Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase

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SUMMARY

Mechanical force or mechanical stress modulates intracellular signal pathways, including the mitogen-activated protein kinase (MAP kinase) cascades. In our system, cell stretching activated and cell contraction inactivated all three MAP kinase pathways (MKK1/2-extracellular signal-regulated kinase (ERK), MKK4 (SEK1)-cJun N-terminal kinase (JNK) and MKK3/6-p38 pathways). However, little is known about the molecular mechanisms that link the mechanical force to the MAP kinase cascades. To test whether Ras and Rap1 are possible components in the stretch-activated MAP kinase pathways, we examined if Ras and Rap1 were activated by cell stretching and if inhibition of their activity decreased the stretch-enhanced MAP kinase activity. Rap1 was activated by cell stretching and inactivated by cell contraction, whereas Ras was inactivated by cell stretching and activated by cell contraction. Rap1GapII and SPA-1, downregulators of Rap1 activity, decreased the stretch-enhanced p38 activity, whereas a dominant-negative mutant of Ras (RasN17) did not inhibit the stretch-initiated activation of MAP kinases. Furthermore, overexpression of Rap1 enhanced p38 activity but not ERK or JNK activity. These results indicate that Rap1 is involved in transducing the stretch-initiated signal to the MKK3/6-p38 pathway, but not to the MEK1/2-ERK or the MKK4 (SEK1)/MKK7-JNK pathway. Thus, Rap1 plays a unique role in force-initiated signal transduction.

Key words: Mechanical stress, Signal transduction, MAP kinase cascade, Ras, Rap1

INTRODUCTION

An increasing number of studies have shown that mechanical stress or force alters growth and differentiation of a number of tissues and cells (Kippenberger et al., 2000; Turner and Pavalko, 1998; Yamazaki et al., 1999). Recently, mechanisms that link the mechanical force with the response of tissues and cells have been intensely investigated and several signaling molecules have been demonstrated to play significant roles in mechanical force-initiated intracellular signal transduction (Ingber, 1998). Among them, MAP kinase cascades, which include MKK1/2-ERK, MKK4(SEK1)/MKK7-JNK and MKK3/6-p38 pathways, are activated by various types of 'mechanical stresses' such as cell stretching (Kippenberger et al., 2000; Yamazaki et al., 1999) and shear stress or fluid flow (Ishida et al., 1996). In fact, recent studies have demonstrated that MAP kinase cascades play an important role in growth, migration, differentiation and apoptosis (Ichijo, 1999; York et al., 1998). However, little is known about how cells sense force and how force-initiated signals are transmitted to MAP kinase cascades.

Small G proteins are known to play roles in adhesion-relevant signal transduction (Sander and Collard, 1999; Tsukamoto et al., 1999). They are also known to mediate signals upstream of MAP kinase cascades (Clark and Brugge, 1995; Ichijo, 1999; Marshall, 1999). Among those signaling mechanisms involving

small G proteins, Ras-MAP kinase pathway has been intensely investigated (Schlaepfer et al., 1994) and the involvement of Ras in the signal transduction from focal adhesions to MAP kinase cascade convincingly documented (Schlaepfer et al., 1998). We postulated that molecules involved in the signal transduction from focal adhesions should also play a significant role in the force-initiated signaling, therefore we tested Ras as a possible upstream molecule of MAP kinase cascades after stretch-dependent activation. We also tested Rap1 because it is known to affect the Ras-MAP kinase pathway (Mochizuki et al., 1999) and also to activate the MAP kinase cascade in some cell systems (York et al., 1998).

In this report, we demonstrate that Rap1 is involved in force-initiated signal transduction to the MKK3/6-p38 MAP kinase pathway and that the activity of Ras is also affected by cell stretching or contraction. Our observations suggest that the 'mechanosensor' of the cells might be associated with the cytoskeleton, since both Rap1 and Ras are shown to be linked to the cytoskeleton (Franke et al., 2000; Tanaka et al., 1999).

MATERIALS AND METHODS

Cell culture

Mouse fibroblastic L-929 cells and human embryonic kidney-derived

293 cells were maintained in DMEM supplemented with 10% FBS and 100 units/ml of penicillin in a 5% CO_2 atmosphere at 37°C.

Cell stretching and contraction

Cells were allowed to adhere to the collagen (type I)-coated silicone dishes as previously described (Komuro et al., 1996) with some modification, which allowed us to expand the dishes biaxially. For the experiments on cell stretching, silicone dishes containing subconfluent cell cultures were expanded by 25% in each dimension for the indicated time period. We confirmed that the cells were stretched, but were not detached from the silicone substrate after stretching (Fig. 1A), and that they survived and grew thereafter. The control samples for each stretch experiment (stretch −) were obtained from cells similarly cultured in silicone dishes that remained unexpanded. For the experiments on cell contraction, cells were cultured in silicone dishes previously expanded by 25%, and at the start of contraction the dishes were allowed to return to the original dimension. Therefore, the ratio of substrate contraction was 20% (25%/125%) (Fig. 1B). The control samples for each contraction experiment (contraction −) were obtained from cells similarly cultured in pre-expanded silicone dishes that remained expanded.

Quantification of phosphorylation of endogenous MAP kinases and MAP kinase kinases by western blot analysis

Cells were lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 12 mM β-glycerophosphate, 1% deoxycholate, 2 mM DTT, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 20 μg/ml aprotinin, homogenized and cleared of insoluble components by centrifugation. The phosphorylation of endogenous MAP kinases (ERK, JNK and p38) and MAP kinase kinases (MKK1/2, MKK4(SEK1) and MKK3/6) was quantified with immunoblotting of equivalent portions of total cell lysates using phosphospecific antibody against each kinase (Cell Signaling Technology). To demonstrate the identical level of total MAP kinases, the antibodies against total ERK1/2 (Cell Signaling Technology), total JNK (sc-571, Santa Cruz, USA) and total p38 (C-20, Santa Cruz, USA) were also used in the immunoblotting. Endogenous JNK activity was also determined with an in vitro kinase assay as described below.

Plasmids

To construct the expression plasmids for hemagglutinin (HA)-tagged MAP kinases, amino-terminal HA-tagged *Xenopus*-ERK, carboxy-terminal HA-tagged human JNK1, and amino-terminal HA-tagged human p38 were incorporated into pCDNA3 by PCR. Vectors for FLAG-tagged Ras and RasN17 in pCXN2 were provided by Dr K. Kaibuchi (Nara Institute of Science and Technology, Ikoma, Japan). FLAG-tagged Rap1 and Rap1GAPII in pCXN2 were provided by Dr M. Matsuda (International Medical Center of Japan, Tokyo, Japan). FLAG-tagged SPA-1 in Spα was provided by Dr M. Hattori (Kyoto University, Kyoto, Japan). A glutathione S-transferase (GST)-fusion protein of Ras-binding domain (RBD) in Raf (GST-RafRBD) was provided by Dr J. L. Bos (Utrecht University, Utrecht, The Netherlands). GST-RalRBD was constructed by incorporating RBD in mouse Ral1 into pGEX4T-1 by PCR.

Transient transfection

2×10^5 293 cells were transfected with the vector(s) dedicated for each experiment using FuGENE 6 (Roche) according to the manufacturer's protocol. Cells were used for the experiments 24 hours after transfection.

In vitro kinase assay

The activity of endogenous JNK or transfected kinase was determined with an in vitro kinase assay as previously described (Nishitoh et al., 1998). In brief, cells were lysed with ice-cold lysis buffer and cleared of insoluble components by centrifugation. Then, the supernatant was subjected to immunoprecipitation with anti-JNK (C-17, Santa Cruz, USA) or anti-HA (12CA5, Roche) antibody and protein A-Sepharose beads (Zymed Laboratories). The beads were washed twice with a Tris-NaCl solution (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EGTA, 1 mM DTT and 1 mM PMSF), mixed with substrates in a kinase buffer (20 mM Tris pH 7.5, 20 mM $MgCl_2$ and 2 μCi [γ-^{32}P]dATP at a final volume of 45 μl) and then incubated for 10 minutes at 30°C. The reaction was stopped by addition of Laemmli's sample buffer. The substrates used were myelin basic protein (MBP, purchased from Sigma) for ERK, GST-cJun (1-79) for JNK and GST-ATF2 (1-109) for p38. Substrate phosphorylation was detected by autoradiography or BAS 2000 (Fuji Film). To confirm the equal expression of transfected constructs, equivalent portions of the cell lysates were subjected directly to SDS-PAGE followed by immunoblotting with anti-HA antibody (3F10, Roche).

GST pull-down assay to quantify the activity of Ras or Rap1

The activity of Ras was determined by a GST pull-down assay using GST-RafRBD fusion protein, which preferentially bound to the GTP-
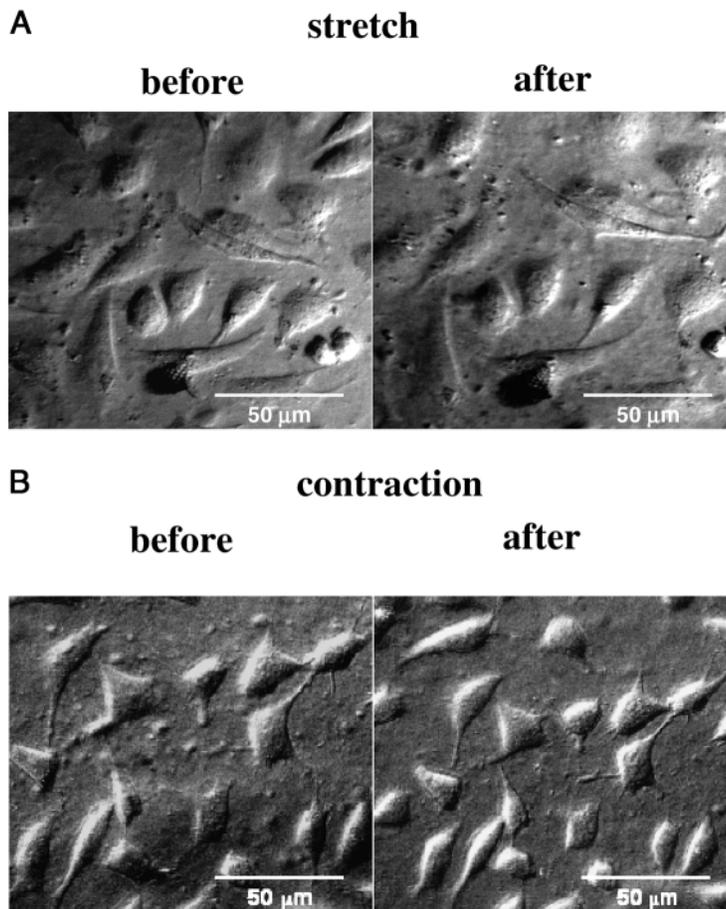

Fig. 1. Microscopic images of cell stretching or cell contraction. L-929 cells cultured in silicone dishes were either stretched or contracted (see Materials and Methods). Differential interference contrast (DIC) images were obtained from the cells before/after cell stretching (A) or before/after cell contraction (B) using a Fluoview Laser Scanning Confocal Microscope (Olympus) in transmission mode. All images were recorded using a 20× LWDC A20PL objective (Olympus) with additional 1.5× hard zoom (scan time, 15.9 seconds for 1024×1024 pixels).
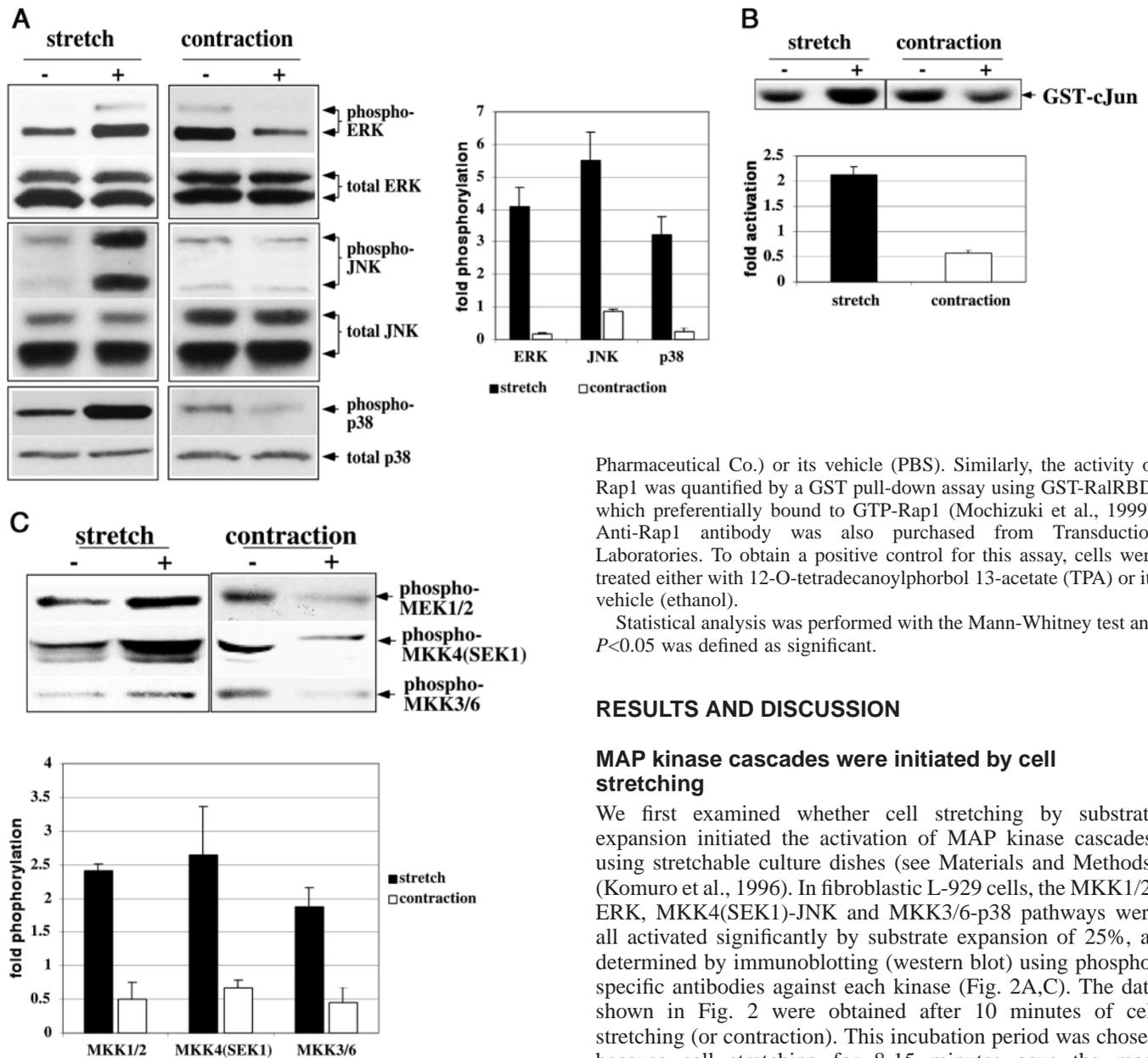
Fig. 2. Effects of stretch or contraction on the phosphorylation/ activity of MAP kinases and MAP kinase kinases. L-929 cells cultured in silicone dishes were used for cell stretching experiments (4×10^5 cells/dish) or cell contraction experiments (6×10^5 cells/dish). After 10 minutes of cell stretching or contraction, the cells were lysed and equivalent amounts of cell lysates were subjected to SDS-PAGE followed by western blot analysis using antibodies against phospho-MAP kinases (A) or MAP kinase kinases (C). To quantify endogenous JNK activity, an in vitro kinase assay was also conducted, using GST-cJun as a substrate (B). Values in the graphs are mean fold activation (stretch +/− or contraction +/−) ± s.d. in at least three independent experiments.

bound, active form of Ras (de Rooij and Bos, 1997). Total Ras in the cell lysate and GTP-Ras in the 'pull-down' beads were quantified by immunoblotting using anti-Ras antibody (Transduction Laboratories). To obtain a positive control for this assay, cells were treated either with FGF2 (also designated as basic FGF, a kind gift from Kaken

Pharmaceutical Co.) or its vehicle (PBS). Similarly, the activity of Rap1 was quantified by a GST pull-down assay using GST-RalRBD, which preferentially bound to GTP-Rap1 (Mochizuki et al., 1999). Anti-Rap1 antibody was also purchased from Transduction Laboratories. To obtain a positive control for this assay, cells were treated either with 12-O-tetradecanoylphorbol 13-acetate (TPA) or its vehicle (ethanol).

Statistical analysis was performed with the Mann-Whitney test and $P<0.05$ was defined as significant.

RESULTS AND DISCUSSION

MAP kinase cascades were initiated by cell stretching

We first examined whether cell stretching by substrate expansion initiated the activation of MAP kinase cascades, using stretchable culture dishes (see Materials and Methods) (Komuro et al., 1996). In fibroblastic L-929 cells, the MKK1/2-ERK, MKK4(SEK1)-JNK and MKK3/6-p38 pathways were all activated significantly by substrate expansion of 25%, as determined by immunoblotting (western blot) using phospho-specific antibodies against each kinase (Fig. 2A,C). The data shown in Fig. 2 were obtained after 10 minutes of cell stretching (or contraction). This incubation period was chosen because cell stretching for 8-15 minutes gave the most prominent phosphorylation, i.e. activation of MAP kinases within times up to 60 minutes. When the cells were contracted by 20%, the MKK1/2-ERK and the MKK3/6-p38 pathways were significantly inactivated (Fig. 2A,C). Although cell contraction gave only a small decrease (approximately 15%) in JNK phosphorylation as determined by western blot analysis (Fig. 2A), it caused a significant (approximately 35%) decrease in MKK4(SEK1) phosphorylation (Fig. 2C). Since the cell contraction-dependent decrease in JNK activity was confirmed by an in vitro kinase assay where the JNK activity was significantly suppressed (approximately 40%) by cell contraction (Fig. 2B), we conclude that cell stretching activates all three MAP kinase kinase to MAP kinase pathways whereas cell contraction suppresses them all.

Since substrate expansion gives rise to the concomitant movement of culture medium, the cells were subjected to fluid flow-derived shear stress simultaneously. Although the fluid shear stress was small, it was difficult to eliminate its effects
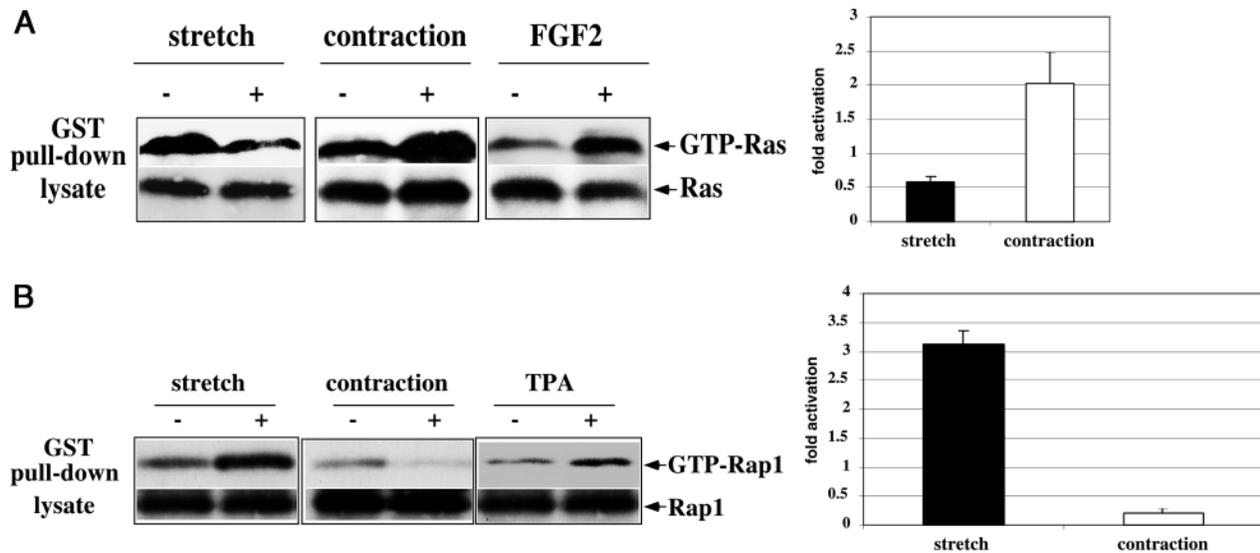
Fig. 3. Effects of stretch or contraction on endogenous Ras and Rap1 activity. (A) L-929 cells were stretched, contracted or treated with FGF2 (10 ng/ml) for 5 minutes. To determine the activity of Ras, a GST-pull down assay was performed using GST-Raf RBD fusion protein, which preferentially bound to GTP-bound, active form of Ras (GTP-Ras). Ras 'pulled-down' by Sepharose beads from cell lysates was quantified by immunoblotting using anti-Ras antibody. (B) L-929 cells were stretched, contracted or treated with TPA (160 ng/ml) for 5 minutes. The activity of Rap1 was determined by measuring GTP-bound, active form of Rap1 (GTP-Rap1) with a GST-pull down assay, using GST-RalRBD. Rap1 was quantified by immunoblotting using anti-Rap1 antibody. Values in the graphs are means of the fold activation (stretch +/−, contraction +/−, FGF2 or TPA +/−) ± s.d. in at least three independent experiments.

in the experiments of substrate expansion. However, fluid flow was equivalent for both contraction and expansion, so the substrate contraction experiments provided an experimental control for possible fluid shear effects of substrate expansion. Therefore, the activation of the three MAP kinase pathways by cell stretching was clearly the result of substrate-applied stress and not fluid-dependent shear stress.

Tensile force on cultured cells that were attached to solid substrate (Galbraith and Sheetz, 1999; Horwitz and Parsons, 1999) was increased with stretch and decreased with contraction. Therefore, we speculate that cell-generated traction forces were responsible for the increase in MAP kinase activity.

Cell stretching enhanced and cell contraction inhibited Rap1 activity, whereas cell stretching inhibited and cell contraction enhanced Ras activity

Since cell stretching should have increased the tensile force to which adherent cells on solid substrate were subjected, we postulated that the signaling molecule(s) involved in adhesion-dependent signaling, e.g. integrin signaling (Reedquist et al., 2000; Renshaw et al., 1999) played a significant role in the stretch-initiated signaling. Small G proteins were possible components in the signaling pathway since they were found to play a role in the signal transduction related to changes in cell adhesion and cell morphology (Boudreau and Jones, 1999; Nobes and Hall, 1999), the regulation of cytoskeleton (Ridley, 1999; Waterman-Storer et al., 1999) and modulation of MAP kinase activity (Ichijo, 1999; Marshall, 1999). Among those small G proteins, Ras was convincingly shown to be involved in the signal transduction from focal adhesions to the MAP kinase cascade (Schlaepfer et al., 1998). We hypothesized that the signaling from focal adhesions to MAP kinase cascades was important for stretch-dependent activation of MAP kinase cascades; therefore, we tested Ras as a possible upstream molecule of MAP kinase

cascades in the cell stretching-initiated signal transduction. We also tested Rap1, another molecule of Ras family small G proteins, because it was found to affect the Ras-MAP kinase pathway (Mochizuki et al., 1999) and to activate the MAP kinase cascade in some cell systems (York et al., 1998).

To evaluate the role of Ras in the force-initiated signaling, we quantified endogenous Ras activity with a GST pull-down assay using GST-RafRBD fusion protein, which preferentially bound to the GTP-bound, active form of Ras (de Rooij and Bos, 1997). As shown in Fig. 3A, the activity of Ras significantly decreased with cell stretching, and conversely, it significantly increased with cell contraction of L-929 cells, suggesting that Ras was not responsible for the stretch-initiated activation of MAP kinases. Since the centripetal transport of focal contacts was reported to follow adhesion through some integrins (Pankov et al., 2000), Ras-dependent, integrin signaling might result from contraction of the cytoskeleton instead of stretching.

To quantify Rap1 activity, we also conducted a GST pull-down using GST-RalRBD fusion protein, which preferentially bound to GTP-Rap1 (Mochizuki et al., 1999). In contrast to Ras, Rap1 was significantly activated (approximately threefold) by cell stretching, whereas it was significantly suppressed (approximately fivefold) by cell contraction in L-929 cells (Fig. 3B). Previous reports described the significant role of Rap1 in cell adhesion and cell spreading (Tsukamoto et al., 1999) and integrin signaling (Reedquist et al., 2000). Our observation and these reports strongly suggest that Rap1 also mediates signals generated by mechanical perturbations of the cells.

In 293 cells, stretching also activated transiently transfected MAP kinases

The correlation of Rap 1 activity with the response of MAP kinase cascades to cell stretching and contraction (Figs 2A-C,
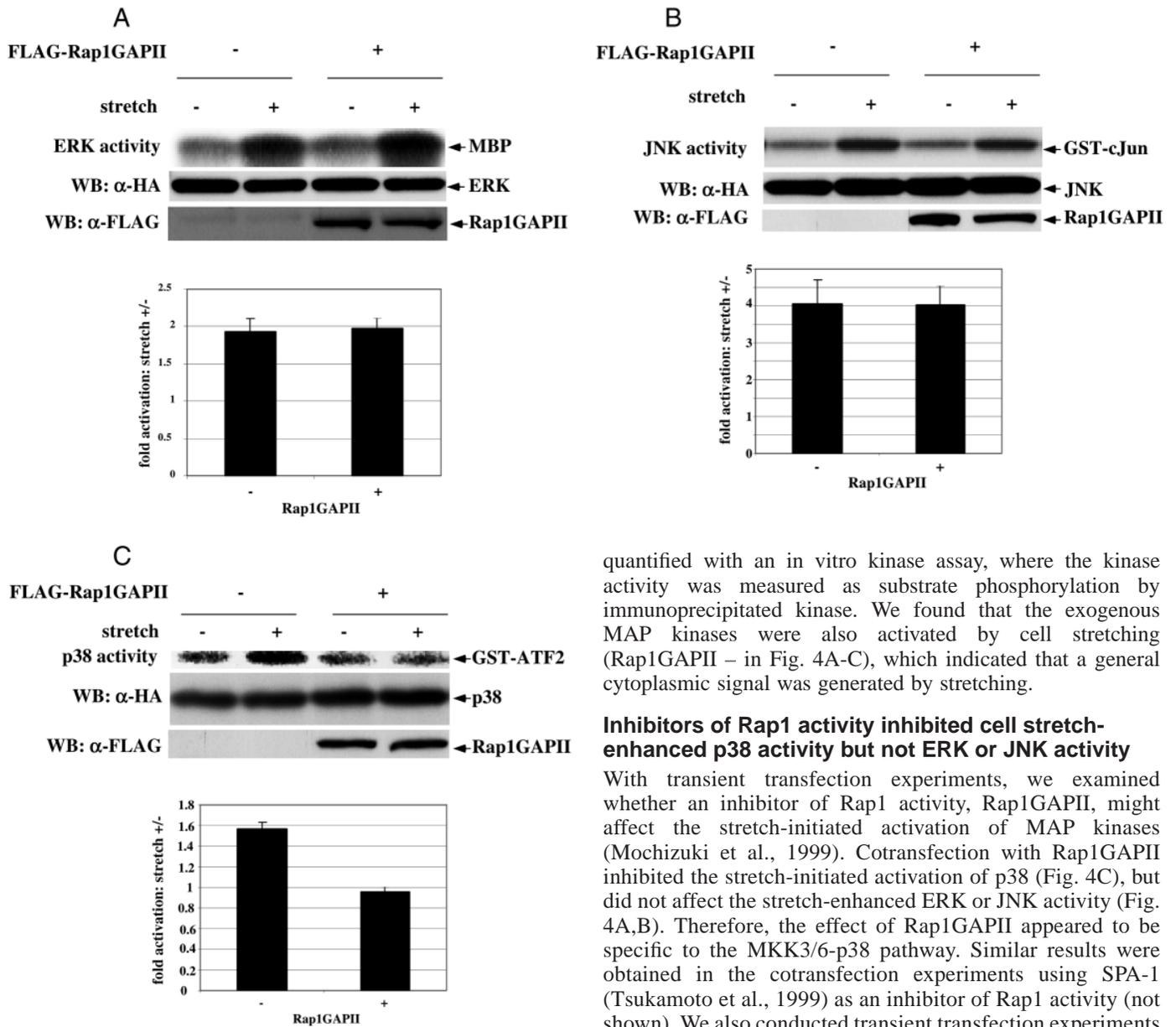
A

B

C

Fig. 4. Effects of Rap1GAPII on the stretch-initiated activation of MAP kinases. Either FLAG-tagged Rap1GAPII or its parent vector (pCXN2) (1 μg) was cotransfected with HA-tagged ERK (A), JNK (B) or p38 (C) expression vector (1 μg) in 293 cells. 24 hours after transfection, the cells were stretched for 10 minutes and then lysed. Cell lysates were subjected to an in vitro kinase assay. Values in the graphs are means of the fold activation (stretch +/−) ± s.d. in at least three independent experiments.

3B) prompted us to further examine the role of Rap1 in the force-initiated activation of MAP kinases with transient transfection experiments. We used 293 cells in these experiments because L-929 cells did not allow sufficient transfection efficiency to monitor the stretch-induced kinase activation and because as in L-929 cells, the activity of Ras and Rap1 was modulated by cell stretching in 293 cells (not shown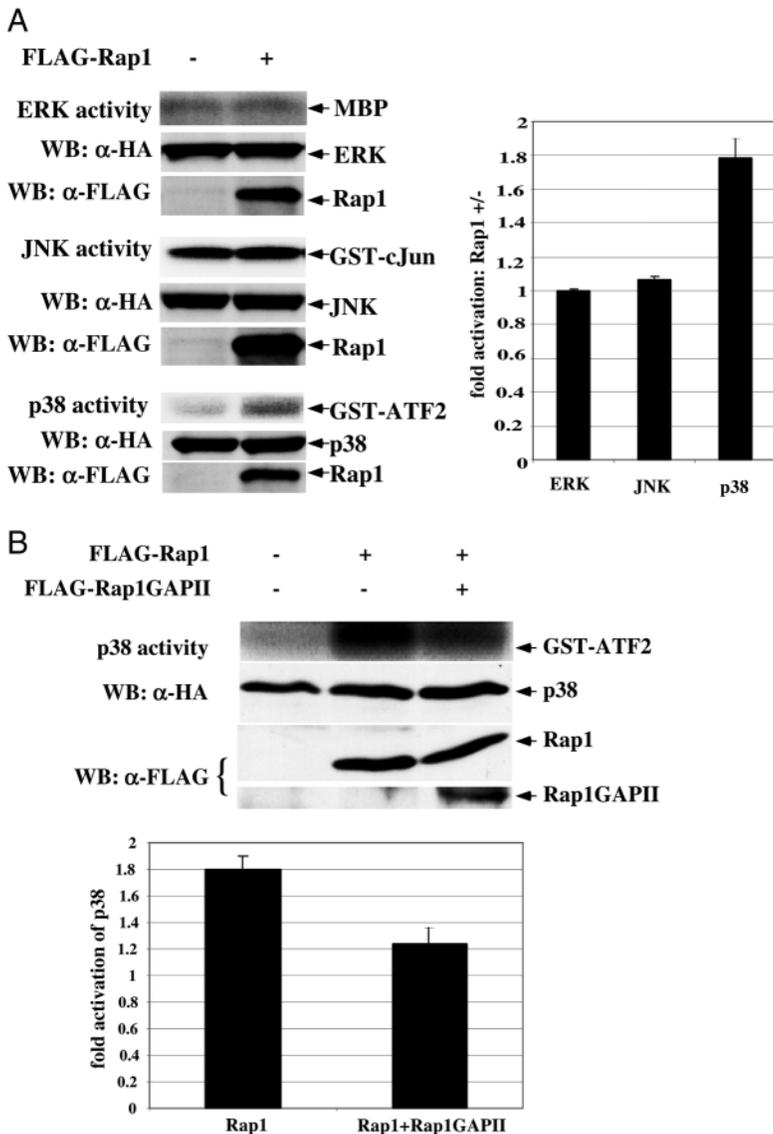). The activity of each kinase introduced in 293 cells was quantified with an in vitro kinase assay, where the kinase activity was measured as substrate phosphorylation by immunoprecipitated kinase. We found that the exogenous MAP kinases were also activated by cell stretching (Rap1GAPII – in Fig. 4A-C), which indicated that a general cytoplasmic signal was generated by stretching.

Inhibitors of Rap1 activity inhibited cell stretch-enhanced p38 activity but not ERK or JNK activity

With transient transfection experiments, we examined whether an inhibitor of Rap1 activity, Rap1GAPII, might affect the stretch-initiated activation of MAP kinases (Mochizuki et al., 1999). Cotransfection with Rap1GAPII inhibited the stretch-initiated activation of p38 (Fig. 4C), but did not affect the stretch-enhanced ERK or JNK activity (Fig. 4A,B). Therefore, the effect of Rap1GAPII appeared to be specific to the MKK3/6-p38 pathway. Similar results were obtained in the cotransfection experiments using SPA-1 (Tsukamoto et al., 1999) as an inhibitor of Rap1 activity (not shown). We also conducted transient transfection experiments using RasN17, a dominant-negative mutant of Ras as an inhibitor of Ras activity. Cotransfection with RasN17 did not give a significant dominant-negative effect on the stretch-initiated activation of MAP kinases (not shown). These results suggest that Rap1 is involved specifically in the stretch-initiated activation of the MKK3/6-p38 pathway but not the ERK or the JNK pathway.

Overexpression of Rap1 enhanced p38 activity, but not ERK or JNK activity

To further test Rap1 as a possible upstream molecule of p38, we examined whether overexpression of Rap1 caused an increase in p38 activity. As shown in Fig. 5A,B, cotransfection with Rap1 (wild type) gave a significant (approximately 80%) increase in p38 activity, but did not affect the activity of ERK or JNK. Moreover, the Rap1-enhancement of p38 activity was significantly diminished in the cells cotransfected with Rap1GAPII (Fig. 5B). These results indicate that Rap1 is a critical signal mediator upstream of p38.

A

in different signaling pathways and a different small G protein is involved in the stretch-initiated activation of JNK.

Cell stretching/contraction modulation of Ras activity

Since Ras was inactivated by cell stretching, it was not considered as an upstream mediator of stretch-dependent activation of MAP kinase cascades. However, stretch-dependent inactivation of Ras might be responsible for some stretch-initiated signaling; it might trigger activation of some molecule, which might be inactivated by active Ras. Moreover, contraction-dependent activation of Ras might also be responsible for contraction-dependent signaling events, for example, some MAP kinases were reported to be activated by contraction in other experimental systems (Lee et al., 2000).

Implications of stretch-initiated signaling

Stretch-initiated signaling mechanisms upstream of MAP kinase cascades are implicated in a number of cellular functions that involve force-sensing by cells. These cascades may be critical in the pathophysiology of physical force-related clinical disorders such as disuse osteoporosis (Jee and Ma, 1999) or cardiac hypertrophy (Yamazaki et al., 1999). Furthermore, a better understanding of the stretch-initiated signaling would help in our understanding of the difference between cell growth on culture plates and growth on soft agar (transformation), since cells generate tensile forces in routine adherent culture conditions (Felsenfeld et al., 1999).

Rap1 mediates cell stretching-initiated signal to p38 MAP kinase pathway

Although it remains unclear how the Rap1-mediated signal is transmitted to p38, the rapid activation (time sequence less than 10 minutes) of p38 by force indicates that Rap1 is an early mediator of the stretch-initiated signal. We hypothesize a pathway in which Rap1 activates a MAP kinase kinase kinase that in turn activates MKK3/6, as some other small G proteins are known to activate MAP kinase kinase kinase (Ichijo, 1999). Since p38 is known to transmit signals related to various cellular functions, such as differentiation and apoptosis (Ichijo, 1999), the Rap1-p38 pathway is likely to be involved in the transformation of physical signals to these biochemical alterations.

Rap1 was involved only in the p38 pathway, which raised the question of whether other small G proteins were involved in the stretch-dependent ERK and JNK pathways. Previous studies demonstrated that Rho and Rac (Aikawa et al., 1999) were involved in the stretch-initiated ERK activation. It is logical to suggest that different small G proteins are involved

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