Extracellular Signal–regulated Kinase and c-Jun NH_2-terminal Kinase Activation by Mechanical Stretch Is Integrin-dependent and Matrix-specific in Rat Cardiac Fibroblasts

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Abstract

Integrins, which connect the cytoskeleton to the extracellular matrix and mediate a variety of signaling cascades, may transduce mechanical stimuli into biochemical signals. We studied integrin- and matrix-dependent activation of extracellular signal–regulated kinase (ERK2), c-Jun NH_2-terminal kinase (JNK1), and p38 in response to 4% static biaxial stretch in rat cardiac fibroblasts. ERK2 and JNK1, but not p38, were rapidly activated by stretch when the fibroblasts were allowed to synthesize their own matrices. When the cells were limited to specific matrix substrates, ERK2 and JNK1 were differentially activated: ERK2 was only activated when the cells were plated on fibronectin, while JNK1 was activated when the cells were plated on fibronectin, vitronectin, or laminin. Plating cells on collagen before stretching did not activate either kinase. Adhesion to all matrices was integrin-dependent because it could be blocked by inhibitors of specific integrins. ERK2 activation could be blocked with a combination of anti-α4 and -α5 antibodies and an arginine-glycine-aspartic acid (RGD) peptide, while the antibodies or peptide used separately failed to block ERK2 activation. This result suggests that at least two integrins, α4β1 and an RGD-directed, non-α5β1 integrin, activate ERK2 in response to mechanical stimulation. Activation of JNK1 could not be blocked with the inhibitors, suggesting that an RGD-independent integrin or integrins other than α4β1 can activate JNK1 in cells adherent to fibronectin. This study demonstrates that integrins act as mechanotransducers, providing insight into potential mechanisms for in vivo responses to mechanical stimuli. (*J. Clin. Invest.* 1998. 101:301–310.) Key words: heart • extracellular matrix • adhesion • signal transduction • mechanotransduction

Introduction

Cells respond in various ways to mechanical stimuli. Mechanical stimuli frequently trigger signals leading to increased gene expression, protein synthesis, or mitogenesis (1–3). In other situations cells respond by differentiating (4), rearranging their cytoskeleton or focal contacts (5–8), or altering the composition of their extracellular matrix. These responses have been studied in vitro by using models that include static or cyclic stretch (tensile stress) and fluid flow over the apical surface of cells (shear stress). Cells also respond to mechanical stimuli in vivo. For example, vascular endothelial cells align with the laminar blood flow and can become disorganized in regions of secondary flow, a possible contributing factor in the pathogenesis of arteriosclerosis. Cardiac fibroblasts respond to changes in ventricular hemodynamic loading by increasing matrix production in models of pressure-overload hypertrophy (11) and myocardial infarction (12).

Recent studies have identified some of the intracellular signaling pathways that mediate the biological effects observed upon mechanical stimulation in vitro. These include extracellular signal–regulated kinase (ERK)[1] pathways (13–18) that are frequently stimulated in response to mitogens. c-Jun NH_2-terminal kinase (JNK), a stress-activated protein kinase, is activated in cardiac myocytes after stretch (14) and in endothelial cells in response to flow (15, 18). JNK is also activated in vivo by reperfusion injury after myocardial ischemia (19, 20).

Integrins are a family of αβ-heterodimeric cell surface receptors that mediate cell adhesion to the extracellular matrix. Integrins associate with signaling molecules in the focal adhesion complex (21–25), which acts both as a signaling device and a connection to the cytoskeleton (25–27). The linkage between the extracellular matrix, integrins, signaling molecules, and the cytoskeleton has lead to the hypothesis that integrins are responsible for sensing and transducing mechanical stimuli (18, 28, 29). In recent years, a rapid accumulation of data have demonstrated that integrin ligation and/or clustering can lead to activation of a variety of signaling pathways (30). Interestingly, integrin-mediated adhesion (another potential mechanical stimulus) leads to transient activation of ERK (31–34) and JNK (23) and phosphorylation of several proteins, including focal adhesion kinase $p125^{FAK}$ (35, 36), $p130^{Cas}$ (22, 37), paxillin (38), and tensin (39; for review see references 27 and 40). Inhibitors of cytoskeletal organization can prevent ERK activation (31–33) and tyrosine phosphorylation (33, 41), but not JNK activation (23). JNK is also activated in epithelial cells in response to disengaging the integrins and disrupting the cytoskeleton (detachment of cells), and restoring attachment can prevent JNK activation and rescue those cells from apoptosis (42). Integrins also interact and synergize with various growth factor receptors to initiate specialized signaling responses that depend on the particular integrin ligated (21, 43–46).The integrin and growth factor pathways also appear to act in parallel, converging at the level of Raf-1 kinase (44, 47, 48).

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1. *Abbreviations used in this paper:* ERK2, extracellular signal–regulated kinase; JNK1, c-Jun NH_2-terminal kinase; RGD, arginine-glycine-aspartic acid; UV, ultraviolet.

To test whether integrins can act as transducers of mechanical signals from the extracellular environment to the nucleus, we studied the activation of ERK2, JNK1, and p38, a third parallel kinase in the mitogen/stress-activated protein kinase cascades in stretched cardiac fibroblasts, and evaluated whether these responses were matrix- and/or integrin-dependent. Cardiac fibroblasts were chosen because they have previously been shown to respond to stretch by increasing transcription and production of the matrix proteins collagen (types I and III) and fibronectin (49), which parallels the fibrotic response to changes in hemodynamic mechanical loading observed in vivo (11). We report here that ERK and JNK1 are rapidly activated by stretch, whereas p38 is unaffected. Activation of the JNK1 pathway appeared to involve integrins that bind to fibronectin, laminin, and vitronectin, but not those that bind to collagen, whereas activation of the ERK pathway only involves fibronectin-binding integrins. Using a variety of anti-integrin antibodies and mixed matrices, we were able to conclude further that multiple integrins feed into each pathway. Activation of these pathways may lead to transcriptional activation of matrix proteins in stretched cardiac fibroblasts, but this remains to be studied.

Methods

Cells and tissue culture. Primary cardiac fibroblasts (initially supplied by Francisco Villarreal, University of California, San Diego) were obtained from ventricular tissue of adult Sprague-Dawley rats as previously described (50). In brief, rats were killed using CO_2, and the hearts were quickly removed under sterile conditions. Ventricular tissue was isolated, minced, and digested with bacterial collagenase (100 U/ml; Worthington Biochemical Corp., Freehold, NJ) and pancreatin (0.6 mg/ml; Sigma Chemical Co., St. Louis, MO). After inactivation of enzymes with FCS (Tissue Culture Biologicals, Tulare, CA), cells were allowed to attach to plastic tissue culture dishes for 30 min. Nonadherent cells (primarily myocytes, blood cells, and endothelial cells) were washed away. The fibroblasts are significantly more proliferative than other cardiac cell types, giving rise to virtually pure cultures by the first passage. Cells were maintained in DMEM (Sigma Chemical Co.) containing 10% FCS, glutamine (290 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). Studies were performed when cells were in passages 3 or 4.

Cell deformation. Cells were stretched using devices designed at the University of California, San Diego (50). The devices apply a uniform, static, equibiaxial stretch to a silastic membrane (Specialty Manufacturing, Saginaw, MI) on which the cells were grown. It has previously been demonstrated that the fibroblasts experience the same deformation as the substrate (50). The amount of stretch applied to the cells can be controlled (and measured) by the user. In these studies, we applied 4% equibiaxial stretch to the fibroblasts because the area change is comparable to the changes that could be experienced in vivo. To reduce the hydrophobicity of the material, the membranes were routinely coated with collagen (3.3 $\mu g/cm^2$; Collaborative Biomedical Products, Bedford, MA) in PBS through passive adsorption overnight at room temperature. Other matrix proteins were also used and coated at concentrations that gave rise to saturated adhesion (see Table I). After matrix coating, the membranes and devices were sterilized by ultraviolet irradiation.

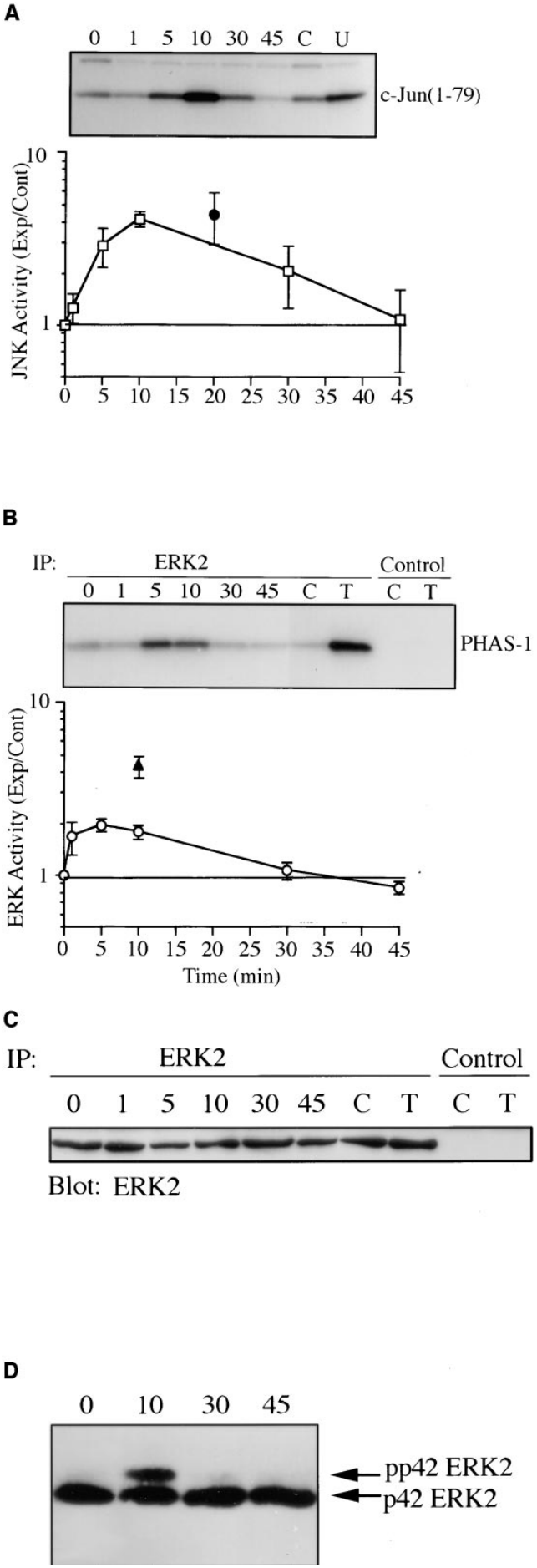
For some studies, cells were plated onto the membranes in normal growth media and were allowed to proliferate, synthesizing their own matrix overnight. The cells were then made quiescent in reduced serum media (containing 0.3% heat-inactivated FCS) for 16–24 h. Cells were mechanically stimulated by stretching the membrane 4%. To study the effects of ligating specific integrins, cells were made quiescent by growth in starvation media as monolayers in standard tissue culture dishes for 16–24 h, detached with 0.05% trypsin (Sigma Chemical Co.), washed once with 100 μg/ml trypsin inhibitor (Sigma Chemical Co.) in starvation media, twice with starvation media alone, and finally plated in the presence of starvation media containing BSA (5 mg/ml). Cells were permitted to spread for 4 or 8 h before they were stretched. This extended delay was intended to promote equal spreading of the cells on all coated matrix proteins without allowing time for new matrix to be synthesized. Additionally, it was intended to allow the kinases to return to basal activity levels after induction by integrin ligation (31–34).

To test whether activation of the kinases was an autocrine/paracrine effect, stretch-conditioned media was applied to cultures that had been prepared in parallel. In these experiments, cells were stretched for various periods (1, 5, or 10 min), and the media was removed from the stretch devices and applied to quiescent monolayers directly, without dilution. The 10 ml of media routinely used in the stretch devices was divided into two 5-ml aliquots and used to replace the media of the quiescent cultures in 6-cm dishes. The cultures were returned to the incubator for 5 or 10 min, and the cells were lysed and processed for kinase activity.

Kinase assays. Kinase assays were performed as described previously (51). In brief, cells were lysed on ice in an NP-40–based lysis buffer (50 mM Hepes pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% NP-40) containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 0.1 U/ml aprotinin, 0.4 μg/ml pepstatin) and the phosphatase inhibitor Na_3VO_4 (1 mM). A total of 50–125 μg of protein was used for each immunoprecipitation (equalized within each experiment). The amount of kinase available to be immunoprecipitated was thus equal for each treatment (data not shown). ERK2, JNK1, or p38 were immunoprecipitated using appropriate antibodies (anti-ERK2 C-14; Santa Cruz Biotechnology, Santa Cruz, CA; anti-JNK1 G151-333; PharMingen, San Diego, CA; anti-p38 C-20; Santa Cruz Biotechnology; or an anti-p38 antibody; a gift from J. Han, The Scripps Research Institute) and protein-A Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden). The anti-ERK2 antibody (C-14) has some cross-reactivity with ERK1, so we will refer to both kinases as ERK. For the ERK kinase assays, myelin basic protein (MBP; Sigma Chemical Co.) or PHAS-1 (Stratagene, La Jolla, CA) were used as the substrate at 5 μg/reaction. As a positive control for ERK activation, quiescent cell cultures were treated with 500 nM TPA (phorbol 12-myristate 13-acetate, Sigma Chemical Co.) for 10 min before lysis. GST-c-Jun (1–79) was used as the substrate for the JNK1 kinase assays. Purified GST-ATF-2 or PHAS-1 was used as the substrate for the p38 kinase assay. Ultraviolet (UV) irradiation with 40 $\mu Ji/cm^2$ followed by incubation at 37°C for 20 min was used as a positive control for JNK1 and p38 activation. Kinase reactions containing the immunoprecipitated kinase, substrate, 10 μM ATP, and 5 μCi [γ-^{32}P] ATP in the kinase buffer (50 mM Hepes pH 7.6, 10 mM $MgCl_2$) were incubated at room temperature for 30 min before separation by SDS-PAGE. Kinase activity was detected by autoradiography (BioMAX MR; Eastman Kodak Co., Rochester, NY) and quantified by scanning the autoradiographs with a flatbed scanner (Hewlett-Packard Co., Palo Alto, CA) and measuring with Image 1.57 software (National Institutes of Health, Bethesda, MD).

Immunoblotting. Activation of the kinases was also evaluated using the mobility shift associated with the active phosphorylated form, or using a phosphospecific antibody in immunoblotting. Equal amounts of protein (10–30 μg equalized within each experiment) were run on a gel using SDS-PAGE, transferred to Immobilon-P (Millipore Corp., Bedford, MA) using a CAPS transfer buffer, and probed with an anti-ERK2 antibody (C-14; Santa Cruz Biotechnology), a phosphospecific anti-p38 antibody (New England Biolabs Inc., Beverly, MA) or phosphospecific anti-ERK2 antibody (New England Biolabs Inc.).

Adhesion assays. Adhesion to various matrix proteins was tested in microtiter plates coated with 0.0005–30 $\mu g/cm^2$ protein (0.0015–100 μg/ml). Cells were allowed to attach for 60 min, and nonadherent cells were washed away with PBS. Cells were fixed with 4%



paraformaldehyde in PBS and stained overnight with crystal violet (5 mg/ml) in 20% methanol. Stained cells were solubilized with 2% SDS, and the OD was measured at 590 nm. Inhibition of adhesion by various antibodies and/or peptides to specific rat integrins was also tested by incubating the suspended cells with various concentrations of the inhibitors for 30 min at 37°C with occasional mixing before plating. The antibodies to α5 (HMα5-1), α4 (MRα4-1), β1 (Ha2/5), and β3 (F11) were from PharMingen, and αvβ5 (P1F6) was from Telios Pharmaceuticals (San Diego, CA). The GRGDSP peptide was from Immuno-Dynamics, Inc. (La Jolla, CA).

Immunoprecipitation. Surface proteins were labeled with either I^{125} or biotin. Cell surface proteins were iodinated using lactoperoxidase. Cells were suspended using EDTA, washed, and mixed with the reaction mixture (1 mCi ^{125}I, 200 μg lactoperoxidase, and 20 μl of 0.12% hydrogen peroxide). After 20 min the reaction was quenched with 0.02% sodium azide, and cells were washed in PBS. To label with biotin, attached cells were incubated with Sulfo-NHS Biotin (Pierce, Rockford, IL) 1 mg/ml in PBS for 20 min on ice. Cells were washed, and free sites were quenched with 100 mM glycine. Whether labeled with iodine or biotin, the cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris pH 8.0) containing protease inhibitors, the insoluble fraction was precipitated, and lysates were used for immunoprecipitation. The labeled integrins were immunoprecipitated with a variety of rabbit antibodies to the cytoplasmic domains of the integrin subunits generated in our laboratory (5 μl of each antisera and 20 μl protein A–Sepharose beads). The α1-containing integrin was immunoprecipitated with AB1934, an antibody from Chemicon International (Temecula, CA). Iodinated integrins were identified by autoradiography. Biotinylated integrins were identified with horseradish peroxidase–linked streptavidin, and were visualized with chemiluminescence substrate (Pierce).

Mixed matrix experiments. To differentiate the contribution of individual integrins, stretch experiments were also performed using mixed matrices and addition of various integrin inhibitors (combinations of collagen and fibronectin or vitronectin and fibronectin; see Table I for coating concentrations). Matrix proteins were added together in PBS and allowed to coat the membrane through passive adsorption overnight. Adhesion to the mixed matrices was tested in parallel with individual matrix proteins to verify that in the combination maximal adhesion was similar to the separate matrix proteins. Moreover, both components in each combination appeared to coat the substrate similarly because antibodies that inhibited adhesion to a single matrix protein were not able to block adhesion to the mixed matrices (see Results). Stretch experiments with addition of various inhibitors were performed only using combinations of antibodies and peptides that inhibited adhesion on one of the individual matrices, but did not affect adhesion to the mixed matrices. As with the adhesion assays,

Figure 1. In vitro kinase assays for (*A*) JNK1 and (*B*) ERK. Cardiac fibroblasts were plated in the presence of growth media overnight, made quiescent by incubation in starvation media for 24 h, and stimulated with static 4% equibiaxial stretching (*open symbols*) for various time points (min). Cells were stimulated with UV irradiation (for JNK1) or TPA (for ERK) as positive controls (*closed symbols*). Equal amounts of total protein were subjected to immunoprecipitation with antibodies to JNK1 or ERK2, and in vitro kinase assays were performed using GST-c-Jun(1–79) for JNK1 or MBP or PHAS1 for ERK as the substrates. Activity was quantified (±SEM) using a flatbed scanner and NIH Image using an unstimulated control as the reference state. *C*, unstimulated control; *U*, UV irradiation; *T*, TPA. (*C*) Immunoblotting for ERK2 after immunoprecipitation with an anti-ERK2 antibody to illustrate equal loading for each kinase assay. (*D*) Immunoblotting for ERK to illustrate shift in mobility due to activation by stretch and TPA. p42 is the inactive ERK2 and pp42 is the phosphorylated, active form of ERK2.

cells were incubated in suspension with the integrin antibodies for 30 min before plating on the matrix protein combinations. In these experiments cells were permitted to spread on the substrate for 8 h before stimulation.

Results

ERK and JNK1, but not p38, are activated by stretch in cardiac fibroblasts. JNK1 (Fig. 1 *A*) and ERK2 (Fig. 1 *B*) were both activated in adult rat cardiac fibroblasts stretched equibiaxially by 4% (a physiologically relevant magnitude) on a deformable membrane. The time courses and magnitudes for the activation of the two kinases were different. JNK1 activity increased within 5 min, reached a fourfold peak at 10 min, and returned to basal levels by 45 min. UV irradiation used as a postive control yielded a similar induction after 20 min. ERK was also activated by stretch with a similar time course and magnitude (peaking with a twofold induction at 5 min and returning to basal levels by 30 min). Immunoprecipitation with an irrelevant antibody (against AKT; Santa Cruz Biotechnology, Santa Cruz, CA) did not demonstrate significant kinase activity. Immunoblotting of the immunoprecipitations demonstrated that equal amounts of ERK2 were present in each kinase assay, and that the differences in kinase activity were caused by intrinsic differences in the kinase activity, not differences in amount of kinase (Fig. 1 *C*). Activation of ERK was further demonstrated by analyzing the change in mobility in SDS-PAGE (Fig. 1 *D*). Stretch and TPA stimulation both caused a shift to a slower-migrating, active form of ERK2. Longer exposures revealed p44-ERK1 as a larger band, which also appeared to be activated (data not shown). Activation of ERK2 was also confirmed through the use of a phosphospecific antibody, which gave the same result as the kinase assay and mobility shift (data not shown). The control ERK2 antibody confirmed that equal amounts of ERK2 were present in the lysate of all treatments (data not shown).

In contrast to the results observed for JNK1 and ERK, p38 did not appear to be appreciably activated by stretch (Fig. 2). Various other interventions were tested for their ability to activate p38, and only UV and osmotic shock demonstrated a capacity to activate p38 in the cardiac fibroblasts. The apparent lack of activation of p38 by stretch was also found by immunoblotting with an antibody that recognizes the activated, phosphorylated form of p38 (data not shown).The control anti-p38 (not phosphorylated) confirmed there were equal amounts of p38 in the lysate of all treatments (data not shown).

Activation of JNK1/ERK is a not an autocrine/paracrine effect. In some cases, changes in growth rate and protein synthesis in response to mechanical stimulation have been shown to be secondary effects caused by secretion of growth factors (PDGF, TGFβ, and angiotensin II in particular; 3, 14, 29, 52–54). To test whether induction of JNK1 and ERK was a direct effect or due to the secretion of some factor(s) into the culture media, conditioned media collected from cells stretched for a variety of time points were applied to quiescent cells in plastic tissue culture dishes. Since the peak of activation in the cells occurred in < 10 min, JNK1 (Fig. 3 *A*) and ERK (Fig. 3 *B*) activity was measured after additional incubation for 5 or 10 min. Any change in kinase activity observed was very small compared with the activation directly induced by the stretch. These data suggest that the JNK1 and ERK activation was the result of direct activation of a signaling cascade rather than the result of a paracrine factor.

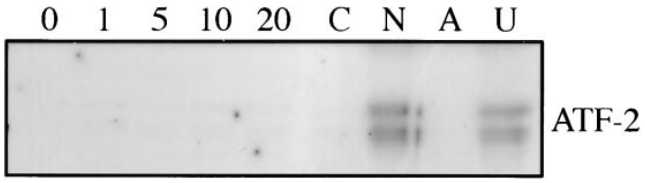


Figure 2. p38 is not activated in response to stretch in cardiac fibroblasts. (*A*) In vitro kinase assays for p38 as described in Fig. 1 using specific p38 antibodies and GST-ATF2 as the substrate. Osmotic stress and UV irradiation were used as positive controls. Anisomycin was also tested for a positive control. *C*, unstimulated control; *N*, 0.7 M NaCl; *A*, anisomycin; *U*, UV irradiation (recover 20 min); *U′*, UV irradiation (recover 45 min); *T*, TPA.

Integrin expression profile in cardiac fibroblasts and adhesion of the fibroblasts to extracellular matrix proteins. The experiments described thus far were conducted with rat cardiac fibroblasts grown in the presence of serum overnight and made quiescent by removing FCS from the media for 24 h before stretching. Although the membranes were initially coated with collagen to reduce hydrophobicity, the fibroblasts have likely produced their own complex matrices in this time frame. To test the matrix and integrin specificity of the responses to stretching, it was necessary plate the cells on purified matrix proteins before stretching. However, that plating required examination of the adhesion characteristics and integrin profiles in these cells.

The rat cardiac fibroblasts attached best to collagen and laminin, followed by vitronectin and fibronectin (Table I). Various integrins were immunoprecipitated from cell lysates of cell surfaces labeled with ^{125}I or biotin (Fig. 4). The most prevalent integrins were α1β1, α4β1, and α5β1. Other prominent integrins were α2β1, α3β1, α6β1, and αvβ3. The presence

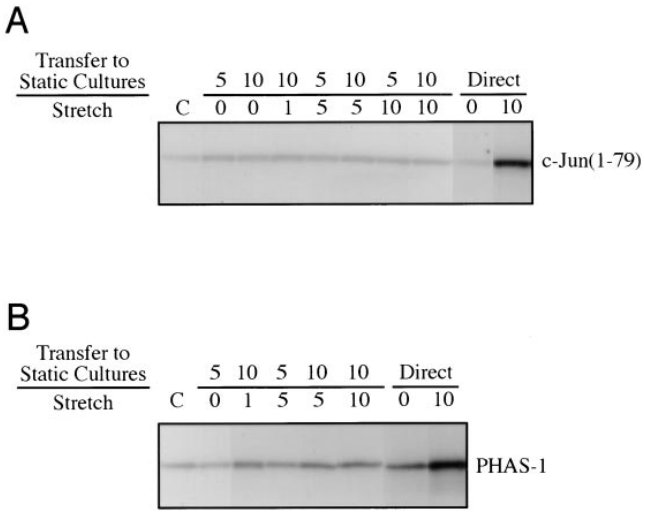


Figure 3. Effect of stretch-conditioned media on in vitro kinase activity of (*A*) JNK1 and (*B*) ERK. Tissue culture media from cardiac fibroblasts exposed to stretch for varying times (0–30 min) were transferred to quiescent fibroblast cultures for 5 or 10 min before lysis and evaluation of kinase activity. The direct activation of JNK1 and ERK in the stretched cultures is shown in the *right lanes* for comparison. *C*, unstimulated control.

Table I. Matrix-dependent Adhesion Characteristics of Rat Cardiac Fibroblasts

Matrix protein	Concentration at 50% adhesion	Coating density
Collagen	0.3 μg/ml	2 μg/ml
	0.1 μg/cm^2	0.67 μg/cm^2
Laminin	1.5 μg/ml	5 μg/ml
	0.5 μg/cm^2	1.67 μg/cm^2
Vitronectin	2.0 μg/ml	10 μg/ml
	0.66 μg/cm^2	3.3 μg/cm^2
Fibronectin	10.0 μg/ml	20 μg/ml
	3.3 μg/cm^2	6.67 μg/cm^2

of some other integrins was evaluated with inhibitory antibody experiments (see below).

To determine which integrins mediated adhesion to the various substrates, anti-rat integrin (α1, α4, α5, β1, β3, and αvβ5) function–blocking antibodies or compounds were tested for their ability to block adhesion to concentrations of the matrix proteins that give rise to saturated adhesion (Table II). In most cases, no particular antibody alone inhibited adhesion to the substrates significantly at the concentrations tested. However, combinations of the antibodies decreased adhesion and combinations of several antiintegrin antibodies with an RGD peptide adhesion could block it completely. This result suggests that cell adhesion in these rat cardiac fibroblasts is completely or in large part mediated by integrins, and that multiple integrins contribute to cell adhesion on any given substrate. For collagen (Table II, column 1) adhesion was blocked by any combination that included anti-β1 antibodies, but not by the RGD peptides or the other integrin antibodies. The α1-blocking antibody alone did not block adhesion to collagen. This result does not preclude the contribution of α1 to adhesion to collagen because both α1β1 and α2β1 integrins are strongly expressed in these cells, and α2-specific reagents

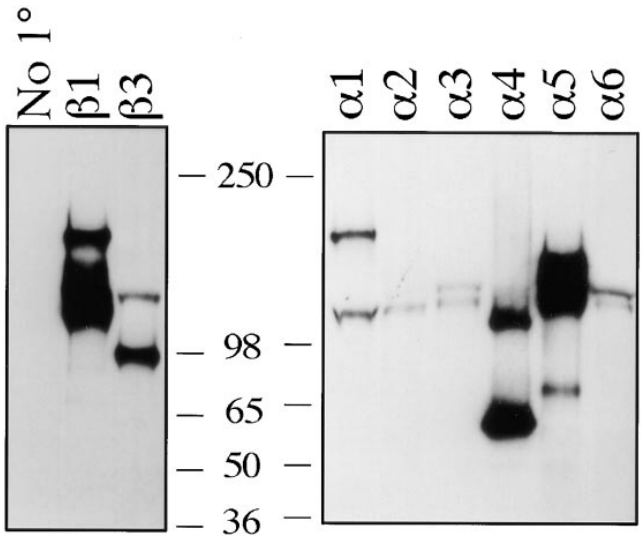


Figure 4. Integrin profile in adult rat cardiac fibroblasts. Immunoprecipitation of integrins from lysates of surface-labeled (biotin) cardiac fibroblasts.

Table II. Percent Inhibition of Adhesion by Antibody and/or Peptide Incubation Before Plating

	Matrices*				
Inhibitors	Coll	VN	FN	FN + Coll	FN + VN
α1	0	—	—	—	—
α4	0	0	0	0	0
α5	0	0	0	0	0
β1	75	0	15	25	0
β3	0	0	0	0	0
β5	—	10	0	—	—
RGD	0	100	0	—	40
α4 + α5	0	0	0	0	0
α4 + RGD	—	100	30	—	60
α4 + β3	—	0	0	—	0
α4 + α5 + RGD	0	100	40	0	60
α4 + β3 + RGD	—	100	20	—	50
β1 + RGD	75	100	95	100	100
β3 + RGD	0	100	25	0	50
β1 + β3	80	30	50	50	0
β1 + β5	—	60	—	—	—
β3 + β5	—	30	—	—	—
β1 + β3 + β5	—	60	—	—	—
α4/α5/β1/β3/RGD	75	100	100	100	100

**Coll*, Collagen; *FN*, fibronectin; *VN*, vitronectin; —, not tested.

for rat integrins were not available for these studies. Adhesion to vitronectin (Table II, column 2) was completely blocked by the RGD peptide. Addition of anti-β1, anti-β3, or anti-αvβ5 antibodies alone did not significantly inhibit adhesion, but the combination of any two inhibited it between 30 and 60%. Simultaneous addition of all three antibodies further decreased adhesion, but did not attain complete inhibition. Therefore, αvβ1, αvβ3, and αvβ5 all appear to mediate adhesion to vitronectin, and another RGD-directed integrin (perhaps αvβ8) also participate(s) in mediating the adhesion. Neither RGD peptides, anti-α4, anti-α5, anti-β3, nor anti-αvβ5 antibodies alone significantly affected adhesion to fibronectin (Table II, column 3). Anti-β1 antibodies alone had a small but consistent effect on adhesion, suggesting that many β1 integrins mediate adhesion to fibronectin, but other non-β1 integrins contribute to it. Combining anti-α4, anti-α5, and RGD peptides together inhibited adhesion by 40%, while the combination of anti-β1 antibodies and RGD peptides blocked it completely. This result suggests that a non-RGD–directed, β1-containing integrin (other than α4β1) can also mediate adhesion to fibronectin (α3β1 is a candidate).

Matrix-dependent activation of JNK1 and ERK in response to stretch. We reasoned that if the JNK1/ERK activation in stretched cells is mediated by integrins, integrins might differ from one another in this regard. This difference would manifest itself as differential responses on various matrices. We therefore examined JNK1 and ERK activation in cells that had been plated on specific matrices.

The cells on all the matrices tested were completely spread by 2–3 h after plating. The adhesion and morphological characteristics were not different between substrates. When the cells were allowed to spread for 4 h before stretch, there was no induction of kinase activity for either JNK1 or ERK on any

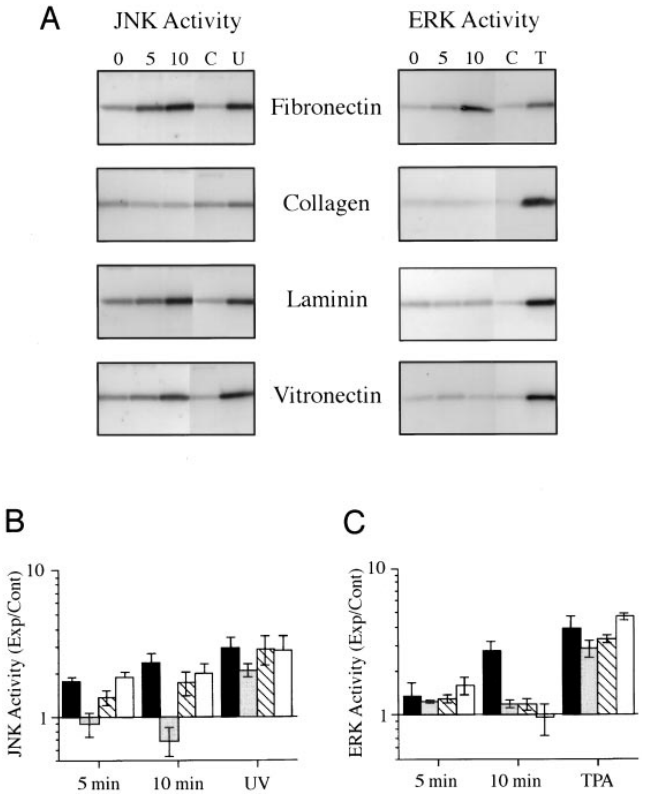


Figure 5. Matrix-dependent regulation of JNK1 and ERK activity in response to stretch. Rat cardiac fibroblasts were made quiescent by growth in starvation media before replating on extracellular matrices. Cells were stretched for 0–10 min, stimulated with UV irradiation for 20 min (positive control for JNK1), or treated with TPA for 10 min (positive control for ERK). (*A*) Representative in vitro kinase assay for JNK1 (*left*) and ERK (*right*) 8 h after plating on the extracellular matrix proteins. Mean (±SEM) induction of JNK1 (*B*) and ERK (*C*) activity for cells plated on fibronectin (*black*), collagen (*stippled*), vitronectin (*striped*), and laminin (*white*) for 8 h before stretching. *C*, unstimulated control; *U*, UV irradiation; *T*, TPA.

of the substrates (data not shown). The cells that had spread for 4 h were able to respond to TPA with ERK activation, but UV caused no stimulation of JNK1. When the fibroblasts were incubated for 8 h before stretching (Fig. 5 *A* shows example kinase assays for each substrate), the ability of both JNK1 (Fig. 5 *B*) and ERK (Fig. 5 *C*) to respond to both stretch and the treatments used as positive controls was restored. Differences between the matrix proteins were observed. When cells were plated on fibronectin, they were able to activate both ERK and JNK1 at levels that were similar to those from the studies when the cells were plated overnight in the presence of serum. Cells plated on vitronectin or laminin were only able to activate JNK1 consistently. In cells plated on collagen, stretch did not stimulate either kinase, but UV and TPA did stimulate their appropriate kinases to the same degree as when the cells were plated on the other matrix proteins. Two independent matrices were tested at random in each experiment. Basal ERK2 and JNK1 activities did not vary with matrix protein in any experiment.

Integrin-dependent activation of JNK1 and ERK in response to stretch. Because adhesion to the individual substrates appears to be mediated in large part if not completely by integrins, it seems possible that the matrix-specific differences were due to activation of the signaling pathways controlled by specific integrins. However, we have also demonstrated that multiple integrins participate in cell adhesion to each of the matrix proteins. Therefore, it is not yet clear whether a single integrin or several different integrins link to the activation of JNK1 and ERK on these substrates. To study this, the stretch experiments were performed using substrates that were combinations of two matrix proteins. Adhesion to only one substrate was blocked with inhibitors to specific integrins to assure that differences in spreading or adhesion were not confounding factors. Cells adhered to the same degree on combinations of fibronectin and collagen as well as fibronectin and vitronectin as they did on fibronectin, collagen, or vitronectin individually (data not shown). In each mixture, both matrix proteins coated the substrate effectively because adhesion to the mixed matrices behaved as one would expect if the fibroblasts were using the full complement of integrins for each matrix protein (Table II, columns 4 and 5). For example, the RGD peptide, which completely blocks adhesion to vitronectin but does not affect adhesion to fibronectin, had an intermediate effect on adhesion to the mixture of fibronectin and vitronectin. Additionally, anti-β1 antibodies, which block adhesion to collagen but only minimally inhibit adhesion to fibronectin, had only a slight effect on a mixture of fibronectin and collagen. Stretch-induced activation of ERK on the mixed matrices (Fig. 6 *A*) was similar to that of fibronectin alone, suggesting that the integrins used to bind collagen or vitronectin were not activating negative regulatory pathways.

Cells plated on a mixture of collagen and fibronectin for 8 h were treated with combinations of anti-α4, anti-α5, and the RGD peptide. This cocktail, which blocks adhesion to fibronectin by 40%, but had no effect on adhesion to collagen or the combination of fibronectin and collagen (Table II, column 4), inhibited activation of ERK by stretch (Fig. 6 *A*). Pretreatment by the RGD peptide alone or by a combination of anti-α4 and α5 antibodies (without the RGD peptide) could not block the activation. ERK activation was also not blocked by the treatment with the anti-β1 antibody when the cells were plated on a combination of vitronectin and fibronectin. Again, the anti-β1 antibody slightly inhibited adhesion to fibronectin and collagen, but did not block adhesion to vitronectin and fibronectin. Altogether, these results suggest that multiple fibronectin binding integrins activate ERK in response to mechanical stimulation. In particular, results from the experiments performed on a mixture of collagen and fibronectin suggest the following: (*a*) a non-RGD-directed integrin is one of the mediators because the RGD peptide does not block activation of ERK, and integrins mediating adhesion to collagen are not capable of activating ERK; (*b*) stimulation through α4β1, which is a non-RGD-directed integrin, can lead to ERK activation because when antibodies to α4 and α5 (which is itself an RGD-directed integrin) are added to the cultures along with the RGD peptide, ERK activation is blocked; (*c*) one or more RGD-directed integrins are used for activating ERK because the combination of the α4 and α5 antibodies without the RGD peptides cannot block activation; and (*d*) finally, as indicated by the experiments performed on a combination of fibronectin and vitronectin, at least one of the RGD-directed integrins that mediates ERK activation is not a β1-containing integrin because although some of the RGD-directed integrins that

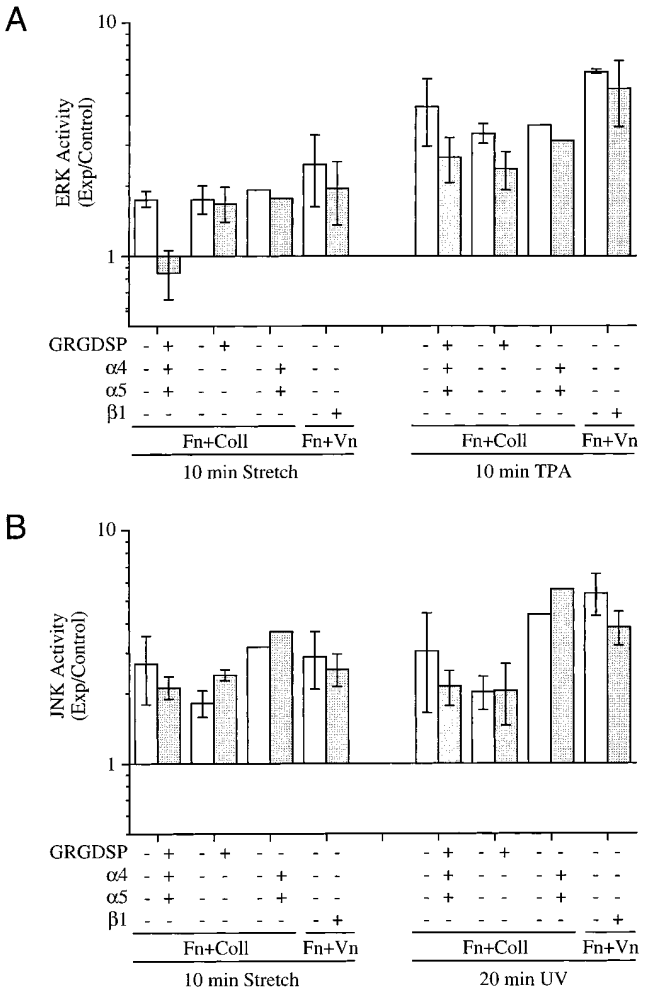


Figure 6. Inhibition of stretch-induced (*A*) ERK and (*B*) JNK1 activation (mean±SEM) on mixed matrices (fibronectin and collagen or fibronectin and vitronectin) by various antibody (anti-α4, anti-α5, or anti-β1) and peptide (*GRGDSP*) inhibitors. Before plating on the mixed substrates for 8 h, the fibroblasts were detached and treated with the indicated antibody and/or peptide inhibitors for 30 min. Cells were stretched for 10 min or treated with UV or TPA as positive controls and lysed, and in vitro kinase assays were performed.

bind fibronectin are β1 integrins, the antibodies to β1 cannot block ERK activation.

Activation of JNK1 by stretch occurs on all matrix proteins except collagen, so in the experiments outlined above, JNK1 activity was only expected to change when the cells were plated on collagen and fibronectin and the fibronectin-binding integrins were blocked. However, pretreatment with the RGD peptide and antibodies to both α4 and α5 integrins together did not inhibit JNK1 activation in response to stretch (Fig. 6 *B*), suggesting that the remaining integrin(s) responsible for adhesion to fibronectin can mediate activation of JNK1. As expected, anti-β1 antibodies did not affect JNK1 activation when the cells were plated on the combination of fibronectin and vitronectin because both substrates elicit an activation of JNK1 individually. These data suggest that JNK1 activation by stretch is also modulated by a variety of integrins, including at least one non-RGD-directed, non-α4β1 integrin (such as α3β1) as well as RGD-directed fibronectin and vitronectin integrins.

Discussion

We show here that cardiac fibroblasts respond to biaxial stretch by transiently activating the JNK1 and ERK cascades, while p38 is not affected by stretching. Activation of JNK1 and ERK is a direct result of stretch, and appears to be mediated by integrins. Moreover, the fibroblasts can differentially regulate the JNK1 and ERK pathways though integrins, and multiple integrins appear to be linked to both of these pathways.

Activation of ERK has previously been demonstrated in various cell types in response to mechanical stimuli (13–18). Recent papers have demonstrated additional activation of JNK (and ERK) in cardiac myocytes in response to stretch (14), and in endothelial cells in response to shear flow (15, 18). Activation of ERK, but not JNK, in cardiac myocytes could be blocked with inhibitors to angiotensin II (14, 16). We found evidence that stimulation by stretching cardiac fibroblasts is directly linked to the ERK and JNK1 pathways, because stretch-conditioned media did not activate the kinases. The relatively modest quantitative induction of the kinases is a consequence of the quantitation method (image analysis of a scanned autoradiograph). The nonlinearity of the radiographic film was difficult to control, as the samples reached saturation quickly. Activation of JNK1 by stretch was as strong as that by ultraviolet irradiation, and the activation of ERK2 was half as strong as that by TPA.

Stretching did not activate p38. This uncoupling of the two stress-activated protein kinases, JNK and p38, is not common. In vitro, most stimuli lead to activation of both pathways (56) while certain stimuli such as osmotic stress lead to activation of p38, but not JNK. Some stimuli that activate p38 in other cell types did not strongly activate p38 in the cardiac fibroblasts, while others, such as osmotic stress, did activate this kinase (Fig. 2 and data not shown). This result suggests that the p38 signaling cascade may not play as important a role in signaling from physiologic stresses in cardiac fibroblasts as does JNK1. During myocardial ischemia, the heart (apparently the myocytes) activates p38, but requires reperfusion to subsequently activate JNK (20). An emerging paradigm is that the balance between mitogen- and stress-activated pathways helps to determine cell fate (57). Transformation (58), apoptosis (57), differentiation (59), and morphologic changes (60) can all be downstream consequences of upregulating these kinases. When static equibiaxial stretch is applied to cardiac fibroblasts, JNK1 and/or ERK activation does not cause apoptosis or proliferation (data not shown). The stimulus used in these studies is physiologically relevant because changes in hemodynamic loading leads to alterations in the extracellular matrix in vivo, and the magnitude of stretch applied here causes modification of the extracellular matrix in vitro (49). Therefore, our results seem to fit with the general paradigm, but extend it by suggesting that the three-way balance between ERK, JNK, and p38 may help to provide multiple outcomes for a variety of cellular signals.

Activation of ERK and JNK1 appears to be controlled delicately by specific integrin-mediated signaling pathways. The basal activities of ERK and JNK1 were similar on all matrix proteins, and had similar maximal activities by the positive controls, demonstrating that the kinases have the same poten-

tial on all matrix proteins tested. However, ERK was only activated when cells were stretched while plated on fibronectin. We found that multiple fibronectin-binding integrins, including α4β1 and additional RGD-directed integrins, regulate ERK. Interestingly, the integrins for collagen, vitronectin, and laminin did not link into this stretch-activated pathway for ERK. The vitronectin and laminin integrins did feed into the JNK pathway; however, we were not able to determine which ones specifically participated. Activation of JNK1 appears at least in part to be through the non-RGD-directed, β1 integrin(s) that are responsible for adhesion to fibronectin in the presence of the inhibitors to α4 and α5 and the RGD peptides. This integrin could be α3β1, but it would not be acting alone because anti-β1 antibodies could not prevent activation of JNK1.

Few studies to date have addressed the contribution of integrins to mechanotransduction. Wilson et al. (29) have performed some studies to test whether plating cells (R22D smooth muscle cells) on different matrices affected stretch-induced proliferation. They had previously shown that the proliferative effect induced by stretch was caused by secretion of PDGF in response to the mechanical signal (3). Similar to our studies, Wilson et al. (29) found that stretching cells adherent to fibronectin produced the greatest effect, while the response to stretch when adherent to vitronectin was modulated by the vitronectin concentration. They were able to inhibit the proliferation in response to stretch on collagen-coated plates by addition of exogenous RGD peptides; the RGD peptide alone had no significant effect on fibronectin-mediated activation of JNK1 or ERK in our study. However, an RGD-directed integrin does participate, because if used in concert with the α4 and α5 antibodies it can block ERK activation. Their cells experienced a slight change in shape after adding the RGD peptides (29) even though collagen binding is generally not RGD-directed (55), suggesting that the cells may have assembled a complex matrix of fibronectin and vitronectin in addition to the collagen coating. We plated the fibroblasts on the matrix proteins for only 8 h, reducing the contribution of matrix synthesis from the cells. Because the antibodies would likely inhibit adhesion to the desired matrix components, we used substrates of mixtures of two matrix proteins. In addition to the differences in experimental setup, the differences in the findings of Wilson et al. (29) and the current study may be due to tissue-specific differences in integrin mechanical signaling pathways, or they may reflect variations in signaling that can lead to numerous biological outputs.

Stretch-induced signaling by integrins appears to link into the established pathways of ERK and JNK. We did not examine which upstream molecules regulate the stretch signaling. Other integrin-dependent signals including simple ligation and/or clustering of integrins initiate a myriad of events that parallels growth factor signaling from tyrosine phosphorylation of various cytoskeletal and signaling molecules such as p130cas (22, 37), paxillin (38), and tensin (39), to activation of kinases such as FAK (61, 62), PKC (63), ERK (31–34), and Src (64). In our cells, stretch-induced activation of ERK and JNK1 did not appear to require dramatic changes in phosphotyrosine-containing proteins since the general phosphotyrosine profile induced by stretch was not significantly affected (data not shown). However, this does not preclude the involvement of tyrosine kinases. In myocytes, stretch causes phosphorylation of various proteins (13), but tyrosine kinase inhibitors cannot block activation of ERK. Therefore, ERK may be acting though a secondary pathway. Many integrin-mediated events can be blocked with cytochalasin (an inhibitor of actin polymerization), suggesting a direct role of the cytoskeleton in integrin signaling (32, 65). However, JNK activation upon integrin clustering (23) and c-fos expression after stretch (66) are both independent of the cytoskeleton, again suggesting alternate pathways. Stretching myocytes causes activation of p21Ras (13), which is frequently found upstream of ERK, and occasionally found upstream of JNK. Whether integrin-mediated activation of ERK or JNK requires signaling through p21Ras is not yet clear. FAK phosphorylation, which occurs upon integrin engagement and activates the kinase, leads to FAK association with the Grb2-Sos complex, which may lead to activation of p21Ras (34, 47). More recent reports suggest that integrin-induced ERK activation can also take place in a FAK-independent manner, suggesting the existence of an additional pathway (67, 68). Lin and co-workers (44, 67) suggest that integrin-dependent ERK activation takes place at the level of Raf-1, not by modulating the Grb2-Sos-Ras connection. Flow-stimulated activation of AP-1 is dependent on p21Ras, but acts through JNK, not ERK (15). Therefore, one possibility is that in response to mechanical stimuli, the fibronectin, vitronectin, and laminin integrins act through the p21Ras pathway to activate JNK, and the fibronectin integrins use a yet unclear pathway to activate ERK.

An interesting effect that was observed in this study is that there is a refractory period associated with activation of JNK1 and ERK. Neither ERK or JNK1 were stimulated by stretch in cells that were plated on the matrix proteins for 4 h before mechanical stretching. Ultraviolet irradiation, which strongly activates JNK1 in cells plated in 10% serum for 24 h, was also not able to activate JNK1 at the 4-h time period. In contrast, TPA (but not stretch) was able to activate ERK at that time frame. Various investigators have demonstrated that ERK is transiently activated in response to cell adhesion and spreading, returning to basal levels by 2 h (68, 69). It is possible that the negative feedback pathway that rapidly inactivates ERK during spreading is still upregulated 4 h after plating, or that some yet undefined intracellular factor is depleted during this process since ERK cannot be restimulated by stretch until later times. The initial stimulus for JNK1 may be detachment or integrin ligation since both can lead to JNK activation in other systems (23, 42). However, the refractory signal appears to be stronger for JNK1 because strong signals such as UV irradiation were not able to activate JNK1 after 4 h.

Using the current biaxial stretch system and rat cardiac fibroblasts, Lee (49) has shown that stretch leads to increased expression of the extracellular matrix proteins: collagen types I and III and fibronectin. A similar result was also found in neonatal cardiac fibroblasts (6) and glomerular mesangial cells (10). Cells may remodel their extracellular matrix to adjust to the change in mechanical environment, perhaps as a feedback mechanism ultimately to decrease the strain. This result closely parallels changes that occur in vivo after alterations in mechanical environment such as aortic banding (11). Activation of JNK1 leads to phosphorylation of c-Jun, which is a component of the transcription factor AP-1. This transcription factor can activate many genes, including collagenase, that may aid in remodeling the matrix (70). JNK has also been identified to be activated in vivo after reperfusion injury associated with myocardial ischemia (19, 20), during which time

cells also experience abnormally high degrees of stretch and remodel their collagen matrix through increased activity of metalloproteinases (71). Therefore, activation of JNK1 through the fibronectin-, laminin- and vitronectin-binding integrins and ERK through the fibronectin-binding integrins may lead to remodeling of the extracellular matrix in response to stretch. Interestingly, the collagen receptors do not partake in this regulation. This fact may reflect the observation that the large collagen fibers maintain the majority of tissue stress during the normal filling cycle of the heart (72).

In summary, the data presented here support the idea that integrins are mechanotransducers, transforming mechanical stimuli into chemical signals. Integrin-mediated signaling uses established signaling cascades that initiate molecules that frequently are localized in the focal adhesion complex, and lead to activation of downstream kinases such as ERK and JNK. We found that integrin–ligand specificity confers additional control over the response of cardiac fibroblasts to stretch. The mechanism of this control is not yet understood; however, existing evidence suggests that association of the various signaling molecules in the focal adhesion complex is probably involved. Understanding the control of stretch-mediated responses in cardiac fibroblasts provides insight into regulation of the fibrotic diseases that occurs in vivo.

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