Cytoprotection against Mechanical Forces Delivered through β₁ Integrins Requires Induction of Filamin A*

Received for publication, March 27, 2001, and in revised form, June 8, 2001
Published, JBC Papers in Press, June 21, 2001, DOI 10.1074/jbc.M102715200

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Cells in mechanically active environments can activate cytoprotective mechanisms to maintain membrane integrity in the face of potentially lethal applied forces. Cytoprotection may be mediated by expression of membrane-associated cytoskeletal proteins including filamin A, an actin-binding protein that increases the rigidity of the subcortical actin cytoskeleton. In this study, we tested the hypotheses that applied forces induce the expression of filamin A specifically and that this putative protective response inhibits cell death. Magnetically generated forces were applied to protein-coated magnetite beads bound to human gingival fibroblasts, cells with constitutively low basal levels of filamin A mRNA and protein. Forces applied through collagen or fibronectin, but not bovine serum albumin or poly-L-lysine-coated beads, increased mRNA and protein content of filamin A by 3–7-fold. Forces had no effect on the expression of other filamentous types or other cytoskeletal proteins. This effect was dependent on the duration of force and was blocked by anti-β₁ integrin antibodies. Force also stimulated a 60% increase in expression of luciferase under the control of a filamin A promoter in transiently transfected Rat2 fibroblasts and was dependent on Sp1 transcription factor binding sites located immediately upstream of the transcription start site. Experiments with actinomycin D-treated cells showed that the increased filamin A expression after force application was due in part to prolongation of mRNA half-life. Antisense filamin oligonucleotides blocked force-induced filamin A expression and increased cell death by >2-fold above controls. The force-induced regulation of filamin A was dependent on intact actin filaments. We conclude that cells from mechanically active environments can couple diverse signals from forces applied through β₁-integrins to up-regulate the production of cytoprotective cytoskeletal proteins, typified by filamin A.

Cells in biomechanically stressful environments can respond to pericellular changes in mechanical force by transducing physical stimuli into chemical signals that subsequently regulate gene expression (1). For example, mechanical force affects the expression of several cytoskeletal genes including the α-skeletal and α-smooth muscle actin isoforms (1) as well as the actin-binding proteins talin and vinculin (2). Mechanical force induction of cytoskeletal protein expression has been examined most prominently reported for smooth muscle cells (3, 4), but the transcriptional regulation of actin-binding proteins in nonmuscle cells has not been evaluated in detail. Notably, cells in mechanically stressed nonmuscle tissues such as bone and periodontium can maintain membrane integrity despite the application of high amplitude applied forces, indicating the existence of adaptive cytoprotective responses that may involve the cytoskeleton. For example, the recruitment of the actin-binding protein filamin A to sites of force transfer in fibroblasts was found to be cytoprotective, since it reduced the potentially lethal influx of calcium ions in response to applied force (5). As filamin A interacts with the cytoplasmic domains of the β₁ and β₂ integrins (6), it may also be able to transduce matrix-derived extracellular signals including those arising from mechanical forces. Currently, there is no evidence for force-induced regulation of filamin A expression in nonmuscle cells.

Filamins are actin-binding proteins originally isolated from chicken gizzard (7) that organize actin filaments into orthogonal networks and enhance the rigidity of the actin cortex (8, 9). Three discrete filamins have been described, and alternative splicing may allow for several additional isoforms (10). Filamin A (actin-binding protein 280) is composed of 23 96-amino acid repeats with flexible hinges and actin binding domains (10–13). The biological importance of filamin A is underscored by evidence showing that the X-linked human disorder periventricular heterotopia is due to mutations at X-q28, the FLN1 locus (14). This condition is characterized by defects in the migration of cerebral cortical neurons and vascular abnormalities that arise because of a lack of filamin-directed actin assembly and deficient rigidity of cell membranes.

While originally described in the context of its ability to organize the actin cytoskeleton, filamin A is now known to interact with many cellular proteins and has multiple functions including its ability to direct the cytosolic sol-gel transitions necessary for lamellopodial extension and cell crawling (8). With the use of the yeast two-hybrid system and other assays, filamin A has been shown to interact with cytoplasmic furin, tumor necrosis factor receptor associated factor-2, the platelet von Willebrand factor receptor glycoprotein Ib-IX, the small GTPase RalA, the FcγR1 receptor, the integral membrane protein presenilin, and the β₁ integrin (10, 15–21). Conceivably, the transmission of mechanical forces from matrix molecules to integrins and finally to filamin A and actin filaments may comprise a global mechanosensory and mechanoprotective signaling network for cells in mechanically active environments.
In view of the dampening of calcium responses conferred by filamin A on cells exposed to high amplitude applied forces (5), we determined if force could directly regulate filamin A gene expression in human gingival fibroblasts. These cells express low basal levels of filamin A but are periodically exposed to high amplitude forces in vivo. Therefore, they may be ideal candidates to assess force-induced induction of filamin A in cytoprotective responses. Our data show that mechanical forces applied through β1 integrins regulate filamin A transcription and that inhibition of filamin A expression by microinjected antisense oligonucleotides increases force-induced cell death. These findings have implications for the design and choice of cells in bioengineered tissues that are designed to withstand high amplitude physical loads.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Mouse anti-filamin A monoclonal antibody (clone MAB1680) was obtained from Chemicon International (Temecula, CA). Mouse anti-β-actin monoclonal antibody (clone AC-15), an anti-smooth muscle actin mAb (clone 1A4), anti-paxillin mAb (clone PXC10), anti-talin mAb (clone 8d4), and anti-vinculin mAb (clone VIN-11-5) were all obtained from Sigma. Mouse anti-cortactin mAb (clone 4F11) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-β1 integrin mAb 4B4 was obtained from Beckman-Coulter and for blocking experiments was used at a dilution of 1:30 as described (22). Actinomycin D, cycloheximide, cytochalasin D, poly-L-lysine, and recombinant fibronectin were all obtained from Sigma.

**Cell Culture**—Human gingival fibroblasts were derived from primary explant cultures as described (23). Cells from passages 6–15 were grown as monolayer cultures in T-25 flasks (Falcon, Becton Dickinson, Mississauga, Ontario, Canada) in α-minimal essential medium containing 10% fetal bovine serum and antibiotics. Twenty-four hours prior to each experiment, cells were harvested and plated at 75% confluence. In experiments requiring transfection, Rat-2 fibroblasts were used as surrogates for gingival fibroblasts as described previously (24). 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 (Canadian Life Technologies, Mississauga, Ontario, Canada) at 75% confluence and then transfected as described below.

**Force Generation**—Force generation through integrins was produced using a previously described model system (25). Briefly, magnetite microparticles (Fe₃O₄; Sigma) were incubated with purified collagen (Vitrogen 100; Cohesion Technologies, Palo Alto, CA; 1 mg/ml), fibronectin (Sigma; 100 μg/ml), or poly-L-lysine (Sigma; 1 mg/ml) or bovine serum albumin (BSA; 1 mg/ml), rinsed with PBS, and incubated with fibroblasts. Following a 45–60 min incubation, excess nonadherent microparticles were removed by vigorous washing, and the cells were supplemented with fresh α-minimal essential 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 (5). The incubation times were specific for each individual experiment as indicated.

**RNA Isolation**—RNA isolation was performed using a modified guanidium thiocyanate procedure (26). Briefly, cells were trypsinized and collected by centrifugation, washed with PBS, and resuspended in 1 ml of guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 100 μM β-mercaptoethanol. Total RNA was purified by phenol/water extraction and isopropyl alcohol precipitation. All RNA preparations were treated with QIAGEN DNase (Qiagen) for 30 min.

**Reverse Transcription (RT) and Polymerase Chain Reaction (PCR) Analysis**—The RT-PCR protocol was performed as described in detail elsewhere (27). RT was conducted on total RNA (1 μg) using 5 units of Moloney murine leukemia virus reverse transcriptase (Canadian Life Technologies) and 10 pmol of oligo(dT) primer. The cDNA product was subjected to 30 cycles of amplification in a PTC 100 MJ Thermal Cycler (Watertown, MA). PCR-amplified products were resolved via agarose gel electrophoresis, transferred to nylon membranes, and validated by probing with 32P-labeled oligonucleotide probes designed to detect filamin A mRNA. Quantification of PCR products was performed using the Ofto 1 system (Light Source Computer Images, Ferguson, MO) and IP Lab Gel (Signal Analytics, Vienna, VA). The density of individual lanes was normalized with the density of the PCR-amplified housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequences of the oligonucleotides used in the RT-PCR analysis were as follows: filamin A forward primer, 5'-GAGTTCTACTGTTGACAGCAGAAGT-3'; filamin A reverse primer, 5'-CTGTGACTATTACCCAGTACCTC-3'; GAPDH forward primer, 5'-AAGATAATTGCGAGAACACAG-3'; GAPDH reverse primer, 5'-TCATCGCATATTATACAGTGAC-3'; filamin C forward primer, 5'-CAAGAAGTCTGACATCAAACCC-3'; filamin C reverse primer, 5'-TCTTCTTCACCACTTCTAAAACCT-3'; GAPDH forward primer, 5'-CCATGAGAGGCTTGGGG-3'; GAPDH reverse primer, 5'-CAAAATTTTGTACATGGACC-3'.

The semiquantitative nature of our RT-PCR protocol, the precautions taken to avoid spurious reaction products, and the controls used have been described previously (28). In each experiment, a non-RT control was used to demonstrate the lack of DNA contamination.

**Northern Blot Analysis**—Molecular RNA was isolated from untreated or force-treated fibroblasts at the times indicated in Fig. 1 using a mRNA isolation kit (Oligotex mRNA Kit, Qiagen, Mississauga, ON). Samples of mRNA (20 μg each) were electrophoresed through a 1.0% agarose formaldehyde gel, transferred onto a nylon membrane by capillary blotting, and cross-linked. The membrane was described above, and PCR products. Prehybridization and hybridization were performed as previously described (28) using the filamin A and GAPDH oligonucleotides described above. The filters were washed using conventional protocols and exposed for standardized lengths of time to permit comparisons between experiments.

**Western Blotting**—Human gingival fibroblasts were grown and treated as described above. Following cell treatments, cells were lysed, and cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (8%) and transferred to nitrocellulose (Schleicher and Schuell) as previously described (5). Protein concentrations in individual samples were analyzed by the Bradford assay using bovine serum albumin (BSA) as a standard. Equal amounts of protein were loaded on individual lanes, and nitrocellulose membranes were analyzed as described previously (5). Chemiluminescent detection was performed according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The radiographic films were exposed for standardized lengths of time using conventional protocols.

**Genomic DNA Isolation, Filamin A Promoter Construction, and Transfection of Rat-2 Fibroblasts**—To generate the 3224-bp filamin A promoter construct, we isolated intact fibroblast genomic DNA using the protocol of Goelz et al. (29). Briefly, whole cell lysates were treated for 8 h at 50 °C with proteinase K in buffer (100 mM NaCl, 10 mM Tris, pH 6.8, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K). Following verification of intact DNA on a 1% agarose gel, 320 ng of DNA was incubated with PCR buffer containing oligonucleotide A1 (5'-GTCGCTCTCAGGAACAGCAGGTGAGGT-3') and oligonucleotide B1 (5'-GAGTTCACTGTGGAGACCAGAAGT-3'). The PCR was performed in an MJ Research PTC 100 Minicycler using 150 μl each of oligonucleotides A and B, 5% (v/v) Me₂SO, 1.5 mM MgCl₂, 200 μM each dNTP, and 1 unit of Expand High Fidelity PCR Enzyme System (Roche Molecular Biochemicals). The thermocycling procedure involved an initial 5-min incubation at 95 °C, followed by 35 cycles of 0.5 min at 94 °C, 1 min at 64.5 °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 with the same conditions with the substitution of nested oligonucleotide A2 (5'-CGCTCTCAGGAACAGCAGGAAGAT-3') and B2 (5'-CGCTCTCAGGAACAGCAGGACGCGCGGCTT-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 and sequencing performed at the DNA Sequencing Facility, Center for Applied Genomics (Hospital for Sick Children, Toronto, Ontario, Canada).

The expression of Rat-2 fibroblasts was achieved using the vector pAMINE-200 (Canadian Life Technologies). Briefly, Rat-2 cells were grown in 35-mm dishes, and 2 μg of Qiagen-prepared vectors (pL2lac Basic negative control, pGL2lac Control-positive control; and pGL2- Nil- Alu) were transfected according to the manufacturer’s instructions. The luciferase assay was used for estimation of force-induced transcriptional activation and has been described previously (30). The experiments were repeated on four independent sets of cells. We used Rat2
cells as surrogates for the human gingival fibroblasts, because previous data indicated that Rat2 cells were phenotypically similar to human gingival fibroblasts (24) and, unlike human fibroblasts, can be readily transplanted (30) for analysis of mass cultures.

**Generation of Filamin Promoter Mutants**—To generate the promoter mutants listed in Fig. 6, B and C, the 3224-bp wild type filamin A promoter construct was digested with the following enzymes. XhoI-XhoI digestion removed 549 bp of the upstream promoter; XhoI-NcoI digestion removed 607 bp of the upstream promoter; SacI-SacI digestion removed 1305 bp of the upstream promoter; KpnI-KpnI digestion removed 1913 of the upstream promoter; Smal-Smal digestion removed 2922 bp of the upstream promoter; and XhoI-PstI digestion removed all but 75 bp of the filamin promoter. To fabricate the final 75-bp promoter construct containing mutations at the Sp1 binding sites, two complementary oligonucleotides (described below) were boiled independently and allowed to slowly cool to room temperature in equimolar amounts. Promoter scanning was used to determine the location of potentially important transcription factor binding sites (31). The Sp1 mutations are indicated in boldface lettering (−75, 5′-TGCCAGATTCCGAGACACTG CAATTCTCGGCTATGAAATGATTAGCTCCACACTTTGGCGGAGACAGGCGCACAGGG-3′). The hybridized oligonucleotides were ligated into the pGL2 basic luciferase vector (Promega), and the correctly ligated vector was verified through restriction enzyme digestion.

**Immunofluorescence**—Human gingival fibroblasts were grown on 10-mm collagen coverslips, incubated with collagen-coated microbeads, and subjected to magnetic force application as described above. Samples were collected at standardized time points and fixed in 3.7% formaldehyde (in PBS) for 15 min followed by permeabilization in 0.3% Triton X-100 (in PBS). Samples were stained with mouse monoclonal anti-human filamin A followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma). Optical sections of filamin fluorescence associated with labeled cells were assessed by confocal microscopy (CLSM, Leica, Heidelberg, Germany) or by conventional immunofluorescence microscopy. In some experiments that examined the relationship of filamin A content to the cell cycle, cells were immunostained in suspension, counterstained with 4′,6-diamidino-2-phenylindole, and analyzed by two-parameter flow cytometry.

**Antisense Oligonucleotides**—The antisense oligonucleotides were synthesized on a 2-μmol scale (MWG Biotech, High Point, NC). The antisense oligonucleotide corresponded to positions +15 to +35 (5′-ttc CGG GGC GTC TCG tcg-3′), and the irrelevant oligonucleotide contained the sequence 5′-aag GCC CGG CCC GAG Agc ag-3′ (lowercase base nomenclature describes phosphorothioate linkages). Both oligonucleotides were generated according to previously described standards. Fibroblasts for microinjection were subcultured on 1-cm glass coverslips and microinjected with −1−4 μl of phosphorothioated oligonucleotides into the cytoplasm at 5 mg/ml in PBS buffer containing Hoescht 33342 to mark the nuclei of microinjected cells. Needles for microinjection were prepared from glass capillaries (Harvard Apparatus, Kent, UK) and pulled on a micropipette puller (Brown-Flaming, Sutter Instruments Co., San Francisco, CA). Following microinjection, cells were incubated with collagen-coated beads as described above and subjected or not subjected to force. Dead cells were evaluated by propidium iodide (10 μg/ml) staining and fluorescence microscopy. The levels and distributions of filamin A were evaluated by immunostaining as described above followed by image analysis. In each assay (n = 6 separate experiments), 60−100 cells were microinjected.

**Statistical Analysis**—For continuous variable data, means and S.E. values of the means were computed. Unpaired Student’s t tests were used for statistical testing, and significance was set at p < 0.05.

**RESULTS**

**Effect of Force on Filamin A Expression**—We examined forcelduced filamin A mRNA expression in human gingival fibroblasts by Northern analysis (Fig. 1A). There was a time-dependent increase in filamin A mRNA that was detected as early as 1 h after force application through collagen beads. Densitometric analysis of the blot showed a 5−7-fold increase in filamin A mRNA following collagen-mediated force induction in the first 4 h of treatment. To determine if the RT-PCR protocol yielded similar results as the Northern blot analysis, 1 μg of total RNA was isolated from cells at different time points following the application of force. A representative Southern blot (Fig. 1A) showed a time-dependent induction of filamin A; densitometric analysis yielded similar results as the Northern blot data (Fig. 1A). To examine whether other filamin isoforms were expressed in these cells and activated by force application, RNA from untreated and force-treated cells was subjected to RT-PCR analysis for filamin isoforms B and C using the identical PCR conditions as for filamin A. There was no detectable
product for filamin C and only a very low amount of filamin B that was not specifically enhanced by force application (data not shown).

We determined if force would enhance filamin A expression by analyzing protein content in Western blots and found a ∼3–4-fold increase in total filamin A protein following 8 h of magnetic force (Fig. 1B; \( p < 0.05 \)). These increases were significant when compared with samples analyzed for other cytoskeletal proteins including α-smooth muscle actin, cortactin, paxillin, talin, vinculin, and \( \beta_1 \) integrin, none of which showed induction by force (Fig. 1B). We assayed filamin A expression at the integrin-microbead locus by immunofluorescence. The results shown in Fig. 1C demonstrate diffuse staining of filamin A in untreated cells (Fig. 1C, b), but in cells subjected to force, there was an increased immunofluorescence signal for filamin A at the integrin-microbead locus (Fig. 1C, d). We also examined if filamin A levels may be dependent on the cell cycle. Using two-parameter flow cytometry, cells in the G1 phase of the cell cycle showed filamin A immunofluorescence that was significantly less than cells in the S phase (G1 phase = 28.5 ± 2 fluorescence units; S phase = 36.4 ± 2 fluorescence units; \( p < 0.05 \)). In a separate experiment to assess the dependence of force-induced filamin A expression on proliferative status, cells in subconfluent cultures (percentage of S phase cells = 12%) and cells in highly confluent cultures (percentage of S phase cells = 3%) were examined. We found by immunoblotting that cells in both conditions exhibited ∼3-fold increases of filamin A content after force application (data not shown). Collectively these results demonstrated that filamin A expression was augmented by mechanical force applied through collagen-coated magnetite microbeads.

*Integrins*—We performed experiments to assess if force activation of filamin A was mediated through \( \beta_1 \) integrins. Previous studies have shown that poly-L-lysine and BSA beads do not attach to cells appreciably through integrins, while collagen and fibronectin specifically bind through the \( \beta_1 \) integrins (5). Microbeads were coated with fibronectin, poly-L-lysine, or BSA and incubated with cells, and then force was applied to cells. While there was no detectable difference in filamin A expression following force application through poly-L-lysine or BSA-coated beads (Fig. 2, A and B), fibronectin-coated beads increased filamin A RNA and protein by 3–4-fold, a similar effect as seen with collagen beads. This result was not due to loss of beads from the dorsal surface of the cell, since microscopic examination and previous data (30) have shown that application of force through poly-L-lysine or BSA-coated beads does not cause increased bead detachment compared with fibronectin or collagen-coated beads (32). To confirm that collagen-mediated activation of filamin A was specifically dependent on \( \beta_1 \) integrins, cells were preincubated with a mouse monoclonal antibody against \( \beta_1 \) integrins (4B4) for 30 min prior to the addition of collagen-coated magnetite beads. This antibody at a 1:30 dilution has been shown to inhibit \( \beta_1 \) integrin-mediated stimulation of α-smooth muscle actin but does not cause inhibition of bead attachment (22). We observed a specific inhibition of force induced filamin A production (Fig. 2C), indicating that the \( \beta_1 \) integrin is involved and is necessary for collagen-mediated induction of filamin A expression by force application.

Filamin-dependent Cellular Survival—Filamin A cross-links actin filaments and greatly increases cortical rigidity (8). Filamin therefore may play a role in stabilizing the cell membrane in response to mechanical forces. We have previously shown that in filamin A-deficient melanoma cells (M2 cells), mechanical force application increases membrane leakage in comparison with the same cells but with stably expressed filamin A expression (A7 cells; Ref. 5). We therefore sought to examine the role of filamin A in protecting fibroblasts from force-induced cell death. Antisense filamin A oligonucleotides or irrelevant oligonucleotides were microinjected into fibroblasts, and force was applied for 6 h through collagen-coated beads. Following immunostaining of filamin A and identification of microinjected cells by Hoechst 33342 fluorescence, filamin A was strongly stained in the cell cortex and in stress fibers of untreated and force-treated cells microinjected with the irrelevant oligonucleotide. Consistent with the immunoblotting results, force increased staining for filamin A. In cells microinjected with the antisense oligonucleotide, filamin A immunofluorescence was greatly reduced in intensity (Fig. 3A). We quantified filamin A staining with an image analyzer and found that the antisense oligonucleotides reduced filamin A staining by >50% (Fig. 3A) and completely blocked the force-induced increase of filamin A (Fig. 3B; \( p < 0.05 \)).

We next examined propidium iodide staining of cells after force application. This dye is excluded from cells with an intact plasma membrane. Quantitative analyses showed that force-treated cells microinjected with the antisense oligonucleotides exhibited >2-fold higher ratios of propidium iodide positive to Hoechst-stained cells than cells microinjected with the irrelevant oligonucleotide or those cells that were not subjected to force (Fig. 3C). These results indicate that force-induced filamin A expression is important for preservation of viability in cells subjected to mechanical force.

*Actin Filaments*—Cell shape is a critical factor for regulating cytoskeletal gene expression in response to stretching (30) and cytokines (22) and is highly dependent on the organization and assembly of actin filaments. Accordingly, we altered cell shape by using culture dishes coated with either poly-L-lysine or fibronectin. Rhodamine phalloidin staining of actin filaments showed that cells grown on fibronectin were well spread and exhibited prominent staining of cortical actin and stress fibers, while cells on poly-L-lysine were rounded and did not exhibit cortical actin staining or stress fibers (Fig. 4A). Following application of mechanical force through collagen-coated microbeads on the dorsal cell surface, there was no induction of filamin A RNA or protein when cells were incubated on poly-L-lysine dishes (Fig. 4, B and C). When the dishes were coated with fibronectin, there was a time-dependent increase of filamin A mRNA after force application, beginning at 15–30 min (Fig. 4B), which persisted for up to 1 h after force application. Immunoblotting showed that mechanical force application to fibroblasts grown on fibronectin-coated dishes induced a 3.5-fold increase in filamin A after 8 h of force, while cells on poly-L-lysine showed no change (Fig. 4C).

In view of the suppression of force-induced filamin expression after cell rounding and loss of actin filaments, we determined if intentional depolymerization of actin filaments would alter the mechanical force induction of filamin A. We pretreated cells with cytochalasin D (5 \( \mu \)g/ml) 45 min prior to the addition of collagen-coated microbeads and examined filamin A expression. Cytochalasin D markedly inhibits actin assembly in human gingival fibroblasts under these conditions but does not cause detachment of beads following force application (33). After pretreatment with cytochalasin D, force-induced production of filamin A mRNA and protein was reduced to levels observed in untreated cells (Fig. 4D). This result, together with the experiments described above, confirms that force application through \( \beta_1 \) integrins and cortical actin filaments enhances production of filamin A.

Transcriptional Regulation—Our observation of filamin A transcriptional activation by mechanical force did not exclude the possibility that force may increase mRNA content by per-
turbing mRNA half-life, an effect that has been shown for α-smooth muscle actin (22). Accordingly, we compared filamin A mRNA half-life in collagen bead-loaded cells with and without force application. Cells were induced for maximal filamin A mRNA production after 4 h of force application; actinomycin D (1 μg/ml) was added to cultures to block further mRNA synthesis. Previous experiments using the same fibroblast type (i.e., early passage human gingival fibroblasts) have established that the addition of actinomycin D at 1 μg/ml is sufficient to block >95% mRNA synthesis (22). For experimental periods <12 h, this protocol does not lead to appreciable cell death. Total RNA was isolated at different time points following the addition of actinomycin D and filamin A, GAPDH, and β-actin mRNAs were analyzed by RT-PCR. Cells treated with actinomycin D but without force application showed a reduction of ~50% filamin A mRNA at 1 h. However, at 2 h, filamin A mRNA was reduced to ~4% of initial levels in controls, while cells exposed to force exhibited ~40% of initial levels of filamin A mRNA (Fig. 5A). Thus, 2 h after force application, filamin A mRNA levels were 10-fold higher in force-treated cells than in control cells (p < 0.001). In contrast to filamin A, the levels of both GAPDH and β-actin mRNA remained relatively constant over this same time period.

We also assessed the role of protein synthesis in the induction of filamin A mRNA by force. Cells were treated with cycloheximide (5 μg/ml) using a protocol that in human gingival fibroblasts blocks >97% of protein synthesis (34). Comparisons of untreated control cells and force-treated cells showed a ~3-fold increase in the level of filamin A mRNA after 60 min of force (Fig. 5B), a similar result as shown in Fig. 1. However, there was no detectable force-induced filamin A mRNA expression in the presence of cycloheximide, indicating that new
Fibroblasts were transiently transfected with a 3224-bp wild type filamin A promoter sequence with “Promoter Scan” software (31) yielded in excess of 50 potential transcription factor binding sites; the most prevalent of these sites was Sp1. Table I shows the most relevant transcription binding sites within the first 75 bp, a region of the promoter, which was still found to be strongly inducible by force application despite the elimination of −3 kbp of promoter (Fig. 6C). Accordingly, we generated a 75-bp filamin promoter vector selectively mutated at the three most proximal Sp1 sites, transfected this into Rat-2 cells, and subjected them to force application. The results show that mutation of Sp1 sites at −15, −21, and −65 specifically eliminates the force-induced production of luciferase (Fig. 6C), demonstrating that these Sp1 sites mediate the force-induced expression of filamin A.

**DISCUSSION**

Throughout life, cells must withstand global biomechanical stresses imposed by gravity and cycles of more intense, muscle-generated local forces, especially the cells in mechanically active tissues. The mechanisms by which nonmuscle cells in mechanically active environments maintain their viability and structure are poorly understood. Our results demonstrate that filamin A mRNA and protein are induced in human gingival fibroblasts following application of mechanical forces through β1 integrins and actin filaments. Notably, we showed that induction of filamin A protects cells against force-mediated death. Thus filamin A may be mechanotranscriptionally coupled in order to maintain cell viability in the face of applied environmental forces.

Fibroblasts, endothelial cells, and a wide variety of stromal cells in mechanically active environments are attached to extracellular matrix molecules by cell surface integrins. Consequently, the organization of the extracellular matrix proteins and their binding to integrins comprise an important communication network in determining biological responses to a wide variety of extracellular signals and stresses. Indeed, cell surface integrins bound to matrix proteins can direct applied physical forces through the cytoskeleton to the nucleus (35). Thus, the cytoskeleton not only functions as a crucial structural support but may also transmit signals required for transcriptional regulation induced by mechanical forces. Our results indicate that in well spread cells with abundant cortical actin, mechanical force application through poly-I-lysine-coated beads did not affect filamin A content, and the force-induced increases of filamin A mRNA and protein were blocked by preincubation with anti-β1 integrin antibodies. As filamin A binds to the cytoplasmic tail of the β1 integrin (10), these results and the immunofluorescence data showing the increased presence of filamin A at the integrin-microbead locus provide evidence for a direct physical linkage between forces applied through the extracellular matrix to filamin A and the transcriptional activation of a protective response.

Our results point to a regulatory mechanism in which a cell can detect cytoskeletal deformations and respond by increasing the transcription and translation of filamin A, an actin cross-linking protein that protects cells against applied forces. This detection system may involve the direct transmission of force from integrins through actin filaments to the nucleus as suggested earlier (35). Cognizant of the importance of cell shape and the ability of cells to transmit forces across the cell mem-

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**Fig. 3. Fibroblast viability following mechanical force application is dependent on filamin A expression.** Fibroblasts were cultured on 1-cm square glass slides and microinjected with antisense (AS) filamin A or irrelevant (IR) phosphorothioate oligonucleotides (described under “Experimental Procedures”) and treated with or without collagen-mediated mechanical force. Cellular filamin A was detected by immunofluorescence (A) and quantified (B, described under “Experimental Procedures”). Note that in cells microinjected with irrelevant oligonucleotides, force application increases fluorescence of immunostained filamin A. In cells microinjected with antisense filamin A oligonucleotides, force-induced increases of filamin A are blocked. C, filamin-dependent cellular survival following mechanical force application. Fibroblasts were microinjected with either the IR or AS oligonucleotides and Hoechst 33342 to mark microinjected nuclei, subjected or not subjected to force, and stained with propidium iodide (10 mg/mL). The ratio of propidium iodide to Hoescht 33342 stained cells gives an estimate of the proportion of dead cells. Note that microinjection of the antisense filamin A oligonucleotides causes doubling of the proportions of propidium iodide-permeable cells (p < 0.05). For B and C, data are mean and S.E. values from n = 6 experiments. In each assay, ~60–100 cells were microinjected.

**Mechanical Force Enhances Filamin A Promoter Activity through the Sp1 Transcription Factor.**—We examined the transcriptional activation of the filamin A gene following force application. Rat-2 fibroblasts were transiently transfected with a reporter vector containing 3224 base pairs of the filamin A promoter (described under “Experimental Procedures”). Twelve hours after transfection, cells were incubated with collagen-coated microbeads, and mechanical force was applied for 6 h. While the filamin A reporter construct exhibited some basal activity, force application induced a 60% increase of luciferase production (Fig. 6A).

After obtaining these initial results, mutant promoter constructs were generated (Fig. 6B, described under “Experimental Procedures”), transfected into Rat-2 cells and subjected to force application. Fig. 6B shows that all of the mutant filamin A promoter constructs demonstrated 2–3-fold induction compared with untreated cells (Fig. 6B). In addition, an analysis of the 3224-bp wild type filamin A promoter sequence with “Promoter Scan” software (31) yielded in excess of 50 potential transcription factor binding sites; the most prevalent of these sites was Sp1. Table I shows the most relevant transcription binding sites within the first 75 bp, a region of the promoter, which was still found to be strongly inducible by force application despite the elimination of −3 kbp of promoter (Fig. 6C). Accordingly, we generated a 75-bp filamin promoter vector selectively mutated at the three most proximal Sp1 sites, transfected this into Rat-2 cells, and subjected them to force application. The results show that mutation of Sp1 sites at −15, −21, and −65 specifically eliminates the force-induced production of luciferase (Fig. 6C), demonstrating that these Sp1 sites mediate the force-induced expression of filamin A.
brane and into the cytoplasm, we conducted experiments in which similar numbers of collagen-coated beads were incubated on the dorsal surface of cells previously plated on poly-L-lysine (PL) or fibronectin (FN). Note that stress fibers are absent in cells on poly-L-lysine and that cells on fibronectin are well spread and show abundant stress fibers. B, RT-PCR analysis of filamin A mRNA. Total RNA was isolated at different time points following the application of force through collagen-coated beads to gingival fibroblasts cultured on poly-L-lysine or fibronectin using collagen-coated magnetite microbeads for force application. RNA (1 µg) was analyzed by RT-PCR for filamin A and GAPDH mRNAs. The product levels were quantified relative to the levels of GAPDH mRNA using the methods described under "Experimental Procedures." Data are mean and S.E. values from n = 3 experiments. Cells on FN but not PL substrates showed increased filamin A mRNA content after force application (p < 0.01). C, filamin A Western blot analysis. Equal amounts of total cellular protein were isolated from cells subjected to forces applied to beads coated with different ligands described above. Cell lysates were analyzed by Western blot. The relative levels of filamin A protein were determined by densitometry. The β-actin control blot is shown below each bar. Application of force through fibronectin but not poly-L-lysine coatings induced increased filamin A content (p < 0.01). D, inhibition of force-induced filamin A production by cytochalasin D. Forty-five minutes prior to the application of force, cells were incubated with cytochalasin D (Cyt-D; 5 µg/ml); cells were then treated with force for 4 h (for mRNA analysis) or 8 h (for protein). Cells were lysed, and total RNA or whole cell proteins were isolated and examined for the expression of filamin A mRNA and protein, respectively. Cytochalasin D treatment completely blocked force-induced filamin A expression (p < 0.01). Data are mean and S.E. values from n = 3 experiments.

filamin A induction, since this plating method causes loss of cortical actin filaments. These results demonstrate the importance of intact actin filaments for delivery of force to the cell interior and are consistent with our data using cytochalasin D to depolymerize actin filaments; cytochalasin D treatment abrogated the induction of filamin A RNA and protein in response to force. Notably, both of these treatments caused marked cell rounding and evidently prevented the transmission of force to intracellular sites that may be important in mediating force-enhanced transcription.

The increased abundance of filamin A mRNA after force application is partially explained by promoter activation. Our results demonstrate that force enhances the basal activity of a
isolated thereafter. Total RNA (1 μg/ml) was added 30 min prior to mechanical force application, and total RNA was prepared with controls (n = 3 experiments). By 120 min after force, there were ~10-fold higher levels of filamin A mRNA in force-treated cells compared with controls (p < 0.001). B, cycloheximide (CHX; 5 μg/ml) was added 30 min prior to mechanical force application, and total RNA was isolated thereafter. Total RNA (1 μg/ml) was analyzed by RT-PCR for filamin A mRNA in both untreated and force-treated cells. Data shown to the left of the vertical bar are cells without CHX treatment and were either untreated (–) or treated with force (+) for 60 min. Data to the right of the vertical line are from cells treated with CHX for the indicated times and either untreated (clear bars) or subjected to force (black bars). Cycloheximide treatment completely blocked the force-induced expression of filamin A mRNA (p < 0.01). Data are mean and S.E. values from n = 3 experiments.

reporter plasmid containing 3.2 kbp of the filamin A promoter. Using a 75-bp mutant filamin A promoter, we determined that Sp1 binding sites located immediately upstream of the start site were responsible for the force-induced up-regulation of filamin A. Notably, Sp1 regulates a large number of genes (>5000; Ref. 37 and references therein), and filamin A can now be added to this list. As an example of a gene that is important in mechanotransduction, the cell surface matrix receptor for collagen, the α2 integrin, is known to be regulated by Sp1/Sp3 binding sites located at position −52 (38). In addition, the epithelial specific keratin gene K14 is also known to contain multiple Sp1 binding sites that bind this protein, as determined by EMSA analysis (39).

Our initial decision to employ a 3.2-kbp promoter construct was prompted by the proximity of FLN-A to the emerin gene located −5 kbp downstream of the FLN-A start site (40). Interestingly, the filamin and emerin genes are separated by a shared promoter sequence of only 5 kbp. It is therefore conceivable that the 3.2-kbp construct that we employed encompasses most of the regulatory sequence that is required for filamin A expression. We based our initial examination of the filamin A promoter on knowledge of the gene that encodes α-smooth muscle actin, another cytoskeletal protein that has been examined in great detail. Previously published studies have utilized

| Name | Location | Weight |
|------|----------|--------|
| Spl  | −15      | 3.29   |
| Spl  | −21      | 3.36   |
| Spl  | −61      | 4.59   |
| ETF  | −63      | 3.93   |
| Spl  | −63      | 3.01   |
| Spl  | −68      | 3.06   |
the inclusion of cells with inducible expression of filamin A. We suggest that bioengineered tissues that may be subjected to exogenous proteins in a variety of cell types that must withstand changes in cell stiffness and extracellular matrix. Our findings raise the likelihood that the cytoprotective protein content of fibroblasts is dependent, in part, on prolonged exposure to mechanical forces. Further, the ability of force to increase filamin A mRNA content in human gingival fibroblasts is mediated through a combination of mechanotranscriptional coupling of a cytoprotective gene and transcription of functionally analogous proteins in a variety of cell types that must withstand intense biomechanical stress in the context of applied biology. We suggest that bioengineered tissues that may be subjected to high amplitude mechanical forces may be optimized for survival by the inclusion of cells with inducible expression of filamin A.

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Cytoprotection against Mechanical Forces Delivered through $\beta_1$ Integrins Requires Induction of Filamin A

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J. Biol. Chem. 2001, 276:31969-31977.
doi: 10.1074/jbc.M102715200 originally published online June 21, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102715200

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