experiments made use of an affinity-binding assay that reports active GTP-Rac and GTP-Cdc42 in cell lysates, based on the ability of GTP-bound, but not GDP-bound, Rac or Cdc42 to bind to the p21-binding domain (PBD) of the 65kD p21-activated kinase, PAK (Manser et al., 1994; Bagrodia et al., 1998; Fig 5 A). PBD binding assays were carried out on extracts of C2C12 cells plated on TSP-1 for times between 15 and 90 min. To relate the effects of TSP-1 to other matrix-adhesion conditions, activity levels were also examined in suspended or fibronectin-adherent cells. Whereas active Cdc42 was negligible in suspended cells, activity increased >25-fold over the first 30 min of adhesion to TSP-1 and remained elevated by fivefold after 60 min (Fig. 5, B and D). In contrast, Cdc42 activity was elevated by 18-fold after 15 min on fibronectin but decreased to negligible levels by 40 or 60 min (Fig. 5, B and D).

Active Rac was present at low levels in suspended cells (Fig 5 B), was elevated after 15 min attachment to TSP-1 (Fig. 5, B and E) and remained increased by ~15-fold in TSP-1–adherent cells at 30 and 45 min (Fig. 5, B and E). Low activity was detectable at 90 min (data not shown). A 25-fold increase in Rac activity was apparent after 15 min adhesion on fibronectin (Fig. 5, B and E) and a 10-fold increase at 30 min. Activity was negligible at 45 min (Fig. 5, B and E). These data establish that Rac and Cdc42 are activated by cell adhesion to TSP-1 and demonstrate extended periods of activity for both GTPases during the period in which cell spreading and spike formation on TSP-1 are taking place (Adams, 1995, and unpublished data).

As a further test of Rac and Cdc42 functionality in cell adhesion to TSP-1, we examined the phosphotransferase activity of PAK as a downstream effector of these GTPases. Although the role of PAK in GTPase signaling has been controversial, its activation should provide an assay for downstream activation of Rac and Cdc42-dependent pathways (reviewed by Knaus and Bokoch, 1998). PAK activity was measured in extracts prepared from C2C12 cells at various times throughout attachment and spreading on TSP-1. Kinase activity was very low in suspended



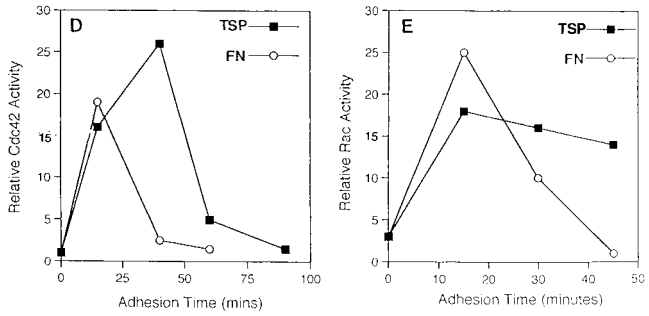


Figure 5. Activation of Rac and Cdc42 by matrix adhesion. (A) Specificity of PDB binding for GTP-Rac and GTP-Cdc42. (B) The activity status of Cdc42 and Rac was examined by PBD binding assay in Triton X-100 or NP-40 extracts, respectively, of C2C12 myoblastic cells after adhesion to TSP-1 (T) or FN (F) for the indicated times, or after suspension for 90 min in BSA-blocked dishes (S). (C) Rac or Cdc42 protein levels were determined by Western blot of cell extracts. (D and E) Quantitation of the stimulation of Cdc42 (D) or Rac (E) activities relative to suspended cells. Data were normalized according to Rac or Cdc42 protein levels. The data are representative of five independent experiments.

C2C12 cells (Fig. 6 A), but increased by 23-fold over the first 30 min of adhesion to TSP-1 and remained elevated for over 60 min (Fig. 6, A and B). These results provide biochemical evidence that an effector of Rac and Cdc42 is activated upon adhesion to TSP-1 in the period when Rac and Cdc42 are active.

Rac and Cdc42 Are Required in Fascin Spike Formation on TSP-1

To determine whether the activities of Rac or Cdc42 documented above are required for cell attachment and fascin spike formation on TSP-1, C2C12 cells were cotransfected with expression plasmids encoding myc-tagged dominant-negative forms of Cdc42, Rac or Rho, plus EGFP-fascin, and analyzed in TSP-1 adhesion assays 48 h after transfection after costaining with rhodamine-phalloidin. As expected, untransfected or mock-transfected cells attached, spread, and developed F-actin-containing sheets and spikes (Fig. 7, A and B). EGFP-fascin colocalized with F-actin in these regions of spike organization (Fig. 7, C and D). EGFP-fascin–expressor cells or cells expressing T19N Rho-myc (Fig. 7, E and F) formed spikes normally, whereas T17N Rac-myc expressors were less spread and exhibited disorganized F-actin–containing structures at cell margins (Fig. 7, G, H, and J). T17N Cdc42 expressors were also less spread than control cells and were smoother-edged than Rac-expressor cells, with peripheral concentrations of F-actin and fascin instead of spikes or other extensions (Fig. 7, K–M). Colocalization of EGFP-fascin with cortical F-actin was apparent under each experimental condition (Fig. 7). The same alterations in morphology in Cdc42 or Rac-expressor cells were apparent in cells costained for the myc-epitope tag and F-actin (data not shown). These data are quantitated in Table II and demonstrate that both Rac and Cdc42 are needed in spreading and the organized assembly of fascin spikes during myoblast adhesion to TSP-1. To determine whether the dominant-negative GTPases altered the ability of C2C12 cells to attach to TSP-1, EGFP-positive cells were quantitated in the starting population and in the TSP-1–adherent population (Fig. 8 A). Transfection with EGFP-fascin alone caused an ~10% decrease in plating efficiency which is most likely due to nonspecific effects of transfection. Expression of dominant-negative Rho had no

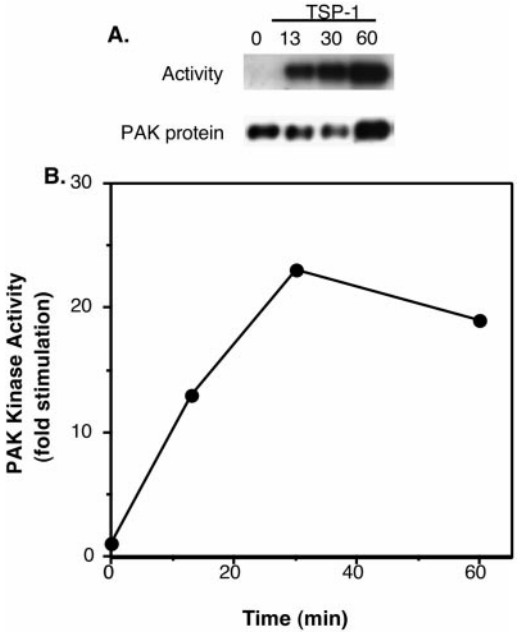


Figure 6. Activation of PAK by TSP-1 adhesion. C2C12 myoblastic cells were suspended for 60 min in BSA-blocked dishes (0 timepoint), or adhered to TSP-1 for times between 13 and 60 min. PAK activity was measured by in gel kinase assay and PAK protein level determined by Western blot (A). Stimulation of kinase activity relative to suspended cells was quantitated by image scanning and is presented normalized according to PAK protein levels (B). The data are representative of three independent experiments.

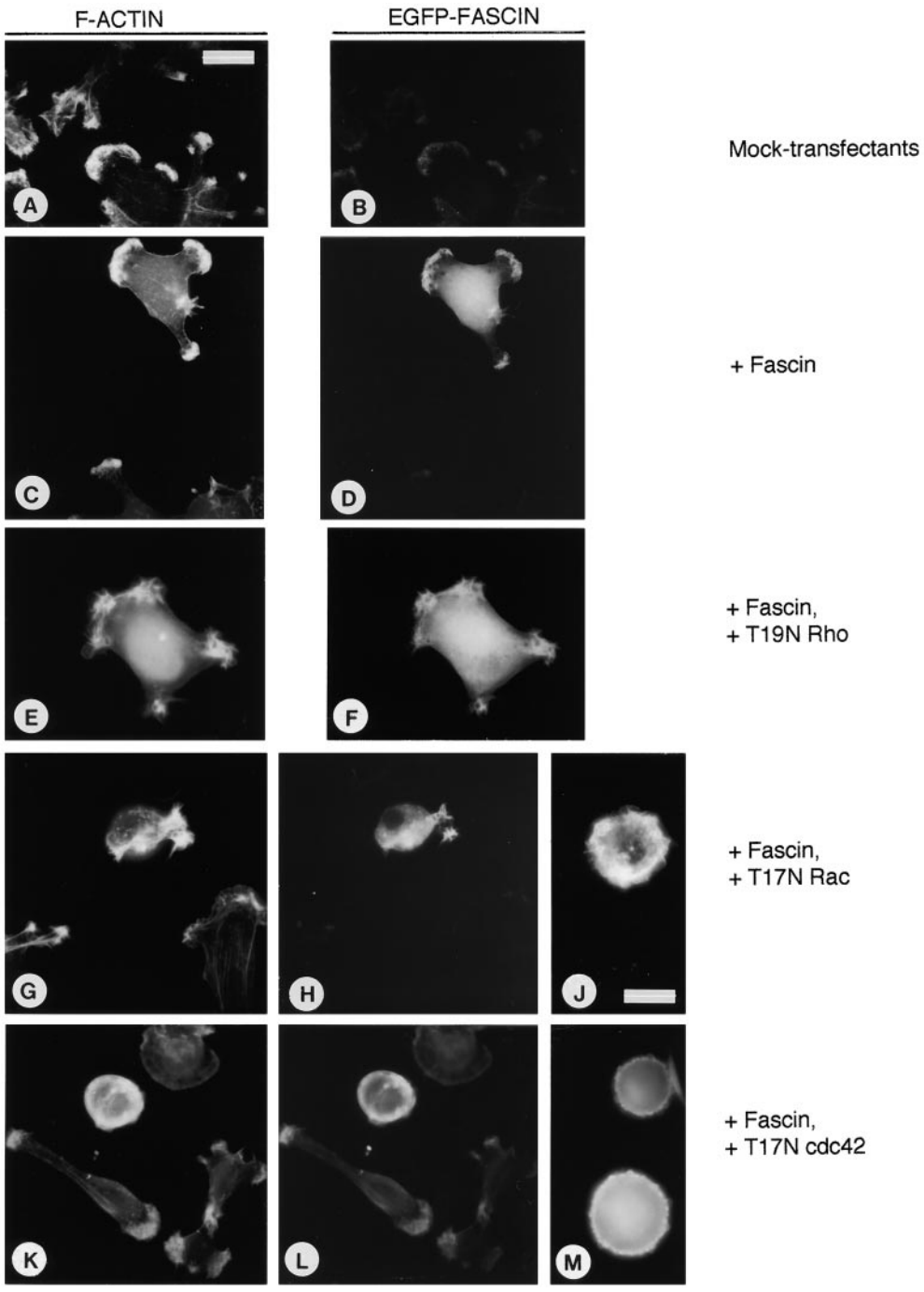


Figure 7. Effects of dominant-negative GTPases on fascin microspike formation by TSP-1-adherent C2C12 cells. Cells transiently transfected with pCMV expression vector (mock-transfectants; A and B), or expression vectors encoding EGFP-fascin alone (C and D), or T19N Rho plus EGFP-fascin (E and F), T17N Rac plus EGFP-fascin (G, H, and J) or T17N Cdc42 plus EGFP-fascin (K–M) were analyzed after 1 h adhesion to 50 nM TSP-1. Transfectants were identified by EGFP-fascin expression and actin organization was determined by TRITC-phalloidin staining. The results shown are representative of those obtained in three independent experiments. Bars: (A, B, G, H, K, and L) 10 μm; (C, D, E, F, J, and M) 5 μm.

further effect. By contrast, expression of either dominant-negative Rac or Cdc42 led to a 40% (T17N Rac) or 55% (T17N Cdc42) decrease in cell attachment (Fig. 8 A). These effects are most likely due to the decrease in spreading seen with dominant-negative Rac and Cdc42 since poorly spread cells tend to detach more readily during washes.

Two types of experiments were carried out to gain further insight into the mechanisms of action of Cdc42 and Rac in spike formation. First, the effects of constitutively active forms of the GTPases on C2C12 attachment or spike formation on TSP-1 were examined. We reasoned that if there were a requirement for normal guanine nucleotide cycling in normal spike assembly, cells expressing the constitutively active proteins might show significant differences in spike morphology to control cells. C2C12 cells expressing either Q61L Cdc42 or Q61L Rac were not altered in their ability to attach to TSP-1 (Fig 8 A). However, cells expressing Q61L Cdc42 typically showed a reduction in spreading and developed fine fascin projections around the periphery (Fig. 8 B). Cells expressing Q61L Rac also appeared less spread with circumferential, irregular fascin-rich lamellipodia rather than the organized arrays of fascin and actin ribs and spikes seen in untransfected or vector-

Table II. Effects of Dominant-negative GTPases on Spike Formation in TSP-1–adherent C2C12 Cells

Treatment	% Transfected cells with spikes at 1 h on TSP-1
	mean ± SEM
Mock transfection	93 ± 5 (n = 400)
T19N Rho-myc	85 ± 11 (n = 180)
T17N Rac-myc	12 ± 8 (n = 230)
T17N Cdc42-myc	7 ± 6 (n = 255)

Data pooled from seven independent experiments.

transfected TSP-1–adherent cells (Fig. 8 B). Thus, constitutive activation of either GTPase adversely affected cell spreading and the organization of cortical F-actin and fascin structures. These results suggest that proper coordination of Rac and CdC42 are required for efficient cell spreading and cytoskeletal organization. The data also show that C2C12 cells spreading on TSP-1 have different requirements for GTPase activation than do quiescent 3T3 cells which are already well spread on their endogenous matrix.

Second, with regard to the roles of Rho family GTPases in F-actin organization, the ability of C2C12 cells to form spikes was examined after treatment with pharmacological inhibitors of actin filament turnover. Latrunculin B inhibits its F-actin polymerization by binding and sequestration of G-actin monomers (Coue et al., 1987). Latrunculin B–treated cells plated on TSP-1 were completely blocked in spreading and showed no spatial organization of F-actin other than a few marginal patches (Fig. 9, A and B). Fascin appeared diffuse throughout the cell body (data not shown). Adhesion to fibronectin also depends on Rho family GTPases (Clark et al., 1998; Price et al., 1998) and in comparison, the latrunculin B–treated cells maintained partial spreading on fibronectin and contained unusual F-actin patches and F-actin–rich cell margins rather than organized microfilament bundles (Fig. 9, C and D). Jasplakinolide binds and stabilizes F-actin and thus inhibits cellular events that depend on filament turnover (Bubb et al., 1994; Sheikh et al., 1997). Jasplakoline-treated cells remained competent to attach to fibronectin, but did not undergo spreading (Fig. 9 E) and were strongly inhibited in attachment to TSP-1. Cells that did attach (<10% of the input population) remained completely round (data not shown). Thus, cell attachment, spreading and fascin spike assembly on TSP-1 are crucially dependent on the exchange activities of Rac and Cdc42 and on F-actin dynamics.

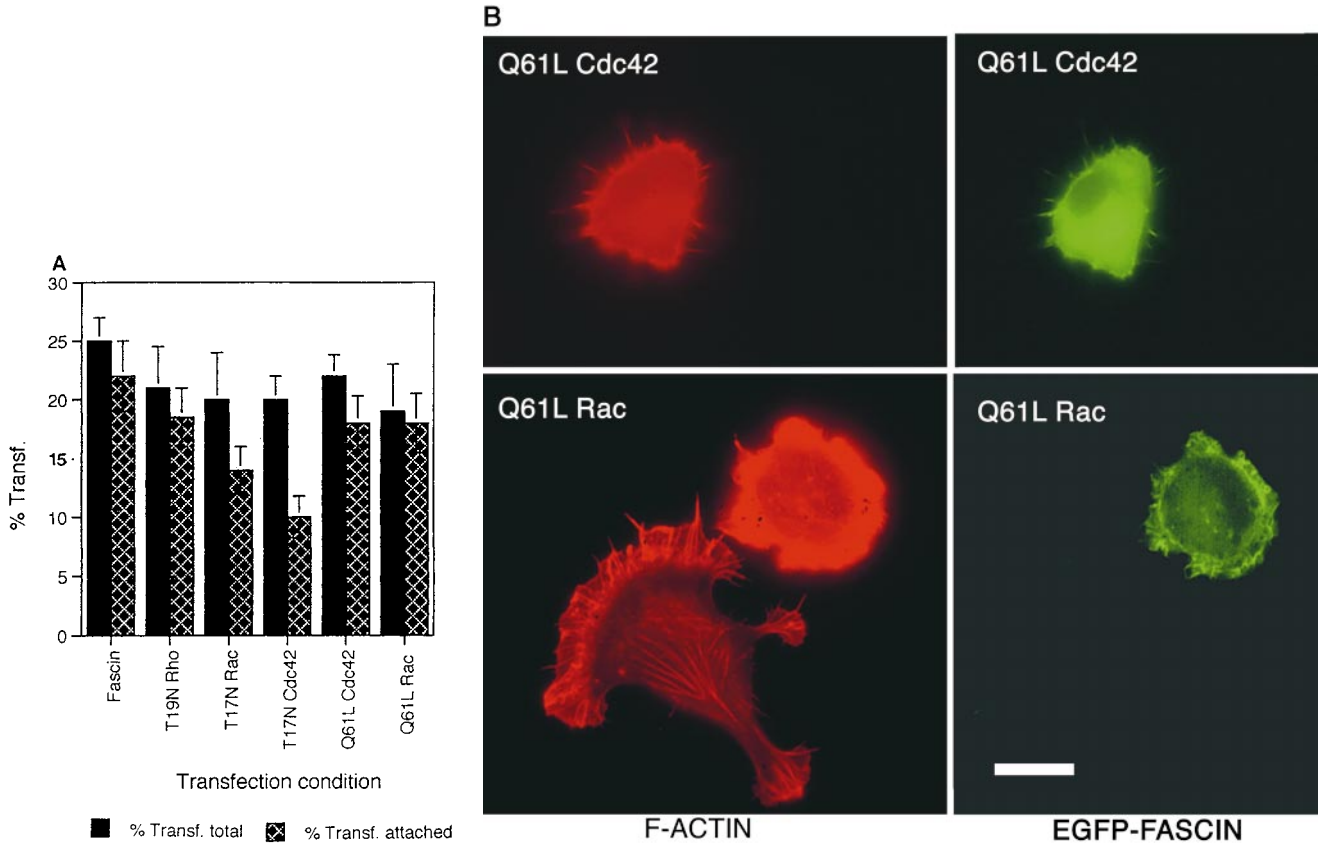


Figure 8. Effects of Rac or Cdc42 constructs on fascin spike formation by TSP-1–adherent C2C12 cells. (A) Effects of dominant-negative or constitutively active GTPases on cell attachment to TSP-1 at 1 h. The graph shows the mean ± SEM of data from three independent experiments and compares the percentage of transfected cells which attached to TSP-1 with the percentage of transfectants in the starting populations. (B) Cytoskeletal organization in C2C12 cells overexpressing Q61L Cdc42 or Q61L Rac. Cells were stained with TRITC-phalloidin after 1 h of adhesion to TSP-1 and positive cells were identified by EGFP-fascin fluorescence. The results shown are representative of those obtained in three independent experiments. Bar, 5 μm.

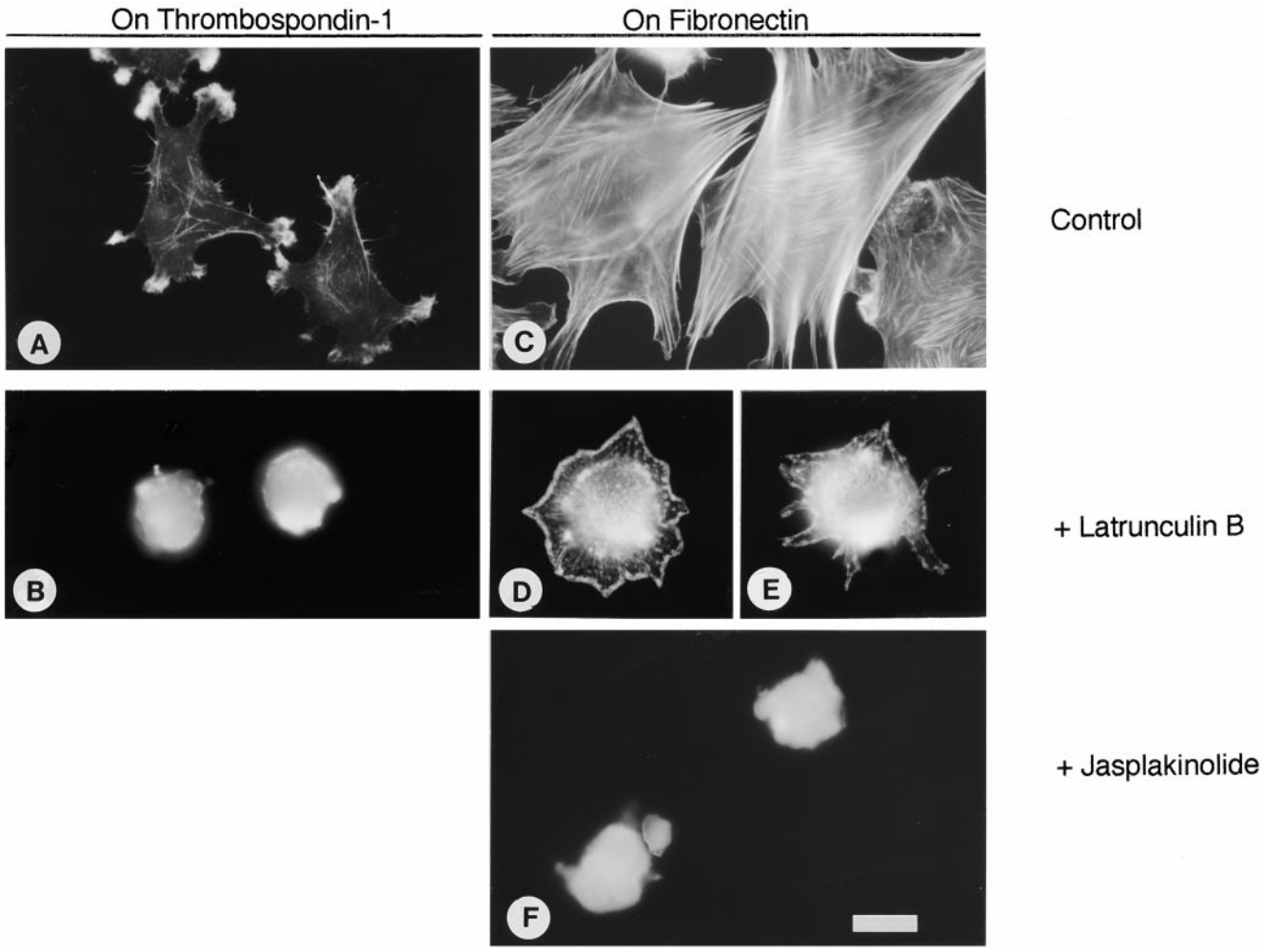


Figure 9. Effects of pharmacological inhibition of actin turnover on cell-matrix spreading and fascin spike formation. C2C12 cells were stained with TRITC-phalloidin after 1 h adhesion to TSP-1 (A and B) or fibronectin (C–F). Control cells (A and C) were compared with cells pretreated with 0.5 μm latrunculin B for 60 min (B and D) or 0.5 μM jasplakinolide for 30 min (E). Bar, 5 μm.

Effects of Fascin Antibodies on Cell Adhesion and Cytoskeletal Organization

Our results describe roles for Rac or Cdc42 in regulating cell adhesion and assembly of fascin spike structures in response to TSP-1. The stable organization of fascin spikes appears intimately linked with cell spreading on TSP-1 (Adams, 1995, and data above), and so, to test the functional significance of fascin spikes in cell adhesion and motility on TSP-1, monospecific anti-fascin IgGs were used to perturb the intracellular interaction of fascin with actin. Two actin-binding sites have been delineated within the fascin molecule, a motif proximal to the NH_2 terminus with similarity to the actin-binding site of MARCKS and a site located within the 27- or 30-kD COOH-terminal tryptic fragments of fascin (Mosialos et al., 1994; Edwards and Bryan, 1995, Yamakita et al., 1996; Ono et al., 1997). The highly conserved peptide sequences used to generate the FAS-N and FAS-C antisera lie within these two regions (Fig. 10 A). The FAS-C reagent has been characterized previously (Adams et al., 1999) and both FAS-N and FAS-C immunoglobulin fractions immunoprecipitated native cellular fascin from radiolabeled cell extracts. These activities were blocked by preincubation of antibody with the cognate immunizing peptide (Fig 10 B). The effects of FAS-N and FAS-C on the ability of fascin to bind to actin were determined directly by a blot overlay assay which reports the direct binding of fascin to actin (Fig. 10 C, lane 2). This interaction was not prevented by addition of nonimmune immunoglobulin, either in the presence or absence of FAS-N and FAS-C peptides (Fig. 10 C, lanes 3 and 4), whereas incubation with FAS-N immunoglobulin, FAS-C immunoglobulin or a mixture of both FAS-N and FAS-C IgGs inhibited the fascin-actin interaction (Fig. 10 C, lanes 5–7). Preincubation of the FAS-N plus FAS-C IgG mixture with the FAS-N and FAS-C peptides blocked the inhibitory activity of the antibodies (Fig. 10 C, lane 8).

The immunoglobulins were next introduced into the cytoplasm of C2C12 cells by shear-loading. Cells loaded with nonimmune or preimmune rabbit IgGs adopted the characteristic organization of F-actin microspikes when adherent to TSP-1 (Fig. 11 A and data not shown). The majority of cells loaded with FAS-N IgG was inhibited in micro-

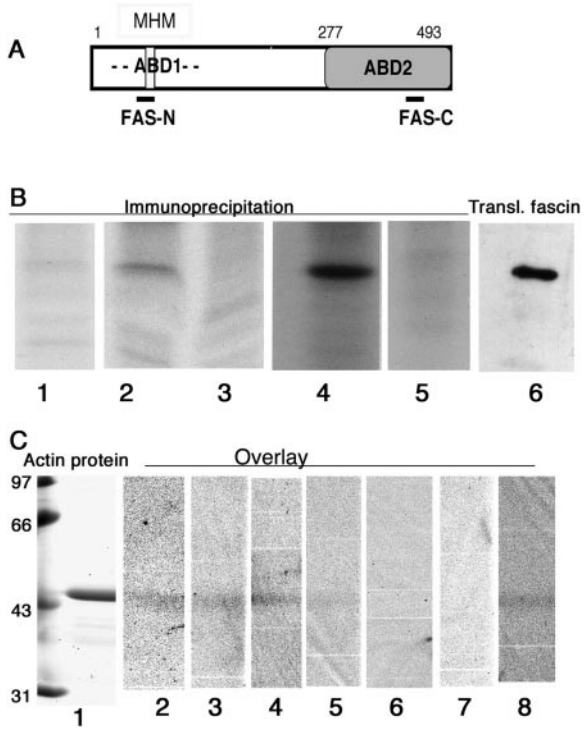


Figure 10. Characterization of anti-fascin immunoglobulins. (A) Schematic diagram of the fascin molecule showing the two actin-binding domains (ABD1 and ABD2), the MARCKS homology motif (MHM) and the locations of the synthetic peptides used to produce fascin polyclonal immunoglobulins. (B) Immunoprecipitation of fascin from metabolically labeled cell extracts by FAS-N and FAS-C immunoglobulins. Lane 1, mixed preimmune FAS-N and FAS-C immunoglobulins; lane 2, FAS-N IgG; lane 3, FAS-N preincubated with 10 ug of FAS-N peptide; lane 4, FAS-C IgG; lane 5, FAS-C IgG preincubated with 10 μg of FAS-C peptide; lane 6, in vitro translated fascin. (C) Effect of the antibodies on the binding of fascin to actin in a blot-overlay assay. Lane 1, 15 μg actin stained with Coomassie blue; lanes 2–8, autoradiographic detection of fascin bound to actin on nitrocellulose membrane. 5 μg of actin was loaded per lane. Overlay conditions were fascin only (lane 2), fascin plus mixed preimmune IgGs (lane 3), fascin plus mixed preimmune IgGs plus 10 μg each FAS-N and FAS-C peptides (lane 4), fascin plus FAS-N IgG (lane 5), fascin plus FAS-C IgG (lane 6), fascin plus FAS-N and FAS-C IgGs (lane 7) and fascin plus FAS-N and FAS-C IgGs plus 10 μg each of FAS-N and FAS-C peptides (lane 8).

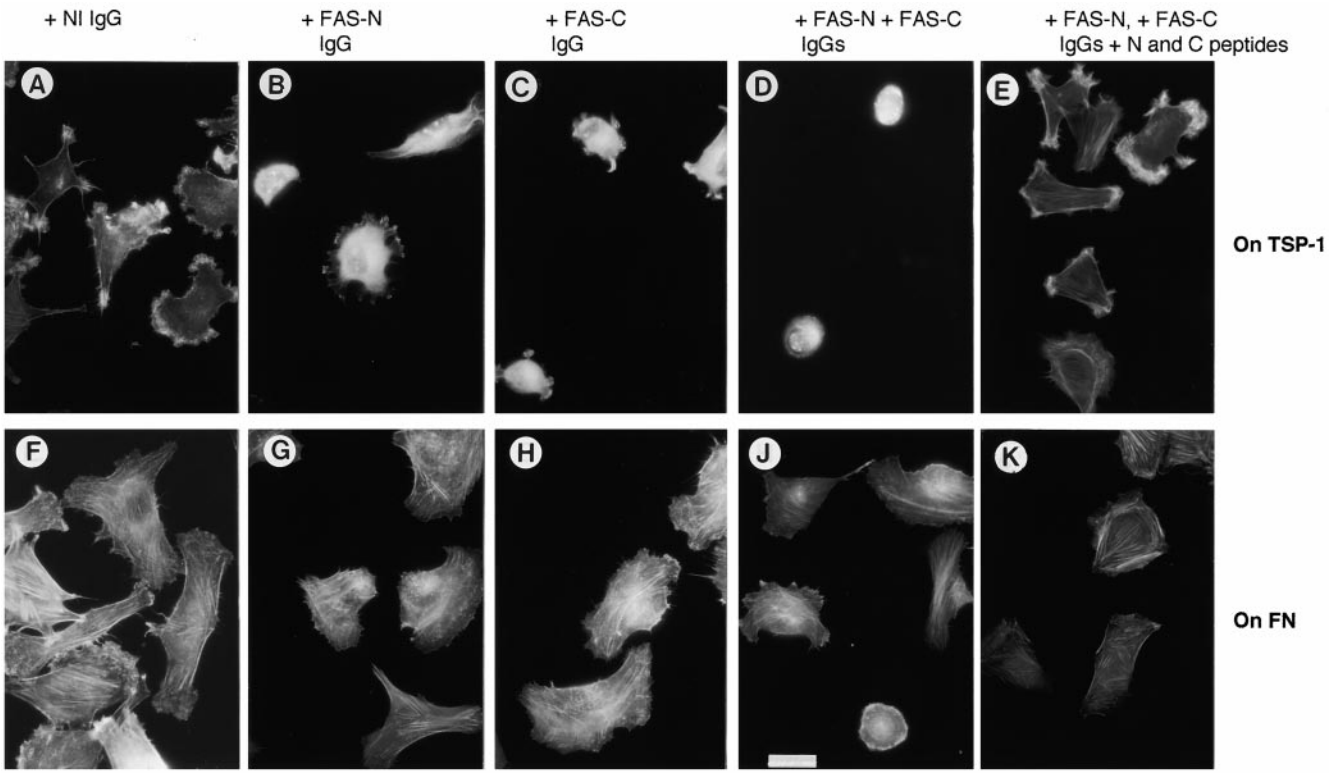
spike formation and showed reduced cell spreading (Fig. 11 B). Cells loaded with FAS-C IgG were more dramatically impaired in spreading and formed only small and poorly organized arrays of spikes at points along cell margins (Fig. 11 C). Cells loaded with a 1:1 mixture of both antibodies lacked spikes entirely and remained completely round (Fig. 11 D). Fascin distribution, visualized by use of a mouse monoclonal antibody, appeared diffuse with small punctate dots in the cytoplasm, suggesting that protein aggregates were formed in the presence of the antibodies (data not shown). Preincubation of the antibody mix with the FAS-N and FAS-C peptides reversed these effects on cell spreading (Fig. 11 E) indicating that the effects of the antibodies were specific.

When plated on fibronectin, which does not support stable formation of microspikes (Adams, 1995), cells loaded with control IgGs spread and showed characteristic formation of prominent actin microfilaments (Fig. 11 F). Loading of FAS-N or FAS-C individually had only slight effects on cell spreading and morphology, however, microfilament bundles appeared less well-organized (Fig. 11, G and H). Introduction of the 1:1 FAS-N/FAS-C mixture resulted in reduced cell spreading and an apparent reduction in organization of actin microfilaments (Fig. 11 J). This effect on microfilament organization was blocked by preincubation of the antibodies with the FAS-N and FAS-C peptide mixture, although the spread area of the cells remained smaller than that of the control cells (compare Fig. 11 K with Fig. 11 F).

To further investigate these effects, cell attachment was quantitated under the various adhesion conditions. Cells loaded with nonimmune rabbit IgG or preimmune IgG were not altered in their ability to attach to TSP-1 (Fig. 11 M). Cells loaded with either FAS-N or FAS-C IgG showed 50% inhibition of attachment to TSP-1 relative to the cells loaded with control IgG. The introduction of the 1:1 mixture of FAS-N and FAS-C IgGs at 0.5 mg/ml resulted in 70% inhibition of attachment to TSP-1, whereas introduction of 0.2 mg/ml of the antibody mixture inhibited attachment by 20% (Fig. 11 M). Loading of the antibody/peptide mixture did not block cell attachment (Fig. 11 M). Attachment to fibronectin was not significantly decreased by either FAS-N or FAS-C IgG, or by 0.2 mg/ml in total of the FAS-N/FAS-C mixture, but was decreased by 32% upon loading of the 0.5 mg/ml FAS-N/FAS-C IgG mixture (Fig. 11 M; significant at $P = 0.001$). This effect was not apparent in cells loaded with the antibody/peptide mixture (Fig. 11 M).

Effects of Fascin Antibodies on Cell Migration

In addition to their role in TSP-1 adhesion, fascin spikes have been functionally implicated in cell migration (Adams, 1997; Yamashiro et al., 1998). The antibodies were therefore used to examine the involvement of fascin in the motile behavior of C2C12 cells on uniform matrix substrata which support random haptokinesis. Cells were recorded by time lapse videomicroscopy from 1 to 6 h after plating. During this time, cells on TSP-1 extended angular processes which bore active, waving, phase dark regions at their tips. These either spread out into fan-like, lamellar sheets which continued rapid, intense membrane activity, or extended and split into separate active processes. In conjunction with these activities, the cells migrated with a mean velocity of 15.9 ± 6.0 μm/h (Fig. 11 N). Cells loaded with FAS-N IgG showed extension of small fans which tended to split into fingerlike zones and regress. These cells migrated with a reduced velocity of 8.5 ± 4.0 μm/h (difference significant at $P = 0.001$). The extension of processes was even more markedly reduced in cells loaded with FAS-C and these cells were strongly inhibited in motility (mean velocity 3.0 ± 1.8 μm/h; difference to cells loaded with nonimmune immunoglobulins significant at $P = 0.001$; Fig. 11 N). Cells loaded with both antibodies remained round and were nonmigratory. Jasplakinolide or latrunculin-B–treated cells were also nonmigratory on TSP-1 (data not shown). Thus, the interaction of fascin



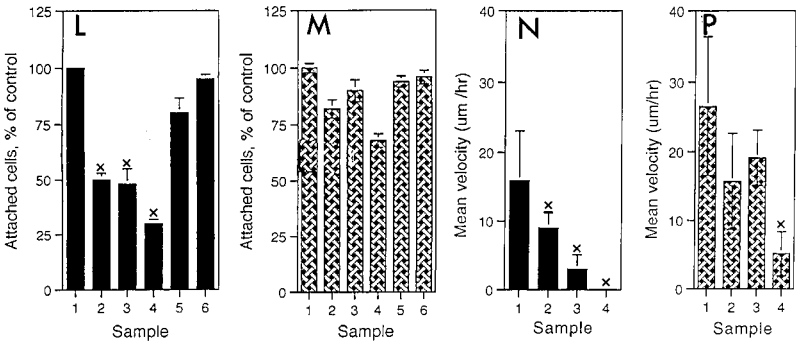


Figure 11. Role of fascin organization in cell adhesion and motility. (A–K) C2C12 cells were loaded with 0.5 mg/ml nonimmune rabbit IgG (A and F); 0.5 mg/ml FAS-N IgG (B and G); 0.5 mg/ml FAS-C IgG (C and H); a 1:1 mixture of FAS-N and FAS-C at 0.5 mg/ml total (D and J), or a 1:1 mixture of 0.5 mg/ml FAS-N and FAS-C containing 50 ug/ml each FAS-N and FAS-C peptides (E and K). Cells were stained with TRITC-phalloidin after 1 h adhesion on 50 nM TSP-1 (A–E) or 50 nM FN (F–K). Bar, 10 um. (L and M) Quantitation of cell attachment at 1 h on 50 nM TSP-1 (L) or 50 nM FN (M). (L–P) Lane 1, cells loaded with 0.5 mg/ml nonimmune rabbit IgG; lane 2, cells loaded with 0.5 mg/ml FAS-N IgG; lane 3, cells loaded with 0.5 mg/ml FAS-C IgG (C and G); lane 4, cells loaded with a 1:1 mixture of FAS-N and FAS-C 0.5 mg/ml total; lane 5, cells loaded with a 1:1 mixture of FAS-N plus FAS-C at 0.2 mg/ml total; lane 6, cells loaded with a 1:1 mixture of 0.5 mg/ml FAS-N and FAS-C plus 14 μg/ml each of FAS-N and FAS-C peptides. Values shown are mean ± SEM from triplicate determinations. N, Effects of fascin antibodies on random C2C12 movement on a 50 nM TSP-1 substratum. P, Effects of fascin antibodies on random C2C12 cell movement on a 50-nM FN substratum. Values are mean ± SEM from duplicate experiments. An asterisk indicates mean value significantly different from control sample at $P = 0.001$.

with F-actin is required in cell spreading and movement on TSP-1.

When adherent to fibronectin, cells loaded with control IgG showed considerable lamellipodial activity. The majority of cells showed repeated extension and regression of lamellae with ruffling margins at different points along their peripheries. These cells underwent random translocation over the fibronectin substratum with a mean velocity of 26.3 um/h (Fig. 11 P). Lamellar ruffling and extension were decreased in the cell populations loaded with FAS-N or FAS-C individually and this correlated with reductions in cell migration, to 15.7 ± 5.0 um/h and 19.0 ± 3.0 um/h, (differences significant at $P = 0.05$; Fig. 11 P). In the cells loaded with the FAS-N/FAS-C mixture, lamellipodial movements were strongly reduced and the mean velocity of cells was 5.1 ± 2.7 um/h, an 81% decrease in comparison with the control IgG-loaded cells (difference significant at $P = 0.001$; Fig. 11 P). Thus, perturbation of cellular fascin also had substantial effects on motility on FN.

Discussion

Our results establish a pathway in which the small GTPases Rac and Cdc42 trigger organization of fascin spikes, which promotes GTPase-mediated cell spreading and mi-

gration on TSP-1. First, we found that adhesion of myoblastic cells to TSP-1 triggered strong activation of Cdc42 and Rac and their common effector PAK. Thus, TSP-1 receptors must be upstream of these GTPases. Second, constitutively active Rac and Cdc42 induced fascin-containing structures in 3T3 cells and dominant-negative GTPases inhibited fascin spikes in C2C12 cells plated on TSP-1. Constitutively active GTPases also caused disorganization of fascin spikes in cells spreading on TSP-1. Third, assembly of fascin spikes was inhibited by blocking actin turnover or by antibodies that block the binding of fascin to actin. Thus, fascin organization depends on actin and lies downstream of the GTPases. Fourth, we showed that anti-fascin IgG strongly inhibited cell migration, a process known to depend on both Rac and Cdc42. Thus, fascin is a functionally important component of this pathway.

Cdc42 and Rac have been reported to regulate the assembly of small focal contact-type structures in several cell types under different matrix-adhesion conditions (for example, Nobes and Hall, 1995; Kozma et al., 1995). In cells undergoing attachment and spreading on fibronectin, this process involves an initial requirement for Cdc42 in the formation of filopodial protrusions and a subsequent involvement of both Cdc42 and Rac during cell spreading and organization of the actin cytoskeleton (Clark et al., 1998; Price et al., 1998). Similarly, disruption of the fibronectin matrix of stably adherent cells leads to morphological alterations involving activation of Cdc42 (Bourdoulous et al., 1998). In TSP-1–adherent cells, focal contacts do not assemble and, in cell types with the capacity to undergo spreading, spikes containing F-actin and fascin form instead (Adams, 1995; 1997; Adams et al., 1999). Our data now establish requirements for the activities of Cdc42 and Rac in the formation of these structures. Thus, Rho family GTPases are required in the induction of these two distinct types of adhesive contacts.

Although Cdc42 and Rac can each act alone to induce formation of cortical fascin protrusions, the resulting structures have distinct morphologies. Constitutively active Cdc42 led to the formation of elongated projections containing F-actin and fascin. In contrast, constitutively active Rac led to the formation of ribbons of short radial fascin spikes within lamellipodia. Expression of either dominant-negative Rac or Cdc42 inhibited normal spreading and spike formation in cells on TSP-1. Interestingly, expression of activated Rac or Cdc42 also disrupted normal cell spreading on TSP-1. These effects indicate a complex dynamic in which the activities of Rac and Cdc42 in cytoskeletal responses to TSP-1 are tightly regulated. Therefore either inhibiting or strongly activating these GTPases interferes with spreading and cytoskeletal organization.

Rac and Cdc42 both activate a number of pathways, including the JNK and p38-MAPK stress activated protein kinase pathways and PAK (Manser et al., 1994; Coso et al., 1995; Minden et al., 1995; reviewed by Knaus and Bokoch, 1998). The simplest explanation is that one of these shared pathways regulates fascin and its ability to bind and bundle actin. Some evidence has been presented for a role of PAK in actin organization (Manser et al., 1997; Sells et al., 1997); however, the morphological activities of Cdc42 and Rac are separable from their ability to bind PAK and overexpression of PAK does not rescue actin organization in cells expressing N17Cdc42 (Lamarche et al., 1996; Westwick et al., 1997, Zhao et al., 1998). One substrate of PAK is LIM–kinase, which phosphorylates and inactivates the actin depolymerizing protein, cofilin (Arber et al., 1998; Yang et al., 1998; Edwards et al., 1999). The resultant accumulation of actin filaments could plausibly facilitate bundle organization by fascin. Our data establish that cell adhesion on TSP-1 leads to sustained PAK activity, indicating that the activation of Rac and Cdc42 results in activation of downstream pathways. However, whether PAK is critical for spike formation cannot be inferred.

Whereas both Rac and Cdc42 can induce actin polymerization in permeabilized cells or extracts, the available evidence suggests that they do so via distinct mechanisms or pathways (Hartwig et al., 1995; Machesky and Hall, 1997; Zigmond et al., 1997; 1998; Ma et al., 1998; Moreau and Way, 1998). The colocalization of cortical F-actin and fascin in TSP-1–adherent cells strongly suggests that both Cdc42 and Rac promote localized bundling of filamentous actin. In vitro, fascin monomers bind and cross-link F-actin to produce stable actin bundles which contain a 1:4 ratio of fascin/actin (Bryan and Kane, 1978; Yamashiro-Matsumura and Matsumura, 1985; Cant et al., 1994; Edwards and Bryan, 1995). The dimensions and overall packing order of such bundles depend on the initial molar ratios of actin and fascin (Stokes and DeRosier, 1991; Edwards et al., 1996). *Drosophila* fascin corresponds to the *singed* gene product and detailed phenotypic analyses of various mutant alleles of *singed* have demonstrated that the actin-bundling activity of fascin is essential to its in vivo roles in bristle cells and nurse cells. In particular, fascin is required for the orderly organization of actin bundles (Cant et al., 1994, 1998; Tilney et al., 1995, 1998). Our data on the consequences of disruption of cellular F-actin, fascin, or GTPases indicate that this bundle-organizing property of fascin is indeed important for the development of actin structures appropriate for cell matrix adhesion and motility. It is possible that the activities of Rac and Cdc42 could directly initiate bundle formation and organization. Alternatively, microfilament bundling might be controlled separately from the initial events of actin polymerization and regulate the shape of the resultant structure.

Inhibition of fascin by introduction of monospecific antibodies into the cytoplasm revealed an important role for fascin in cell spreading and migration on TSP-1. These effects were highly specific as they were completely reversed by including the antigenic peptides with the antibodies and were much less dramatic in cells on FN. These weaker effects on FN, however, were also prevented by the peptides, indicating specificity. Notably, migration of cells on FN was inhibited only by the combination of blocking antibodies. In this context, highly transient formation of fascin-containing structures during initial cell spreading on fibronectin has been noted (Adams, 1995). Furthermore, our biochemical data revealed a transient activation of GTPases on fibronectin in contrast to the prolonged activation of Rac and Cdc42 in TSP-1–adherent cells. These results highlight differing requirements for fascin structures in supporting cell adhesion to TSP-1 or fibronectin. Therefore, we hypothesize that on TSP-1 stable fascin-containing structures maintain adhesion through a molec-

ular mechanism that involves the sustained activities of Rac and Cdc42. Under these conditions, high levels of actin-associated fascin are needed to maintain cell spreading and cytoskeletal organization, thus even partial blockade of fascin impairs these processes. On fibronectin, fascin-dependent structures participate in the initial Cdc42- and Rac-dependent formation of cell extensions or adhesions but are rapidly lost as initial focal complexes or focal adhesions that lack fascin are formed. This process involves phosphorylation of fascin (Adams et al., 1999). These results suggest that, on FN, lower levels of fascin are required so that cell spreading and actin cytoskeletal organization is less sensitive to perturbation of fascin.

Our results therefore establish a novel and unexpected role for actin-associated fascin in cell adhesion, migration and cytoskeletal organization downstream of Rac and Cdc42. Fascin is most likely necessary for assembly of normal actin bundles in GTPase-induced matrix-contact structures and for the adhesive function of those structures. These events are highly transient upon adhesion to many ECM proteins, but are stabilized on TSP-1, where fascin spikes become major cytoskeletal structures and are important in cell migration. Future work will be directed toward identifying the pathways downstream of Rac and Cdc42 that regulate fascin function and toward understanding the role of fascin in adhesion and migration.

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