Src and focal adhesion kinase mediate mechanical strain-induced proliferation and ERK1/2 phosphorylation in human H441 pulmonary epithelial cells

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Chaturvedi LS, Marsh HM, Basson MD. Src and focal adhesion kinase mediate mechanical strain-induced proliferation and ERK1/2 phosphorylation in human H441 pulmonary epithelial cells. Am J Physiol Cell Physiol 292: C1701–C1713, 2007. First published January 10, 2007; doi:10.1152/ajpcell.00529.2006.—Pulmonary epithelial cells are exposed to repetitive deformation during physiological breathing and mechanical ventilation. Such deformation may influence pulmonary growth, development, and barotrauma. Although deformation stimulates proliferation and activates extracellular signal-regulated kinases (ERK1/2) in human pulmonary epithelial H441 cells, the upstream mechanosensors that induce ERK activation are poorly understood. We investigated whether c-Src or focal adhesion kinase (FAK) mediates cyclic mechanical strain-induced ERK1/2 activation and proliferation in human pulmonary epithelial (NCI-H441) cells. The H441 and A549 cells were grown on collagen I-precoated membranes and were subjected to an average 10% cyclic deformation in many (23, 25, 34, 40, 57, 59) but not all (19, 24) other cell types.

We therefore sought to determine whether ERK mediates the mechanical effects of cyclic strain on human pulmonary epithelial cells. We further sought to define the upstream mediators responsible for such ERK activation. In particular, we further hypothesized that strain activation of non-receptor tyrosine kinases, specifically focal adhesion kinase (pp125FAK, also known as protein tyrosine kinase 2) and Src, might mediate both strain-induced ERK activation and proliferation in H441 pulmonary epithelial cells. In the present study, we investigated FAK, Src, and ERK signaling in response to cyclic mechanical strain in H441 pulmonary epithelial cells. We then evaluated the effects of inhibiting these signals to begin to trace the mechanotransduced pathway that links these signals into a mitogenic cascade.

EXPERIMENTAL PROCEDURES

Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, Oligolectamine, and Plus reagent were obtained from Invitrogen (Carlsbad, CA). Western blot stripping reagent was obtained from Chemicon International (Temecula, CA). Human transferrin was obtained from Roche Applied Science (Indianapolis, IN). Trypsin was obtained from Sigma (St. Louis, MO). Phosphospecific polyclonal antibodies to FAK at Tyr197 or at Tyr576 were obtained from Biosource International (Camarillo, CA). Phosphospecific polyclonal antibodies to p44/p42 (p-ERK1/2) Thr202/Tyr204, Src at Tyr116 (which recognizes human phospho-Src Tyr416), rabbit polyclonal antibody to total p44/p42 (t-ERK1/2), and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were obtained from Cell Signaling (Beverly, MA). Mouse monoclonal anti-FAK (clone 4.47) and rabbit monoclonal anti-Src antibodies were obtained from Upstate Cell Signaling Solutions (Charlottesville, VA). 2′-Amino-3′-methoxyllavone (PD-98059), 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine (PP2), and α-tubulin monoclonal antibody were obtained from EMD Biosciences (San Diego, CA). Double-stranded short interfering

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RNAs (siRNAs) targeting human forms of FAK, Src, and control nontargeting 1 (NT1) sequences were purchased from Dharmacon (Lafayette, CO). Sequences targeted by siRNAs were selected using Dharmacon SMART design: human FAK1, NGGCAUGUGGC-CUGCUAUGGA; human FAK2, UUUGCGGUGCAAUAAA ATT; human Src1, NNCUCGGCUCUAGAAGACCA; and human Src2, NNUGGCUACUACUCAAACA.

**Cell Culture**

The NCI-H441 and A549 human lung epithelial cell lines used for this work were obtained from the American Type Culture Collection (ATCC; Manassas, VA). H441 cells were maintained at 37°C with 5% CO2 in RPMI 1640 medium (Invitrogen) with 25 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, pH 7.4, and 1.5 g/l NaICO3 supplemented with 10% heat-inactivated fetal bovine serum (FBS). The A549 cells were grown in Ham’s F12K medium (ATCC) containing 2 mM L-glutamine supplemented with 1.5 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS as per ATCC protocol and were maintained similarly to H441 cells.

**Inhibitors**

PD-98059, a specific inhibitor of the activation of ERK kinase by mitogen-activated protein kinase (MAPK/ERK) kinase (MAPKK or MEK), and PP2, a potent and selective inhibitor of Src tyrosine kinases, were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20°C. These inhibitors were then diluted immediately before use in cell culture medium. Cells were pretreated with PD-98059 (50 μM), PP2 (10 μM), or an equivalent amount of DMSO (vehicle control) for 45 min before exposure to cyclic mechanical strain. FAK, Src, or NT1 siRNA sequences were dissolved in the buffer (Dharmacon) at 40 μM, aliquoted, and stored at −80°C.

**Application of Mechanical Strain**

Cells were plated on elastomer membranes coated with type I collagen Flex I six-well culture dishes (Flexcell International, Hillsborough, NC). A sterile cloning ring was placed in the center of each well before the cells were seeded to attain maximal strain effects. Cells were not plated in the center because the strain effect is minimal at the center and maximal at the periphery (3, 10, 14). Similar plates were subjected to cyclic mechanical strain using the Flexercell Strain Unit (FX-3000; Flexercell International). Cells were subjected to cyclic mechanical strain using the Flexercell Strain Unit (FX-3000; Flexercell International) as previously described (33) with slight modifications. Briefly, cells were exposed to continuous cycles of strain/relaxation generated by a cyclic vacuum produced by a computer-driven system (Flexcell 3000; Flexcell International). Cells were subjected to a 20-kPa vacuum at 20 cycles/min, with a stretch/relaxation ratio of 1:1 (1.5 s of deformation alternating with 1.5 s in neutral conformation), creating maximum 20% strain at periphery of the well with an average 10% strain for the indicated times. The vacuum applies negative pressure that stretches the membranes to a known percentage elongation. Strain is transmitted to adherent cells cultured on the upper surface of the membrane, which experience similar elongation (3).

**Proliferation Studies**

Proliferation was assessed by using two different methods. In some studies, we counted cells directly after trypsinization, using an automated cell counter. In other studies, we used a crystal violet absorption assay, demonstrated to correlate linearly with cell number over the range of cell numbers studied.

**Direct cell counting.** H441 cells were seeded at 150,000 cells/well on precoated type I collagen Flex I six-well culture plates for 24 h. Subconfluent (30–40% confluent) cells were serum-starved for 24 h and then switched back to normal growth medium with 10% FBS. At this point, cells from one six-well plate were trypsinized and counted to provide a time 0 measurement. The remaining cells were then cultured for 48 h under either static conditions or conditions of cyclic mechanical strain before trypsinization and cell counting. Cell number was determined by counting each of the six wells independently (Coulter Electronics, Luton, UK). Data from each experiment were analyzed with six observations in each group.

**Crystal violet staining.** H441 or A549 cells were seeded at 150,000 or 100,000 cells/well, respectively, on precoated type I collagen Flex I six-well culture plates for 24 h. Subconfluent (30–40%) cells were serum-starved for 24 h for H441 and 12 h for A549 cells, a single six-well plate was reserved for a time 0 measurement, and the remaining serum-starved cells were switched back to normal growth medium with 10% FBS under static or cyclic mechanical strain conditions for 24–72 h before staining with crystal violet. Cell proliferation was assessed by a colorimetric assay using crystal violet (Sigma), a cytochemical stain that binds to chromatin (5), as described previously (21) with slight modifications. Briefly, viable cells were rinsed in warm PBS and fixed in absolute ethanol-glacial acetic acid (3:1, vol/vol) for 10 min at room temperature and left to air dry (eventually stored at 4°C, wrapped in aluminum paper). The cells were stained with 0.1% crystal violet (wt/vol) for 10 min at room temperature. Excess dye was removed by decantation and washed twice with distilled water. The dye was extracted in 10% acetic acid (vol/vol), and optical density was measured at 550 nm using a Thermomax microplate reader (Molecular Devices, Ramsey, MN). Data from each experiment were analyzed with six observations in each group. The crystal violet absorption assay, performed on both H441 and A549 cells under our laboratory conditions, correlated linearly with cell number over the range of cell numbers studied (data not shown).

**Western Blot Analysis**

Cells were cultured as previously described (59), grown to confluence, and changed to serum-free medium for 24 h to reduce background phosphorylation due to serum growth factors and thereby facilitate delineation of deformation-related signals. After treatment, cells were lysed on ice in modified radioimmunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1% deoxycholic acid, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM Na3VO4, 50 mM NaF, 10 mM sodium pyrophosphate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). Lysates were centrifuged at 15,000 g for 10 min at 4°C, and supernatants were stored at −80°C. Protein concentrations were determined by bicinchoninic acid analysis (BCA assay; Pierce Chemical, Rockford, IL). Twenty micrograms of protein were loaded per well on an SDS polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences). Nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline with 1 ml of Tween 20 per liter for 1 h at room temperature. The immunoblots were probed with appropriate primary and secondary antibodies and detected using the enhanced chemiluminescence method (ECL; Amersham Biosciences). Nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline with 1 ml of Tween 20 per liter for 1 h at room temperature. The immunoblots were probed with appropriate primary and secondary antibodies and detected using the enhanced chemiluminescence method (ECL; Amersham Biosciences). Nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline with 1 ml of Tween 20 per liter for 1 h at room temperature. The immunoblots were probed with appropriate primary and secondary antibodies and detected using the enhanced chemiluminescence method (ECL; Amersham Biosciences). Nonspecific binding sites were blocked with 5% BSA in Tris-buffered saline with 1 ml of Tween 20 per liter for 1 h at room temperature. The immunoblots were probed with appropriate primary and secondary antibodies and detected using the enhanced chemiluminescence method (ECL; Amersham Biosciences).
Transfection Studies

For transfection with siRNA, H441 cells were plated on type I collagen-coated Flex I six-well culture dishes at 40–50% confluence 1 day before transfection. siRNAs were combined with Plus reagent in Opti-MEM as previously described (45). Oligofectamine in Opti-MEM was used for transfection at 10 μg/ml according to the manufacturer’s protocol. The final siRNA concentration was 100 nM unless otherwise indicated. After 6–8 h of transfection, 0.5 volume of RPMI medium containing 20% serum was added to the cells, and transfection was continued 48 h. The cells were serum-starved overnight before the study. The effectiveness of the siRNA transfection was verified in each study by parallel transfections in which the cells were lysed at the conclusion of the study and the resulting lysates were immunoblotted for the target protein of interest. For proliferation studies, cells were transfected with siRNA for 24 h followed by 48 h with and without cyclic strain.

Statistical Analysis

All experiments were done independently at least three times unless indicated otherwise. Data are presented as means ± SE and were analyzed using Student’s unpaired t-test. P < 0.05 was considered significant.

RESULTS

Mechanical Strain Induces Lung Epithelial Cell Proliferation

H441 cells exposed to cyclic mechanical strain at 24, 48, and 72 h significantly increased cell numbers after strain initiation compared with static control cell monolayers at the same time points, as assessed by a colorimetric assay using crystal violet absorbance assay over the linear range of the assay, with interpolation against a standard curve (Fig. 1, A and B, n = 6, P < 0.05 for 24 h, P < 0.005 for 48 and 72 h). For instance, the increase in cell number at 48 h in monolayers subjected to repetitive deformation was 36.4 ± 7.1% greater than that in their static control counterparts at the same time point. We chose the 48-h time point for further proliferation studies, either by crystal violet absorbance or, in some studies, by direct cell counting, because the proliferative response was more obvious at 48 h than at an earlier time point. These cells approximately doubled by 58 h in medium with 10% serum (6). Indeed, parallel studies by direct cell counting confirmed that cyclic strain stimulates H441 proliferation. For instance, cell numbers increased 29.8 ± 4.4% more in cells exposed to cyclic mechanical strain than in their static control counterparts (n = 4, P < 0.003) (see Fig. 4B, left).

Mechanical Strain Stimulates ERK1/2 Phosphorylation

To confirm a previous report (10) that mechanical strain activates ERK1/2, we either maintained confluent serum-starved H441 cells under static conditions or subjected them to cyclic strain for 0–60 min. Figure 2A depicts rapid and sustained induction of ERK1/2 phosphorylation in cells exposed to repetitive deformation, as assayed by Western blotting with an antibody that detects phosphorylated ERK1/2 at the Thr202 and Tyr204 residues, normalized to GAPDH band intensity as a protein loading control. ERK phosphorylation was significantly increased within 5 min after the initiation of cyclic strain (143.1 ± 21.8%, n = 5, P < 0.05). Maximal ERK phosphorylation occurred at 15–30 min after the initiation of deformation (197.06 ± 7 and 177.06 ± 9.99%, respectively).

MEK Inhibition Blocks Strain-Induced Proliferation

We therefore hypothesized that the mitogenic effect of mechanical deformation might be mediated by ERK activation. In vehicle-treated (0.1% DMSO) control cells, cyclic strain increased the proliferative response 23.6 ± 9.2% compared with unstretched cells (n = 3, P < 0.003). The MEK inhibitor PD-98059 (50 μM) blocked strain-induced proliferation (Fig. 2B). Treatment with PD-98059 during the 48-h incubation period did not alter basal cell proliferation compared with DMSO vehicle-treated control cells, and the PD-98059 concentration used is consistent with that previously used to inhibit ERK in human peripheral bronchial epithelial cells (44).

Fig. 1. Cyclic mechanical strain induces H441 pulmonary epithelial cell proliferation. A: cyclic mechanical strain increases H441 proliferation as measured by crystal violet staining. H441 cells were maintained under static conditions or exposed to cyclic mechanical strain at 20 cycles/min for 0–72 h. The clear area in the center of each well occurred because no cells were seeded into these areas at the beginning of each study as described in EXPERIMENTAL PROCEDURES. A representative crystal violet-stained type I collagen-coated Flex I well for each time point demonstrates that cyclic mechanical strain increases crystal violet stain over static conditions for 24–72 h. The lighter crystal stain represents fewer cells and darker crystal stain represents more cells as measured by absorbance at 550 nm (n = 6). B: cyclic mechanical strain increases H441 cell number. H441 cells maintained under static conditions or exposed to cyclic strain for 24–72 h exhibited significantly increased proliferation as measured by cell number, assessed by crystal violet absorbance assay over the linear range of the assay, with interpolation against a standard curve (means ± SE, n = 6, *P < 0.05 and **P < 0.005 compared with unstrained controls).

n = 5, P < 0.05 for each) compared with parallel static controls.
Mechanical Strain Stimulates Site-Specific Tyrosine Phosphorylation of FAK at Tyr397 and Tyr576

To determine whether exposure of lung epithelial cells to mechanical strain increases tyrosine phosphorylation of FAK, we maintained confluent serum-starved H441 cells under static conditions or conditions of cyclic strain for 2–60 min. Whole cell lysates were immunoblotted with antibodies specific for different tyrosine phosphorylation sites for FAK at Tyr397, or at Tyr576. Mechanical strain induced FAK tyrosine phosphorylation at both Tyr397 and Tyr576 in pulmonary epithelial cells in a time-dependent manner (Fig. 3, A and B). Phosphorylation of FAK at Tyr397 increased significantly as rapidly as 2 min after the initiation of strain, and this effect was sustained for up to 60 min (n = 5, P < 0.05). Phosphorylation of FAK at Tyr576 also increased by 2 min after strain initiation, with maximal phosphorylation at 5–15 min. However, in contrast to the sustained FAK phosphorylation at Tyr397 after strain initiation, FAK phosphorylation at Tyr576 was not detectably different from baseline by 60 min after initial activation (n = 5, P < 0.05).

Role of FAK in Strain-Induced ERK1/2 Activation

To determine whether FAK is required for strain-induced ERK modulation, we transfected H441 cells with a nontargeting (NT1) control siRNA or siRNA targeted to FAK. Cells were serum-deprived and either subjected to cyclic strain for 15 min or maintained under static conditions. Transfection with each of two sequences of siRNA targeted to FAK reduced total FAK protein by ~70% (n = 3, P < 0.05, Fig. 3C, top). Since these two sequences for FAK yielded similar results, we then performed subsequent studies of the effects of FAK reduction on strain-associated mitogenicity and signaling using an siRNA pool derived by combining the two FAK-targeted sequences. Mechanical strain significantly increased ERK1/2 phosphorylation in cells transfected with NT1 control siRNA compared with cells transfected with NT1 control siRNA under static culture conditions (n = 5, P < 0.05, Fig. 3C, bottom left). In contrast, although cells transfected with FAK siRNA exhibited similar basal levels of phosphorylation, FAK siRNA transfection substantially attenuated strain-induced ERK activation (Fig. 3C, bottom right).

Role of FAK in Mechanical Strain-Induced Proliferation

To further evaluate the role of FAK in the mechanical strain-induced mitogenic response, we assessed proliferation in cells by transient transfection with NT1 nontargeting sequences. Mechanical strain significantly increased ERK1/2 phosphorylation in cell transfected with NT1 control siRNA compared with cells transfected with NT1 control siRNA under static culture conditions (n = 5, P < 0.05, Fig. 3D, left). However, FAK reduction by siRNA transfection inhibited strain-induced proliferation (Fig. 3D, right), suggesting that FAK is necessary for the strain-induced mitogenic response in H441 pulmonary epithelial cells.
magnitude and at a substantially higher frequency (25% elongation, 60 cycles/min for 10 min) increases Src activation in A549 lung epithelial cells.

**Src Inhibition Inhibits Mechanical Strain-Induced Proliferation**

Having observed that Src is phosphorylated by cyclic mechanical strain in these cells, we next hypothesized that Src activation might contribute to mitogenic signaling in response to mechanical deformation. Semiconfluent H441 cells were pretreated for 45 min with PP2 (10 μM) or a DMSO vehicle control (0.1% vol/vol) and then cultured under static conditions or conditions of cyclic strain for 48 h before crystal violet staining and absorbance measurement. Strain stimulated cell proliferation in cells transfected with the NT1 sequence but not in cells in which FAK had been reduced (means ± SE, n = 3, *P < 0.05).
proliferation and prevented any stimulation of cell proliferation by cyclic mechanical strain compared with unstretched static cells (n = 4, P < 0.003, Fig. 4B). We sought to confirm this key result in a second cell line and studied the effects of similar mechanical deformation in A549 cells at 24 h, because these cells double more rapidly, approximately within 22 h (13). A549 cells exposed to cyclic mechanical strain for 24 h significantly increased cell numbers after strain initiation com-

Fig. 4. Role of Src in cyclic mechanical strain-induced proliferation. A: cyclic mechanical strain stimulates Src phosphorylation. The effect of cyclic strain on H441 cell phosphorylation of Src at Tyr^{418} was assessed by Western blotting in cells subjected to cyclic strain for 0–60 min. Typical blots are represented at top, whereas graph at bottom summarizes densitometric analysis. Strain resulted in rapid and sustained phosphorylation of Src at Tyr^{418} (means ± SE, n = 5, *P < 0.05). B: PP2 inhibits strain-induced H441 proliferation. Cells were maintained under static conditions or conditions of cyclic strain for 48 h after pretreatment with vehicle control (0.1% DMSO) or PP2 (10 μM) for 45 min. Cell numbers were assayed by direct counting after time 0 values were subtracted. The Src inhibitor PP2 prevented any stimulation of cell proliferation by cyclic strain compared with unstretched cells (n = 6 from 1 of 4 similar studies, *P < 0.003), and it also reduced basal cell proliferation (n = 6 from 1 of 4 similar studies, **P < 0.05). Values are means ± SE. C: PP2 inhibits strain-induced A549 proliferation. Cells were maintained under static conditions or conditions of cyclic strain for 24 h after pretreatment with vehicle control (0.1% DMSO) or PP2 (10 μM) for 45 min. Cell numbers were assayed by direct counting after time 0 values were subtracted. The Src inhibitor PP2 prevented any stimulation of cell proliferation by cyclic strain compared with unstretched cells (n = 4, *P < 0.002), and it did not affect basal cell proliferation (n = 4, P = 0.5). Values are means ± SE. D: Src reduction by siRNA reduces Src protein. Cells transfected with either NT1 siRNA or siRNAs targeted to Src (Src-1 and Src-2) demonstrate ~50% reduction in Src protein levels by siRNAs targeted to Src but not by NT1 siRNA (n = 6, P < 0.05). E: Src reduction by siRNA inhibits strain-induced proliferation. Cells transfected with either NT1 siRNA or siRNA targeted to Src were cultured under static conditions or conditions of cyclic strain for 48 h before crystal violet staining and absorbance measurement. Strain stimulated proliferation in cells transfected with the NT1 sequence but not in cells in which Src had been reduced (means ± SE, n = 3, *P < 0.05).
pared with static control cell monolayers (31.4 ± 6.5%; n = 4, P < 0.002, Fig. 4C, left). This is consistent with previous observations by McAdams et al. (30). Vlahakis et al. (50) did not observe increased cell numbers when A549 cells were exposed to repetitive deformation, but these authors studied deformation at the substantially higher frequency of 40 cycles/min, as well as using a different amplitude of strain and a different device to deliver it. Our laboratory (31) previously reported that the stimulation of intestinal epithelial proliferation by repetitive deformation is frequency dependent and is abolished at high strain frequencies, so this could have contributed to the different observation by Vlahakis et al. Treating A549 cells with PP2 did not affect basal cell proliferation under static conditions but abrogated mechanical strain-induced cell proliferation compared with static controls (n = 4, Fig. 4C). These results suggest that the mitogenic effects of cyclic mechanical strain are mediated by Src in A549 pulmonary epithelial cells as well.

We further confirmed these results by assessing proliferation in H441 cells after transient transfection with nontargeting NT1 siRNA or siRNA targeted to Src. Transfection with either of two sequences of siRNA targeted to Src yielded similar reduction of total Src protein by ~50 ± 3.8% (n = 6, P < 0.05, Fig. 4D). Since these two sequences for Src yielded similar results, we performed subsequent studies of the effects of Src reduction on strain-associated mitogenicity using an siRNA pool derived by combining the two Src-targeted sequences. Reduction of Src by transient transfection with siRNAs targeted to Src blocked strain-induced H441 proliferation compared with static controls without affecting basal proliferation compared with NT1-transfected controls (n = 4, P < 0.005, Fig. 4E). Thus activation of Src may be required for the mitogenic effects of strain in pulmonary epithelial cells.

Mechanical Strain-Induced ERK Phosphorylation is Inhibited by Src Inhibition

We next investigated whether Src kinase was required for strain-induced ERK activation. For these studies, confluent serum-starved H441 cells were pretreated for 45 min with PP2 (10 μM) or a DMSO vehicle control (0.1% vol/vol) and then cultured under static conditions or conditions of cyclic strain for 15 min. Cell lysates were immunoblotted with an antibody specific for phosphorylated ERK1/2 and then stripped and reprobed for t-ERK1/2 and GAPDH antibodies as well. Src inhibition prevented ERK activation in response to cyclic mechanical strain (Fig. 5). Thus Src may play a key role in the mechanotransduction leading to ERK phosphorylation.

Effect of Src Inhibition on Mechanical Strain-Induced FAK Activation

We next sought to determine whether Src activation might be required for FAK phosphorylation. Confluent serum-starved H441 cells were similarly pretreated with PP2 and then cultured under static conditions or conditions of cyclic strain for 15 min. Whole cell lysates were immunoblotted with antibodies specific for phosphorylated FAK at Tyr576, a Src-dependent phosphorylation site on FAK. Src inhibition by PP2 did not inhibit strain-induced phosphorylation of FAK at Tyr576 (n = 5, P < 0.05, Fig. 6A).

However, PP2 completely blocked the strain-induced phosphorylation of FAK at Tyr576 without affecting basal FAK phosphorylation at Tyr576 (Fig. 6B).

Role of FAK in Strain-Induced Src Phosphorylation

We also sought, conversely, to determine whether reducing FAK would alter Src phosphorylation in response to mechanical strain. H441 cells transfected with NT1 (control) or FAK-specific siRNAs were either subjected to cyclic mechanical strain for 15 min or maintained under static conditions. Src phosphorylation at Tyr418 was significantly increased in cells transfected with NT1 siRNA exposed to mechanical strain compared with cells transfected with NT1 siRNA but not exposed to cyclic mechanical strain (n = 5, P < 0.05). Cells transfected with FAK siRNA exhibited basal Src phosphorylation equivalent to that of NT1-transfected cells and a similar increase in Src phosphorylation at Tyr418 in response to cyclic mechanical strain (n = 5, P < 0.05, Fig. 7, right). Together with the effects of PP2 on FAK phosphorylation, these results are consistent with a model in which initial phosphorylation of FAK at Tyr397 and phosphorylation of Src at Tyr418 are independent, but Src activation is then required for further FAK phosphorylation at Tyr576 in response to cyclic mechanical strain.

Longer Exposure to Mechanical Strain Also Stimulates FAK, Src, and ERK Phosphorylation Without Altering FAK, Src, or ERK Protein Expression

We performed additional experiments assaying for both phosphorylation and expression of FAK, Src, and ERK in H441 cells exposed to 24 h of cyclic mechanical strain to evaluate the effects of longer term exposure to cyclic strain. Rhythmic deformation did not significantly affect the protein expression levels of FAK, Src, or ERK kinases (Fig. 8).
Although we did not observe a statistically significant increase in the phosphorylation of FAK at Tyr\(^{397}\) after 24 h (Fig. 9A), we did observe that cyclic mechanical strain for 24 h induced the sustained phosphorylation of FAK at Tyr\(^{576}\), as well as that of Src at Tyr\(^{418}\) and ERK at Thr\(^{202}\)/Tyr\(^{204}\) (Fig. 9B). These results suggest that the phosphorylation of Src and ERK persist over at least 24 h after the initiation of repetitive deformation. Although the initial phosphorylation of FAK at Tyr\(^{397}\) may eventually decrease toward baseline, phosphorylation of FAK at Tyr\(^{576}\) may display a biphasic activation curve in which early phosphorylation returns to baseline by 60 min (Fig. 3B) but then increases again thereafter (Fig. 9B). Such biphasic activation curves also occur in response to repetitive deformation in other epithelial cell types (17).

**DISCUSSION**

The present studies were aimed at identification of upstream intracellular signaling pathways mediating the mitogenic effects of mechanical strain in pulmonary epithelial cells. Src activation and translocation to the cytoskeleton after mechanical strain has been described in rat lung cells (12, 16, 29), and FAK- and Src-mediated signaling have been implicated in mechanotransduction in other cell types (4, 25, 32, 47). In contrast, however, Chess et al. (10) have reported that FAK is not activated in response to mechanical strain in pulmonary epithelial cells. Moreover, whether Src interacts with ERK in response to repetitive deformation has not previously been studied in human pulmonary epithelial cells. The findings presented begin to delineate a pathway by which Src and FAK may mediate cyclic mechanical strain-induced ERK1/2 phosphorylation and consequent proliferation in human pulmonary epithelial cells. This conclusion is based on the observations that cyclic mechanical strain of pulmonary epithelial cells induced both ERK activation and proliferation and that these effects were inhibited either by inhibiting Src or MEK or by siRNA targeted to FAK.

We studied the effects of repetitive deformation at a frequency of 20 cycles/min and an average mechanical strain of 10%. These values are similar to the frequencies of deformation that pulmonary epithelial cells might experience in vivo. For instance, normal respiratory rates vary by age. An adult normally breathes at 12–20 breaths/min, a child normally breathes at 15–30 breaths/min, and an infant normally breathes at 25–50 breaths/min (43). Although strain frequency is uniform across the membrane in the Flexcell system, it must be uniform across the membrane in the Flexcell system, it must be

**Fig. 6.** Src inhibition does not inhibit cyclic mechanical strain-induced FAK phosphorylation at Tyr\(^{397}\) but blocks FAK phosphorylation at Tyr\(^{576}\). Cells were pretreated with vehicle (DMSO) or PP2. The effect of PP2 on phosphorylation of FAK at Tyr\(^{397}\) (A) and at Tyr\(^{576}\) (B) was assessed by Western blotting in cells subjected to cyclic strain for 15 min. Typical blots are represented at top, whereas graph at bottom summarizes densitometric analysis. Strain stimulated FAK phosphorylation at Tyr\(^{397}\) (A) and at Tyr\(^{576}\) (B) in vehicle-treated cells. PP2 did not inhibit strain-induced FAK phosphorylation at Tyr\(^{397}\) (A); however, PP2 significantly inhibited strain-induced FAK phosphorylation at Tyr\(^{576}\) (B) (means ± SE, n = 5, *P < 0.05).

**Fig. 7.** Reduced FAK does not inhibit cyclic mechanical strain-induced Src phosphorylation. Cells were transfected with either NT1 siRNA or siRNA targeted to FAK. Cells were maintained under static conditions or conditions of cyclic strain for 15 min before lysis and Western blotting. The graph summarizes densitometric analysis from 5 similar studies. Strain significantly increased Src phosphorylation in NT1-transfected cells as well as in FAK-reduced cells (means ± SE, n = 5, *P < 0.05).
acknowledged that the amplitude of mechanical strain applied by the Flexcell system is nonuniform. When the average deformation is set to 10%, cells may be subjected to up to 20% strain at the periphery of the monolayer while deformation at the center is very low. This may add complexity to the interpretation of the data that we have derived, since we are studying heterogeneously deformed cell populations. However, local distension patterns within the lung parenchyma in vivo during ventilation also are likely to be complex and heterogeneous (48). Previous investigators have generally used cyclic axial cell elongation factors ranging from 5 to 30% to study the effects of repetitive deformation on pulmonary epithelial cells in vitro (39, 48, 54). These parameters are consistent with morphometric studies performed in ventilated animals, at least for pulmonary epithelial cells (2, 48).

We observed maximal ERK activation at about 15–30 min following cyclic mechanical strain, similar to that previously reported by Chess et al. (10) as well as reports of ERK activation in various other cell types (1, 4, 20, 22, 25). However, Chess et al. also described a reduction of total ERK1 in H441 cells following exposure to mechanical strain for 10 min. Unless ERK protein is degraded very rapidly, a decrease in protein levels within 10 min of initiation of a stimulus would seem unlikely. We did not observe such a decrease in our studies.

Although Chess et al. (10) did not study the effects of blocking strain-induced ERK activation in H441 cells, we found that the effects of cyclic strain on H441 proliferation were blocked by MEK inhibition with PD-98059. Although ERK activation has not previously been demonstrated to mediate the mitogenic effect of strain in human pulmonary epithelial cells, ERK activation has been linked to mechanical strain-induced proliferation in mouse lung epithelial cells and other epithelial cells (8, 25) and to the mechanical strain-induced cytokine release in BEAS-2B bronchial epithelial cells (33). These results contrast with one report (19) that ERK is not required for the mitogenic effects of strain in endothelial cells. The disparities may reflect fundamental differences between pulmonary epithelial cells and the endothelial cells examined in that study.

FAK phosphorylation at tyrosine also has previously been reported to be critical for stretch-induced ERK activation in some other epithelial (1, 25) and nonepithelial (4, 7, 51, 52, 58) cell types exposed to mechanical stress. However, FAK is not activated by mechanical forces in MC3T3-E1 osteoblasts (18), and Chess et al. (10) reported ERK activation in H441 cells without FAK activation. In contrast, we demonstrated that exposure to cyclic mechanical strain rapidly and robustly stimulated FAK phosphorylation in H441 pulmonary epithelial cells at Tyr397, the major autophosphorylation site, and at Tyr576, within the kinase activation loop of FAK and Src-dependent phosphorylation site. This discrepancy might reflect potential important methodological differences. First, Chess et al. studied pulmonary epithelial cells at 60 cycles/min. This frequency might not be as physiologically relevant to the pulmonary epithelial deformation induced by human ventilation as the 20 cycles/min deformation frequency we used. Indeed, in studies of Caco-2 intestinal epithelial cells, our group (31) has previously observed strain effects at 10–20 cycles/min that were absent at frequencies of 60–90 cycles/min, less relevant to gut peristalsis. In addition, those investigators (10) used an anti-phosphotyrosine antibody for their studies, whereas we used site-specific FAK antibodies that may have been more sensitive or accurate. Mechanical strain does stimulate FAK tyrosine phosphorylation in several other cell types, including colonic epithelial cells (25), lung endothelial cells (41), and other cell types (4, 47, 52).

Fig. 8. Effect of 24-h exposure of cyclic mechanical strain on FAK, Src, and ERK expression. The effect of cyclic strain on H441 cell expression of FAK (A), Src (B), and ERK (C) was assessed by Western blotting in cells subjected to cyclic strain for 24 h. Typical blots represented at top, whereas graphs at bottom summarize densitometric analysis. Strain did not significantly affect the expression of protein levels of FAK, Src, and ERK (means ± SE, n = 8).
Having defined FAK activation, we investigated the effects of siRNA targeted to FAK on ERK activation and proliferation induced by cyclic mechanical strain to determine whether strain-induced FAK phosphorylation was important in strain-mediated ERK activation and mitogenicity. Indeed, FAK siRNA strongly attenuated strain-induced ERK activation, consistent with the hypothesis that FAK is upstream of ERK in the mechanical strain-induced signal pathway that stimulates cell proliferation in H441 cells, although it remains possible that a minor FAK-independent pathway also may contribute to ERK activation by strain. FAK has been presumed to be required for the mitogenic effects of strain in other cell types because it is upstream of ERK. However, this was not certain, since it was possible that parallel mitogenic pathways could have compensated for the lack of FAK activation. Indeed, fibroblasts transfected with the FAK-inhibitory molecule FRNK decreased ERK phosphorylation in response to adhesion, perhaps because of such compensation (27, 53). Our results now demonstrate directly that significantly reducing FAK protein level blocks mechanical strain-induced proliferation in H441 pulmonary epithelial cells.

Liu et al. (29) weakly implicated Src activation in mechanotransduction in fetal rat lung cells by demonstrating that the relatively nonspecific tyrosine kinase inhibitor herbimycin prevented strain-induced DNA synthesis. We found activation of c-Src in H441 pulmonary epithelial cells in response to cyclic mechanical strain similar to that reported by Liu et al. and demonstrated that more specific Src family tyrosine kinase inhibition with PP2 and specific reduction of Src by targeted siRNA each prevented the mitogenic effects of cyclic mechanical strain in pulmonary epithelial cells. Moreover, Liu et al. did not address the relationship between Src and strain-induced ERK activation, whereas our present data suggest that Src is required for the mitogenic activation of ERK by repetitive strain. These observations further delineate a role of Src in strain-induced mitogenicity by placing it within the context of a mitogenic signal cascade also involving ERK. Although Src activation by repetitive strain has not previously been studied in epithelial cells, these observations are consistent with reports of Src activation by mechanical forces in other cell types (15, 24, 35) and the activation of Src by constant mechanical pressure in human colonic adenocarcinoma cells (45).
If both FAK and Src are important upstream mediators of ERK activation and proliferation in response to strain in pulmonary epithelial cells, then the relationship between these two upstream mediators requires elucidation. Han et al. (16) have suggested alternate models for mechanotransduced signal pathways in which cytoskeletal proteins such as actin filament-associated protein (AFAP) might bind to and activate Src directly or in which FAK might activate Src. Although we did not explore the role of AFAP in this study, our data suggest that Src may be proximal to FAK, rather than FAK activating Src, in at least one mechanotransduced pathway, because Src inhibition prevents FAK-Tyr576 phosphorylation, whereas Src itself is phosphorylated even when FAK is reduced. However, FAK-Tyr397 phosphorylation occurs in response to cyclic strain in H441 pulmonary epithelial cells even when Src is blocked. Thus two mechanotransduced pathways may converge upon FAK in these cells. One pathway appears independent of Src and stimulates FAK-Tyr397 phosphorylation. A second Src-dependent pathway seems required for the further activation of FAK by phosphorylation at Tyr576. Indeed, our group (46) recently reported that pharmacological manipulation of the cytoskeleton tends to ablate the effects of increased constant pressure on FAK-Tyr397 phosphorylation in human colon adenocarcinoma cells without altering Src activation. The details of how the cytoskeleton may interact with FAK and Src signaling in pulmonary epithelial cells subjected to repetitive deformation await elucidation but are beyond the scope of the current investigation.

The relationship between FAK and Src in mechanical signaling may vary even among cell types in which both FAK and Src are activated. Kumar et al. (24) suggested that cyclic mechanical strain induces cell proliferation by a FAK-Src-Rac1-NF-κB signaling pathway in C2C12 skeletal myoblasts but believed FAK to be the most proximal mediator in the pathway, with Src downstream of FAK. FAK has similarly been reported upstream of Src (11, 26). In contrast, our data suggest that reducing FAK does not prevent Src activation in H441 cells but that Src is required for strain induced FAK phosphorylation at Tyr397 in these cells. This is consistent with a report by Liu et al. (29) that strain-induced Src translocation and activation in fetal rat lung cells occur independently of FAK. Kumar et al. (24) also reported that although cyclic mechanical strain increases MAPK activity in C2C12 myoblasts, inhibition of MAPK did not affect strain-induced proliferation, whereas our results demonstrate that ERK inhibition blocks strain-induced proliferation in pulmonary H441 epithelial cells. The apparent discrepancy between the results of Kumar et al. and the results of our own studies and those of Liu et al. suggests that mechanotransduced signal pathways may differ between myoblasts and epithelial cells.

In conclusion, our results indicate that Src and FAK mediate the mitogenic response induced in lung epithelial cells by mechanical strain through an ERK MAPK signaling cascade. These observations both explain and refine previous reports that mechanical strain is mitogenic for pulmonary epithelial cells, since we are now able to delineate at least part of the mechanotransduced signal pathway responsible for this mitogenic effect. We speculate that our definition of FAK and deformation-induced ERK signals not previously observed by some others studying pulmonary epithelial cells may reflect more physiologically relevant strain parameters and more specific antibodies now available for immunoblotting. Mechanical strain-induced phosphorylation of FAK at Tyr576 (within the FAK kinase activation loop) is mediated by a pathway in which Src appears to be upstream. How Src is initially activated and the separate pathway by which FAK is phosphorylated at Tyr397 in response to cyclic mechanical strain independently of Src awaits further study. Tracing the signaling pathways initiated by mechanical strain in lung epithelial cells may guide the development of targeted pharmacological or molecular strategies aimed at optimizing strain-induced lung growth while mitigating ventilator-induced lung injury.

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