Repetitive Deformation Activates Focal Adhesion Kinase and ERK Mitogenic Signals in Human Caco-2 Intestinal Epithelial Cells through Src and Rac1*

Lakshmi S. Chaturvedi†**, H. Michael Marsh†**, Xun Shang†, Yi Zheng†, and Marc D. Basson†**†

From the †Surgical Service, John D. Dingell Veterans Affairs Medical Center and Departments of **Surgery, †Anatomy and Cell Biology, Wayne State University, Detroit, Michigan 48201 and †Division of Experimental Hematology, Children’s Hospital Research Foundation, Cincinnati, Ohio 45229

Intestinal epithelial cells are subject to repetitive deformation during peristalsis and villous motility, whereas the mucosa atrophies during sepsis or ileus when such stimuli are abnormal. Such repetitive deformation stimulates intestinal epithelial proliferation via focal adhesion kinase (FAK) and extracellular signal-regulated kinases (ERK). However, the upstream mediators of these effects are unknown. We investigated whether Src and Rac1 mediate deformation-induced FAK and ERK phosphorylation and proliferation in human Caco-2 and rat IEC-6 intestinal epithelial cells. Cells cultured on collagen-I were subjected to an average 10% cyclic strain at 10 cycles/min. Cyclic strain was blocked by inhibition of Src (PP2 or short interfering RNA) or Rac1 (NSC23766). Src or Rac1 inhibition also prevented strain-induced FAK phosphorylation at Tyr576 and Tyr397, and ERK1/2 at Thr202/Tyr204. The mitogenic effect of cyclic strain was blocked by inhibition of Src (PP2 or short interfering RNA) or Rac1 (NSC23766). Src or Rac1 inhibition also prevented strain-induced FAK phosphorylation at Tyr576 and Tyr397, and ERK phosphorylation but not FAK phosphorylation at Tyr397. Reducing FAK using short interfering RNA blocked strain-induced mitogenicity and attenuated ERK phosphorylation but not Src or Rac1 phosphorylation. Src inhibition blocked strain-induced Rac1 phosphorylation, but Rac inhibition did not alter Src phosphorylation. Transfection of a two-tyrosine phosphorylation-deficient FAK mutant Y576F/Y577F prevented activation of cotransfected myc-ERK2 by cyclic strain. Repetitive deformation induced by peristalsis or villus motility may support the gut mucosa by a pathway involving Src, Rac1, FAK, and ERK. This pathway may present important targets for interventions to prevent mucosal atrophy during prolonged ileus or fasting.

The intestinal mucosa is subjected to a wide variety of physical forces during normal function and in pathophysiologic states (1). These physical forces include deformation, pressure, and shear stress engendered by villous motility, peristalsis, interaction with luminal contents, and mucosal remodeling and healing (2–6). Such repetitive deformation induces intestinal epithelial proliferation in vitro (1, 7–11) and in vivo (12), as well as differentiation (13) and intracellular signaling (1, 10, 11, 14, 15). Mechanical forces such as repetitive deformation, pressure, and shear stress also influence many other cell types (16), although the mechanism(s) by which cells sense and respond to physical forces are not fully understood. We have reported previously that rhythmic mechanical strain-induced deformation activates focal adhesion kinase (FAK) and extracellular signal-regulated kinases (ERK1/2) in human Caco-2 intestinal epithelial cells (15) and tyrosine kinase signaling in rat small and large bowel mucosa in vivo (17). ERK appears to be required for the mitogenic effect in human Caco-2 intestinal epithelial cells, and transfection with a dominant negative FAK construct inhibits ERK activation by strain (15). However, the upstream mediators of these effects have not been delineated.

Src has been shown to be upstream of FAK in pressure-induced rat mesenteric arteries and is part of a mechanosensory protein complex linking integrins with the cytoskeleton (18). Cyclic mechanical deformation induces rapid Src activation by translocation to the membrane or phosphorylation of the active site in many cell types (19–23). Several lines of evidence suggest that activation of Rho family guanosine triphosphatases (GTPases, including Rho, Rac, and Cdc42) may also modulate FAK activation in response to certain stimuli (24–27). However, the involvement of the small G-protein Rac1 in mechanosensing and signaling in response to repetitive deformation is not as well understood. Mechanical strain activates Rac1 in muscle cells (28–30), aortic smooth muscle cells (31), fibroblasts (32), and cardiomyocytes (33), but it may not be universally true. Different cell types and different physical forces may be associated with different force-activated signal pathways. For instance, Rac is actually inhibited in response to equibiaxial stretch or tensile strain in aortic vascular smooth muscle cells.

The abbreviations used are: FAK, focal adhesion kinase; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; RGM-1, rat gastric epithelial cell line; ERK, extracellular signal-regulated kinases; PP2 (4-amino-5-(4-chlorophenyl)-7-(1-butyl)pyrazolo[3,4d]pyrimidine); HA, hemagglutinin; siRNAs, short interfering RNAs; NT1, nontargeting siRNA sequence; Ms2, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* This work was supported in part by a Veterans Affairs merit research award (to M. D. B.), National Institutes of Health Grant RO1 DK067257 (to M. D. B.), and developmental funds from the Department of Anesthesiology, Wayne State University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Chief, Surgical Service (115), John D. Dingell Veterans Affairs Medical Center, 4646 John R. St., Detroit, MI 48201. Tel.: 313-576-3598; Fax: 313-576-1002; E-mail: marc.basson@va.gov.
Src and Rac1 Mediate Mitogenic Effects of Strain

Although Rac1 activation has not been investigated in the gut in response to strain, application of repetitive mechanical strain to a rat gastric epithelial cell line (RGM-1) at lower strain parameters has been reported not to influence Rac1 protein levels except in cells migrating at the edge of circular wounds in which Rac1 immunoreactivity may be decreased (35). Thus, Rac1 involvement in regulating the FAK and ERK activation that mediates the mitogenic effects of repetitive mechanical strain in intestinal epithelium is not well understood.

We therefore sought to determine what upstream mediators might be responsible for such FAK and ERK activation. In particular, we sought to determine whether strain activation of Src and the small G-protein Rac1 mediates strain-induced FAK and ERK activation and cell proliferation in human (Caco-2) and rat (IEC-6) intestinal epithelial cells. We used the Flexercell apparatus (Flexcell, McKeesport, PA) to rhythmically deform Caco-2 and IEC-6 cell monolayers cultured on collagen-coated flexible-bottomed wells at an average 10% repetitive deformation at 10 cycles/min (7), similar in magnitude and frequency to the irregular repetitive deformation that the mucosa experiences in vivo (36, 37). We characterized time-dependent site-specific FAK phosphorylation and Rac1 and Src activation in response to repetitive deformation in Caco-2 intestinal epithelial cells in order to begin tracing a mechanotransduced pathway that links these signals into a mitogenic cascade.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), Oligofectamine, Lipofectamine, and Plus Reagent were obtained from Invitrogen. The rat intestinal cell line, IEC-6 cells, was obtained from American Type Culture Collection (Manassas, VA). Western blot stripping reagent was obtained from Chemicon International (Temecula, CA). Human transferrin was obtained from Roche Applied Science. β-Actin, bovine insulin, and trypsin were obtained from Sigma. Phosphospecific polyclonal antibodies to FAK at Tyr397 or Tyr576 were obtained from BIOSOURCE. Phosphospecific polyclonal antibodies to p42/p44 (p-ERK1/2), Tyr(P)202/Thr(P)204, Src-Tyr418, which recognized the phosphorylated form of human Src-Tyr416, Rac1-cdc42 Ser(P)71, rabbit polyclonal antibody to p42/44 (total ERK1/2), and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were obtained from Cell Signal-

was obtained from Clontech. The HA-tagged FAK wild type and HA-tagged FAK mutant changed codons for phosphoacceptor Tyr576—Tyr577 to Phe576—Phe577 were generous gifts from Dr. Steven K. Hanks (Vanderbilt University School of Medicine, Nashville, TN). The wild type myc-ERK2 expression vector was a generous gift from Dr. Christopher Marshall (Institute of Cancer Research, London, UK). Double-stranded short interfering RNAs (siRNAs) targeting human forms of FAK and control nontargeting siRNA 1 (NT1 siRNA) were purchased from Dharmacon (Lafayette, CO). The sequences targeted by siRNA were selected using Dharmacon Smartdesign as follows: human FAK1, UUUGCGGUUGCAUAUGGA; human FAK2, UUUUGCGGUUGCAUAUAAT; human Src1, NNCUGCGCUAUGGAACAA; and human Src2, NNUGGCCAUCACUCACAAC. We used two different sequences targeted to FAK and Src for our initial studies of the effects of siRNA on FAK or Src protein. Because these two sequences yielded similar results, we then performed subsequent studies of the effects of FAK or Src reduction on strain-associated mitogenicity and signaling using an siRNA pool derived by combining the two FAK- or Src-targeted sequences.

Cell Culture—The Caco-2BBE intestinal epithelial cell line used for this study was a subclone of the original Caco-2 cell line that was selected for its ability to differentiate in culture as indicated by formation of an apical brush border and expression of brush border enzymes (39). Caco-2 cells were maintained at 37 °C with 8% CO2 in DMEM with 25 mM glucose, 4 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml transferrin, 10 μM HEPES, pH 7.4, and 3.7 g/liter NaHCO3 supplemented with 10% heat-inactivated fetal bovine serum (FBS). IEC-6 cells were maintained at 37 °C with 5% CO2 in DMEM with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 unit/ml insulin and 1.5 g/liter NaHCO3 supplemented with 10% heat-inactivated FBS.

Inhibitors—PP2, a potent and selective inhibitor of the Src family tyrosine was dissolved in dimethyl sulfoxide (Me2SO), and NSC23766, a specific inhibitor of Rac activation was dissolved in sterile distilled water. All inhibitors were aliquoted and stored at −20 °C and were diluted immediately prior to use in cell culture medium. Cells were treated with PP2 (10 μM) or an equivalent amount of Me2SO (vehicle control) for 45 min or NSC23766 (50 μM) or cell culture medium (vehicle control) for 60 min prior to exposure to repetitive cyclic strain. FAK, Src, or NT1 siRNAs were dissolved in 1× siRNA buffer (Dharmacon, Lafayette, CO) at 40 μM. All siRNAs were aliquoted and stored at −80 °C.

Application of Cyclic Strain—The cells were subjected to repetitive deformation using the Flexercell Strain Unit (FX-3000; Flexercell), as described previously (10). Briefly, Caco-2 cells were plated on elastomer membranes coated with type I collagen Flex I 6-well culture plates (Flexcell International Corp, Hillsborough, NC) and were exposed to continuous cycles of strain/relaxation generated by a cyclic vacuum produced by a computer-driven system (Flexcell 3000; Flexcell International Corp.). Caco-2 cells were subjected to a 20-kilopascal vacuum at 10 cycles/min, with a stretch/relaxation ratio of 1:1 (3.0-s deformation alternating with 3.0 s in neutral conformation), creating an average 10% strain for the indicated times.
Src and Rac1 Mediate Mitogenic Effects of Strain

as described under “Experimental Procedures.” The vacuum applies negative pressure that stretches the membranes to a known percentage elongation. We have previously demonstrated that strain is transmitted to adherent cells cultured on the upper surface of the membrane, which experience similar elongation (7, 8). The 6-well plates were maintained in a 37 °C humidified incubator with 5% CO₂ during the application of repetitive strain. Similar plates containing control cultures were kept in the same incubator but were not subjected to strain regimens.

Proliferation Studies—Proliferation was assessed by two different methods. In some studies, we counted cells directly after trypsinization with 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—Caco-2 cells were seeded at 100,000 cells/well on type I collagen Flex I 6-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 6-well plate were trypsinized and counted to provide a time 0 measurement. The remaining cells were then cultured for 24 h under either static conditions or conditions of repetitive strain before trypsinization and cell counting. Cell number was determined by counting each of the 6 wells independently (Coulter Electronics, Luton, UK). Data from each experiment were analyzed with six observations in each group.

Crystal Violet Staining—Caco-2 or IEC-6 cells were seeded at 100,000 cells/well on precoated type I collagen Flex I 6-well culture plates for 24 h. Subconfluent (30–40%) cells were serum-starved for 24 h (Caco-2 cells) or for 6 h (IEC-6 cells), and a single 6-well plate was reserved for a time 0 measurement. The remaining serum-starved cells were switched back to normal growth medium with 10% FBS under static or repetitive strain conditions for 24 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, as described elsewhere (40). The crystal violet staining was carried out as described previously (41), with slight modifications. Briefly, viable cells were rinsed in warm phosphate-buffered saline (PBS) and fixed in absolute ethanol/glacial acetic acid (3:1, v/v) for 10 min at room temperature and left to air dry (eventually stored at 4 °C, wrapped in aluminum foil). The cells were stained with 0.1% crystal violet (w/v) for 10 min at room temperature. Excess dye was removed by decantation and two subsequent washings with distilled water. The dye was extracted in 10% acetic acid (v/v), and absorbance 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.

Western Blot Analysis—Cells were cultured as described previously (10), grown to confluence, and changed to serum-free media for 24 h. After treatment, cells were lysed on ice in modified radiolymphocine precipitation 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 phenylmethylsulfonyl fluoride (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). 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% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) with 0.1% Tween 20 for 1 h at room temperature. The immunoblots were probed with appropriate primary and secondary antibodies and detected by ECL (Amersham Biosciences) with a Kodak Image Station 440CF PhosphorImager (Kodak Scientific Imaging Systems, Rochester, NY).

Rac1 Translocation Assays—The Rac1 translocation assay was performed as described previously (42) with slight modifications. The static control cells or cells exposed to cyclic strain were scraped into a lysis buffer containing 50 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM MgCl₂, 2 mM EDTA, 5 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na₃VO₄, and 1 mM dithiothreitol. The cell lysates were sonicated at 20% duty cycles 10 times to disrupt the cell membranes and release cytosolic proteins (Sonifier 250, Branson Ultrasonic Corp., Danbury, CT). The lysates were ultracentrifuged at 10⁵ × g for 1 h at 4 °C. The supernatant was collected as the “cytosolic fraction.” The resulting pellet “membrane fraction” was washed twice with PBS and resuspended in the above lysis buffer. The pellet was sonicated at 20% duty cycles 10 times, incubated 30 min on ice, and then stored at −80 °C. Protein concentrations were determined by the BCA method as above. An equal amount of cell lysates (5–10 μg protein) of cytosolic and membrane fractions was loaded per well on a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with monoclonal anti-Rac1 antibody to assess the amounts of Rac1 in each fraction. The blots were also probed with Rho-GDI and E-cadherin as protein loading controls for cytosolic and membrane fractions, respectively.

Rac1 GTPase Activity Assays—Rac1 activity was assessed by pulldown assay as described previously with slight modifications (38). Cells were grown to confluence and were serum-starved for 24 h before unstimulated cells at time 0 were compared with cells stimulated by cyclic strain for 2, 5, and 15 min. Cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM MgCl₂, 2 mM NaF, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Extracts were sonicated and clarified by centrifugation (10 min, 14,000 rpm at 4 °C), and the protein concentration was determined by the BCA method as above. Active Rac1 levels were determined by GST-PBD (glutathione S-transferase-conjugated p21 binding domain) of p21-activated kinase 1 pull-down assays. The cell lysates were incubated with PAK-PBD beads for 1 h at 4 °C on a rotator, and the beads were pelleted by centrifugation at 5000 × g for 3 min at 4 °C. The resulting pellet was then resuspended in Laemmli buffer resolved electrophoretically, transferred to nitrocellulose, and immunoblotted with monoclonal anti-Rac1 antibody (Pierce). Fifteen to 20 μg of cell lysates were used for Western blots for total Rac1 or β-actin antibodies.
Transfection with siRNA—For these studies, cells were plated on type I collagen Flex I 6-well culture plates at 30–40% confluence 1 day before transfection. siRNAs were combined with Plus reagent in Opti-MEM as described previously for plasmid DNA transfection (43). 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 DMEM containing 20% heat-inactivated FBS was added to the cells, and transfection was continued for 48 h. The cells were serum-starved overnight prior to signal studies. For proliferation studies, cells were transfected for 24 h followed by 24 h with and without cyclic strain. The effectiveness of the siRNA transfection was verified in each study by parallel transfections in which the cells were then lysed at the conclusion of the study, and the resulting lysates were immunoblotted for the target protein of interest.

FAK-ERK Cotransfection Studies—Caco-2 cells were transfected as described previously with slight modifications (44). To compare the effect of transfection with wild type FAK or FAK mutant Phe576–Phe577 on ERK2 activity, cells at 70–80% confluence were cotransfected with 4.8 µg of HA-tagged FAK-wild type or HA-tagged FAK mutant Phe576–Phe577 DNA and 1.2 µg of DNA of Myc-tagged ERK2 expression vector prior to study. Thus, cells in each well received a total of 1.0 µg of DNA with FAK and ERK constructs at a 4:1 ratio. The DNA was mixed with 60 µl of Plus reagent in 1 ml of Opti-MEM for 15 min, and then Lipofectamine (30.0 µl in 1 ml of Opti-MEM) was added. This mixture was incubated at room temperature for 20 min, diluted with 6.0 ml of Opti-MEM, and added at 1.0 ml per well to cells for 6 h. Cells were rinsed twice with Opti-MEM prior to addition of the transfection mixture. Following transfection, cells were incubated with normal medium for 20–24 h and then incubated with medium containing no FBS for an additional 18–24 h prior to exposure to cyclic strain. 20–25% of cells are transfected using this procedure (45).

Myc Tag Monoclonal Antibody 9E10 Immunoprecipitation and Western Analysis—Immunoprecipitation was performed as described previously with slight modifications (44). Briefly, Caco-2 cells cotransfected with FAK and ERK were lysed in immunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 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), and protein was measured by BCA as above. 20 µl of protein G-Sepharose was preblocked with 1% heat-inactivated bovine serum albumin in PBS for 1 h at room temperature for each immunoprecipitation reaction. The preblocked protein G-Sepharose was then rinsed twice with immunoprecipitation buffer, and 3.0 µg of Myc-tagged monoclonal 9E10 antibody was incubated for each immunoprecipitation reaction for 2 h at 4°C on a rotator. The protein G-Sepharose-bound Myc-tagged antibody was rinsed twice with immunoprecipitation buffer. 50 µl of the complex was added to 500 µg of protein-matched samples diluted to equal volumes with immunoprecipitation buffer for each immunoprecipitation reaction, and the resulting mixture was incubated at 4°C overnight on a rotator. The resultant immunocomplexes were rinsed four times with 0.5 ml of immunoprecipitation buffer by centrifugation at 5000 rpm for 2 min at 4°C. The final pellets were resuspended in sample loading buffer, boiled for 4 min, resolved by 10% SDS-PAGE, and transferred to nitrocellulose membrane. The membranes were probed with polyclonal phospho-ERK and total ERK antibodies. Fifteen to 20 µg of protein-matched aliquots were used for Western blots of cell lysates for Myc, HA, and α-tubulin as additional protein loading controls.

Statistical Analysis—All experiments were done independently at least three times unless indicated otherwise. Data are presented as means ± S.E. and analyzed by Student’s unpaired t test. p < 0.05 was considered significant.

RESULTS

Repetitive Deformation Stimulates Site-specific FAK Phosphorylation in a Time-dependent Manner—We have shown previously that repetitive deformation induced by cyclic strain stimulates FAK tyrosine phosphorylation (15) as well as site-specific FAK-Tyr397 phosphorylation on collagen I, collagen IV, or laminin substrates in Caco-2 cells (10). However, the time course over which cyclic strain induces this site-specific phosphorylation of FAK in Caco-2 cells was not known. Increased phosphorylation of both tyrosine moieties was observed as rapidly as 2 min (132.21 ± 10.76% (Fig. 1A), n = 5, p < 0.5 for FAK-Tyr397p and 138.84 ± 5.72% (Fig. 1B), n = 5, p < 0.001 for FAK-Tyr576p, respectively) after the initiation of strain. The FAK-Tyr397p phosphorylation was stable for the first 15 min and then declined somewhat, but remained higher than base line after an hour. In contrast, FAK-Tyr576p phosphorylation increased over the first 15 min to 176.46 ± 21.5% of control values after strain initiation (n = 5, p < 0.005 versus time 0). FAK-Tyr576p phosphorylation was also statistically greater at 15 min after strain initiation than at 2 min (n = 5, p < 0.05 versus the 2-min time point). After 15 min, FAK-Tyr576p phosphorylation declined gradually but still remained above base line. Thus, repetitive deformation stimulates FAK phosphorylation at Tyr397 and Tyr576 in a rapid and sustained manner, but over a somewhat different time course.

FAK Is Required for the Stimulation of Proliferation by Cyclic Strain—We have previously shown that transfection of Caco-2 cells with dominant negative FAK inhibits strain-induced mitogenic ERK activation. To further confirm the role of FAK in this mitogenic response, we transfected Caco-2 cells with siRNA targeted to FAK or a nontargeting siRNA sequence (NT1) for 24 h and then assessed proliferation with and without repetitive strain for 24 h. FAK-specific siRNA significantly reduced total FAK protein by 67.0 ± 3.02% (Fig. 1A), n = 4, p < 0.05.

Repetitive Deformation Stimulates Src Phosphorylation—The activity of c-Src is regulated by tyrosine phosphorylation at multiple sites. The autophosphorylation of c-Src at Tyr418 of the activation loop is a critical step leading to its full activation (46). We observed a threshold increase of Src activation within
SRC and RAC1 MEDIATE MITOGENIC EFFECTS OF STRAIN

FIGURE 1. Role of FAK in repetitive deformation-induced proliferation in Caco-2 cells. A and B, the effect of cyclic strain on FAK-Tyr397 and Tyr576 phosphorylation was assessed by Western blot in cells subjected to repetitive deformation for 0–60 min. Strain induces rapid and sustained FAK phosphorylation at both Tyr397(A) and Tyr576(B). Total FAK (t-FAK) and GAPDH served as controls. Bars represent densitometric analysis (n = 5, *p < 0.05 for each); representative Western blots are shown above the graph. C, cells were transiently transfected with siRNA targeted to FAK or with nontargeting NT1 sequences prior to lysis and Western blot for FAK protein. Either the FAK-1 or FAK-2 siRNA sequence achieved ~70% reduction in FAK protein levels. A typical immunoblot showing FAK protein level reduction in cells transiently transfected with siRNA targeted to FAK in comparison with cells transfected with a nontargeting sequence (NT1). D, cells transiently transfected with pooled siRNA targeted to FAK or nontargeting NT1 sequences were maintained under static conditions (open bars) or repetitive deformation (shaded bars) for 24 h prior to crystal violet staining. Deformation stimulates proliferation as quantitated by crystal violet staining (550 nm absorbance) in NT1-treated cells but not in cells in which FAK was reduced (n = 4, *p < 0.05).

2 min (Fig. 2A, 123.67 ± 4.13%, n = 5, p < 0.05) after the initiation of repetitive deformation with maximal activation at 15 min (132.46 ± 5.97%, n = 5, p < 0.05). Src-Tyr418 phosphorylation was increased for up to 30 min after repetitive deformation but decreased to base line by 60 min after initial activation.

Src Is Required for Deformation-Induced Proliferation—We next studied the involvement of Src in the mitogenic response to repetitive deformation using PP2 to inhibit Src. Semi-confluent Caco-2 or IEC-6 cells were pretreated with PP2 (10 μM) or vehicle control Me2SO (0.1% v/v) for 45 min and were then exposed to repetitive deformation for 24 h or cultured without repetitive strain. Me2SO-treated Caco-2 or IEC-6 cells treated with Me2SO exhibited increased proliferation in response to deformation compared with static controls (n = 4, p < 0.005; Fig. 2, B and C). PP2 blocked strain-induced proliferation in comparison with unstretched cells without affecting basal proliferation (Fig. 2D). Reduction of Src by transient transfection with siRNA targeted to Src also blocked strain-induced Caco-2 proliferation in comparison with static control without affecting basal proliferation compared with NT1-transfected controls (n = 4, p < 0.005; Fig. 2E).

Cyclic Strain Induces Rac1 Phosphorylation and Translocation—Cells exposed to repetitive deformation exhibited increased Rac1 phosphorylation within 2 min (121.97 ± 6.27%, n = 5, p < 0.05; Fig. 3A). This effect was maximal at ~5–15 min (142.42 ± 14.27%, n = 5, p < 0.05; Fig. 3A). Translocation of Rho family GTPases (Rho, Rac, and cdc42) from cytosol to the cell membranes has been widely used as an indirect measure of activation (28, 47). To further confirm that Rac1 is activated in response to cyclic strain, confluent serum-starved Caco-2 cells were subjected to strain for 0–30 min. Cytosolic and membrane fractions derived from cell lysates harvested at each time point were resolved by SDS-PAGE and immunoblotted for total Rac1. Repetitive deformation stimulated Rac1 translocation from the cytosol to cell membranes in a time-dependent manner (Fig. 3B, upper panel). Blots were also probed with antibodies that recognize the abundant cytosolic Rho-GDI (Fig. 3B, lower panel) and the membrane-abundant protein E-cadherin (lower panel) to confirm fraction enrichment and equal loading. We observed significantly increased Rac1 translocation as early as 5 min (168 ± 15.6%, n = 4, p < 0.05) and maximal at 30 min after strain initiation (182 ± 17.8%, n = 4, p < 0.05; Fig. 3B).

Strain Induces Rac1 Activation—To further confirm that Rac1 is activated in response to mechanical strain, we performed a Rac1 pulldown assay (Fig. 3C). Rac1 activation increased 3.7 ± 0.85-fold (n = 3, p < 0.05) as early as 2 min after strain initiation, appeared maximal 5 min after strain initiation (4.1 ± 0.83-fold increase, n = 3, p < 0.05), and decreased by 15 min but was still significantly greater than basal levels at that time (1.64 ± 0.30-fold, n = 3, p < 0.05). Consistent with our observations of Rac1 phosphorylation and translocation, these results strongly suggest that Rac1 is activated by mechanical strain in intestinal epithelial cells.

Rac1 Is Required for Repetitive Deformation-Induced Proliferation—Repetitive deformation induces Rac1 phosphorylation, translocation, and activation. We next determined Rac1 involvement in strain-induced proliferation. The 50 μM Rac1-specific inhibitor NSC23766 (38) completely blocked repetitive deformation-induced Rac1 phosphorylation without affecting basal Rac1 phosphorylation (n = 5, p < 0.05; Fig. 3D).
We therefore pretreated Caco-2 and IEC-6 cells with NSC23766 or with vehicle (complete DMEM) for 60 min and then studied proliferation for the subsequent 24 h. Rac1 inhibition blocked strain-induced Caco-2 and IEC-6 proliferation (Fig. 3, E and F) and inhibited basal proliferation in Caco-2 cells (n = 4, p < 0.05; Fig. 3E) in comparison with static control cells. However, in contrast to Caco-2 cells, the Rac1 inhibitor did not affect the basal IEC-6 proliferation (Fig. 3F).

Effect of siRNA Targeted to FAK on FAK Phosphorylation—Pooled siRNAs targeted to FAK markedly reduced the amount of phosphorylated FAK for each tyrosine studied at baseline. Although FAK phosphorylation increased in response to repetitive strain for each tyrosine studied in the NT1-transfected cells (Fig. 4A, n = 4 from six similar studies, p < 0.05 for each), strain did not induce a detectable increase in the amount of phosphorylated FAK in the FAK-reduced cells.

FAK Is Required for Strain-induced ERK Phosphorylation—Cells transfected with NT1 siRNA displayed increased ERK phosphorylation in response to repetitive strain (Fig. 4B, left two bars, n = 10, p < 0.05). In contrast, cells transfected with FAK siRNA displayed slightly increased basal ERK phosphorylation, but ERK phosphorylation was not further stimulated by repetitive strain in the FAK-reduced cells (Fig. 4B, right two bars).

Deformation-induced Src Phosphorylation Is Independent of FAK—Src phosphorylation was significantly increased by repetitive deformation in NT1-transfected cells in comparison with cells transfected with NT1 siRNA but not exposed to repetitive strain (Fig. 4C, left two bars, n = 5, p < 0.05). FAK reduction did not inhibit strain-stimulated Src phosphorylation (Fig. 4C, right two bars, n = 5, p < 0.05).

FAK Reduction Stimulates Basal Rac1 Phosphorylation but Does Not Prevent Rac1 Induction by Deformation—Cells transfected with NT1 siRNA displayed significantly increased Rac1 phosphorylation in response to 15 min of repetitive deformation in comparison with static controls (Fig. 4D, left two bars, n = 5, p < 0.05). Cells transfected with FAK siRNA displayed significantly increased
FIGURE 3. Role of Rac1 activation in repetitive deformation-induced proliferation in Caco-2 and IEC-6 cells. A, cyclic strain (0–60 min) promotes a rapid and transient Rac1 phosphorylation (p-Rac1) as assessed by Western blot. Bars document densitometric analysis (n = 5, *p < 0.05) of the ratio of p-Rac1 to the GAPDH control; representative blots are shown above the graph. B, cyclic strain (0–30 min) also stimulates rapid Rac1 translocation to the cell membrane. Rho-GDI and E-cadherin served as controls for cytosolic and membrane fractions, respectively. Typical blots are shown in the upper panel and densitometric analysis in the lower bars (n = 4, *p < 0.05). C, Rac1 GTPase is rapidly activated by cyclic strain. The effect of cyclic strain on Rac1 activation was quantitated by pulldown assays of the GST-PBD domain of p21-activated kinase 1 as described under “Experimental Procedures.” Total Rac1 (t-Rac1) or β-actin served as controls; representative blots are shown above the bars summarizing densitometric analysis (n = 3, *p < 0.05). D, pretreatment (60 min) with the Rac1 inhibitor NSC23766 (50 μM) inhibits rapid (15 min) repetitive deformation-stimulated Rac1 phosphorylation. Densitometric analysis of the ratio of p-Rac1 to total Rac1 (n = 5, *p < 0.05) is documented in the bars; typical Western blots are shown above the graph. E, Rac1 inhibition also blocks deformation-induced proliferation as assayed by crystal violet absorbance. After 24 h under static (open bars) or cyclic strain (shaded bars), proliferation was stimulated in vehicle (DMEM)-treated cells (n = 4, *p < 0.05) but not in cells pretreated with 50 μM NSC23766. The Rac inhibitor also affected basal proliferation (n = 4, #p < 0.05). F, Rac1 inhibition also blocks deformation-induced proliferation of IEC-6 cells as assayed by crystal violet absorbance. After 24 h under static (open bars) or cyclic strain (shaded bars), proliferation was stimulated in vehicle (DMEM)-treated cells (n = 4, *p < 0.05), but not in cells pretreated with 50 μM NSC23766. In contrast to Caco-2 cells, the Rac inhibitor did not affect the IEC-6 basal proliferation (n = 4, p > 0.05).
Src and Rac1 Mediate Mitogenic Effects of Strain

**A** Basal Rac1 phosphorylation (Fig. 4D, n = 5, p < 0.05) as well as further induction of Rac1 phosphorylation in response to repetitive deformation (Fig. 4D, right two bars n = 5, p < 0.05).

**B** SRC inhibition selectively blocks strain-induced FAK phosphorylation—SRC inhibition by 45 min of PP2 pretreatment did not inhibit the strain-induced FAK-Tyr397 phosphorylation at 15 min (n = 5, p < 0.05; Fig. 5A), although PP2 increased basal FAK-Tyr397 phosphorylation significantly (n = 5, p < 0.05; Fig. 5A). In contrast, PP2 completely blocked strain-stimulated FAK-Tyr397 phosphorylation even after repeating basal FAK-Tyr397 phosphorylation (n = 5, p < 0.05; Fig. 5B). Reducing SRC did not block strain-induced FAK-Tyr397 phosphorylation (n = 5, p < 0.05; Fig. 6A) similarly to PP2, although a significant effect of reducing SRC on basal FAK-Tyr397 phosphorylation was not observed. Reducing SRC also prevented strain-induced FAK-Tyr576 phosphorylation similarly to PP2 (n = 5, p < 0.05; Fig. 6B).

**C** SRC is required for deformation-induced ERK and Rac1 phosphorylation—PP2 and reducing SRC by siRNA transfection (n = 5) each blocked deformation-induced ERK phosphorylation at 30 min (Figs. 5C and 6C, respectively; p < 0.05 for each), PP2 and SRC-targeted siRNA also each blocked deformation-induced Rac1 phosphorylation at 15 min (Figs. 5D and 6D, respectively; n = 5, p < 0.05 for each).

**D** Rac inhibition selectively modulates strain-induced FAK phosphorylation—Cyclic strain again stimulated FAK-Tyr397 and FAK-Tyr576 phosphorylation in comparison with static controls at 15 min (Fig. 7, A and B, n = 5, p < 0.05 for both, left two bars). Rac1 inhibition by NSC23766 slightly increased basal FAK-Tyr397 phosphorylation but did not prevent further FAK-Tyr397 phosphorylation in response to repetitive strain (Fig. 7A, n = 5, p < 0.05, right two bars). In contrast, Rac1 inhibition completely blocked strain-induced FAK-Tyr576 phosphorylation without affecting basal FAK-Tyr576 phosphorylation (Fig. 7B).

**E** Rac is required for repetitive deformation-induced ERK phosphorylation—We next asked whether Rac1 is required for ERK activation in response to deformation. Cyclic strain stimulated ERK phosphorylation in comparison with static controls at 30 min (Fig. 7C, n = 5, p < 0.05, left two bars). Rac1 inhibition by NSC23766 (50 μM) prevented ERK phosphorylation in response to repetitive deformation (Fig. 7C, right two bars).}

---

**FIGURE 4.** FAK reduction with siRNA inhibits repetitive deformation-induced FAK and ERK but not Src or Rac1 phosphorylation. A, treatment of Caco-2 cells with pooled siRNA targeted to FAK lowers FAK expression and site-specific FAK phosphorylation. Cells were cultured for 48 h after transfection with either the nontargeting (NT1) control or FAK siRNA and then serum-starved for 24 h before being subjected to cyclic strain for 15 min. Deformation stimulated FAK-Tyr397 and Tyr576 phosphorylation in NT1-treated cells but not in cells where FAK had been reduced (n = 4 from six similar studies). In the subsequent panels, densitometric analysis of the ratio of activated to total (α-tubulin or GAPDH control) is depicted in the bars, and representative Western blots are shown above the graph. B, lowering FAK attenuates deformation-induced ERK phosphorylation. Cells transfected with either NT1 or FAK siRNA were maintained under static conditions (open bars) or conditions of cyclic strain (shaded bars) for 30 min prior to lysis and Western blot analysis. Strain stimulated ERK phosphorylation in NT1-transfected cell but did not significantly affect p-ERK in FAK-reduced cells (n = 10, *, p < 0.05), C, FAK reduction does not inhibit strain-induced Src phosphorylation. In these experiments, cells transfected with NT1 or FAK siRNA were subjected to cyclic strain for 15 min. Strain significantly increased Src phosphorylation in NT1 as well as FAK siRNA-treated cells. The graph summarizes densitometric analysis from five similar studies (*, p < 0.05). D, strain also significantly increases Rac1 phosphorylation in both NT1- and FAK siRNA-treated cells. The graph summarizes densitometric analysis from five similar studies (*, p < 0.05). The FAK-reduced cells also displayed increased Rac1 phosphorylation in comparison with cells transfected with NT1 but not stimulated with strain (n = 5, p < 0.05).
Src and Rac1 Mediate Mitogenic Effects of Strain

Src phosphorylation of FAK at Tyr$^{576}$ and Tyr$^{577}$ might also be required for ERK activation. We compared the effects of cotransfection of cells with a Myc-tagged wild type ERK2 expression vector and HA-tagged FAK wild type expression vector or HA-tagged FAK phosphorylation-deficient mutant constructs (48), in which the tyrosine phosphorylation sites of FAK at Tyr$^{576}$ and Tyr$^{577}$ are mutated to phenylalanine (Phe$^{576}$–Phe$^{577}$) to prevent tyrosine phosphorylation. Expression of the FAK mutant Phe$^{576}$–Phe$^{577}$ blocked strain-induced ERK2 activation (Fig. 8), although Myc-tagged ERK2 was activated by cyclic strain after cotransfection with wild type FAK ($n = 3, p < 0.05$), indicating that Src-mediated FAK-Tyr$^{576}$–Tyr$^{577}$ phosphorylation is also necessary for strain-induced ERK activation.

**DISCUSSION**

These studies were aimed at identification of the upstream intracellular signaling pathways that mediate the mitogenic effects of repetitive mechanical deformation in Caco-2 intestinal epithelial cells. The findings presented here demonstrate two apparently independent signaling events that converge upon the mitogenic signal of ERK activation in response to repetitive strain. FAK-Tyr$^{397}$ phosphorylation occurs independently of Src and Rac1. However, Src is activated independently of FAK in response to repetitive deformation and seems to be required for the downstream phosphorylation of Rac1. Both Src and Rac1 activation are required for FAK-Tyr$^{576}$ phosphorylation, which also seems to become maximal somewhat later than FAK-Tyr$^{397}$ phosphorylation. Finally, this convergent FAK activation results in ERK activation and increased cell proliferation (Fig. 9).

Our results strongly implicate Src kinase signaling in the intestinal epithelial mitogenic response to rhythmic mechanical stimulation. Src is activated in a time-dependent manner in response to cyclic strain, and blocking Src with PP2 prevented both ERK activation and the mitogenic effects of repetitive deformation, something functional link between Src and ERK signaling. This is consistent with general observations that Src mediates a variety of mechanotransduced effects in rabbit proximal tubular epithelial cells (49), as well as in other nonepithelial cell types (19–23, 50–52). Our results show that Src mediates at least some mitogenic mechanotransduced signals in intestinal epithelial cells as well.

The coincidence of Src activation with that of FAK and ERK raised the possibility that deformation-induced FAK and ERK activation in Caco-2 cells is mediated, at least in part, through Src activation. Indeed, pretreatment of cells with the Src inhibitor PP2 blocked both deformation-induced FAK phosphorylation and the mitogenic effects of deformation, suggesting that Src activates FAK at Tyr$^{576}$ and that both Src and downstream ERK activation are required for the mitogenic effects of strain.
These data thus suggest that Src might be a proximal kinase in the signal pathway that leads through FAK and ERK to the increased proliferation of Caco-2 cells in response to rhythmic mechanical deformation.

We reported previously that FAK phosphorylation at Tyr397 is required for strain-induced ERK activation in response to repetitive deformation in Caco-2 cells by transient transfection of a dominant negative mutant of FAK that cannot be phosphorylated at Tyr397 (15), and we had therefore assumed that FAK was required for the mitogenic effect of strain. We tested this hypothesis directly in this study that FAK-specific siRNA not only attenuated the ERK activation induced by repetitive deformation but also prevented the mitogenic effects of strain. Although we had previously assumed this to be true, because FAK seemed required for ERK activation and ERK activation for strain-stimulated proliferation, this had not been directly demonstrated and could even have been false if the loss of FAK had unmasked a compensatory signal pathway that could bypass FAK and ERK.

The question then arose as to how FAK was being regulated in response to strain. We had previously reported that FAK is globally time-dependently more tyrosine-phosphorylated in response to repetitive deformation in Caco-2 cells (15) and that FAK is phosphorylated at Tyr397 in response to cyclic strain at 10–30 min (10). The current implication of Src in this pathway prompted us to compare FAK autophosphorylation at Tyr397 with FAK phosphorylation at Tyr576, a Src phosphorylation site. Exposure to repetitive strain rapidly and robustly stimulated site-specific FAK phosphorylation at Tyr397, the major autophosphorylation site, as well as at Tyr576, the putative activation loop of the kinase domain in Caco-2 intestinal epithelial cells, although there were differences in the time course over which these two phosphorylations occurred that could represent their different control mechanisms.

Although we had previously reported that FAK autophosphorylation was required for ERK activation, the cotransfection studies described here demonstrate that further phosphorylation of FAK by Src is also required for downstream activation of this pathway.

FAK is an important component of integrin-dependent signaling events that control various cellular responses to the extracellular matrix, including migration, survival, spreading, differentiation, and proliferation (53–55). Site-specific FAK
phosphorylation is critical for regulation of FAK catalytic activity, interaction with other focal adhesion proteins, and focal adhesion turnover (48, 56–58). Elevation of the phosphorylation content of FAK has been shown to be essential for both cell adhesion to extracellular matrix substrates and to cell transformation by Rous sarcoma virus, which correlates directly with an increase in kinase activity (48). The maximal kinase activity of FAK immune complexes requires phosphorylation of tyrosine 576 and tyrosine 577 in addition to tyrosine 397 (48).

Approximately 30% of FAK protein remains after siRNA transfection. Densitometric analysis of the FAK that remains suggests that the proportion of FAK that is phosphorylated on tyrosine 397 and tyrosine 576 under static conditions is increased in response to this overall reduction of FAK and that no further increase is detectable in response to repetitive deformation (data not shown). We have previously observed increased phosphorylation of FAK and paxillin in response to a reduction in overall levels of each signal protein by siRNA (43, 61). This may represent the effects of some intracellular attempt to compensate for the overall reduction in the total amount of the phosphorylated protein. Whether this remaining population of FAK is truly not deformation-responsive, already maximally phosphorylated, or exhibits phosphorylation changes below the detection limit of our assay awaits further investigation but is beyond the scope of this study.

Interestingly, Haussinger et al. (62) described the stimulation of choleresis by osmotically induced swelling in isolated perfused rat liver via integrin-dependent Src, FAK, and MAPK activation. Osmotic swelling likely also involves mechanotransduction, at least in part. In contrast to our findings, these authors reported that FAK and ERK activation occurred independently of Src. However, these authors used lower concentrations of PP2 (250 nM). They were able to demonstrate blockade of osmotically induced p38 activation, but not that of ERK, and investigated FAK activation only by examining FAK-Tyr397 autophosphorylation rather than phosphorylation of the Src-dependent phosphorylation sites on FAK. Thus, their studies of FAK activation are actually consistent with our finding that FAK-Tyr397 autophosphorylation is blocked by PP2, but FAK-Tyr397 phosphorylation is not. The differences between our studies of the Src dependence of ERK activation and those of Haussinger et al. (62) may reflect variations in signaling between hepatocytes and intestinal epithelial cells, differences in the direct cellular effects of osmotic swelling and repetitive strain, species differences, differential sensitivity to PP2, or indirect paracrine consequences of the application of the osmotic agent within an entire liver.
Although mechanotransduction by other members of the small GTP-binding protein Rho family has been evaluated in other cell types (63), the involvement of Rac1 in mechanosensing and signaling in response to repetitive deformation in epithelial cells is not as well understood. Although our results do not exclude a role for other small GTP-binding proteins, the activation of the small G-protein Rac1 seems to contribute to deformation-induced activation of FAK and ERK and proliferation of Caco-2 cells because Rac1 is phosphorylated, translocated, and activated in response to repetitive strain.

Our observation that Rac1 phosphorylation paralleled Rac1 activation and translocation in Caco-2 cells subjected to repetitive deformation differs from a previous report that Rac1 phosphorylation at Ser71 by activated recombinant Akt kinase may inhibit Rac1-GTP binding through its phosphorylation without affecting its GTPase activity in SK-MEL28 melanoma cells (64). However, the relationship between Akt and Rac1 is complex, and Rac1 may also modulate Akt in some systems (65). Moreover, preliminary observations suggest that Akt is not activated in response to repetitive deformation in Caco-2 cells,3 so Rac1 may be modulated differently in response to different stimuli. In contrast, Rac1 phosphorylation is stimulated (66, 67) and Rac1 is activated (68, 69) by both epidermal growth factor and fibroblast growth factor-2. These studies do correlate with our present observation that Rac1 phosphorylation is stimulated together with Rac1 activation in response to repetitive deformation.

We further observed that inhibition of Rac by NSC23766 significantly reduced deformation-stimulated FAK phosphorylation at Tyr576, ERK activation, and the strain-induced mitogenic response. Thus, Rac1 may be a key upstream regulator of these mechanically induced signals in this model. Activation of the small G-protein Rac1 has also been implicated in mechanotransduction in some other cell types (28–32), although this may not be true for all cells. For instance, Rac is inhibited in rat aortic smooth muscle cells in response to equibiaxial stretch or tangential strain, although it may be increased in these cells in response to radial strain (34). Moreover, Rac1 activation appears downstream of Src in the mechanotransduced pathway in these cells and is in turn required for downstream strain activation of FAK at Tyr576 and then of ERK. The interactions of Rac1 and FAK are variable in the literature. Rac1 has been shown by many investigators to be required for FAK activation in other cell types in response to various stimuli (70–73). In contrast, FAK has also been reported to be upstream of Rac1 in response to several stimuli in different cell culture systems in diverse complex signaling pathways (74–77). Src has often been observed to be upstream of Rac1 in other settings (78–80), consistent with our present observation, although Rac1 has been reported to

3 L. S. Chaturvedi, H. M. Marsh, X. Shang, Y. Zheng, and M. D. Basson, unpublished observations.
Src and Rac1 Mediate Mitogenic Effects of Strain

target Src toward focal adhesion complexes, a preliminary step for its activation in fibroblast cells (81). These differences likely reflect not only differences in cell type but also the complexity of signal protein interactions in pathways that may transduce signals in either direction depending upon the nature of the stimulus. Intestinal epithelial Caco-2 cells appear to activate FAK at Tyr576 independently of Rac1, but they require Rac1 activation for the further phosphorylation of FAK at Tyr576. Our further observation that Rac1 is required for mechano-transduced MAPK activation does seem consistent with reports of Rac1 interactions with ERK in various other cell types (29, 32). However, this relationship has not been investigated previously in epithelial cells.

The present results are different in important ways from three previous similar investigations in other cell systems. Kumar et al. (30) reported that cyclic mechanical strain induces proliferation in C2C12 skeletal myoblasts via FAK, Src, and Rac1 signaling, but they postulated a FAK-Src-Rac1 pathway in which FAK was the most proximal mediator in the pathway, with Src and Rac1 downstream of FAK. These authors did not characterize Src or FAK phosphorylation in response to strain in the setting of inhibition of the other signal, however. In contrast, our data suggest that reducing FAK does not prevent Src or Rac1 activation but that Src and Rac1 are required for strain-induced FAK-Tyr576 phosphorylation in Caco-2 cells. These authors also did not address the interaction between FAK or Src and Rac1.

Liu et al. (19) also implicated Src in the mitogenic response to repetitive strain in fetal rat lung cells. These investigators found no evidence of FAK phosphorylation or Src association with paxillin or FAK in response to cyclic strain, and they concluded that these cells respond to strain independently of FAK or paxillin. In contrast, the present results demonstrate that FAK does transduce deformation-induced signals in Caco-2 intestinal epithelial cells. We have previously described increased paxillin phosphorylation in these cells in response to strain as well (15). Others have also described FAK and paxillin signaling in response to strain in other cell types (82, 83). Liu et al. (19) did not study Rac1. The differences between our own results and those of Liu et al. (19) may reflect differences in mechanotransduced signal pathways between fetal rat lung cells and intestinal epithelial cells.

Osada et al. (35) described inhibition of proliferation in response to repetitive strain at 5 cycles/min in RGM1 cells, a rat gastric epithelial cell line. Although these authors did not study signal protein activation, they did report that Rac1 immunoreactivity is actually decreased by 24–48 h of repetitive deformation in gastric RGM1 cells at the edge of a wound in the monolayer, although unchanged in the RGM1 cells distant from the wound edge. Whether the change in Rac1 expression in response to strain in migrating cells was a direct consequence of strain or an indirect consequence of the slow down of wound closure in response to strain was not clear (35). Although we did not investigate the chronic effects of repetitive strain on Rac1 expression here, our observations suggest that mechanical strain rapidly activates Rac1 in Caco-2 intestinal epithelial cells. The difference between the anti-proliferative responses to strain in RGM1 cells described by Osada et al. (35) and the mitogenic effect of strain that we have observed in Caco-2 cells (7, 14, 15) and in primary human intestinal epithelial cells (11) may reflect a fundamental difference in the response of gastric and intestinal epithelial cells to repetitive deformation. The differences in strain frequencies studied could also have contributed to the differences between the results of Osada et al. (35) and our own results. We have previously reported that Caco-2 cell proliferation is not demonstrably altered in response to repetitive 10% strain at 5 cycles/min (13).

Although the magnitude of the effects that we have observed here are relatively small, typically in the range of 30–100%, signals of similar magnitude have been studied and are reported to be biologically significant by others in response to repetitive deformation in other cell types (22, 51, 84–90) and in intestinal epithelial cells in response to various mitogens (91–94). Intestinal epithelial proliferation is tightly regulated. Small changes in signal intensity may alter the rate of cell proliferation, and small changes in the rate of intestinal epithelial proliferation could have important consequences for this highly biologically active mucosal barrier.

In conclusion, our results suggest that c-Src, the small G-protein Rac1, FAK, and ERK mediate the mitogenic response to repetitive deformation in intestinal epithelial cells. Two deformation-activated signal pathways converge upon FAK, one Src- and Rac1-independent, which stimulates FAK-Tyr576 phosphorylation, and a second, Src- and Rac1-dependent, which is required for the further activation of FAK by phosphorylation at FAK-Tyr576 (within the FAK kinase activation loop). How Src is initially activated and the separate pathway by which FAK is phosphorylated at Tyr576 in response to mechanical strain independently of Src and Rac1 await further investigation. Tracing the signaling pathways initiated by repetitive mechanical deformation in intestinal epithelial cells may identify important targets for therapeutic interventions designed to prevent mucosal atrophy during prolonged ileus or fasting.

Acknowledgments—We thank Dr. Mary F. Walsh for helpful discussion. We also thank Dr. Steven K. Hanks (Vanderbilt University School of Medicine, Nashville, TN) for kindly providing the HA-tagged expression plasmids.

REFERENCES
1. Basson, M. D. (2003) Digestion 68, 217–225
2. Womack, W. A., Barrowman, J. A., Graham, W. H., Benoit, J. N., Kviety, F. R., and Granger, D. N. (1987) Am. J. Physiol. 252, G250–G256
3. Gutierrez, J. A., and Perr, H. A. (1999) Am. J. Physiol. 277, G1074–G1080
4. McNeil, P. L., and Ito, S. (1989) Gastroenterology 96, 1238–1248
5. Friedman, H. L., and Cardell, R. R., Jr. (1977) Anat. Rec. 188, 77–101
6. Schlegel, T. F., Hawkins, R. J., Lewis, C. W., Motta, T., and Turner, A. S. (2006) Am. J. Sports Med. 34, 275–280
7. Basson, M. D., Li, G. D., Hong, F., Han, O., and Sumpio, B. E. (1996) J. Cell Physiol. 168, 476–488
8. Basson, M. D., Turowski, G., and Emenaker, N. J. (1996) Exp. Cell Res. 225, 301–305
9. Basson, M. D., Modlin, I. M., and Madri, J. A. (1992) J. Clin. Investig. 90, 15–23
10. Zhang, J., Li, W., Sanders, M. A., Sumpio, B. E., Panja, A., and Basson, M. D. (2003) FASEB J. 17, 926–928
11. Zhang, J., Li, W., Sumpio, B. E., and Basson, M. D. (2003) Biochem. Bio-

JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 282 • NUMBER 1 • JANUARY 5, 2007
26
Src and Rac1 Mediate Mitogenic Effects of Strain

Biocen. Biophys. Res. Commun. 259, 8–14
85. Chen, Q., Li, W., Quan, Z., and Sumpio, B. E. (2003) J. Vasc. Surg. 37, 660–668
86. Li, L. F., Ouyang, B., Choukroun, G., Matyal, R., Mascarenhas, M., Jafari, B., Bonventre, J. V., Force, T., and Quinn, D. A. (2003) Am. J. Physiol. 285, 1464–1475
87. Lehoux, S., Esposito, B., Merval, R., and Tedgui, A. (2005) Circulation 111, 643–649
88. Wang, J., Sridurongrit, S., Dudas, M., Thomas, P., Nagy, A., Schneider, M. D., Epstein, J. A., and Kaartinen, V. (2005) Dev. Biol. 286, 299–310
89. Shikata, Y., Rios, A., Kawkitinarong, K., DePaola, N., Garcia, J. G., and Birukov, K. G. (2005) Exp. Cell Res. 304, 40–49
90. Hornberger, T. A., Armstrong, D. D., Koh, T. J., Burkholder, T. J., and Esser, K. A. (2005) Am. J. Physiol. 288, C185–C194
91. Fenton, J. I., Hord, N. G., Lavigne, J. A., Perkins, S. N., and Hursting, S. D. (2005) Cancer Epidemiol. Biomark. Prev. 14, 1646–1652
92. Potoka, D. A., Upperman, J. S., Zhang, X. R., Kaplan, J. R., Corey, S. J., Grishin, A., Zamora, R., and Ford, H. R. (2003) Am. J. Physiol. 285, G861–G869
93. Barone, M., Berloco, P., Ladisa, R., Ierardi, E., Caruso, M. L., Valmerti, A. M., Notarnicola, M., Di, L. A., and Francavilla, A. (2002) Scand. J. Gastroenterol. 37, 88–94
94. Fitzgerald, A. J., Jordinson, M., Rhodes, J. M., Singh, R., Calam, J., and Goodlad, R. A. (2001) Aliment. Pharmacol. Ther. 15, 1077–1084