Regulation of Tension-induced Mechanotranscriptional Signals by the Microtubule Network in Fibroblasts*

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Mechanical loading of connective tissues induces the expression of extracellular matrix and cytoskeletal genes that are involved in matrix remodeling. These processes depend in part on force transmission through β1 integrins and actin filaments, but the role of microtubules in regulating mechanotranscriptional responses is not well defined. We assessed the involvement of microtubules in the mechanotranscriptional regulation of filamin A, an actin-cross-linking protein that protects cells against force-induced apoptosis by stabilizing cell membranes. Collagen-coated magnetite beads and magnetic fields were used to apply tensile forces to cultured fibroblasts at focal adhesions. Force enhanced recruitment of α-tubulin and the plus end microtubule-binding protein cytoplasmatic linker protein-170 (CLIP-170) at focal adhesions. Immunoprecipitation studies demonstrated no direct binding of tubulin to actin or filamin A, but CLIP-170 interacted with tubulin, filamin A, and β-actin. The association of CLIP-170 with β-actin was enhanced by force. Force activated the p38 mitogen-activated protein kinase, increased filamin A expression, and induced the relocation of p38 and filamin A to focal adhesions. Disruption of microtubules with nocodazole, independent of force application, enhanced filamin A expression and Sp1-mediated filamin A promoter activity, while stabilization of microtubules with taxol inhibited force induction of both filamin A mRNA and protein. We conclude that in response to tensile force applied through β1 integrins and actin the microtubule network modulates mechanotranscriptional coupling of filamin A.

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During application of mechanical loads to connective tissues, the extracellular matrix distributes forces to cellular adhesive structures including integrins; these forces are in turn directed to structural components including the cytoskeleton (for reviews, see Refs. 1–3). Forces applied through integrins are transduced into intracellular signals that mediate the redistribution of cytoskeletal proteins and the expression of several cytoskeletal genes including α-skeletal and α-smooth muscle actins, filamin A, talin, and vinculin (4–9). Although the mechanotranscriptional regulation of these genes has not been analyzed in depth, force-induced activation of mitogen-activated protein kinases can mediate increased binding of transcription factors to regulatory sites on the promoters of some of these genes (7, 9), thereby regulating cytoskeletal gene expression.

Filamin A is an example of a cytoskeletal gene that is induced by mechanical forces (9). Filamins are actin-binding proteins that organize actin filaments into orthogonal networks and enhance the rigidity of the actin cytoskeleton (10). Tensile force application through β1 integrins induces enhanced expression of filamin A (7, 9); the subsequent increase of filamin A protein facilitates cell survival by mechanical stabilization of cortical actin and by prevention of cell depolarization due to excessive membrane distortion caused by high amplitude tensile forces (11). As filamin A binds integrins and other proteins enriched at cell adhesion sites (10), filamin A thereby provides a good model for determining mechanotranscriptional responses transduced through β1 integrins.

Cellular responses to tensile forces applied through integrins require an intact actin cytoskeleton (7, 8), but the role of the microtubule network in mediating mechanical signaling is currently not defined. Microtubules can modulate contraction and can also affect cell attachment to the extracellular matrix through regulation of turnover at adhesion sites (12–15). Locally applied forces promote growth of microtubules toward substrate attachment sites (15) as has been shown in Aplysia in which beads coated with matrix ligands induce microtubule assembly adjacent to the beads (16). Thus microtubules may be involved in modulating mechanotranusduction.

Recent studies have established functional links between actin filaments and microtubules in a broad array of cellular processes including vesicle and organelle movement, cytokinesis, nuclear migration, contractile ring formation, and mitotic spindle alignment (17). An important element of actin-microtubule interactions that has not been characterized in detail is the interaction of these two networks in integrin-dependent mechanotranusduction. We have previously shown that mechanical forces applied through β1 integrins activate the filamin A gene (9). Here we describe the involvement of microtubules in regulating the activation of the filamin A gene after application of mechanical forces.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human gingival fibroblasts were derived from primary explant cultures as described previously (18). Cells from passages 6–10 were grown as monolayer cultures in α-modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. Experiments involving promoter analyses used Rat-2 fibroblasts as surrogates for gingival fibroblasts as described previously (19).

Mouse anti-filamin A antibodies were obtained from Serotec (Cedarlane Laboratories, Hornby, Ontario, Canada). Mouse monoclonal antibodies to β-actin, vinculin, and α-tubulin were from Sigma. Antibodies to pp38, p38, and Sp1 were obtained from Cell Signaling Technologies (New England Biolabs, Mississauga, Ontario, Canada). Rabbit anti-
cytoplasmic linker protein-170 (CLIP-170) was obtained from Dr. N. Galjart (Erasmus University, Rotterdam, Netherlands). Nocodazole (1 μM, Sigma) and the microtubule stabilization agent paclitaxel (Taxol, 0.5 μM, Sigma) were used as indicated. Monoclonal antibody to α,β, was obtained from Calbiochem, and anti-β integrin was obtained from Beckman Coulter, Inc., and its use has been described elsewhere (7). 

**Force Generation**—Tensile forces were applied to integrins using a model system described previously (20). In brief, magnetic microparticles (Fe₃O₄, Sigma) were incubated with purified type I bovine collagen (Vitrogen 100, Cellectix, Palo Alto, CA; 1 mg/ml); following 30-min incubations, excess non-adherent microparticles were removed by vigorous washing, and cells were supplemented with fresh α minimum Eagle’s medium. A ceramic permanent magnet (Jobmaster, Mississauga, Ontario, Canada) was placed on top of the dish to generate a perpendicular tensile force of ~0.48 piconewtons/μm² cell area; this force level is comparable to that applied to cells in vivo during normal function (20). The incubation times were specific for each individual experiment as indicated.

**RNA Isolation, Reverse Transcription (RT), and PCR Analysis**—RNA isolation was performed with RNAeasy reagents (Qiagen, Mississauga, Ontario, Canada). All RNA preparations were treated with RQ1 DNase (Promega Corp., Madison, WI) for 30 min. The RT-PCR protocol and the oligonucleotides used in the procedure have been described in detail in previous studies (7, 9, 21).

The semiquantitative nature of the RT-PCR protocol, the precautions taken to avoid spurious reaction products, and the controls used have been described previously (7, 21). In each experiment, a non-RT control demonstrated the lack of DNA contamination.

**Immunoblotting, Immunofluorescence, and Immunoprecipitation**—Cells were lysed, and cellular proteins were separated by SDS-PAGE and transferred to nitrocellulose, and analyzed as described previously (22). Chemiluminescent detection was performed according to the manufacturer’s instructions (Amersham Biosciences), and radiographic films were exposed for standardized luminographs on an conventional processor.

For immunofluorescence, gingival fibroblasts were grown on 10-mm glass coverslips, incubated with collagen-coated microbeads, and subjected to magnetic force application as described above. Samples were collected at standardized time points and stained as described previously (7). The protocol used to detect protein-protein interactions was immunoprecipitation of target proteins with β-actin, filament A, CLIP-170, and α-tubulin has been described previously (22). In brief, samples were treated with radiimmune precipitation assay buffer containing sodium vanadate (1 mM) and a protease inhibitor mixture (Sigma). Isolated proteins were incubated with protein G-Sepharose beads (Zymed Laboratories Inc.) that had been preincubated with antibodies to CLIP-170 or α-tubulin overnight at 4 °C. Samples were resolved by 5–20% gradient SDS-PAGE and transferred to nitrocellulose. Blots were probed with the specific antibody indicated in each figure, and ECL was carried out according to the manufacturer’s instructions (Amersham Biosciences).

**Genomic DNA Isolation and Filamin A Promoter Construction**—To generate the 3224-bp filament A luciferase promoter construct, we isolated intact fibroblast genomic DNA using the protocol of Goelz et al. (23). The construction of the amplified promoter has been described in detail elsewhere (7, 9). The construct was verified by restriction enzyme digestion and sequencing performed at the DNA Sequencing Facility, Center for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada).

To generate the final wild type 75-bp filament A luciferase construct (pFil75wtluc), the original 3.2-kb filament A promoter component was cut out and isolated from agarose gel, and the construction containing the 75-bp Sp1 fragment was fused to the luciferase reporter construct. To synthesize the final 75-bp promoter construct containing mutations at the Sp1 binding sites (pFil75mutluc), two complementary oligonucleotides (MWG Biotech, described in Refs. 7 and 9) were hybridized in equimolar amounts and ligated into pGL2lac. Promoter scanning was used to determine the location of potentially important transcription factor binding sites, and these locations were specifically mutated (24). The hybridized oligonucleotides were ligated into the pGL2 Basic luciferase vector (Promega Corp.), and the correctly ligated vector was verified through restriction enzyme digestion.

**Bead and Protein Isolation**—Cells in normal growth medium that had reached 80–90% confluence on 60-mm tissue culture dishes were incubated with collagen (coated or bovine serum albumin-coated) and the beads were isolated from dishes as described previously (25). Cells were gently washed three times with ice-cold phosphate-buffered saline to remove unbound beads and scraped into ice-cold cytoskeleton extraction buffer (CSKB; 0.5% Triton X-100, 50 mM NaCl, 500 mM sucrose, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM PIPES, pH 6.8). The cell bead suspension was sonicated for 10 s, and the beads were isolated from the lysate using a magnetic separation stand. The beads were resuspended in fresh, ice-cold CSKB and homogenized with a Dounce homogenizer (20 strokes), and the magnetic isolation was repeated. The beads were washed thoroughly in CSKB, sedimented with a microcentrifuge, resuspended in Laemmli sample buffer, and placed in a boiling water bath for 10 min to allow the collagen-associated complexes to dissociate from the beads. The beads were pelleted, and the lysate was collected for immunoblot analysis.

**Cell Transfections**—Rat-2 cells were transfected using Effectene transfection reagent (Qiagen) as described previously (7, 26). Briefly, following titration experiments to determine the optimal concentration of vector needed, cells were transfected, incubated for 18–24 h, and then subjected to specific treatments (described for each individual experiment). Following each treatment, cells were processed for luciferase activity as recommended by the manufacturer (luciferase assay system, Promega Corp.). The luciferase vectors pFil15.2lac, pFil175wtluc, and pFil175mutluc are described above. To establish transfection efficiency and to provide experimental controls, a green fluorescent protein vector (pEGFPluc) was used (Clontech).

**Statistical Analysis**—For continuous variables, means and S.E. were computed. Unpaired Student’s t tests were used for comparing means between two experimental groups. Analysis of variance was used for multiple comparisons followed by Tukey’s test for post hoc comparisons. Significance was set at p < 0.05. In each assay, n = 3, and each experiment was repeated at least three times.

**RESULTS**

**Force-induced Recruitment of CLIP-170, α-Tubulin, and Filamin A to Focal Adhesions**—As force-induced increases of filamin A rely on transmission of force through focal adhesions to actin (7, 9), we determined whether the plus ends of growing microtubules are targeted to focal adhesions in response to force applied at these sites by collagen-coated magnetite beads. We examined CLIP-170, which is implicated in the targeting of the plus ends of growing microtubules to focal adhesions (27, 28). The relative enrichment of CLIP-170 and α-tubulin was quantified after force application. Immunoblotting of proteins isolated from collagen beads was evaluated by densitometry and normalized to vinculin, an actin-binding protein that is a marker for focal adhesions. The relative amounts of bead-associated CLIP-170 and tubulin were increased substantially after force (>5-fold, p < 0.01; Fig. 1Ai). These data indicated that CLIP-170 and tubulin are recruited to focal adhesions by force, and they implicate microtubules in the global cytoskeletal response to tensile forces.

We have shown previously that when force is applied to bovine serum albumin- or poly-L-lysine-coated magnetite beads there was no increase of filament A transcription or enhanced filament A protein production. In contrast, fibronectin-coated beads exhibited increased filament A RNA and protein by 4- and 3-fold, respectively, after force application (7). The participation of β integrins was confirmed by preincubating fibroblasts with a monoclonal antibody (4B4) that blocks ligand-β integrin interactions without inhibiting cell attachment (22). Preincubation of cells with monoclonal antibody 4B4 (at 1:30 dilution) for 30 min followed by application of force blocked the force-induced production of filament A protein (1-fold increase of filament A by immunoblotting and analysis by densitometry; no...
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Mechanical force increases CLIP-170 accumulation at focal adhesions. A, adherent fibroblasts were cultured in normal serum-containing medium, incubated with collagen-coated magnetic beads, and either untreated or subjected to a vertically directed force (0.48 piconewton/μm², 4 h) using a permanent ceramic magnet. Bead-associated proteins were prepared as described previously (25), loaded on 6–10% denaturing PAGE, transferred to nitrocellulose, and immunoblotted for vinculin, α-tubulin, and CLIP-170. Lane 1, bead loading without force; lane 2, bead loading plus force. Data in the histogram are means ± S.E. of relative protein levels adjusted for constant numbers of beads. Lane 1, no force; lane 2, force; lane 3, Taxol and no force; lane 4, Taxol plus force; lane 5, nocodazole and no force; lane 6, nocodazole plus force. B, proteins in collagen bead-associated complexes immunoblotted for filamin A and vinculin. Data in the histogram are means ± S.E. of relative protein levels adjusted for constant numbers of beads. Lane 1, no force; lane 2, force; lane 3, Taxol and no force; lane 4, Taxol plus force; lane 5, nocodazole and no force; lane 6, nocodazole plus force. Nocodazole strongly enhanced filamin A recruitment to beads independent of force application.

As tensile force is applied to cells through focal adhesions formed adjacent to the collagen beads, we assessed whether there was force-induced recruitment of filamin A protein to the bead complexes. Collagen bead-associated proteins were examined by immunoblotting and quantified by densitometry following adjustment for bead numbers. Application of tensile force caused a 3-fold increase of filamin A to the bead within 4 h (p < 0.01, Fig. 1B). Taxol treatment exerted no effect on force-induced recruitment of filamin A. Nocodazole strongly enhanced filamin A recruitment to beads independent of force application.

The results described above demonstrate that force application recruits CLIP-170, α-tubulin, and filamin A to sites of force application, but they do not show whether α-tubulin or CLIP-170 is associated with actin/filamin A and whether this association is affected by force. Cell lysates were immunoprecipitated with antibodies to CLIP-170 or α-tubulin, and the immunoprecipitates were blotted for filamin A and β-actin (Fig. 1C). The specificity of the monoclonal antibody to CLIP-170 for 1 integrin. Accordingly we isolated bead-associated proteins from untreated (no force) and force-treated fibroblasts and analyzed this material for β1 and αβ3 integrins (Fig. 1Aii). We detected a 2–2.5-fold increase in the level of β integrin associated with collagen-coated magnetite beads following force application. We found very little detectable αβ3 integrin with or without force application in bead-associated protein. Confirmation of the ability of the monoclonal antibody to detect αβ3 integrin in these cells was confirmed by immunoblotting whole cell lysates (Fig. 1Aii, lane c).

We assessed whether the collagen beads remained on the cell surface or were potentially internalized over time. Cells were incubated with magnetite beads coated with fluorescein isothiocyanate-labeled collagen for up to 4 h. Trypan blue quenching of the cell surface fluorescence showed that the difference in the pre- and postquench fluorescence (an estimate of the amount of cell surface fluorescence isothiocyanate-labeled collagen) at 30 min compared with 4 h of force was not significantly different (7.14 ± 1.66 fluorescence units at 30 min and 8.69 ± 2.31 fluorescence units at 4 h, p > 0.2). Therefore the collagen beads remained largely on the cell surface during force application.
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Fig. 2. Force application causes redistribution of filamin A. Cells were grown on glass slides, incubated with collagen-coated magnetite beads, and either untreated (A, C, and E) or subjected to a vertically directed force (B, D, and F) (0.48 piconewtons/μm², 4 h) with a magnet. Cells were fixed with methanol, permeabilized with Triton X-100, and immunostained for filamin A. Force application increases total filamin A staining. Pretreatment with nocodazole (C and D) increases filamin staining in the cell periphery that is further enhanced by force application. There is little increase of staining intensity for filamin A in cells treated with Taxol (E and F).

ment of microtubules in regulating the distribution and expression of filamin A after application of tensile forces through collagen receptors. Preliminary experiments with nocodazole (1 μM) and the microtubule stabilization agent paclitaxel (Taxol, 0.5 μM) were conducted, and cells were immunostained with antibodies to α-tubulin to verify that the microtubule network was depolymerized (nocodazole) or was stabilized (Taxol). When cells were incubated with these agents for 2–8 h (the duration of the force application experiments), microtubules were either poorly stained (after nocodazole) or were largely unaffected (Taxol, data not shown). Immunohistochemical examination of filamin A in force-treated cells showed increased overall staining intensity within 4 h of force application (Fig. 2). Pretreatment with nocodazole caused redistribution of filamin A to the cell periphery, an effect that was enhanced by force application. Taxol blocked force-induced redistribution of filamin A.

Application of exogenous tensile force reproducibly increased the levels of filamin A mRNA 5–6-fold (p < 0.01, Fig. 3A). Cells treated with Taxol (to stabilize the microtubule network) but without force showed no change of filamin A mRNA, while Taxol reduced the force-induced filamin A increase by 50% (p < 0.05, Fig. 3A). As depolymerization of microtubules by nocodazole increases endogenously generated cellular tension (12) and promotes the formation of actin stress fibers (data not shown), we treated cells with nocodazole and measured filamin A mRNA. Nocodazole treatment without exogenous force application increased filamin A mRNA by 4-fold, while nocodazole with force increased filamin A mRNA by 6-fold (p < 0.001).

Filamin A protein content was examined by immunoblotting, quantified by densitometry, and normalized to β-actin content. After 4 or 8 h of exogenous force application, filamin A expression was increased by 5–6-fold (Fig. 3Bi, p < 0.01). Cells treated with force plus nocodazole showed >2-fold increase of filamin A at 4 h and 5–6-fold increase by 8 h (p < 0.01). In contrast, cells treated with Taxol exhibited only small increases after force application (∼2-fold at 4 and 8 h). Notably, treatment of cells with nocodazole alone (i.e. without force) was sufficient to enhance filamin A protein levels by ∼3-fold within 3 h of incubation (Fig. 3Bii). Nocodazole accelerated the force-induced increase of filamin A over a 4-h period. Wash-out of nocodazole caused a 50% reduction of filamin A content and also strongly dampened the force-induced increase of filamin A.

Regulation of the Filamin A Promoter by Force Is Abolished by Microtubule Disruption—The filamin A promoter contains ∼3.2 kbp of upstream sequence that is activated by tensile force (7), and this force-induced activation requires an intact actin cytoskeleton (9). To examine the involvement of microtubules in force-mediated activation of the filamin A promoter, we transfected Rat-2 fibroblasts with a vector containing 3.2 kbp of the filamin A promoter fused to luciferase. In cells incubated with collagen beads but without force application, the pFIl3.2Luc vector exhibited low basal activity levels. Force increased activity of the filamin A promoter by >6-fold (Fig. 4A, p < 0.01). Treatment with nocodazole alone increased basal levels of pFIl3.2Luc by 3-fold, which were further increased by force (total of 6-fold increase, p < 0.01). Taxol treatment alone did not change basal promoter activity but strongly inhibited force-induced activation of the 3.2-kbp luciferase vector (∼50% of force-induced levels, p = 0.05).

Sp1 has been implicated as a critical transcription factor in force-induced regulation of filamin A (9). The proximal 75 bp of the filamin A promoter is enriched with Sp1 binding sites, which are responsible for >50% of the force-induced activation of the filamin A promoter (9). Accordingly, to determine the relative importance of Sp1 activation and microtubules in the force-induced activation of filamin A, cells were transfected with an Sp1-dependent vector (pFIl75wtLuc) or its mutated homologue (pFIl75mutLuc). These vectors encompass the 75-bp sequence immediately upstream of the filamin A translational start site and contain up to seven Sp1 transcription factor binding sites (7). The pFIl75mutLuc contains two mutations in the most heavily weighted Sp1 binding sites according to a promoter scan analysis (24). After pFIl75wtLuc was transfected into fibroblasts and subjected to mechanical force, luciferase levels increased ∼8–9-fold (Fig. 4B, p < 0.01). Nocodazole pretreatment caused increases in luciferase levels that were ∼35% less than those with force alone (p < 0.05) but not significantly increased by force application (p > 0.2), suggesting that nocodazole treatment alone can stimulate filamin A promoter activity. Cells treated with Taxol showed force-induced increases of promoter activity that were ∼40% less than those in force-treated cells (p < 0.05) without the drug.

When the mutant promoter vector (pFIl75mutLuc) was introduced into Rat-2 fibroblasts, force application increased the levels of luciferase significantly less than those observed with the wild type 75-bp fragment (only 3-fold increase after force, Fig. 4C, p < 0.05). In addition, nocodazole alone increased promoter activity ∼3-fold, but there was no further enhancement by force. In cells treated with Taxol, there was no change in promoter activity after force application.

Microtubule Disruption Attenuates Force-induced Sp1 Phosphorylation—Sp1 phosphorylation is indicative of Sp1-mediated activation of cellular transcription (30–32). We have previously shown that Sp1 is phosphorylated on serine/threonine residues after force-induced activation of the filamin A gene (9). Further we demonstrated that force application increases interactions of Sp1 with p38 mitogen-activated protein kinase and β-actin (9). In the present study, total Sp1 phosphorylation was determined following force application or after preincubation with either nocodazole or Taxol (Fig. 4D). Force application increased Sp1 phosphorylation by ∼3-fold (p < 0.01), while nocodazole exerted no significant effect (p > 0.2). These results were not due to direct microtubule-Sp1 interactions as we were unable to show protein association by immunoprecipitation and immunoblotting (Fig. 1C). Microtubule stabilization by Taxol abrogated force-induced Sp1 phosphorylation.
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Microtubule Disruption Increases p38 Activation and Induces Relocation to Bead-Integrin Loci—Application of tensile forces through β1 integrins regulates filamin A expression by mechanisms that may involve interactions of Sp1 with pp38 and β-actin with pp38 (9). Previous data have also shown that p38 (but not extracellular signal-regulated kinase 1/2 or c-Jun NH2-terminal kinase) is phosphorylated in response to tensile forces applied to adult rat cardiac fibroblasts (26). Accordingly, we examined phosphorylation of p38 in cells subjected to tensile forces with or without microtubule-disrupting reagents. Whole cell extracts were immunobotted for p38 and pp38, and the blot density of pp38 was normalized to p38. Within 5 min of force application, p38 was phosphorylated, an effect that was maximal at 15 min (Fig. 5Ai). After force application, phosphorylation of p38 was similar to that observed in cells pretreated with nocodazole. In contrast, cells treated with Taxol alone, or with Taxol plus force, showed no significant changes of p38 phosphorylation. We also assessed the effect of nocodazole alone (i.e. without force) on p38 phosphorylation. Within 15 min of addition of nocodazole, there were maximal increases of p38 phosphorylation that decreased rapidly to control levels within 10 min after wash-out of nocodazole (Fig. 5Aii).

Previous data have shown that application of tensile forces to collagen beads attached to cells causes recruitment of pp38 to focal adhesions (7). We assessed the relative enrichment of pp38 and vinculin at focal adhesions after force application using immunoblotting of proteins isolated from collagen beads as described previously (20). Quantitative analysis of bead-associated proteins was done by densitometry of immunoblots. The relative amounts of bead-associated pp38 were adjusted to bead-associated vinculin, which we used here as a marker of focal adhesions. When vinculin was adjusted to approximately constant levels by appropriate protein loading of the gels (Fig. 5B), the relative abundance of phosphorylated p38 was strongly increased by force; Taxol exerted no significant effect (p > 0.2). Incubation of cells with nocodazole or with nocodazole plus force increased phosphorylated p38 by 4-fold in bead-associated proteins.

We next assessed whether force or nocodazole could induce redistribution of phosphorylated p38 within the cell. Cells were permeabilized with methanol and immunostained for pp38 to visualize the relative abundance of nuclear associated phosphorylated p38. Both force alone and nocodazole alone caused greatly increased staining for phosphorylated p38 within the nucleus (Fig. 5C).

DISCUSSION

Tensile forces applied to cell surface β1 integrins induce transcriptional activation of the actin-binding protein filamin A, a response that requires intact actin filaments (7, 9). These reports have not addressed the involvement of microtubules in mechanotranscriptional regulation, a notable gap since recent evidence suggests that microtubules are involved in regulating several actin-dependent processes including cell motility and

Fig. 3. Mechanical force activation of filamin A is modulated by microtubule-disrupting agents. A, adherent fibroblasts were cultured in normal serum-containing medium, incubated with collagen-coated magnetite beads at a ratio of ~10 beads/cell, and subjected to vertically directed tensile forces for the indicated times. Taxol (0.5 μM) or nocodazole (1 μM) was added 15 min prior to application of force. Total RNA was isolated after 3 h of force, and 1 μg RNA was subjected to RT-PCR analysis for filamin A and glyceraldehyde-3-phosphate dehydrogenase (GADPH). RT-PCR products (at top) show filamin A mRNA from cells loaded with beads but not subjected to force or with beads and subjected to force. The histogram shows mean ± S.E. of relative mRNA levels from triplicate RT-PCRs adjusted to glyceraldehyde-3-phosphate dehydrogenase. Lane 1, indicates bead loading without force; lane 2, cells were subjected to force application; lane 3, cells were incubated with Taxol for 15 min with no force; lane 4, cells were incubated with Taxol for 15 min, and force was then applied for 4 h; lane 5, cells were incubated with nocodazole for 15 min without force application; lane 6, cells were pretreated with nocodazole for 15 min prior to force application for 6 h. Data are densitometric analyses normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase in each lane. Histograms show mean ± S.E. from n = 3 independent experiments. Bii, time course of cells treated with Taxol or nocodazole, reagents were added to cultures 15 min prior to force application. Equal amounts of total cellular protein were loaded in each lane, separated on denaturing polyacrylamide gels, immunoblotted for the indicated protein, scanned, quantified, and shown on the graphs below each section. The time points below each blot indicate the duration of force application. Data are means ± S.E. from three separate experiments. Bii, time course of cells treated with Taxol or nocodazole, reagents were added to cultures 15 min prior to force application. Equal amounts of total cellular protein were loaded in each lane, separated on denaturing polyacrylamide gels, immunoblotted for the indicated protein, scanned, quantified, and shown on the graphs below each section. The time points below each blot indicate the duration of force application. Data are means ± S.E. from three separate experiments.
The novel features of this report are that disruption of the microtubule network by nocodazole leads to the transcriptional activation of the filamin A gene and that microtubule stabilization by Taxol blocks force-induced filamin A activation. An earlier report demonstrated a possible functional relationship between actin and microtubules: microtubule disassembly enhanced fibroblast contractility and formation of actin stress fibers within minutes of microtubule disruption (33). In the same context, Swiss 3T3 fibroblasts treated with nocodazole exhibited rapid increases of tyrosine phosphorylation of focal adhesion kinase and paxillin as well as the assembly of paxillin and vinculin into focal adhesions, while Taxol exerted little effect (12). Our current results show that microtubule disruption by nocodazole or application of tensile force can increase filamin A mRNA and protein within minutes and hours, respectively. More direct evidence for promoter activation was obtained when a filamin A reporter vector was transfected into fibroblasts and subsequently treated with nocodazole. Following nocodazole treatment, luciferase levels were elevated and were enhanced further by subsequent force application. This effect was not detected after Taxol treatment. Thus microtubule depolymerization has an effect on the expression of a mechanotranscriptionally sensitive gene (filamin A) similar to the effect of the application of exogenous force. In contrast, preincubation of cells with Taxol caused only minor changes in unstimulated cells, while Taxol treatment blocked increases of filamin A mRNA and protein in cells treated with force.

We considered that the force-induced activation of filamin A may require interactions between microtubules and actin mediated by CLIP-170. The CLIP family of proteins was originally described in the context of their ability to interact with endosomes (27, 37), but CLIP-170 is now known to interact with the growing end of microtubules (28) and treadmill to the cell periphery from the microtubule organizing center (38). Our results demonstrated increased abundance of CLIP-170 at focal adhesions following force application, suggesting that CLIP-170 may mediate force-induced microtubule-actin interactions and that microtubule plus ends are shuttled to force-responsive bead loci.

We also assessed binding of CLIP-170 with $\beta$-actin or filamin A and found that, while both of these proteins were bound by CLIP-170, force increased CLIP-170 interactions with $\beta$-actin. These results confirm previous in vitro reconstitution assays using purified brain tubulin, purified muscle actin, and microtubule-binding proteins from Xenopus oocytes in which interactions were demonstrated but without directly assessing which microtubule-binding proteins were required (39). We now demonstrate that CLIP-170 may be a molecular linker.
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between the actin and microtubule networks when exogenous forces are applied to cells. Our results are consistent with data from total internal reflection fluorescence microscopy showing that green fluorescent protein-CLIP-170 was targeted to peripheral cell adhesions marked by zyxin and that microtubule tracking to peripheral adhesions is dependent on actin filaments (27). Accordingly, during force application to cells, the targeting of microtubules to focal adhesions may be mediated in part by CLIP-170.

We have suggested earlier that activation of the filamin A promoter was associated with p38 since mechanical force causes rapid phosphorylation of p38 and migration of p38 to focal adhesions and nuclei (7, 9). Our current data show that nocodazole-induced microtubule disruption alone (without force) is sufficient to phosphorylate p38 and to promote its movement to focal adhesions and nuclei. This effect was accentuated by force application. Notably nocodazole-induced activation of p38 was transient: following wash-out of this agent, phosphorylated p38 levels returned to basal levels, indicating rapid adaptation to the environment. Previous demonstrations of enrichment of mitogen-activated protein kinases in the microtubule fraction of cells (34) indicated potential interactions between signal transduction molecules and cytoskeletal components. This possibility has been shown specifically for binding of α- and β-tubulin subunits with the p85 subunit of phosphatidylinositol 3-kinase (35). By analogy with the results shown here, nocodazole causes redistribution of the p85 subunit from the perinuclear region to the cell periphery (36), and this translocation is blocked by Taxol. As our results with CLIP-170 did not demonstrate any detectable interaction of α-tubulin with either actin filaments or p38, we suggest that the p38 migration may be attributable to an actin-p38 association as we have shown previously (9).

Previous data have shown that stress activation of p38 in macrophages enhances Sp1 binding to the interleukin-10 promoter (43). Force-induced activation of the filamin A promoter also requires phosphorylation of Sp1 on serine/threonine residues and promoter binding (9). The novel finding shown here is that treatment with Taxol abrogated force-induced phosphorylation of Sp1 but that force could induce phosphorylation of Sp1 in cells treated with nocodazole. Our current data indicate that, in contrast to p38 phosphorylation, depolymerization of microtubules is not sufficient alone to mediate phosphorylation.

In conclusion, disruption of the microtubule network by nocodazole activates the filamin A gene in fibroblasts similar to application of exogenous forces applied through β1 integrins. This result may be due to liberation of the microtubule-bound Rho guanine nucleotide exchange factor (GEF-1), which is known to activate RhoA and regulate actin assembly (40, 41). Notably GEF-H1 mutants that cannot bind microtubules alter cell morphology and actin organization in a manner similar to nocodazole-induced changes, suggesting that these two cytoskeletal systems are interconnected (42). However, when microtubule treadmilling was blocked by Taxol, filamin A was not significantly induced even after force application. Thus the microtubule cytoskeleton can modulate the filamin A mechanotranscriptional network that is mediated by actin filaments. Conceivably interactions between the actin and microtubule cytoskeletons are mediated by CLIP-170, which is enriched in focal adhesions subjected to tensile forces.

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