Force activates smooth muscle α-actin promoter activity through the Rho signaling pathway

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Summary

In pressure or volume overload, hypertrophic growth of the myocardium is associated with myofibroblast differentiation, a process in which cardiac fibroblasts express smooth muscle α-actin (SMA). The signaling mechanisms that mediate force-induced myofibroblast differentiation and SMA expression are not defined. We examined the role of the Rho–Rho-kinase pathway in force-induced SMA expression in fibroblasts using an in vitro model system that applies static tensile forces (0.65 pN/μm^2) to integrins via collagen-coated magnetite beads. Force maximally induced RhoA activation at 10 minutes that was localized to force application sites and required intact actin filaments. Force application induced phosphorylation of LIM kinase (5-10 minutes) and an early dephosphorylation of cofilin (5 minutes) that was followed by prolonged cofilin phosphorylation. These responses were blocked by Y27632, an inhibitor of Rho kinase. Force promoted actin filament assembly at force application sites (10-20 minutes), a process that required Rho kinase and cofilin. Force application induced nuclear translocation of the transcriptional co-activator MRTF-A but not MRTF-B. Nuclear translocation of MRTF-A required Rho kinase and intact actin filaments. Force caused 3.5-fold increases of SMA promoter activity that were completely blocked by transfection of cells with dominant-negative MRTF-A or by inhibition of Rho kinase or by actin filament disassembly. These data indicate that mechanical forces mediate actin assembly through the Rho–Rho-kinase–LIMKcofilin pathway. Force-mediated actin filament assembly promotes nuclear translocation of MRTF and subsequent activation of the SMA promoter to enhance SMA expression.

Key words: Mechanotransduction, MRTF, Integrins, Force, Promoter

Introduction

Excessive hemodynamic loading leads to the development of cardiac hypertrophy, a process characterized by enlargement of cardiomyocytes, activation and proliferation of cardiac fibroblasts, differentiation of fibroblasts into myofibroblasts and excessive deposition of extracellular matrix (Eghbali, 1992; MacKenna et al., 2000; Nicoletti and Michel, 1999). The conversion of cardiac fibroblasts into myofibroblasts is of crucial importance in hypertrophic processes (Eghbali, 1992) because of their central role in the synthesis and secretion of extracellular matrix proteins that stiffens the myocardium (Chien, 1999).

Myofibroblast differentiation is marked by strong expression of smooth muscle α-actin (SMA) (Darby et al., 1990; Leslie et al., 1991; Sun and Weber, 1996) and depends on the concerted action of cytokines (e.g. TGFβ1), matrix proteins (e.g. the splice variant ED-A fibronectin) and mechanical tension. A two-stage model of myofibroblast differentiation proposes that mechanical tension and TGF-β1 are required for the sequential expression of ED-A fibronectin and SMA (Tomasek et al., 2002). For myofibroblasts, mechanical force is essential for maintenance of myofibroblast contractility (Grinnell and Ho, 2002; Hinz et al., 2001) and is a prerequisite for the expression of SMA (Tomasek et al., 2002).

Force-dependent processes involve signaling through Rho, a small GTPase that directs the assembly and stabilization of the actin cytoskeleton (Nobes and Hall, 1995). In vitro studies have shown that exogenous forces can regulate Rho activity in different cell types, thereby enhancing the ability of RhoA to increase cell contractility (Smith et al., 2003; Wojciak-Stothard and Ridley, 2003). Downstream of Rho is the Rho-kinase pathway which, through phosphorylation of LIM kinase and cofilin, can lead to increased actin assembly. Cofilin is an abundant and widely expressed actin-binding protein (Lappalainen and Drubin, 1997; Theriot, 1997). When cofilin is phosphorylated (e.g. by LIM kinase), the actin-binding activity of cofilin is diminished, actin filaments are stabilized and total cellular actin filament content is increased (Bamburg et al., 1999). ROCK-induced phosphorylation of LIM kinase can thus regulate actin dynamics through phosphorylation of cofilin (Arber et al., 1998).

The relative concentration of actin filaments and actin monomers can control serum response factor (SRF) activity (Miralles et al., 2003), an important determinant of force-induced SMA expression (Wang et al., 2002). Myocardin-related transcription factor (MRTF, also termed MAL, BSAC or MKL) is a broadly expressed, actin monomer-associated SRF co-activator which relocates from the cytoplasm to the nucleus in response to Rho-induced actin assembly (Miralles et al., 2003). The N-terminal sequence of MRTF contains two

RPEL motifs that are required for signaling and that associate with actin monomers (Miralles et al., 2003). Importantly, the relative abundance of actin monomers and filaments in a cell can impact the ability of MRTF to relocate to the nucleus and there regulate transcription.

We considered that force may be able to promote MRTF nuclear localization and, as a result, increase the expression of genes that depend on SRF-mediated promoter activity, specifically SMA. Accordingly, we examined whether tensileforces activate the Rho→Rho-kinase→LIM-kinase→cofilin→actin filament assembly pathway and whether this in turn leads to MRTF-dependent co-activation of the SMA promoter.

Results

Mechanical force activates RhoA

In cells subjected to tensile force the proportion of active RhoA to total Rho was increased by 50% above no-force controls at 5 minutes and by 100% above no-force controls by 10 minutes ($P<0.01$; Fig. 1). RhoA activity returned to control levels by 15 minutes. When cells were incubated with BSA-coated beads or collagen beads co-incubated with 4B4 (β1-integrin-blocking antibody) and then subjected to tensile force, there was no change of RhoA activity. The absence of force-induced RhoA activation by BSA-coated beads was not attributable to reduced numbers of bound beads because visual inspection of bead density on the cells showed no obvious reduction compared with collagen beads with or without force application for these time intervals (i.e. 30 minutes, but see below for longer time periods). Treatment of cells with latrunculin B blocked force-induced RhoA activation in cells that were incubated with collagen-coated beads, indicating that force requires intact actin filaments to promote RhoA activation. Cells subjected to force showed no detectable Rac or Cdc42 activity (Fig. 1), suggesting that force selectively activates Rho in this system.

To assess the sites of RhoA activation induced by mechanical loading, an in situ rhotekin-binding assay was used. Cells were incubated with collagen beads, subjected to tensile forces for 10 minutes (the optimal time for RhoA activation described above), permeabilized, fixed and analyzed. Active RhoA induced by force application was detected around collagen-coated magnetite beads. In controls with attached beads but no force application, there was no enhanced staining. In cells subjected to force but incubated with GST alone, there was no detectable staining above background (Fig. 1). In cells examined at 15 minutes after force application, very little staining was detected around beads (data not shown), consistent with Rho activation data obtained from cell lysates. These data indicated that tensile force transiently activates RhoA at sites of force application applied through integrins.

Mechanical loading activates LIMK and cofilin

Cells were incubated with collagen-coated beads, subjected to tensile force (5-60 minutes) and equal amounts of cell lysates were immunoblotted for LIMK1, phosphorylated LIMK1, cofilin, and phosphorylated cofilin. Force enhanced phosphorylation of LIMK within 5 minutes by ~30% ($P<0.05$) an effect that largely dissipated by 15 minutes (Fig. 2). As a positive control, lysophosphaditic acid increased LIMK phosphorylation fivefold within 5 minutes. Force caused an initial decrease of cofilin phosphorylation at 5 minutes followed by subsequent increases at 10-30 minutes (twofold

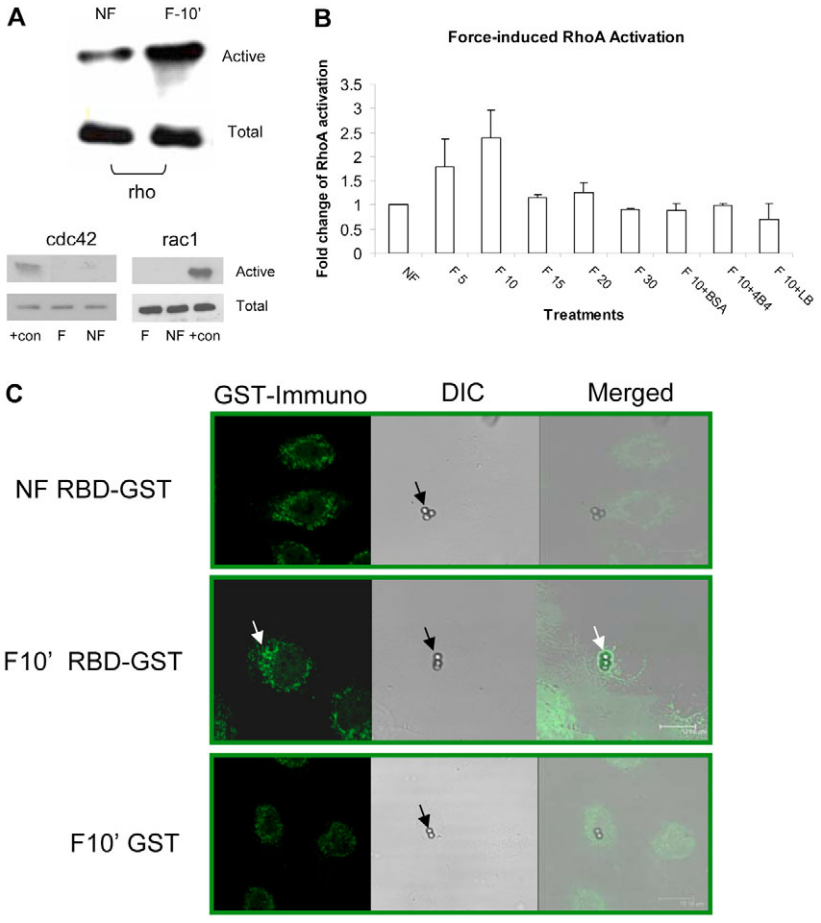

Fig. 1. Mechanical force increases RhoA activation. (A) Rat-2 cells were stretched with collagen or BSA-coated magnetite beads. RhoA activation was measured by rhotekin binding and total RhoA was measured by immunoblotting cell lysates. Rac and Cdc42 activity were measured as described in the Materials and Methods. NF, no force; +con, positive control with 5% serum addition; F and F-10', applied force for 10 minutes. (B) The ratio of active to total Rho was computed from densitometry measurements of immunoblots and are given as the mean ± s.d. compared with the no force ratio (designated as 1). NF, no force; F5, F10, F15, F20 and F30, applied force for 5, 10, 1 and 20 minutes, respectively; F10+BSA, applied force for10 minutes with BSA-coated beads; F10+4B4, applied force for 10 minutes in the presence of 4B4 antibody (1 μg/ml); F10+LB, applied force for 10 minutes after pre-treatment with latrunculin B (1 μM, 30 minutes before treatment). (C) Collagen-coated beads incubated with cells and treated with and without force (10 minutes). Cells were probed with rhotekin-binding domain (RBD)-GST and immunostained with anti-GST antibody.

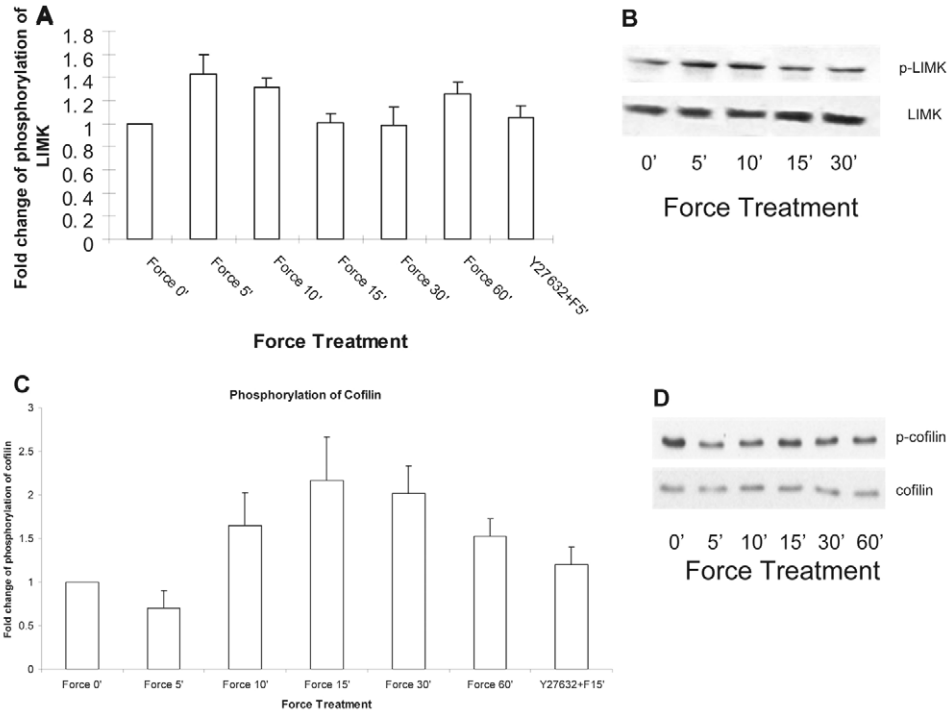

Fig. 2. Force enhances phosphorylation of LIMK1 and cofilin. (A,B) Immunoblots of LIMK1 and phosphorylated LIMK (p-LIMK) in lysates of Rat-2 cells. The ratio of phosphorylated to total LIMK was computed from densitometry measurements of immunoblots. The data are mean ± s.d. compared with the no-force ratio (designated as 1). For cells treated or not with Y27632, the difference between force and no force at 5 minutes (A) or 15 minutes (B) is statistically significant (P<0.05). (C,D) Immunoblots of cofilin and phosphorylated cofilin (p-cofilin) in Rat-2 cells as measured for LIMK1.

at 15 minutes; P<0.01); by 60 minutes cofilin phosphorylation returned to 1.1-fold of initial levels. As a control, lysophosphatidic acid promoted a sevenfold increase of cofilin phosphorylation 15 minutes after incubation. Pre-incubation of the cells with the ROCK inhibitor Y27632 (10 μM; 30 minutes prior to treatment) blocked force-induced phosphorylation of both LIMK1 (at 5 minutes after force) and cofilin (at 15 minutes after force, Fig. 2A,C).

Force effect on phosphorylation of myosin light chain

Enhanced phosphorylation of myosin light chain increases actomyosin contractility, stress fiber formation and the recruitment of integrins into focal adhesion structures (Chrzanowska-Wodnicka and Burridge, 1996). We determined whether tensile force can increase phosphorylation of the myosin light chain. Force mediated a small but significant (P<0.05) reduction in phosphorylation of myosin light chain that was maximal at 15 minutes, indicating that this pathway may not be directly involved in stretch-induced actin assembly. These data, taken together with the observed rapid reduction of active RhoA after force application, suggests that proteins regulating myosin light chain phosphorylation may, in some way, be induced by force. Indeed, the scaffold protein $p116^{Rip}$ associates with the actomyosin cytoskeleton where it recruits and activates the MLC phosphatase complex, enhances the catalytic activity of myosin-light-chain phosphatase and downregulates RhoA-GTP levels through its Rho GAP activity (Clark et al., 2007). Conceivably, $p116^{Rip}$ might be regulated by force application to cells after which it subsequently facilitates downregulation of myosin-light-chain phosphorylation and reduction of GTP-loaded RhoA. As a positive control for phosphorylation of myosin light chain, cells were treated with the Ca^{2+} ionophore ionomycin (2 μM), and this treatment strongly enhanced phosphorylation of myosin light chain (sixfold at 15-30 minutes; Fig. 3).

Force-mediated actin assembly requires ROCK and cofilin

Application of tensile forces through collagen-coated magnetite beads causes localized increases of actin filaments around the beads (Glogauer et al., 1997), the sites of force application used here. Similarly, in cells attached to flexible membranes and subjected to whole-cell stretch, actin assembly is observed in subcortical regions (Pender and McCulloch, 1991). We confirmed these results (Fig. 4) and determined

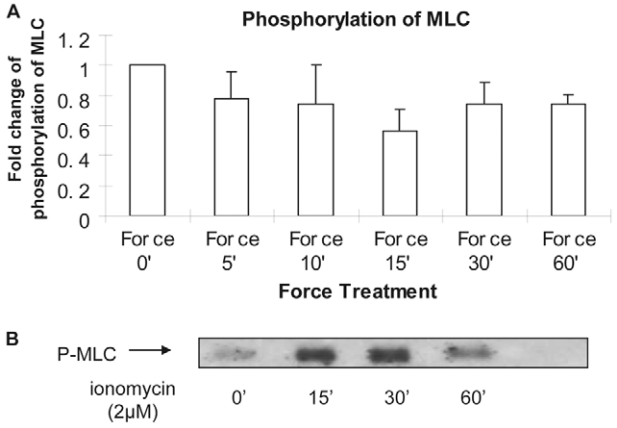

Fig. 3. Phosphorylation of myosin light chain (MLC) after mechanical loading. (A) Immunoblots of phosphorylated MLC were prepared from Rat-2 cells treated with force. The ratio of phosphorylated to total MLC was computed from densitometry measurements of immunoblots. Data are given mean ± s.d. compared with the no-force ratio (designated as 1). (B) Positive control for phosphorylation of MLC. Cells were treated with ionomycin (2 μM) for 0, 15, 30 or 60 minutes (0′, 15′, 30′ or 60′, respectively). Cell lysates were immunoblotted for phosphorylated MLC (P-MLC).

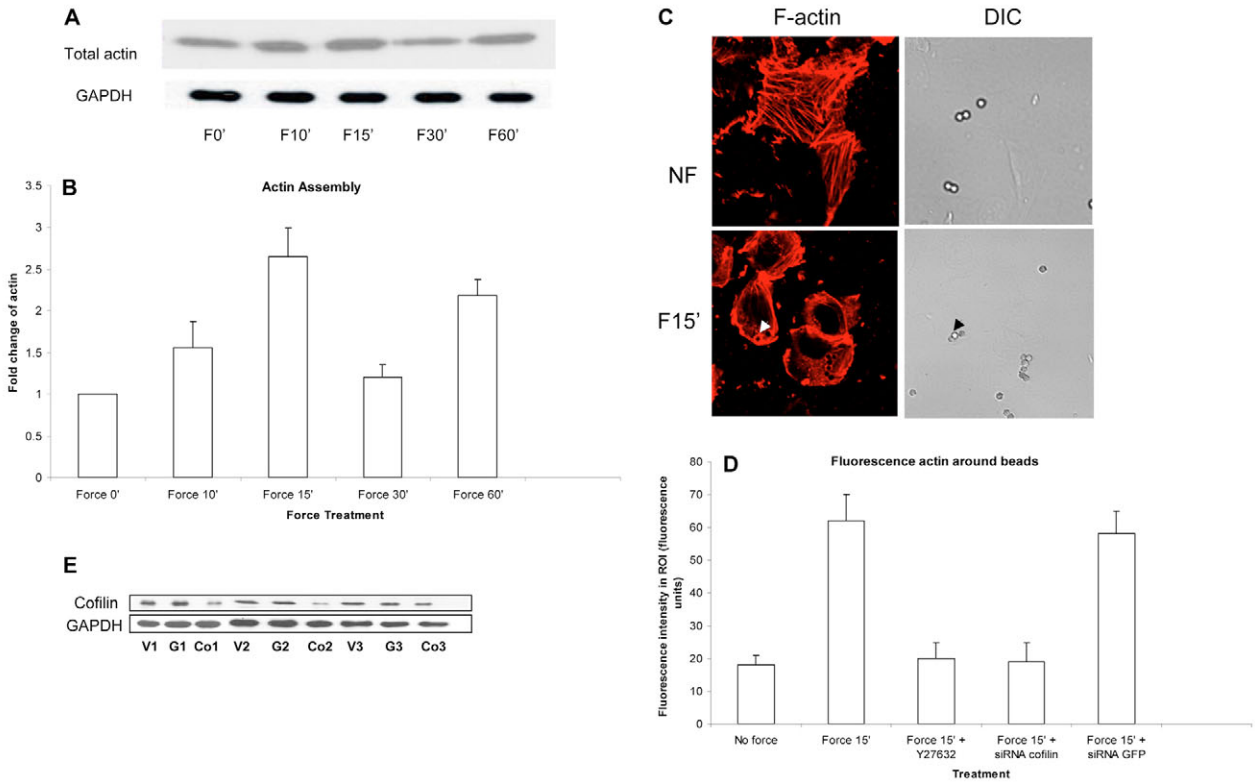

Fig. 4. Force increases actin in collagen-bead-associated attachment complexes. (A) Bead-associated proteins from Rat-2 cells treated with force. Cells were incubated with collagen-coated beads. Cells were lysed in cytoskeletal stabilizing buffer containing phalloidin (1 μM). Soluble material was immunoblotted for GAPDH. Beads were removed magnetically and counted. Proteins from equal numbers of beads were immunoblotted for β-actin. (B) The relative change of bead-associated actin was computed from densitometry measurements of immunoblots. Data are given as the mean ± s.d. compared with the no-force ratio (designated as 1). Force increased the amount of actin filaments 2.5-fold above controls at 15 minutes (P<0.01). (C) Rhodamine-phalloidin staining of cells not subjected to force (NF) or subjected to force for 15 minutes (F15′). Notice the staining around beads in force-treated sample. (D) Fluorescence intensity around collagen beads was quantified in 4-μm circular regions of interest around beads with a CCD camera. The results are given as the mean ± s.d. fluorescence units and are derived from the counts of beads in 100 cells for each treatment in each experiment. (E) Cells were transiently transfected with vehicle (V), siRNA targeting cofilin (Co) or siRNA targeting GFP (G) for 1, 2 or 3 days. Immunoblots show that siRNA targeting cofilin suppresses cofilin protein by >80% after 2 days transfection.

whether force-induced actin assembly is regulated by the ROCK-LIMK-cofilin pathway. Isolation of proteins from collagen-coated beads demonstrated an ~2.5-fold increase of bead-associated actin within 15 minutes of force application followed by a decrease to control levels at 30 minutes. Consistent with an earlier report (Pender and McCulloch, 1991), stretch caused a second increase of actin assembly at 60 minutes. Rhodamine-phalloidin staining showed that actin assembly was localized to force application sites. Quantification of fluorescence intensity attributable to Rhodamine-phalloidin staining immediately around beads showed a more than fivefold increase (P<0.01) compared with controls. Measurement of fluorescence attributable to Alexa Fluor-488 DNase staining of actin monomers in 4-μm zones around beads was performed. There was reduced staining (50%; P<0.01) at 15 and 60 minutes compared with baseline (fluorescence intensity per area of measurement; 0.21±0.04 for baseline; 0.12±0.02 for 15 minutes; 0.14±0.03 for 60 minutes).

In cells pre-incubated with Y27632, there was no detectable actin assembly around the collagen-coated beads after 15 minutes of stretch. We also assessed whether cofilin was involved in the stretch-induced actin-assembly pathway. Cells were transiently transfected with small interfering RNA (siRNA) targeting cofilin or an irrelevant siRNA (targeting GFP) and immunoblotted to assess the effectiveness of the knockdown. Compared with controls, knockdown of cofilin reduced cofilin protein content approximately threefold (2 days of siRNA treatment; P<0.01) compared with control (siRNA targeting GFP). In cells treated with siRNA cofilin for 2 days, incubated with collagen-beads and subjected to force, knockdown of cofilin blocked the force-mediated actin assembly around force application sites (Fig. 4).

MRTF A but not MRTF B localizes to the nucleus after force application

When localized to nuclei, MRTF is believed to function as a co-activator of SRF (Pipes et al., 2006) and can thereby mediate serum response element (SRE)-regulated gene expression, for example, SMA. In cells immunostained for endogenous MRTF A, MRTF A translocated from the cytoplasm to the nucleus after force application (Fig. 5). Force-induced MRTF A nuclear translocation was blocked by

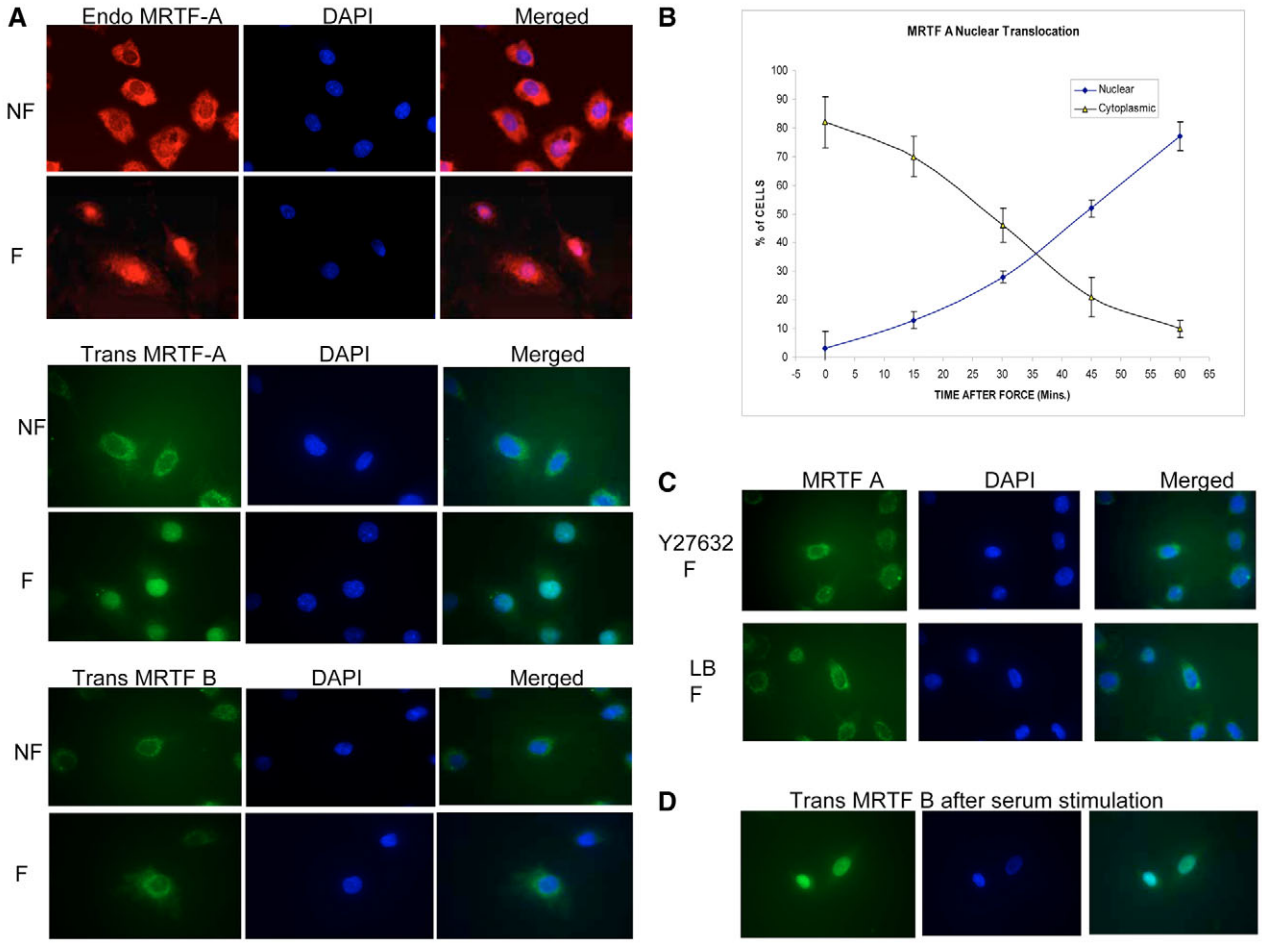

Fig. 5. (A-D) MRTF translocation to the nucleus after force application. Representative images show NF (no force) and F (after 60 minutes of force). (Left panels, top rows) Endogenous MRTF A (red) in serum-reduced cells without force application was predominantly cytoplasmic. After force application endogenous MRTF A relocated to the nucleus. (Left panels, middle rows) Rat-2 cells transfected with MRTF-A–FLAG-tagged constructs for 20 hours, subjected to force and immunostained for FLAG. MRTF A (green) in serum-reduced cells without force application or with force application was predominantly nuclear. (Bottom left panels) Rat-2 cells transfected with MRTF-B–FLAG-tagged constructs for 20 hours. Nuclear translocation was never observed between 0 and 60 minutes. Cells were stained with DAPI for nuclear localization. (B) Percentage of cells in which transfected MRTF A was scored as predominantly cytoplasmic or predominantly nuclear after indicated times of force application. Results are given as the mean ± s.d. (C) Rat-2 cells transfected with MRTF A-FLAG (green). Blocking of ROCK with Y27632 (Y27632 F) and of actin filament assembly with latrunculin B (LB F) interfered with the nuclear translocation of MRTF A after force application. (D) Rat-2 cells transiently transfected with MRTF B-FLAG (green) were stimulated with serum for 30 minutes and show nuclear translocation of MRTF B.

Y27632 and by treatment of cells with latrunculin B (Fig. 5; Table 1). In cells transfected with either MRTF A or MRTF B and immunostained for the FLAG marker in these constructs, force induced nuclear translocation in MRTF A transfected cells but not MRTF B-transfectants (Fig. 5). Serum stimulation was used as a positive control and MRTF B-transfected cells exhibited nuclear translocation after 30 mins of serum stimulation.

Rho kinase, actin assembly and MRTF regulate force-induced SMA promoter activity

SMA promoter activity is increased by tensile forces in osteoblastic cells (Wang et al., 2002). In the fibroblasts used here, application of force caused a 3.5-fold increase of SMA promoter activity up to 3 hours (P<0.01), which was completely blocked by transfection of cells with dominant-negative MRTF A, by inhibition of Rho kinase with Y27632 or by actin filament disassembly with latrunculin B (Fig. 6A). FLAG immunostaining of cells transfected with dominant-negative MRTF A showed significant expression of MRTF A (Fig. 6B). As a control for the dominant-negative MRTF A, we co-transfected cells with the SMA promoter and an irrelevant plasmid (a Rous sarcoma virus construct) and found after 2 hours of force application that there was similar fold-induction of the SMA promoter (3.4±0.3) and in cells not transfected with the irrelevant plasmid (3.5±0.4; P>0.5).

As there was a significant (P<0.05) reduction of luciferase protein between 180 and 240 minutes after continuous force application that was unlikely to be caused by luciferase turnover or degradation, we examined cell loss from the plates after force exposure for this time interval. We found by phase-contrast microscopy a ~40% reduction of cell numbers at 240

Table 1. Nuclear translocation of endogenous MRTF A after force application

Time (minutes)	Untreated	Cells exhibiting nuclear staining for endogenous MRTF A (%)	
		Latrunculin B (1 μM, 30 minutes before force treatment)	Y27632 (10 μM, 30 minutes before force treatment)
0	6±2	5±2	4±2
15	12±3	6±2	7±3
30	27±5	9±3	10±4
45	48±6	11±4	12±4

Cells were incubated with collagen beads and force was applied for 0, 15, 30 or 45 minutes. Cells were fixed and stained for endogenous MRTF A using a polyclonal antibody generously donated by H. Nakano (Juntendo University School of Medicine, Tokyo, Japan). Percentage of cells that exhibited nuclear staining for endogenous MRTF A was evaluated by fluorescence microscopy. A significantly higher percentage of MRTF-A-stained nuclei was found 15, 30 or 45 minutes after force application in untreated (control cells) compared with those treated with latrunculin B or Y27632 ($P<0.05$).

minutes compared with 180 minutes, presumably owing to the cells being detached from the substratum by the magnetically induced force and also by force-induced cell death, as we have reported earlier with this system (Kainulainen et al., 2002).

Our previous data (Fig. 3) showed that force did not increase myosin-light-chain phosphorylation. Accordingly, we asked whether actomyosin contractility is required for stretch-dependent induction of the gene encoding SMA. Cells were treated with blebbistatin (Straight et al., 2003) (100 μM for 1 hour prior to force application for 90 minutes) and SMA promoter activity was measured with the luciferase assay. We found an equivalent increase of luciferase in force-treated cells with or without blebbistatin (Fig. 6A; $P>0.5$), indicating that the actomyosin system is not required for force-induced SMA induction.

Discussion

The expression of SMA is an important marker for the differentiation of myofibroblasts (Serini and Gabbiani, 1999) but there is no defined mechanism establishing the link between mechanical loading and SMA expression. We demonstrate here a pathway that links mechanical force, the RhoA–Rho-kinase–LIMK–cofilin pathway, actin-filament assembly and nuclear translocation of MRTF A, subsequently activates the SMA promoter and thereby enhances SMA expression. These data provide evidence for a mechanical-force-induced transcriptional response that may contribute to the development of cardiac hypertrophy.

Force-induced RhoA activation

RhoA plays a crucial role in the transduction of mechanical signals into cellular responses that increase contractility of airway smooth muscle cells (Smith et al., 2003). We showed that in serum-reduced fibroblasts, RhoA activity was selectively activated within 10 minutes after force application, after which it diminished to baseline. By contrast, RhoA, Rac1 and Cdc42 are all activated during the early stages of endothelial actin cytoskeletal remodelling induced by shear stress (Wojciak-Stothard and Ridley, 2003). Similarly, we found that activation of RhoA was very transient and returned to control levels by 10 minutes. Further, force-induced RhoA activation required integrins and intact actin filaments. These data suggest the existence of a positive feedback loop involving an interaction of active RhoA and ROCK that leads to actin polymerization.

With an in situ rhotekin-binding assay we determined the localization of active RhoA. These data showed that force-induced RhoA activation at 10 minutes was localized to sites of force application and this localization required actin assembly. Other studies have reported that Rho and Cdc42 translocate to the plasma membrane in response to shear stress, and mediate the activation of the transcription factor AP1 through Jun N-terminal kinases (Li et al., 1999). Whereas we have not assessed whether mechanical force stimulates the redistribution of RhoA into the cytoskeleton, previous studies were not able to demonstrate cytoskeletal translocation of Rho GTPases in response to chemical stimulation, e.g. smooth muscle cells responding to phenylephrine (Gong et al., 1997) and in response to shear stress, e.g. endothelial cells (Li et al., 1999).

Force-induced actin assembly via the RhoA–Rho-kinase–LIMK–cofilin pathway

As previous studies have shown that mechanical loading induces actin polymerization at force-transfer sites (Glogauer et al., 1997; Pender and McCulloch, 1991), we asked whether force mediates actin assembly via a RhoA-dependent pathway. Cofilin is an actin-binding protein that, when dephosphorylated, can generate free actin barbed ends, thereby facilitating the growth of actin filaments (Lappalainen and Drubin, 1997; Theriot, 1997). Conversely, Rho-induced cofilin phosphorylation abolishes the actin-binding activity of cofilin, thereby stabilizing existing actin filaments (Bamburg et al., 1999). As phosphorylation of cofilin can be mediated by LIM kinase, which, in turn, requires phosphorylation of LIM kinase (Arber et al., 1998), we immunoblotted cells that had been subjected to force application. These results demonstrated that externally applied force promoted phosphorylation of LIM kinase (within 5-10 minutes). There was an early dephosphorylation of cofilin (possibly to mediate generation of free actin barbed ends) followed by phosphorylation of cofilin (5-30 minutes). Force-induced phosphorylation of cofilin was blocked by the Rho kinase inhibitor Y27632. As Rho kinase is an effector of Rho (Ishizaki et al., 1997), inhibition of force-induced LIMK and cofilin by Y27632 indicates that the activity of phosphorylation depends on RhoA activation, which was promoted by mechanical loading. Since shear stress can induce sequential signaling through the Rho-Rock-LIMK-cofilin pathway in endothelial cells (Lin et al., 2003), our data are consistent with and extend these observations to tensile force-induced regulation of the gene encoding SMA.

We determined the relative abundance of actin filaments at force application sites by isolating bead-associated proteins and

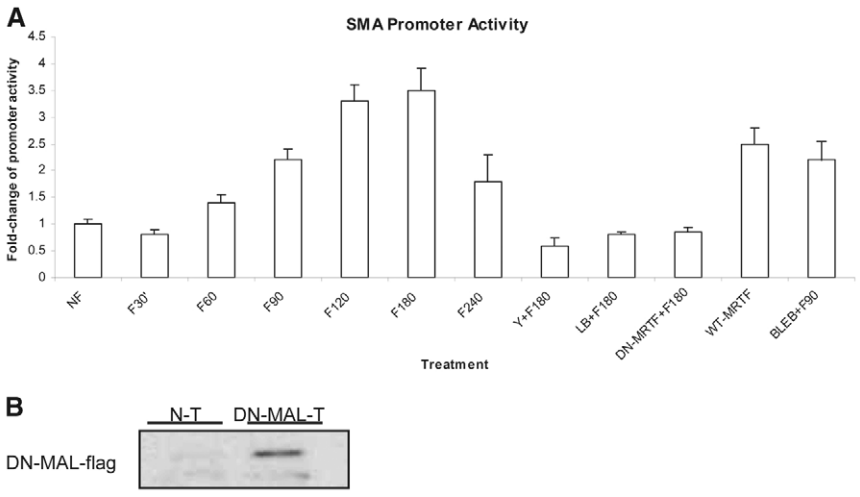

Fig. 6. Force-induced SMA promoter activity. (A) Rat-2 cells were co-transfected with a construct containing a SMA-promoter–luciferase construct and a β-gal vector as a loading control. Cells were stimulated with force over a time course of 30 minutes to 4 hours. In some experiments, inhibitors were included as indicated. Promoter activities are shown as fold change compared with basal promoter activity and are adjusted for loading based on the β-gal-control loading vector. The results are given as the mean ± s.d. and are derived from four replicates. For experiments with wild-type MRTF A, cells were transfected with the SMA-promoter–luciferase construct and then, 5 hours prior to luciferase activity assay, with the constitutively active MRTF A plasmid. (B) Immunoblots for FLAG show expression of dominant-negative FLAG-tagged MRTF A in cells co-transfected with SMA promoter constructs and dominant-negative MRTF A-FLAG.

measuring actin by immunoblotting. These results demonstrated that force induced 2.5-fold increases of actin filaments at force application sites, which was confirmed by Rhodamine-phalloidin staining for actin filaments. Blocking of Rho kinase with Y27632 or knockdown of cofilin inhibited the actin assembly driven by mechanical force, indicating that – at least in part – mechanical force induces actin assembly through the RhoA-ROCK-LIMK-cofilin pathway. Notably, actin assembly can be regulated by other pathways, including the diaphanous-related formin mDia1. In the context of regulation of the promoter activity of the gene encoding SMA (see below), mDia1 can control SRF activity through its effects on actin polymerization, possibly through interactions with profilin (Copeland and Treisman, 2002; Geneste et al., 2002). Further, a previous report that examined the impact of tensile-force application on generation of focal contacts showed that external force can bypass the requirement of ROCK activity for focal adhesion formation as long as there is active mDia (Riveline et al., 2001). Thus, under certain circumstances when external forces are applied, the segment of the Rho-dependent pathway involving activation of ROCK to enhance myosin-light-chain phosphorylation, can apparently be bypassed in the formation of focal complexes. Taken together these results may indicate that exogenous force can promote Rho activation and that active Rho induces actin polymerization through two effector pathways: the ROCK-LIMK-cofilin pathway stabilizes actin filaments, thereby inducing SRF-mediated promoter activity, whereas the mDia1 pathway promotes actin assembly and generates tensile force to enhance focal contact assembly. These two pathways are probably not independent and may positively regulate each other. Whichever pathway is most important, as a result of force application there was a net increase in the abundance of actin filaments.

Role of MRTF in regulating SMA expression in response to force

Application of exogenous force can increase SMA expression in fibroblasts through activation of the promoter of the gene encoding SMA (Wang et al., 2002). The regulation of SMA expression is dependent on CArG elements in the SMA promoter (Mack and Owens, 1999; Wang et al., 2002). The CArG element is a core sequence of the SRE and plays a pivotal role in SMA transcription (Mack and Owens, 1999). SRF is one of the factors that can activate SRE-mediated promoter activity. The transcriptional co-activator MRTF regulates SRF binding to SRE-binding sites; MRTF is also an actin-monomer-binding protein that translocates from the cytoplasm to the nucleus as a result of its dissociation from actin monomers (Miralles et al., 2003). Because external-force-induced actin assembly promotes accumulation of actin filaments subjacent to the plasma membrane and a decrease of actin monomers in the cytoplasm, we considered that force might induce MRTF to translocate to the nucleus. Indeed, immunostaining showed that, upon force application, MRTF A but not MRTF B translocated from the cytoplasm to the nucleus. This translocation was blocked by Y27632 and latrunculin B, indicating that the force-induced nuclear translocation of MRTF A required Rho-actin signaling. MRTF B is a human MRTF-A-related gene. MRTF A and MRTF B exhibit redundant functions in response to serum and RhoA induction of reporter genes (Cen et al., 2003). However, in force-induced actin assembly leading to regulation of genes regulated by SRF, MRTF A and MRTF B evidently exhibit distinctly different regulatory roles.

In migrating *Drosophila* cells, tension at the edge of the migrating cell sheet, which is associated with increased actin assembly, promotes nuclear translocation of MRTF. Along with SRF, MRTF is thought to be required for cell migration in *Drosophila* cells (Somogyi and Rorth, 2004). In migrating *Drosophila* cells and in mammalian fibroblasts subjected to tensile forces, MRTF exhibits similar changes in sub-cellular localization. These findings indicate that MRTF-associated signaling pathways are evolutionarily conserved and may play a crucial role in force-induced SMA expression and possibly, cardiac hypertrophy.

Consistent with earlier data (Wang et al., 2002), we found that force enhanced the activity of the promoter activity of the gene encoding SMA. The pathway leading to this activation involved ROCK, intact actin filaments and MRTF A, because inhibitors of these processes completely blocked force-induced SMA promoter activity. Previous data indicated that tensile force activates SRF to form a complex with unidentified transcriptional factor(s); this SRF complex bound to the CArG-B box but not to TATA and CArG-A boxes, and regulated the promoter activity of the gene encoding SMA in response to

mechanical loading (Wang et al., 2002). In view of our current findings, MRTF A might be one of the unidentified transcriptional activators that are required for force-induced regulation of the SMA promoter.

Collectively, our data suggest a relatively simple model by which externally applied forces induce SMA expression in fibroblasts. In this model, force application through integrins induces RhoA activation at force-transfer sites. As a result of RhoA-ROCK signaling, force promotes phosphorylation of LIM kinase and cofilin, thereby enhancing actin assembly. In response to force-induced actin assembly, MRTF is no longer sequestered by actin monomers and redistributes from the cytoplasm into the nucleus where it functions as a SRF co-activator to activate SRE-mediated promoter activity of the gene encoding SMA. This increased promoter activity induces SMA expression. Finally, elevated SMA expression marks the transition of cardiac fibroblasts to myofibroblasts in response to mechanical loading.

Materials and Methods

Reagents

RhoA- and Rac-activation assay kits and RhoA- and Rac-specific antibodies were purchased from Cytoskeleton (Denver, CO). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Chemicon (Temecula, CA). Antibodies against myosin light chain 2, phosphorylated myosin light chain 2, LIMK1, phosphorylated LIMK1 (Thr508), cofilin and phosphorylated cofilin (Ser3), and GST (26H1) were purchased from New England BioLabs (Pickering, ON). Rhotekin GST and GST-fusion proteins were obtained from Upstate (Lake Placid, NY). Y27632, latrunculin B and blebbistatin were obtained from Calbiochem (San Diego, CA). Ionomycin, phalloidin, Flag® M2 HRP-conjugated antibody, monoclonal Flag® M2-FITC-conjugated antibody, goat anti-rabbit whole molecule IgG-FITC-conjugated antibody and antibody against actin were obtained from Sigma-Aldrich (Oakville, ON). Polyclonal BSAC antibody that recognizes MRTF A was generously donated by H. Nakano (Juntendo University School of Medicine, Tokyo, Japan).

Cell culture and treatments

Rat cardiac fibroblasts (Wang et al., 2003) or Rat-2 cells were incubated at 37°C in complete DME medium 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% air and 5% CO_2, and were passaged with 0.01% trypsin (Gibco, Burlington, ON). Prior to force experiments, cells were incubated in DME medium containing 1% serum, incubated with beads and force was applied for various time periods.

Bead coating and force application

Fibrillar collagen-coated magnetite beads (3 μm mean diameter; Sigma-Aldrich) (Glogauer and Ferrier, 1998; Glogauer et al., 1995) were prepared as described (Glogauer et al., 1995; Lew et al., 1999) and 0.4 g of beads were incubated with cells in 60-mm dishes. A ceramic permanent magnet was used to generate perpendicular, tensile forces on beads attached to the dorsal surface of cells. For all experiments the pole face was parallel with and 2 cm from, the culture dish surface. At this distance the force on a single cell with ~750 μm^2 area of dorsal bead coverage was 480 pN per cell or 0.65 pN/μm^2 (Glogauer and Ferrier, 1998). A constant force of varying duration was used for all experiments.

Isolation of focal adhesion proteins

Collagen-coated magnetite beads were incubated with cells and the bound focal adhesion complexes were isolated in ice-cold cytoskeleton extraction buffer (CKSB, 0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM PIPES, 1 μM phalloidin, pH 6.8). The cell-bead suspension was sonicated. Beads were isolated from the lysate using a magnetic separation stand and re-suspended in fresh, ice-cold CSKB, homogenized with a Dounce homogenizer and isolated magnetically. The beads were washed in CSKB, sedimented and the collagen-associated proteins were dissociated from the beads by boiling in Laemmli buffer.

Immunoblotting

Protein concentrations of cell lysates were determined using the BCA™ protein assay kit (Pierce). Equal amounts of protein were loaded on to SDS-polyacrylamide gels (7.5% acrylamide), resolved by electrophoresis, transferred to nitrocellulose membranes and immunoblotted and quantified as described (Wang et al., 2005).

Actin monomers and filaments

For measurements of actin filaments around beads after force application, cells were fixed and stained with Rhodamine-phalloidin. The fluorescence attributable to bead-associated actin filaments was measured in 4-μm regions around beads with a CCD camera optically interfaced to a fluorescence microscope. The bead-associated fluorescence was divided by the area of each measurement to provide an estimate of area-adjusted fluorescence intensity. The relative amount of actin filaments associated with beads was also measured by immunoblotting bead-associated proteins for β-actin as described (Glogauer et al., 1997). To estimate the relative amount of actin monomers around beads, cells were stained with Alexa Fluor-488 conjugated to DNase as described in an earlier method using FITC-DNase (Knowles and McCulloch, 1992) and the fluorescence was measured in 4 μm regions around beads. For experiments involving block of actin assembly, latrunculin B was used (1 μM, 30 minutes prior to treatment).

RhoA activation assay

RhoA, Rac and Cdc42 activities in cell lysates were measured with RhoA or Rac activation assay kits according to the manufacturer's recommendations (Cytoskeleton) with minor modifications. Rat-2 cells cultured in 150-mm dishes overnight without serum were subjected to force at 37°C. Control cells with beads but no force were also assayed for RhoA activation. Lysophosphatidic acid (LPA; 1 μM for 30 minutes) was used as a positive control for active RhoA, whereas serum was used as a positive control for Cdc42 and Rac in previously serum-reduced cells. Cells were lysed in lysis buffer containing protease inhibitor, and cell lysates were homogenized by sonication. Supernatants were incubated with rhotekin-RBD or PBD beads for 1 hour at 4°C on a rotator. Bead-associated proteins were immunoblotted. The amount of GTP-bound RhoA was quantified by immunoblotting with antibody against RhoA and by comparing the bead-bound protein (active RhoA) with total RhoA in the cell lysates. For experiments requiring inhibition of ROCK, Y27632 (10 μM; 30 minutes prior to force) was administered to cells.

In situ Rho affinity assay

Detection of active Rho in situ was based on a previous report (Berdeaux et al., 2004). Cells on glass coverslips were washed in PBS, fixed with 4% paraformaldehyde, incubated in 0.1 M glycine and 0.05% Triton X-100, and washed and incubated with soluble GST-RBD or GST. Cells were immunostained with anti-GST mouse monoclonal antibody. The signal was detected by incubation with FITC-conjugated goat anti-mouse antibody followed by DAPI staining and confocal microscopy.

Short interfering RNA

Specific inhibition of rat cofilin was conducted with Silencer® siRNAs (Ambion) with the following sequences: 3′-GCACGAAUUACAAGCUAACtt-5′, 3′-GGG-CAAGGAGAUUCUGGUAtt-5′, and 3′-CGCAAGAAGGCAGUGCUCUtt-5′. Cells were transfected with 200 nM of siRNA targeting cofilin or negative control siRNA targeting GFP (Ambion) using oligofectamine (Invitrogen) for 24, 48 and 72 hours. Cells were washed in PBS, lysed with Laemmli buffer, and immunoblotted to assess the efficacy of the siRNA-dependent knockdown. After transfection and before force experiments, cells were incubated in DME medium containing 1% serum.

Transfections and determination of reporter activity

Rat-2 cells were transfected with a 765-base-pair rat SMA-luciferase construct (a generous gift of Raphael Nemenoff, University of Colorado, Denver, CO). Cells were co-transfected with a β-galactosidase construct as a control to normalize for variations of transfection efficiency. Transfections were done with Fugene 6 (Roche Applied Science) according to the manufacturer's instructions. Cells were incubated with normal growth medium (5% serum in DME medium) within the first 36 hours and then cells were cultured in serum-reduced conditions (1% serum in DME medium) overnight. After a total transfection time of 5 hours (WT-transfection data in Fig. 6A) or 20 hours (data in Fig. 5), cells were loaded with collagen-coated beads and magnetic force was applied for specific time periods. Cells were harvested and luciferase and β-galactosidase activities were determined as described (Wang et al., 2002). Transfection data were computed as the fold change compared with basal promoter activity normalized to β-galactosidase activity. For MRTF A, MRTF B and dominant-negative MRTF (kind gifts of Eric Olson, University of Texas Southwestern Medical Center, Dallas, TX), cells were transfected with Fugene 6 transfection reagent in accordance with the manufacturer's recommendations and analyzed after 5 or 24 hours, depending on the experiment. For dominant-negative MRTF, the transcription activation domain was deleted so that transcriptional activity was abolished in transfected cells (Kuwahara et al., 2005).

Statistical analyses

For quantitative data, means and standard errors were computed. When appropriate, comparisons between groups were evaluated by Student's *t*-test or one-way ANOVA. Post-hoc testing was carried out using Tukey's test. Statistical significance

was set at $P<0.05$. For all analyses, data from $n \geqslant 3$ independent experiments were used and in each experiment, three replicates were used.

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