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ERK Regulates Strain-Induced Migration and Proliferation From Different Subcellular Locations

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ABSTRACT

Repetitive deformation like that engendered by peristalsis or villous motility stimulates intestinal epithelial proliferation on collagenous substrates and motility across fibronectin, each requiring ERK. We hypothesized that ERK acts differently at different intracellular sites. We stably transfected Caco-2 cells with ERK decoy expression vectors that permit ERK activation but interfere with its downstream signaling. Targeting sequences constrained the decoy inside or outside the nucleus. We assayed proliferation by cell counting and migration by circular wound closure with or without 10% repetitive deformation at 10 cycles/min. Confocal microscopy confirmed localization of the fusion proteins. Inhibition of phosphorylation of cytoplasmic RSK or nuclear Elk confirmed functionality. Both the nuclear-localized and cytosolic-localized ERK decoys prevented deformation-induced proliferation on collagen. Deformation-induced migration on fibronectin was prevented by constraining the decoy in the nucleus but not in the cytosol. Like the nuclear-localized ERK decoy, a Sef-overexpressing adenovirus that sequesters ERK in the cytoplasm also blocked the motogenic and mitogenic effects of strain. Inhibiting RSK or reducing Elk ablated both the mitogenic and motogenic effects of strain. RSK isoform reduction revealed isoform specificity. These results suggest that ERK must translocate to the nucleus to stimulate cell motility while ERK must act in both the cytosol and the nucleus to stimulate proliferation in response to strain. Selectively targeting ERK within different subcellular compartments may modulate or replace physical force effects on the intestinal mucosa to maintain the intestinal mucosal barrier in settings when peristalsis or villous motility are altered and fibronectin is deposited into injured tissue. J. Cell. Biochem. 109: 711–725, 2010. Published in 2010 Wiley-Liss, Inc.

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The intestinal epithelium is exposed to numerous mechanical forces during normal bowel function. These forces include bowel peristalsis [Hennig et al., 1999], shear stress from endoluminal chyme [McNeil and Ito, 1989], and villous motility [Womack et al., 1987]. Since normal function leads to mucosal injury, the gut mucosal barrier must constantly repair itself to maintain its integrity [McNeil and Ito, 1990]. Deformation patterns are profoundly altered or even eliminated in conditions such as postoperative or septic ileus, or prolonged fasting states, and these conditions are associated with decreased mucosal healing, bacterial translocation, and breakdown of the mucosal barrier [Jones et al., 1990; Van Leeuwen et al., 1994].

In vitro, repetitive deformation is trophic for intestinal epithelial cells. However, the intestinal epithelial response to deformation depends upon the matrix in which the cells are cultured. Human Caco-2 intestinal epithelial cells [Basson et al., 1996], rat IEC-6 intestinal epithelial cells [Chaturvedi et al., 2007], and primary human intestinal epithelial cells from surgical specimens [Zhang et al., 2003] all proliferate more rapidly when exposed to strain on collagen or laminin substrates. If fibronectin is added, however, either to the medium or the matrix substrate, these effects are reversed [Zhang et al., 2003]. In contrast, strain stimulates Caco-2 and IEC-6 intestinal epithelial cell migration across a fibronectin matrix but inhibits migration across a collagen I matrix [Zhang

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et al., 2006], suggesting that deformation stimulates the intestinal mucosa in a complex fashion depending upon fibronectin levels. Although the mechanisms of either effect is incompletely delineated, ERK is required for both the mitogenic [Chaturvedi et al., 2007] and motogenic [Zhang et al., 2006] effects of deformation on intestinal epithelial cells. This poses the question of how activation of the same molecule leads to different effects in response to cyclic strain when cells are cultured on different matrices. Deformation increases activated ERK diffusely within the cytosol and nucleus of Caco-2 cells on collagen, but also produces readily discernable selective increases in phosphorylated ERK at both the lamellopodial edge and within the nucleus in Caco-2 cells migrating across fibronectin and subjected to repetitive deformation [Zhang et al., 2006]. We therefore hypothesized that ERK may act differently depending upon its subcellular localization to mediate these different effects.

Recently investigations have looked into subcellular relationships with ERK. ERK may enter the nucleus via a nuclear translocation sequence that then interacts with an importing protein [Zehorai et al., 2009] though others suggest ERKs movement is related to diffuse and its interaction with other binding partners in specific compartments [Burack and Shaw, 2005]. This ability may contribute to its diverse spectrum of cell responses. ERK movement within the cell is dependent on its specific stimulus. While glutamate stimulates ERK nuclear accumulation [Trifilieff et al., 2009], activation of Sef, a FGF feedback inhibitor, results in cytosolic accumulation [Kovalenko et al., 2003]. ERK activation and subsequent translocation seems to be cell-type and stimulus-type specific as evidenced by its role in liver versus gill cells of trout [Ebner et al., 2007].

ERK shuttles between the cytoplasm and the nucleus and is predominantly found within the cytoplasm in quiescent cells, but its function may be dictated by its subcellular location [Ebisuya et al., 2005]. Other molecules are also known to function both inside and outside the nucleus, including ILK [Acconcia et al., 2007] and Dok1 [Niu et al., 2006]. We therefore investigated the effects of repetitive deformation on intestinal epithelial cells on collagen and fibronectin substrates in which the effects of ERK had been selectively blocked on a subcellular level. We created stably transfected Caco-2 cell sub-lines expressing ERK decoy plasmids that competitively inhibit phosphorylated ERK in either the nucleus or the cytosol or control plasmids containing the localization sequence without the ERK decoy, and studied two different sub-lines for each construct with similar results. In parallel studies, we used viral transfection to overexpress Sef (*s*imilar *e*xpression to *F*GF gene), which constrains ERK within the cytosol, thus preventing its nuclear activity.

METHODS

MATERIALS

Dulbecco's minimal essential medium (DMEM), lipofectamine, Plus reagent, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, geneticin, carboxyfluorescein diacetate succinimifyl ester (CFSE), Hoechst stain, and anti-hemagglutinin (HA) Alexa Fluor 488 were obtained from Invitrogen Corporation (Carlsbad, CA), Western blot stripping reagent from Chemicon International (Temecula, CA), human transferrin was from Roche Applied Science (Indianapolis, IN), and trypsin and horseradish peroxidase-conjugated rabbit anti-mouse IgG from Sigma Chemical Co (St. Louis, MO). Phosphospecific polyclonal antibodies to Elk-1-Ser383 and p90RSK-Ser380 (ribosomal S6 kinase) and horseradish peroxidase-conjugated anti-mouse IgG were obtained from Cell Signaling (St. Louis, MO), as were total polyclonal antibodies to the same molecules. Anti-V5 tag IgG conjugated to R-Phycoerythrin (PE) was from Columbia Biosciences (Columbia, MD). The Sef-adenovirus was a generous gift from Dr. Robert Friesel from Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, Maine and Cooperative Graduate Program in Molecular Genetics and Cell Biology, University of Maine, Orono, Maine. SL0101, a non-specific RSK inhibitor, was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada).

PLASMID VECTORS FOR SELECTIVE INHIBITION OF ERK ACTIVITY

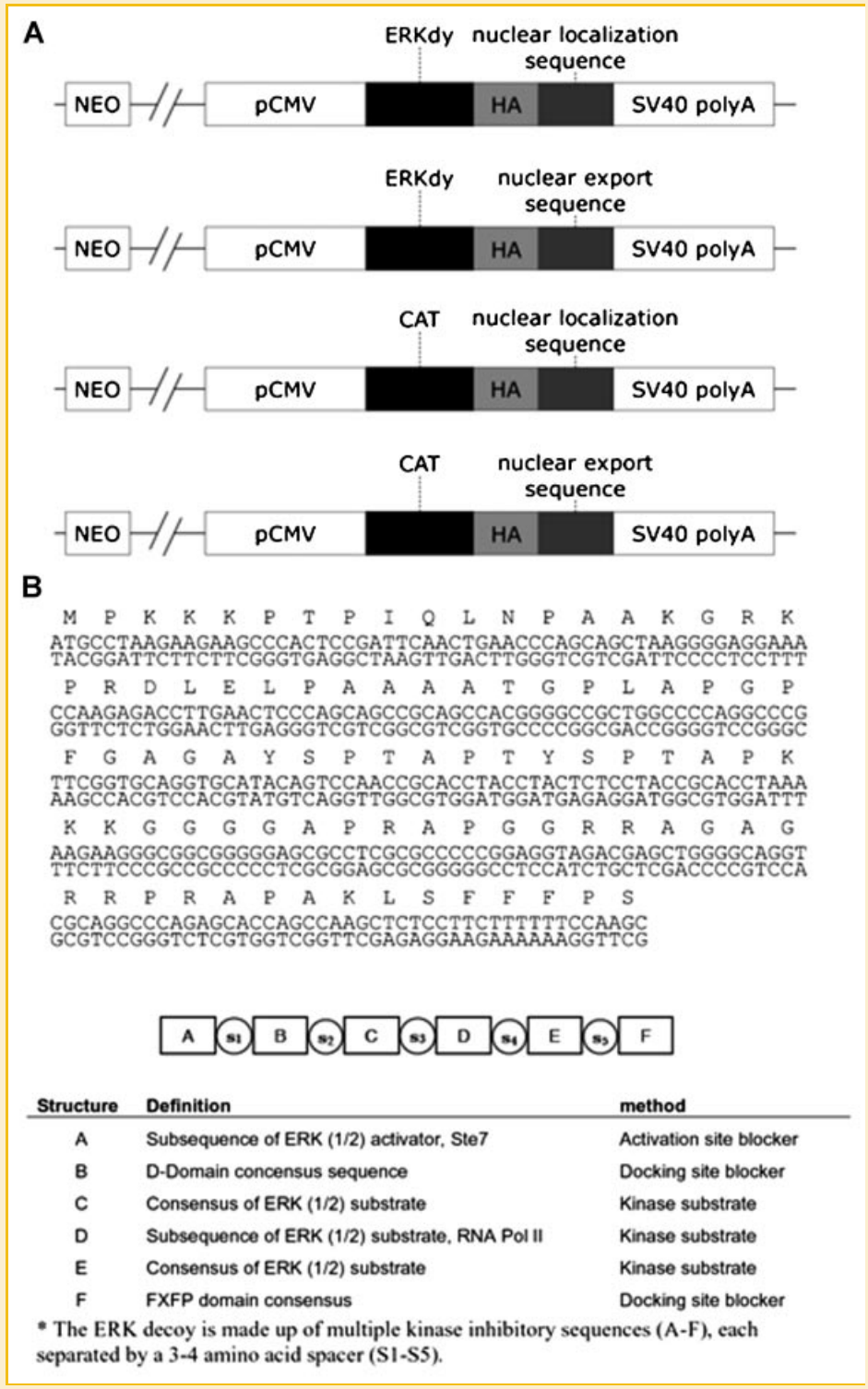
A series of transcriptional and protein modular domains was assembled in combinatorial fashion into a pUC plasmid-based backbone to yield four parallel vectors illustrated in Figure 1. The nuclear localization sequence (NLS2), the nuclear export sequence (NXP2), and chloramphenicol acetyltransferase (CAT) coding sequences were all generated as PCR amplification products. The HA epitope tag dimer (influenza hemagglutinin protein) sequence and the ERK decoy sequence were synthesized de novo. The neomycin resistance gene was supplied as a fragment containing the SV40 early promoter and the HSV TK-polyA.

MATRIX PRECOATING AND MEDIUM TREATMENTS

Flexwell plates were purchased precoated with collagen I (Flexcell International Corp, Hillsborough, NC) or Flexwell amino plates were precoated with 12.5 μg/ml tissue fibronectin (Sigma Chemical Co) at saturating concentrations in ELISA buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) as previously described [Zhang et al., 2006]. All wells were rinsed twice with sterile PBS prior to seeding at 300,000/well and studied sub-confluent for signaling experiments.

CELL CULTURE AND STABLE TRANSFECTIONS

We studied a subclone of the original human intestinal epithelial Caco-2 cell line that had been originally selected for its ability to differentiate in culture, as indicated by formation of an apical brush border and expression of brush border enzymes. The parent line was maintained at 37°C with 8% CO_2 in DMEM with 25 mM D-glucose, 4 mM glutamine, 1 mM sodium pyruvate, 100 unit/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml transferrin, 10 mM HEPES, pH 7.4, and 3.7 g/L $NaHCO_3$ supplemented with 10% heat inactivated fetal bovine serum (FBS). For some studies, these cells were stably transfected with each of the ERK decoy plasmids described above. To accomplish this, a total of 1.0 μg DNA per reaction was used with 10 μl per reaction Plus reagent and 5 μl per reaction lipofectamine after 15-min room temperature incubation. Opti-MEM was used to dilute the mixture so that 1 ml could be placed per well. After 6 h, media was replaced with normal media. Forty-eight hours later the medium was supplemented with geneticin 1 mg/ml as a selection



Structure	Definition	method
A	Subsequence of ERK (1/2) activator, Ste7	Activation site blocker
B	D-Domain concensus sequence	Docking site blocker
C	Consensus of ERK (1/2) substrate	Kinase substrate
D	Subsequence of ERK (1/2) substrate, RNA Pol II	Kinase substrate
E	Consensus of ERK (1/2) substrate	Kinase substrate
F	FXFP domain consensus	Docking site blocker

* The ERK decoy is made up of multiple kinase inhibitory sequences (A-F), each separated by a 3-4 amino acid spacer (S1-S5).

Fig. 1. A: ERK decoy expression plasmids containing localization sequences to isolate the gene product into a particular subcellular area. Decoys all contain HA tags. Panel B shows the decoy sequence and an illustration of the decoy structure and proteins used to derive the ERK decoys.

drug and cells were serially seeded into a 96-well plate. After single colonies were established, they were trypsinized and passed into progressively larger flasks. At this time media was changed to 0.6 mg/ml geneticin for maintenance to ensure continued selectivity for the transfected cells. Two separate stable transfectants were used for analysis for each plasmid (eight cell lines in total), with duplicates yielding similar results that were pooled together for statistical analysis.

siRNA TRANSFECTIONS

Caco-2 cells were plated to 30–40% confluence on Flexwell plates 1 day prior to transfection. Non-targeting siRNA (NT1), RSK1, RSK2, RSK3, and Elk siRNAs were purchased from Dharmacon (Lafayette, CO). The sequence for siRSK1 SMARTpool is: CUACAAGUGUUGC-UAGUUU, UAGCAAUCGUAGAUACUUA, CCAGGAAGGUAUAUG-CUAU, GCCAAUGA-CUUACUUAGGA. The sequence for siRSK2 SMARTpool is: CAACUGGCUCGUUCAAUU, UAGAAUAUGUGGC-CUAGAA, GAAACUAAUAGGACACUAA, CAAACUUGGUAAAG-AAUUG. The sequence for siRSK3 SMARTpool is: GAGGGAUUCU-GAACGACUA, CUUG-AAAUGAGACGUGCUA, CAUAUAGGCGG-GAGCAGAA, UGGAAUGGUAUUAGUCAA-A. The sequence of siElk SMARTpool is: GGACUACGCAAGAACAAGA, GCCAGAAGUU-CG-UCUACAA, UAGAAGGGCCCAAGGAAGA, GCAAGAACAAGACC-AACAU. siRNA was combined with Plus reagent in Opti-MEM with a final concentration of 60 μg/ml The final concentration of siRNA was 40 nM. Oligofectamine in Opti-MEM was used for transfection at 10 μg/ml according to the manufacturer's protocol. After 6 h of transfection, 0.5 volume of DMEM with 20% FBS was added for overnight incubation and the transfection continued for 24–48 h. Cells were exposed to serum free media 24 h prior to study for proliferation experiment. Effectiveness of the transfection was verified in parallel using cells similarly transfected and lysed at the conclusion of the study.

VIRAL TRANSDUCTION

Sef adenovirus was transduced as previously described by Kovalenko et al. [2003]. Briefly, plates were seeded at 50,000 cells/well with 10,000 virus particles per cell and incubated with 1,500 ng lipofectamine per well for 30 min followed by addition of serum-free DMEM addition to place 1 cc/well. After 6-h incubation at 37°C, the medium was aspirated and normal medium was added. Cells were used 24–48 h later for study.

STRAIN APPLICATION

Cells on Flexwell plates were subjected to mechanical deformation using the Flexcell Strain Unit (FX-3000; Flexcell, McKeesport, PA) as described previously [Zhang et al., 2006]. Briefly, cells were subjected to cyclic strain by a computer-controlled vacuum manifold using a 20 kPa vacuum at 10 cycles/min, producing an average 10% strain. Plates were maintained at 37°C humidified incubator with 5% CO_2 during repetitive deformation. Control plates were placed in the same incubator but not attached to the Flexcell Unit. Non-uniformity of strain in the center of the flexible wells was addressed by placing a Plexiglas ring in the center to exclude this area. Thus, only cells placed around the periphery of the wells were studied, where strain is more uniform. Previous studies have demonstrated that cells experience reproducible elongation and relaxation in parallel with the repetitive deforming membrane with this technique [Basson et al., 1996].

PROLIFERATION ASSAYS

Proliferation was assayed as previously described [Chaturvedi et al., 2007]. Briefly, stably transfected Caco-2 cells were plated on collagen-coated flex-well plates at 30% confluence, cultured overnight, and then serum-deprived for 24 h with one plate reserved for cell counting at the initial 0 h time. Cells were then re-exposed to serum and subjected to 24 h of either static conditions or repetitive deformation. After fixation, cells were counted via crystal violet staining and elution. Using 10% acetic acid, dye was extracted and absorbance at 550 nm was measured using a Thermomax microplate reader (Molecular Devices, Ramsey, MN). Each experiment contained six observations of six separate wells per condition. Alternatively, cells infected with the Sef adenovirus were assessed for proliferation using a flow cytometry-based CFSE proliferation assay in which the fluorescent CFSE label is inherited by daughter cells and is serially reduced with each cell division. Here, after 24 h of serum starvation, cells were incubated with 12.5 μM CFSE for 15 min at 37°C. Following 24-h exposure to cyclic strain or static conditions, cells were fixed and assessed for proliferation by relative CFSE signal in Sef-PE positive and negative cell populations by flow cytometric analysis.

MIGRATION ASSAY

Stably transfected Caco-2 cells were plated on Flexwell plates precoated with fibronectin and cultured to confluence. The cells were then serum-starved for 24 h and circular wounds were created using a 1,000-μl pipette tip as previously described [Zhang et al., 2006]. Cells were then subjected to 0–24 h of repetitive deformation. Wound areas were measured at each time interval using digital photographs taken on an inverted light microscope. The area of each photographed wound was quantitated using a Kodak Image Station (Perkin Elmer, Boston, MA) and compared to migration without strain.

WESTERN BLOT ANALYSIS

Cells were plated and cultured as described above, grown to confluence, serum-starved for 24 h, and then subjected to 0–60 min of cyclic strain. Cells were lysed on ice with fresh lysis buffer (50 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 2 μg/ml aprotinin, and 2 μg/ml leupeptin [pH 7.4]). After the lysates had been sonicated and centrifuged at 15,000*g* for 10 min at 4°C, protein concentrations were determined by bicinchoninic acid analysis (BCA assay, Pierce Chemical, Rockford, IL). Equal amounts of protein were loaded in a 10% sodium dodecyl sulfate polyacrylamide gel. Gels were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, Piscataway, NJ). After transfer, the membranes were blocked for at least 1 h at room temperature in 5% bovine serum albumin (BSA) in Tris-buffered saline with 1 ml/L Tween-20 prior to Western blot with appropriate primary and secondary antibodies. Immunoblots were probed and detected with ECL Plus reagent (Amersham Biosciences) on the Kodak Image Station 440 CF Phosphoimager (Kodak Scientific Imaging Systems, Rochester, NY). All exposures used for densitometric analysis were within the linear range.

FLOW CYTOMETRY

Cells were fixed with 2% paraformaldehyde for 5 min, permeabilized with 0.25% Triton-X, blocked with 5% BSA for 1 h and washed with

staining buffer containing 0.2% BSA and 0.02% sodium azide in PBS. Sef expression was determined by incubation with anti-V5/PE-conjugated antibody for 1 h at room temperature. Cells were then washed twice with staining buffer and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and WinMDI software.

CONFOCAL IMAGING

Cells were grown to confluence on Flexwell plates and washed twice with cold calcium-free phosphate-buffered saline (PBS). After 10 min fixation in cold methanol/acetone mixture, cells were washed again with PBS followed by blocking for 1 h with blocking buffer (0.5% BSA, 0.1% Triton in PBS). Anti-HA conjugated to Alexa Fluor 488 was diluted 1:200 in blocking buffer and added to the cells for 2 h at room temperature in the dark. Following PBS wash, cells were exposed to Hoechst strain diluted 1:20,000 from stock in blocking buffer for 45 min. Cells were washed and cover slips were placed. The samples were maintained in the dark at 4°C and then were viewed on the LSM 510 LASER scanning microscope from Carl Zeiss, Inc. (Thornwood, NY) the following day.

SUBCELLULAR FRACTIONATION

Caco-2 cell monolayers were exposed to either cyclic strain or static conditions for 60 min. Cell monolayers were then washed with ice-cold PBS, lysed, and cytosolic and nuclear cell fractions were isolated using the Qproteome Nuclear Protein Kit (Qiagen, Valencia, CA) as recommended by the manufacturer. Equal protein aliquots of cytosolic and nuclear fractions were resolved by SDS–PAGE, transferred to nitrocellulose, and probed for the proteins of interest. Successful fractionation and equal protein loading was verified by blotting for Rho-GDI (Cell Signaling) in cytosolic fractions and Histone H1 (Santa Cruz Biotechnology) in nuclear fractions.

STATISTICAL ANALYSIS

Results were compared using Student's *t*-test or Wilcoxon signed ranks test for non-parametric data and $P < 0.05$ was considered statistically significant. Data shown are expressed as the mean ± SEM of a minimum of three independent but similar experiments.

RESULTS

SUBCELLULAR LOCALIZED ERK DECOY EXPRESSION CONSTRUCTS

Design and synthesis of polypeptide ligands that modulate protein kinase activity in a specific subcellular compartment was previously demonstrated [Ji et al., 2003]. In that study, delivery of the polypeptide ligands was achieved by cellular expression from DNA vectors encoding sequences that localized the protein kinase inhibitory polypeptide ligands to the sarcoplasmic reticulum through fusion of a sarcoplasmic reticulum localization signal (derived from phospholamban) to a polypeptide inhibitory ligand. This expression led to inhibition of calcium/calmodulin-dependent protein kinase only in the targeted subcellular compartment, the sarcoplasmic reticulum. A similar strategy was employed in the design and expression of polypeptide ligands to modulate extracellular-signal-regulated kinase (ERK). Here, the kinase inhibitory sequence was targeted to the nuclear compartment through fusion to nuclear localization sequences (NLS) derived from the SV40 large T antigen [Kalderon et al., 1984]. These NLS sequences function in the nuclear import pathway through binding importin-alpha [Adam, 1999]. Targeting to the cytoplasmic compartment was accomplished through fusion of the kinase inhibitory sequence to nuclear export sequences (NES) derived from nuclear export protein of influenza B virus [Paragas et al., 2001]. These kinase inhibitory sequences are comprised of multiple protein domains that can potentially interact with phosphorylated ERK, and competitively interfere with its interaction with multiple substrates in different subcellular locations. Domains were derived from ERK substrates normally found in both the nucleus and the cytoplasm, but which have been modified to prevent phosphorylation by ERK. Other domains within the decoy were derived from sequences that serve as activation site or docking site blockers. This design approach of an inhibitory polypeptide with multiple inhibitory domains aims at maximal opportunities to inhibit ERK activity in whatever compartment the decoy encounters the ERK protein. Control vectors contained the same localization sequences, but with the ERK inhibitory sequence replaced by a biologically neutral sequence derived from the CAT protein. Immunological detection of all four proteins was mediated by an epitope tag sequence from the influenza hemagglutinin protein, 2× (YPYDVPDYA). Expression of the vectors was regulated by the CMV promoter and the 3′ regulatory sequence from SV40.

STABLE TRANSFECTION OF SUBCELLULAR LOCALIZED ERK DECOYS AND VERIFICATION OF SUBCELLULAR LOCALIZATION

The ERK decoys were designed to selectively block ERK on a subcellular level by competing with phosphorylated ERK for its downstream substrates. The four constructs used were the nuclear decoy, blocking ERK activity in the nucleus; the cytosolic decoy, blocking ERK activity in the cytosol; and localization control vectors for each of the two decoys. These controls contained the localization sequences that isolate the gene product within the relevant subcellular space, but no functional ERK decoy. Cells expressing each construct were fixed onto glass slides, permeabilized, and stained using with anti-HA antibody coupled to Alexa Fluor 488. These were viewed under the confocal microscope to verify the subcellular location of the ERK decoys (Fig. 2). To further assess the localization of these decoys, stable transfection cells were subcellularly fractionated and resultant lysates were probed for the HA tag. Nuclear fractions showed eightfold higher HA staining in the nuclear decoy and control versus cytosolic while cytosolic fractions showed eightfold higher HA staining in the cytosolic decoy and control versus nuclear.

RSK AND Elk ARE PHOSPHORYLATED IN RESPONSE TO STRAIN

RSK, a cytosolic protein and a known downstream target of ERK, and Elk, a nuclear protein and a known downstream target of ERK [Torii et al., 2004], were tentatively chosen as markers to test the functionality of the ERK decoys. We first confirmed that both RSK and Elk are activated in Caco-2 cells in response to cyclic strain. Confluent, serum-deprived Caco-2 cells were subjected to strain at 0, 15, 30, and 60 min and cell lysates were probed for phosphorylated RSK (pRSK) and phosphorylated Elk (pElk). RSK phosphorylation

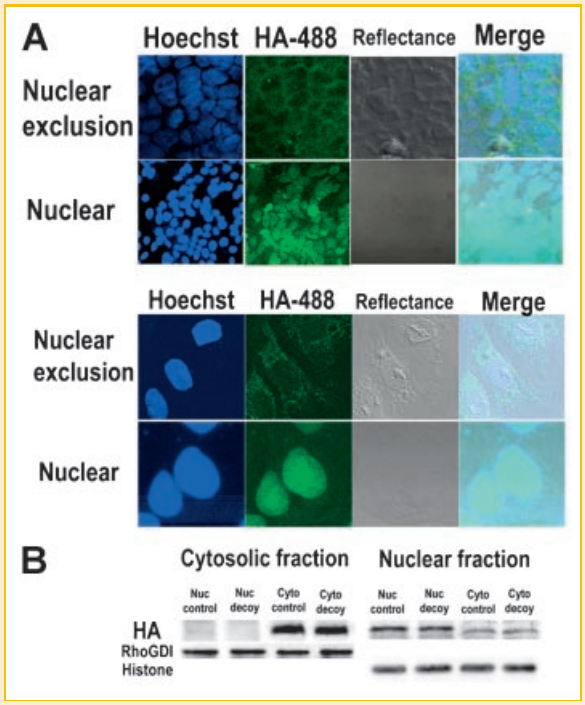


Fig. 2. ERK decoys are enriched in specific subcellular compartments. A: Confocal microscopy was used to verify subcellular location of ERK decoys. The cytosolic ERK decoy shows HA staining only extranuclearly while the nuclear ERK decoy shows staining only in the cell nucleus. Two different magnifications are shown. B: Cytosolic and nuclear fractions were immunoblotted for the HA antigen to further confirm the subcellular specificity of these decoys using a RhoGDI (cytosolic) and histone (nuclear) control.

increased in a time-dependent manner with a maximum observed increase of $75 \pm 16\%$ ($P < 0.001$) on collagen and $218 \pm 58\%$ ($P < 0.02$) on fibronectin ($n = 6$, Fig. 3A,B). Similarly, Elk phosphorylation also increased in a time-dependent manner with a maximum increase of $61 \pm 27\%$ ($P < 0.02$) on collagen and $60 \pm 12\%$ ($P < 0.001$) on fibronectin ($n = 6$, Fig. 3C,D).

SUBCELLULAR LOCALIZED ERK DECOYS ARE FUNCTIONAL

We therefore assessed the phosphorylation of Elk as a marker for nuclear ERK activity and RSK phosphorylation as a marker for cytosolic ERK activity. Western blots of cells maintained after confluence under static conditions or cyclic strain for 30 min demonstrated increased Elk phosphorylation in all transfected cell lines except for those expressing the nuclear decoy (Fig. 4A, $n = 9$, $P < 0.001$ for the nuclear localization control, $P < 0.05$ for the cytosolic decoy and the cytosolic control). Conversely, Western blots for phosphorylated RSK demonstrated increased RSK phosphorylation in response to strain in all the cell lines except for those expressing the cytosolic decoy (Fig. 4B, $n = 5$, $P < 0.05$ for each). Caco-2 cells subjected to strain were then fractionated into nuclear and cytosolic fractions and the resulting lysates were probed for phosphorylation of RSK and Elk. Cytosolic fractions showed a $23 \pm 8\%$ increase in phosphorylation of RSK while nuclear fractions showed a $43 \pm 10\%$ increase in phosphorylation of Elk with strain ($n = 4$, $P < 0.05$ for all, Fig. 4C,D). Elk was not detected in cytosolic fractions while RSK was detected in very low levels in the nucleus making analysis difficult. When cells were pretreated with the ERK inhibitor PD98059, this strain-induced increased RSK and Elk phosphorylation was blocked. These results confirmed the functionality and specificity of the decoys as expressed in these cells.

EFFECTS OF SUBCELLULAR BLOCKADE OF ERK ON CELL PROLIFERATION IN RESPONSE TO STRAIN

We next sought to determine the effect of inhibiting ERK activity within each subcellular compartment on the mitogenic effect of strain. Proliferation was assayed under static conditions or after cyclic strain in transfected cells on collagen-coated Flexwell plates. The nuclear and cytosolic localization control cell lines exhibited similar approximately 30% increases in proliferation in response to strain, as expected ($n > 25$, $P < 0.0001$ for each). However, no mitogenic response to strain was observed in cells expressing either the nuclear or cytosolic decoy (Fig. 5A).

EFFECTS OF SUBCELLULAR BLOCKADE OF ERK ON CELL MIGRATION IN RESPONSE TO STRAIN

We similarly assessed the stimulation of migration across fibronectin in the transfected cells. Cells expressing each of the four constructs were individually seeded on Flexwell plates coated with fibronectin and migration assays were performed with and without 24 h of cyclic strain. The nuclear and cytosolic controls showed the expected approximately 15% increase in migration with strain, and cells expressing the cytosolic decoy displayed a similar increase in migration with strain ($n > 55$, $P < 0.0001$ for each, Fig. 5B). However, strain-induced migration was blocked in cells expressing the nuclear decoy.

OVEREXPRESSION OF Sef BEHAVES LIKE THE CYTOSOLIC LOCALIZED ERK DECOY

To confirm these results, we overexpressed Sef by adenoviral infection. Sef blocks nuclear translocation of ERK without interrupting its cytoplasmic activity [Torii et al., 2004]. Targeting the V5 antigen on the Sef adenovirus with an anti-V5/PE antibody, we demonstrated an 88% transduction efficiency (Fig. 6A). Proliferation was assessed in transduced cells by flow cytometric analysis of CFSE fluorescence in Sef/PE-positive populations. The proliferative effect was quantified using the percent decrease in CFSE mean fluorescence intensity (MFI) between 0 and 24 h. Strain induced a $24 \pm 5\%$ increase in proliferation among cells exposed to adenovirus but not transduced (no V5 antigen expression) ($n = 3$, $P < 0.05$, Fig. 6B). Uninfected control cells studied in parallel yielded similar results (data not shown). This effect is blocked in cells transduced with the adenovirus (Fig. 6C). To assess migration on fibronectin, parallel transduced cells were grown to confluence and circular wounds were created. After 24 h of cyclic strain, control cells transfected with an adenovirus containing only a lacZ protein showed a $21 \pm 2.5\%$ increase in migration with strain that was blocked by cells overexpressing Sef ($n = 20$, $P < 0.001$, Fig. 6D). The proliferative effect was quantified using the percent decrease in CFSE MFI between 0 and 24 h. Among cells exposed to adenovirus but not transduced (no V5 antigen expression), strain induced a $24 \pm 5\%$ (MFI of 248 ± 12) increase in proliferation compared with

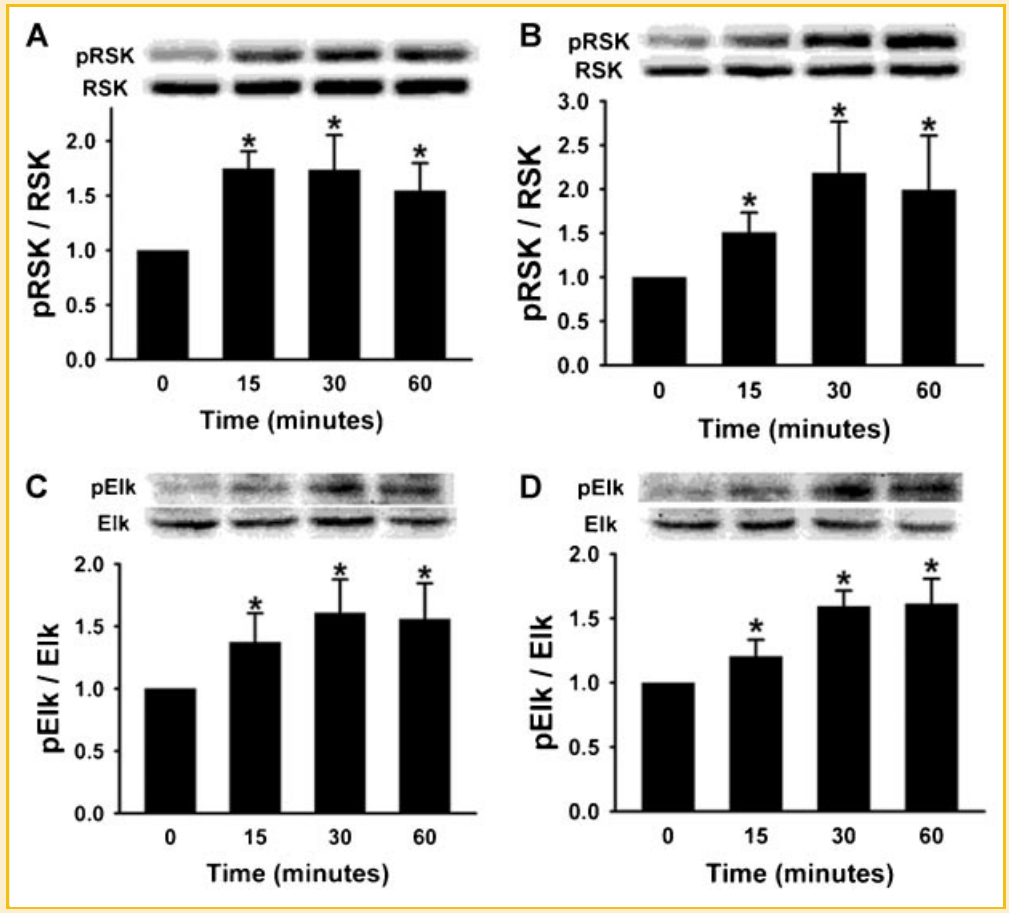


Fig. 3. RSK and Elk are activated in response to strain. RSK, a cytosolic target of ERK, is phosphorylated in a time-dependent fashion when subjected to cyclic strain on both a collagen (A) or a fibronectin (B) matrix ($n = 6$, $P < 0.001$ and $P < 0.02$ respectively). Similarly, Elk, a nuclear target of ERK, is also phosphorylated in a time-dependent fashion when subjected to cyclic strain on both a collagen (C) or a fibronectin (D) matrix ($n = 6$, $P < 0.02$ and $P < 0.001$ respectively).

cells maintained under static conditions (MFI of 308 ± 19) ($n = 3$, $P < 0.05$, Fig. 6B). To confirm that Sef was indeed tethering ERK to the cytoplasm, cells exposed to Sef adenovirus infection were fractionated and the resultant lysate was immunoblotted for phosphorylated ERK. ERK phosphorylation is increased $30 \pm 15\%$ in the cytosol in Sef-infected cells while there is no ERK increase in the nuclear fraction ($n = 4$, $P < 0.05$ for all, Fig. 6E).

RSK AND Elk ARE INVOLVED IN STRAIN-INDUCED CELL MIGRATION AND PROLIFERATION

The ERK targets RSK (a cytosolic target) and Elk (a nuclear target) used earlier as markers of ERK phosphorylation were then investigated as to their role in strain-induced cell migration and proliferation. The RSK inhibitor SL0101 (100 μM) blocked the $17 \pm 3\%$ increase in proliferation stimulated by strain ($n = 5$, $P < 0.01$) and reversed the $17 \pm 3\%$ increase in migration ($n = 3$, $P < 0.001$, Fig. 7A,B). When RSK isoform levels were reduced using specific siRNA, we found that while RSK1 knockdown reversed the strain effect of migration seen in NT1 controls, RSK2 and 3 knockdown did not alter the strain effect suggesting isoform-specificity ($n = 5$, $P < 0.01$ for all effects, Fig. 7C). Proliferation experiments were then done using the same specific RSK isoform siRNA knockdowns. We found that all three RSK isoforms blocked the $23 \pm 6\%$ strain-induced increase in proliferation on collagen ($n = 5$, $P < 0.05$, Fig. 7D). Knockdown of 50–80% was achieved (Fig. 7E). Similar to RSK blockade by SL0101, reduction of Elk levels by specific siRNA transfection also blocked strain-induced proliferation of $23 \pm 5\%$ ($n = 3$, $P < 0.01$) and reversed strain-induced migration of $28 \pm 4\%$ ($n = 4$, $P < 0.001$, Fig. 8A,B). Knockdown was approximately 70% (Fig. 8C).

DISCUSSION

The intestinal epithelium is subjected to constant injury and requires repair to maintain barrier function [McNeil and Ito, 1989]. Mechanical forces such as peristalsis may contribute to maintenance of the mucosal barrier by modulating proliferation [Basson et al., 1996; Murnin et al., 2000] and cell migration [Zhang et al., 2006; Chaturvedi et al., 2008] of the intestinal epithelial cells to incite restoration. Indeed, when physical forces are diminished or absent, as in sepsis, ileus, or prolonged fasting, the mucosal barrier breaks down [Jones et al., 1990; Van Leeuwen et al., 1994]. Repetitive deformation also influences proliferation and migration of other cell

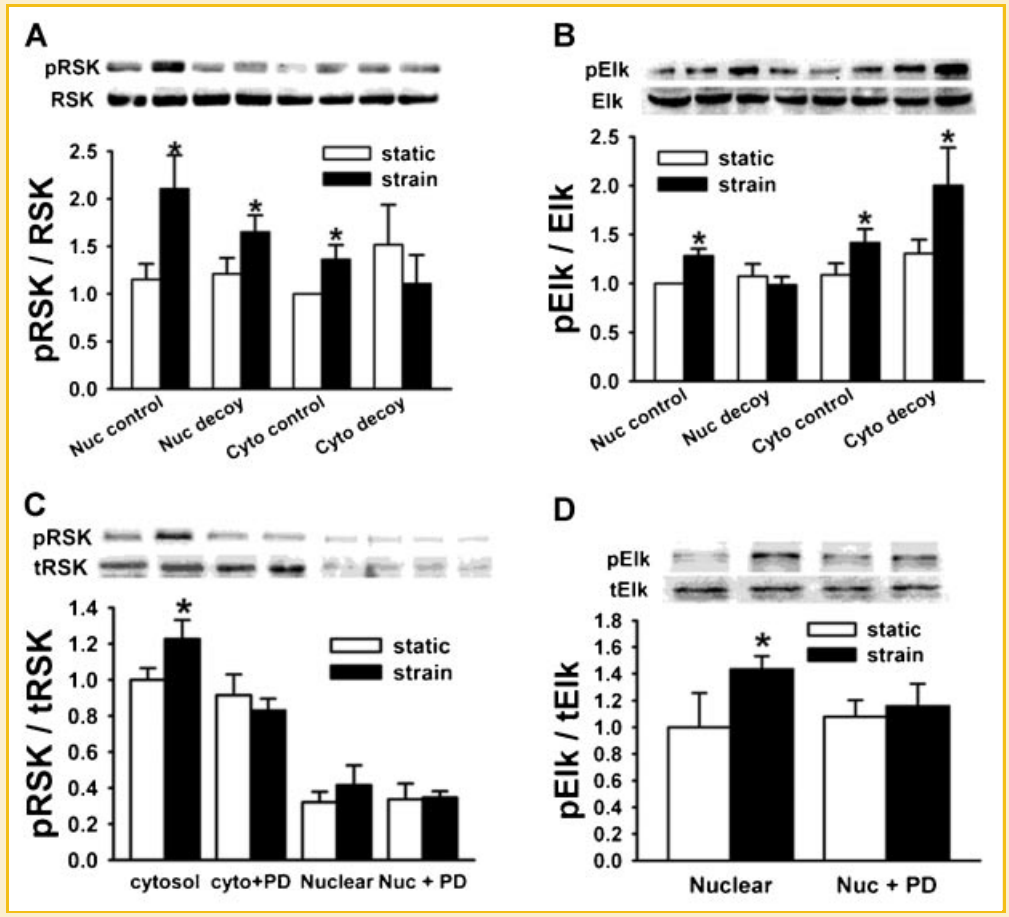


Fig. 4. ERK decoys are functional. Stable transfection cells lines were independently subjected to strain and lysates were immunoblotted for RSK phosphorylation (A) or Elk phosphorylation (B). Increased RSK phosphorylation in response to strain was prevented only in the cell lines expressing the cytosolic decoy ($n = 5$, $P < 0.05$ for nuclear decoy, nuclear control and cytosolic control) while strain-induced Elk phosphorylation was prevented only in cells expressing the nuclear decoy ($n = 9$, $P < 0.001$ nuclear control, $P < 0.05$ nuclear decoy and cystolic control). Fractionated cell lysates were then probed for RSK and Elk. Strain phosphorylated RSK in the cytosol (C) and Elk in the nucleus (D), both of which were blocked with by exposure to the ERK inhibitor PD98059 (PD). While RSK was detected in the nucleus, it was in very low levels while Elk was not detected at all in the cytosol.

types, including vascular endothelial cells [Shimizu et al., 2008], osteocytes [Song et al., 2007], pulmonary epithelial cells [Chess et al., 2000], and myocytes [Kumar et al., 2004] suggesting that mechanical forces play a key role in regulating cell biology. ERK is activated in response to repetitive intestinal epithelial deformation and is required for strain-induced cell proliferation on collagen [Chaturvedi et al., 2007] and strain-induced cell migration on fibronectin [Zhang et al., 2006]. Furthermore, phosphorylated ERK is localized to the lamellopodial edge of the migrating front and the nucleus in Caco-2 cells subjected to cyclic strain on a fibronectin substrate, while phosphorylated ERK is found in the nucleus and is diffusely dispersed through the cytosol of cells subjected to cyclic strain on collagen [Zhang et al., 2006]. Here, we show that ERK differentially determines the intestinal epithelial cell response to deformation based upon its subcellular location. The mitogenic effects of strain require functional ERK in both the nucleus and the cytosol. However, the motogenic effects of strain require only nuclear ERK to be functional. Taken together with the observation that phosphorylated ERK is localized to the lamellopodial edge of migrating cells, this suggests that ERK is activated at the lamellipodial edge in response to strain on fibronectin and then translocates to the nucleus while ERK activity is required both in the cytosol and nucleus to stimulate strain-induced proliferation on collagen.

A number of factors went into the construction of the ERK decoys. Although protein–protein interaction studies can prove informative on the potential inhibitory function, such results are not always correlative. The strength of the interaction in vitro does not necessarily correlate with in vivo function, and similarly in vitro specificity may not predict in vivo functionality [Wu et al., 2009]. Inhibitory proteins can have a high on–off rate with a target protein and still effectively function to inhibit the activity of that target. There are studies using deletion-based analysis of functional domains that demonstrate within a protein that one domain coordinates the interaction and another elicits the affect [Prijatelj et al., 2002]. The focus of this study was to introduce a protein-based inhibitor, designed to inhibit ERK, demonstrate that its expression reduces phosphorylation levels of known ERK targets, and show that

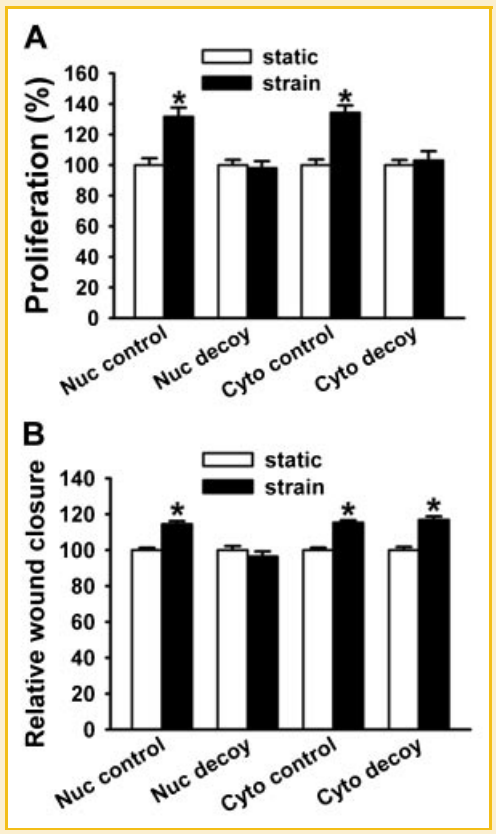


Fig. 5. Effects of ERK decoys on the mitogenic and motogenic effects of cyclic strain. Independently each stably transfected cell line was subjected to proliferation assays on a collagen matrix (A) and migration assays on a fibronectin matrix (B) to determine the effects blocking subcellular ERK has on strain effects. While cells expressing nuclear and cytosolic control plasmids exhibited an increase in proliferation in response to strain ($P < 0.0001$), this effect was blocked in cells expressing either the nuclear or cytosolic decoy ($n > 25$). In contrast, the cells expressing the nuclear and cytosolic control along with the cytosolic decoy showed an increase in migration with strain ($P < 0.0001$) while only expression of the nuclear decoy blocked this effect ($n > 55$).

constraining this inhibitory activity to discreet subcellular locales is associated with alteration of a physiological effect in response to mechanical strain. The possibility of differential ERK isoform functions is interesting, but beyond our current scope.

ERK has numerous cytoplasmic substrates, including myosin light-chain kinase, cytosolic phospholipase A2, and p90RSK while phosphorylated ERK in the nucleus controls Elk-1 activity and the expression of ERK target genes such as *Fos* and *Jun* [Ebisuya et al., 2005]. The potential role of these and other ERK downstream signals in mediating the effects of mechanical deformation on intestinal epithelial cells awaits further study. Here, we studied p90RSK and Elk-1 as markers for cytoplasmic and nuclear ERK activity respectively. Each of these molecules has been shown to be activated in response to mechanical stress on diaphragm muscle [Kumar et al., 2002]. Cyclic strain also activates ERK and Elk phosphorylation in endothelial cells [Wung et al., 1999].

ERK is predominantly cytoplasmic in most quiescent cells due to MEK or other cytoplasmic anchors. Upon phosphorylation, ERK dissociates from these anchors and can translocate to the nucleus, although translocation of activated ERK does not always occur [Ebisuya et al., 2005]. Sef blocks ERK nuclear translocation by binding activated MEK and preventing ERK dissociation, tethering the complex to the Golgi without blocking ERK cytoplasmic activity [Torii et al., 2004]. In this study, we used a Sef-overexpressing adenovirus to keep ERK in the cytoplasm and prevent nuclear translocation. Sef overexpression mediated by this virus also blocks fibroblast growth factor-induced cell proliferation and inhibits ERK activation in NIH3T3 murine fibroblasts [Kovalenko et al., 2003]. We used Sef overexpression to verify our nuclear localized ERK decoy results and found that Sef-overexpressing cells behave similarly to cells stably transfected with the nuclear localized ERK decoy, displaying neither the mitogenic nor motogenic effect of strain.

ERK contributes to diverse cellular functions including cell-cycle progression, cell differentiation, migration, and proliferation [Marshall, 1995; Huang et al., 2004]. Although often tonically quiescent in the cytoplasm, ERK can translocate to the nucleus upon activation [Fukuda et al., 1996]. In primary human fibroblasts, ERK is activated within the cytosol in a dose-responsive fashion in response to EGF or the phorbol ester PMA, activating RSK within the cytosol. However, ERK translocates to the nucleus in these cells and activates Elk only in response to PMA, suggesting that ERK translocation may be regulated independently of its extra-nuclear activation [Whitehurst et al., 2004]. This is consistent with our own observation here that strain activates ERK in intestinal epithelial cells on either fibronectin or collagen, but that nuclear translocation of the active ERK seems promoted by strain predominantly on fibronectin. Taken together, these observations suggest that the identity of the specific stimulus that activates ERK, as opposed to simply phosphorylation itself, dictates the ultimate consequence of ERK activity.

On collagen, cyclic strain appears to stimulate intestinal epithelial proliferation in a manner requiring both cytosolic and nuclear ERK. This is consistent with the observation that proliferating cells within a gastric ulcer bed display increased phosphorylated ERK in the cytosol as well as evidence of nuclear ERK translocation [Tarnawski et al., 1998]. ERK activation and relocation to the nucleus is impaired in senescent human fibroblasts but providing nuclear ERK activity can bypass critical senescence checkpoints and increase their lifespan [Tresini et al., 2007]. Excluding ERK from the nucleus but allowing its activity in the cytosol prevents the stimulation of proliferation in rat renal epithelial cells, which is reversed when ERK is allowed to translocate and function in the nucleus [DeFea et al., 2000]. Indeed, several reports [DeFea et al., 2000; Smith et al., 2004] suggest that sequestering ERK in the cytosol and preventing its nuclear translocation and nuclear activity can block cell proliferation in various cells in response to various stimuli. However, there is little evidence that cytosolic ERK activity prior to nuclear translocation is also important. Here, we show that preventing ERK activity in the cytosol while maintaining the ability of activated ERK to translocate to the nucleus and act there suffices to block strain-induced proliferation. The importance of ERK cytosolic activity may reflect loss of activation of cytosolic transcription factors that ERK is known to activate in the cytosol such as RSK [Sturgill et al., 1988], or to the loss of activation of some other cytosolic substrate of ERK that

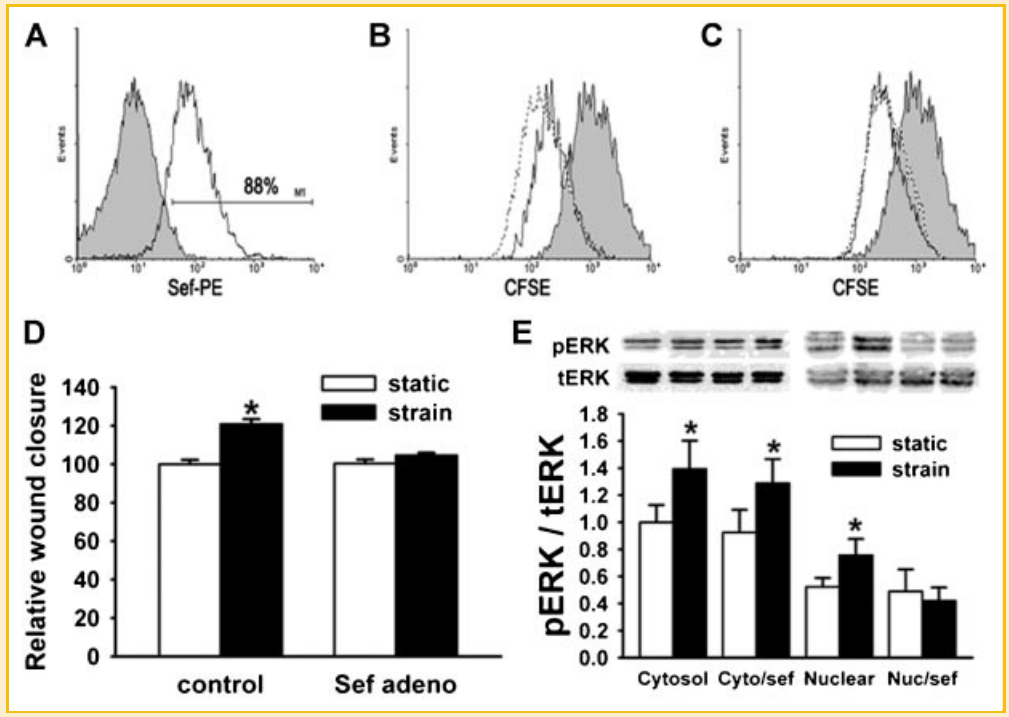


Fig. 6. Sef overexpression behaves like the nuclear ERK decoy. To verify our results we overexpressed Sef, a molecule that tethers ERK in the cytosol without altering its cytosolic activity, via adenoviral transduction. Our transduction efficiency was 88% by flow cytometry (A). Flow cytometric analysis of CFSE fluorescence in Sef/PE-negative populations showed increased proliferation in response to strain (B) while Sef/PE-positive populations which represent Sef-overexpressing cells showed a blockade of this effect (C) (filled histogram is 0 h, open solid line histogram is static, and open dashed histogram is strain). Cells transduced in parallel with either a LacZ adenovirus (control) or the Sef-adenovirus were grown to confluence and subjected to migration assays. Strain-induced migration was blocked by Sef-overexpressing cells (D, $n = 20$, $P < 0.001$). Cytosolic fractions of cells exposed to Sef show an increase in pERK similar to fractions not exposed to Sef while nuclear fractions exposed to Sef do not show an increased in ERK phosphorylation (E). Overall ERK phosphorylation is lower in the nuclear fraction.

itself may then translocate to the nucleus and stimulate proliferation there.

In contrast to the mitogenic effect of strain, strain-induced migration on fibronectin requires ERK translocation to the nucleus and ERK nuclear activity, but does not require ERK to act within the cytosol. This is consistent with the previous observation that ERK is involved in cell restitution in the intestinal epithelium and is activated in response to injury with evidence of translocation to the nucleus and subsequent activation of transcription factors [Dieckgraefe et al., 1997]. In pulmonary artery smooth muscle cells, HMG-CoA reductase inhibitors inhibit serotonin-induced ERK nuclear translocation, consequently reducing cell migration even though serotonin-induced initial activation of ERK was unaffected [Li et al., 2007]. Similarly, in kidney and endothelial cell lines, a large portion of phosphorylated ERK stimulated via stromal cell-derived factor 1 (through Src and Rho-kinase) can be found in the nucleus, resulting in increased Elk activation as well as increased cell migration [Zhao et al., 2006]. Interestingly, ERK and Elk nuclear accumulation was blocked by Src and Rho inhibition in that study. We have previously implicated Src in the mediation of strain signals in intestinal epithelial cells on both collagen [Chaturvedi et al., 2007] and fibronectin [Chaturvedi et al., 2008], while others have described Rho activation by repetitive deformation in other cell types including fibroblasts [Maier et al., 2008] and pulmonary epithelial cells [Thomas et al., 2006]. Protein kinase C alpha induces nuclear ERK activation and regulates proliferation while the protein kinase C epsilon isoform activates ERK at the focal adhesion and regulates cell adhesion and migration in glioma cells [Besson et al., 2001]. This again suggests that independent upstream regulators converge upon ERK activation to determine the consequences of this activation depending upon its location within the cell.

Although phosphorylated ERK localizes to the lamellipodial edge as well as to the nucleus in intestinal epithelial cells stimulated to increased migration across fibronectin by cyclic strain [Zhang et al., 2006], our present results suggest that the stimulation of motility by strain requires only nuclear ERK activity. Activated ERK also localizes to the leading edge of pancreatic adenocarcinoma cells stimulated with LPA [Stahle et al., 2003], but this report did not investigate whether the activated ERK at the leading edge of the LPA-stimulated cells actually functions at that site. EPLIN (epithelial protein lost in neoplasm) is a novel ERK substrate that contributes to actin filament reorganization and cell motility by localizing to the lamellopodial edge of fibroblasts stimulated by PDGF [Han et al., 2007]. Although these investigators did not address where within the cell ERK phosphorylates EPLIN, this seems more likely to have occurred outside the nucleus than within it, suggesting a role for extra-nuclear ERK activity in that model that contrasts with our present observation that the nuclear exclusion decoy did not prevent the stimulation of migration by cyclic strain in Caco-2 epithelial cells. Different motogenic stimuli may well involve ERK in different

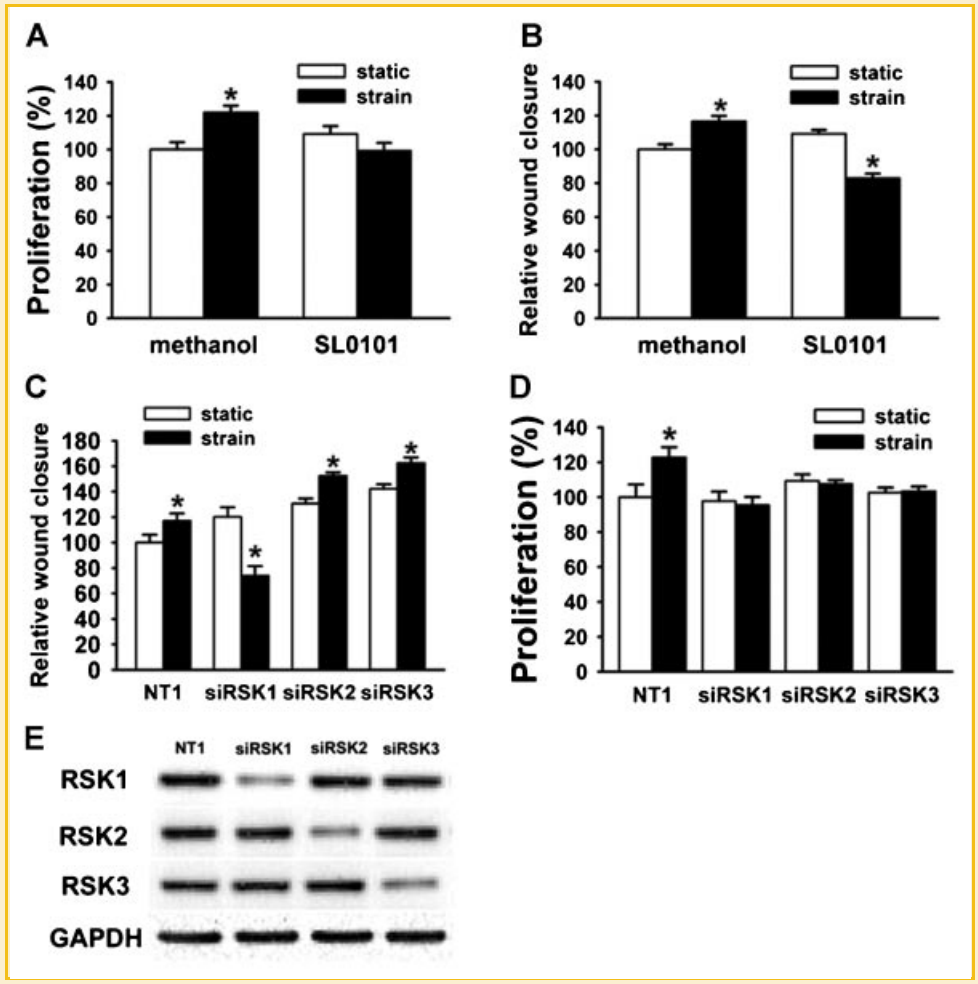


Fig. 7. RSK is involved in strain-induced cell migration and proliferation. Blockade of RSK by the pharmacologic inhibitor SL0101 blocked strain-induced proliferation (A) and reversed strain-induced migration (B) on Caco-2 cells ($n \geq 3$, $P < 0.01$ for each). Reduction of RSK1 by siRNA showed a reversal of the strain effect of strain on migration while reduction of RSK2 and 3 had no effect (C, $n = 5$, $P < 0.01$ for all effects). Approximately 50–80% knockdown was achieved (D).

ways. Moreover, epithelial sheet migration is manifestly different from the motility of individual fibroblasts [Kolega, 1981].

Though we initially used RSK and Elk simply as markers for subcellular ERK activity, we decided to investigate what roles these two molecules have in the mitogenic and motogenic effect of cyclic strain. Blocking either molecule on collagen results in a loss of the mitogenic effect of strain, similar to results from both the nuclear and cytosolic ERK decoy studies. Elk reduction and global RSK blockade also blocks strain-induced migration on fibronectin. While this was expected for Elk reduction (similar to the nuclear ERK decoy results), it was not expected for RSK blockade (given the cytosolic ERK decoy results). RSK has four known isoforms that are expressed in variable amounts in various tissues, although the GI tract has not been examined for specific relative amounts of each isoform [Anjum and Blenis, 2008]. RSK4 is of significantly lower levels versus the other three isoforms, however [Anjum and Blenis, 2008]. When we reduced specific RSK isoforms 1–3 using siRNA we found only RSK1 activity was required for strain-induced migration while RSK2 and 3 were not. Although the cytosolic ERK decoy did not prevent strain-induced migration, this was prevented by RSK inhibition or reduction of RSK1 by specific siRNA. This may reflect a requirement for a tonic level of RSK activity in order for strain to stimulate motility. Alternatively, some other kinase may also contribute at a lower level to RSK activation in the cytosol in response to strain in the setting of ERK blockade. Ser/Thr kinases, such as PDK1 (3-phosphoinositide-dependent kinase-1), other receptor tyrosine kinases, such as FGR (fibroblast growth factor), and even other MAPKs, such as p38, have all been reported to phosphorylate RSK independently of the ERK pathway [Zaru et al., 2007; Anjum and Blenis, 2008; Xian et al., 2009]. In fact, RSK activation is seen with PDK1 alone without ERK upstream [Richards et al., 1999]. Interestingly, RSK-null Drosophila showed enhanced ERK-regulated differentiation while overexpressing RSK suppressed ERK-dependent differentiation [Kim et al., 2006]. Clearly the relationship between RSK and ERK is complex. Distinguishing among these competing possibilities awaits further study beyond the scope of the current manuscript.

It is interesting and not clear what is specifically unique about the cell matrix that results in such varying reactions to similar stimuli. The matrix regulates static intestinal epithelial cells via integrins

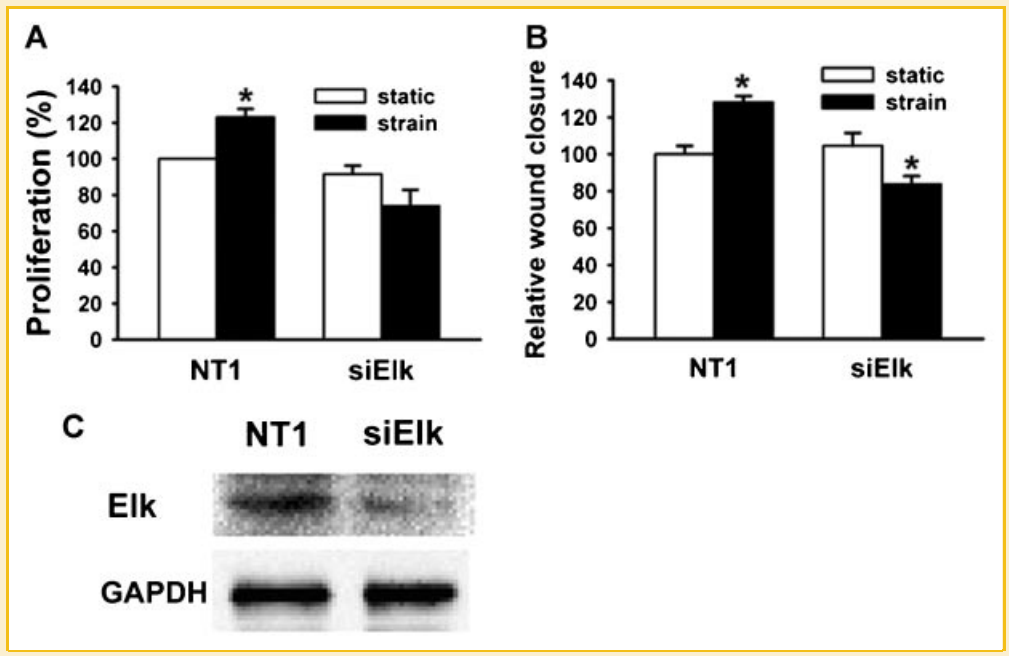


Fig. 8. Elk is involved in strain-induced cell migration and proliferation. Reduction of Elk by siRNA showed a similar result as RSK blockade on proliferation (A) where it blocked the strain effect and migration (B, $n \geq 3$, $P < 0.01$ for each) where it reversed the strain effect. Approximately 60–80% knockdown was achieved (C).

[Basson et al., 1992; Emenaker and Basson, 1998]. Physical forces may alter surface expression and integrin localization with subsequent activation of different intracellular signals [Rozzo et al., 1997; Basson et al., 2000]. This may lead to differing matrix binding and thus different responses. The integrin αv subunit, which contributes to fibronectin-binding by αv-containing integrins is upregulated at the lamellipodial edge in migrating cells on fibronectin in response to strain [Zhang et al., 2006]. However, the mitogenic effects of strain on collagen are prevented by blocking the α2 integrin subunit, which contributes to integrin–collagen interactions [Zhang et al., 2003], while blocking the fibronectin-binding subunits αv and α5 does not interfere with this mitogenic effect on collagen. Thus, the matrix-specific effects of strain are likely to be determined at least in part by the involvement of different integrin heterodimers. Integrins themselves are not signaling molecules but associate with a variety of kinases and adaptor proteins in the focal adhesion complex [Wozniak et al., 2004]. Whether these different integrin heterodimers associate with differently composed or organized focal adhesion complexes that may then respond differently to strain on the cytoskeleton is an attractive hypothesis that awaits further study beyond the scope of the current manuscript.

Although the signal activation by cyclic strain and changes in proliferation and motility that it drives in intestinal epithelial cells may be viewed as modest, they are both consistent and highly statistically significant. Relatively minor changes in the rates of proliferation or cell migration may have profound consequences in a tightly regulated intestinal epithelium that experiences constant injury to the mucosal layer. A wide variety of external factors influence proliferation and migration and can tip the balance between an intact mucosal barrier and barrier breakdown if the effects of deformation are absent. We [Basson et al., 1996; Zhang et al., 2006; Chaturvedi et al., 2007] and others [Chen et al., 2003; Fenton et al., 2005] have previously described changes in cell proliferation and migration in intestinal epithelial cells of similar magnitudes in response to various stimuli. Furthermore, we studied a 10% average deformation here in vitro. This amplitude reflects a limitation of the available in vitro strain apparatus. However, the effects of strain on intestinal epithelial cells are amplitude dependent [Basson et al., 1996]. In vivo, the bowel mucosa is subject to much greater amplitudes of deformation [Miftakhov and Wingate, 1994], so it is likely that the effects of deformation on the gut mucosa in vivo are greater than those observed here in vitro. In addition, although the level of ERK activation that we have studied here seems relatively small, this is both statistically significant and physiologically relevant since blockade of this activation results in a loss of the proliferative and migratory effects of strain. Similar increases in activation of 30–50% have been shown to be important mediators of strain-regulated migration and proliferation for both ERK itself and other intracellular signals such as AKT and PI3K [Chaturvedi et al., 2008; Gayer et al., 2009a,b]. This level of stimulation is sufficient to have substantial effects in other systems as well. For instance, ERK is stimulated approximately 50% by media conditioned with α-2-adrenoreceptor agonist in Caco-2 cells resulting in increased peptide absorption and cell proliferation [Buffin-Meyer et al., 2007]. Intestinal cells stimulated with CCL20, a chemokine, exhibit increases in ERK phosphorylation of approximately 30–80% although mRNA levels are stimulated by as much as eightfold [Brand et al., 2006]. The rather large increases in mRNA levels that are often observed for such signal proteins do not necessarily equate to protein increases of similar magnitude [Schrattenholz and Soskic, 2008].

Taken together, these results demonstrate differing roles for ERK in different subcellular locations, based upon input from the extracellular matrix. Whole cell observations of kinase activation or activity may not have sufficient resolution to delineate such intracellular signal pathways. Furthermore, intestinal epithelial cells subjected to deformation in inflamed fibronectin-rich tissue may display a very different ERK pathway than enterocytes on a healthy collagen-rich basement membrane. Although we studied a collagen I substrate here for convenience, intestinal epithelial cells respond to cyclic deformation similarly on collagen I and collagen IV [Zhang et al., 2003]. During normal function, cyclic deformation results in increased phosphorylated ERK in both the cytosol and the nucleus, and ERK activity in each compartment is required for the proliferative effects of strain. Previous observations [Zhang et al., 2006] suggest that the stimulation of intestinal epithelial wound closure by cyclic deformation begins when ERK associates with and is activated by focal adhesion complex kinases within the migrating lamellipodial edge. However, active ERK must then translocate to the nucleus to stimulate cell motility. These results contrast with what might have previously been expected and raise the possibility that ERK may be modulated either at the focal adhesion complex or within the nucleus to induce wound healing in settings of chronic mucosal inflammation when tissue fibronectin is increased.

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