

Cell-Matrix Adhesions Differentially Regulate Fascin Phosphorylation

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Cell adhesion to individual macromolecules of the extracellular matrix has dramatic effects on the subcellular localization of the actin-bundling protein fascin and on the ability of cells to form stable fascin microspikes. The actin-binding activity of fascin is down-regulated by phosphorylation, and we used two differentiated cell types, C2C12 skeletal myoblasts and LLC-PK1 kidney epithelial cells, to examine the hypothesis that cell adhesion to the matrix components fibronectin, laminin-1, and thrombospondin-1 differentially regulates fascin phosphorylation. In both cell types, treatment with the PKC activator 12-tetradecanoyl phorbol 13-acetate (TPA) or adhesion to fibronectin led to a diffuse distribution of fascin after 1 h. C2C12 cells contain the PKC family members α , γ , and λ , and PKC α localization was altered upon cell adhesion to fibronectin. Two-dimensional isoelectric focusing/SDS-polyacrylamide gels were used to determine that fascin became phosphorylated in cells adherent to fibronectin and was inhibited by the PKC inhibitors calphostin C and chelerythrine chloride. Phosphorylation of fascin was not detected in cells adherent to thrombospondin-1 or to laminin-1. LLC-PK1 cells expressing green fluorescent protein (GFP)-fascin also displayed similar regulation of fascin phosphorylation. LLC-PK1 cells expressing GFP-fascin S39A, a nonphosphorylatable mutant, did not undergo spreading and focal contact organization on fibronectin, whereas cells expressing a GFP-fascin S39D mutant with constitutive negative charge spread more extensively than wild-type cells. In contrast, C2C12 cells coexpressing S39A fascin with endogenous fascin remained competent to form microspikes on thrombospondin-1, and cells that expressed fascin S39D attached to thrombospondin-1 but did not form microspikes. Blockade of PKC α activity by TPA-induced down-regulation led to actin association of wild-type fascin in fibronectin-adherent C2C12 and LLC-PK1 cells but did not alter the distribution of S39A or S39D fascins. The association of fascin with actin in fibronectin-adherent cells was also evident in the presence of an inhibitory antibody to integrin $\alpha 5$ subunit. These novel results establish matrix-initiated PKC-dependent regulation of fascin phosphorylation at serine 39 as a mechanism whereby matrix adhesion is coupled to the organization of cytoskeletal structure.

INTRODUCTION

Cell adhesion to extracellular matrix macromolecules is mediated by specific cell surface receptors, of which integrins and proteoglycans form major families (reviewed by Hynes, 1987, 1992; Ruoslahti, 1988, 1989; Hardingham and Fosang, 1992). Interactions with individual matrix components lead to distinct outcomes in terms of subsequent cell behavior (reviewed by Adams and Watt, 1993). In cell types for which

this phenomenon has been analyzed in depth, the association of individual integrins with cytoplasmic adaptor molecules has been demonstrated to provide linkage to specific intracellular signaling pathways (Wary *et al.*, 1996; Schneller *et al.*, 1997; Pozzi *et al.*, 1998). In other experimental systems, activation of specific intracellular signals upon ligation of certain integrins has been described (Mackenna *et al.*, 1998).

Another aspect of the specificity of cell-matrix interactions, which to date has received less attention, involves the morphological organization and biochemical composition of the substratum contacts formed by matrix-adherent cells.

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Thus, integrin-activated cell spreading on fibronectin, vitronectin, or collagens results in the assembly of focal contact or focal adhesion structures, which are of functional importance in cell-adhesive and motile behavior and in the integration and networking of intracellular signals (reviewed by Jockusch *et al.*, 1995; Schwartz *et al.*, 1995; Burridge and Chrzanowska-Wodnicka, 1996). Ligation of $\alpha 6 \beta 4$ integrin in epithelial cells leads to the formation of a specialized adhesive structure, the hemidesmosome (reviewed by Borradori and Sonnenburg, 1996; Giancotti, 1996). Cells that spread on thrombospondin-1 (TSP-1) or tenascin-C form substratum contacts consisting of radial actin microspikes that contain the actin-bundling protein fascin. The same cell types do not form microspikes when adherent on fibronectin or vitronectin (Adams, 1995, 1997; Fischer *et al.*, 1997).

An important question arising from such correlative analyses concerns the molecular mechanisms by which cell-matrix adhesions differentially regulate fascin microspike formation. In this regard, it is of interest that the subcellular localization of fascin is dramatically altered according to the matrix substratum provided. Whereas cells adherent on TSP-1 or tenascin-C form cortical microspikes that contain fascin and F-actin and cells adherent on laminin-1 also show codistribution of fascin with actin microfilament bundles, cells adherent on fibronectin or vitronectin have a uniform diffuse distribution of fascin that does not coincide specifically with F-actin bundles (Adams, 1995, 1997; Fischer *et al.*, 1997). In random cell populations, microspikes are formed at the leading edge of motile cells (Tao *et al.*, 1996; Adams, 1997) and have been functionally implicated in cell motile behavior (Adams, 1997). Furthermore, overexpression of fascin in kidney epithelial cells leads to increased transfilter migratory activity (Yamashiro *et al.*, 1998). These findings raise the possibility that the regulation of fascin distribution by cell-matrix interactions is of importance for the physiological coordination and polarization of the cytoskeleton in cell-adhesive and motile behavior.

In mammalian cells, the polymerization and organization of F-actin is regulated by numerous actin-binding proteins. These can be grouped into families according to their structures or to functional properties with respect to actin nucleation, capping, severing, cross-linking, or bundling (reviewed by Matsudaira, 1991; Vanderkerckhove and Vancompernelle, 1992). Within the functional category of actin-bundling proteins, the 55-kDa fascin polypeptide is a structurally unique and evolutionarily conserved type of actin cross-linking protein (reviewed by Edwards and Bryan, 1995). The actin-binding and -bundling activities of fascin *in vitro* are reduced upon phosphorylation of fascin by PKC (Yamakita *et al.*, 1996; Ono *et al.*, 1997). Treatment of intact SK-N-SH cells with the PKC activator 12-tetradecanoyl phorbol 13-acetate (TPA) also stimulates fascin phosphorylation. This correlates with relocation of fascin from microfilaments and membrane ruffles to a diffuse cytoplasmic compartment (Yamakita *et al.*, 1996). Given that fascin is also noncoincident with F-actin in fibronectin-adherent cells, we wished to test the hypothesis that cell-matrix interactions differentially regulate phosphorylation of fascin. We report a biochemical mechanism in myoblasts and kidney epithelial cells by which matrix adhesion regulates fascin phosphorylation at serine 39 by a process dependent on PKC α . Surprisingly, the ability of cells to phosphorylate fascin is

needed in $\alpha 5 \beta 1$ integrin-mediated spreading and cytoskeletal organization on fibronectin.

MATERIALS AND METHODS

Reagents and Antibody Preparation

C2C12 mouse skeletal myoblasts (Blau *et al.*, 1985) were grown in DMEM containing 20% FCS in a humidified 5% CO₂ atmosphere. Stable clonal transfectant cell lines expressing green fluorescent protein (GFP)-fascin were derived as described previously and evaluated for expression by fascin Western blot (Adams *et al.*, 1998). LLC-PK1 pig kidney epithelial cells (American Type Culture Collection [Rockville, MD] CL-101) were grown in medium 199 (Life Technologies, Grand Island, NY) containing 3% FCS. LLC-PK1 cells stably expressing GFP-fascin were cultured in the same medium with 500 μ g/ml G418 (Sigma Chemical, St. Louis, MO). Plasma fibronectin, Engelbreth Holm-Swarm laminin (laminin-1), rabbit skeletal muscle actin, TPA, and 4- α -phorbol were obtained from Sigma Chemical. Chelerythrine chloride and calphostin C were from LC Laboratories (Läufelfingen, Switzerland). Cell-permeable PKC α peptide inhibitor was from Calbiochem-Novabiochem (Nottingham, United Kingdom). The rabbit polyclonal antiserum FAS-C was raised against a synthetic peptide corresponding to amino acid residues 467–479 of human fascin (Mosialos *et al.*, 1994) conjugated to keyhole limpet hemocyanin (immunization and serum collection carried out according to standard procedures at Zenaca/CRB, Northwich, Cheshire, United Kingdom). Mouse mAbs to β -catenin and PKC family members were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antiserum to PKC α and mouse mAb to β -actin (Gimona *et al.*, 1994) were from Sigma Chemical. Rat antibody 5H10-27 to mouse $\alpha 5$ integrin subunit (Kinashi and Springer, 1994) was from PharMingen (San Diego, CA).

Two-Dimensional Gel Electrophoresis and Western Blotting

Dishes containing 1.5×10^6 C2C12 myoblasts were treated with 50 nM TPA or the equivalent volume of DMSO for 1 h at 37°C, rinsed in PBS, and lysed directly into isoelectric focusing (IEF) sample buffer (9.95 M urea, 4% NP-40, 2% ampholines, pH 3–10 [Pharmacia Biotech, Piscataway, NJ], 100 mM DTT) (O'Farrell, 1975). Sample volumes corresponding to 1×10^5 cell equivalents (100 μ g of protein) were loaded onto 100-mm \times 3-mm (inner diameter) rod gels, prepared with pH 5–7 and pH 3–10 ampholines in a 1:3 ratio. In other experiments, 10^6 cells were allowed to adhere to BSA-blocked dishes coated with matrix proteins for 1 h, washed in PBS, lysed in IEF sample buffer, equalized, and loaded onto rod gels as described above. Three independent experiments were carried out for each adhesion condition. IEF was performed according to the method of O'Farrell (1975). Rods were prerun for 15 min at 200 V, 30 min at 330 V, and 30 min at 400 V before the samples were loaded. Subsequent electrophoresis was at 400 V for a total of 7000 volt-hours, followed by 800 V for 15 min. The pH gradient established was measured by cutting rods run with sample buffer alone into 0.5-cm segments, eluting overnight into 0.5 ml of water, and measuring the pH of the eluant. In the second dimension, proteins were resolved from the rods on 1.5-mm-thick 12.5% polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose (0.22- μ m pore size; Bio-Rad, Richmond, CA), and probed with FAS-C antiserum. Bound antibody was visualized by the ECL detection technique using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (reagents from Clontech [Palo Alto, CA] and Perkin Elmer-Cetus [Norwalk, CT]; detection on Hyperfilm ECL [Amersham, Arlington Heights, IL]).

Cell Adhesion Assays and Immunofluorescence

Cell adhesion assays were carried out as described (Adams, 1995) for 1 h at 37°C. Some experiments involved a modified protocol in

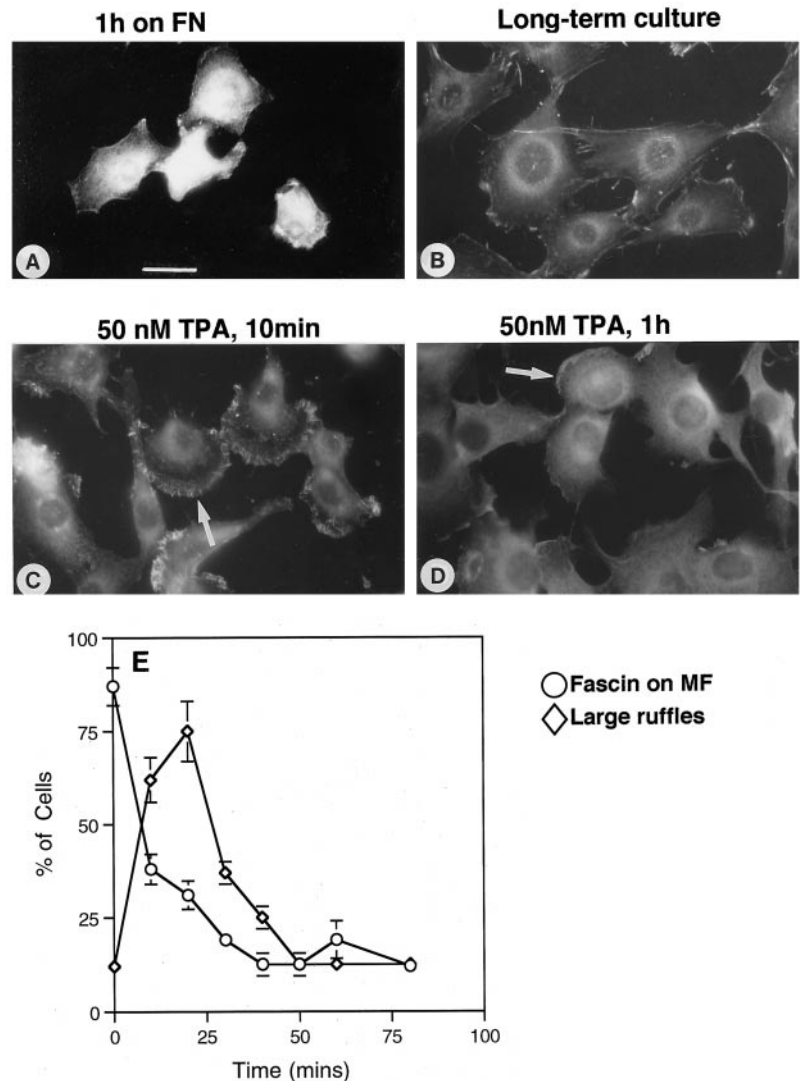


Figure 1. Fibronectin adhesion and TPA treatment alter fascin localization in C2C12 cells. C2C12 cells were stained for fascin after 1 h of adhesion on fibronectin in serum-free medium (A), in long-term culture 1 h after addition of DMSO solvent control (B), after 50 nM TPA for 10 min (C), and after 50 nM TPA for 1 h (D). An example of a large ruffle is arrowed in C, and the residue of a ruffle is arrowed in D. Bar, 10 μ m. (E) The percentage of cells with microfilament-associated fascin (\circ) or large ruffles (\diamond) was quantitated with time of TPA treatment. Each point is the mean \pm SEM of four independent experiments.

which cells were treated with pharmacological inhibitors or activators of PKC, either before and during the adhesion assay or after cells had adhered to a specific matrix for 45 min. In pilot experiments, these inhibitors were tested at a range of concentrations for their effects on cell adhesion or cell viability. The concentrations used in the main experiments were 50 nM TPA, 100 nM calphostin C, 320 nM chelerythrine chloride, and 80 μ M myristoylated PKC α peptide inhibitor. These values represent the lowest concentrations needed to achieve clear effects on cell adhesion. Down-regulation of PKC α was achieved by 24-h treatment with 100 nM TPA (LLC-PK1 cells) or 24-h treatment with 500 nM TPA (C2C12 cells) and was confirmed on Western blots of whole cell extracts using rabbit antibody specific to PKC α . In some assays, antibody 5H10-27 to mouse α 5 integrin subunit was added at 5 μ g/ml at the start of the adhesion period. Adherent cells were quantified, fixed and processed for fascin immunofluorescence, and costained with TRITC-phalloidin or monoclonal VIN 11.5 to vinculin (Sigma Chemical) as described (Adams, 1995). Staining with antibody to β -actin was carried out on methanol-fixed cells and visualized as double staining with GFP-fascin. For staining with PKC antibodies, cells were fixed in 3.7% formyl saline and then permeabilized for 10 min with

0.2% Triton X-100 in PBS. Primary antibodies were detected with the use of appropriate species- and class-specific TRITC- or FITC-conjugated secondary antibodies (ICN Biomedical, Costa Mesa, CA).

RESULTS

Fibronectin Adhesion and TPA Treatment Have Similar Effects on Fascin Localization in Diverse Cell Types

We used C2C12 myoblasts to examine whether adhesion to fibronectin or TPA treatment would have equivalent effects on fascin localization in a single cell type. As demonstrated for other cell types (Adams, 1997), C2C12 cells adherent on fibronectin showed a diffuse distribution of fascin (Figure 1A). In long-term adherent C2C12 cells spread on endogenous matrix, fascin was present on microfilament bundles and in small cortical ruffles and extended projections. Diffuse perinuclear staining was also noticeable (Figure 1B).

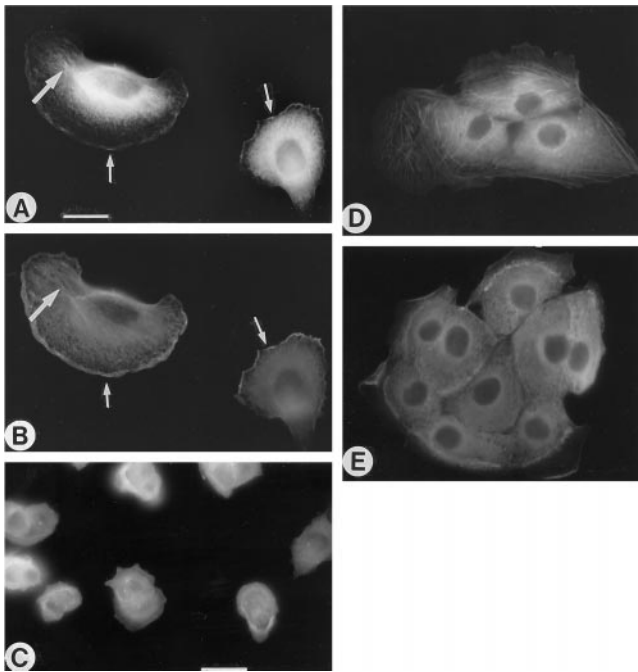


Figure 2. Effects of matrix adhesion or TPA treatment on GFP-fascin localization in LLC-PK1 cells. Cells adherent on laminin-1 for 1 h were double-stained with GFP-fascin (A) or antibody to β -actin (B). (C–E) GFP-fascin visualized after 1 h on fibronectin (C), in long-term adherent cells (D), or after treatment with 50 nM TPA for 1 h (E). Large arrows in A and B indicate coincidence of fascin with microfilaments, and small arrows indicate examples of coincidence in membrane ruffles. Bar, 5 μ m for A and B, 10 μ m for other panels.

Treatment with 50 nM TPA to activate PKC resulted in initial intense membrane ruffling, with localization of fascin into short, radial ribs within the ruffles (Figure 1C), followed by a major reallocation of fascin to a diffuse distribution after 1 h of treatment (Figure 1D). These redistributions were confirmed quantitatively by scoring cells for large ruffles or for microfilament-associated fascin with time of TPA treatment. A sharp peak of ruffling activity occurred at 10–20 min of TPA treatment, and over time, between 10 and 40 min of TPA treatment, there was a gradual loss of microfilament-associated fascin. Diffuse fascin staining was apparent in 87% ($\pm 5.1\%$; four separate experiments) of cells after 1 h of TPA treatment (Figure 1E). A few cells showed residues of marginal ruffles (Figure 1E; example arrowed in Figure 1D).

To determine the generality of these effects, we also examined fascin localization in LLC-PK1 pig kidney epithelial cells. These cells have low endogenous expression of fascin, so GFP-fascin was used as a reporter in a stable transfectant LLC-PK1 cell line. First, to establish that the localization of GFP-fascin was regulated by matrix adhesion conditions in a manner similar to endogenous fascin (Adams, 1997), we compared GFP-fascin distributions in LLC-PK1 cells adherent on laminin-1 and fibronectin. In cells adherent to laminin-1, GFP-fascin codistributed with actin in cortical ruffles and showed partial colocalization with microfilament bundles in the central regions of cells (Figure 2, A and B;

GFP-fascin and β -actin double-stained images). In cells adherent to fibronectin, GFP-fascin had a uniform diffuse distribution (Figure 2C; see also Figure 8C). LLC-PK1 cells did not spread or form projections on TSP-1 (our unpublished results).

We next examined the effect of TPA treatment on GFP-fascin distribution. Long-term adherent LLC-PK1 cells on endogenous matrix spread extensively and displayed codistribution of GFP-fascin with actin microfilament bundles and cortical actin structures as well as with diffuse GFP-fascin in the perinuclear region (Figure 2D). The greater colocalization of fascin with microfilaments in long-term adherent LLC-PK1 cells than in C2C12 cells (compare Figure 1B and 2D) is very likely due to the different compositions of extracellular matrix laid down by these differentiated cell types. C2C12 cells secrete fibronectin, laminins, TSP-1, and the heparan sulfate proteoglycan perlecan (Larrain *et al.*, 1994, 1997; Adams, 1997), whereas LLC-PK1 cells produce fibronectin, collagen IV, and laminins (Low *et al.*, 1994; Kruidering *et al.*, 1998). Treatment with 50 nM TPA for 1 h correlated with loss of microfilament-associated GFP-fascin and the enhancement of diffuse cytoplasmic GFP-fascin (Figure 2E). These results demonstrate that either adhesion to a pure fibronectin substratum (Figures 1A and 2C) or sustained activation of PKC by TPA treatment (Figures 1D and 2E) results in the noncoincidence of fascin with F-actin bundles, ruffles, or projections in two physiologically diverse cell types.

Role of PKC in the Organization of Substratum Contacts in Matrix-adherent Cells

PKC is activated upon integrin ligation and has an important general role in cell spreading, with effects on focal contact assembly (Vuori and Ruoslahti, 1993; reviewed by Schwartz *et al.*, 1995; Kolanus and Seed, 1997). In vitro, fascin is a substrate for PKC and is phosphorylated at the same site as in vivo (Yamakita *et al.*, 1996). We set out to examine the effects of PKC inhibitors or agonists on the localization of fascin in matrix-adherent C2C12 cells. Because several components of focal contacts are also substrates for PKC (Werthe *et al.*, 1983; Beckerle, 1990), we also used vinculin staining to examine the balance of matrix-adhesive contacts assembled under the various experimental conditions. Pretreatment of C2C12 cells for 15 min with the specific PKC inhibitors chelerythrine chloride or calphostin C, either before adhesion assay or for 15 min after cell spreading had proceeded for 45 min, resulted in cell rounding. TSP-1-adherent cells showed reduced fascin microspikes, and vinculin-positive focal contacts were reduced or absent in fibronectin-adherent cells (our unpublished results). Up-regulation of PKC activity by treatment with 50 nM TPA for 20 min before the adhesion assay did not alter the distribution of fascin in fibronectin-adherent cells (Figure 3A; compare with Figure 1A), and vinculin was highly localized to focal contacts (Figure 3B). In marked contrast, TPA treatment correlated with the complete loss of fascin microspikes from TSP-1-adherent cells and a diffuse localization of fascin (Figure 3, C and D). A similar redistribution of fascin also took place in cells that had adhered and formed microspikes on TSP-1 for 40 min before the addition of 50 nM TPA for 15 min (our unpublished results). TPA treatment also correlated with the

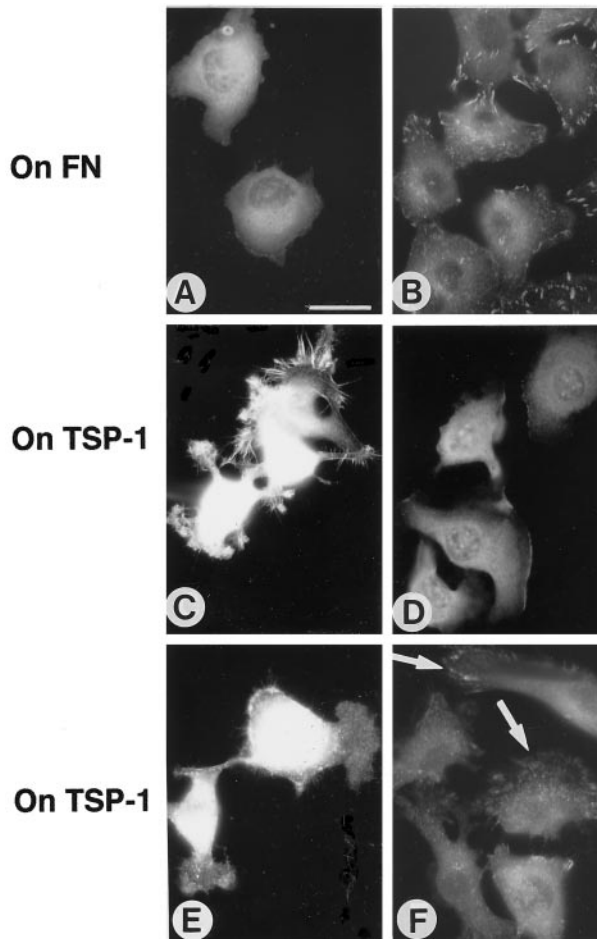


Figure 3. Effect of PKC activation on microspikes and focal contacts in matrix-adherent C2C12 cells. (A and B) Cells adherent for 1 h on fibronectin in the presence of 50 nM TPA. (C and E) Control C2C12 cells adherent for 1 h on TSP-1. (D and F) Cells adherent on TSP-1 for 1 h in the presence of 50 nM TPA. (A, C, and D) Fascin stain. (B, E, and F) Vinculin stain. Arrows in F indicate localization of vinculin in focal contacts. Bar, 10 μ m.

organization of vinculin into focal contacts by TSP-1-adherent cells (Figure 3, compare E and F).

Because activation of PKC led to the loss of microspikes in cells adherent to TSP-1, these results raised the possibility that the activation of PKC family members does not have the central role in microspike formation that it does in focal contact assembly and cell spreading. To investigate this possibility, we examined the localization of PKC family members in matrix-adherent cells on the basis that membrane translocation of PKC is intimately linked with enzyme activation (reviewed by Newton, 1995). Because multiple PKC gene family members are activated by TPA, it was first necessary to determine which forms of PKC are expressed in C2C12 cells. C2C12 cells were found to express the conventional PKCs α and γ and the atypical family member λ . The cells did not express PKCs β , δ , or ϵ (Figure 4A) (our unpublished results). Of these enzymes, the atypical PKCs are not susceptible to regulation

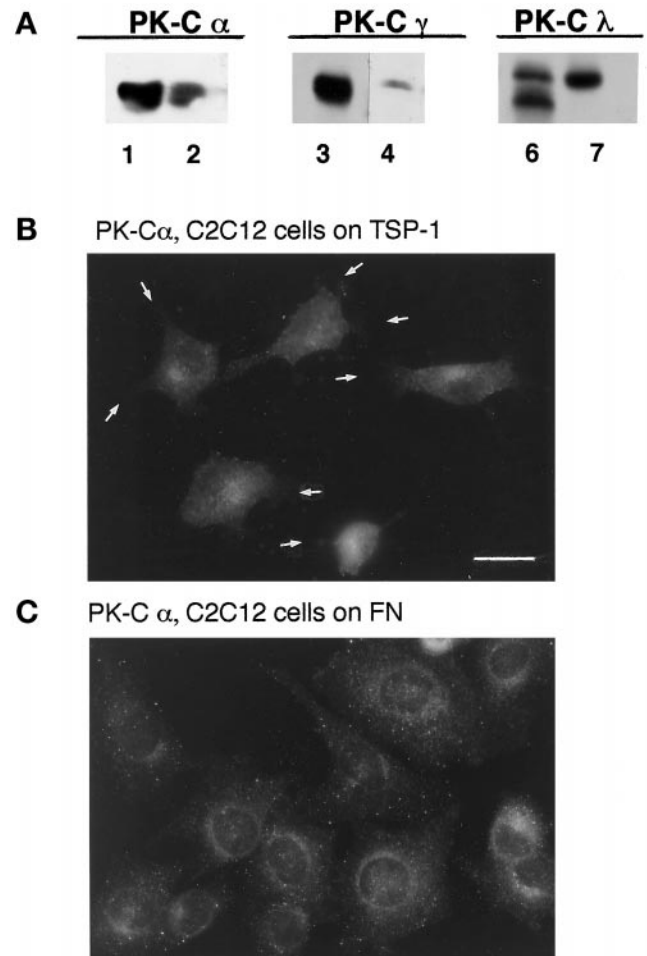


Figure 4. Expression of PKC family members in C2C12 cells and effect of matrix adhesion on PKC α localization. (A) Western blots of brain extract (lanes 1, 3, and 6) and C2C12 whole cell extract (lanes 2, 4, and 7) were probed with antibodies to PKC α , PKC γ , and PKC λ . (B and C) C2C12 cells adherent for 1 h on TSP-1 (B) or fibronectin (FN) (C) were stained with antibody to PKC α . Arrows indicate regions of microspike formation in TSP-1-adherent cells that do not stain for PKC α . Bar, 8 μ m.

by diacylglycerol or phorbol esters (reviewed by Dekker and Parker, 1994).

The localization of PKC γ was diffuse in cells adherent to either fibronectin or TSP-1 and was not altered under different matrix adhesion (our unpublished results). In cells adherent on TSP-1, PKC α was perinuclear and was undetectable in the cell cortex (Figure 4B). Thus, PKC α was not located within zones of microspike formation (compare Figures 4B and 3C) (Adams *et al.*, 1998). In fibronectin-adherent cells, perinuclear staining was also apparent, but PKC α was also concentrated in small focal contact-like patches that were distributed to cell margins and thus appeared to overlap diffusely distributed fascin (Figure 4C). To further explore the functional involvement of PKC α in cell adhesion, cells were treated with a cell-permeant PKC α pseudosubstrate peptide inhibitor and then used in adhesion assays.

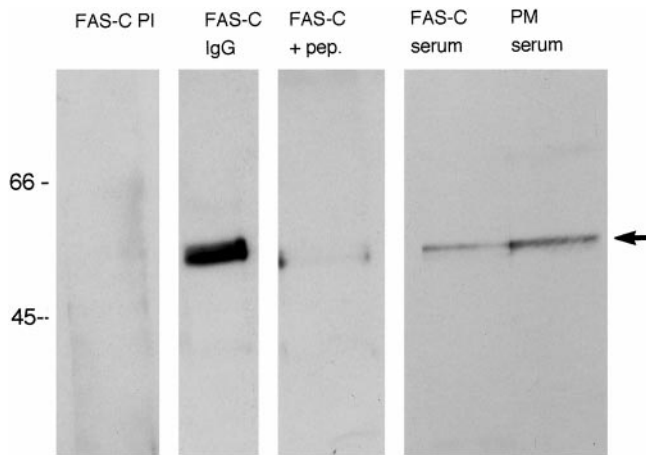


Figure 5. Characterization of anti-fascin serum. Western blots of C2C12 whole cell extracts probed with preimmune or FAS-C serum in the presence or absence of FAS-C peptide, as indicated. PM serum, anti-fascin reagent of Pierre McCrea.

Peptide treatment correlated with a 90% reduction in the number of cells attached to fibronectin and a 40% reduction in attachment to TSP-1. Cells adherent to TSP-1 still formed cortical spikes. This difference was statistically significant ($p = 0.005$; $n = 3$) (data not shown).

Matrix Adhesion Conditions Regulate PKC-dependent Phosphorylation of Fascin

To permit analysis of the biochemical processes underlying regulation of fascin localization, we raised a rabbit polyclonal antiserum (FAS-C) against a synthetic peptide corresponding to amino acid residues 467–479 of human fascin (Mosialos *et al.*, 1994). Reactivity of the antiserum with fascin polypeptide was demonstrated by immunoprecipitation of *in vitro* translated human fascin. FAS-C also recognized native fascin protein, as determined by immunoprecipitation of fascin from Triton X-100 extracts of metabolically labeled C2C12 myoblasts (our unpublished results). On Western blots of whole C2C12 cell extracts, FAS-C immune serum or IgG fraction reacted specifically with 55-kDa fascin protein (Figure 5). Preimmune serum did not exhibit reactivity, and reactivity of immune serum was abolished in the presence of immunizing peptide (Figure 5). The synthetic peptide immunogen corresponded to a sequence motif highly conserved between vertebrate fascins, and indeed, FAS-C serum reacted on Western blot with fascin in extracts derived from human, rat, mouse, green monkey, pig, dog, and chicken (our unpublished results). The peptide motif is not conserved in invertebrate fascins (reviewed by Edwards and Bryan, 1995).

To analyze the biochemical processes associated with matrix-regulated fascin relocalization, whole cell urea extracts of C2C12 cells were resolved on two-dimensional IEF/SDS-PAGE gels, blotted, and probed with FAS-C antibody to detect fascin isoelectric variants. The calculated isoelectric point of mouse fascin is 6.1, and in extracts of long-term adherent cultures, fascin resolved as two isoelectric variants in the isoelectric point 6.1–6.2 range (Figure 6A). The more

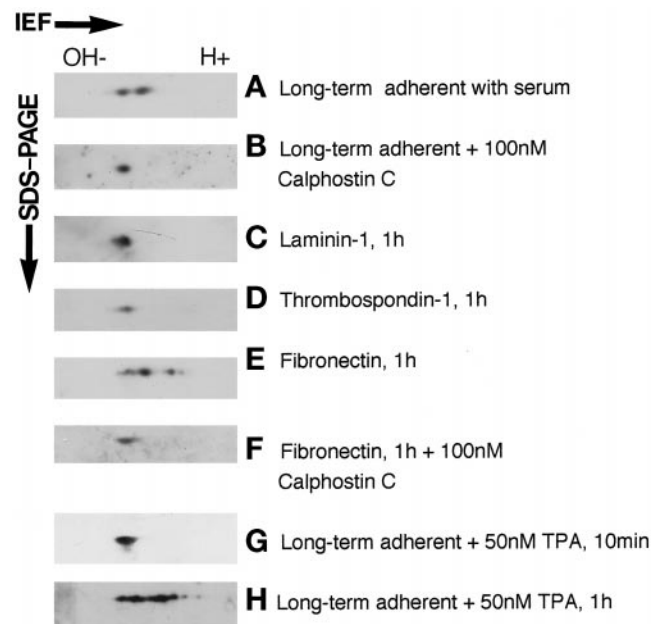
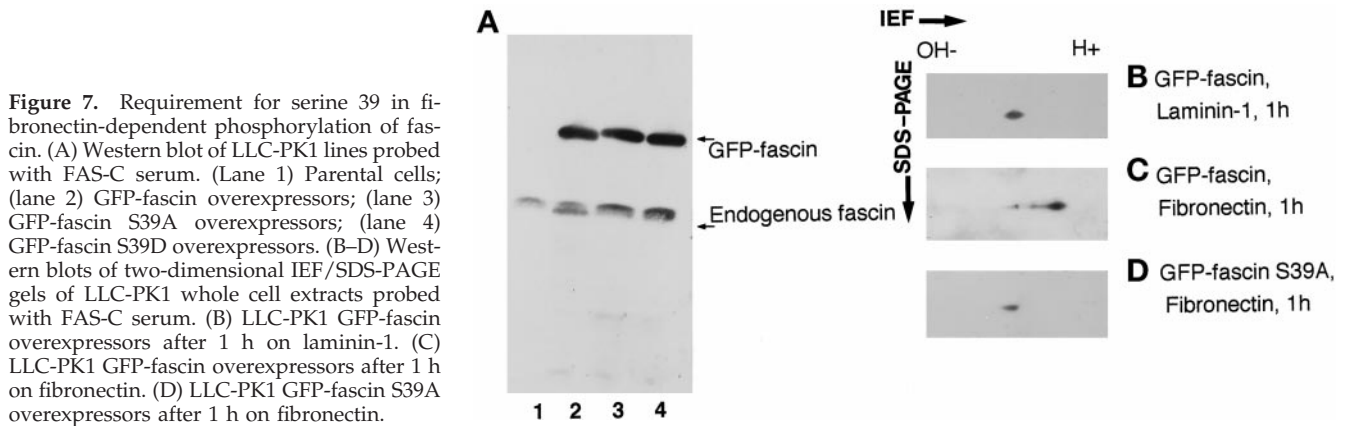


Figure 6. Matrix adhesion molecules differentially affect fascin phosphorylation. Whole cell extracts of C2C12 cells prepared under different adhesion conditions, as indicated, were resolved in two dimensions by IEF and SDS-PAGE, blotted, and probed with FAS-C IgG.

acidic spot corresponded to phosphorylated fascin and was not present in extracts of cells treated with either of the specific PKC inhibitors chelerythrine chloride or calphostin C (Figure 6B; shown for calphostin C only).

We then analyzed the effects of cell-matrix adhesion on the phosphorylation status of fascin. Cells adherent on laminin-1 or TSP-1 contained unphosphorylated fascin (Figure 6, C and D). In marked contrast, fibronectin-adherent cells contained the acidic fascin variant and two additional minor acidic spots (Figure 6E). Previous studies have indicated the existence of minor acetylated variants of fascin; phosphorylation of such isoforms likely gives rise to the multiple isoelectric variants detected here (reviewed by Edwards and Bryan, 1995). To determine whether the appearance of acidic fascin variants in fibronectin-adherent cells resulted from PKC-dependent phosphorylation, extracts were prepared from cells treated with 100 nM calphostin C during the course of the fibronectin adhesion assay. In these extracts, fascin appeared as a single spot equivalent to the spot detected in laminin-1- or TSP-1-adherent cells (Figure 6F). The same result was obtained from cells treated with 320 nM chelerythrine chloride (our unpublished results). For comparison, C2C12 cells were treated with 50 nM TPA to artificially activate PKC. At 10 min, when large ruffles containing fascin ribs were formed (Figure 1C), fascin appeared as the single spot (Figure 6G). After 1 h, when fascin appeared diffuse (Figure 1D), all three acidic variants had appeared (Figure 6H). Because fascin phosphorylation reduces its actin-binding activity (Yamakita *et al.*, 1996) and because β -catenin has been reported as a second binding partner for fascin (Tao *et al.*, 1996), we explored whether 1 h of fibronectin adhesion or 1 h of TPA treatment might result in up-regulation of the pool of fascin associated with β -catenin.



Although a small fraction of the total pool of β -catenin was detectable by immunoblot of fascin immunoprecipitates, we did not detect an alteration in the amount of β -catenin that coprecipitated with fascin upon TPA treatment in three replicate experiments (our unpublished results).

Serine 39 of Fascin Is Required in Fibronectin-dependent Phosphorylation

The major site on fascin that is phosphorylated by $\text{PKC}\alpha$ in vitro or in response to TPA is serine 39 (Yamakita *et al.*, 1996; Ono *et al.*, 1997). To determine whether fibronectin-stimulated phosphorylation of fascin involves serine 39, we examined whether fibronectin adhesion led to the appearance of phosphorylated fascin in LLC-PK1 cells transfected with GFP-fascin or a nonphosphorylatable mutant, GFP-fascin S39A. The proteins were expressed stably at around 10-fold the level of endogenous fascin in LLC-PK1 cells (Figure 7A, lanes 1–3). GFP-fascin exhibited matrix regulation of phosphorylation in that it was not phosphorylated in cells adherent to laminin (Figure 7B) but became phosphorylated in LLC-PK1 cells adherent on fibronectin (Figure 7C). However, GFP-fascin S39A migrated as a single spot after 1 h of adhesion to fibronectin (Figure 7D). These results identify serine 39 as the required site for fascin phosphorylation in response to fibronectin and confirm that phosphorylation at serine 39 is responsible for the appearance of acidic isoelectric variants of fascin.

Mutation of Serine 39 of Fascin Results in Anomalous Cell Spreading and Cytoskeletal Organization in Matrix-adherent Cells

To determine whether stimulation of fascin phosphorylation is required in cell spreading and morphological organization on fibronectin, we compared the adhesive behavior of LLC-PK1 cells that stably expressed wild-type or mutant GFP-fascins. For these experiments, the mutant fascins included the nonphosphorylatable GFP-fascin S39A, which has wild-type actin-binding activity, and GFP-fascin S39D, which generates a protein with a constitutive negative charge compromised in actin binding and bundling. All three proteins were expressed at comparable levels, and all three cell lines

showed quantitatively similar attachment to fibronectin (Figure 7A, lanes 2–4, and data not shown).

For optimal visualization of microfilament organization, cells were stained with rhodamine-phalloidin after 1 h of adhesion to fibronectin under serum-free conditions. GFP-fascin expressor cells spread and assembled circumferential actin arcs and microfilament bundles. Radial actin ribs were present in the cell cortex. These did not correspond morphologically to filopodia because they lay within the lamellipodial margins and thus formed part of the complex actin network of the lamellipodium (Figure 8, A and B). In marked contrast, cells expressing GFP-fascin S39A spread poorly, showed little organization of microfilament bundles, and contained prominent ring-like concentrations of F-actin in the cell cortex (Figure 8D). Surprisingly, GFP-fascin S39D cells spread more extensively than wild-type cells and tended to assemble large actin microfilament bundles and arcs (Figure 8F).

Because phalloidin staining is not preserved in methanol-fixed preparations, we examined GFP-fascin distribution in matched replicate samples of fibronectin-adherent cells. As expected, wild-type GFP-fascin had a diffuse distribution in fibronectin-adherent cells (Figure 8C). GFP-fascin S39A cells displayed distinctive circumferential arrays of small fascin-containing projections at the substratum level and also apically (Figure 8E). In GFP-fascin S39D cells, fascin was diffuse and cortical projections were not apparent (Figure 8G). LLC-PK1 parental cells, GFP-fascin cells, and GFP-fascin S39D cells all assembled vinculin-positive focal contacts on fibronectin, whereas GFP-fascin S39A cells did not do so (our unpublished results). On laminin-1, GFP-fascin S39A cells spread less well than cells containing wild-type fascin and showed irregular fascin projections at cell margins. The localization of GFP-fascin S39D was diffuse, and these cells also spread less well than wild-type expressors on laminin-1 (our unpublished results).

LLC-PK1 cells do not spread on TSP-1, and so effects of the mutant fascins on TSP-1-stimulated microspike formation were examined by generating transfectant C2C12 clonal cell lines. The highest expressing lines, which expressed the mutant fascins at around 50% of the level of endogenous fascin, were chosen for functional analysis (Figure 9A). Cells expressing fascin S39A underwent partial spreading on TSP-1 and formed arrays of cortical actin and fascin projec-

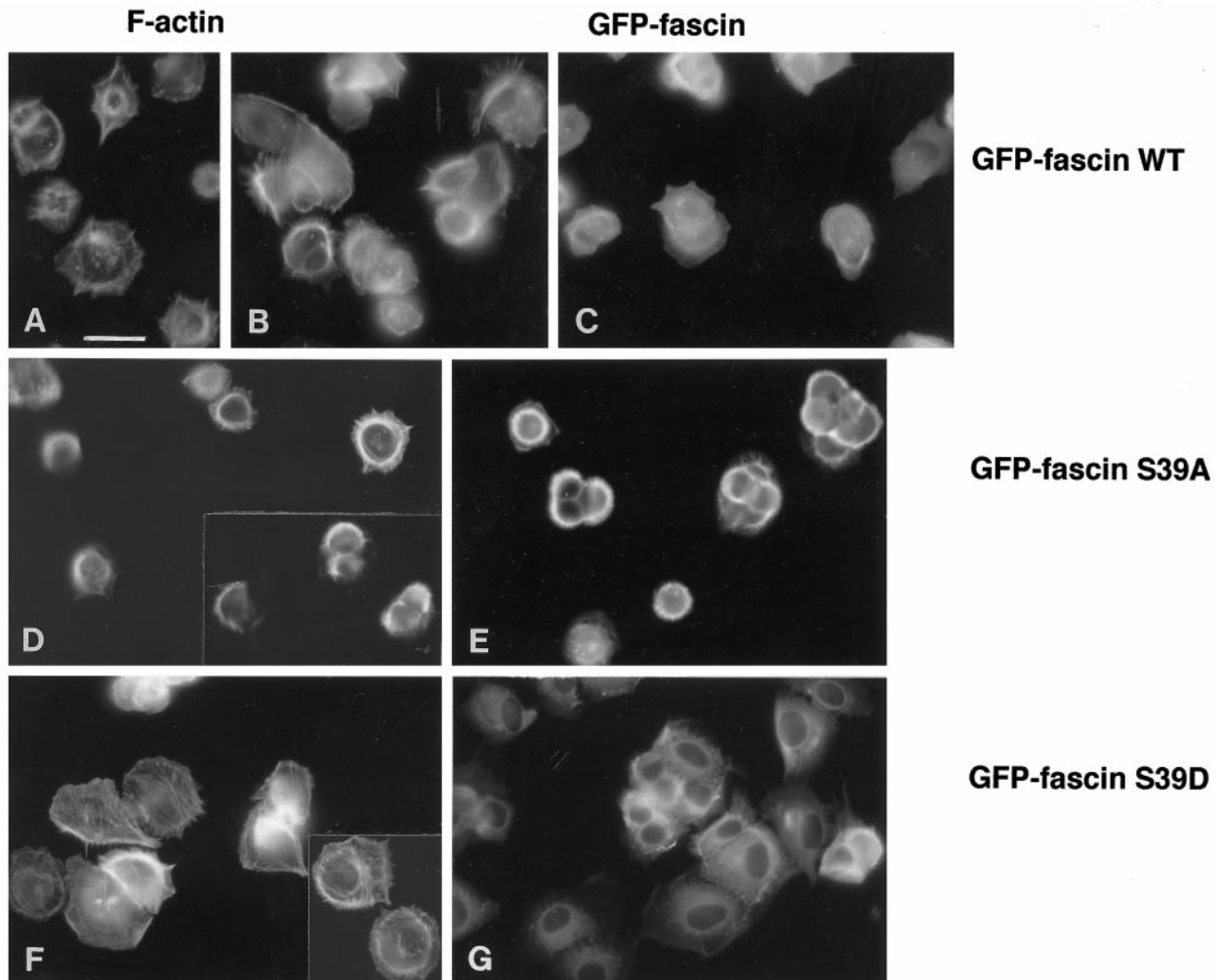


Figure 8. Mutant fascins affect cytoskeletal organization in fibronectin-adherent LLC-PK1 cells. Cells expressing GFP-fascin (A–C), GFP-fascin S39A (D and E), or GFP-fascin S39D (F and G) were stained with TRITC-phalloidin (A, B, D, and F) or fixed to visualize GFP-fascin (C, E, and G) after 1 h of adhesion to fibronectin. Bar, 10 μ m.

tions. These did not appear as well ordered as the projections formed by parental or vector-transfected C2C12 cells (Figure 9B). Fascin S39D cells attached to TSP-1 but remained round and did not form cortical fascin spikes (Figure 9B). On fibronectin, fascin S39D cells spread in a similar manner to wild-type cells (our unpublished results). Fascin S39A cells appeared round or poorly spread and displayed many F-actin- and fascin-containing projections (Figure 9C; compare with Figure 1A) (our unpublished results). Thus, fascin S39A has general inhibitory effects on fibronectin-initiated spreading.

PKC α Activity Is Necessary for Matrix-dependent Localization of Fascin

The results described above demonstrated that activation of PKC α causes relocalization of fascin from actin structures (Figures 1–3) and that matrix adhesion conditions differen-

tially regulate fascin phosphorylation by a PKC α -dependent process (Figures 4, 6, and 7). Three approaches were taken to demonstrate that PKC α activation is the stimulus for the dissociation of fascin from actin. First, C2C12 cells were treated with different concentrations of TPA for 24 h to establish conditions that caused down-regulation of PKC α . Down-regulation of PKC α was achieved by treatment with 500 nM TPA. The inactive compound 4- α -phorbol had no effect at this concentration (Figure 10A). Treatment with 500 nM TPA did not alter the level of fascin protein (Figure 10A). The cells in which PKC α was down-regulated spread less well on fibronectin and showed increased localization of fascin to actin microfilaments, membrane ruffles, and short spike projections (Figure 10B) compared with the 4- α -phorbol-treated cells. Double staining for F-actin and vinculin showed that the populations of TPA-down-regulated cells also had less well-organized microfilaments, increased actin

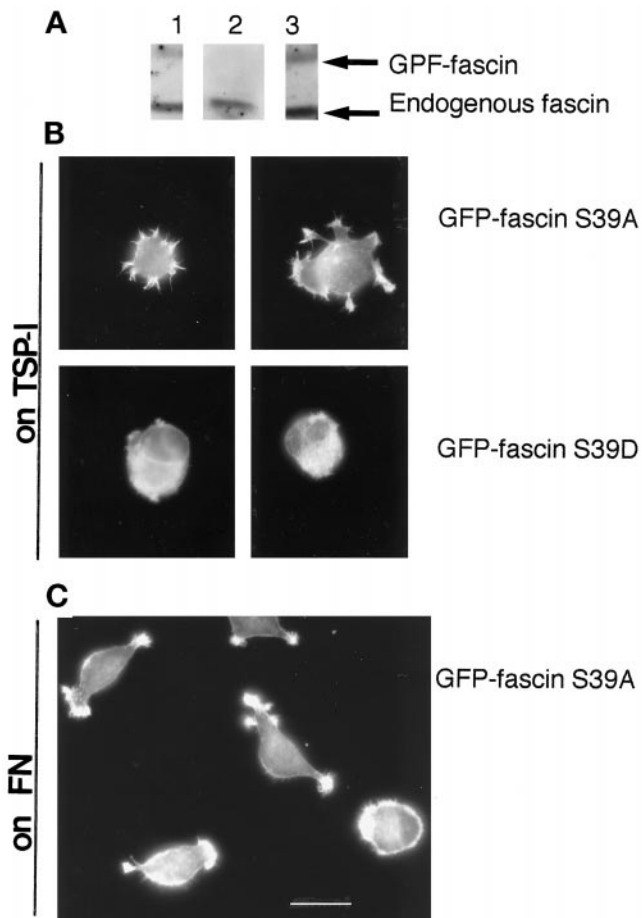


Figure 9. Mutant fascins affect cytoskeletal organization in fibronectin-adherent C2C12 cells. (A) Western blots of whole cell extracts of C2C12 stably expressing GFP-fascin S39A (lane 1), C2C12 (lane 2), or GFP-fascin S39D (lane 3). (B) F-actin organization in C2C12 cells expressing GFP-fascin S39A or S39D after 1 h of adhesion to TSP-1. (C) F-actin organization in C2C12 cells expressing GFP-fascin S39A after 1 h of adhesion to fibronectin. Bar, 10 μ m.

ruffles and projections at cell margins, and decreased focal contacts (Figure 10B). These cells formed spikes on TSP-1 (our unpublished results).

The attachment of skeletal myoblasts to fibronectin is mediated primarily by the $\alpha 5\beta 1$ integrin (Enomoto *et al.*, 1993; Adams and Lawler, 1994), and PKC α is activated during $\alpha 5\beta 1$ -mediated cell spreading (Vuori and Ruoslahti, 1993). As a second means to inhibit PKC α , C2C12 cells were plated on fibronectin in the presence of an adhesion-blocking antibody to the $\alpha 5$ integrin subunit, which was used at a concentration that blocked cell spreading without preventing cell attachment. The antibody-treated cells adopted irregular, partially spread morphologies and showed localization of fascin to microfilament bundles, ruffles, spikes, and filopodial projections of various sizes (Figure 10B). Sister cells double stained for F-actin and vinculin showed decreased organization of microfilaments and focal contacts and increased numbers of cortical actin projections (Figure 10B).

To link the effects of PKC α down-regulation on fascin distribution to phosphorylation of fascin at serine 39, the GFP-fascin-expressing LLC-PK1 cell lines were compared for adhesion to fibronectin after treatment for 24 h with 100 nM 4- α -phorbol or 100 nM TPA. LLC-PK1 cells express PKC α and PKC ϵ (Middleton *et al.*, 1993; Amsler *et al.*, 1996; Dibas *et al.*, 1996). The TPA treatment specifically down-regulated PKC α (Figure 11A). Levels of fascin protein were unchanged (our unpublished results). The cells expressing GFP-fascin in which PKC α had been down-regulated spread more extensively and showed a dramatic increase in fascin-containing cortical ruffles in which fascin colocalized with β -actin. The cells also showed partial colocalization of fascin with actin microfilaments in the perinuclear region (Figure 11B). Localization of vinculin to focal contacts was reduced (our unpublished results). TPA-treated GFP-fascin S39A cells also spread more than control cells but were unchanged in their ability to form cortical arrays of short fascin- and actin-containing projections (Figure 11B). In contrast, GFP-fascin S39D cells did not show enhanced spreading after down-regulation of PKC α , nor did they show localization of fascin or actin to cortical projections or ruffles. As in the 4- α -phorbol-treated or untreated control GFP-fascin S39D cells, fascin appeared diffuse and did not colocalize with the most prominent actin bundles (Figure 11B). Thus, PKC α activity is required to achieve a diffuse localization of fascin and, depending on the differentiated cell type, acts to inhibit localization of fascin to cortical spikes or ruffles. Additionally, the results in LLC-PK1 cells confirm that the phosphorylation of fascin at serine 39 is a target of activated PKC α .

DISCUSSION

We report a novel mechanism concerning the differential phosphorylation of fascin in response to cell-matrix adhesion conditions. We have used biochemical, pharmacological, and molecular genetic approaches to establish that $\alpha 5\beta 1$ integrin-mediated adhesion to fibronectin correlates with stimulation of fascin phosphorylation and that the molecular mechanism involves a PKC-dependent process in which serine 39 of fascin is the required site for phosphorylation. Adhesion to TSP-1 or laminin-1, conditions under which cells assemble stable microspikes or ruffles containing fascin and F-actin, does not stimulate fascin phosphorylation. Furthermore, the ability of cells to regulate fascin phosphorylation and thereby their actin-binding activity is of functional significance in matrix attachment, spreading, and cytoskeletal organization under different matrix-adhesion conditions.

Fascin was discovered as an actin-bundling protein in sea urchin egg extracts. Species orthologues in *Drosophila* and in mammalian cells have also been demonstrated to bind and bundle actin into tightly packed, highly ordered arrays (reviewed by Edwards and Bryan, 1995). More recently, the localization of fascin to cell surface spikes and projections at the leading edges of migratory mammalian cells and of fascin-transfected cells has suggested a major role in the formation of cellular protrusions (Tao *et al.*, 1996; Adams, 1997; Yamashiro *et al.*, 1998). Several independent analyses have indeed indicated that the assembly of fascin spikes or projections is of functional significance for cell motile behavior (Adams, 1997; Yamashiro *et al.*, 1998). Fascin-containing

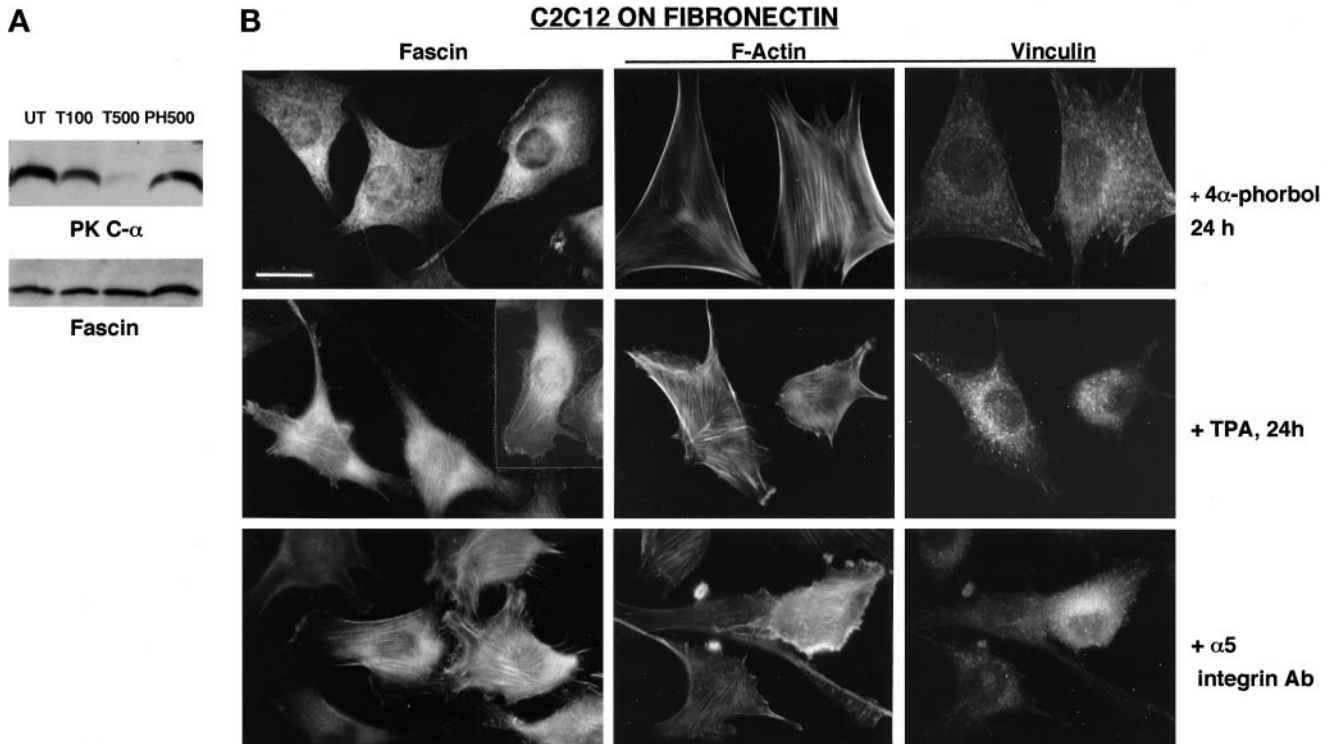


Figure 10. Requirement for PKC α activity in matrix-dependent fascin localization in C2C12 cells. (A) Western blot of C2C12 whole cell extracts probed with antiserum to PKC α or FAS-C to fascin. (UT) Untreated cells; (T100) cells treated with 100 nM TPA for 24 h; (T500) cells treated with 500 nM TPA for 24 h; (PH500) cells treated with 500 nM 4- α -phorbol for 24 h. (B) Comparison of the localizations of fascin, F-actin, and vinculin in cells treated with 500 nM 4- α -phorbol, 500 nM TPA, or 5 μ g/ml 5H10 antibody to integrin α 5 subunit after 1 h of adhesion to fibronectin under serum-free conditions. Bar, 5 μ m.

projections may also participate in other cellular activities. The antigen-presentation interactions of dendritic cells with T-cells involve the formation of close cell-to-cell appositions that are mediated by the finger-like dendritic projections of dendritic cells. Treatment of epidermal Langerhans cells with fascin antisense oligonucleotides inhibits the formation of these dendrites (Ross *et al.*, 1998). The presence of fascin-rich spikes and membrane projections on neuronal growth cones may indicate a role in axon guidance or adhesion (Edwards and Bryan, 1995; Adams, unpublished observations).

It has been established that cell adhesion to specific extracellular matrix macromolecules provides potent regulation of fascin distribution and microspike formation (Adams, 1995, 1997). On fibronectin, fascin-containing projections are formed transiently during the initial, postattachment spreading and are rapidly lost as cells adopt a polygonal, spread morphology. Fascin then appears uniformly diffuse. On TSP-1, fascin microspikes are formed in large arrays and remain stably adherent during cell spreading (Adams, 1995). The data presented here identify a biochemical mechanism that underlies these correlative effects. Adhesion to fibronectin mediated by α 5 β 1 integrin leads to PKC α -dependent phosphorylation of fascin at serine 39. This down-regulates the ability of fascin to bind and bundle actin (Ono *et al.*, 1997). In the absence of PKC α , these events do not take

place, and spikes and actin-associated fascin are retained at later times of adhesion. Interestingly, LLC-PK1 cells expressing the nonphosphorylatable fascin S39A formed many small fascin-containing projections when attached on fibronectin but were impaired in cell spreading. Yet, cells expressing fascin S39D spread more extensively than cells expressing wild-type fascin. Thus, the ability to modulate the actin-binding activity of fascin through phosphorylation appears to be an important required step in cell adhesion and focal contact assembly on fibronectin. Our data demonstrate that phosphorylation at this site is an *in vivo* target of PKC α activity, because PKC α down-regulation strongly altered the distribution of wild-type fascin yet did not markedly affect the distribution of fascin S39A or S39D. LLC-PK1 cells contain little endogenous fascin and provide a low background for observation of the effects of mutant fascins.

The experiments also demonstrate that adhesion of C2C12 cells on TSP-1 does not lead to phosphorylation of fascin. This suggested a possible mechanism for the stable nonpolarized formation of microspikes, in that fascin remains competent to bind actin in spread cells. This hypothesis was tested by expression of the mutant fascins in C2C12 cells. C2C12 cells expressing fascin S39A spread and formed arrays of spikes that appeared disorganized compared with the spikes of control C2C12 cells, whereas C2C12 cells expressing fascin S39D remained round. Thus, nonphospho-

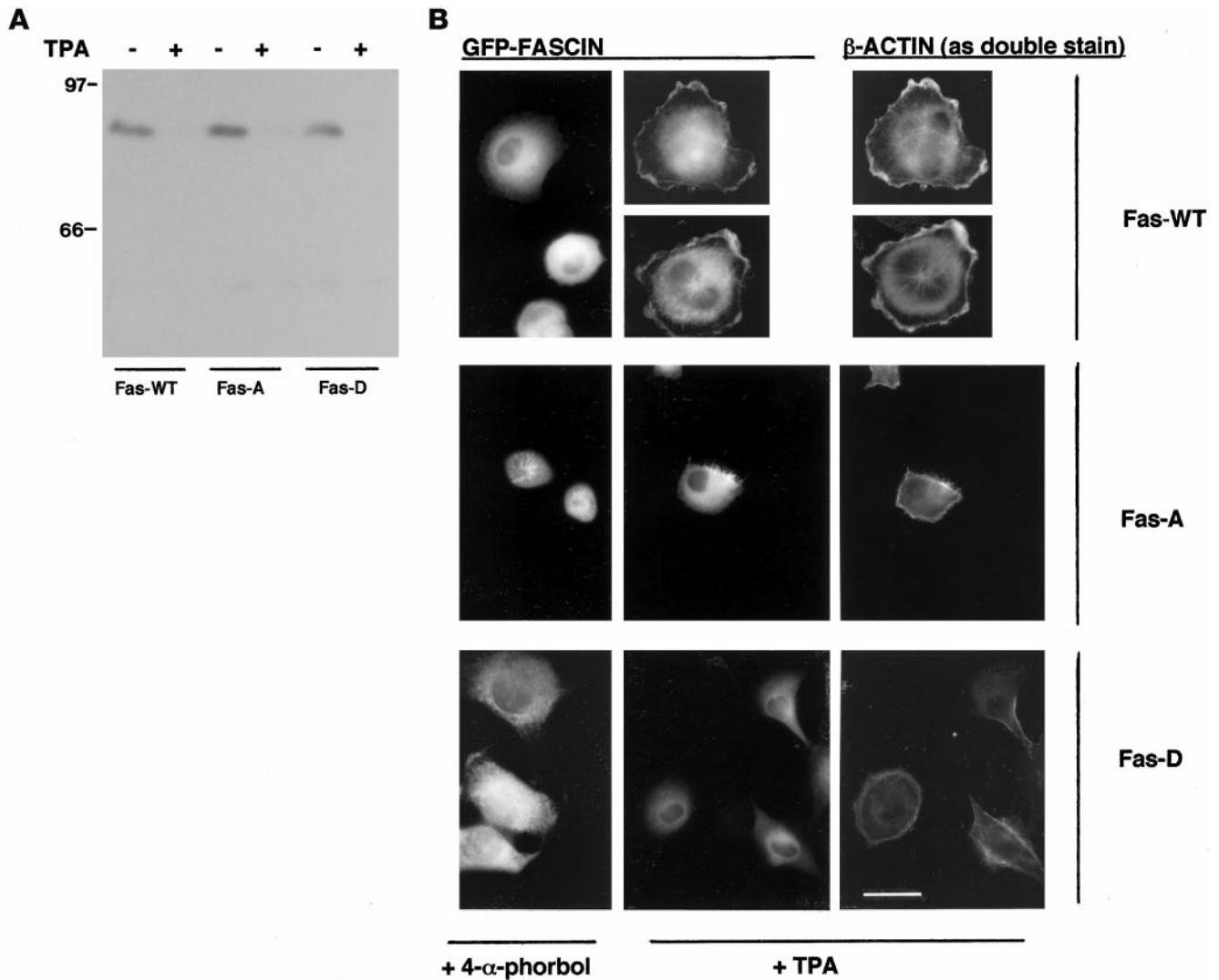


Figure 11. Requirements for PKC α activity and fascin phosphorylation at serine 39 in matrix-dependent fascin localization in LLC-PK1 cells. (A) Western blot of whole cell extracts of LLC-PK1 cell lines expressing GFP-fascin (Fas-WT), GFP-S39A fascin (Fas-A), or GFP-S39D fascin (Fas-D) prepared after 24 h of treatment with 100 nM 4- α -phorbol (TPA -) or 100 nM TPA (TPA +). The blot was probed with antiserum to PKC α . (B) Comparison of GFP-fascin localization in cells treated with 100 nM 4- α -phorbol or TPA for 24 h after 1 h of adhesion to fibronectin under serum-free conditions. TPA-treated cells are shown double stained for β -actin. Bar, 5 μ m.

rylated fascin is required for cells to form microspikes on TSP-1. The ability to cycle fascin phosphorylation could be important for the initial organization of adherent fascin spikes when cells contact TSP-1; however, because the activities of the mutant fascins in C2C12 cells are displayed against a large pool of endogenous fascin, it was not possible to define this point further in this experimental system.

The lack of specific colocalization of fascin with actin bundles or microfilaments in fibronectin-adherent cells raises the possibility that fascin interacts with other protein(s) under these conditions. A reported second binding partner is β -catenin (Tao *et al.*, 1996). We were unable to obtain evidence for an increase in the amount of fascin binding to β -catenin in fibronectin-adherent or TPA-treated cells. Interactions with skeletal muscle tropomyosin revers-

ibly inhibit fascin-actin binding, and it is possible that such interactions may be dominant for phosphorylated fascin, which has low affinity for actin (Ishikawa *et al.*, 1998). Alternatively, fascin may have additional noncytoskeletal binding partners.

Activation of PKC α is recognized as an early signaling event consequent to α 5 β 1 integrin-mediated attachment to fibronectin and has also been correlated with ligation of the vitronectin-binding integrins α v β 3 and α IIb/ β 3 (Vuori and Ruoslahti, 1993; Lewis *et al.*, 1995; reviewed by Kolanus and Seed, 1997). PKC α localizes to focal contacts in long-term adherent cells and preferentially interacts with active β 1 integrins (Jaken *et al.*, 1989; Ng *et al.*, 1999). Stimulation or inhibition of PKC, respectively, promotes or inhibits the organization of focal adhesions in prespread cells (Woods

and Couchman, 1992). Similarly, activation of PKC by TPA or overexpression of a constitutively activated PKC α mutant correlates with enhanced cell spreading and motility (Vuori and Ruoslahti, 1993; Rigot *et al.*, 1998; Miranti *et al.*, 1999; Sun and Rotenberg, 1999). Cells in which PKC is inhibited pharmacologically or down-regulated typically show reduced spreading (Chun and Jacobson, 1993; Gao *et al.*, 1996; Miranti *et al.*, 1999). We have obtained similar results in C2C12 cells. The dramatically increased spreading on fibronectin of GFP-fascin expressor LLC-PK1 cells in which PKC α is down-regulated is thus an unusual and surprising response. To our knowledge, this is the first examination of the effects of PKC α down-regulation on the matrix adhesion of these kidney epithelial cells. One possible explanation could be that secondary effects of PKC α down-regulation on other regulatory molecules or elements of the cytoskeleton result in an abnormal state of cell contraction. Indeed, certain mRNAs are stabilized in LLC-PK1 cells upon down-regulation of PKC (Nanbu *et al.*, 1994).

Several components of the submembranous cytoskeleton are substrates of PKC; these include profilin (Hansson *et al.*, 1988), MARCKS, an actin-binding protein that functions in initial spreading on fibronectin (Myat *et al.*, 1997), and the focal contact components vinculin and talin (Werth *et al.*, 1983; Werth and Pastan, 1984; Beckerle, 1990; reviewed by Jaken, 1996). The mechanism by which PKC promotes focal contact assembly may involve stabilization of interactions between the integrin cytoplasmic domain and talin (Burn *et al.*, 1988; Woods and Couchman, 1992). In addition, phosphorylation of vinculin tail domain by PKC is increased in the presence of acidic phospholipids. It has been established that acidic phospholipids inhibit the intramolecular interactions of vinculin head and tail domains, thereby exposing the actin- and paxillin-binding sites. Phosphorylation of unfolded vinculin tail domain by PKC may further facilitate its incorporation into focal contacts. This activity may underlie the poor formation of focal contacts by cells in which PKC α is down-regulated or inhibited by blockade of fibronectin binding to $\alpha 5\beta 1$ integrin (Schweinbacher *et al.*, 1996; Weekes *et al.*, 1996; Huttelmaier *et al.*, 1998). Further experiments will examine whether such regulation of the vinculin-actin interaction contributes indirectly to the regulation of microspike formation by extracellular matrix.

Such promotion of focal contact organization by activated PKC contrasts with its inhibitory effects on actin and/or fascin spikes. We postulate that cell adhesion on TSP-1 may involve low activity of PKC in the cell cortex. Evidence in support of this view is provided by (a) the absence of phosphorylated fascin in TSP-1-adherent cells; (b) the loss of microspikes from TSP-1-adherent cells upon strong activation of PKC α by phorbol ester; and (c) localization studies that indicate that PKC α is not detectable in the cortical regions of TSP-1-adherent cells. However, the impairment of adhesion to TSP-1 in cells treated with PKC α pseudosubstrate peptide is suggestive of a role for PKC α in the early stages of attachment. A likely scenario may be that PKC α is transiently or weakly activated when cells attach to TSP-1 and may be needed to phosphorylate substrates that play a role in receptor clustering or actin nucleation. In the absence of sustained PKC α activity in the cell cortex, cross-linking of actin by fascin would take place and lead to microspike formation. Indeed, in another experimental system, TSP-1

has been found to activate distinct signaling events involving a Gi-type heterotrimeric G protein (Gao *et al.*, 1996). Alternatively, PKC activity may form part of a parallel pathway that can be overridden in the context of adhesion to TSP-1. One interesting possibility is that adhesion to TSP-1 may activate a serine phosphatase. These possibilities will be addressed in further experiments.

Cell-staining studies have shown that fascin projections and microspikes are not uniformly present on all cells under standard tissue culture conditions. When formed, the projections tend to be restricted to localized domains of the cell surface (Yamashiro-Matsumura and Matsumura, 1986; Adams, 1995; Tao *et al.*, 1996). These observations imply the existence of physiological mechanisms that modulate the formation of fascin spikes and projections in conjunction with other adhesive contacts such as focal contacts. Experimental 1:1 mixed fibronectin/TSP-1 substrata stimulate concurrent formation of focal contacts and fascin spikes, and this phenomenon depends on both integrins and proteoglycans (Adams, 1997). Here we have established a molecular mechanism, namely the promotion or inhibition of PKC-dependent fascin phosphorylation, by which cell adhesion to specific extracellular matrix macromolecules differentially regulates the ability of cells to assemble fascin projections. In the context of a complex tissue extracellular matrix, specific microenvironments may either facilitate or disfavor the localized or polarized formation of fascin spikes and filopodia and thereby modulate cell-adhesive and migratory behavior.

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