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Specific inhibition of skeletal α-actin gene transcription by applied mechanical forces through integrins and actin

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Skeletal α-actin (skA), a prominent fetal actin isoform that is re-expressed by adult cardiac myocytes after chronic overload *in vivo*, provides a model for studying cytoskeletal gene regulation by mechanical forces *in vitro*. We have determined the mechanisms by which perpendicular applied forces acting through integrins and the actin cytoskeleton regulate the expression of skA. Rat-2 fibroblasts were transiently transfected with plasmids containing 5′-regulatory regions of the *skA* gene fused to luciferase coding sequences. A constant, perpendicular force (0.2 $pN/\mu m^2$) was applied by using a collagen–magnetic bead model; a 25 % deformation was obtained on the dorsal cell surface. In this system, force is applied through focal adhesion integrins and strongly induces actin assembly [Glogauer, Arora, Yao, Sokholov, Ferrier and McCulloch (1997) J. Cell Sci. **110**, 11–21]. *skA* promoter activity was inhibited by 68 % in cells subjected to 4 h of applied force, whereas Rous sarcoma virus promoter activity was unaffected. In cells transiently transfected with a skA expression vector there was also a parallel 40 % decrease in skA protein levels by force, as shown by Western blotting. In L8 cells, constitutive skA expression was decreased by more than 50 %. Analyses of specific motifs in the *skA* promoter revealed that transcriptional enhancer factor 1 and Yin and Yang 1 sites, but not serum response factor and Sp1 sites, mediated inhibitory responses to force. In cells treated with cycloheximide the force-induced inhibition was abrogated, indicating a dependence on new protein synthesis. Inhibition of actin filament assembly with either cytochalasin D or Ca^{2+}-depleted medium blocked the inhibitory effect induced by the applied force, suggesting that actin filaments are required for the regulation of *skA* promoter activity. Western blot analysis showed that p38 kinase, but not Jun N-terminal kinase or extracellular signal-regulated protein kinase 1/2, was activated by force; indeed, the p38 kinase inhibitor SB203580 relieved the force-induced inhibition of skA. We conclude that the force-induced inhibition of *skA* promoter activity requires an intact actin cytoskeleton and can be mapped to two different response elements. This inhibition might be mediated through the p38 kinase.

Key words: calcium, mechanotransduction, p38 kinase, *skA* promoter.

INTRODUCTION

Cellular processes such as motility, wound contraction and phagocytosis are dependent on alterations in the organization of the actin cytoskeleton [1,2]. A wide variety of agonists, including externally applied force, can induce actin assembly and cause qualitative and quantitative changes in the distribution and proportion of actin filaments [3]. When force is applied through focal adhesions in fibroblasts, actin assembly and filament cross-linking occur preferentially at force transfer sites [4]. Although the molecular details of how physical signals influence actin assembly are not completely understood [5], it is notable that the relative proportions of actin monomer and filaments can also regulate actin gene expression [6]. Thus constitutive, cell-generated forces directed along actin filaments might be able to affect transcription. Indeed, applied physical stretching forces can regulate myosin expression in smooth-muscle cells but the regulation in an upwards or downwards direction is dependent on the myosin isoform [7].

Little is known at present about the signalling pathways that link externally applied mechanical stress and the cellular effectors that mediate the requisite nuclear events. Indeed, a wide variety of second-messenger systems, cytoskeletal elements or stress-activated kinases might mediate the effects of mechanical stress on actin gene expression [8]. For example, actin might regulate its own synthesis through an autoregulatory feedback loop that is dependent on the state of actin assembly and the level of β-actin mRNA [6]. Alternatively, the regulation of actin promoter activity by force might be mediated by intracellular mediators such as stress-activated kinases [8,9]; however, the nuclear targets of these kinases and their contribution to the up-regulation of actin gene expression have not been identified. Extracellular signal-regulated protein kinase 1/2 (ERK1/2) can translocate into the nucleus after external stimulation to phosphorylate nuclear proteins directly [10]. There is evidence that c-Jun, Elk-1 and activating transcription factor 2 are nuclear targets of Jun N-terminal kinase (JNK) [11–14] and that activating transcription factor 2 [15], Max [16] and ERK1/activating protein kinase-2 [17,18] are p38-dependent substrates. It has yet to be determined whether any of these transcription factors mediate the induction of actin gene transcription by mechanical stress.

Extensive mapping of the skeletal α-actin (skA) promoter region has revealed that within the proximal 400 bases of the *skA* promoter there are motifs for the serum response factor (SRF), which binds to a serum response element (SRE) as well as the glioblastoma–Kruppel protein Yin and Yang 1 (YY1), a basic-helix–loop–helix protein Sp1 and the simian virus 40 enhancer-binding protein transcriptional enhancer factor 1 (TEF-1) [19]. *skA* gene transcription is augmented by different members of the transcription factor family AP-1, which consists of Fos and Jun

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, Jun N-terminal kinase; RSV, Rous sarcoma virus; RSV/L, RSV/Luciferase construct; skA, skeletal α-actin; skA/L, skeletal α-actin–luciferase construct; SRE, serum response element; SRF, serum response factor; TEF-1, transcriptional enhancer factor 1; YY1, Yin and Yang 1.

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proteins, despite the lack of AP-1-binding sites within the *skA* promoter region [20]. The SRE site might be required for AP-1 factors to activate *skA* promoter activity but the interaction was evidently indirect because these factors did not bind to the SRE site or the SRF itself. None of the transcription-factor-binding elements within the *skA* promoter are known targets for JNK or p38 kinase, which can be regulated by shear force and hyper-osmolar conditions in other model systems [21,22].

Increased *skA* gene expression can be induced by longitudinal mechanical stretching of cardiac myocytes *in vitro* [23] but it is unclear whether this is a response to a passive stretching of the whole cell that is independent of actin filaments or to an integrin-dependent stretch that is mediated through actin filaments. As load-induced alterations of actin genes in cardiac cells are thought to be mediated by actin-dependent transduction mechanisms [8] that rely on the attachment of myocytes to a collagenous matrix, we wished to assess the role of actin-mediated stretch on *skA* transcription. In view of this objective we examined the *skA* promoter to determine its responsiveness to perpendicular forces applied through actin in Rat-2 fibroblast cells. These cells were used because, with a collagen–magnetic bead system, forces of defined amplitude and direction can be applied through an $\alpha_2\beta_1$-integrin-dependent mechanism to the associated actin cytoskeleton [4]. Further, the amplitude and direction of the forces generated in this model closely mimic those encountered by force-loaded fibroblasts *in vivo* [4]. Finally, unlike cardiac myocytes, which can be difficult to transfect and can contain highly variable amounts of endogenous skA, Rat-2 cells are easily transfected and do not express skA. These features of Rat-2 cells facilitate transcriptional analyses, particularly when transfection approaches are used. The application of this model has permitted the study of *skA* gene transcription in response to quantified linear forces as well as the role of the stress-activated kinase family, which can mediate transcriptional regulation.

MATERIALS AND METHODS

Reagents

Cytochalasin D was purchased from Calbiochem (San Diego, CA, U.S.A.). Cycloheximide and tetramethyl rhodamine isothiocyanate (TRITC)–phalloidin were purchased from Sigma (St. Louis, MO, U.S.A.). Anti-p38, anti-ERK1/2 and anti-JNK as well as the phospho-specific antibodies against each of these kinases were purchased from New England BioLabs (Beverly, MA, U.S.A.). Anti-vinculin and anti-skA were obtained from Sigma. Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Research Diagnostics (Flanders, NJ, U.S.A.). Anti-annexin antibodies were from the Hybridoma Bank (Iowa City, IA, U.S.A.).

Plasmids

The chicken *skA* promoter (-394 to $+24$)–luciferase construct (skA–L) as well as the linker-scanning mutations of this promoter were generously provided by M. D. Schneider [20]. The linker-scanning mutants used contained mutations in the SRF-, YY1-, TEF-1- and Sp1-binding sites. All of the promoter–luciferase plasmid DNA species for transfections were purified with Maxi-prep columns from Qiagen (Chatsworth, CA, U.S.A.). The Rous sarcoma virus (-124 to $+34$)–luciferase construct (RSV/L) was provided by H. P. Elsholtz and has been described [24].

Cell culture and transfections

Subconfluent (60 % on 35 mm dishes) Rat-2 fibroblasts, a kidney-derived cell line (A.T.C.C. CRL 1764), were transiently transfected by using the transfection reagent Lipofectamine (Gibco–BRL, Grand Island, NY, U.S.A.) in accordance with the manufacturer's recommendations. In some experiments, L8 cells (A.T.C.C. CRL 1769) were grown to confluence and then plated at low serum concentration (1 %, v/v) to promote the expression of the endogenous *skA* gene. After 16 h of incubation, the medium was replaced with serum-free medium. Collagen- or BSA-coated ferric oxide microparticles (henceforth referred to as beads; Aldrich Chemical, Milwaukee, WI, U.S.A.) [25] were added to the transfected cells for 15 min at 37 °C. Excess beads were removed by three washes with PBS; the cells were then resuspended in serum-free medium. A ceramic magnet (Jobmaster, Mississauga, ON, Canada) was used to generate a perpendicular force of 0.2 pN/μm^2 when placed 2 cm from the cell culture dish surface [4]. As assessed by optical sectioning with confocal microscopy, this force application caused an approx. 25 % deformation of the dorsal cell surface in a vertical direction for both collagen and BSA-coated beads. After a 4 h incubation at 37 °C, the magnet was removed and cells were washed with PBS. Harvest buffer [50 mM Tris/Mes (pH 7.8)/1 mM dithiothreitol/0.1 % (v/v) Triton X-100 (Sigma)] (0.25 ml) was added to each plate, cells were scraped and collected in 1.5 ml Eppendorf tubes. The tubes were vortex-mixed briefly and centrifuged at 8000 ***g*** for 3 min. The supernatants (200 μl) were placed in 12 mm × 75 mm tubes, mixed with 10 mM $MgCl_2$/6 mM ATP and assayed for luciferase activity with a Berthold Lumat LB 9501 luminometer. The supernatant was immediately mixed in the measuring chamber after the addition of luciferin (ICN, Costa Mesa, CA, U.S.A.) (0.14 mg/ml luciferin in 5 mM potassium phosphate, pH 7.5; 30 μl per tube). Protein content was determined for each transfection with the use of the Bradford assay, to normalize the activity of each promoter. In some experiments, transfection efficiency was estimated by transfection with a β-galactosidase-expression vector followed by histochemical staining for β-galactosidase. The transfection efficiency ranged from 4 to 9 % depending on the experiment. Western blotting of Rat-2 cell lysates showed no detectable native skA expression, so the experiments were conducted in a null background.

Western blot analysis

In brief, cell lysates were prepared from subconfluent Rat-2 fibroblast cells (60-mm-diameter dishes) that were either untreated or subjected to an applied force for various times. Cells were rinsed with PBS, lysed by the addition of 200 μl of SDS sample buffer [62.5 mM Tris/HCl (pH 6.8)/2 % (v/v) SDS/10 % (v/v) glycerol/50 mM dithiothreitol/0.1 % Bromophenol Blue] and transferred to a Microfuge tube. The samples were kept on ice, sonicated for 10 s and boiled for 5 min; aliquots (12 μl) were then separated by SDS/PAGE [10 % (w/v) gel] and transferred to nitrocellulose. The blots were incubated for 1 h in blocking solution [5 % (w/v) skimmed milk/0.1 % (v/v) Tween 20/PBS] and incubated overnight at 4 °C with the indicated antibodies. The membranes were washed in 0.1 % (v/v) Tween 20/PBS for 30 min and incubated in blocking solution with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blots were washed in 0.1 % (v/v) Tween 20/PBS for 15 min and developed by chemiluminescence with LumiGLO (New England BioLabs, Beverly, MA, U.S.A.).

Statistical analysis

Except where noted, all individual experiments contained three to five replicates and each experiment was performed at least twice. Data for stretch effects were calculated on the basis of

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percentage luciferase activity as a function of either unstretched controls or with and without drug treatment. Data are displayed as means ± S.D.

RESULTS AND DISCUSSION

Applied mechanical force through actin filaments specifically inhibits *skA* promoter activity

To determine whether forces perpendicular to the dorsal cell surface delivered through the actin cytoskeleton can regulate *sKA* gene transcription, transient transfection studies were performed in Rat-2 fibroblasts with the use of skA/L, a construct containing 394 bp of the 5′-flanking sequence of the *skA* gene linked to the promoterless luciferase coding region [20]. A perpendicular applied force of 0.2 pN/μm^2 delivered through collagen-coated magnetic beads inhibited skA/L promoter activity maximally at 4 h by 68 % (Figure 1A; mean baseline luminometer units 7132 ± 550; $n = 5$). This force level and the direction of force were chosen on the basis of preliminary experiments showing that the largest inhibitory effect of applied force on skA protein levels was optimized under these conditions. This same protocol has also been shown to promote local actin assembly maximally [4]. The force-induced regulation of promoter activity was specific in that no inhibition of activity was observed with another promoter–luciferase construct (RSV/L) under the same conditions (baseline 15175 ± 1554; $n = 5$). Further, the force-induced variation of luciferase activity was apparently not due to variation in transfection efficiencies because separate experiments with concurrent transfections with a β-galactosidase-expressing vector as well as adjustment for protein loading did not account for the consistent inhibition that was seen ($n = 10$ separate experiments, each with three to five replicates; transfection efficiency 7 %). Although maximal inhibition was observed at 4 h, luciferase levels increased over time so that by 24 h basal levels had been restored (Figure 1A). The re-establishment of basal levels might be indicative of an adaptive mechanism in which compensatory signalling pathways are activated to allow *skA* gene transcription to return to normal levels. The inhibition of skA/L activity by a perpendicular stretching force through the actin cytoskeleton in Rat-2 cells is opposite to the results obtained in a different model in which stretching of rat cardiac myocytes in a direction parallel to the substrate stimulated *skA* promoter activity [23]. These differences could be due to differential regulation of signalling pathways between Rat-2 fibroblasts and cardiac myocytes in response to

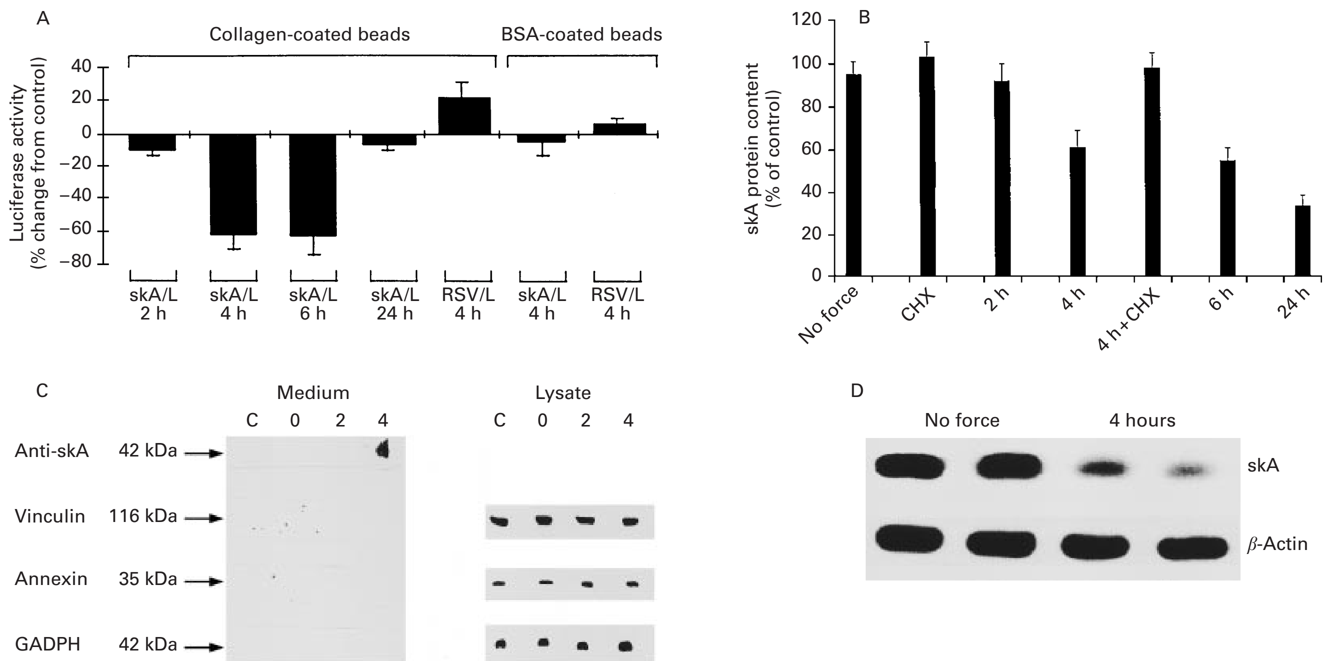

Figure 1 Analysis of the contribution of mechanical force to skA reporter activity

(**A**) skA/L and RSV/L constructs were transiently transfected into Rat-2 fibroblasts. At 24 h after transfection, collagen-coated or BSA-coated magnetic beads were added to each culture as indicated, subjected to force (0.2 pN/μm^2) for 2, 4, 6 and 24 h as indicated, then harvested for luciferase assays. The luciferase levels of stretched and unstretched cultures were measured in a luminometer. The results are relative luminometer readings of stretched samples compared with control samples and are expressed as a percentage change from unstretched controls. Equality of protein loading was verified by Bradford assays. Similarity of transfection efficiencies was assessed by concurrent transfections with a β-galactosidase expression vector and histochemical staining. Transfection efficiencies ranged between 5% and 9%. Collagen- and BSA-coated beads caused similar levels of vertical deformation of cells (approx. 25%). Force caused a signficant inhibition of *skA* promoter activity at 4 and 6 h ($P < 0.01$). Results are means ± S.D. (**B**) Lysates from Rat-2 cells were transiently transfected with a skA expression vector, incubated with collagen-coated beads and treated without force application (no force), with no force and cycloheximide (CHX), or with force for the indicated durations and cycloheximide, as shown. Cell lysates were analysed by Western blotting with anti-skA antibodies. The blot was reprobed with a β-actin antibody to determine equivalent amounts of protein loading. Densitometry was performed and results are shown as the blot densities of experimental groups as a percentage of control cells without collagen beads. Applied force caused an inhibition of skA protein content at 4, 6 and 24 h after the application of force ($P < 0.05$; results are means ± S.D.; $n = 3$). (**C**) Mechanical force induces the leakage of skA into cell culture medium but no detectable leakage of vinculin, annexin or GAPDH from cells. Results for untreated cells (C) or cells, loaded with beads, that were either unstretched (0) or stretched for 2 or 4 h (2, 4) are shown. Cell culture medium and lysates were immunoblotted for vinculin, annexin and GAPDH, whereas immonoblotting for anti-skA was performed only on the medium. (**D**) Mechanical force decreases the skA content of L8 muscle cells. L8 muscle cells were grown to confluence, changed to 1 % (v/v) serum for the induction of skA expression, incubated with collagen beads and exposed to 4 h of force or not (no force). Duplicate cell lysates were immunoblotted for skA content, showing a marked (more than 50%) inhibition of skA content in L8 cells after stretching.

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the direction and the site of the applied stretch (i.e. perpendicular stretching through bead-engaged integrins or whole-membrane stretching parallel to the substrate). We also note that non-muscle and muscle myosin heavy chains are differentially regulated in rat smooth-muscle cells after stretch [7], indicating that closely related genes can exhibit very different force effects.

To determine whether the force-mediated inhibition of *skA* promoter activity corresponded to an inhibition of skA protein levels, extracts of Rat-2 fibroblasts transiently transfected with a skA expression vector were analysed by Western blotting (Clone 5C5; Sigma). Applied force for 2, 4, 6 and 24 h showed significant decreases in skA protein by 4 h compared with control extracts (Figure 1B; 40 % decrease; $P < 0.05$) that were preserved over 24 h if force was applied continuously. Inhibition of protein synthesis by cycloheximide (20 μg/ml) abolished the force-induced decreases in skA protein levels. We considered that the actin turnover rate might be rapid; however, in previous reports [25,26], radiolabelling of cells with [^{35}S]methionine followed by fluorography of immunoprecipitates showed that the loss of radiolabelled (i.e. nascent) α-actins was virtually undetectable over 4 h in the absence of force. Further, in unstretched cells treated with cycloheximide, skA protein content remain unchanged. These results point to a very slow turnover rate of skA in these cells.

If the turnover rate of skA was slow, as suggested by the results of treatment with cycloheximide, we considered that the force-induced loss of skA was because of leakage from cells as a result of the stretching. This was confirmed by the immunoblotting of 10-fold concentrated cell medium by Centricon filters. After stretching there were very large increases in skA (from undetectable at 0 and 2 h to the presence of a well-defined band at 42 kDa at 4 h). However, there was no detectable vinculin, annexin or GAPDH in the medium and no loss of these proteins from the cell lysates (Figure 1C). Because there was no inhibition of skA protein levels after cycloheximide treatment with or without force application, we suggest that the force-mediated effects on skA protein were dependent on the net loss of protein as a result of physical stretching.

In view of these results, we examined the validity of the Rat-2 model by examining the effects of force on skA protein in L8 muscle cells, which constitutively express skA when they are grown to confluence and then incubated in a low-serum medium. Compared with β-actin, the skA content was decreased by more than 50 % after 4 h of force application (Figure 1D); again, skA was detected in concentrated cell-culture medium.

Previous work has shown that the application of force through collagen-coated beads [25] induces the formation of actin filaments at focal adhesions and that there is selective enrichment of actin, vinculin, talin and the α_2 subunit of the integrin receptor in bead-associated proteins [4]. These results are consistent with the binding of collagen-coated cells at focal adhesions. Actin filaments insert into focal adhesions and bind specifically to the α_2 integrin subunit [28], indicating that the force effects are mediated through focal-adhesion-associated actin filaments. To determine whether the force-induced inhibition of skA was mediated specifically through an integrin-dependent mechanism, transfected cells were loaded with BSA-coated beads and assayed for luciferase activity. This protocol assesses the effect of forces applied through non-specific, low-affinity binding sites and stretches the cell membrane through integrin-independent adhesions [4]. No force-induced effect was observed in cells subjected to force application through BSA-coated beads (Figure 1A), indicating that the regulation of *skA* promoter activity by physical forces is mediated at focal adhesion sites specifically through integrin collagen receptors. Separate experiments established that this lack of effect was not simply due to pulling beads off the cells because abundant numbers of BSA-coated beads remained on the cells after the application of the magnetic force. Further, as assessed by confocal microscopy, both collagen and BSA-coated beads caused an approx. 25 % upward movement of the dorsal surface of the cells, indicating that both types of bead caused similar levels of deformation.

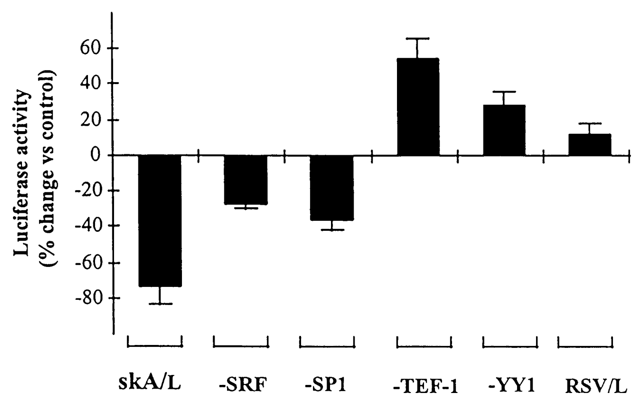

Figure 2 Inhibition of the *skA* promoter by force requires TEF-1 and YY1

Rat-2 cells were transfected with 2 μg of each of the luciferase reporter genes as indicated. These linker-scanning mutations of the *skA* promoter impair the binding of SRR, SP1, TEF-1 and YY1 respectively. For each reporter plasmid with a linker-scanning mutation, luciferase activity (mean $\pm$ S.D.) is shown as a percentage change relative to the same deletion mutant but without stretch ($n = 5$ for each condition tested). The RSV promoter was used as a control. TEF-1 and YY1 caused significant ($P < 0.01$) increases in luciferase activity, whereas SRF and SP1 caused signficant decreases ($P < 0.01$).

Force-mediated inhibition of *skA* gene expression is mediated through specific DNA-binding motifs

To distinguish between the contributions of various transcription factors that bind to the *skA* promoter, linker-scanning mutations of the promoter that impair SRF, Sp1, TEF-1 and YY1 binding [19] were transfected into Rat-2 cells. The effects of these mutants on the total transcriptional activity of the *skA* promoter before stretching was evaluated by comparing baseline luciferase activities. All mutants caused decreased transcriptional activities of the *skA* promoter (SRF, 76 % decrease; SP1, 66 % decrease; TEF-1, 59 % decrease; YY1, 28 % decrease). In a typical example of a transfection experiment, the baseline luminometer readings in unstretched cells were: skA, 2638 ± 559; SRF, 633 ± 52; SP1, 894 ± 571; TEF-1, 1069 ± 1035; YY1, 1892 ± 257 ($n = 5$ replicates). To estimate the importance of the individual mutations on the force-induced decrease in *skA* promoter activity, the luciferase activities of the deletion mutants exposed to force were compared with the activities of the same mutants that were not subjected to force. Luciferase constructs with mutations that abolished SRF and Sp1 binding partly blocked (up to 60 %) the ability of the applied force to inhibit *skA* promoter activity (Figure 2). Disruption of either TEF-1- or YY1-binding elements completely abolished the inhibitory effect of force on *skA* promoter activity and indeed led to increased activity. Cell counts were performed to determine that equal number of cells were plated per sample. Force-induced inhibition was not due to a loss of cells through cell death or cell detachment because neither cell viability, as determined by staining with Trypan Blue ([4], and results not shown), nor total protein levels were affected by the treatment. We also observed that the degree of force-induced inhibition was less in the SRF and SP1 mutants than in

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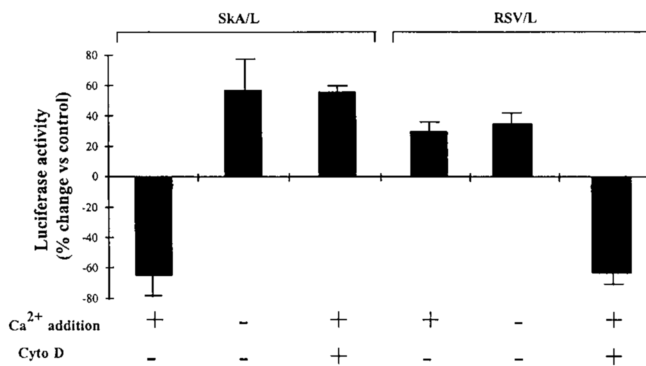

Figure 3 Filamentous actin and $[Ca^{2+}]_i$ are important mediators of the force-induced inhibition of *skA* promoter activity

Cells were depleted of Ca^{2+} with EGTA in the bathing buffer (1 mM) or treated with cytochalasin D (Cyto D; 500 ng/ml for 15 min and 1 ng/ml for 4 h) and subjected to an applied force for 4 h. Cell extracts were harvested for luciferase assays. Luciferase activities (means ± S.D.) are expressed as a percentage change relative to that of cells not subjected to force. Ca^{2+} depletion or cytochalasin both caused significant increases in *skA* promoter activity compared with baseline values ($P < 0.05$; $n = 5$). Cytochalasin also signficantly inhibited RSV promoter activity ($P < 0.05$; $n = 5$). In a separate experiment to examine the effect of Ca^{2+} depletion on unstretched cells, EGTA did not alter baseline *skA* promoter activity ($n = 5$).

the cells transfected with full-length constructs; however, the degree of relief was much less than that in the TEF-1 and the YY1 mutants.

These results indicate that the co-ordinated action of two transcription factors, TEF-1 and YY1, mediate the force-induced inhibition of *skA* promoter activity and that SRF and SP1 seem to be less important. It was unexpected that TEF-1 would be a negative regulator of this gene, as it has been shown to be a positive mediator of *skA* transcriptional activity by transforming growth factor β [20]. It is possible that TEF-1 might mediate opposing effects on the same promoter with the recruitment of different cofactors, selected in response to stimulatory or inhibitory signals. Although evidence for this reversible role for TEF-1 has yet to be determined, it has been suggested that the activation of the *skA* promoter by SRF is in concert with TEF-1 [19,29]. The identification of an inhibitory cofactor that is regulated by force would be of great interest. Alternatively, there might be specific isoforms of TEF-1 that contribute to the preferential regulation of *skA* promoter activity. Unlike TEF-1, the transcription factor YY1 has been proposed as a negative regulator of *skA* transcription [30]. Our results substantiate this role and suggest an additional role as a nuclear mediator for applied forces. Interestingly the binding site for YY1 overlaps another site, SRE, and in HeLa cells the predominant binding activity to the *skA* promoter was YY1, whereas the SRF binding detected was slight [19]. It is therefore possible that the inhibitory action of both TEF-1 and YY1 in mediating the force effect on *skA* promoter activity might be due to increased levels of the negative factor YY1 as well as a smaller amount of SRF protein to bind to TEF-1 to supplement its role as a positive regulator.

Ca^{2+}-dependent regulation of *skA* promoter activity

Ca^{2+} mediates the regulation of several actin-binding proteins such as the actin-severing protein gelsolin [31]. Applied forces acting through integrins sharply elevate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human fibroblasts [25]; depletion of Ca^{2+} in the bathing buffer leads to actin disassembly [3,4]. Because the force-induced inhibition of *skA* promoter activity described above was observed in Ca^{2+} buffers, we assessed whether the force-induced elevations of $[Ca^{2+}]_i$ levels might be required for the inhibition of *skA* promoter activity. In contrast with the inhibition observed in the presence of Ca^{2+}-containing buffer, the transcriptional activity of the *skA* promoter in response to force was increased when elevations of $[Ca^{2+}]_i$ were blocked by 1 mM EGTA (Figure 3; baseline luminometer units 2891 ± 352; $n = 5$). Notably, by blocking the force-induced elevations of $[Ca^{2+}]_i$ or by eliminating the contribution of either TEF-1 and YY1, there was a stimulation in *skA* promoter activity. Controls that were not subjected to force and incubated in EGTA-containing buffer showed no significant change in luciferase activity compared with cells in normal Ca^{2+}-containing buffer. These results indicated that both factors might require Ca^{2+}-dependent mechanisms to regulate *skA* transcription. Preventing elevations of $[Ca^{2+}]_i$ might alter the binding of either transcription factor to its respective binding element or might inhibit the binding of any co-regulator. Notably, because specific mechanotransduction events involved in actin assembly require cytoplasmic Ca^{2+} [3,4], a cytoskeletal component regulated by intracellular Ca^{2+} levels might be required for the regulation of *skA* gene transcription by mechanical forces.

Depolymerization of actin filaments blocks the force-induced inhibition of *skA* promoter activity

The intracellular levels of polymerized actin might regulate actin synthesis by a polymerization-dependent feedback loop [6]. To determine whether the level of actin filaments regulates *skA* gene expression, transfected Rat-2 cells were treated with cytochalasin D to cause the disruption of microfilament organization and thereby increase the pool of actin monomer. This protocol decreases the content of filamentous actin to low levels [3]. Cytochalasin D treatment not only blocked the ability of the force to inhibit *skA* promoter activity but caused a stimulation of transcription as well. This stimulatory effect was in marked contrast with the RSV promoter because cytochalasin D inhibited RSV promoter activity in the presence of Ca^{2+} (Figure 3). We also observed that the inhibition of new protein synthesis blocked the inhibition of *skA* promoter activity by applied forces (Figure 1A). However, as the half-life of actin is quite long [25,26], it is doubtful that newly synthesized actin mediates the force-induced inhibition of *skA* promoter activity, although the experiments with cytochalasin D indicate a requirement for actin filaments.

A physical connection might exist between integrins, cytoskeletal filaments and the nuclear scaffold [32], thereby providing a mechanism by which nuclear events are mediated by applied mechanical forces. Our results support this view: we found that mechanical force regulates gene transcription through actin filaments. The cytochalasin-induced disassembly of actin filaments might in effect break these molecular 'strings', thereby blocking the signals to the nucleus that are required for actin gene regulation.

p38 kinase activity is activated by applied force

To investigate the possible role of some of the intracellular mediators in force-induced inhibition of the *skA* promoter, cell lysates from Rat-2 cells were analysed by Western blotting with phospho-independent and phospho-specific antibodies against ERK1/2, JNK and p38 kinase. The phospho-specific antibodies recognize only the phosphorylated forms of their respective kinase and therefore detect the activated form of that kinase. With the use of phospho-independent ERK1/2 and JNK antibodies, it was evident that these kinases were expressed in Rat-

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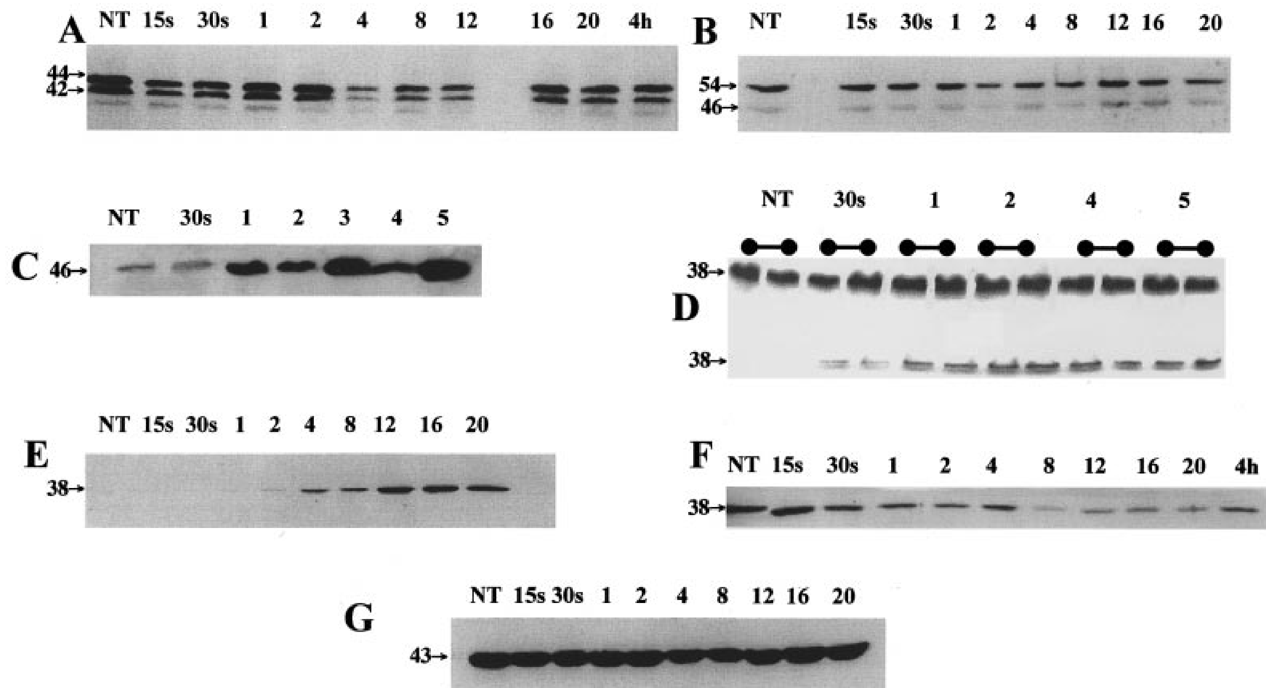

Figure 4 p38 kinase regulates skA reporter activity by mechanical force

Cell lysates prepared from Rat-2 cells exposed to mechanical force at various times were used to determine the activation of ERK1/2, JNK or p38 kinase (NT, no treatment; s, seconds; 1–20 are minutes). (**A**, **B**) Western blots probed with phospho-independent antibodies against ERK1/2 (**A**) and JNK (**B**). (**C**) Cells were exposed to UV for various durations as indicated; lysates were analysed by Western blot analysis with JNK phospho-specific antibodies. (**D**) Cells were exposed to UV for the indicated durations. Lysates were immunoblotted with a p38 phospho-independent antibody (top blot), to determine the total amount of p38, or with phospho-specific antibody (bottom blot), to estimate phosphorylated p38. (**E**, **F**) Phospho-specific p38 and total p38 blots of cells after mechanical force application for indicated times: phospho-p38 antibody (**E**); phospho-independent antibody (**F**) to quantify the phosphorylation of p38. To determine whether equal amounts of protein were loaded, blots were reprobed with β-actin antibodies (**G**). Western blots representative of at least three independent studies are shown, all of which showed late, force-induced variations in total p38 and ERK1/2 immunoreactive protein compared with internal β-actin density.

2 cells (Figures 4A and 4B); however, no signal was detected with the phospho-specific ERK1/2 or JNK antibodies (results not shown), suggesting that neither kinase is stimulated by applied force in Rat-2 cells. Because stresses such as UV and hyperosmolarity have been shown to regulate JNK but not ERK1/2 [12,22,33,34], any effect by applied forces might be mediated by JNK but might not be observed because of an inability to regulate this kinase in Rat-2 cells. To demonstrate that JNK could be activated, lysates from cells subjected to UV irradiation were analysed with the phospho-specific JNK antibody. Under these conditions UV did induce phosphorylation of JNK (Figure 4C). Similarly, the p38 kinase was also activated by UV (Figure 4D). In contrast with JNK and ERK1/2, the p38 kinase was phosphorylated within 2 min of force application and was still active after 20 min (Figure 4E). Reprobing the blot with p38 phospho-independent antibodies, which recognize the kinase independently of its phosphorylation state, showed that total levels of p38 did vary over the time course (Figure 4F) but equal protein loading was confirmed by reprobing these same Western blots with a β-actin antibody (Figure 4G). In all three experiments that were conducted, time-dependent variations in total p38 kinase levels were noted, in spite of the equality of β-actin levels.

To determine whether p38 mediated the force-induced inhibitory effect on *skA* promoter activity, a specific p38 inhibitor was tested in our transfection assays with the *skA* promoter construct. The p38 inhibitor SB203580 selectively inhibits p38 kinase activity but does not alter the activity of either ERK1/2 or JNK [35]. We found that SB203580 (10 μM) did not alter *skA* promoter activity, independently of its effect on force (i.e. luciferase activity was not different in unstimulated cells treated with or without the inhibitor) but the inhibitor completely blocked the force-induced inhibition of *skA* promoter activity at concentrations that prevented the activation of p38 kinase activity (Figure 5, upper panel). Therefore we surmise that the activation of p38 kinase regulates the inhibition of *skA* promoter activity by applied forces. The discrepancy between the time of maximal activation and the time at which transcriptional inhibition is observed (4 h) is consistent with the possibility that another inhibitory protein is synthesized that mediates the inhibition. Presumably, the time required for the synthesis of this protein accounts for the delay between the time of maximal p38 activation and the inhibition of the skA. We assessed this possibility by exposing cells transfected with skA/L to stimuli that are known to activate the p38 kinase. Cells were transfected with skA/L, exposed to either UV (as described for Figure 4) or with 10 μg/ml anisomycin; luciferase assays then were conducted at 0, 2 or 4 h after stimulation. Significant decreases in luciferase activity ($P < 0.05$) were not detected until 4 h after stimulation with UV (Figure 5, lower panel); similarly with anisomycin, a period of 4 h was required before luciferase activity compared with controls was decreased by 50 %. These results indicate that agents that induce p38 activity also repress *skA* promoter activity, suggesting that p38 is a key molecule in the signalling pathway required for inhibition of skA.

We proposed above that *skA* promoter regulation by applied physical force is dependent on the nature of the force. Per-

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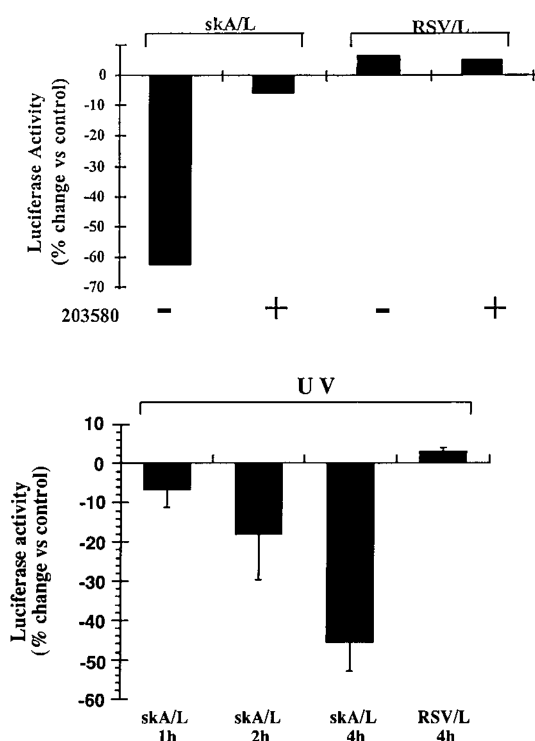

Figure 5 p38 kinase and *skA* promoter activity

Upper panel: p38 kinase inhibitor abrogates the effect of force on *skA* promoter activity. skA/L and RSV/L constructs were transiently transfected in the presence or absence of SB203580 (a p38 kinase inhibitor); the difference in luciferase activity due to force application is plotted as a percentage of the control. SB203580 did not alter *skA* promoter activity in unstretched cells compared with cells treated with vehicle only. Untreated control cells and SB203580-treated cells (4 h) were harvested for luciferase activity. The experiment is representative of two. Lower panel: UV causes a slow decrease in *skA* promoter activity. Cells were transfected with either skA/L or RSV/L and subjected to UV as described in the legend to Figure 4. Significant decreases ($P < 0.05$) in *skA* promoter activity were detected only by 4 h after UV stimulation. No apparent change in RSV activity was detected after 4 h.

pendicular forces applied through the actin cytoskeleton evidently have different effects from forces applied to integrin-independent sites (i.e. via BSA-coated beads) or in forces that passively stretch the cell in a plane parallel to the substrate [9]. Therefore inhibition of the *skA* promoter might be determined by the regulation of specific signalling pathways that rely on the engagement of the actin cytoskeleton and force-induced actin assembly. Forces applied parallel to myocyte cell membranes activate ERK1/2 and JNK [9]. We have shown that forces applied perpendicular to the cell membrane through the cytoskeleton activate only the p38 kinase. To elucidate the upstream regulatory mechanisms in these processes would be of particular interest, to identify the intracellular targets as well as to determine whether TEF-1 and YY1 are nuclear targets for p38 kinase. The stress-activated kinase, p38, might be a potential component of the mechanotransduction system in which external forces are translated into nuclear signals that regulate *skA* promoter activity. Although the conversion of external stresses into biochemical signalling pathways might be similar to those found in other cell types, there is evidently considerable heterogeneity between cell types in response to stretch. Therefore mechanoreceptors are probably cell-type-specific.

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