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Force-induced apoptosis mediated by the Rac/PAK/p38 signaling pathway is regulated by filamin A

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Short title: Force-induced activation of p38 signaling
Abstract

Cells in mechanically challenged environments cope with high amplitude exogenous forces that can lead to cell death but the mechanisms that mediate force-induced apoptosis and the identity of mechanoprotective cellular factors are not defined. We assessed apoptosis in 3T3 and HEK cells exposed to tensile forces applied through β1 integrins. Apoptosis was mediated by Rac-dependent activation of p38α. Depletion of Pak1, a downstream effector of Rac, prevented force-induced p38 activation and apoptosis. Rac was recruited to sites of force transfer by filamin A, which inhibited force-induced apoptosis mediated by Rac and p38α. We conclude that in response to tensile force, filamin A regulates Rac-dependent signals, which induce apoptosis through Pak1 and p38.

Key words: Filamin A, p38 MAP kinase, Rac, Pak1, apoptosis, mechanical force, small GTPases

Introduction

In many types of mechanically loaded tissues, pathophysiological force levels can induce cell death. For example, high amplitude forces promote apoptosis in cardiac myocytes [1], rat alveolar type II cells [2], and mineralized connective tissues [3]. Cell death and the loss of adaptation to mechanical forces caused by cell depletion may contribute to pathological states such as cardiac hypertrophy [4], atherosclerosis [5] and osteoarthritis [6]. Currently, the nature of the mechanisms that mediate, or alternatively, prevent death of cells in mechanically active environments, is not defined.

One of the earliest cellular responses to mechanical stress involves phosphorylation of the mitogen-activated protein kinase p38 [7]. Stress kinases in general, and p38 in particular, are critical components of the cellular responses to a variety of extracellular stimuli and determine cell fates under stressful conditions [8]. Force-induced activation of p38 also appears to be one of the signaling pathways that regulate the expression of cytoprotective genes in response to mechanical stress [9, 10]. One potential cytoprotective protein is filamin A (FLNa), the expression of which can be induced by mechanical force [11]. Tensile forces promote transcriptional upregulation of the FLNa gene through p38-induced activation of the transcription factor Sp1 [12]. After force application, FLNa is re-distributed to force transfer sites where it facilitates cell survival by mechanical stabilization of cortical actin and prevention of force-induced cell depolarization [13].

The mechanoprotective properties of FLNa are associated with its ability to act as a scaffolding factor by recruiting the Rac GAP protein FilGAP, which suppresses excessive Rac activation in mechanically-challenged cells [14]. Rac plays a dual role in cell survival, depending on the cell type and the nature of the extracellular signals. Active Rac can protect cells from apoptosis [15] but higher levels of Rac expression induce apoptosis in fibroblasts [16] and neurons [17]. This pro-apoptotic function of Rac is related to its ability to regulate the activation of mitogen activated protein kinases, including p38 [18]. Further, activation of Rac stimulates p21-activated Ser/Thr kinases (Paks) [19], which are likely integral parts of the signaling pathway that leads to activation of p38 [20]. Currently it is not known whether Pak mediates the stress response in force-challenged cells.

In this report we show that Rac and its downstream effector, Pak1, regulate the activity of p38 in response to tensile forces. This signaling pathway, which is modulated by the scaffolding properties of FLNa, may be an important mechanism for regulating force-induced apoptosis in mechanically-challenged cells.
Materials and methods

Antibodies and reagents

Mouse monoclonal antibodies were purchased from Sigma-Aldrich (Oakville, ON; β-actin; clone AC-15), Cell Signalling (Danvers, MA; phospho-ATF-2, cleaved caspase-3, p38, phospho p38) and Millipore (Etobicoke, ON; Pak1, Rac, GAPDH). Anti-mouse rat monoclonal antibody to cell-surface CD29 (β1 integrin; clone KMI6) and (activated β1 integrin; 9EG7) were obtained from BD Biosciences (Burlington, ON). Anti-rabbit polyclonal antibodies were obtained from Calbiochem (EMD, Darmstadt, Germany; p38α, p38β) and Epitomics (Burlingame, CA; p38γ). Anti-mouse HRP-conjugated goat IgG was purchased from Sigma-Aldrich. Anti-rabbit HRP-conjugated goat IgG was purchased from Amersham (GE Healthcare, QC). Purified proteins were obtained from Cell Signalling (p38α, p38β p38γ) and Millipore (p38δ). The p38 inhibitor SB203580 and the Rac1 inhibitor NSC23766 were obtained from Calbiochem.

Plasmids

Constitutively active (Q61 c-myc) Rac, dominant negative (N17 c-myc) Rac and dominant negative p38 (pCMVp38DF) were obtained from A. Kapus (St. Michael's Hospital Research Institute, Toronto, ON). Constitutively active p38 (pCMV-p38FLAG) was obtained from R.J. Davis (University of Massachusetts, Amherst, MA).

Cell culture and transfection

3T3 cells that express FLNa or were null for FLNa [14] (obtained from David Calderwood, Yale University, New Haven, CT) and HEK293 cells [14] were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. HEK cells were used in most experiments as they exhibited more consistent collagen bead loading and consequently more reproducible apoptotic responses to tensile forces. HEK293 cells stably transfected with a shRNA against FLNa [14] were grown in the presence of puromycin (1 µg/ml). For transfection, cells were trypsinized, plated on 100-mm dishes and immediately transfected using Fugene 6 transfection reagent, as described by the manufacturer (Roche, Mississauga, ON).

Preparation of coated beads and force application

Tensile mechanical forces were applied to cells using a previously described model system [21, 22]. Briefly, magnetite microparticles (Fe₃O₄, modal diameter = 5 µm; Sigma-Aldrich) were incubated with 1 mg/ml purified collagen (Vitrogen 100, Cohesion Technologies, Palo Alto, CA), neutralized to pH 7.4 or with bovine serum albumin (BSA, Sigma-Aldrich, 1.3 mg/ml) and rinsed with PBS. Beads were incubated with cells in a serum-free medium for 15 minutes to prevent bead internalization. Excess non-adherent beads were removed by washing and the cells were supplemented with fresh serum-containing medium. A ceramic permanent magnet (Jobmaster, Mississauga, ON) was used to generate perpendicular, tensile forces on beads attached to the dorsal surface of cells. The pole face was oriented parallel with the culture dish surface at a distance of 20 mm from the surface. At these distances, single cells of ~250 µm² surface area are exposed to tensile forces of 160 pN [21]. For all experiments involving force application, controls involved incubation of cells with collagen beads but without force application.

Rac activation assay

We estimated GTP loading of Rac in HEK 293 cells grown on 10 cm dishes and transiently transfected with relevant plasmids for 48 h. After treatments indicated for each individual experiment, cells were washed in ice-cold PBS and lysed in RIPA buffer (20 mM Tris-HCl at pH 7.5, 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl₂, 0.2 M PMSF, 10 µg ml⁻¹ aprotonin and 10 µg ml⁻¹ leupeptin) at 4°C. Cell lysates were cleared and supernatants were sampled for the determination of total GTPases or incubated with GST fusion protein, corresponding to the p21-binding domain (PBD) of human Pak1, expressed in...
*Escherichia coli* and bound to glutathione agarose beads (Millipore). The beads were washed and boiled in 2 x Laemli buffer. Samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with Rac antibody. Supernatants were also separated on SDS-PAGE gels and immunoblotted for total Rac.

**Caspase 3 activity**

Cells were grown on 10 cm dishes and allowed to attach overnight. After indicated treatments, cells were washed with ice-cold PBS and centrifuged at 150 x g for 5 min at 4°C. Pellets were lysed in 100 μl lysis buffer (50 mM HEPES, pH 7.4; 5 mM CHAPS; 5 mM DTT) for 15 min on ice, followed by two freeze-thaw cycles. Cell lysates were centrifuged at 20,000 x g for 15 min at 4°C and the supernatants were collected. Cell lysates (20 μl) were combined with assay buffer (79 μl; 20 mM HEPES, pH 7.4; 2 mM EDTA; 0.1% CHAPS; 5 mM DTT) and caspase 3 substrate (Ac-DEVD-pNA, Sigma-Aldrich; 1 μl; 2 mM) and incubated in a 96-well microplate for 2 h at 37°C. Caspase-3 activity was measured at 405 nm using a microplate reader (Microplate reader II, Labsystems, Finland). Caspase 3 activities were calculated and normalized against the amount of protein in each sample, as measured by BCA™ protein assay kit (Pierce, Nepean, ON). One unit is the amount of enzyme that will cleave 1.0 nmol of the substrate per hour at 37°C under saturated substrate concentrations.

**p38 activity**

Cells grown in 6-well plates overnight were treated as indicated, washed with ice-cold PBS, pelleted by centrifugation at 150 x g for 5 min at 4°C and lysed in 400 μl RIPA buffer (20 mM Tris-HCl pH 7.5, 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl2, 0.2 M PMSF) by two freeze-thaw cycles. Lysates were centrifuged at 20,000 x g for 15 min at 4°C and the supernatants were incubated with p38 antibody (15 μg) and immobilized protein G resin slurry (30 μl; Pierce) for 3 h at 4°C with gentle rocking. Bead pellets were collected by centrifuging the samples at 8000 x g for 30 sec and washed twice with RIPA buffer. For the kinase assay pellets were re-suspended in kinase buffer (50 ml; 25 mM Tris-HCl pH 7.5; 5 mM β-glycerophosphate, pH 7.3, 10 mM MgCl2, 2 mM DTT, 0.1 mM Na vanadate) and incubated with ATP (200 μM) and ATF-2 fusion protein (1 μg; Cell Signaling) for 30 min at 30°C. The reaction was stopped by adding Laemli buffer (25 μl) and boiling. Samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with phospho-ATF-2 antibody. Samples of cell lysates were also immunoblotted for total p38.

**Immunoblotting and immunostaining**

Cells were lysed and proteins were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Protein concentrations of cell lysates were determined using the BCA™ protein assay (Pierce). Equal amounts of protein were loaded on individual lanes. Nitrocellulose membranes were probed for the indicated antibody followed by incubation with anti-mouse or anti-rabbit HRP-conjugated secondary antibody. Chemiluminescent detection was performed according to the manufacturer's instructions (ECL, GE Healthcare, Oakville, ON). The radiographic films were exposed for standardized lengths of time using conventional methods. In experiments to examine cell surface expression of β1 integrin, cells were detached quickly (<20 secs) from dishes with versene, fixed, immunostained with KMI6 antibody, counter-stained with FITC-conjugated anti-rat IgG and analyzed by flow cytometry [23].

**Collagen bead-associated proteins**

Collagen bead-associated proteins were isolated and immunoblotted as described [24]. Briefly, after designated incubation times, cells and attached collagen-coated magnetic beads were collected by scraping into ice-cold extraction buffer (CSK-EB: 0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 2 mM AEBSF, 1 mM EDTA, 30 μM bestatin, 14 μM E-64, 1 μM...
leupeptin, 0.3 µM aprotinin, and 10 mM PIPES, pH 6.8), and pelleted using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, NY). Supernatants were collected and isolated beads were re-suspended, sonicated, homogenized, and washed three times in extraction buffer before PAGE and Western blot analysis.

RNA interference

For depletion of endogenous Pak1, cells were transfected with a mixture of SignalSilence Pak1 siRNA I and II (Cell Signaling) using Oligofectamine transfection reagent (Invitrogen, Burlington, ON). The transfection efficiency of siRNA was determined by immunofluorescence microscopy using SignalSilence Control siRNA (fluorescein conjugate; Invitrogen). At 2 days after transfection levels of Pak1 were analyzed by immunoblotting with anti-Pak1 antibody.

Quantification of apoptosis

Apoptotic cells were detected using TUNEL staining. Briefly, paraformaldehyde-fixed cells were permeabilized with Triton X-100, stained with TUNEL (Invitrogen) as described by the manufacturer, washed, counter-stained with DAPI and examined with a fluorescence microscope at ×250. The number of TUNEL-positive cells and the total number of DAPI-stained cells were counted in three different sampling grids for each sample to yield the percentage of apoptotic cells. Data from three separate areas were obtained and the mean ± standard deviation was computed for each sample.

TIRF microscopy

Analysis of activated β1 integrins on the ventral surface of attached cells was performed using TIRF microscopy of cells as described [23] using the 9EG7 antibody [25] that recognizes an epitope on the activated β1 integrin, counter-staining for total β1 integrins with KIM6 antibody and computation on a per cell basis (100 cells per time point) of the % of 9EG7 staining as a function of total β1 integrin staining.

Statistical analysis

For all studies, experiments were repeated at least three times for each experiment. For quantitative data, means and standard deviations were computed. When appropriate, comparisons of two samples were analyzed by Student's unpaired t-test, or with multiple samples, with ANOVA followed, when appropriate, by application of Tukey’s test. Statistical significance was set at p<0.05.

Results

Force-induced p38 activation and cell death

High amplitude tensile forces applied to cells through β1 integrins can induce apoptosis [13], a process that may involve activation of p38 and regulation by FLNa [7]. We examined the relationship between FLNa and force-induced apoptosis in wild-type and FLNa knockdown 3T3 cells. By immunoblotting we determined that FLNa expression in the knockdown cells was reduced by >95% (Fig. 1A). β1-integrin, a protein whose ligand-binding affinity is thought to be regulated by FLNa [26] [27], remained unchanged (Fig. 1A). Vertically-directed forces (0.6 pN/µm²) [24] applied through collagen-coated magnetite beads enhanced the phosphorylation of p38, which was more marked in cells with depletion of filamin A (Fig. 1B,C).

We determined whether the presence of FLNa affects activation of β1 integrins after application of tensile force. Wild-type and FLNa knockdown cells were subjected to tensile force and assessed by immunostaining with the 9EG7 antibody, a neo-epitope antibody that recognizes activated β1 integrins [25], counter-staining with KIM6 to label all β1 integrins, and by quantification of TIRF-based images. When cells were subjected to force, for all time points, there were reduced percentages of staining for activated β1-integrins as a function of total β1 integrins.
integrin staining in FLNa knockdown cells compared to FLNa-expressing cells (Fig. 1D, 1E; p<0.05). Notably, there was a time-dependent reduction of the percentage of cells with activated \( \beta_1 \)-integrins in FLNa-expressing cells but not in FLNa knockdown cells. These results were not due to variations of collagen receptor expression in the whole cell populations as flow cytometry analysis of surface-stained \( \beta_1 \) integrins showed no differences of fluorescence between the FLNa expressing and FLNa knockdown cells (mean fluorescence channel number ± standard deviation; FLNa wild-type cells = 4.7 ± 0.26 fluorescence units; FLNa KD cells = 5.3 ± 0.18; p>0.2).

We used TUNEL assays to assess apoptosis in response to force application. The percentage of apoptotic cells was greater in FLNa knockdown cells compared to WT cells for all time points (Fig. 1F; p<0.02); by 8 hrs the percentage of apoptotic cells was 8 times larger in FLNa knockdown cells than the WT cells (p<0.001) (1G).

We found that while phospho-p38 analyses were readily conducted in 3T3 cells, interpretations of experiments with these cells were complicated by differences of cell size within the cell population, variation of bead loading between replicated experiments and most importantly, difficulties with transfection. Due to the more consistent bead loading, uniform cell size and ease of transfection of HEK cells compared with 3T3 cells [14], we used FLNa-expressing HEK cells or HEK cells in which FLNa expression was stably knocked down by shRNA (FLNa-KD), as we showed earlier [14]. Activation of p38 is associated with induction of cell death in several different cell types [28] but different isoforms of p38 (\( \alpha, \beta, \gamma, \delta \)) can have opposing effects on downstream signaling [29]. Accordingly by immunoblotting we found that HEK cells expressed the p38\( \alpha \) and p38\( \delta \) isoforms but the p38\( \beta \) and p38\( \gamma \) isoforms were not detectable (Fig. 2A). With the use of recombinant proteins as standards and by comparing blot densities with measured numbers of cells, the abundance of p38\( \alpha \) and p38\( \delta \) was estimated. These data showed that p38\( \alpha \) was \( \sim 10 \)-fold more abundant than p38\( \delta \) (\( \sim 2.63 \times 10^{-6} \) pmol/cell for p39\( \alpha \); 2.63 \times 10^{-7} \) pmol/cell for p38\( \delta \)). Notably, as different p38 isoforms can have opposing effects on apoptosis [30], we focused our subsequent analyses on p38\( \alpha \) as it was 10-fold more abundant than p38\( \delta \). We evaluated force-induced changes of p38\( \alpha \) activity by measuring phosphorylation of the transcription factor ATF-2, a downstream target of activated p38 [31], which we found provided more reproducible measurements of p38 activation that phospho-p38 in HEK cells. Notably, in HEK cells, vertically-directed forces (0.6 \( \)pN/\( \mu \)m\(^2 \)) promoted a 4-fold increase in the level of phosphorylated ATF-2 in FLNa wild-type cells and a >5-fold increase in FLNa-KD cells (p<0.03; Fig. 2A,B). We evaluated the specificity of force-induced p38 phosphorylation of ATF-2 by pre-treatment with SB203580 (p38i, 1 \( \mu \)M), a specific inhibitor of p38 activity [32]. For both wild type and FLNa-KD cells, the inhibitor reduced ATF-2 phosphorylation to baseline levels of un-stimulated cells (Fig. 2 B,C).

Tensile forces applied through integrins induce markedly greater Rac activation in FLNa-deficient cells compared with FLNa-expressing cells [14] and Rac has been implicated in the signaling pathways in which cell stressors induce p38 phosphorylation [20]. Conceivably, force-induced Rac activation may also regulate p38 function. Accordingly, cells were transfected with constitutively active (CA) Rac. In some experiments we pre-treated cells with NSC23766 (50 \( \mu \)M; Raci) to inhibit Rac1 binding and activation by Rac-specific GEFs. This inhibitor does not interfere with the closely related Cdc42 or RhoA [33]. By pull-down assays with GST-PBD and immunoblotting we found that CA-Rac-transfected cells exhibited abundant GTP-Rac while cells treated with the Rac inhibitor exhibited low amounts of GTP-Rac (Fig. 2D). Pre-treatment of FLNa-expressing and FLNa-deficient cells with NSC23766 (50 \( \mu \)M; 30 min prior to force application) blocked the increase of phosphorylated ATF-2 in response to force (Fig. 2E), and
this was particularly marked in the FLNa-deficient cells. Next, we transfected wild type and FLNa-KD cells with CA-Rac, incubated cells with collagen beads and subjected them to force or no force. In the absence of force, transfection with CA-Rac increased levels of ATF-2 phosphorylation in FLNa-expressing cells and even more so in FLNa-deficient cells (Fig. 2F), but there was no further increase with force application in the FLNa-deficient cells. The enhanced ATF-2 phosphorylation in FLNa-deficient cells with CA-Rac transfection exhibited a similar trend as that observed in force-induced p38 phosphorylation in 3T3 cells (Fig. 1B) and in force-treated HEK cells that did not express FLNa (Fig. 2A,B).

**Force-induced p38 activity promotes apoptosis**

As tensile forces applied through collagen receptors induce apoptosis in cultured fibroblasts [13, 14], we assessed the effect of force-induced p38 activity on caspase-3 activity as a measure of apoptosis. Caspase-3 activity was increased 4-fold (p<0.01) by force in FLNa-expressing cells to levels that were ~30% lower than treatment with staurosporine (3 h: 1 μM; Fig. 3A) whereas force-treated FLNa-deficient cells exhibited 20% higher caspase activity than staurosporine-treated cells. Pre-treatment of wild-type and FLN-KD cells with the p38 inhibitor SB203580 (1 μM) reduced force-induced caspase-3 activity to control levels; p<0.01; Fig. 3A). We also analyzed caspase-3 cleavage in wild-type and FLNa-KD cells subjected to force (Fig. 3B). Treatment of cells with staurosporine or force promoted the cleavage of pro-caspase-3 (35 kDa) and the formation of active caspase-3 (17 kDa). Pre-treatment with SB203580 (1 μM) inhibited force-induced formation of active caspase-3 but did not affect staurosporine-induced formation of active caspase-3 (Fig. 3B).

Measurements of caspase-3 activity to estimate apoptosis were extended with TUNEL staining (Fig. 3C). Consistent with previous reports [13, 14], exposure to tensile forces for 16 hours promoted marked increases of the % of TUNEL-stained cells (6-fold increase for FLNa-expressing cells; no force-1.5±0.3%; force-9.2±1.1%; 14-fold increase for FLNa-deficient cells; no force-5.1±0.7%; force-71.4±4.1; mean±S.E. n = 3; all differences were p<0.001). Inhibition of p38 with SB203580 reduced force-induced apoptosis (Fig. 3C). The effect of p38 inhibition on force-induced cell death was especially notable in FLNa-deficient cells: pre-treatment with SB203580 reduced (by 10-fold) the % of TUNEL-stained FLNa-KD cells (Fig. 3D; force without inhibitor-71.8±6.9%; force with inhibitor-6.8±1.9%).

We examined the effect of altering p38 activity on apoptosis. Wild type cells were transfected with CA p38 or dominant-negative (DN) p38 expression vectors, subjected to force and the levels of p38 activity in transfected cells were estimated by measuring ATF-2 phosphorylation (Fig. 4A). As expected, dominant-negative p38 suppressed, while constitutively active p38 enhanced, ATF-2 phosphorylation (Fig. 4A). In cells that were not stimulated by force, transfection with CA-p38 increased caspase-3 activity by 3-fold compared to untransfected controls (p<0.05, Fig. 4B). In cells transfected with CA-p38, force promoted no additional increase of caspase-3 activity compared to CA-p38-transfected cells without force. In the absence of force, cells transfected with dominant negative p38 exhibited low levels of caspase-3 activity, which was comparable to that of untransfected controls. Inhibition of p38 activity with dominant negative p38 completely blocked force-induced caspase-3 activation (Fig. 4B). Thus in mechanically-stimulated cells, elevated levels of active p38 contribute to force-induced caspase 3 activity.

**Rac promotes force-induced apoptosis by activating p38**

Activation of Rac GTPases can induce apoptosis in several different cell types including fibroblasts, neurons, and epithelial cells [16, 17]. We examined whether Rac activity may contribute to force-induced apoptosis of wild-type and FLNa-KD cells treated with CA-Rac or the Rac inhibitor NSC23766 [33] (50 μM, 30 min prior to force application). The effect of these
treatments on promotion of apoptosis was evaluated by caspase-3 activity (Fig. 5A). Without force application, transfection of FLNa-expressing and FLNa-deficient cells with CA-Rac promoted a >2-fold increase of caspase-3 activity compared to untransfected cells that were not exposed to force (Fig. 5A). Notably, in wild-type cells but not in FLNa-KD cells, force application induced an additional 1.5-fold increase of active caspase-3 compared to unstimulated cells that had been transfected with CA-Rac (Fig. 5A). Incubation of cells with the Rac inhibitor reduced caspase 3 activity to control levels for all treatments.

We noted that the increased abundance of phosphorylated ATF-2 (a measure of p38 activity) in cells transfected with CA-Rac (Fig. 2F) was similar in magnitude to the elevated caspase-3 activity observed in cells with the same treatments (Fig. 5A,B). Accordingly, we tested whether Rac promotes force-induced apoptosis through p38 in FLNa-expressing cells. Inhibition of p38 by SB203580 reduced the high levels of caspase-3 activity in cells transfected with CA-Rac to control levels (Fig. 5B). When tensile force was applied, cells that were pre-treated with SB203580 exhibited no significant increase of caspase-3 activation and caspase activity was similar to cells without force treatment (p>0.2). Notably, SB203580 exerted no inhibitory effect on levels of GTP-Rac in control and CA-Rac transfected cells with or without force treatment, indicating that Rac activation is upstream of p38 in this system (Fig. 5C).

FLNa interacts functionally with a diverse array of proteins including Rac [34]. In force-stimulated cells, FLNa is redistributed to force application sites (i.e. the β1 integrin-collagen bead locus) [11]. As wild-type cells (but not FLNa-KD cells) transfected with CA-Rac and stimulated with force exhibited an additional 1.5-fold increase of caspase-3 activity compared to unstimulated cells that were transfected with CA-Racs (Fig. 5A), we considered that FLNa may regulate downstream signaling by localizing Rac to force application sites. Accordingly, we assessed the recruitment of Rac to the β1 integrin–collagen bead locus in unstimulated and force-treated wild type and FLNa-KD cells. Even in the absence of force, FLNa-expressing cells exhibited >4-fold higher amounts of Rac associated with beads compared with FLNa-deficient cells (Fig. 6A,B). In wild type cells, force promoted an additional 2-fold enrichment of Rac associated with collagen beads (p<0.05; Fig. 6B) but not in cells incubated with BSA-coated beads (Fig. 6A). In FLNa-KD cells there was no significant (1.2-fold; p>0.2) force-induced enrichment of Rac (Fig. 6A, B) indicating that FLNa may regulate apoptosis through collagen adhesion-associated sequestration of Rac.

**Force-induced p38 activation is regulated through Pak1**

Rac and its downstream effector p21-activated kinase (Pak1) can regulate the activity of p38 [20]. We examined whether Pak1 is involved in force-induced activation of p38 by Rac by reducing the expression of endogenous Pak1 in wild-type and FLNa-KD cells by siRNA (Fig. 7A); cells were then subjected to force. Depletion of Pak1 reduced force-induced caspase-3 activation in FLNa-KD and wild-type cells to control levels (Fig. 7B). FLNa wild-type cells that were depleted of Pak1 also exhibited low levels of force-induced p38 activity as measured by p-ATF2 (Fig. 7C).

We transfected control siRNA cells and Pak1-siRNA cells with (or without) CA-Rac and examined force-induced caspase 3 activity. Compared to unstimulated CA-Rac-transfected cells treated with control siRNA, the caspase-3 activity in cells with Pak1-siRNA was reduced 6-fold (p<0.001), which was similar to control cells without CA-Rac transfection (Fig. 7D). In cells transfected with control siRNA, force promoted an additional 20% increase of active caspase-3 above that of cells expressing CA-Rac (p<0.05). In Pak1-siRNA cells that were transfected with CA-Rac and treated with force or with no force, caspase activity was comparable to non-transfected, unstimulated control cells (p>0.2; Fig. 7D).
Discussion

Mechanical forces play central roles in regulating cell behavior including the control of cell differentiation and death. Failure of mechanically-challenged tissues to adapt to applied forces because of cell death may provide insights into the loss of homeostasis and tissue destruction that occurs in heavily loaded connective tissues such as osteoarthritis [6]. Currently, little is known about how forces mediate cell death or how cells protect themselves against force-induced apoptosis. Our central findings are that Rac, through its downstream effector Pak1, stimulates activation of p38 MAP kinase in response to mechanical force. FLNa in turn regulates the overall levels of Rac activity, possibly by targeting Rac to the site of force application. As FLNa may act as a scaffolding factor for Rac and p38 at force transfer/cell adhesion sites [12], we suggest that the Rac-Pak1-p38 pathway as modulated by FLNa, may be a key mechanism for determining apoptosis in mechanically-challenged cells (Fig. 8).

Activation of p38 has been implicated in pro-apoptotic and growth inhibitory signaling as well as pro-inflammatory responses [8]. Previous studies suggested that the small GTPase Rac may activate the stress kinases JNK and p38; this activity is thought to be pro-apoptotic [35]. Recently we reported that FLNa suppresses Rac activation in cells challenged by tensile forces, thereby contributing to the ability of cells to resist force-induced apoptosis [14]. Here we determined the roles of p38 and Rac in cell death induced by tensile forces.

Tensile forces promote cell death through Rac-Pak1-p38 pathway

Tensile forces applied through β1-integrins can induce specific phosphorylation of p38, one of the earliest steps in the cell stress response [7, 36]. Consistent with these data we found that application of force to FLNa-expressing and FLNa-deficient cells led to p38 activation, as indicated by phosphorylation of p38 or the transcription factor ATF-2. These force-induced changes of p38 activity were correlated with increased levels of active caspase 3, a measure of apoptosis. We found that constitutive activation of p38 was pro-apoptotic even in the absence of mechanical stimuli. In contrast, suppressing p38 activity by a dominant-negative mutant or with a specific inhibitor increased resistance to force-induced apoptosis. These data suggest that high levels of p38 activity are sufficient to trigger a pro-apoptotic response. Thus the cellular levels of active p38 are important determinants of force-induced apoptosis.

The force-induced p38 activation and apoptosis that we observed were dependent on Rac activity, which is in agreement with previous reports showing that Rac can regulate the mitogen activated protein kinase signalling pathway in various cell systems [37]. Recently we reported a temporal correlation between force-induced Rac activation and increased apoptosis in FLNa-deficient cells [14]. Previous studies suggested that the pro-apoptotic activity of Rac may arise from its ability to activate JNK and p38 [35]. Consistent with these data, we found that while suppression of Rac activity (by a specific small-molecule inhibitor) abolished force-induced p38 activation, high levels of Rac activity promoted p38 activation and apoptosis even in cells that were not exposed to mechanical force. When tensile forces were applied, p38 activation was evidently essential for Rac-induced apoptosis. Thus Rac emerges as a critical regulator of p38 activation and apoptosis by tensile forces.

We found that p38 activation by tensile forces and the pro-apoptotic effect of elevated Rac activity in force-challenged cells required the expression of Pak1. Previous studies reported that GTP-bound Rac binds to and stimulates the activity of Pak1 [19], and this activation controls p38 signaling [20, 38]. Pak1 activity has been implicated in protecting cells from intrinsically generated pro-apoptotic signals [39], while Pak2 is considered as the only member of the p21 activated kinase family that exhibits pro-apoptotic functions [40, 41]. Here we found that tensile forces are able to induce apoptosis through Pak1. Depletion of Pak1 with siRNA abolished...
apoptosis in cells expressing constitutively active Rac and stimulated with force. Pak1 may therefore be an essential component in the Rac-p38 signaling pathway that leads to force-induced cell death.

**FLNa as a scaffolding protein for the Rac-Pak1-p38 pathway**

While high levels of p38 and Rac activity are pro-apoptotic in both FLNa-expressing and FLNa-deficient cells, FLNa evidently promotes some additional p38 activation and cell death when tensile forces are applied. We found that FLNa was necessary for force-induced enrichment of Rac at β1 integrin binding sites. FLNa is widely cited as a scaffolding protein that is involved in signal transduction and cytoskeletal reorganization [42, 43]. Some of these signal regulating factors include Trio, a FLNa-binding GEF, and FilGAP, a Rac GTPase-activating protein [44, 45]. Previous reports showed that FLNa is recruited to the sub-membrane cortex after mechanical stimulation [7, 46] and specifically targets FilGAP to the sites of force transfer [14]. This targeting apparently plays a role in protecting cells from force-induced apoptosis [14]. Therefore it is conceivable that by promoting the association of Rac with FilGAP and Trio, FLNa maintains levels of active Rac in mechanically-stressed cells, which enhance their resistance to force-induced apoptosis.

We demonstrated that the depletion of FLNa was associated with overall higher levels of active p38 and apoptosis, even in unstimulated cells, which is in agreement with previous data [14]. Notably, while force could promote additional p38 activation in FLNa-expressing cells, this effect was not as evident in FLNa-deficient cells. Previous studies show that in addition to recruiting FLNa to the sites of force transfer, mechanical stress causes re-distribution of phosphorylated p38 from the cytoplasm to the integrin/collagen bead locus, as well as to the nucleus [12]. Accordingly we suggest that FLNa may also regulate levels of p38 and Rac activity in cells by targeting them to specific cellular locations. Notably, Pak1 interacts with FLNa and this interaction is essential for its kinase activity [47]. Conceivably, while overall high levels of p38 and Rac activity may contribute to apoptosis, specific force-induced targeting of phosphorylated p38, Rac and possibly Pak1 by FLNa may improve their accessibility to upstream and downstream regulatory factors.

In conclusion, Rac-induced p38 activation by Pak1 is modulated by the scaffolding properties of FLNa, which is an important determinant of cellular responses to mechanical force.

**Acknowledgments**

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**Funding**

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**Figure Legends**

**Fig. 1.** Force-induced apoptosis. (A) Immunoblot characterization of filamin A wild-type (FLNa WT) and knockdown (FLNa KD) NIH 3T3 cells. Lanes were loaded with equal amounts of protein and probed for filamin A, β1-integrin or GAPDH as loading control. These data are representative of three separate and independent experiments. (B) FLNa-WT and FLNa-KD NIH 3T3 cells were incubated with collagen-coated beads for 3h prior to force application for 30 min. Equal amounts of protein were loaded and immunoblotted for phospho-p38 (pp38) and p38. (C) Quantification of phosphorylated p38. Band intensities were measured using ImageJ densitometry software. Phospho-p38 quantification is represented by peak intensity and normalized to p38 blot densities of total cell lysates. Data are expressed as the means and standard deviations of three independent experiments. (D) FLNa WT and KD 3T3 cells were subjected to force over an 8 hr time-course, stained with 9EG7 and KIM6 in situ to label activated and total β1 integrins and imaged by TIRF. Images show 9EG7 staining. (E) Quantified data are means±standard deviations of % 9EG7 staining intensity as a function of KIM6 staining for 100 cells per time point. These data are representative of 4 independent experiments. (F) Time-course assessment of force-induced apoptosis in FLNa-WT and FLNa-KD 3T3 cells. Data are mean±standard deviations of percentages of TUNEL-positive cells of the total number of cells counted at indicated time points. (G) Representative DAPI and TUNEL images of FLNa-WT and FLNa-KD 3T3 cells after 8-hour force application. This experiment was repeated three times.

**Fig. 2.** Force-induced p38 activation. (A) HEK cell lysates were immunoblotted for the p38 isoforms α,β,γ and δ and compared to recombinant protein standards of p38 α,β,γ and δ. These data are representative of three separate and independent experiments. (B) FLNa-WT and FLNa-knock down HEK cells were incubated with collagen-coated magnetite beads and were either untreated or subjected to tensile force for 1h. Where indicated, cells were pre-treated with 1 μM SB203580 (p38i) 1h prior to force application. Total cell lysates were immunoprecipitated with anti-p38 antibody and endogenous p38 kinase activity was measured by an immunocomplex kinase assay using ATF2-fusion protein as a substrate (described in Materials and Methods). The amounts of p38 in crude cell extracts and immunoprecipitated phosphorylated ATF-2 were detected by immunoblotting. (C) Quantification of phosphorylated ATF-2. Intensities of bands were measured using ImageJ densitometry software. Quantification of phosphorylated ATF-2 protein is represented by peak intensity and normalized to p38 blot densities of total cell lysates. Data are expressed as the means and standard deviations of three independent experiments. *p<0.05 versus respective control. (D) FLNa-WT and FLNa-KD cells were transfected with constitutively active Rac (CA-Rac); 24h after transfection cells were treated with 50 μM NSC23766 (Raci) for 30 min (where indicated). Cell extracts were then incubated with GST-PBD immobilized on glutathione-Sepharose beads. The amount of Rac in cell lysates and GST-PBD bound Rac by pull-downs were measured by immunoblot, which is representative of 4 independent experiments. (E) Wild-type and FLNa-KD cells were pre-treated with 50 μM NSC23766 (Raci) for 30 min (where indicated), subjected to 1h force and analyzed as in B. (F) FLNa-WT and FLNa-KD cells were transfected with constitutively active Rac construct (CA-Rac) and subjected to force 24 h after the transfection. p38 kinase activity in transfected cells was determined by kinase assay. Levels of p38 and immunoprecipitated phosphorylated ATF-2 were detected by immunoblotting. Data shown are representative of 3 independent experiments.

**Fig. 3.** Relationship of force-induced p38 activity to caspase activity. (A) FLNa-WT and FLNa-KD cells were subjected to tensile force for 16h, or, alternatively, incubated with 1 μM staurosporine (positive control). Caspase-3 activity in cell lysates was measured with a
colorimetric assay. Where indicated, cells were pre-treated with SB203580 (p38i) 1h prior to force/staurosporine application. Data represent % relative to the level of caspase-3 activity in staurosporine-treated cells and are expressed as the means and standard deviations of four independent experiments. (B) Immunoblot shows levels of cleaved caspase-3 in crude extracts of cells treated as in A. *p<0.01 versus respective control. (C) FLNa-WT and FLNa-KD cells were subjected to force as in A. Apoptotic cells were detected with TUNEL staining. These data are representative of three separate and independent experiments.

Fig. 4. Requirement of p38 activity for apoptosis. (A) FLNa-WT cells were transfected with either dominant negative (DN-p38) or constitutively active (CA-p38) p38 constructs or vector control (Control). After 24 h, cells were incubated with collagen-coated magnetite beads and subjected to mechanical force for 1h or, alternatively, treated with 1μM staurosporine for 3h. Endogenous p38 kinase activity in cell lysates was determined by an immunocomplex kinase assay as described above. Activated p38 in cell lysates was detected by immunoprecipitation of ATF-2 and immunoblotting. GAPDH was probed as a loading control. (B) Cells transfected as in A were subjected to 16 h of force, or, alternatively, incubated with 1μM staurosporine (stau; positive control). Caspase-3 activity in cell lysates was measured with a colorimetric assay and is presented as % relative to the level of caspase-3 activity in staurosporine-treated cells. Data are expressed as the means and standard deviations of three independent experiments. *p<0.05 versus respective control.

Fig. 5. Rac promotes apoptosis through p38. (A) Cells were transfected as indicated and after 24 h subjected to force (1 h) or, alternatively, treated with staurosporine for 3 h. Where indicated, the transfected cells were pre-treated with Rac inhibitor (Raci) 30 min prior to force application. Cells were lysed and caspase-3 activity was measured with a colorimetric assay. Data represent % relative to level of caspase-3 activity in staurosporine-treated cells and are expressed as the means and standard deviations of three independent experiments. *p<0.05 versus respective control. (B) Cells were transfected with CA-Rac as in A and subjected to mechanical force for 16 h or staurosporine treatment as described above. Where indicated, cells were pre-treated with SB203580 (p38i) 1h prior to force or staurosporine application. Caspase-3 activity in lysates was measured and expressed as % relative to level of caspase-3 activity in staurosporine-treated cells. Data are expressed as the means and standard deviations of three independent experiments. *p<0.05 versus respective control. (C) Immunoblot of FLNa-WT cells transfected with CA-Rac and treated with p38i. GTP-bound Rac was measured with pull-down as described. These data are representative of three separate and independent experiments.

Fig. 6. Recruitment of Rac to bead complexes. (A) FLNa-WT and FLNa-KD cells were subjected to tensile force for 1h. Proteins associated with collagen- or BSA-coated beads were obtained by magnetite bead isolation described in Materials and Methods and analyzed by immunoblotting. Proteins from whole cell lysates were immunoblotted for Rac. (B) Quantification of focal adhesion (FA) coated bead-associated Rac. Collagen-coated beads were used for purification. Intensities of bands were measured by densitometry. Abundance of bead-associated Rac is shown as % relative to levels of Rac in cell lysates. Data are expressed as the means and standard deviations of three independent experiments. *p<0.05 versus respective control.

Fig. 7. Pak1 mediates force-induced p38 activation and apoptosis by Rac. (A) Immunoblot of Pak1 48 h after siRNA treatment of FLNa-WT cells. Cell lysates were immunoblotted for β-actin as loading control. These data are representative of three separate and independent experiments. (B) FLNa-WT and FLNa-KD cells were treated with siRNA against Pak1 as in A. Cells were loaded with collagen-coated magnetite beads and were either untreated or subjected to tensile
force for 16h. Caspase-3 activity was measured as described and data are mean ± standard deviation of % relative to level of caspase-3 activity in staurosporine-treated cells. (C) FLNa-WT cells were treated with control siRNA or Pak1 siRNA and then subjected to force as in B. Overall levels of p38 and immunoprecipitated phosphorylated ATF-2 were examined by immunoblotting. These data are representative of three separate and independent experiments. (D) FLNa-WT cells were treated with control siRNA or Pak1 siRNA alone (as in A) or, after 48 h, transfected with constitutively-active Rac (CA-Rac). Force treatment was for 1 h as indicated. Caspase-3 activity in cells was measured by colorimetric assay and presented as mean % ± standard deviation relative to level of caspase-3 activity in staurosporine-treated cells (n=3). *p<0.05 versus respective control.

**Fig. 8.** Proposed model of filamin A regulation on force-induced apoptosis. The model illustrates the mechanoprotective function of filamin A (FLNa) in the presence and absence of force. (A) In the absence of force, FLNa and talin compete for binding to cytoplasmic tails of β1-integrins, acting to repress or activate, respectively, their function for downstream processes. Rac participates in normal cell function such as lamellae formation and cell survival. (B) In the presence of force, FLNa recruits the GTPase FilGAP to sites of force application. Here, FilGAP serves to activate cell retraction pathways through Rho while suppressing the activation of Rac. This regulation inhibits lamellae formation and prevents Rac/Pak/p38-mediated apoptosis after application of tensile forces. In the absence of FLNa, when cells are subjected to tensile force, increased Rac activation is not inhibited, thereby leading to increased levels of force-induced apoptosis and suggesting a role of FLNa as a mechanoprotective protein.
References


Fig. 1

A

<table>
<thead>
<tr>
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<tr>
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B

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<tr>
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<tr>
<td>-</td>
<td>+</td>
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<tr>
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C

![Graph showing Phospho-p38 (% of total p38 cell lysate) over time (hr).](image)

D

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E

![Bar graph showing % of activated β-integrins over time (hr).](image)

F

![Graph showing % of Apoptotic cells over time (hr).](image)

G

![Images showing DAPI and TUNEL staining.](image)
**Fig. 2**

**A** Standards (ng) and Lysates (μg)

- FLNa-WT
  - p38α
  - p38β
  - p38γ
  - p38δ

- FLNa-KD
  - p38α
  - p38β
  - p38γ
  - p38δ

**B**

- FLNa-WT
  - force
  - p38i
  - p-ATF2
  - Cell lysate (p38)

- FLNa-KD
  - force
  - p38i
  - p-ATF2
  - Cell lysate (p38)

**C**

ATF-2 activity (band intensity)

- force
- p38i
- FLNa-WT
- FLNa-KD

**D**

- CA-Rac
- CA-Rac + Raci
- PBD (GTP-Rac)
- Cell lysate (Rac)

**E**

- FLNa-WT
  - force
  - Raci
  - p-ATF2
  - Cell lysate (p38)

- FLNa-KD
  - force
  - Raci
  - p-ATF2
  - Cell lysate (p38)

**F**

- HEK control
- FLNa-WT/FLNa-KD
- CA-Rac
- CA-Rac + force
- p-ATF2
- Total cell lysate (p38)
**Fig. 3**

**A**

Caspase-3 activity (% of positive control) for FLNa-WT and FLNa-KD with different treatments: force, p38i, and stauro.

**B**

FLNa-WT and FLNa-KD with different treatments: force, p38i, and stauro. Cleaved caspase 3 and total cell lysate (GAPDH) are shown.

**C**

TUNEL staining for FLNa-WT and FLNa-KD under No Force, Force, and p38i + Force conditions.
<table>
<thead>
<tr>
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**Fig. 4**

**A**

**B**

[Graph showing caspase 3 activity (% of positive control) for different conditions]
**Fig. 6**

A

<table>
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<tr>
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Collagen-coated beads-associated Rac

BSA-coated beads-associated Rac

Cell lysate (Rac)

B

![Bar graph showing FA-associated Rac (as % of total Rac) for FLNa-WT and FLNa-KD under different forces.](image)

- FLNa-WT
- FLNa-KD

* indicates significance.
Fig. 8