Lysophosphatidic Acid and Microtubule-destabilizing Agents Stimulate Fibronectin Matrix Assembly through Rho-dependent Actin Stress Fiber Formation and Cell Contraction

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Fibronectin (FN) matrix assembly is a cell-dependent process mediated by cell surface-binding sites for the 70-kDa amino-terminal region of FN. We have shown recently that lysophosphatidic acid (LPA) is a stimulator of FN matrix assembly. Disruption of microtubules has been shown to mimic some of the intracellular effects of LPA including the formation of actin stress fibers and myosin light chain phosphorylation. We compared the effects of microtubule disruption and LPA on FN binding and actin cytoskeleton organization. The disruption of microtubules by nocodazole or vinblastine increased FN binding to adherent cells. The modulation of binding sites was rapid, dynamic, and reversible. Enhanced binding was due to increases in both the number and affinity of binding sites. These effects are similar to the effects of LPA on FN binding. Binding induced by nocodazole was inhibited by the microtubule-stabilizing agent Taxol but not by pretreatment with a concentration of phospholipase B that totally abolished the stimulatory effect of LPA. Fluorescence microscopy revealed a close correlation among actin stress fiber formation, cell contraction, and FN binding. Blockage of the small GTP binding protein Rho or actin–myosin interactions inhibited the effects of both nocodazole and LPA on FN binding. These observations demonstrate that Rho-dependent actin stress fiber formation and cell contraction induce increased FN binding and represent a rapid labile way that cells can modulate FN matrix assembly.

INTRODUCTION

The immediate microenvironment[1] surrounding cells has vital importance to cellular growth and differentiation. The extracellular matrix, through its binding receptors on the cell surface, activate various intracellular signals that specifically modulate events like mitogenesis and cell movement (Hynes, 1992). One of the major extracellular matrix proteins is fibronectin (FN), a dimeric glycoprotein found at high concentrations in plasma or other body fluids and in an insoluble form in connective tissues and basement membranes (Mosher, 1989; Hynes, 1990). Deposition of soluble FN into the extracellular matrix is a cell-dependent event initiated by binding of the FN to cell surface sites in a specific and saturable manner (McKeown-Longo and Mosher, 1983; Peters and Mosher, 1987; Allio and McKeown-Longo, 1988). Reversibly bound FN then is polymerized into high molecular weight multimers (McKeown-Longo and Mosher, 1984; Barry and Mosher, 1988, 1989). Insoluble FN appears to have a role in a wide variety of physiologic events, e.g., in embryogenesis and wound healing

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[1] Abbreviations used: BDM, butanedione-2-monoxime; FN, fibronectin; LAMMs, large apparent molecular mass molecules; LPA, lysophosphatidic acid; PMSF, phenylmethylsulfonyl fluoride; rFXIII, recombinant factor XIII; 70-kDa fragment, 70-kDa amino-terminal fragment of fibronectin; TBS, Tris-buffered saline; Vzg-1, ventricular zone gene-1.

(Thiery et al., 1989; Gailit and Clark, 1994). In vivo studies have shown that inhibiting the interaction of mesodermal cells with FN fibrils in amphibian embryos inhibits cell adhesion and migration and blocks gastrulation (Boucaut et al., 1984). Transgenic mice lacking FN die early in embryogenesis, demonstrating that FN is essential for normal development (George et al., 1993).

Serum has long been known to influence the elaboration of FN matrix by cultured cells (Mautner and Hynes, 1977; Mosher and Vaheri, 1978; Rennard et al., 1981). We have shown that lysophosphatidic acid (LPA) is the serum enhancement factor of FN matrix assembly (Checovich and Mosher, 1993; Zhang et al., 1994). LPA rapidly up-regulates the FN matrix assembly binding sites. This up-regulation is reversible and closely correlated with LPA-induced actin stress fiber formation and cell contraction (Zhang et al., 1994). The 70-kDa amino-terminal region of FN (70-kDa fragment) mediates binding to the FN matrix assembly sites (McKeown-Longo and Mosher, 1985, 1989). The FN matrix assembly receptor has not been characterized. Both the FN binding integrin $\alpha5\beta1$ (Akiyama et al., 1989; Giancotti and Ruoslahti, 1990; Wu et al., 1993) and preexisting FN (Aguirre et al., 1994; Hocking et al., 1994, 1996) have been implicated as the assembly receptor. We have recently characterized cell surface binding sites for 70-kDa fragment regulated by LPA by using cross-linking strategies (Zhang and Mosher, 1996). Target cell surface molecules migrated in discontinuous SDS-PAGE at the top of the stacking gel and near the top of the separating gel giving an estimated size of approximately 3-MDa (with reduction). Ability of cells to support formation of the cross-linked complexes is rapidly up-regulated by LPA, and the complexes are not recognized by antibodies recognizing $\alpha5\beta1$ or FN (outside the 70-kDa region).

LPA is unique among a number of agonists in causing enhanced assembly of FN (Zhang et al., 1994). This result suggests that specific signal transduction mechanisms mediate the regulation of FN matrix assembly sites. Cytochalasin has been shown to cause loss of FN binding sites, indicating a role for actin polymerization (Barry and Mosher, 1988; Wu et al., 1995). Activation of protein kinase C up-regulates FN binding (Somers and Mosher, 1993), while increased cellular c-AMP down-regulates the binding (Allen-Hoffmann and Mosher, 1987). LPA has a complex intracellular action on cells activating several signaling pathways (Moolenaar et al., 1997). In the nanomolar dose range, LPA induces actin stress fiber and focal adhesion formation through the activation of the small GTP binding protein Rho (Moolenaar et al., 1997). Recent data indicate that LPA-induced formation of focal adhesion is dependent upon the activation of myosin light chain kinase and actin-myosin interactions (Chrzanowska-Wodnicka and Burridge, 1996). A candidate gene

product for the LPA receptor called ventricular zone gene-1 (Vzg-1) was cloned from neural tissue (Hecht et al., 1996). Vzg-1 is a seven transmembrane spanning G protein-coupled receptor. When transfected into cells, Vzg-1 mediated both LPA-induced cell contraction and lowering of cAMP through two different G proteins (Hecht et al., 1996). A second seven-membrane spanning receptor that mediates oscillatory Cl⁻ currents in response to LPA has been cloned from Xenopus oocytes (Guo et al., 1996).

Microtubule destabilizing agents have been shown to induce the formation of actin stress fibers (Danowski, 1989; Kajstura and Bereiter-Hahn, 1993), and contraction and phosphorylation of myosin light chain (Kolodney and Elson, 1995) in fibroblasts. Furthermore, disruption of microtubules has been shown to activate the integrin-dependent signaling cascade involving tyrosine phosphorylation of focal adhesion kinase and paxillin and formation of focal adhesions (Bershadsky et al., 1996). These effects of microtubule-destabilizing agents are similar to the effects seen after LPA activation or microinjection of dominant active Rho (Ridley and Hall, 1992).

In this article, we compare the effects of microtubule-destabilizing agents and LPA on binding of FN or 70-kDa fragment to labile cell surface binding sites, the formation of actin stress fibers, and cell contraction. We found that microtubule-destabilizing agents mimic the effects of LPA in enhancing binding of FN to cell surfaces. Both enhancers of FN binding require Rho-dependent stress fiber formation and actin–myosin interactions.

MATERIALS AND METHODS

Materials

Nocodazole {methyl-[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]-carbamate}, vinblastine, Taxol (paclitaxel), 2,3-butanedione 2-monoxime (BDM), bovine serum albumin, phospholipase B, hirudin, and mouse monoclonal antibodies to α-tubulin (clone B-5-1-2) were purchased from Sigma (St. Louis, MO). 1-Oleoyl-LPA was obtained from Avanti Polar Lipids (Birmingham, AL). KT5926 was obtained from Calbiochem (La Jolla, CA). [^{32}P]NAD was purchased from DuPont New England Nuclear (Boston, MA). Recombinant factor XIII (rFXIII), human thrombin, and C3 exotransferase were generous gifts from Dr. Paul Bishop at Zymogenetics (Seattle, WA), Dr. John Fenton at New York State Department of Health (Albany, NY), and Dr. Connie Lebakken at University of Wisconsin (Madison, WI), respectively. Rhodamine-phalloidin and fluorescein isothiocyanate (FITC) were obtained from Molecular Probes (Eugene, OR). Anti-FN antiserum was produced in our laboratory. FITC-conjugated goat anti-mouse IgG was obtained from Cappel (Durham, NC).

Human plasma FN and the 70-kDa amino-terminal gelatin-binding fragment of FN generated by cathepsin D were isolated, iodinated, and reisolated as previously described (McKeown-Longo and Mosher, 1985). The labeled protein was stored in portions at −70°C in TBS [Tris(hydroxymethyl)aminomethane hydrochloride, 20 mM, pH 7.4; sodium chloride, 150 mM] containing 0.2% bovine serum albumin. Purity of labeled protein was assessed by SDS-PAGE with and without reduction followed by autoradiography. FN was la-

beled with FITC as described previously (McKeown-Longo and Mosher, 1985).

Cell Cultures

The MG63 human osteosarcoma cell line and fetal bovine heart endothelial cells were obtained from the American Type Culture Collection (Rockville, MD). Human foreskin fibroblasts were a strain derived by Dr. Lynn Allen-Hoffmann (University of Wisconsin-Madison). Cells were cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 5% (for MG63) or 10% (for human foreskin fibroblasts) fetal bovine serum (Intergen, Purchase, NY). Fetal bovine heart endothelial cells were cultured in DMEM supplemented with 20% fetal bovine serum and 20 ng/ml basic fibroblast growth factor. For binding studies, cells were seeded in 24-well cluster tissue culture plates (Costar, Cambridge, MA) and analyzed 1 d after they reached confluency (3–5 d after seeding).

FN Binding and Cross-linking

FN binding to cells was assayed using ^{125}I-labeled 70-kDa fragment (^{125}I-70-kDa fragment) as described previously (Zhang et al., 1994). Briefly, after washing in TBS, cells were incubated with the agent being tested and radiolabeled 70-kDa fragment (150,000 cpm/well in 0.5 ml, approximately 0.2 μg/ml) for the duration of binding assay (usually 45 min). Cells were then washed and solubilized in 0.2 N NaOH, and the amount of cell layer-associated radioactivity was quantified. Nonspecific binding (in the presence of unlabeled 70-kDa fragment, 50 μg/ml) was subtracted from total binding to calculate specific binding, and binding was calculated as nanograms of specifically bound ligand per milligram of cellular protein. Cellular protein was assessed using BCA protein assay reagent (Pierce, Rockford, IL).

For binding isotherms at equilibrium, confluent cell layers in the absence or presence of nocodazole were incubated at 37°C for 45 min with increasing concentrations of ^{125}I-70-kDa fragment as described previously (Zhang et al., 1994).

To characterize the molecular nature of binding sites, cell-associated ^{125}I-70-kDa fragment was cross-linked by 10 μg/ml thrombin-activated rFXIII in the presence of 2 mM Ca^{2+} at 37°C for 5 min, as described previously (Zhang and Mosher, 1996). The cell lysate samples were reduced and analyzed by SDS-PAGE and phosphor-imaging.

Fluorescent Microscopy

Cells cultured on coverslips were incubated with 20 μg/ml FITC-labeled FN in the absence or presence of various additives for 45 min at 37°C, washed with TBS, and fixed in 3% paraformaldehyde. For actin localization, cells were fixed in 3% paraformaldehyde for 30 min, permeabilized with 0.2% Triton X-100 for 5 min, and incubated with 0.1 μg/ml rhodamine-labeled phalloidin for 20 min. For tubulin visulization and staining of preformed FN matrix, cells were incubated with 1:1000 diluted anti-α-tubulin or anti-FN for 60 min followed by detection with FITC-conjugated secondary antibody. Tubulin staining was performed on cells treated with paraformaldehyde and Triton X-100, whereas the the Triton step was omitted prior to FN staining. Coverslips were mounted with glycerol gel and cells were viewed on a Nikon epifluorescence microscope. All experimental permutations using a given fluorochrome were photographed at the same settings.

ADP-Ribosylation Assay

To assess the ADP-ribosylation state of Rho induced by C3 transferase, cells were pretreated with the transferase for 24 h, and extracts were assayed for residual Rho available for ADP-ribosylation with excess transferase (Aktories and Just, 1995). Briefly, cells were washed in TBS and lysed in ice-cold buffer containing 10 mM triethanolamine hydrochloride (pH 7.5), 2 mM $MgCl_2$, and 1 mM

phenylmethylsulfonyl fluoride (PMSF). The cell lysate (30 μl) was then incubated for 30 min at 37°C with C3 transferase (final concentration 1 μg/ml), [^{32}P]NAD (10 μCi), 50 mM triethanolamine hydrochloride (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 0.2 mM PMSF (total volume 50 μl). After stopping the reaction with SDS sample buffer and heating at 95°C for 10 min, the lysate was subjected to SDS-PAGE and analyzed by phosphorimaging. The 21-kDa band corresponding to Rho was the only band specifically labeled in presence of C3 transferase and was quantified by phosphorimaging.

RESULTS

Microtubule Disruption Increases FN Binding to Adherent Cells

LPA has been shown to stimulate FN binding concomitant with changes of cell shape and actin-containing cytoskeleton (Zhang et al., 1994). Disruption of microtubules by nocodazole or vinblastine results in the rapid assembly of focal adhesion and of microfilament bundles (Bershadsky et al., 1996) that are similar to the changes induced by LPA. We therefore asked whether disruption of microtubules would induce expression of binding sites for FN. For these studies, the ^{125}I-70-kDa amino-terminal fragment of FN was used in binding assays, since the initial reversible binding of soluble FN to substrate-attached cells is mediated principally by the 70-kDa amino-terminal region of FN, and the fragment does not become irreversibly bound to cell surfaces without cross-linking (McKeown-Longo and Mosher, 1985; Barry and Mosher, 1989; Zhang and Mosher, 1996). Binding of ^{125}I-70-kDa fragment to human MG63 osteosarcoma cell monolayers was enhanced in the presence of 10 μM nocodazole (Figure 1). This concentration of nocodazole has been
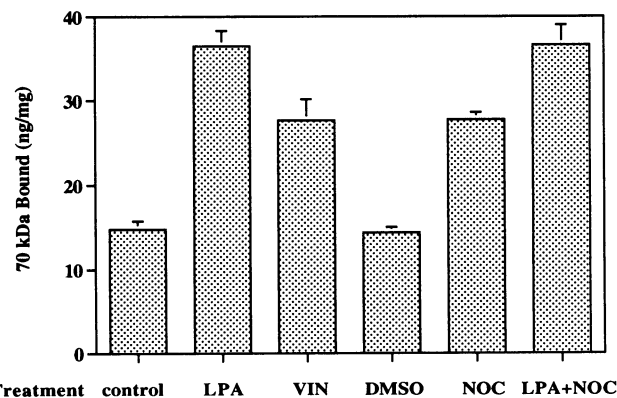

Figure 1. Effects of microtubule-disrupting agents and LPA on ^{125}I-70-kDa fragment binding to MG63 cells. Cells were incubated with ^{125}I-70-kDa fragment and the various agents: LPA (500 nM), vinblastine (50 μM, VIN), DMSO (0.1%), nocodazole (10 μM, NOC), or the combination of 500 nM LPA and 10 μM nocodazole (LPA+NOC) for 45 min. Nocodazole was dissolved in DMSO at a final concentration of 0.1%. Each bar represents the mean ± SD of triplicate values. Data represent specific binding as described in MATERIALS AND METHODS.

reported to cause dissociation of microtubules and induce an increase in the number and size of microfilament bundles in serum-starved cells (Kajstura and Bereiter-Hahn, 1993; Bershadsky *et al.*, 1996). The stimulatory effect of nocodazole was comparable with that of LPA, and the effects of the two agents were not additive (Figure 1). The presence of dimethyl sulfoxide (DMSO) did not affect 70-kDa fragment binding (Figure 1). Vinblastine, which disrupts microtubule by a different mechanism than that of nocodazole (Wilson and Jordan, 1994) and produces tubulin "paracrystals" (Bershadsky *et al.*, 1996), had a stimulatory effect similar to that of nocodazole on binding of 70-kDa fragment (Figure 1). Binding of 70-kDa fragment to human TJ6F foreskin fibroblasts and fetal bovine heart endothelial cells was also enhanced by nocodazole treatment (our unpublished results). Although LPA- and microtubule-destabilizing agents both increased binding of 70-kDa fragment to cell surfaces in the 45-min assay, these agents had no discernible effect on preformed FN matrix as assessed by immunofluorescence of similarly treated cells using antibodies against human FN. When freshly seeded MG63 cells were treated with 10 μM nocodazole for 16 h, there was a marked increase in FN matrix formed by the cells as assessed by immunofluorescence. There was no difference in the FN matrix formed by cells incubated with 500 nM LPA compared with control cells (our unpublished observations). This is presumably due to the rapid breakdown of LPA by cells in culture (Zhang *et al.*, 1994).

The enhancement of 70-kDa fragment binding to MG63 cells by nocodazole was observed within 10 min after the addition of nocodazole and binding ligand and sustained for hours (Figure 2). Just like LPA,
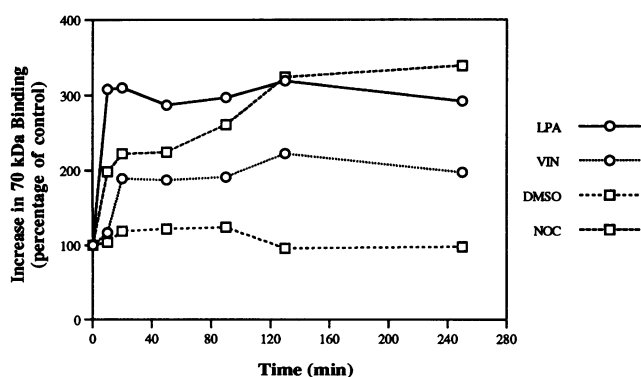

Figure 2. Time course of ^{125}I-70-kDa fragment binding to MG63 cells after stimulation with microtubule-disrupting agents and LPA. Cells were incubated with ^{125}I-70-kDa fragment and the various agents: 500 nM LPA, 50 μM vinblastine (VIN), 0.1% DMSO, or 10 μM nocodazole (NOC) for the time indicated. Data are presented as specific binding in presence of additive divided by specific binding in the absence of any additives as percentage of control. Each point represents the average of duplicate values (varied < 10%).
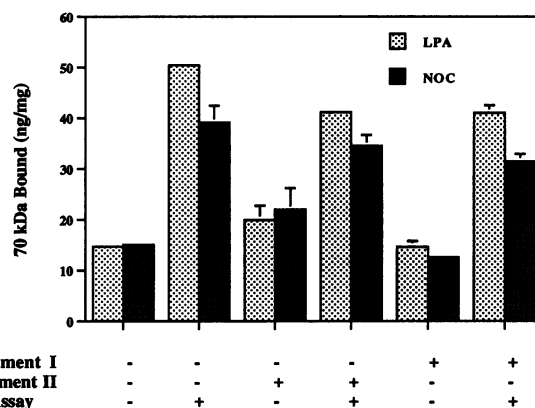

Pre-Treatment I	-	-	+	-	+	+
Pre-Treatment II	-	-	+	+	-	-
Binding-Assay	-	+	-	+	-	+

Figure 3. Effect of incubation order on the enhancement by nocodazole or LPA on ^{125}I-70-kDa fragment binding to MG63 cells. Cells were pretreated for two 1-h periods, with three quick washes of TBS between each pretreatment, and then incubated for 45 min with ^{125}I-70-kDa fragment during the binding assay. When present (+), the concentrations of LPA or nocodazole during the pretreatments and/or binding assay were 500 nM or 10 μM, respectively.

the stimulatory effect on 70-kDa fragment binding by nocodazole was reversible (Figure 3). Cells preincubated with nocodazole, with or without an intervening interval, responded to nocodazole during the binding assay as well as previously untreated cells. Cells from which nocodazole had been withdrawn at the beginning of the binding assay bound the same low level of 70-kDa fragment as control cells that had not been exposed to nocodazole at all. Similar results were seen when the cells were incubated with LPA with or without pretreatments (Figure 3). These result indicate that the cellular binding sites are labile, in that LPA or nocodazole must be present at the time of the binding assay and removal of the stimulatory agent results in rapid down-regulation of the binding sites.

Three independent analyses of the isotherms of binding of 70-kDa fragment to nocodazole-treated and control MG63 cells for 45 min at 37°C indicated that nocodazole caused changes in both the number of the binding sites per cell (166,000 ± 23,000 for nocodazole-treated cells and 93,000 ± 10,000 for control cells) and K_d (4.8 ± 0.8 nM for nocodazole-treated cells and 8.3 ± 1.7 nM for control cells). The magnitudes of the increases in binding sites and affinity induced by nocodazole were similar to those previously reported for LPA (Zhang *et al.*, 1994).

LPA treatment causes increased cross-linking of bound 70-kDa fragment to molecules of large apparent molecular mass (LAMMs) proposed to represent the FN matrix assembly receptor (Zhang and Mosher, 1996). Cross-linking of bound ^{125}I-70-kDa fragment to molecules on nocodazole- or vinblastine-treated cells was also to LAMMs of 3-MDa and >>3-MDa (Figure 4). The amounts of cross-linked products correlated with the ability of cell layers to bind ^{125}I-70-kDa frag-
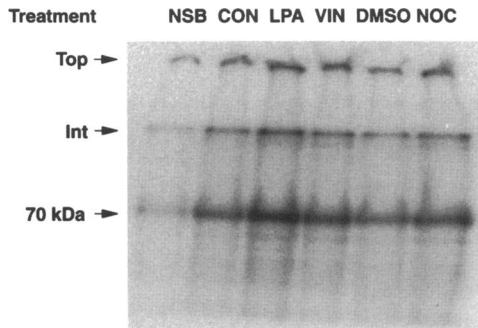
Figure 4. Cross-linking of [125]I-70-kDa fragment to MG63 cells. Newly confluent cells were incubated for 60 min with [125]I-70-kDa fragment (200 ng/ml) in medium containing 50 µg/ml unlabeled 70-kDa fragment (NSB), no additives (CON), 500 nM LPA, 50 µM vinblastine (VIN), 0.1% DMSO, or 10 µM nocodazole (NOC). Cell layers were then washed and incubated for additional 5 min with the cross-linking agent rFXIIIa, 10 µg/ml. Samples were examined by SDS-PAGE under reducing conditions (8% running and 3% stacking gels). Top, top of the stacking gel; Int, interface between the running and stacking gels; 70 kDa, 70-kDa fragment. Cross-linked [125]I-70-kDa fragment at the top and interface, and uncross-linked 70-kDa fragment were quantified by phosphorimaging. Quantitative counts in the three bands (Top, Int, 70 kDa), expressed as a percentage of experimental compared with control in each of the bands, respectively, were as follows: NSB, 20, 25, and 28; LPA, 271, 301, and 333; VIN, 205, 165, and 184; DMSO, 65, 81, and 79; and NOC, 177, 156, and 187.
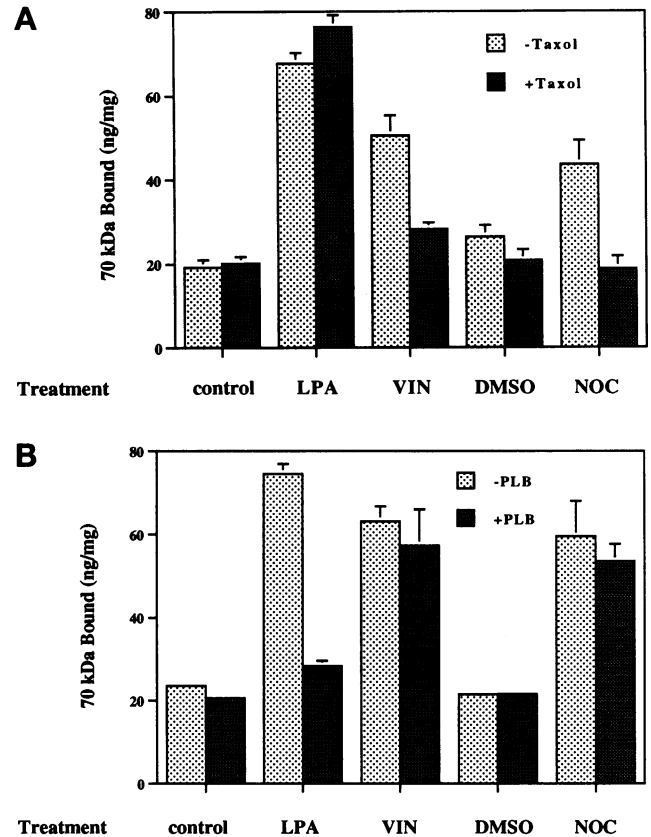

Figure 5. Effects of Taxol (A) or phospholipase B (B) pretreatment on [125]I-70-kDa fragment binding. MG63 cells were pretreated with or without Taxol (20 µM) for 2 h (A) or phospholipase B (PLB, 3 U/ml) for 15 min (B) prior to adding stimulants (final concentration: LPA, 500 nM; vinblastine, 50 µM [VIN]; DMSO, 0.1%; nocodazole, 10 µM [NOC]). Each bar represents the mean ± SD of triplicate values. All bars represent specific binding as described in MATERIALS AND METHODS.

ment (Figure 4, legend), and this cross-linking was markedly inhibited by excess unlabeled 70-kDa fragment, indicating specific cross-linking. These results suggest that the molecules mediating the enhanced binding of 70-kDa fragment to cell monolayers in response to nocodazole or LPA are similar.

Differentiation of the Stimulatory Effects on 70-kDa Fragment Binding by LPA or by Microtubule Disruption

When the cells were preincubated for 2 h with 20 µM Taxol, a microtubule-stabilizing agent (Wilson and Jordan, 1994), before addition of 10 µM nocodazole or 50 µM vinblastine, enhancement of 70-kDa fragment binding was totally abolished (Figure 5A). The stimulatory activity of LPA on 70-kDa fragment binding, in contrast, was not affected by pretreatment with Taxol (Figure 5A). These results indicate that the effects of nocodazole or vinblastine on 70-kDa fragment binding are due to microtubule disruption, inasmuch as microtubule stabilization efficiently and specifically prevented the stimulatory activity exerted by nocodazole or vinblastine.

Secretory phospholipase A2 has been shown to generate LPA in membrane microvesicles shed from activated cells (Fourcade et al., 1995), and increased amounts of LPA are synthesized by cells in response to a variety of stimuli (Billah et al., 1981; Lapetina et al.,

1981; Watson et al., 1985; Gerrard and Robinson, 1989; Exton, 1990). To test whether nocodazole- or vinblastine-enhanced 70-kDa fragment binding is dependent upon endogenous LPA production, cells were pretreated with phospholipase B, which hydrolyzes acyl bonds at the 1 position of LPA and renders LPA inactive. Phospholipase B treatment totally abolished the stimulatory activity of added LPA on binding of 70-kDa fragment, whereas the enhancement of binding by nocodazole or vinblastine was not affected at all (Figure 5B). This result argues that the stimulatory activity on FN binding by microtubule disruption is not secondary to extracellular LPA production.

Correlation between Actin Stress Fiber Formation and FN Binding

Changes in actin-containing cytoskeleton induced by nocodazole or LPA were correlated with enhanced
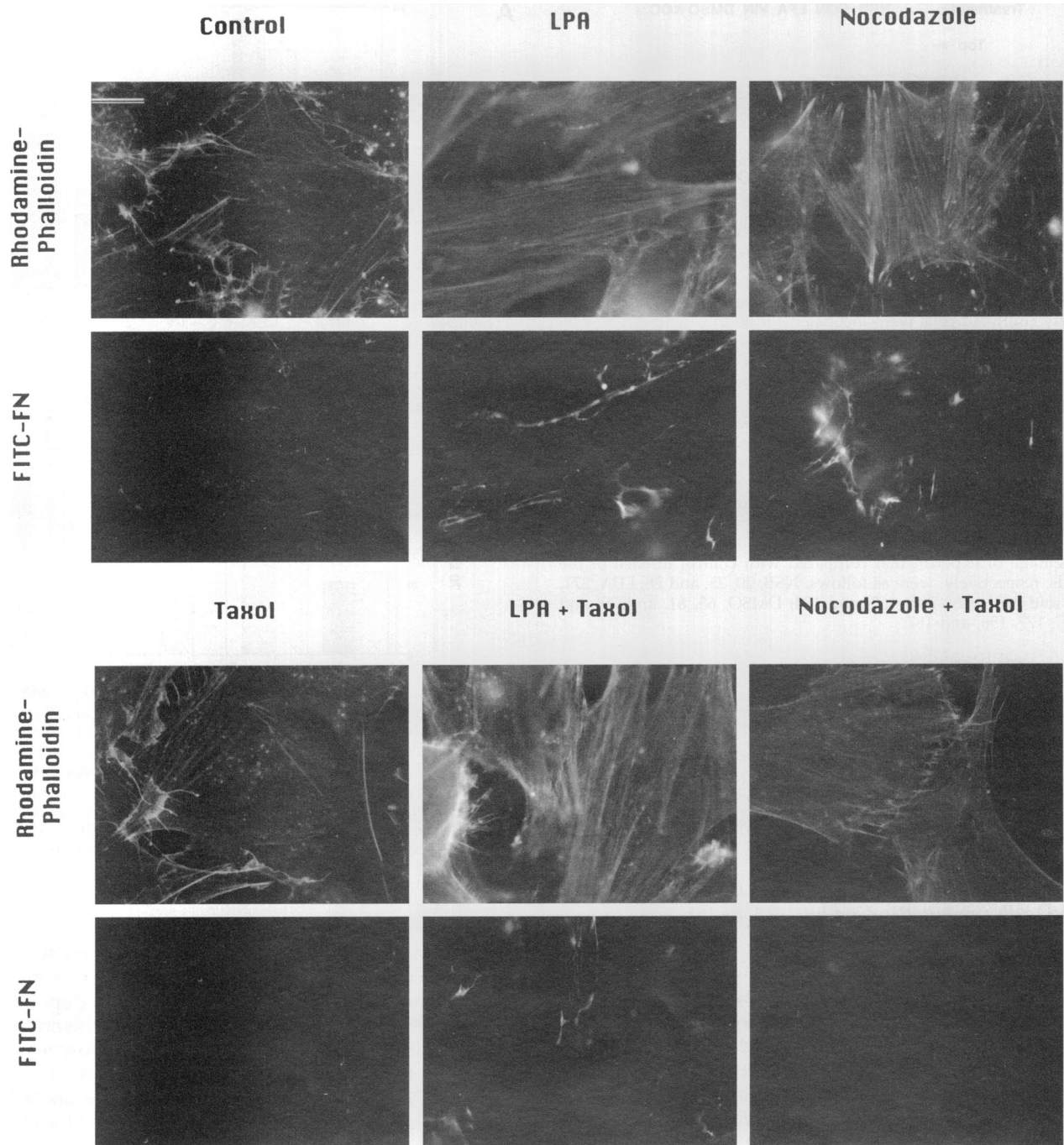
Figure 6. Effects of nocodazole or LPA on actin cytoskeleton and FN binding without (top) or with (bottom) Taxol pretreatment. MG63 cells were pretreated without or with Taxol (20 μM) for 2 h prior to incubation for 45 min with FITC-labeled FN (20 μg/ml) in the presence of LPA (500 nM), nocodazole (10 μM), or 0.1% DMSO (control). Cellular actin was stained with rhodamine-labeled phalloidin as described in MATERIALS AND METHODS.

binding of FN to MG63 cells by double fluorescence staining with FITC-labeled FN, 20 μg/ml, and rhodamine-phalloidin (Figure 6). After addition of 10 μM nocodazole or 500 nM LPA, MG63 cells contracted and

had a better developed network of phalloidin-stained microfilament bundles when compared with control cells (Figure 6). After a 45-min incubation with cells, FITC-FN was found in linear arrays on the edges of

cells and where cells overlapped (Figure 6). More labeled protein was present on the nocodazole- or LPA-treated cells than on control cells (Figure 6). This pattern represented specific binding since little fluorescence was detected when 500 μg/ml unlabeled FN was included during the incubation with FITC-labeled FN.

The microtubular system of MG63 cells, as detected by immunofluorescence staining, was well developed and typically had a radial organization (Figure 7). The addition of 10 μM nocodazole to the cells resulted in almost complete disruption of microtubules (Figure 7). Pretreatment of MG63 cells with 20 μM Taxol for 120 min preserved microtubules when nocodazole was added (Figure 7). There was no difference in microtubule appearance between LPA-treated and control cells, whereas cells pretreated with Taxol had more abundant microtubular structures (Figure 7).

When MG63 cells were treated with Taxol, the actin-containing cytoskeleton and FITC-FN binding were not affected (Figure 6). Cell contraction, actin stress fiber formation and increases of FITC-FN binding induced by nocodazole were blocked by the pretreatment with Taxol (Figure 6), whereas the similar effects of LPA on cell contraction, actin stress fiber formation and FN binding were not significantly changed (Figure 6). These results agree with the binding studies using the 70-kDa fragment (Figure 5A) and indicate that there is a close correlation between actin stress fiber formation and FN binding.
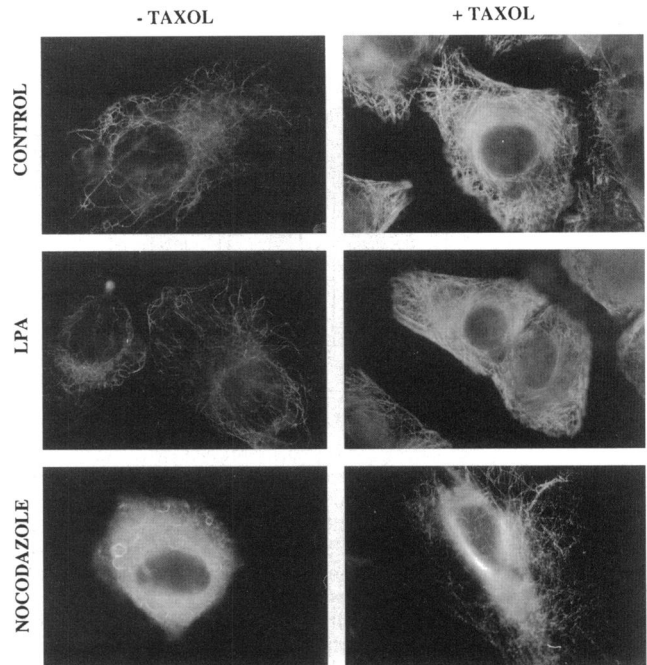

Figure 7. Effects of nocodazole or LPA on microtubular networks, without (left) or with (right) Taxol pretreatment. MG63 cells were pretreated without or with Taxol (20 μM) for 2 h prior to stimulating for 45 min with LPA (500 nM), nocodazole (10 μM), or 0.1% DMSO (control). Cells were then incubated with monoclonal anti-α-tubulin antibodies followed by staining with FITC-labeled goat anti-mouse IgG to detect microtubules as described in MATERIALS AND METHODS.

Involvement of Rho-dependent Contractility in FN Binding

Activated RhoA, a small GTP-binding protein, stimulates the appearance of stress fibers, focal adhesions, and tyrosine phosphorylation in quiescent cells (Ridley and Hall, 1992). Treatment of fibroblasts with LPA activates Rho, induces myosin light chain phosphorylation, and stimulates contractility, driving the formation of stress fibers and focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). We tested the involvement of this signal transduction pathway in the regulation of FN binding by either LPA or nocodazole.

C3 transferase, which inactivates Rho via ADP-ribosylation (Narumiya et al., 1988), was tested for its influence on FN assembly. After a 24-h incubation of the MG63 cells with 10 or 100 μg/ml C3 exotransferase, FN binding was inhibited in a dose-dependent manner. With 100 μg/ml C3, the 70-kDa fragment binding was inhibited to 44% in the presence of LPA and 43% in the presence of nocodazole (Figure 8A). This correlated well with the level of residual ADP-ribosylation in extracts of control and transferase-treated cells (Figure 8B) and suggests that the failure of treatment of cells with C3 transferase (100 μg/ml)

to inhibit stimulation of 70-kDa fragment binding completely to baseline is due to incomplete ribosylation of Rho. These results indicate that the modulation of binding of ^{125}I-70-kDa fragment to cells by both LPA and microtubule disruption is Rho-dependent.

BDM was tested for ability to affect nocodazole and LPA induced events. It inhibits cell contraction by muscle myosin or nonmuscle myosin II by slowing the release of phosphate from myosin after ATP hydrolysis (Higuchi and Takemori, 1989; McKillop et al., 1994; Cramer and Mitchison, 1995). BDM dose-dependently inhibited 70-kDa fragment binding in response to LPA and nocodazole (Figure 9). At 40 mM BDM, the 70-kDa fragment was reduced to 21% of the value obtained in the presence of LPA and 33% of the value in the presence of nocodazole (Figure 9). Fluorescent microscopy studies confirmed that BDM had similar dose-dependent effects on LPA- and nocodazole-induced cell contraction and actin stress fiber formation. At 10 and 20 mM, there were less abundant stress fibers present after stimulation with LPA or nocodazole, while 40 mM BDM further inhibited their formation. These results indicate that modulation of FN matrix assembly sites by microtubule disruption or LPA shares similar pathway and is mediated by Rho-
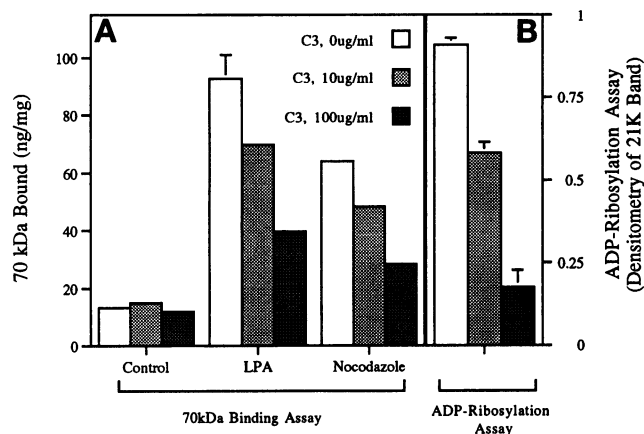
Figure 8. Effects of C3 transferase pretreatment on LPA- or no-codazole-stimulated [125]I-70-kDa fragment binding (A) and ADP-ribosylation of Rho (B). Confluent MG63 cells were incubated without or with C3 transferase (C3, 10 or 100 μg/ml) in DMEM containing 5% serum for 24 h. In A, the cells were incubated with [125]I-70-kDa fragment in the presence of 500 nM LPA, 10 μM nocodazole (NOC), or without additions (Control) for 45 min. The bars represent the mean ± SD of triplicate values. All bars represent specific binding as described in MATERIALS AND METHODS. In B, the cells were lysed, and an in vitro ADP-ribosylation assay was carried out with ^{32}P-labeled-NAD as described in MATERIALS AND METHODS. The samples were then analyzed by SDS-PAGE with a 12% separating gel. The ADP-ribosylated band at 21 kDa corresponding to Rho was quantitated by phosphorimaging. The data represent the mean ± SD from three independent experiments.

Figure 9. Effects of BDM on LPA- or nocodazole (NOC)-stimulated [125]I-70-kDa fragment binding. Confluent MG63 cells were pretreated with increasing doses of BDM for 10 min and then incubated with [125]I-70-kDa fragment in the presence of 500 nM LPA or 10 μM nocodazole (NOC) or without additions (Control) for 45 min. The bars represent the mean ± SD of triplicate values as percentage of control. All bars represent specific binding as described in MATERIALS AND METHODS.

dependent actin stress fiber formation and cell contraction requiring actin and myosin interactions.

DISCUSSION

Organization of soluble FN into extracellular matrix is a regulated pathway relevent to embryogenesis, wound healing, inflammation, and degenerative disease processes such as atherosclerosis and fibrosis. We have shown previously that LPA is a stimulator of FN matrix assembly. This effect is associated with LPA-induced actin stress fiber formation (Zhang *et al.*, 1994). We now demonstrate that microtubule disruption closely mimicked the effects of LPA on FN binding. Disruption of microtubules by nocodazole or vinblastine was associated with cellular contraction and increased FN-binding sites on adherent cells. The modulation of binding sites was rapid, dynamic, and reversible. Enhanced binding was due to increases in both the number and affinity of binding sites and was associated with increased cross-linking of bound ligand to LAMMs. Fluorescence microscopy revealed a correlation between actin stress fiber formation and FN binding. These results indicate that the regulatory mechanisms of the initial binding of FN to cell monolayers by either microtubule depolymerization or LPA
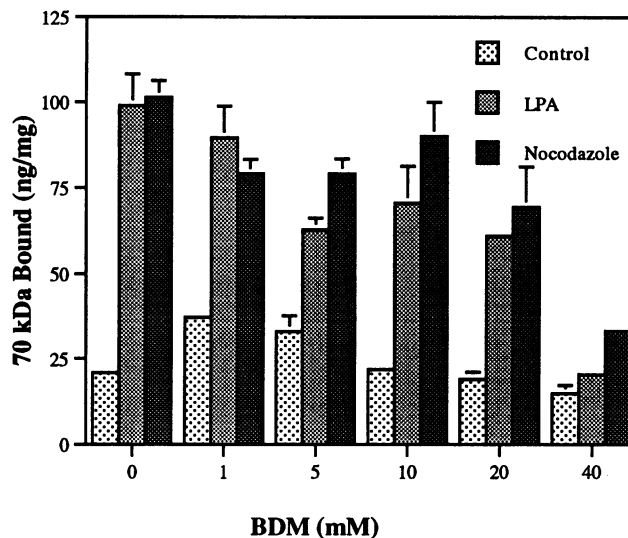
are similar and mediated by Rho-dependent actin stress fiber formation and cell contraction.

The depolymerization of cellular microtubular system elucidates diverse pleiotropic effects on cells, including the assembly of prominent actin cables (Danowski, 1989; Kajstura and Bereiter-Hahn, 1993), an increase in the number and size of vinculin-containing focal adhesions (Lloyd *et al.*, 1977), local tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin (Bershadsky *et al.*, 1996), and the stimulation of DNA synthesis (Crossin and Carney, 1981; Shinohara *et al.*, 1989). The impairment of microtubules may block intracellular vesicular transport and affect signaling by blocking receptor internalization or recycling (Kapeller *et al.*, 1993). Microtubule depolymerization may change the distribution of kinases or phosphatases normally associated with microtubules (Kapeller *et al.*, 1993; Reszka *et al.*, 1995; Sontag *et al.*, 1995). The most immediate cellular effect of microtubule disruption seems to be generalized cell contraction (Danowski, 1989) that is associated with an increase in the phosphorylation of the myosin regulatory light chain (Kolodney and Elson, 1995). Little is known about how microtubule disassembly causes the formation of actin stress fibers and cell contractility (Bershadsky *et al.*, 1996). Our demonstration that the FN binding induced by nocodazole can be inhibited by C3 transferase suggests that activation of the small G protein Rho is involved. Tubulin dimers with

bound GTP or a nonhydrolyzable GTP analogue have been shown to complex with and transfer GTP to the α-subunits of the heterotrimeric G proteins G_s and G_i, causing activation (Roychowdhury and Rasenick, 1994; Yan et al., 1996). Nocodazole acts by destabilizing polymerized tubulin and increasing the intracellular free tubulin concentration (Wilson and Jordan, 1994). This free tubulin might thus activate heterotrimeric G proteins, e.g., $G_{\alpha12}$ or $G_{\alpha13}$, that have been shown to specifically activate Rho (Buhl et al., 1995).

Extracellular LPA has many biological effects (Moolenaar et al., 1997). Its action involves activation of complex signal transduction pathways including activation of the phosphoinositide system with mobilization of intracellular Ca^{2+} (Moolenaar et al., 1986; Jalink et al., 1990) and activation of protein kinase C (van Corven et al., 1989), inhibition of adenylate cyclase (van Corven et al., 1989), regulation of p21ras (van Corven et al., 1993), and activation of the small GTP-binding protein Rho with downstream formation of actin stress fibers and recruitment and phosphorylation of different focal adhesion proteins with subsequent formation of focal adhesion (Ridley and Hall, 1992; Miura et al., 1993; Kumagai et al., 1993; Jalink et al., 1994). Chrzanowska-Wodnicka and Burridge (1996) have shown that blocking both myosin light chain phosphorylation and actin–myosin interactions by using KT5926 and BDM results in inhibition of LPA-induced cell contraction, stress fiber and focal adhesion formation, tyrosine phosphorylation of paxillin and focal adhesion kinase, and recruitment of integrins to focal adhesion points. Furthermore, a recently identified Rho-associated kinase (Matsui et al., 1996) has been shown to activate the actin–myosin contractile machinery both through direct phosphorylation of myosin light chain (Amano et al., 1996) and inhibition of myosin phosphatase (Kimura et al., 1996). Rho-associated kinase provides a possible link among Rho activation, actin–myosin interactions, and focal adhesion formation. In our study, ADP-ribosylation of Rho or inhibition of actin–myosin interactions decreased the effects of both microtubule depolymerization and LPA on FN binding. The myosin light chain kinase inhibitor KT5926 had a smaller but significant inhibitory effect (our unpublished results). Thus, stimulation of Rho-dependent cell contractility by two different agents results in enhanced binding of FN or its 70-kDa amino-terminal fragment to assembly sites. Cross-linking studies show that for both LPA (Zhang and Mosher, 1996) and microtubule-destabilizing agents the assembly sites are composed of LAMMs containing multiple binding motifs for amino-terminal region of FN (Zhang and Mosher, 1996). Contractility of the adherent cells induced by LPA or microtubule depolymerization may stretch the large assembly site molecules and open multiple cryptic binding sites for the amino-terminal region of FN. This mechanism

would explain the rapid up- and down-regulation of FN matrix assembly sites on cell surface.

When FN is assembled, the FN binding integrin $\alpha5\beta1$ is organized in focal contacts rather than diffusely on the cells basal surface (Roman et al., 1989). Similarly, assembled FN induces an organization of actin into stress fibers and colocalization of $\alpha5\beta1$, focal adhesion kinase, vinculin, and paxillin to regions of cell–matrix contact (Sechler and Schwarzbauer, 1997). These outside-in effects of assembled FN on the cytoskeleton are slow (Sechler and Schwarzbauer, 1997) compared with the acute inside-out effects of the cytoskeleton on FN matrix assembly sites. Thus, one can envision a complex reciprocal relationship in which the state of the cytoskeleton influences the formation of the extracellular matrix that in turn influences the state of the cytoskeleton.

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