Smooth Muscle Actin Determines Mechanical Force-induced p38 Activation*

The mitogen-activated protein kinase p38 is activated by mechanical force, but the cellular elements that mediate force-induced p38 phosphorylation are not defined. As α-smooth muscle actin (SMA) is an actin isoform associated with force generation in fibroblasts, we asked if SMA participates in the activation of p38 by force. Tensile forces (0.65 pN/μm²) generated by magnetic fields were applied to collagen-coated magnetite beads bound to Rat-2 cells. Immunoblotting showed that p38α was the predominant p38 isoform. Analysis of bead-associated proteins demonstrated that SMA enrichment of collagen receptor complexes required the α2β1 integrin. SMA was present almost entirely as filaments. Knockdown of SMA (70% reduction) using RNA interference did not affect β-actin but inhibited force-induced p38 phosphorylation by 50%. Inhibition of Rho kinase blocked SMA filament assembly, force-induced increases of SMA, and force-induced p38 activation. Force application increased SMA content and enhanced the association of phosphorylated p38 with SMA filaments. Blockade of p38 phosphorylation by SB203586 abrogated force-induced increases of SMA. In cells transfected with SMA promoter-β-galactosidase fusion constructs, co-transfection with constitutively active p38 or MKK6 increased SMA promoter activity by 2.5-3-fold. Dominant negative p38 blocked force-induced activation of the SMA promoter. In SMA negative cells, there was no force-induced p38 phosphorylation. We conclude that force-induced p38 phosphorylation is dependent on an SMA filament-dependent pathway that uses a feed-forward amplification loop to synergize force-induced SMA expression with p38 activation.

The mechanisms by which mechanical forces regulate gene expression is of considerable biomedical importance, but the force-transduction circuits have not been defined. Mechanical force transmission through the extracellular matrix, integrins, and the cytoskeleton has been convincingly demonstrated using matrix protein-coated magnetic beads bound to integrins (1–4). With these experimental approaches, critical mechanotransduction processes have been studied including force-induced activation of the mitogen-activated protein (MAP) kinase (5) kinases (2, 3, 5–7). Activation of the MAP kinase p38 has been associated with increased binding of transcription factors to regulatory sites on the promoters of cytoskeletal genes, including the actin-binding protein filamin A (2, 5, 8), thereby suggesting a potential mechanotranscriptional circuit in which force application leads to p38 activation and, ultimately, regulation of gene expression.

α-Smooth muscle actin (SMA) is an actin isoform that is normally restricted to vascular smooth muscle cells. In healing wounds and fibro-contractive lesions, SMA is also expressed by certain non-muscle cells such as myofibroblasts (9–11). In cultured myofibroblasts, SMA expression is associated with the generation of increased contractile forces and stress fiber formation (12–14). Interference with SMA expression inhibits force generation and the formation of focal adhesions and stress fibers (15). In myofibroblasts, induction of SMA by TGF-β is dependent on the compliance of the substrate (16), suggesting an important role for cell-generated tension in regulating gene expression (17). SMA in myofibroblasts is largely assembled into actin filaments (15, 18), and some of these SMA filaments are localized to focal adhesions (19). These are important observations because the transmission of tensile forces involved in the induction of force-regulated genes requires actin filaments (16–18), and focal adhesions are thought to function as mechanosensors (20). Accordingly, we hypothesized that specific actin isoforms such as SMA, when associated with focal adhesions, may provide a selective physical link by which mechanical forces induce specific patterns of gene expression.

Currently, it is unknown if SMA can mediate mechanical signals such as force-induced activation of MAP kinases. In certain cell types, TGF-β-induced SMA expression is dependent on p38 activity (21). Force-induced regulation of SMA expression is also associated with p38 activation, depending on the extent of actin assembly in the specific types of cells examined (3, 22). As p38 activation is a prominent cellular response to applied tensile forces (2, 3, 5, 6), we examined the role of SMA

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The abbreviations used are: MAP, mitogen-activated protein; SMA, smooth muscle actin; RNAI, RNA interference; TGF, transforming growth factor; PBS, phosphate-buffered saline; RSV, Rous sarcoma virus; ROS, reactive oxygen species; TRITC, tetramethylrhodamine isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH² terminal kinase; CMV, cytomegalovirus.
SMA Determines Mechanical Force-induced p38 Activation

in mediating force-induced activation of p38, and conversely, we studied the importance of p38 in regulating force-induced SMA expression. Our data indicate that when tensile forces are applied to α2β1 integrins, phosphorylated p38 binds to SMA, and there are p38-dependent increases of SMA expression. As our results showed that cells with abundant SMA exhibit enhanced force-induced activation of p38, we suggest that SMA and p38 participate in a feed-forward amplification loop to synergize force-induced SMA expression with p38 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies to α-smooth muscle actin (clone 1A4), β-actin (clone AC-15), total-actin (clone AC-40), vimentin (clone VIM-13.2), vinculin (clone hVIN-1), TRITC-phallodin, TgF-β1, DNase I, and bovine serum albumin were from Sigma. Goat anti-mouse IgG2a, goat anti-mouse IgM, and goat anti-mouse IgG1 antibodies were purchased from Caltag Laboratories (Burlingame, CA). Antibodies to p38α, p38γ, p38, ERK1/2, and JNK and the respective phospho-specific antibodies for each of these kinases were purchased from Cell Signaling Technology (New England Biolabs, Mississauga, Ontario, Canada). Antibodies to p21 (clone C-18), p27 (clone KIP-1), vinculin (clone hVIN-1), TRITC-phalloidin, TGF-β1 (Santa Cruz, CA), Mouse monoclonal antibody to GAPDH (clone 6C5) was purchased from Chemicon International (Temecula, CA). Soluble type I bovine collagen was obtained from Celltrix (Palo Alto, CA). The Rho kinase inhibitor Y27632, rat polyclonal antibodies to RhoA, RhoB, and RhoC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to GAPDH (clone 6C5) was purchased from Caltag Laboratories (Burlingame, CA). The Rho kinase inhibitor Y27632, rat polyclonal antibodies to p1, α2, α3 integrins, DR1 receptor, and mouse monoclonal antibody to the α2β1 integrin (clone PIE8) were purchased from Calbiochem. The Rho attack assay kit was purchased from Cytoskeleton, Inc. (Denver, CO).

**siRNA Preparation**—We designed target-specific siRNA duplexes based on the sequence of the rat SMA gene. A 21-nucleotide SMA siRNA sequence relative to the start codon (876–896, accession number X06801) was submitted to a BLAST search against the rat genome to ensure that only the SMA gene of the rat genome was targeted. An siRNA sequence (5′-GAGCUAACAGGCACACAAGUGC-3′) for green fluorescent protein was synthesized as an irrelevant control. All siRNAs were produced by Qiagen.

**Cell Culture and Transfection for RNAs**—Rat-2 cells were incubated at 37 °C in complete Hg-DMEM containing 5% fetal bovine serum and a 1:10 dilution of an antibiotic solution (0.17% w/v penicillin V, 0.1% gentamycin sulfate, and 0.01 μg/ml amphotericin). Cells were maintained in a humidified incubator gassed with 95% O2, 5% CO2 and were passed with trypsin. One day before transfection with siRNAs, cells were trypsinized, diluted with fresh medium without antibiotics, and transferred to 6-well plates for immunoblotting and 12-well plates for immunostaining. Transient transfection of siRNAs was conducted with FuGene6 (Roche Molecular Biochemicals). Cells were assayed 48 h after transfection. Triplicate assays were conducted for a minimum of three independent experiments.

**Mechanical Force Application**—A force delivery system employing collagen-coated magnetite beads and magnetic fields was used to apply exogenous tensile forces in vitro as described previously (8). Briefly, magnetite beads (400 mg; Sigma) were incubated for 10 min with 1 ml of an acidic bovine collagen solution (95% type I collagen, 5 mg/ml) at 37 °C and neutralized to pH 7.4 with 100 μl of 1 N NaOH. Under these conditions, collagen polymerizes and forms fibrils around the beads within 30 min. The beads were sonicated to eliminate clumps and were then dispersed. Analysis of bead size was performed by electronic particle counting (Coulter Channelzer, Coulter Electronics, Hialeah, FL). Prior to incubation with cells, beads were rinsed in PBS, washed three times, resuspended in Ca++-free buffer, and added to attached cells in complete medium for 10 min. Cells were washed three times to remove unbound beads prior to exposure to force. A ceramic permanent magnet was used to apply perpendicular forces to beads attached to the dorsal surface of cells. For all experiments, the pole face was parallel with and 2 cm from the surface of the cell culture dish. As the surface area of the magnet was larger than the culture dishes, and as the bead positioning was relatively uniform for all cells, the forces applied to cells across the width of the culture dish were relatively uniform (23). Constant forces (0.65 pN/μm2 project cell area) but of varying duration were used for all experiments.

**Immunofluorescence**—After transfection (48 h), cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 0.2% bovine serum albumin, stained for SMA, and counterstained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. For double staining of SMA and actin filaments, cells were incubated with antibody to SMA, counterstained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody, stained with TRITC-phallodin, and imaged with wide field imagefluorescence microscopy.

**Immunoblotting**—Magnetite beads with the associated focal adhesion complexes were isolated in CSSB buffer, and bead-associated proteins were recovered by magnetic selection. Beads were sonicated and washed in PBS, and the associated proteins were eluted by boiling in SDS sample buffer (62.5 μg Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% w/v bromophenol blue). For cell lysates, the cells were washed with PBS and proteins solubilized in 200 μl of SDS sample buffer without bromophenol blue. Total protein was measured using the RC-DC protein assay (Bio-Rad), and equal quantities were loaded on SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. Blots were blocked for 1 h with 5% skim milk in TBS and incubated with the indicated antibody (SMA, vinculin, β-actin, GAPDH, JNK, ERK1/2, and p38) diluted 1:1000 in 0.5% Tween/TBS for 1 h at room temperature or overnight at 4 °C. Blots were washed with 0.5% Tween/TBS, incubated with appropriate secondary antibodies for 1 h, washed four times in Tween/TBS, and developed by chemiluminescence (ECL; Amersham Biosciences). The blots were exposed to Kodak X-Omat film, and band density was analyzed by IP Lab Gel Software (Signal Analytics Corporation, Santa Cruz, CA).

**G-actin and Immunodepletion**—We determined the G-actin content using the G-actin depletion method. First, because no SMA was detected in the G-actin fraction, we measured G-actin content using the procedure described by Firtel et al. (4). Briefly, the assay relies on the specific binding of the phorbolester ST25 to α-thymocyte polyamine receptors on RBCs. The amount of GT-P-Rho was quantified by immunoblotting with a Rho monoclonal antibody and by comparing the bead-bound protein (active Rho) with total Rho in the cell lysates.

**Rho Activation**—Rho activation was measured in Rat-2 cells cultured overnight without serum and subjected to force for 15 min. Control cells with beads but no force were also assayed for Rho activation. Lysophosphatidic acid treatment of these cells was performed as a positive control for Rho. Briefly, the assay relies on the specific binding of the phorbolester ST25 to α-thymocyte polyamine receptors on RBCs. The amount of GT-P-Rho was quantified by immunoblotting with a Rho monoclonal antibody and by comparing the bead-bound protein (active Rho) with total Rho in the cell lysates.

**G-actin and Immunodepletion**—We determined G-actin content utilizing the specific binding of G actin to DNase I (25, 26). Briefly, DNase I was coupled to Aminolink (Pierce) as per the manufacturer’s protocol. Cells were lysed at −10 °C in cell lysis buffer (1% Triton X-100, 10 mM Tris, 2 mM MgCl2, 0.2 mM dithiothreitol, pH 7.4) with 1 μg/ml phallodin to stabilize F-actin and sedimented at 14,000 × g for 1 min to remove cell debris, and the cell lysate was incubated with DNase beads for 2 min at 4 °C. Beads were rapidly washed three times, and bound protein (G-actin, supernatant (F-actin), and the total cell lysate (F plus G actin) were quantified by immunoblotting using monoclonal antibodies that recognize all actin isoforms, including β-actin and SMA. For estimation of the relative proportion of SMA in the total actin pool, we used an immunodepletion method. First, because no SMA was detected in the G-actin fraction by immunoblotting, we incubated the F-actin fraction overnight with SMA antibodies at 4 °C. SMA immunocomplexes were removed with ImmunoPure® Plus (G) (Pierce) using incubation for 1.5 h at 4 °C followed by centrifugation. The remaining actins in the supernatant were processed for total actin immunoblotting as described above.

**Cytoskeletal Pellets**—Pellets were prepared by lysing cells in PBS containing 1% Triton X-100, 1 μg/ml phallodin, and inhibitors. The detergent-insoluble pellet was obtained by centrifugation (20,000 × g for 30 min) and solubilized by boiling in 2% SDS sample buffer. Equivalent amounts of proteins were loaded in each lane and immunoblotted for β-actin and SMA.
SMA Determines Mechanical Force-induced p38 Activation

Transfections and Reporter Gene Assays—ROS 17/2.8 cells were grown in α-DMEM with 10% fetal calf serum. Prior to transfection, cells were plated at $1 \times 10^4$ cells/well in 6-well plates and incubated in complete α-DMEM for 24 h. Cells were transiently transfected with reporter plasmids containing p547/LacZ structures (from Dr. G. Owens) in which the whole SMA promoter is present. Cells were co-transfected with a pCMV-p38 structures. The vectors used in the co-transfection are the following, and their source is indicated in parentheses: pCMV-MKK6 (constitutively active from Dr. J. Woodgett, University of Toronto), pCMV-p38FLAG and pCMV-38WT (constitutively active; Dr. R. J. Davis, University of Massachusetts), pCMV-p38DN (dominant negative p38 from Dr. Andras Kapas). Rous sarcoma virus (RSV) expression plasmid was normalized for equal loading using the effecting reagent according to the supplier’s instructions (Qiagen). Cell extracts were prepared with a detergent lysis method. For estimates of promoter activity, β-galactosidase reporter enzyme activity was measured with an enzyme assay system (Roche Applied Science). β-Galactosidase activities were normalized to RSV luciferase activity as a transfection control. The RSV (−124 to +34) luciferase construct was provided by H. P. Elsholtz (University of Toronto) and was used as described earlier (8). RSV luciferase assays were conducted using a luciferase assay system (Promega) according to the manufacturer’s instructions. RSV luciferase activity (reflecting promoter activity) was unaffected by all treatments at all experimental times.

Statistical Analyses—For quantitative data, means and standard errors were computed. When appropriate, comparisons between groups were evaluated by Student’s t test or analysis of variance with statistical significance set at $p < 0.05$. At least three independent experiments were conducted for each condition described, and in each experiment, there were at least three replicates.

RESULTS

SMA Incorporation into Collagen Receptor Complexes—We used Rat-2 fibroblasts and ROS 17/2.8 cells as cell models to examine the role of SMA in mechanotransduction. These cells can be induced to express SMA with appropriate stimuli (27). They express abundant cell surface collagen receptors that provide attachment for collagen-coated beads and are readily transfected (2, 5, 6). We first examined the collagen receptors that were associated with collagen beads bound to Rat-2 cells. Immunoblotting of bead-bound proteins showed that the α2 integrin, α3 integrin, and the discoidin domain receptor 1 were associated with attached collagen beads (Fig. 1A), although in contrast, the α1 integrin was not expressed.

As SMA actin filaments are associated with mature focal adhesions in human fibroblasts (19), we asked if SMA was also enriched at collagen receptor adhesion complexes in Rat-2 cells and if this enrichment required the α2β1 integrin or α3β1 integrin. Incubation of cells with α3 integrin antibody did not block the association of SMA or the focal adhesion protein vinculin with collagen-coated magnetite beads (Fig. 1B). In contrast, incubation of cells with α2β1 integrin antibody blocked the association of SMA and vinculin with collagen-coated magnetite beads. By densitometry, there was a small (18%) inhibitory effect of the α2β1 integrin on the association of β-actin with collagen beads (Fig. 1C). Fluorescence microscopy showed rings of immunohistochemically stained SMA around collagen beads that co-localized with actin filaments stained by
rhodamine phalloidin (Fig. 1D). Incubation with the α2β1 integrin antibody blocked the formation of SMA-stained rings around the collagen-coated beads but did not affect staining for β-actin. Thus the incorporation of SMA into the adhesion complexes that form around collagen beads, the site of force application to cells in this model system (6), is dependent on the α2β1 integrin.

**SMA Incorporation into Actin Filaments Is Sensitive to Swinholide**—We determined whether swinholide A and latrunculin B, actin filament disrupting agents, could be used to block the association of SMA and β-actin with collagen receptor complexes. After washing away unattached beads, only cell-attached beads were used for analysis of bead-associated proteins. Pretreatment with latrunculin B (1 μM; 30 min) was not sufficient to dissociate SMA, β-actin, and vinculin from collagen beads. In contrast, swinholide A (0.1 μM; 20 min) strongly blocked the incorporation of SMA and vinculin from collagen bead complexes, while preserving the binding of β-actin (Fig. 2A). This result was not because of a lack of bead binding to cells because only proteins from bound beads were analyzed. Immunohistochemically stained SMA around collagen beads that co-localized with actin filaments stained by rhodamine phalloidin were found after latrunculin B treatment, whereas swinholide A blocked the formation of SMA-stained rings around the collagen-coated beads (Fig. 2B). Thus swinholide A but not latrunculin can be used reliably to dissociate SMA filaments from the sites of force application in Rat-2 cells.

In some well differentiated fibroblast sub-types cultured in vitro, SMA comprises up to 14% of total actin content (14). As the rate of assembly of SMA into actin filaments during cell spreading can be quite different from that of β-actin filaments (29), we considered that SMA, even as a relatively low abundance actin isoform, may have an impact on the transmission of filament-dependent mechanical signals. We estimated the relative proportions of SMA and other actin isoforms in monomeric (G-actin) form by measuring the binding of G-actin to DNase I (30) followed by actin isoform-specific immunoblotting and densitometry. In untreated Rat-2 cells, ∼20% of total actin bound to DNase I and was therefore monomeric (Fig. 2C). Treatment with swinholide A (0.1 μM; 20 min) or with latrunculin B (1 μM, 30 min) greatly increased the abundance of total actin monomers (to ∼50%) in comparison to untreated controls. In untreated samples and in samples pretreated with latrunculin B, SMA monomers were present at extremely low levels, whereas swinholide A treatment increased the amount of monomeric SMA to ∼70% (Fig. 2C).
SMA Determines Mechanical Force-induced p38 Activation

To determine the relative abundance of SMA in actin filaments, we removed G-actin with DNase I followed by SMA immunodepletion and immunoblotting for total actins. This approach showed that based on the blot densities before and after immunodepletion, there was 18.1% SMA filaments in the total actin filament pool. Collectively these results indicate that in untreated fibroblasts, SMA is almost exclusively as filaments and that SWINHOLIDE converts relatively more SMA to monomers than other actin isoforms.

**Actin Filaments and p38 Activation**—To study the impact of actin filaments on p38 activation, we first examined which p38 isoforms are expressed in Rat-2 cells. Immunoblotting showed that p38α is the predominant p38 isoform, whereas p38b was expressed at much lower levels. p38β and p38γ isoforms were not detected in Rat-2 cells (Fig. 3A). Therefore, in Rat-2 cells, force induction of p38 is largely restricted to the p38α isoform.

As the relative proportion of SMA assembled into filaments is greater than the relative proportion of other actin isoforms assembled into filaments, we asked if p38 kinase activation after force is influenced by the relative abundance of actin filaments. Previous reports (3, 6, 7, 22) have shown that MAP kinase activation following application of mechanical forces requires intact actin filaments. We employed an alternative strategy to reduce actin filament content by using short term plating of Rat-2 cells prior to examining force-induced p38 phosphorylation. When Rat-2 cells were incubated overnight on tissue culture plastic, they showed abundant SMA expression and exhibited prominent actin filaments as assessed by rhodamine phalloidin staining. In contrast, 45 min after plating, cells showed no significant staining of actin filaments (Fig. 3D). With this same approach, cells were plated for 20 min or were plated overnight, incubated with beads (10 min), and subjected to force application for 15 min (total time = 45 min). Phase contrast microscopy showed that the relative abundance of bead binding to cells after short term plating or overnight plating was equivalent (data not shown). Immunoblotting of cell lysates showed that cells plated overnight (with abundant actin filaments, Fig. 3D) exhibited greatly increased p38 phosphorylation after force application, whereas cells with minimal levels of filaments (short term plating) showed little increase of force-induced phosphorylation of p38. Collectively, these results suggest that p38 activation by applied force depends on actin filaments.

As TGF-β can increase SMA content in fibroblasts (16), we determined whether increasing SMA levels with TGF-β alters force-induced phosphorylation of p38. Immunohistochemistry showed low but detectable levels of SMA in unstimulated cells (Fig. 4A). After 48 h of incubation with TGF-β (10 ng/ml), there was greatly enhanced SMA staining (Fig. 4B). Immunoblotting
confirmed that in Rat-2 cells, SMA was increased 2-fold by TGF-β1 treatment, but there was no significant effect of TGF-β1 on phosphorylation of p38 (Fig. 4C). As TGF-β1 treatment could also alter the number of bound collagen beads available for force application, we used flow cytometry to measure binding of fluorescent collagen beads to cells. In this assay, collagen bead binding was reduced 2.1-fold by TGF-β1, indicating that TGF-β1 could not increase force-induced SMA by simply increasing the number of bound collagen beads available for force generation. Densitometry of immunoblots for p38 and phospho-p38 showed increased p38 phosphorylation after 15 min in cells pretreated with TGF-β1 compared with untreated control cells with lower levels of SMA (p < 0.05; n = 3 replicates). Data are mean density ratios ± S.E. Bottom, immunoblots of phospho-p38 and p38 in untreated Rat-2 cells and in TGF-β-induced cells after 15 min of force application. NF, no force; F, force.

**Inhibition of SMA by RNAi**—We used RNAi to reduce SMA selectively and thereby assess its role in force-induced p38 phosphorylation. We first determined whether RNAi for SMA could inhibit SMA expression in Rat-2 cells using duplexes of 21-nucleotide SMA siRNA. Immunohistochemical staining of Rat-2 cells showed abundant SMA in cells treated with the vehicle control or with an irrelevant 21-nucleotide siRNA to green fluorescent protein (Fig. 5A). Cells treated with SMA RNAi exhibited little or no detectable staining for SMA. We next induced higher levels of SMA expression by incubation with TGF-β1, and we evaluated the effect of RNAi on SMA content. There was strong staining for SMA in vehicle-treated controls and in controls treated with irrelevant RNAi (Fig. 5A). In contrast, cells treated with SMA RNAi showed only very weak staining for SMA that was comparable with the staining intensity of unstimulated Rat-2 cells that were subsequently treated with RNAi. In cultures treated with RNAi for SMA, there was no detectable effect on vimentin content, a marker of mesenchymal cells (Fig. 5B). Northern analysis of SMA and β-actin in cells previously transfected with SMA RNAi showed that the relative abundance of SMA transcripts was reduced by RNAi, but there was no effect on β-actin mRNA (Fig. 5C). Immunoblots of TGF-β-induced cells probed for SMA, β-actin, and vimentin showed that SMA RNAi selectively reduced SMA protein content (Fig. 5D). We quantified the effect of SMA RNAi in immunoblots by densitometry and normalized blot density to β-actin content. In unstimulated fibroblasts, SMA RNAi reduced SMA content by 70% compared with controls (p < 0.01). In TGF-β1-treated cells, SMA RNAi reduced SMA content by 85% (p < 0.01; Fig. 5E). Thus RNAi can selectively suppress SMA expression to low levels.

**SMA RNAi Inhibits Tension-induced p38 Phosphorylation**—We determined whether knockdown of SMA affects phosphorylation of p38 and other MAP kinases in response to applied tensile forces. Cells that were pretreated with RNAi were exposed to exogenous force application through magnetite...
beads and immunoblotted for phosphorylated and total p38, JNK, and ERK as well as SMA and β-actin. As expected, immunoblots of SMA and β-actin showed reduced SMA content by RNAi, but there was no effect on β-actin content (Fig. 6A). In cells transfected with RNAi to SMA or vehicle control, after force application (0–45 min), there were time-dependent increases of phosphorylated p38 (Fig. 6A). In control cells, p38 phosphorylation was increased to maximum levels at 20 min after force, whereas in cells treated with SMA RNAi, p38 phosphorylation was 50% weaker and peaked later (p < 0.05; 30 min to peak; Fig. 6D). In cells transfected with RNAi to SMA, force did not affect ERK phosphorylation, whereas cells treated with phorbol 12-myristate 13-acetate (as a positive control) showed abundant phospho-ERK (Fig. 6C). Similarly, force had no effect on JNK, whereas stimulation with ultraviolet light (positive control) activated JNK (Fig. 6D).

As the data above (Fig. 3) indicated that force-induced p38 phosphorylation was dependent on the presence of well developed actin filaments, we determined whether cells transfected with SMA RNAi showed marked loss of actin filaments. Simultaneous immunostaining for SMA and rhodamine phallolidin showed that whereas RNAi greatly reduced SMA, there was abundant rhodamine phallolidin staining, nearly equivalent to that of controls (Fig. 6E). Thus the reduction of force-induced p38 phosphorylation was not due to a global loss of actin filaments as shown in the experiments in Fig. 3 but was attributable to selective reduction of SMA.

Because mechanical force-induced regulation of SMA content is dependent on the basal levels of SMA in cardiac fibroblasts (22), we determined whether exogenously applied tensile forces can regulate SMA in Rat-2 fibroblasts. Cells were subjected to applied static tensile forces using collagen-coated magnetite beads. Densitometric analysis of immunoblots for SMA and β-actin showed that SMA was increased by mechanical force (Fig. 7A). Preincubation of cells with the p38 inhibitor SB203580, or with swinholide A or the Rho-associated kinase inhibitor Y27632, blocked force-induced increases of SMA. In contrast, latrunculin B exerted no significant effect. These results indicated that force-induced SMA requires p38 and Rho activation as well as SMA filaments. Furthermore, the data suggested that SMA and p38 phosphorylation may be mutually dependent. Accordingly, we considered that SMA might mediate force-induced p38 phosphorylation by providing docking sites for phospho-p38 in the mechanotransduction pathway. Rat-2 cells were incubated with collagen beads and were subjected to force. Cell extracts were immunoprecipitated with antibody to SMA. Immunoblotting of immunoprecipitates for phospho-p38 showed that force application enhanced the association of phospho-p38 with SMA but not of p38 with SMA. Furthermore, the force-induced association of SMA with phospho-p38 was insensitive to latrunculin (Fig. 7B), whereas swinholide completely disrupted binding of phospho-p38 to SMA (data not shown).

Role of Rho in Force-induced p38 Phosphorylation—The generation of contractile forces by fibroblasts is enhanced by SMA (12, 15) but is dependent also on Rho, a Ras-related GTPase that regulates actin filament assembly (32, 33). As the ROCK inhibitor Y27632 blocked force-induced increases of SMA content, we asked if force induces Rho activity. Rho activation assays showed that within 15 min after force application there was a significant increase (>50%; p < 0.01) of activated Rho compared with cells without force (Fig. 8A). As the Rho-associated kinase is an effector for Rho (34) and helps to maintain sustained contractile forces in cells (35), we examined the impact of ROCK on the assembly of SMA into actin filaments and stress fiber formation in Rat-2 cells. Rat-2 cells were incubated with Y27632 (10 μM) without serum in overnight cultures. Rhodamine phallolidin staining showed that Y27632 slightly
FIG. 6. RNAi for SMA inhibits tensile force-induced p38 phosphorylation. A, in cells transfected with vehicle RNAi, after force application for 0–45 min, there are sharp, time-dependent increases of phospho-p38 in vehicle controls (0–20 min). After RNAi for SMA, p38 phosphorylation was attenuated and slower to peak. B, line graphs of mean ± S.E. of ratios of phospho-p38/p38 blot density show that RNAi for SMA reduces p38 activation at 10 and 20 min after force application (p < 0.05; three replicates). C and D, in cells transfected with RNAi to SMA or with vehicle controls (VC), force does not affect phosphorylation of ERK or JNK in response to force. Cells treated with phorbol 12-myristate 13-acetate (PMA) or ultraviolet light (UV) are positive controls and exhibit abundant phospho-ERK and phospho-JNK. E, immunostaining of SMA and rhodamine phalloidin staining of actin filaments in RNAi-treated Rat-2 cells show no change of actin filaments despite decreased SMA staining by RNAi.

FIG. 7. A, force-induced SMA requires p38 and Rho activation and SMA filaments. Immunoblots of SMA in cells treated with tensile force (4 h) with vehicle or with p38 inhibitor SB203580 (10 μM), swinholide A (Swin A; 0.1 μM, 20 min), latrunculin B (Lat B; 1 μM, 30 min), or ROCK inhibitor Y27632 (10 μM; overnight). Histograms of densitometric scans of immunoblots for SMA and β-actin. Calculation of ratio of SMA:β-actin (n = 3 separate experiments; data are means ± S.E.). B, force promotes association of phospho-p38 with SMA. Rat-2 fibroblasts were preincubated with vehicle control or with latrunculin B (Lat B; 1 μM, 30 min) and then subjected to force or no force. Cell extracts were immunoprecipitated with antibody to SMA. The immunoprecipitated material (IP) and remaining supernatant (Sup) were separated by 10% SDS-PAGE and immunoblotted for phospho-p38 and p38. A whole cell lysate of UV-treated cells is shown as a positive control for phospho-p38.
inhibited stress fiber formation compared with untreated controls (Fig. 8B). Cytoskeletal pellets were prepared and immunoblotted for SMA. Compared with vehicle-treated controls, treatment with Y27632 reduced SMA incorporation into actin filaments by 3-fold (Fig. 8B). Furthermore, Y27632 increased the amount of SMA binding to DNase from undetectable levels in controls to readily detectable amounts. There were no significant changes in the relative amounts of β-actin in the cytoskeletal pellet or the fraction that bound to DNase (Fig. 8B), consistent with the rather small impact of this inhibitor on rhodamine phalloidin-stained filaments and stress fibers. As ROCK appeared to be important for the assembly of SMA into actin filaments by 3-fold (Fig. 8B), Y27632 increased the amount of SMA binding to DNase from undetectable levels in controls to readily detectable amounts. There were no significant changes in the relative amounts of β-actin in the cytoskeletal pellet or the fraction that bound to DNase (Fig. 8B), consistent with the rather small impact of this inhibitor on rhodamine phalloidin-stained filaments and stress fibers. As ROCK appeared to be important for the assembly of SMA into actin filaments but did not exert a strong effect on β-actin in Rat-2 cells, we asked if inhibition of ROCK would block force-induced p38 activation. Immunoblot analysis of phospho-p38 in cells preincubated with Y27632 (10 μM; overnight) prior to force application showed that inhibition of ROCK strongly blocked force-induced p38 phosphorylation (Fig. 8C).

**Activation of SMA Promoter Activity by p38**—The data described above showed that exogenously applied tensile forces increased both SMA expression and p38 phosphorylation in Rat-2 fibroblasts. Furthermore, knockdown of SMA inhibited force-induced p38 activation, whereas blocking phosphorylation of p38 inhibited force-induced increases of SMA. In view of these findings, we asked if phosphorylation of p38 alone was sufficient for enhanced SMA promoter activity. To eliminate the potentially confounding effect of p38 binding to endogenous SMA on the p38 regulation of the SMA promoter, we conducted transfection experiments using ROS 17/2.8 cells that do not express SMA in the absence of force (8). Cells were co-transfected with plasmids containing an SMA promoter (547 bp) construct fused to β-galactosidase and either pCMV-MKK6+ (constitutively active MKK6), pCMV-p38FLAG (constitutively active p38), pCMV-p38WT (constitutively active p38), or pCMVP-p38 DF (dominant negative p38) in which either MKK6 or p38 expression is under the control of a CMV promoter. Cells were also co-transfected with an RSV luciferase plasmid as an internal loading control. Co-transfection of cells with either pCMV-MKK6+ or pCMV-p38+ WT, or pCMV-p38FLAG expression vectors with SMA p547 increased β-galactosidase reporter enzyme activity. In contrast, transfection of the dominant negative pCMV-p38 slightly reduced SMA promoter activity (Fig. 9). These results indicated that activation of the SMA promoter requires p38 phosphorylation.

We next asked if force can stimulate p38 acutely with or without SMA. We used TGF-β (10 ng/ml for 48 h) to increase SMA levels and then applied force to either TGF-β-treated cells (SMA-positive) or to untreated cells (SMA-negative). Immunoblotting showed very low levels of SMA in untreated cells (Fig. 9B), and after force application (0–45 min), there were no detectable increases of phosphorylated p38. Stimulation of cells with ultraviolet light as a positive control showed phosphorylation of p38 in these cells. After 48 h of incubation with TGF-β (10 ng/ml), immunoblotting showed abundant SMA, and in
these cells, following exposure to exogenous force, there were
time-dependent increases of phosphorylated p38 (Fig. 9B). We
also used cell culture-induced tensile force to determine
whether tensile force-induced SMA could enhance phosphoryl-
ation of p38 by applied force. Cells were cultured overnight
(undetectable SMA) or for 4 days to induce SMA by cell culture-
application of p38 by applied force. Cells were cultured overnight
whether tensile force-induced SMA could enhance phosphoryl-
tion of p38 (Fig. 9B). We determined whether force-induced activation of the SMA
promoter was regulated by p38. Cells were transfected with
pCMV-MKK6+ (constitutively active MKK6), pCMV-p38FLAG (constitutively active p38), pCMV-38WT (constitutively active p38), and
pCMV-p38DN (dominant negative p38). In all constructs, p38 and MKK6 expression are under the control of a CMV promoter. RSV-luciferase
construct was co-transfected as an internal loading control. Cell extracts were prepared with a detergent lysis method. β-Galactosidase reporter
ezyme activity was measured with an enzyme assay system (Roche Applied Science) and normalized to RSV luciferase activity as a transfection
control. Data are mean ± S.E. of ratios of β-galactosidase to RSV luciferase activities (n = 3 replicates for all experiments; p < 0.01 for MKK-6,
p38 WT and p38 FLAG constructs compared with p547 or dominant negative p38). B, TGF-β-induced SMA enhances phosphorylation of p38
mediated by tensile force. ROS 17/2.8 cells were treated with vehicle control (minimal SMA) or with TGF-β (10 ng/ml; 48 h; abundant SMA). After
force application for 0–45 min, there are time-dependent increases of phospho-p38 in SMA abundant cells. Ultraviolet light (UV) was used as a
positive control for phosphorylation of p38. C, force synergizes SMA-dependent p38 phosphorylation. ROS 17/2.8 cells were cultured overnight
(undetectable SMA) or were cultured for 5 days on rigid culture dishes to induce tensile forces on cells. Tensile forces induced SMA (Force-induced
SMA). Cells were incubated with collagen beads and forces applied for the indicated times. Cells were immunoblotted for β-actin, SMA, and
phosphorylated p38 as shown. D, force-induced activation of the SMA promoter is inhibited by dominant negative p38. Cells were co-transfected
with the SMA promoter and RSV or with the SMA promoter, RSV, and pCMV-p38DN (dominant negative p38), subjected to force for 2 h, and
analyzed as described above. Dominant negative p38 blocks force-induced increases of SMA promoter activity (n = 3 replicates; p < 0.01 for cells
without dominant negative p38 transfection).

DISCUSSION

SMA is an actin isoform that is associated with increased contractile force generation during wound healing (21) and is a
phenotypic marker of myofibroblasts (36). We considered here
that SMA is also a critical element in the mechanotransduction
pathway. Our data show that SMA is a central component of a
mechanically induced, feed-forward amplification loop that syn-
ergizes force-induced SMA expression with p38 activation. Previous
work has established that p38 activation is an important
mechanotransduction process in fibroblasts (2, 5, 6, 19). Con-
versely, our findings of a specific requirement for SMA in medi-
ing p38 activation suggest that this actin isoform could partici-
pate in a feed-forward amplification loop; cells that express SMA
are more likely to activate p38 after force application, and these
cells will consequently exhibit even greater SMA expression
when stimulated by tensile forces. Thus force-induced SMA ex-
pression and p38 activation are mutually co-dependent.

SMA Incorporation into Matrix Receptor Complexes Requires
α2β1 Integrin—Integrins physically associate with cytoskeletal
proteins and may be mechanosensory (37, 38). Collagen can
bind to several integrins, one of which, the α2β1 integrin, is
required for matrix contraction (39) and for mechanotranscrip-
tional coupling of cytoskeletal proteins such as filamin A (2, 6).
Our immunohistochemical and immunoblotting data of colla-
gen bead-associated proteins showed that both SMA and the
focal adhesion protein vinculin are enriched in collagen recep-
tor adhesion complexes mediated by the α2β1 integrin. In
contrast, β-actin recruitment into collagen adhesion complexes
was not specifically mediated by the α2β1 integrin and is
instead possibly associated with other collagen receptors in-

FIG. 9. A, transcriptional activation of SMA requires constitutive activation of p38. Cells were transfected with β-galactosidase reporter
constructs driven by the SMA promoter (P547) in which the whole promoter is present. Cells were co-transfected with CMV-p38 constructs
including pCMV-MKK6+ (constitutively active MKK6), pCMV-p38FLAG (constitutively active p38), pCMV-38WT (constitutively active p38), and
pCMV-p38DN (dominant negative p38). In all constructs, p38 and MKK6 expression are under the control of a CMV promoter. RSV-luciferase
construct was co-transfected as an internal loading control. Cell extracts were prepared with a detergent lysis method. β-Galactosidase reporter
e enzyme activity was measured with an enzyme assay system (Roche Applied Science) and normalized to RSV luciferase activity as a transfection
control. Data are mean ± S.E. of ratios of β-galactosidase to RSV luciferase activities (n = 3 replicates for all experiments; p < 0.01 for MKK-6,
p38 WT and p38 FLAG constructs compared with p547 or dominant negative p38). B, TGF-β-induced SMA enhances phosphorylation of p38
mediated by tensile force. ROS 17/2.8 cells were treated with vehicle control (minimal SMA) or with TGF-β (10 ng/ml; 48 h; abundant SMA). After
force application for 0–45 min, there are time-dependent increases of phospho-p38 in SMA abundant cells. Ultraviolet light (UV) was used as a
positive control for phosphorylation of p38. C, force synergizes SMA-dependent p38 phosphorylation. ROS 17/2.8 cells were cultured overnight
(undetectable SMA) or were cultured for 5 days on rigid culture dishes to induce tensile forces on cells. Tensile forces induced SMA (Force-induced
SMA). Cells were incubated with collagen beads and forces applied for the indicated times. Cells were immunoblotted for β-actin, SMA, and
phosphorylated p38 as shown. D, force-induced activation of the SMA promoter is inhibited by dominant negative p38. Cells were co-transfected
with the SMA promoter and RSV or with the SMA promoter, RSV, and pCMV-p38DN (dominant negative p38), subjected to force for 2 h, and
analyzed as described above. Dominant negative p38 blocks force-induced increases of SMA promoter activity (n = 3 replicates; p < 0.01 for cells
without dominant negative p38 transfection).
including the α3β1 integrin or the discoidin domain receptors (40). These data indicate that the selective incorporation of SMA into α2β1 integrin collagen receptor complexes may provide mechanical linker or mechanosensory functions (20).

**SMA and Actin Assembly**—Force transmission from the extracellular matrix through focal adhesions and into the cytoskeleton is evidently enhanced by SMA filaments. SMA is an abundant protein of stress fibers (17) and focal adhesions (19). We have shown here and others have found (18) that in fibroblasts SMA is present almost exclusively as filaments. This is notably different compared with other actin isoforms, much of which is in monomeric form (our data and see Ref. 15). For study of the functional importance of actin filaments in force-mediated p38 activation, we used swinholide A, a dilactone ring macrolide that sequesters actin dimers and severs actin filaments (31). Our immunoblotting data showed that after severing actin filaments with swinholide A, the incorporation of SMA and vinculin into collagen receptor adhesion complexes was blocked. We also used latrunculin B, an actin-monomer sequestering agent that can depolymerize actin filaments (41), in an attempt to promote actin filament disassembly in collagen receptor complexes. However, possibly because of its relatively selective effects on actin filaments rapidly turning over (42), latrunculin B treatment did not dissociate SMA and vinculin in collagen receptor complexes. These adhesion complexes are evidently more resistant to latrunculin and are not subject to the depolymerization of filaments induced by swinholide. Indeed, low-dose latrunculin B treatment may selectively preserve more stable actin filaments that insert into cell attachments on the cell surface (43, 44).

SMA is required for focal adhesion maturation and for the initial formation of cortical filament bundles in spreading rat lung myofibroblasts (29). SMA comprises up to 14% total actin content (14) in well differentiated fibroblasts and up to 60–70% of total actin in smooth muscle cells (45, 46). By using G-actin binding to DNase I, we estimated the relative SMA filament content in Rat-2 cells. In untreated cells, ~80% of the total actin pool was polymerized, whereas virtually all of the SMA pool was polymerized. Swinholide A rapidly converted a much larger fraction of SMA filaments to monomers than it did for the total actin pool. These results indicate that in untreated cells SMA is almost entirely present as filaments and that proportionally more SMA is converted to monomers by swinholide A than the other actin filament isoforms.

**Actin Filaments and p38**—The MAP kinase pathway is a crucial mediator of cell survival during environmental stress. Tensile forces activate p38 and induce p38 relocation to the nucleus (5). In endothelial cells, actin stress fiber formation is linked to increased p38 activity (47). We found that in Rat-2 cells the tensile forces used here selectively activated p38 but not JNK or ERK, as has been shown earlier in cardiac fibroblasts (3). We also found that swinholide A strongly inhibited p38 phosphorylation after force application, whereas latrunculin B (which did not depolymerize SMA and β-actin in adhesion complexes) did not suppress force-induced p38 activation. The importance of actin filaments for p38 activation after force application was confirmed in experiments using recently plated cells to reduce actin filament content. These data showed that actin filaments may provide a selective link by which mechanical forces can activate specific mechanotransduction elements.

**p38 Activation and SMA**—Analysis of SMA null mice has established a central role for this actin isoform in regulating vascular contractility and blood pressure homeostasis (48), but prior to the present report, the functional importance of SMA in mediating force-induced activation of p38 has not been demonstrated. We used RNAi (49) to selectively knockdown SMA and thereby determine its functional role in force-induced p38 activation. Our results show that RNAi can selectively suppress SMA expression to low levels; the specificity of this approach was demonstrated by its lack of effect on other marker genes for fibroblasts (e.g. vimentin and β-actin).

Tension-induced activation of specific MAP kinases is dependent on basal levels of SMA (22), and activation of p38 in particular increases fibronectin polymerization in fibroblasts (19). We measured activation of MAP kinases in response to exogenous tension after treatment of cells with RNAi for SMA. Phosphorylation of p38 was abrogated by SMA RNAi. Thus, p38 kinase activation and basal levels of SMA may be functionally interdependent because we showed that tensile force-induced production of SMA requires p38 phosphorylation. We also found that RNAi for SMA had no detectable effect on the integrity of filaments containing other actin isoforms, because in cells treated with RNAi for SMA, filament formation (as judged by rhodamine phalloidin staining) was indistinguishable from controls. This finding implies that although SMA filaments do not markedly influence overall cell morphology, they contribute significantly to force sensing in the mechanotransduction system (19) that leads to phosphorylation of p38.

Previous work has demonstrated force-sensitive elements in the SMA promoter (8), but upstream signaling elements were not identified. We have shown here in transfection experiments using cells with very low levels of endogenous SMA that p38 phosphorylation is an important requirement for activation of the SMA promoter. Conversely, our data also show that SMA is important in regulating force-induced p38 activation. Accordingly, we asked if SMA filaments might contribute a scaffolding function for mediating the force response as has been shown earlier for β-actin (24). Immunoprecipitation data showed that force application increased the relative amount of phospho-p38 binding to SMA, although in contrast, binding of p38 to SMA was almost undetectable after force. These findings indicate that SMA may play a critical structural role in mediating early mechanotransduction events by facilitating appropriate localization of phosphorylated p38 in the cell (16).

**Rho- and SMA-dependent p38 Activation**—Fibroblasts respond to either exogenous mechanical loading or cell-generated tension by increased synthesis of matrix proteins and by a switch to a myofibroblastic phenotype in which cells express SMA (28, 50, 51). Our current results indicate that exogenously applied mechanical forces can increase p38 activation over a very short time scale, a feature that facilitates experimental design. With the use of this model, and with the knowledge that Rho and ROCK are involved in regulating actin assembly and cell-generated tension (34, 35), we studied the mechanism by which upstream regulators of actin assembly are involved in the force-induced p38 activation pathway. Our results showed that tensile forces significantly increase Rho activity and that inhibition of ROCK blocks SMA filament assembly. As SMA assembly into actin filaments may be important in the force-induced p38 response, our finding that the ROCK inhibitor strongly blocked force-induced p38 phosphorylation suggests that the ability of ROCK to mediate SMA assembly is a critical requirement for force-induced activation of p38.

In summary, we found that mechanical forces applied to collagen beads activate p38 through an α2β1 integrin-SMA adhesion complex. This signaling pathway is dependent on ROCK-mediated incorporation of SMA into actin filaments and is associated with the selective binding of phospho-p38 to SMA filaments. As force-induced p38 activation and force-induced SMA expression are mutually dependent, we suggest that SMA and p38 interact to synergize structural and sensory functions in the tensile force mechanotransduction circuit.
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