Connective tissue cells in mechanically active environments survive applied physical forces by modifying actin cytoskeletal structures that stabilize cell membranes. In fibroblasts, tensile forces induce the expression of filamin-A, a mechanoprotective actin-binding protein, but the mechanisms and protein interactions by which force activates filamin-A transcription are not defined. We found that in fibroblasts, application of tensile forces through collagen-coated magnetite beads to cell surface β1 integrins induced filamin-A expression. This induction required actin filaments and selective activation of the p38 mitogen-activated protein kinase. Force promoted the redistribution of p38 to the integrin/bead locus and the nucleus as well as enhanced binding of the transcription factor Sp1 to proximal, regulatory domains of the filamin-A promoter. Force application increased association of Sp1 with p38 and phosphorylation of Sp1. Transcriptional activation of filamin-A in force-treated fibroblasts was subsequently mediated by Sp1-binding sites on the filamin-A promoter. These results provide evidence for a mechanically coupled transcriptional circuit that originates at the magnetite bead/integrin locus, activates p38, tethers p38 to actin filaments, promotes binding of p38 to Sp1 in the nucleus, and induces filamin-A expression.

Connective tissue cells are subjected to high amplitude mechanical forces that in part are directed through cell surface integrins to the cell interior (1). Some of the cellular responses to extracellular forces include reorganization of subcortical actin and alteration of gene expression (reviewed in Refs. 2 and 3), suggesting that integrin-mediated signals can mediate adaptations to forces that extend from the plasma membrane to the nucleus. We have shown previously that mechanical forces directed through β1 integrins promote redistribution of filamin-A to the integrin/bead locus (4) and enhance filamin-A expression (5). Although these findings support a role for integrin-based cell signaling in response to physical forces, the protein interactions and transcriptional regulation involved in this pathway have not been examined in detail.

Integrin-mediated forces activate several signaling pathways including phosphorylation of focal adhesion structural proteins such as α-actinin, vinculin, talin, tensin, filamin, and paxillin as well as the focal adhesion kinase and Src family protein tyrosine kinases (reviewed in Refs. 2, 3, and 6). Filamins are actin-binding proteins originally isolated from rabbit macrophages that organize actin filaments into orthogonal networks and enhance the rigidity of the actin cytoskeleton (reviewed in Ref. 7). Filamins bind a large number of membrane-associated and cytoplasmic proteins at their carboxyl- and amino-terminal ends and help tether the actin cytoskeleton to numerous cytoplasmic structures (7). The enhanced transcription and expression of filamin-A in response to mechanical force directed through cell surface integrins is dependent on de novo protein synthesis, an intact actin cytoskeleton, and Sp1 transcription factor binding sites in the filamin-A promoter (5). These studies suggested the outline of a mechanism in which extracellular mechanical forces can regulate the expression of filamin-A.

The ability of cells to respond to exogenous and endogenously generated mechanical forces has prompted the examination of integrin-matrix interactions and the organization of the actin cytoskeleton. In this context, fibroblasts grown on fibronectin but not poly-L-lysine exhibit basal ERK1/2 activation (8), a response that requires intact filaments. Similarly, ERK 1/2 activation occurs at focal adhesions in fibroblasts grown on laminin or fibronectin but not poly-L-lysine (9). Mechanical force-induced DNA synthesis and down-regulation of the platelet-derived growth factor promoter also require integrin ligation (10, 11), processes that are mediated in part by NF-κB and Sp1-binding sequences in the platelet-derived growth factor promoter (11). Force-induced filamin-A expression is found in cells plated on collagen or fibronectin but not poly-L-lysine (5). Collectively, these studies suggest that cellular adhesion to extracellular matrices affects the organization of the actin cytoskeleton and accordingly regulates force-induced gene expression through specific transcription factors and distinct signaling pathways.

Whereas these studies indicate the existence of force-induced activation pathways that originate at the cell membrane, the protein interactions that mediate downstream transcriptional regulation of cytoskeletal genes have not been defined. In this report we examined the regulation of the actin-binding protein filamin-A.

1 The abbreviations used are: ERK, extracellular signal-regulated kinase; MAP, p38 mitogen-activated protein; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; wt, wild type; EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; CREB, cAMP-response element-binding protein; NE, nuclear extracts; BSA, bovine serum albumin.
filamin-A in response to tensile forces applied through integrin receptors (1, 4). We describe a functional pathway that is initiated at the extracellular matrix-β integrin locus, drives bidirectional migration of p38 and pp38 to integrins and to the nucleus, and promotes the interaction of p38 with actin filaments. The activation of this pathway enhances Sp1 phosphorylation and mediates the interaction of Sp1 with the p38 kinase, processes that are essential for force-induced filamin expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human gingival fibroblasts were derived from primary explant cultures as described (12). Cells from passages 6–15 were grown as monolayer cultures in T-25 flasks (Falcon, BD Biosciences) in α-minimum Eagle’s medium containing 10% fetal bovine serum and antibiotics. Twenty four hours prior to each experiment, cells were harvested and plated at 75% confluence. The experiments involving promoter analyses utilized Rat-2 fibroblasts as surrogates for gingival fibroblasts as described previously (13). Cells were maintained in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum and antibiotics. Prior to transfection, the cells were cultured in Opti-MEM (Invitrogen) at 75% confluence and then transfected as described below.

Alexa-fluor filamin-A monoclonal antibody was obtained from Sero-tec (Cedarlane Laboratories, Hornby, Ontario, Canada). Mouse monoclonal antibodies to β-actin, pp38, p38, Sp1, and phosphothereonine/threonine were obtained from Cell Signaling Technologies (New England Biolabs, Mississauga, Ontario, Canada). For immunoprecipitation of p38, a second monoclonal antibody to p38 was obtained from BD Biosciences. The anti-β- integrin mAb 4B4 was obtained from Beckman Instruments. Latrunculin-B, SB203580, and PD98059 were obtained from Calbiochem. Monoclonal antibodies to FLAG and talin were obtained from Sigma.

Force Generation—Force generation through integrins was produced using a model system described previously (14). Briefly, magnetite microparticles (Fe3O4, Sigma) were incubated with purified collagen (Vitrogen 100, Cohesion Technologies, Palo Alto, CA; 1 mg/ml), neutralized to pH 7.4, rinsed with phosphate-buffered saline, and incubated with fibroblasts. Following a 30-min incubation, excess non-adherent microparticles were removed by vigorous washing, and the 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 mechanical force of ~0.48 piconewtons/µm2 cell area, a force that is comparable with that which may be experienced by cells in vivo during normal function (14). The incubation times were specific for each individual experiment as indicated.

RNA Isolation, Northern Blot, Reverse Transcription (RT), and PCR Analysis—RNA isolation was performed with RNeasy reagents (Qia-gen, Mississauga, ON). All RNA preparations were treated with RNasefree DNase (Promega Corp., Madison, WI) for 30 min. The RT-PCR protocol was conducted as described in detail elsewhere (15). RT was performed using 1 unit of M-MLV reverse transcriptase (MBI Fermentas, Mississauga, Ontario, Canada) and 10 pmol of oligo(dT) primer. The cDNA product was verified through diagnostic restriction enzyme cleavage digestion of the amplified fragment.

The semi-quantitative nature of the RT-PCR protocol, the precautions taken to avoid spurious reaction products, and the controls used have been described previously (15). In each experiment, a non-RT control was run with each individual cDNA. A dot blot analysis of filamin-A and GAPDH has been described previously (5).

Western Blotting, Immunofluorescence, and Immunoprecipitation—Cells were lysed, and cellular proteins were separated by SDS-PAGE (8% gels were used for filamin-A and β-actin Westerns, and 12% gels were used for pp38 and p38 blots) and transferred to nitrocellulose (Schleicher & Schuell) as described previously (4). Protein concentrations were determined using the Bradford assay and bovine serum albumin as a standard. Equal amounts of protein were loaded on individual lanes, and nitrocellulose membranes were analyzed as described previously (4, 5). Chemiluminescent detection was performed according to the manufacturer’s instructions (Amersham Biosciences). Immunoblotting films were exposed for standardized lengths of time using conventional protocols.

For immunofluorescence, gingival fibroblasts were grown on 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 (5). Immunofluorescence was visualized using a Leica DMIRE2 microscope.

GAPDH-primersForward, 5′-CTCTCCTCAGGCGAGGTGAGATCT-3′; reverse primer, 5′-GAGTTCACTGTGACTTAT-3′.

The amplified fragment was used to generate a nested PCR product containing oligonucleotide A1 (5′-GTCGCC-TCTCAGGAGACGACGGTGGAGCT-3′) and oligonucleotide B1 (5′-GGAGTTCACTGTGACTTAT-3′). The correctly amplified product was verified through diagnostic restriction enzyme cleavage and ligated into the pGL2 Basic Vector (Promega). The nested PCR product was used as the same components with the substitution of oligonucleotide A2 (5′-GTGCTTCCAGGACGACGGTGGAGCT-3′) and B2 (5′-GGAGTTCACTGTGACTTAT-3′). The correctly amplified product was verified through diagnostic restriction enzyme cleavage and ligated into the pGL2 Basic vector between HindIII and BglII, and the correct orientation of the insert was verified through diagnostic restriction enzyme digestion sequencing and the Sequencing Facility, Center for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada).

To generate the final wild type 75-bp filamin-A luciferase construct [pFil75(wt)luc], the original 3.2-kbp filamin-A luciferase vector was digested with Xhol-PstI (which effectively removed 3.15 kbp of upstream promoter). The fragments were blunt-ended and isolated from an agarose gel, and the portion containing the 75-bp fragment fused to the luciferase reporter construct was re-ligated. To fabricate the final 75-bp promoter construct containing mutations at the Sp1-binding sites [pFil75(mut)luc], two complementary oligonucleotides (described below and made by MWG Biotech) were boiled independently and allowed to cool to room temperature before being equal amounts. Priming 5-min incubation at 95 °C, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 54 °C, and 3 min at 68 °C with a final extension at 68 °C for 7 min. The amplified fragment was used to generate a nested PCR product that contained BglII and HindIII restriction sites for directional cloning into the pGL2 Basic Vector (Promega). The nested PCR product was used as the same components with the substitution of oligonucleotide A1 (5′-GTCGCC-TCTCAGGAGACGACGGTGGAGCT-3′) and B1 (5′-GGAGTTCACTGTGACTTAT-3′). The correctly amplified product was verified through diagnostic restriction enzyme cleavage and ligated into the pGL2 Basic vector between HindIII and BglII, and the correct orientation of the insert was verified through diagnostic restriction enzyme digestion sequencing and the Sequencing Facility, Center for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were isolated to determine Sp1 binding activity in force-treated and control fibroblasts according to an isolation protocol established previously (19). Briefly, cells were treated as indicated for 30 min and then harvested. Nuclear extracts were prepared by incubating the cell pellet in buffer containing 10 mM Hepes, pH 8.0, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM PMSF, 200 mM sucrose, 0.5% Nonidet P-40, and 1 mg/ml of both aprotonin and leupeptin (both from Sigma). Nuclei were lysed in buffer

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Rat 2 fibroblast cells were transfected using the pCMV-MKK6AL (pCMV-MKK6AL-dominant repressor; both from Dr. R. Tjian, University of California), pCMV-NF-κBp50 and pCMV-NF-κBp50 (both from Dr. N. Rice, National Cancer Institute, Frederick, MD). All vectors have been described elsewhere (20–22), and a dominant negative MKK6 (pCMV-MKK6ΔL) has been described (23). The luciferase vectors pFIL3.2Luc, pFIL175/WTLuc, and pFIL175/MutLuc are described above. To establish transfection efficiency and as a control, a green fluorescent protein vector (pEGFP1Luc) was used (Clontech).

Statistical Analysis—For continuous variable data, means ± S.E. from n = 3 experiments were computed. Unpaired Student’s t tests were used for statistical testing, and significance was set at p < 0.05. In each assay n = 3.

RESULTS

Induction of Filamin-A Expression by Force Application—We assessed the requirement of cell surface β1 integrins in force-mediated filamin-A gene regulation. Fibroblasts were incubated with either collagen- or BSA-coated magnetite beads and were pretreated with either integrin blocking antibodies (mAb 4B4) or vehicle prior to force application (Fig. 1A). Densitometric analysis of the RT-PCR products showed a 6-fold induction of filamin-A in force-treated cells through activated integrins (Fig. 1A).

Fig. 1. Mechanical force-induced activation of filamin-A. 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 (0.48 piconewtons/μm²). Total RNA was isolated after 6 h of force, and 1 μg was subjected to RT-PCR analysis for filamin-A and GADPH. Lane 1 indicates bead loading without force; lane 2, cells were subjected to force application; lane 3, same as lane 2 but cells were pretreated with mAb 4B4 (anti-β1 integrin); lane 4, cells were incubated with BSA-covered beads (1 mg/ml) and force-applied; lane 5, same as lane 2 but cells were pretreated with latrunculin-B (1.0 μM) prior to force application; lane 6, same as lane 2 but cells were pretreated with SB203580 (2.0 μM), and lane 7, same as lane 2 but cells were pretreated with PD98059 (5 μM). Histograms show data from n = 3 independent experiments and are densitometric analyses relative to the levels of GAPDH in each lane. For the Northern blot analysis shown below, lane 1 indicates bead-loaded cells without force application and lane 2 demonstrates RNA from force-treated cells. On the graph, the Northern blot data is shown in solid black bars. B, Western blot analysis of filamin-A, β-actin, pp38, and p38 protein content in untreated (--) and force-treated fibroblasts. In samples treated with force, cells were untreated (force alone) or preincubated with SB203580 or PD98059 prior to force application for the times indicated below each blot. Equal amounts of total cellular protein were loaded in each lane, separated on denaturing polyacrylamide gels that were subsequently scanned, quantified, and shown on the graphs below each section. Data are means ± S.E. from n = 3 experiments.

Cell Transfections—Rat 2 fibroblast cells were transfected using the Effectene transfection reagent (Qiagen). Briefly, following titration experiments to determine the optimum concentration of vector needed, cells were transfected, left for 36–48 h, and then subjected to various treatments described for each individual experiment. Following each treatment, cells were processed for luciferase activity using the manufacturer’s instructions (Luciferase Assay System, Promega Corp). The vectors used in the transfection are following and their source is indicated in parentheses: pCMV-MKK6ΔL (constitutively active) and pCMV-MKK6ΔL (pCMV-MKK6ΔL-dominant repressor; both from Dr. J. Woodgett, University of Toronto), pCMV-p38FLAG (constitutively active; Dr. R. J. Davis, University of Massachusetts); pCMV-Sp1 and pCMV-Sp1(SA21) (positive and negative Sp1 controls, respectively; both from Dr. R. Tjian, University of California), pCMV-NF-κBp50 and pCMV-NF-κBp65 (both from Dr. N. Rice, National Cancer Institute, Frederick, MD). All vectors have been described elsewhere (20–22), and a dominant negative MKK6 (pCMV-MKK6ΔL) has been described (23). The luciferase vectors pFIL3.2Luc, pFIL175/WTLuc, and pFIL175/MutLuc are described above. To establish transfection efficiency and as a control, a green fluorescent protein vector (pEGFP1Luc) was used (Clontech).

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Actin filaments are important for transmission of extracellular signals to the nucleus required for transcriptional activation (24). Accordingly, we examined the role of p38 in force-induced activation of filamin-A. Inhibition of the p38 kinase pathway by SB203580 (2 μM) suppressed the transcriptional activation of filamin-A, whereas the ERK-specific inhibitor PD98059 (5 μM) had no effect (Fig. 1A). Within 15 min of force, there was a 3–4-fold increase in pp38 when lysates were analyzed by immunoblotting. Similar increases of pp38 were also found in cells pretreated with PD98059 but not in cells preincubated with SB203580 (Fig. 1B). To assess the involvement of other MAP kinase pathways in this force model system, we analyzed lysates for force-induced phosphorylation of ERK and JNK but found no increases following force application (data not shown).

We determined whether force would also increase filamin-A protein content. Whole cell extracts were prepared from fibroblasts treated with force for increasing lengths of time and examined by Western blot. There was a 5–7-fold increase in filamin-A protein content (Fig. 1B). Similar to our findings for filamin-A mRNA, the force-induced increase of filamin-A was eliminated by pretreatment with SB203580 but not by PD98059. We also evaluated the role of other, non-collagen receptors in force-mediated activation of filamin-A. Magnetite beads were coated with bone sialoprotein (1 mg/ml) that binds αvβ3 integrin subunits. After several hours, force application did not appreciably increase protein levels of filamin-A (data not shown). As these results indicated that force-mediated increases of filamin were not obtained when force was delivered through αvβ3 integrins, there is an apparent requirement for β1 integrins in this process.

Force Induces p38 and pp38 Redistribution within the Cell—As activated ERK translocates to newly established focal adhesions in freshly plated fibroblasts (9), we assessed pp38 and p38 MAP kinase localization by immunofluorescence in bead-loaded cells that were bead-loaded with or without force (Fig. 2). In cells fixed with methanol to permit nuclear access for antibodies, immunostaining for pp38 in UV-irradiated fibroblasts showed redistribution of pp38 to the nucleus (Fig. 2A.i). Equivalent nuclear aggregation of FLAG-tagged p38 has been detected in UV-irradiated COS cells (20). In untreated cells, p38 and pp38 were diffusely distributed throughout the cytoplasm (Fig. 2A.iii, panel b), but after 1 h of force, both p38 and pp38 were aggregated to the nucleus (Fig. 2A.iii, panels e and h). When we fixed cells with paraformaldehyde (i.e. without nuclear permeabilization), immunostaining showed that force caused selective recruitment of pp38 and p38 to the integrin/magnetite bead locus (Fig. 2B). These results demonstrate force-induced migration of p38 and its active phosphorylated form (pp38) to cell nuclei and the integrin/magnetite bead locus in a manner analogous to the localization of activated ERK at newly formed focal adhesions (9). We also assessed the relative enrichment of p38 and pp38 to focal adhesions after force application using isolated, bead-associated proteins. With the use of a protocol established previously (4, 26) followed by immunoblotting, we found that pp38, talin, and filamin-A were increased by force, whereas β-actin was relatively constant (Fig. 2B), results which were consistent with the immunofluorescence data.

We assessed the role of actin filaments in the localization of filamin-A and the activation of p38 by treatment of cells with latrunculin-B prior to application of force. In view of the apparent migration of pp38 and p38 in force-treated cells, we examined pp38 and p38 localization by immunofluorescence. Rhodamine phalloidin staining of control cells demonstrated characteristic actin stress fibers that were disrupted by pretreatment with latrunculin-B (Fig. 3A). Latrunculin-B-treated cells also showed diffuse pp38 and p38 staining patterns, a distribution that did not change appreciably following force application. Quantitative evaluation of filamin-A protein content after force application showed a 5-fold increase after 12 h (p < 0.01) that was consistent with data from Fig. 1B. After treatment with latrunculin-B followed by force, filamin-A protein content was not increased significantly above base line, demonstrating a requirement for intact actin filaments (Fig. 3B). The restricted movement of pp38 and p38 with latrunculin-B pretreatment after force parallels the results of Aplin et.al. (27) in cytochalasin D-pretreated fibroblasts in which nuclear localization of ERK and phosphorylation of Elk-1 were suppressed by actin filament depolymerization.

Activation of Filamin-A Transcription by the MKK6-p38 Kinase Pathway—Functional Sp1 transcription factor-binding sites in the filamin-A promoter were necessary for force-mediated activation of a filamin-A reporter vector (5). However, the signaling mechanisms required for transmitting a tensile force-mediated signal from cell surface integrins to the nucleus have not been defined. In view of previous studies describing a role for the MKK6 (MAP kinase kinase 6)/p38 MAP kinase in force-induced gene regulation (28), we assessed the involvement of this pathway using MKK6 or p38 expression vectors. The introduction of pCMV-MKK6+ and pCMV-p38+ expression vectors by transfection increased endogenous filamin-A mRNA (Fig. 4), whereas transfection of the dominant negative pCMV-MKK6AL had no effect. Furthermore, application of force following transfection with either MKK6+ or p38+ did not significantly (p > 0.2) increase the levels of filamin-A mRNA and did not abrogate the inhibitory effects of the dominant negative MKK6AL (Fig. 4).

To determine the effects of these expression vectors on protein expression, we assessed the cellular content of filamin-A, Sp1, and pp38 in transfected and force-treated transfected transfectants (Fig. 5, A–C). Notably, in Chinese hamster ovary cells, introduction of pCMV-MKK6+ causes specific phosphorylation of p38 without any effect on JNK or ERK (20). We found that transfection with pCMV-MKK6+ augmented the production of filamin-A, Sp1, and pp38; there were further increases when cells were subsequently treated with force (Fig. 5A). A similar pattern was observed when the constitutively active p38+ vector was introduced into fibroblasts (Fig. 5C). In both experiments, pretreatment with SB203580 significantly decreased production of filamin-A and Sp1, whereas treatment with PD98059 had no effect (data not shown). We are aware that the effects of SB203580 on pCMV-MKK6+–driven expression of filamin, Sp1, and pp38 may be due to overexpression and autoregulatory feedback mechanisms involving kinase activation downstream of MKK6 and p38 because several downstream kinases are stimulated (29–32). Our assays
required 24–36 h for full expression following transfection, as determined by the production of FLAG from pCMV-p38FLAG. Accordingly, the overexpression of MKK6 may lead to the subsequent activation or suppression of downstream kinases that stimulate p38 indirectly leading to the results observed in our experiments. Furthermore, as the efficiency of transfection was ~50–60%, we also detected endogenous filamin, Sp1, or p38 in the untransfected cell populations. Notably, transfection with the dominant negative MKK6AL vector reduced filamin-A, Sp1, and pp38 content in those cells that were subsequently treated with force (Fig. 5B). Collectively, these data demonstrate that constitutive activation of the MKK6/p38 MAP kinase pathway enhances the basal levels of filamin-A, pp38, and Sp1.

Regulation of the Filamin-A Promoter by MKK6 and p38 MAP Kinase—The data from Figs. 1 and 5 suggested that the MKK6/p38 MAP kinase pathway regulates filamin-A expression in response to tensile force. Furthermore, previous results (5) indicated that a reporter vector containing 3.2 kbp of the filamin-A upstream sequence is regulated by Sp1 transcription factor-binding sites. Accordingly, MKK6+, MKK6AL, or p38+ expression vectors were transiently transfected into Rat-2 fibroblasts in conjunction with reporter vectors specific for the filamin-A promoter. By using the full-length 3.2-kbp filamin-A reporter vector (pFil3.2luc), we assessed base-line levels of luciferase activity and found a 4–6-fold induction by force application that was inhibited by SB203580 but not by PD98059 (Fig. 6A). When MKK6+ and p38+ expression vectors were co-transfected with pFil3.2luc, we noted a reproducible 6–8-fold induction that was further augmented following force application. A control assay using the dominant negative MKK6 (MKK6AL) suppressed basal luciferase activity and decreased force-induced activation of pFil3.2luc. Control co-transfection experiments with expression vectors for wild type Sp1 (pCMV-Sp1, 24-fold increase of pFil3.2luc activity) and an Sp1 DNA-binding mutant pCMV-Sp1 (SA21, no induction of pFil3.2luc) showed the specificity of Sp1-dependent activation. Notably, SA21 dimerizes but is unable to bind DNA and is therefore an ideal Sp1 negative control (from Dr. R. Tjian).

The specificity of the MKK6/p38 MAP kinase pathway was assessed using SB203580. This agent specifically inhibited MKK6+ and p38+-dependent activation of the pFil3.2luc reporter vector, whereas the ERK-specific inhibitor PD98059 produced no similar inhibition (Fig. 6). We have reported previously (18) that a truncated version of the filamin-A promoter containing 75 bp of upstream sequence contains several transcription factor-binding sites based on a promoter scan analysis. This 75-bp filamin-A reporter vector (pFil75(wt)luc) was regulated by force in a manner similar to the 3.2-kbp sequence;
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**Fig. 3.** Mechanical force induction of filamin-A is dependent on an intact actin cytoskeleton. A, fibroblasts were grown on glass slides, preincubated with latrunculin-B for 20 min, and then either untreated or subjected to force. Cells were fixed with paraformaldehyde and stained for pp38 or p38. Rhodamine phalloidin was used to stain actin filaments. Note that disruption of the actin cytoskeleton by latrunculin-B blocked mobilization of pp38 and p38 to the integrin/mag- 

[Diagram of fibroblasts showing actin filaments and pp38/p38 localization]

**Fig. 4.** Filamin-A gene transcription is increased in p38- and MKK6-transfected cells. Fibroblasts were cultured at 75% confluence in 6-well plates and transfected with the vectors indicated below. Total RNA was isolated, and 1 μg was subjected to RT-PCR analysis for filamin-A and GADPH.

- Lane 1, bead-loaded cells, no force (mock-transfected); lane 2, bead-loaded cells with force (mock-transfected); lane 3, cells transfected with pCMV-MKK6; lane 4, cells transfected with pCMV-MKK6; lane 5, cells transfected with pCMV-p38; lane 6, cells transfected with pCMV-Sp1; lane 7, cells transfected with pCMV-MKK6 and treated with SB203580; and lane 8, cells transfected with pCMV-p38 and treated with PD98059. Data are the means ± S.E. from n = 3 experiments.

Mutation of Sp1-binding sites in pFil75(wt)luc abolished force induction (5). To assess the involvement of the p38 MAP kinase pathway in the force-induced regulation of pFil75(wt)luc, co-transfection experiments were performed with MKK6AL, MKK6+ and p38+ expression vectors (Fig. 6B). The pFil75(wt)luc was equally responsive to constitutively active MKK6+ and p38+ expression vectors and was similarly enhanced by force application. The use of SB203580 strongly suppressed all basal and force-induced activation of pFil75(wt)luc alone and in co-transfection assays, whereas PD98059 had little effect. Similar to pFil3.2luc, the use of pCMV-MKK6AL alone or with force strongly reduced all luciferase activity to minimal levels (Fig. 6B). Control co-transfection experiments with pCMV-Sp1 or pCMV-Sp1(SA21) (described above) demonstrated the specificity of Sp1-dependent activation in pFil75(wt)luc (i.e. <30% induction above background; data not shown). Furthermore, co-transfection of pFil75(wt)luc with expression vectors for NF-κB subunit p50 (pCMV-NF-κBp50) and NF-κB subunit p65 (pCMV-NF-κBp65, both provided by Dr. N. Rice) demonstrated minimal luciferase activity with or without force application (data not shown). These findings indicated a specific requirement of pFil(wt)luc for Sp1-dependent activation.

To establish the importance of the Sp1 sites at position −15 and −25, we generated a 75-bp filamin-A reporter vector with mutations at these sites (described under "Experimental Procedures" as pFil75(mut)luc). The Sp1 sites mutated in pFil75(mut)luc were chosen based on their predicted impact on transcriptional regulation according to methods described by Prestridge (18). This mutated 75-bp filamin reporter vector, however, still possesses several heterologous upstream transcription factor-binding sites when examined with a promoter scan analysis (18) and hence could potentially be regulated by other transcription factors. When pFil75(mut)luc was transfected into Rat-2 cells either alone or co-transfected with MKK6/p38 expression vectors, a decrease in luciferase activity was detected (Fig. 6C). pFil75(mut)luc demonstrated a basal level of activity that was inducible by force application or after co-transfection with pCMV-MKK6 and pCMV-p38 expression vectors. However, the relative levels of induction were significantly lower than the luciferase levels obtained with pFil75(wt)luc (compare the relative levels of induction along each axis). The residual inducibility of pFil75(mut)luc by force, pCMV-MKK6, or pCMV-p38 may be explained by the presence of other transcription factor-binding elements upstream of the mutated Sp1 sites at positions −15 and −25.

**Force Induces Binding of Sp1 to the Filamin-A Promoter**—

Our previous results demonstrated that force-induced filamin-A expression is mediated through Sp1 sites on the filamin-A promoter (5). Whereas the pFil75luc vector contains several potential transcription factor-binding sites (18), we specifically examined the role of Sp1 because up to six binding sites for this factor exist and are located immediately upstream of the transcription start site of filamin-A. Nuclear extracts (NE, 5 μg) were isolated from bead-loaded controls and force-treated cells and analyzed by EMSA (Fig. 7). The migration patterns of most Sp1-DNA complexes show that although control cells exhibited basal Sp1 binding levels, force application for increasing lengths of time induced a 4–6-fold increase in...
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Sp1 binding within 2 h (Fig. 7A). To determine the specificity of the interaction of Sp1 with the filamin-A oligonucleotide, competition assays were performed. These experiments established that authentic wild type Sp1, and not sequences corresponding to NF-κB or CREB, could diminish or eliminate Sp1 binding to the −15 filamin-A oligonucleotide (Fig. 7B). To determine whether the enhanced Sp1 binding following force application was a generalized phenomenon for other transcription factors, CREB and AP-1 binding assays were performed. The results showed <5% increase in binding of either CREB or AP-1 following 8 h of force application. To confirm the authenticity of Sp1 in the binding complex, supershift analyses were performed. The addition of mAbs specific to Sp1 (75 ng and 1 µg) was sufficient to create a protein-DNA complex that migrated more slowly; these complexes were not detected when antibodies to NF-κB or CREB were used (Fig. 7C).

Tensile Force Induces p38 Association with Sp1 and β-Actin and p38 Phosphorylation—The data above suggested that the p38 MAP kinase pathway is involved in the transcriptional activation of filamin-A. Furthermore, tensile forces evidently induce the migration of both pp38 and p38 to the integrin/magnetite bead locus and the nucleus (Fig. 2) through an undefined mechanism. To provide information on potentially important protein associations involved in this signal transduction pathway, we immunoprecipitated proteins interacting with p38 or pp38. Force application caused a 3–4-fold increase in the association of p38 and pp38 with β-actin (Fig. 8), a cytoskeletal protein enriched in focal adhesions (Fig. 2Aii). Control immunoblotting for p38 confirmed that equivalent amounts of p38 were immunoprecipitated in the force and no-force samples. Other controls using an irrelevant immunoprecipitating antibody (anti-nebulin) showed no immunoprecipitation of actin (data not shown), thereby establishing the specificity of the association. To confirm the physical association between actin and p38/pp38, we transfected pCMV-p38FLAG into fibroblasts, immunoprecipitated lysates with anti-β-actin mAb, and immunoblotted these extracts with an antibody to FLAG. The results confirmed that extracts from force-treated cells showed much more abundant association of p38 with actin than cells without force (Fig. 8).

To assess whether Sp1 is activated following force application, we analyzed total Sp1 protein content and the level of serine/threonine-phosphorylated residues on Sp1, a modification that has been shown to be indicative of Sp1 transcription factor activation (33–35). We found that in response to force, there was increased phosphorylation of Sp1 in cell lysates that were initially immunoprecipitated with anti-Sp1 antibody and then immunoblotted with antibody to phosphoserine/threonine (Fig. 9). Immunoblotting of the immunoprecipitates with a different Sp1 antibody showed equal amounts of immunoprecipitated protein in force-treated and untreated cells (Fig. 9). These results show that force application induces an increase in phosphorylation of Sp1 at serine/threonine residues.

As force application enhanced the movement of p38 and pp38 into the nucleus (Fig. 2A.i), we assessed the ability of p38 to interact with Sp1 because p38 can activate other transcription factors including ATF-2, NF-κB, Elk-1, and MEF-2C (2, 3, 22, 25). To examine the involvement of p38 in Sp1 activation, we assessed Sp1 protein interactions with p38 and pp38. We immunoprecipitated p38- and pp38-bound material, divided these
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Materials into two sets of blots, and immunoblotted with either anti-Sp1 or anti-phosphoserine/threonine antibodies. There was a 2–3-fold increase in the association of p38 and pp38 with Sp1 in force-treated cells (Fig. 10i). Force treatment also increased the amount of phosphoserine/threonine-phosphorylated protein that co-migrated with Sp1 in the immunoprecipi-
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We have shown previously that application of mechanical force through cell surface β1 integrins increases filamin-A production (5) and that the expression of this protein protects cells against force-induced apoptosis (36). Here we demonstrate that force-mediated filamin-A expression involves activation of p38 and its localization to nuclei and integrin/magnetite beads at the plasma membrane. Furthermore, we show that activation of filamin-A is dependent on Sp1 transcription factor binding elements in the filamin-A promoter and that the p38 MAP kinase-signaling pathway mediates this activation through interactions with Sp1. These data provide evidence for a novel, force-induced transcriptional response that promotes cell survival by elaboration of a mechanoprotective protein, filamin-A. This response involves the association of the actin cytoskeleton with force-sensitive signaling molecules of which the p38 MAP kinase is apparently a critical element.

Many signaling molecules involved in response to extracellular stimuli are sequestered into discrete macromolecular complexes. The spatiotemporal organization of these aggregates can determine the specificity and the intensity of responses to a wide range of extracellular stimuli including force (37). The MAP kinases are a group of serine/threonine kinases that transduce signals from cell membrane receptors into intracellular regulatory signals that control gene expression. Our present analysis showed that intact actin filaments were required for force-induced activation of p38 and the migration of p38 to nuclei and integrin/magnetite bead loci. In addition, tensile forces promoted interactions of p38 and pp38 with actin, suggesting that the cytoskeleton may tether signaling molecules into protein complexes that participate in mechanically induced signaling. These complexes can increase the efficiency of signaling by decreasing the distance over which intermediates must interact to exert their effects. Thus ERK is activated at actin filament-enriched focal adhesions in response to spreading (9, 27), whereas disruption of actin filaments abrogates ERK activation (8) and blocks nuclear translocation/phosphorylation of the transcription factor Elk-1 (27). Notably, as filamin-A cross-links actin filaments and is recruited to the submembrane cortex after mechanical stimulation (4), filamin-A may also provide a scaffolding role in p38 signaling as has been shown for MKK4 and TRAF2 (38). Although we have not determined the mechanisms underlying the migration of p38 to the nucleus, the Ran GTPases may be involved based on their contribution to nuclear localization of ERK (39, 40). Furthermore, Whitehurst et al. (41) recently demonstrated that GFP-ERK2 enters the nucleus in a saturable, time-, and temperature-dependent manner through its interaction with nucleoporin.

Activation and nuclear localization of MAP kinases can regulate gene expression by phosphorylation of a number of transcription factors including SAP-1, Elk-1, c-Jun, ATF-2, MEF2C, CHOP, and NF-κB (22, 42, 43). The p38 MAP kinase is particularly responsive to cellular stressors and can specifically phosphorylate Elk-1, ATF2, and MEF2C (38). Our data show that application of tensile force promotes a 3–5-fold increase in Sp1 serine/threonine phosphorylation and concomitant association with p38, the first demonstration of a mechanically induced signaling system involving this transcription factor and activation by p38. Sp1 is a zinc finger DNA-binding protein that binds a putative GC-rich element, originally thought to be ubiquitously present in core promoter elements (reviewed in Refs. 35 and 44). Following phosphorylation by protein kinases including DNA-dependent protein kinase, casein kinase II, protein kinase A, and the cell cycle-regulated Sp1-associated protein kinase (34), Sp1 binds DNA and regulates transcription. Here we show that Sp1 can regulate an important mechanoprotective gene after stimulation by tensile forces. We have identified previously several binding sites for Sp1 on the filamin-A promoter (5), and we show here that these

**FIG. 8.** Fibroblasts subjected to force exhibit increased Sp1 phosphorylation and enhanced binding of p38 to β-actin and Sp1. Fibroblasts were cultured in 6-well dishes and were untreated (−) or subjected to force (+). Cell extracts were immunoprecipitated (IP) with antibody to either pp38, p38 (i), or FLAG (ii). The immunoprecipitated material was separated on a 5–20% gradient-denaturing PAGE and transferred to nitrocellulose and then immunoblotted for β-actin. lysates were immunoprecipitated (with antibody to FLAG) from cells transfected with pCMV-pp38FLAG, separated on a 5–20% gradient-denaturing PAGE, and immunoblotted for β-actin. Force application increased the interaction of p38/pp38 with β-actin. Each histogram shows the means and range from two independent experiments.

**FIG. 9.** Force application to fibroblasts increases levels of phosphorylated Sp1. Cell extracts from untreated (−) and force-treated (+) cells were immunoprecipitated (IP) with antibody to Sp1, separated on a 5–20% gradient denaturing PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphoserine/threonine antibody (i). As a standard, immunoprecipitated material from force-treated and untreated cells was immunoblotted for Sp1 with a different Sp1 antibody (ii). Each histogram shows the means and range from two independent experiments.

**FIG. 10.** Sp1 and pp38 are associated more strongly following force treatment. Cell extracts from untreated and force-treated cells were immunoprecipitated (IP) with pp38 and p38 antibodies, separated on a gradient denaturing PAGE, and immunoblotted for Sp1 (i) or for phosphoserine/threonine in adjacent lanes (ii). In all experiments, compared with untreated cells, extracts from force-treated cells showed enhanced p38 and pp38 interaction with Sp1 and phosphoserine/threonine residues that co-migrated with Sp1. Histograms are means ± S.E. and range from two independent experiments.

**DISCUSSION**

We have shown previously that application of mechanical force through cell surface β1 integrins increases filamin-A production (5) and that the expression of this protein protects cells against force-induced apoptosis (36). Here we demonstrate that force-mediated filamin-A expression involves activation of p38 and its localization to nuclei and integrin/magnetite beads at the plasma membrane. Furthermore, we show that activation of filamin-A is dependent on Sp1 transcription factor binding elements in the filamin-A promoter and that the p38 MAP kinase-signaling pathway mediates this activation through interactions with Sp1. These data provide evidence for a novel, force-induced transcriptional response that promotes cell survival by elaboration of a mechanoprotective protein, filamin-A. This response involves the association of the actin cytoskeleton with force-sensitive signaling molecules of which the p38 MAP kinase is apparently a critical element.

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binding sites contribute to regulation of force-induced filamin-A expression. Ablation of critical Sp1-binding sites in the filamin-A promoter strongly decreased the tensile force- and p38-mediated activation of a filamin-A reporter vector. Furthermore, we demonstrate that both CREB and AP-1 are not equally activated by force application demonstrating the specific activation of Sp1. Notably, Sp1 may not regulate filamin-A transcription alone. Sp1 may interact with other transcription factors including the insulin-responsive binding protein, NF-κB, and c-Jun (45–48) to regulate a multitude of genes. Furthermore, Sp1 shares DNA-binding elements with NF-κB, NF-1, and p33 proteins, indicating that cooperative or obstructive binding to specific promoter sequences may confer additional transcriptional regulation (49–52).

In the tensile force model system described here, we demonstrate the activation of filamin-A gene expression through phosphosine/threonine-mediated stimulation of Sp1. Our proposed model therefore describes a mechanistic circuit that originates at the integrin-magnetite bead locus and induces the activation of p38 and pp38 (Fig. 7). The activation of p38/pp38 enhances its interaction with the actin cytoskeleton and promotes their localization to the nucleus and the integrin-magnetite bead locus. In the nucleus, we propose that p38/pp38 phosphorylates Sp1 and enhances its interaction with the filamin-A promoter to augment gene transcription. In this manner, filamin-A production in our mechanical stress model protects the cells against lethal applied forces by promoting the re-distribution of the actin cytoskeleton through a cytoprotective mechanism.

Depending on the target gene, activation of Sp1 could occur through either phosphorylation or dephosphorylation. For example, serine/threonine phosphorylation of the amino terminus of Sp1 enhances the CDK activity of cyclin A by 3–4-fold (34). Phosphorylation also increases Sp1-mediated activation of the platelet-derived growth factor B-chain and tissue factor genes by shear stress (11, 19). However, terminal liver cell differentiation is down-regulated by Sp1 phosphorylation (53), whereas glucose-mediated activation of the acetyl-CoA carboxylase gene activation is down-regulated by Sp1 phosphorylation (53), whereas glucose-mediated activation of the acetyl-CoA carboxylase gene (53, 54). Indirect evidence that cell and gene-specific mechanisms are involved in Sp1-mediated gene regulation. Our data clearly indicate, however, that cell and gene-specific mechanisms are involved in Sp1-mediated gene regulation.

In conclusion, we have demonstrated that tensile force applied in the tensile force model system described here, we demonstrate the activation of filamin-A gene expression through phosphosine/threonine-mediated stimulation of Sp1. Our proposed model therefore describes a mechanistic circuit that originates at the integrin-magnetite bead locus and induces the activation of p38 and pp38 (Fig. 7). The activation of p38/pp38 enhances its interaction with the actin cytoskeleton and promotes their localization to the nucleus and the integrin-magnetite bead locus. In the nucleus, we propose that p38/pp38 phosphorylates Sp1 and enhances its interaction with the filamin-A promoter to augment gene transcription. In this manner, filamin-A production in our mechanical stress model protects the cells against lethal applied forces by promoting the re-distribution of the actin cytoskeleton through a cytoprotective mechanism.

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Interaction of p38 and Sp1 in a Mechanical Force-induced, \( \beta_1 \) Integrin-mediated Transcriptional Circuit That Regulates the Actin-binding Protein Filamin-A

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