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Extracellular Signal-Regulated Kinase Promotes Rho-Dependent Focal Adhesion Formation by Suppressing p190A RhoGAP▽

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Cell migration is critical for normal development and for pathological processes including cancer cell metastasis. Dynamic remodeling of focal adhesions and the actin cytoskeleton are crucial determinants of cell motility. The Rho family and the mitogen-activated protein kinase (MAPK) module consisting of MEK–extracellular signal-regulated kinase (ERK) are important regulators of these processes, but mechanisms for the integration of these signals during spreading and motility are incompletely understood. Here we show that ERK activity is required for fibronectin-stimulated Rho-GTP loading, Rho-kinase function, and the maturation of focal adhesions in spreading cells. We identify p190A RhoGAP as a major target for ERK signaling in adhesion assembly and identify roles for ERK phosphorylation of the C terminus in p190A localization and activity. These observations reveal a novel role for ERK signaling in adhesion assembly in addition to its established role in adhesion disassembly.

Cell migration is a highly coordinated process essential for physiological and pathological processes (69). Signaling through Rho family GTPases (e.g., Rac, Cdc42, and Rho) is crucial for cell migration. Activated Rac and Cdc42 are involved in the production of a dominant lamellipodium and filopodia, respectively, whereas Rho-stimulated contractile forces are required for tail retraction and to maintain adhesion to the matrix (57, 58, 68). Rac- and Cdc42-dependent membrane protrusions are driven by the actin cytoskeleton and the formation of peripheral focal complexes; Rho activation stabilizes protrusions by stimulating the formation of mature focal adhesions and stress fibers. Active Rho influences cytoskeletal dynamics through effectors including the Rho kinases (ROCKs) (2, 3).

Rho activity is stimulated by GEFs that promote GTP binding and attenuated by GTPase-activating proteins (GAPs) that enhance Rho's intrinsic GTPase activity. However, due to the large number of RhoGEFs and RhoGAPs expressed in mammalian cells, the molecular mechanisms responsible for regulation of Rho activity in time and space are incompletely understood. p190A RhoGAP (hereafter p190A) is implicated in adhesion and migration signaling. p190A contains an N-terminal GTPase domain, a large middle domain juxtaposed to the C-terminal GAP domain, and a short C-terminal tail (74). The C-terminal tail of ~50 amino acids is divergent between p190A and the closely related family member p190B (14) and thus may specify the unique functional roles for p190A and p190B revealed in gene knockout studies (10, 11, 41, 77, 78). p190A activity is dynamically regulated in response to external cues during cell adhesion and migration (5, 6, 59). Arthur et al. (5) reported that p190A activity is required for the transient decrease in RhoGTP levels seen in fibroblasts adhering to fibronectin. p190A activity is positively regulated by tyrosine phosphorylation (4, 5, 8, 17, 31, 39, 40, 42): phosphorylation at Y1105 promotes its association with p120RasGAP and subsequent recruitment to membranes or cytoskeleton (8, 17, 27, 31, 71, 75, 84). However, Y1105 phosphorylation is alone insufficient to activate p190A GAP activity (39). While the functions of p190A can be irreversibly terminated by ubiquitinylation in a cell-cycle-dependent manner (80), less is known about reversible mechanisms that negatively regulate p190A GAP activity during adhesion and motility.

The integration of Rho family GTPase and extracellular signal-regulated kinase (ERK) signaling is important for cell motility (48, 50, 63, 76, 79). Several studies have demonstrated a requirement for ERK signaling in the disassembly of focal adhesions in migrating cells, in part through the activation of calpain proteases (36, 37) that can downregulate focal adhesion kinase (FAK) signaling (15), locally suppress Rho activity (52), and sever cytoskeletal linkers to focal adhesions (7, 33). Inhibition of ERK signaling increases focal adhesion size and retards disassembly of focal adhesions in adherent cells (57, 64, 85, 86). It is also recognized that ERK modulates Rho-dependent cellular processes, including membrane protrusion and migration (18, 25, 64, 86). Interestingly, ERK activated in response to acute fibronectin stimulation localizes not only to mature focal adhesions, but also to peripheral focal complexes (32, 76). Since these complexes can either mature or be turned over (12), ERK may play a distinct role in focal adhesion assembly. ERK is proposed to promote focal adhesion formation by activating myosin light chain kinase (MLCK) (21, 32, 50).

Here we find that ERK activity is required for Rho activation and focal adhesion formation during adhesion to fibronectin and that p190A is an essential target of ERK signaling in this context. Inspection of the p190A C terminus reveals a number of consensus ERK sites and indeed p190A is phosphorylated by recombinant ERK only on its C terminus *in vitro*,

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and on the same C-terminal peptide *in vivo*. Mutation of the C-terminal ERK phosphorylation sites to alanine increases the biochemical and biological activity of p190A. Finally, inhibition of MEK or mutation of the C-terminal phosphorylation sites enhances retention of p190A in peripheral membranes during spreading on fibronectin. Our data support the conclusion that ERK phosphorylation inhibits p190A allowing increases in RhoGTP and cytoskeletal changes necessary for focal adhesion formation.

MATERIALS AND METHODS

Cell culture, plasmids, transfection, and antibodies. Standard cell culture methods were followed. REF52 and COS1 cells were transfected with Superfect (Qiagen, CA) and Lipofectamine (Invitrogen, CA), respectively. Plasmids encoding glutathione *S*-transferase (GST)–RhoA (wild type and 63L and 17N mutants), and enhanced green fluorescent protein (EGFP)-p190A RhoGAP (wild type and catalytically inactive R1258A mutant) were gifts from William T. Arthur and Keith Burridge (University of North Carolina, Chapel Hill). FLAG-RhoA (human wild type, plasmid 11750) and myc-RhoA-63L (human constitutively active, plasmid 15900) were purchased from Addgene (www.addgene.org). myc-tagged ROCKII-Δ3 (constitutively active ROCKII) was a gift from Shuh Narumiya (Kyoto University, Japan). UO126 and Y27632 were purchased from Calbiochem. Membrane-permeable C3 transferase (Cytoskeleton, Boulder, CO) and antibodies against FLAG epitope (M2), active MEK (pSer218/222), MEK1, vinculin (Sigma-Aldrich), RhoA (Santa Cruz Biotechnology), p190A RhoGAP (clone 30), p190B RhoGAP (clone 54), RhoGDI (BD Biosciences), active ERK (12D4; Upstate), and EGFP (Clontech monoclonal antibody [MAb] JL-8) were used as suggested by commercial suppliers. Antibodies against ERK1 and -2 (clone B3B9 MAb) and myc epitope (9E10 MAb) were gifts from Michael Weber (University of Virginia, Charlottesville). Phospho-specific pT696 MYPT1 (Millipore) and pT853 MYPT1 (Cell Signaling) were used at a 1:1,000 dilution. Antibodies that recognize phospho-cofilin (pSer3) were purchased from Cell Signaling. Cofilin antibody was obtained from Cytoskeleton (ACFL02, 1:1,000, rabbit polyclonal antibody). All other chemicals were from Sigma-Aldrich unless stated otherwise.

siRNA knockdown of p190A expression. Stealth small interfering RNAs (siRNAs) targeting rat p190A RhoGAP were synthesized by Invitrogen. Two siRNAs targeting the open reading frame (no. 1 and 2) and two targeting the 3′ untranslated region (UTR) (no. 3 and 4) and a mismatched control oligonucleotide to siRNA 1 (see Fig. 5) were used to transfect REF52 cells using the calcium phosphate method. Seventy-two hours after transfection, cells were lysed and analyzed for p190A knockdown or subjected to replating on fibronectin as described below.

Site-directed mutagenesis. Mutagenesis of four potential p190A mitogen-activated protein kinase (MAPK) sites (S1451, S1476, T1480, and S1483) to alanines was carried out using the QuikChange XL mutagenesis kit (Stratagene) and oligonucleotides synthesized at the University of Virginia, Charlottesville. The quadruple-mutant sequence was subcloned to p190A constructs using standard techniques. All plasmids were sequenced to verify that the right mutations were introduced and the correct fragments and reading frames were restored.

RhoGTP assay. Cellular RhoGTP was measured by pulldown with the Rho binding domain (RBD) of Rhotekin fused to GST (GST-RBD) as described previously (65, 66). Briefly, freshly transformed *Escherichia coli* BL21(DE3) cells were grown overnight at 37°C in LB medium (with 100 μg/ml ampicillin), diluted 1:50 into fresh LB-ampicillin and grown until the A_{600} was 0.2. Cultures were then induced with 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at room temperature for 16 h. To determine RhoGTP levels, clarified cell lysates prepared in buffer A (50 mM Tris, pH 7.4, 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, and 0.5 mM $MgCl_2$ supplemented with 10 μg/ml leupeptin, aprotinin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) were incubated with 30 to 50 μg of GST-RBD bound to glutathione-Sepharose beads (GE Health Care) for 30 min at 4°C. Beads were washed four times in buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.5 mM $MgCl_2$ supplemented with 10 μg/ml leupeptin, aprotinin, and 1 mM PMSF) and analyzed by immunoblotting with either anti-FLAG antibody to detect transfected FLAG-RhoA (M2; Sigma) or anti-RhoA to detect endogenous RhoA.

p190A RhoGAP assay. Recombinant GST-RhoA-63L and -17N and the wild type were produced in *E. coli* (BL21) cells as described previously (34), with the following modifications. Expression was induced at room temperature for 16 h and purification was done in the absence of dithiothreitol (DTT). After measurement of the protein concentration (Pierce bicinchoninic acid [BCA] reagent kit with bovine serum albumin [BSA] as a standard), DTT was added to a final concentration 2 mM. Purified GST-RhoA-63L was stored at 4°C and used within 10 days or was stored in 50% glycerol at −20°C and used within 6 weeks of preparation. Active p190A RhoGAP was precipitated from cell lysates as previously described (34, 59).

Replating assay. Cells were trypsinized (with 0.05% Trypsin-EDTA; Invitrogen) and collected with serum-free medium containing soybean trypsin inhibitor (1 mg/ml) as described previously (76). Cells were centrifuged (500 × *g* for 5 min), washed once with serum-free medium, and resuspended gently in serum-free medium. After incubation for 90 min in a cell culture incubator at 37°C (5% CO_2), suspensions were replated on fibronectin (10 μg/ml)-coated coverslips (for immunofluorescence) or bacterial petri dishes (biochemical assays) for the indicated times at 37°C (5% CO_2). Cells were lysed in ice-cold lysis buffer (20 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1%Triton X-100, 1 mM PMSF, 3 mM benzamidine, 10 μg/ml each leupeptin and pepstatin, 10 nM microcystin-LR, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride) and centrifuged at 13,000 × *g* for 5 min. Supernatants were normalized for protein concentration (Pierce BCA reagent kit) with lysis buffer and assayed as described for each experiment.

Metabolic and *in vitro* labeling. Metabolic labeling with $^{32}P_i$ and *in vitro* phosphorylation reactions were carried out essentially as described previously (16, 26), except that FLAG p190A immunoprecipitates destined for *in vitro* phosphorylation by ERK were pretreated with calf intestinal alkaline phosphatase (1 U; NEB) for 60 min at 37°C according to the manufacturer's instructions. After phosphatase treatment, immunoprecipitates were washed in kinase buffer (20 mM Tris, pH 7.8, 10 mM $MgCl_2$,) and suspended in 50 μl of kinase buffer supplemented with 1 mM DTT, 10 μM ATP, 10 μCi [γ-^{32}P]ATP (EazyTide; Perkin Elmer) and 0 to 100 ng of recombinant ERK1 (MBL International Corporation). After 30 min of incubation at 30°C, kinase reactions were stopped by adding 2× SDS-PAGE sample buffer and analyzed by PhosphorImager or exposure to X-ray film. Tryptic phosphopeptides prepared as described by Luo et al. (38) were resolved on 16.5% Tris-Tricine-SDS gels (73) and transferred to Immobilon P^{SQ} membrane (Millipore). *In silico* trypsin digestion was performed with PeptideCutter (ca.expasy.org/tools/peptidecutter).

Immunofluorescence. Cells were fixed with 3.7% paraformaldehyde (in 4% sucrose and 1× phosphate-buffered saline [PBS] pH 7.4), permeabilized with 0.5% Triton X-100 (for 15 min), washed with PBS (plus 0.05% Igepal CA630), and blocked with 3% bovine serum albumin (in PBS; Igepal CA630). Blocked cells were washed thoroughly with PBS and incubated with primary antibody against vinculin (1:200; Sigma, mouse monoclonal clone hVin1) and detected with Fluor-conjugated secondary antibodies (Jackson Laboratory). F-actin was stained with phalloidin-Oregon green 488 or phalloidin-595 (Invitrogen), rinsed with PBS, and mounted on glass slides with ProLong Antifade with DAPI (4′,6-diamidino-2-phenylindole) mounting medium (Invitrogen). In double-staining experiments, antibodies against vinculin (1:200), zyxin (1:100; Sigma, rabbit polyclonal), and phospho-paxillin (pY118) conjugated to Alexa Fluor 488 (1:50; Biosource 44-722A1, rabbit polyclonal antibody) were used for indirect or direct immunofluorescence with the appropriate secondary antibodies conjugated to Cy3 or Cy5. Cells transfected with myc-tagged RhoA-63L or ROCKII-Δ3 ("active" ROCKII) were detected with rabbit monoclonal anti-myc antibody (Cell Signaling; clone 71D10, 1:200). Immunofluorescence images were captured with a Leica DM RA2 epifluorescence microscope equipped with the appropriate filters, excitation sources, and a motorized z-stage controlled by Slide Book software (Intelligent Imaging Innovations).

Image analysis, quantitation, and statistical analyses. Immunofluorescent images were converted to 32-bit gray scale image in Image J. Threshold values were set between 45 and 225. Masks generated from threshold images were quantitated using plug-ins in ImageJ (http://rsb.info.nih.gov/ij/plugins). Tabulated values were manually filtered by comparing the *x-y* position of each object from the table with that present in the image. Filtered data were exported to Excel, and statistical analyses were performed with Prism Graphpad with an unpaired *t* test or one-way analysis of variance (ANOVA). Pairwise comparisons were made posttest (when P is ≤ 0.05) with Tukey's multiple comparison test. Pixel values were converted to micrometers using the constant pixel^2 = 0.101 μm^2 in our setting (a function of charge-coupled device [CCD] capabilities and objectives). Chemiluminescent images of immunoblots obtained with Fujifilm Intelligent Dark Box II run through LAS1000Plus software were quantitated using ImageQuant application. Densitometry of digital images was converted to intensity (arbitrary units) and exported to Prism GraphPad for statistical analysis. In experiments where cells were quantitated for mature focal adhesion or for spreading, at least 20 nonoverlapping fields were counted for each experimental condition. We defined mature adhesions as those that resembled clustered ad-

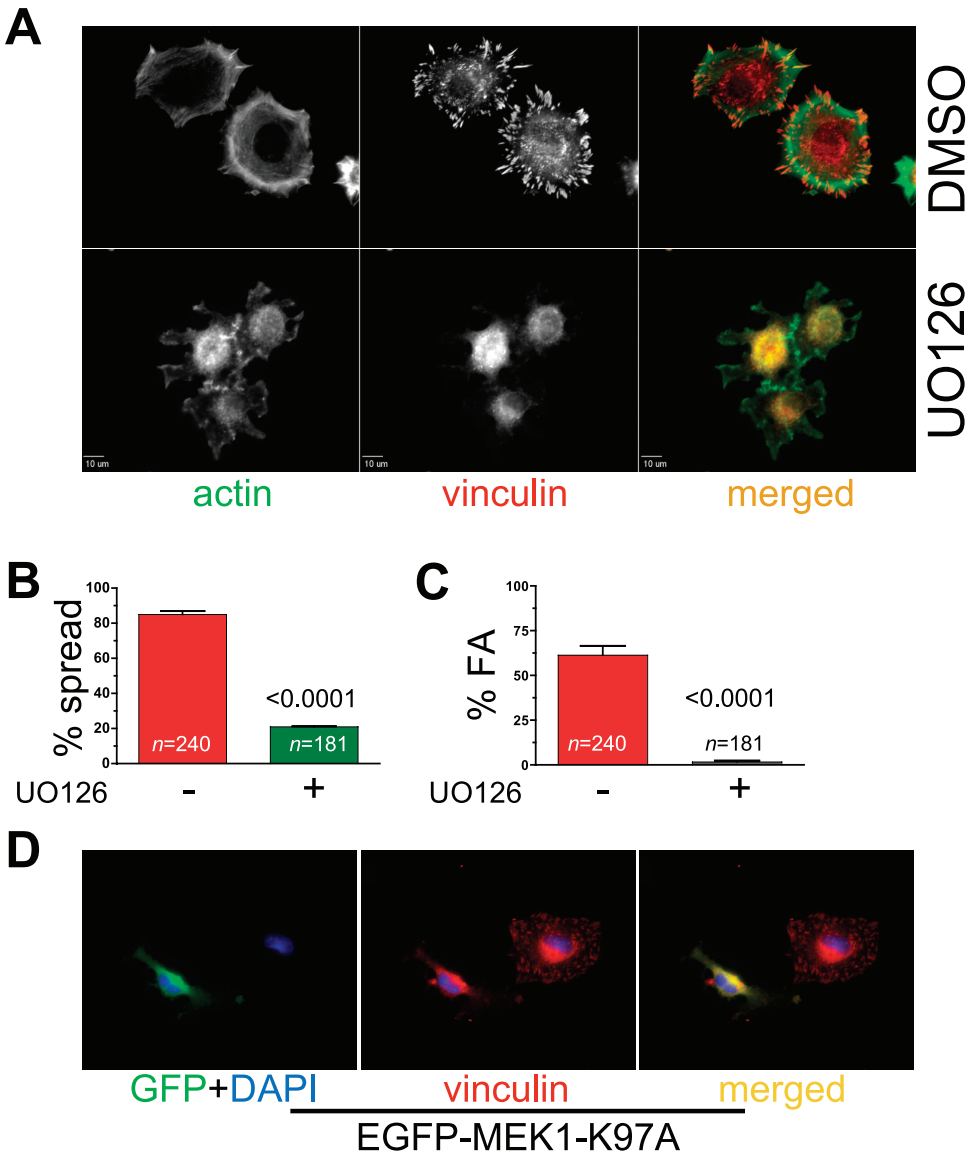


FIG. 1. MEK activity is required for cell spreading and focal adhesion assembly. REF52 cells were plated on fibronectin-coated coverslips for 30 min with or without prior treatment with the MEK inhibitor UO126 (25 μM). Cells were fixed and stained for focal adhesions (vinculin) and F-actin (phalloidin-Oregon green 488) (A) and scored for spreading (B) and for mature focal adhesions (C). At least 20 random fields were counted for each experimental condition. Experiments were performed on at least three different occasions with similar results. Data were analyzed by Student's *t* test, and *P* values and number of cells (*n*) in this experiment are indicated. (D) REF52 cells were transfected with EGFP-tagged kinase-defective MEK1 (EGFP-MEK1-K97A) and replated on fibronectin-coated coverslips for 30 min. Cells were fixed and imaged as in panel A.

hesions present in control cells (see the example in Fig. 1A, top of the middle panel) and fully spread cells as those that contained a major lamellipodium and distinct stress fibers. In some experiments with cells of higher passage (>20), even control cells contained diffused vinculin staining that was atypical to the expected punctate or focal adhesion staining. Such cells were excluded from analysis from all experimental conditions. For GAP phenotype analysis, REF52 cells transfected with GFP-tagged constructs were counted 24 to 48 h posttransfection with a Nikon Eclipse TS100 microscope fitted with a GFP filter. Rounded cells in each bright field were counted (20× objective), and GFP-positive cells in the same field were scored by switching to the GFP channel.

RESULTS

ERK activity is required for focal adhesion formation and cell spreading. To test if MEK activity is required for focal adhesion formation, we treated REF52 cells with the MEK inhibitor UO126 (29) and allowed them to adhere to fibronectin. Vehicle-treated control cells spread radially and formed numerous vinculin-containing focal adhesions, whereas cells treated with UO126 lacked focal adhesions and spread with multiple broad membrane protrusions (Fig. 1A). Approximately 85% of control cells spread radially (Fig. 1B), and ~60% produced focal adhesions following adhesion to fibronectin for 30 min (Fig. 1C); pretreatment with UO126 reduced these percentages to ~20% and 2%, respectively. A similar phenotype was seen in cells transiently expressing dominant-negative MEK1 (MEK1-K97A; Fig. 1D). Together these data indicate a requirement for MEK-ERK signaling in the formation of focal

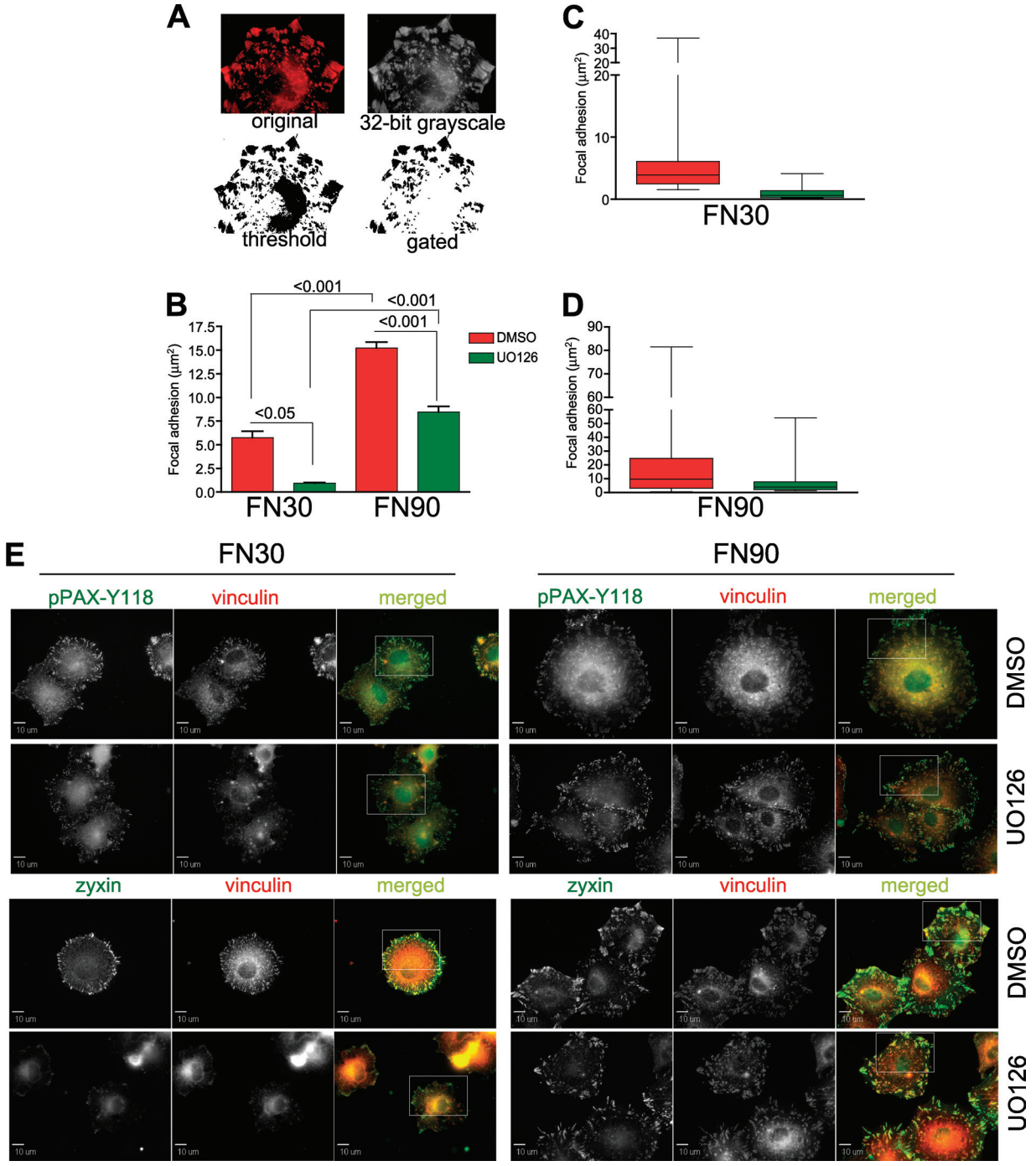


FIG. 2. MEK signaling facilitates maturation of focal adhesions. Cells plated on fibronectin for 30 or 90 min (FN30 and FN90, respectively) in the presence or absence of UO126 were stained for vinculin. Digital images were converted to 32-bit gray scale, threshold set and semiautomatically gated (A). Pixel values were converted to micrometers and analyzed by one-way ANOVA (B). (C and D) Box-whisker plot representation of the distribution of focal adhesion areas in cells plated for 30 of 90 min, respectively. The top and bottom edges of the rectangle are 75th and 25th percentiles, and the horizontal line within the rectangle is the median value. The means ± standard errors (SE) from this analysis are presented in panel B. (E) Cells allowed to spread on fibronectin for 30 or 90 min (FN30 and FN90, respectively) in the presence or absence of UO126 were stained for zyxin, a marker for mature adhesions, or phospho-Y118 paxillin, found in both mature and nascent adhesions. (F) Cropped and magnified details from insets shown in panel E.

adhesions during adhesion to fibronectin, consistent with previous reports (32, 50).

Quantitative analysis of immunofluorescent images of vinculin staining (Fig. 2A) revealed that the average size of adhesions was significantly decreased in UO126-treated cells at 30 min (0.94 ± 0.07 μm^2 compared to 5.76 ± 0.66 μm^2 in control cells; $P < 0.05$) (Fig. 2B and C) with some recovery seen at 90 min (8.46 ± 0.6 μm^2 compared to 15.24 ± 0.61 μm^2

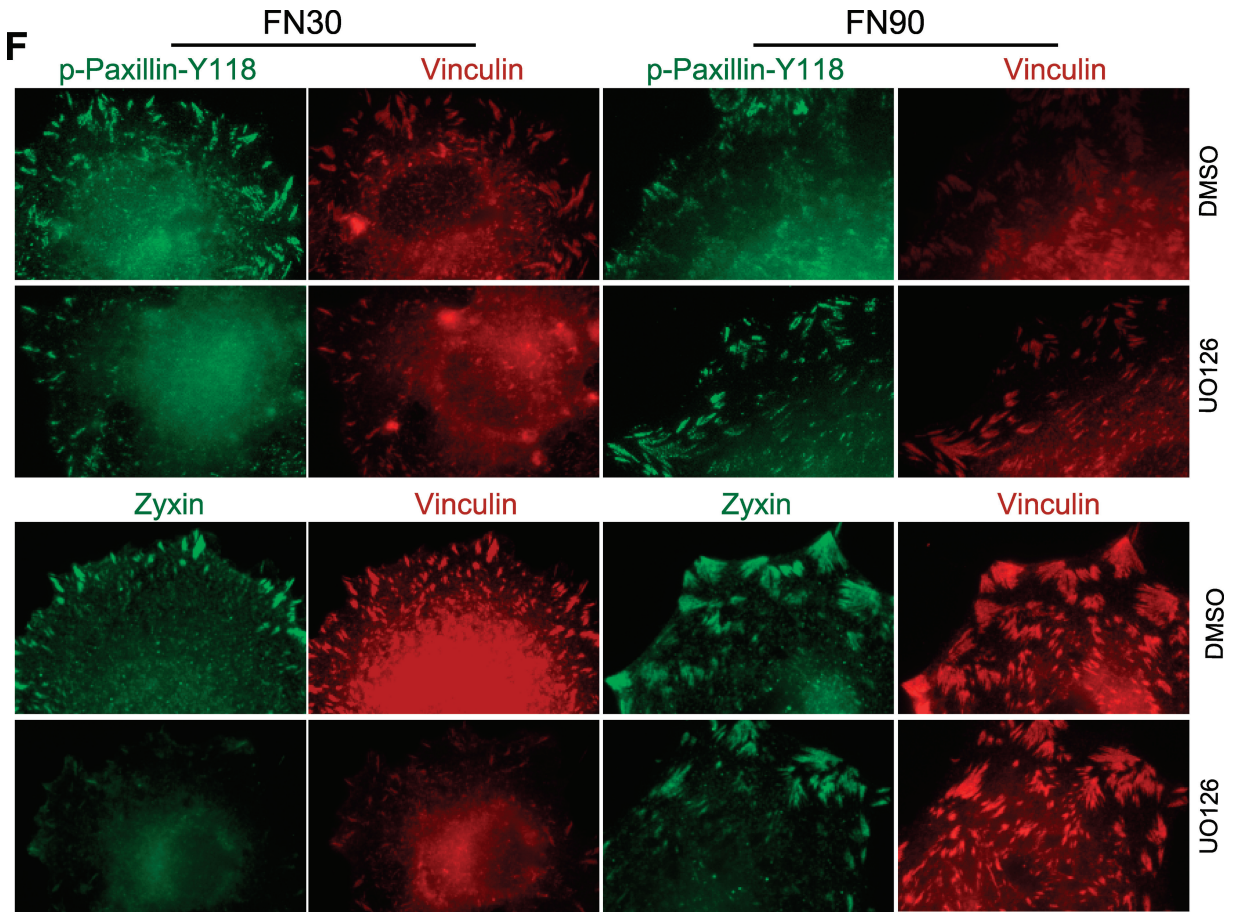


FIG. 2—*Continued*.

in control cells; $P < 0.001$) (Fig. 2B and D), suggesting a defect in the maturation of nascent adhesions. Nascent adhesions contain paxillin phosphorylated on tyrosine 118, but little zyxin, whereas mature adhesions contain both markers (35, 90). Zyxin was largely absent from adhesions in UO126-treated cells at 30 min but recovered somewhat at 90 min (Fig. 2E and F). These observations indicate that MEK/ERK activity facilitates the maturation of nascent adhesions.

ERK inhibition phenocopies Rho and ROCK inhibition. Inhibition of MEK signaling (Fig. 1) causes a phenotype reminiscent of inhibition of Rho/ROCK signaling in fibroblasts (64) and THP-1 leukocytes (87). To begin to test this possibility directly, we pretreated cells with membrane-permeable *Clostridium* C3 to inactivate Rho proteins (1) or with Y27632 to inhibit ROCK (45). Cells incubated with C3 toxin or Y27632 typically failed to spread normally or elaborate mature focal adhesions and exhibited phenotypes closely resembling those of UO126-treated cells (Fig. 3A and B).

To determine if MEK/ERK signaling is required upstream or downstream of Rho/ROCK as cells adhere to fibronectin, we performed two experiments. First, we asked whether the contractile morphology induced by constitutively active Rho or ROCK could be reversed by UO126. Cells transiently transfected with constitutively active Rho (myc-RhoA-63L) or ROCK (myc-ROCKII-Δ3) were plated on fibronectin in the presence or absence of UO126. UO126 had little effect on the morphology of cells transiently expressing active Rho or ROCK but inhibited focal adhesion formation in neighboring untransfected cells (Fig. 3C and D). Thus, MEK/ERK activity is not required downstream of constitutively active Rho or ROCK. Second, we asked whether endogenous RhoGTP loading and ROCK function require MEK/ERK activity. Cells pretreated with UO126 or solvent control were plated on fibronectin for 30 min. RhoGTP was measured by pulldown with the Rho-binding domain of Rhotekin (65, 66); ROCK activity was measured by assessing phosphorylation of a direct ROCK substrate, myosin phosphatase targeting subunit (MYPT) (30, 43, 49), and by phosphorylation of a ROCK-dependent but indirect target, cofilin (81, 82, 88). In vehicle-treated controls, RhoGTP levels are elevated over baseline following 15 and 30 min of adhesion to fibronectin (Fig. 4A and B) in concert with the appearance of focal adhesions and stress fibers (Fig. 1) (64). However, cells treated with UO126 exhibited substantially lower RhoGTP levels at 15 min, with some recovery seen at 30 min (Fig. 4A and B). Furthermore, UO126 substantially inhibited ROCK-dependent phosphorylation of both MYPT and cofilin (Fig. 4C and D). Note that neither Y27632 (Fig. 4B) (64) nor C3 transferase (data not shown) inhibits ERK activation stimulated by fibronectin. Together, these data support a requirement for MEK upstream but not downstream of the

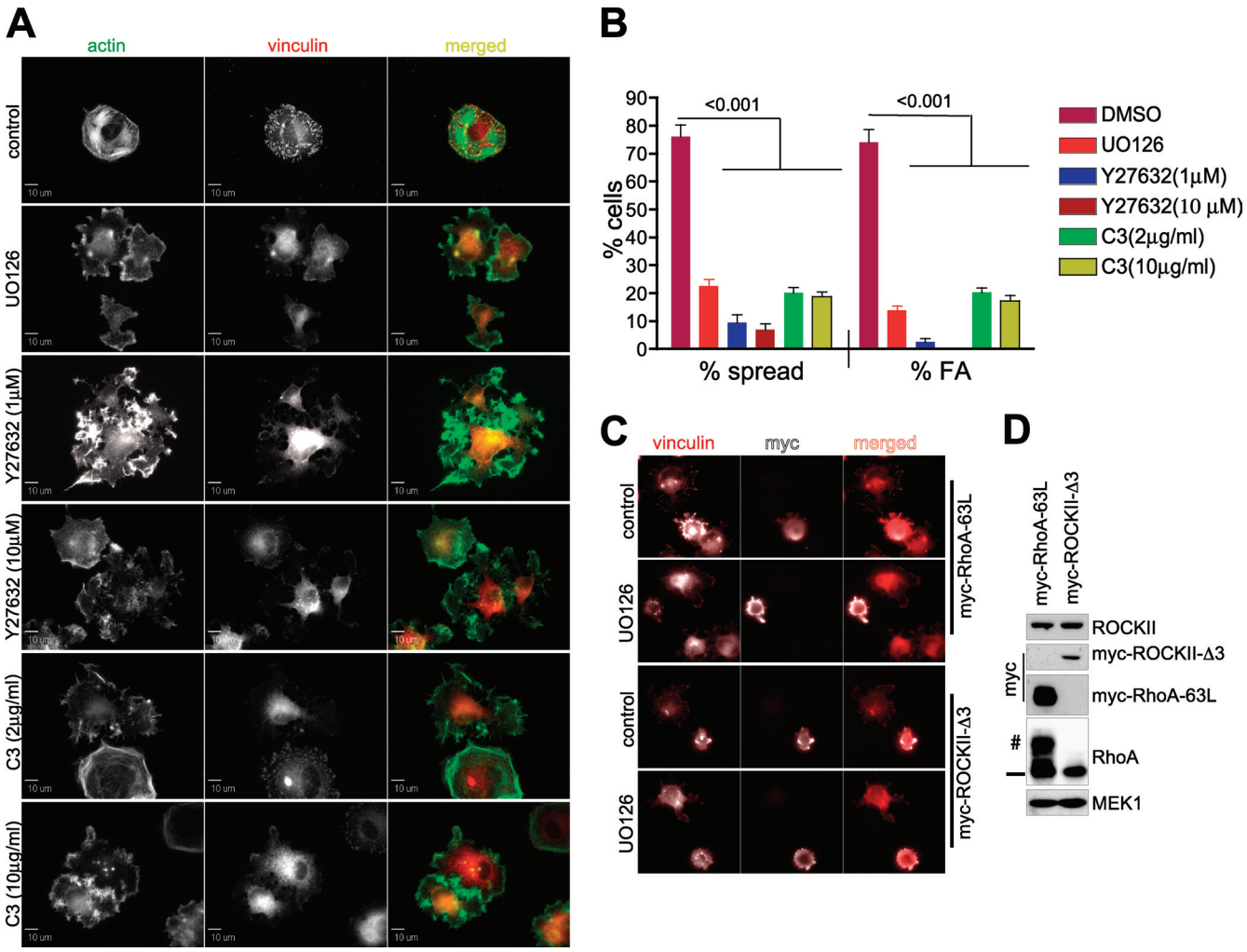


FIG. 3. MEK inhibition phenocopies Rho or ROCK inhibition during acute spreading. Cells in suspension were treated with MEK inhibitor UO126, ROCK inhibitor Y27632, or the Rho inhibitor C3-transferase at the indicated concentration and then allowed to adhere and spread on fibronectin for 60 min. Cells were stained for vinculin and F-actin (A). The fractions of cells spreading normally and exhibiting mature focal adhesions were quantitated (B). All images were at the same magnification, and at least 20 random fields were counted for each experimental condition. (C) The requirement for MEK activity is upstream of Rho and ROCK activities. REF52 cells were transfected with constitutively active RhoA (myc-RhoA-63L) or ROCK (myc-ROCKII-Δ3) and replated on fibronectin-coated coverslips (30 min) with or without prior treatment with UO126. Fixed cells were stained for myc epitope (to identify transfected cells) and costained with vinculin. (D) Western blot of expression of endogenous and exogenous proteins from the experiment shown in panel C. The symbols **#** and — denote exogenous and endogenous RhoA, respectively.

Rho/ROCK activity necessary for maturation of focal adhesions during spreading on fibronectin.

p190A is a major target for ERK during focal adhesion formation. Fibroblasts exhibit a transient decrease and subsequent recovery of RhoGTP levels following adhesion to fibronectin, in concert with membrane spreading and subsequent focal adhesion formation (4–6, 56, 64, 65). Since ERK activity is required for RhoGTP loading in this recovery phase (Fig. 4A and B), we hypothesized that ERK suppresses p190A RhoGAP activity to allow Rho activation necessary for focal adhesion formation. We first asked whether p190A activity in cells adhering to fibronectin was elevated by pretreatment with the MEK inhibitor UO126. Active RhoGAPs and effectors were enriched from cell lysates by virtue of their affinity for RhoGTP (34, 59). p190A RhoGAP was detected by blotting GST-Rho63L pulldowns with p190A antisera; preliminary experiments established the selectivity of this assay (data not shown). Approximately 50% more endogenous p190A was pulled down from UO126-treated cells than control cells following 20 min of adhesion to fibronectin (Fig. 4E and F), consistent with the hypothesis that ERK activity suppresses p190A Rho-binding activity.

A key prediction of this hypothesis is that depletion of p190A should render focal adhesion maturation resistant to the MEK inhibitor UO126. We designed four siRNAs targeting the p190A open reading frame or 3′ untranslated region and a mismatched nontargeting control (Fig. 5A), and siRNA-transfected cells were plated on fibronectin for 30 min in the presence or absence of UO126. All four p190A siRNAs substantially reduced p190A levels, while none affected p190B,

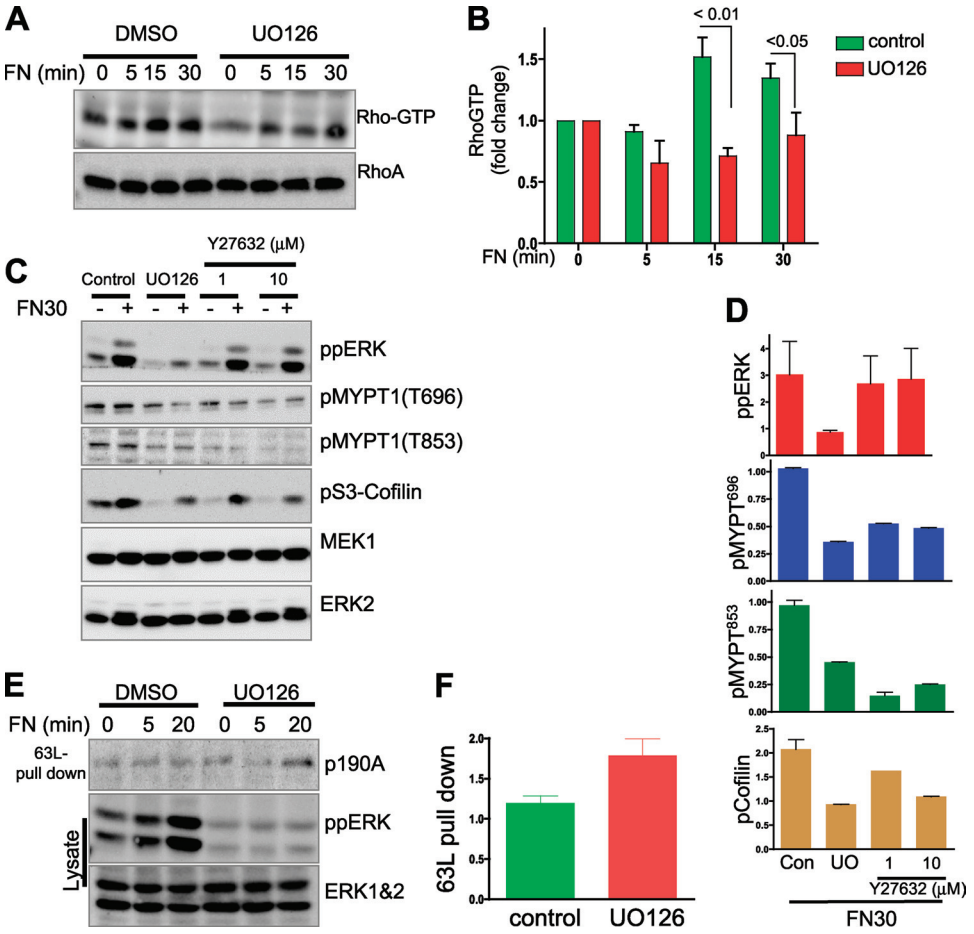


FIG. 4. MEK activity is required for RhoGTP loading and ROCK activity during adhesion to fibronectin. (A) Suspended REF52 cells were treated with dimethyl sulfoxide (DMSO) or UO126 and replated on fibronectin. RhoGTP was pulled down with the Rho binding domain of Rhotekin. (B) Quantitation of RhoGTP normalized to total Rho from experiments represented in panel A (n = 3). (C) Cells replated on fibronectin for 30 min (FN30) with or without prior treatment with UO126, Y27632, or DMSO vehicle were assessed for ROCK-dependent phosphorylation of MYPT (pMYPT1-T696 or -T853) and cofilin (pS3-Cofilin). (D) Quantitation of experiments represented in panel C. Values from FN30 were normalized to those in control suspended cells for each condition. (E and F) Inhibition of MEK activity increases p190A RhoGAP binding to active RhoA. Cells were treated with DMSO or UO126 and replated on fibronectin. Lysates were subjected to GST-RhoA-63L pulldown and blotted with p190A antibody to determine binding of endogenous p190A-RhoGAP to active RhoA. (F) Quantitation of p190A bound to RhoGTP at 20 min expressed as fold increase over suspended cells (0 min).

ERK2, or MEK1/2 (Fig. 5B). As expected, control siRNA-transfected cells treated with UO126 failed to spread and form mature focal adhesions (Fig. 5C to F). In striking contrast, cells transfected with any of the four p190A siRNAs spread and formed focal adhesions even in the presence of UO126 (Fig. 5C to F). Collectively, these data support the hypothesis that ERK activity suppresses p190A RhoGAP function to allow RhoGTP loading and focal adhesion formation.

p190A RhoGAP is phosphorylated on ERK phosphorylation motifs. The p190A primary sequence contains at least four potential ERK sites clustered in the C-terminal tail following the RhoGAP domain (Fig. 6A). Amino acids 1451 (PGS*P), 1476 (PQS*P), 1480 (PPT*P), and 1483 (PQS*P) conform to a consensus PXS/T*P ERK phosphorylation motif, where * tags the phosphorylated residue (23), and are conserved in all vertebrate p190A isoforms analyzed (Fig. 6A) but are absent from the closely related p190B protein (13). Unfortunately, attempts to identify C-terminal phosphorylation sites in full-length p190A by mass spectrometry were unsuccessful (data not shown), likely because all four phosphorylation sites reside in a large (~8.5 kDa) and chemically nonoptimal tryptic peptide. Given its size and given that all other predicted tryptic peptides from p190A are smaller than 3.75 kDa, we were able to analyze *in vitro*- and *in vivo*-labeled p190A using a Tris-Tricine gel electrophoresis/transfer approach that exploits the large size of the C-terminal tryptic peptide. To determine whether ERK can phosphorylate p190A, FLAG-tagged wild-type p190A or p190A-4A (in which serines 1451, 1476, and 1483 and threonine 1480 are mutated to alanine) were transiently expressed in COS1 cells. FLAG-p190A and FLAG-p190A-4A from transfected cells were immunoprecipitated and phosphorylated *in vitro* with [γ-^{32}P]ATP and recombinant active ERK. In initial experiments, recombinant ERK phosphorylated both wild-type p190A and p190A-4A to similarly low levels (data not shown). We reasoned that poor *in vitro* phosphorylation might result from high occupancy of the C-

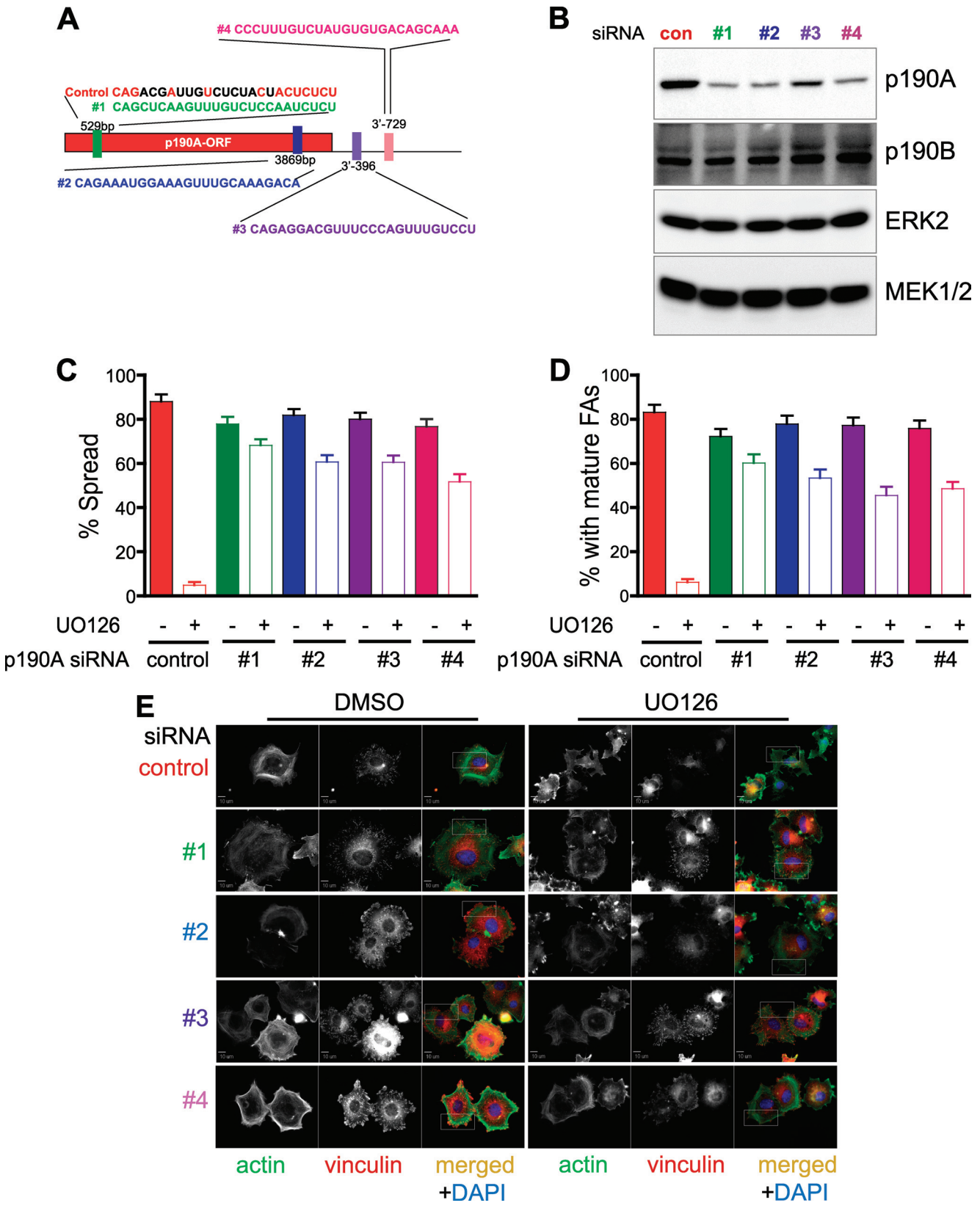


FIG. 5. p190A RhoGAP is a major target of MEK signaling during focal adhesion maturation and cell spreading. Cells transfected with stealth siRNA targeting one of four different regions in the p190A open reading frame or 3′UTR, or a mismatched nontargeting control (see panel A; mismatched residues in the control are indicated in black letters) were treated with DMSO or UO126 and replated on fibronectin-coated coverslips for 30 min. (B) Stealth p190A siRNAs suppress p190A expression without altering expression of the closely related p190B homologue. (C and D) Quantitation of cell spreading and focal adhesions. Cells in at least 20 nonoverlapping fields were counted and subjected to one-way ANOVA. (E and F) Cells were imaged for focal adhesions or F-actin. Selected regions from images in panel E are magnified in panel F. Numbers 1 to 4 denote the siRNA used (see panel A).

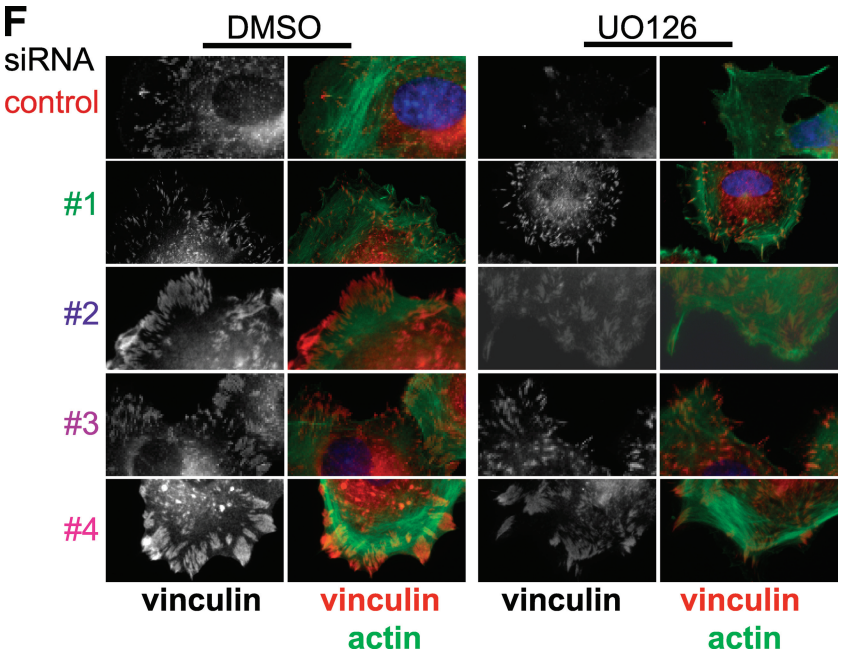


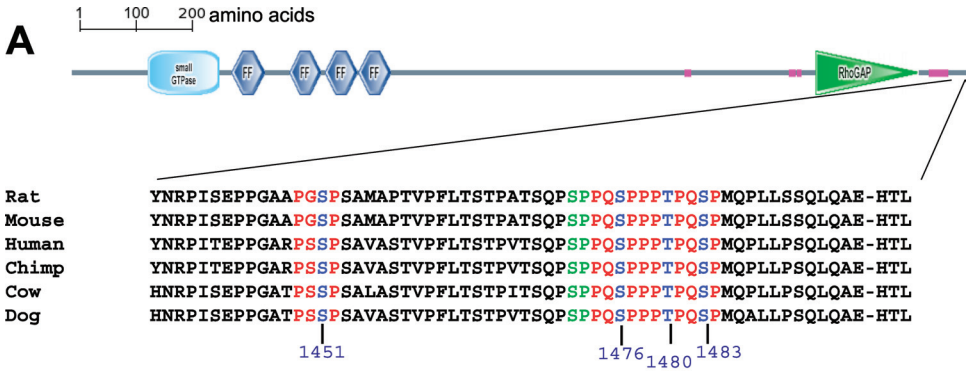
FIG. 5—*Continued*.

terminal sites in the immunoprecipitated wild-type protein, and indeed prior incubation of the immunoprecipitates with alkaline phosphatase allowed ERK to robustly phosphorylate wild-type p190A but not the p190A-4A mutant (Fig. 6C). These data indicate that serines 1451, 1476, and 1483 and/or threonine 1480 are the only residues in p190A appreciably phosphorylated by ERK. To determine if these sites are phosphorylated in cells, FLAG-tagged wild-type p190A or p190A-4A or empty vector was transiently expressed in REF cells and cultures were labeled with $^{32}P_i$. Given the technical difficulties associated with performing this type of labeling experiment in acutely adhering cells, we instead labeled adherent cultures. FLAG-p190A proteins were recovered by immunoprecipitation and resolved by SDS-PAGE. A nonspecific labeled protein was reproducibly present in all lanes including the vector control (Fig. 6D), but Coomassie blue staining of the membrane (not shown) allowed for precise excision of the p190A proteins. After trypinization of either *in vitro*-or *in vivo*-labeled proteins, an ~8- to 10-kDa tryptic phosphopeptide was reproducibly detected in wild-type p190A but not in p190A-4A (Fig. 6E), demonstrating that one or more of the sites phosphorylated by ERK *in vitro* are used *in vivo*. Note that trypsinization of the nonspecific band yielded no phosphopeptides in the ~8- to 10-kDa size range (Fig. 6E): i.e., the observed ~8- to 10-kDa phosphopeptides are derived exclusively from p190A. Treatment of stably adherent cells expressing wild-type p190A with UO126 did not substantially decrease phosphorylation of the C-terminal tryptic phosphopeptides (Fig. 6E). We speculate that this is due to decreased turnover of the C-terminal sites in densely packed, stably adherent cells or in the absence of active ERK, other kinases such as p38 and glycogen synthase kinase β (GSKβ) may phosphorylate the C-terminal sites as observed by Jiang et al. *in vitro* (47).

Together the *in vitro* and *in vivo* phosphorylation data demonstrate that ERK selectively phosphorylates the C-terminal ERK consensus motifs in full-length p190A and that these sites are used in REF cells. These data extend the observations made by Settleman and colleagues, who found that serines 1476 and 1483 are phosphorylated when the isolated C terminus of p190A is expressed in cells and serine 1476 and T1480 are phosphorylated when this fragment is incubated with active ERK *in vitro* (47).

Mutation of the ERK consensus phosphorylation sites increases p190A functional activity. We next asked whether the C-terminal phosphorylation sites affect p190A biological function by using a well-established cellular assay (4, 47). Cells transfected with EGFP-p190A constructs or the EGFP vector control were incubated in a monolayer culture, and GFP-positive cells were scored for the round-cell "GAP" phenotype (4, 17, 47). As expected, <15% of GFP vector- or GAP-deficient p190A-RA-expressing cells were round, as compared to ~55% of wild-type p190A-expressing cells (Fig. 7A). Importantly, the GAP phenotype was more prevalent (~85%) in cells expressing p190A-4A (Fig. 7A). Levels of expression of the wild type and GAP- and phosphorylation-deficient mutants were similar (Fig. 7B).

To refine this analysis, we asked whether mutation of the putative C-terminal ERK phosphorylation sites influenced focal adhesion formation in cells acutely spreading on fibronectin. Cells expressing GFP alone spread normally and elaborated focal adhesions following adhesion to fibronectin for 30 min (Fig. 7C). In contrast, ~55% of all cells expressing GFP-tagged wild-type p190A exhibited reduced focal adhesion formation and defects in spreading (Fig. 7C and D). These phenotypes were more prevalent (~82%) in cells expressing GFP-tagged p190A-4A (Fig. 7C and D). This phenotypic difference was most obvious in cells expressing relatively low levels of the p190A constructs (Fig. 7E).



B

Site	Kinase	Score	Percentile	Source
S1451	ERK	-	-	manual
S1472	GSK3α	0.4126	0.052%	Scansite
S1476	ERK	0.2807	0.003%	Scansite
	GSK3β	0.2042	0.0%	Scansite
	GSK3α	0.2211	0.0%	Scansite
T1480	ERK	-	-	manual
	GSK3α	0.4599	0.11%	Scansite
S1483	ERK	-	-	manual
	p38	0.3051	0.186%	Scansite
L341	ERK docking site	0.5212	0.068%	Scansite
L344	ERK docking site	0.4590	0.017%	Scansite

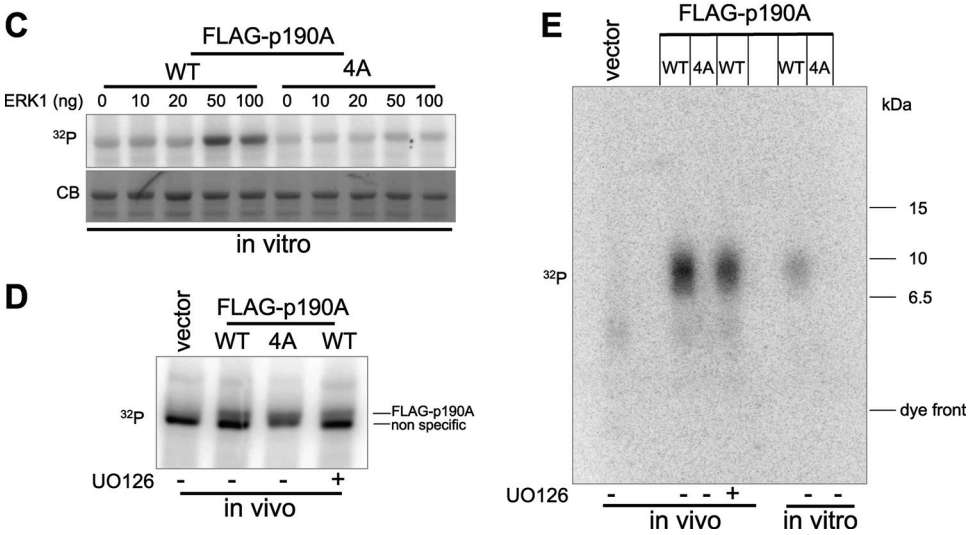


FIG. 6. p190A RhoGAP is an ERK substrate. (A) Four potential ERK phosphorylation motifs are highly conserved in the p190A C terminus. Vertebrate p190A sequences are aligned to show potential ERK phosphorylation (PXSP and PXTP) motifs. (B) Collation of putative phosphorylation and docking sites identified either manually or through computational methods (ScanSite). (C and D) p190A is phosphorylated on one or more putative C-terminal ERK sites *in vitro* and *in vivo*. FLAG-tagged wild-type (WT) or p190A-4A mutant (4A; where the four putative ERK sites shown in panel A have been replaced with alanines) was immunoprecipitated, pretreated with alkaline phosphatase, and then phosphorylated *in vitro* with recombinant active ERK in the presence of [γ-^{32}P]ATP (C). Alternatively, REF52 cells transfected with FLAG-p190A, FLAG-p190A-4A, or empty vector were metabolically labeled with $^{32}P_i$ and FLAG-p190A forms were recovered by immunoprecipitation (D). (E) Tryptic digests of *in vitro*- and *in vivo*-labeled p190A were resolved by electrophoresis (with low-molecular-mass markers) and transferred to membrane. The positions of the dye front and ~6.5-, 10-, and 15-kDa markers are indicated. ^{32}P denotes phosphorimages, and CB in panel C represents a Coomassie blue-stained image of the same blot.

These biological assays support the hypothesis that C-terminal phosphorylation of p190A by ERK suppresses RhoGAP function in the context of focal adhesion formation. We therefore asked whether the enhanced functional activity of p190A-4A correlated with an increase in p190A biochemical activity. EGFP-tagged p190A constructs were cotransfected with FLAG-RhoA and lysates assayed for both EGFP-p190A binding to recombinant active Rho and for FLAG-RhoGTP.

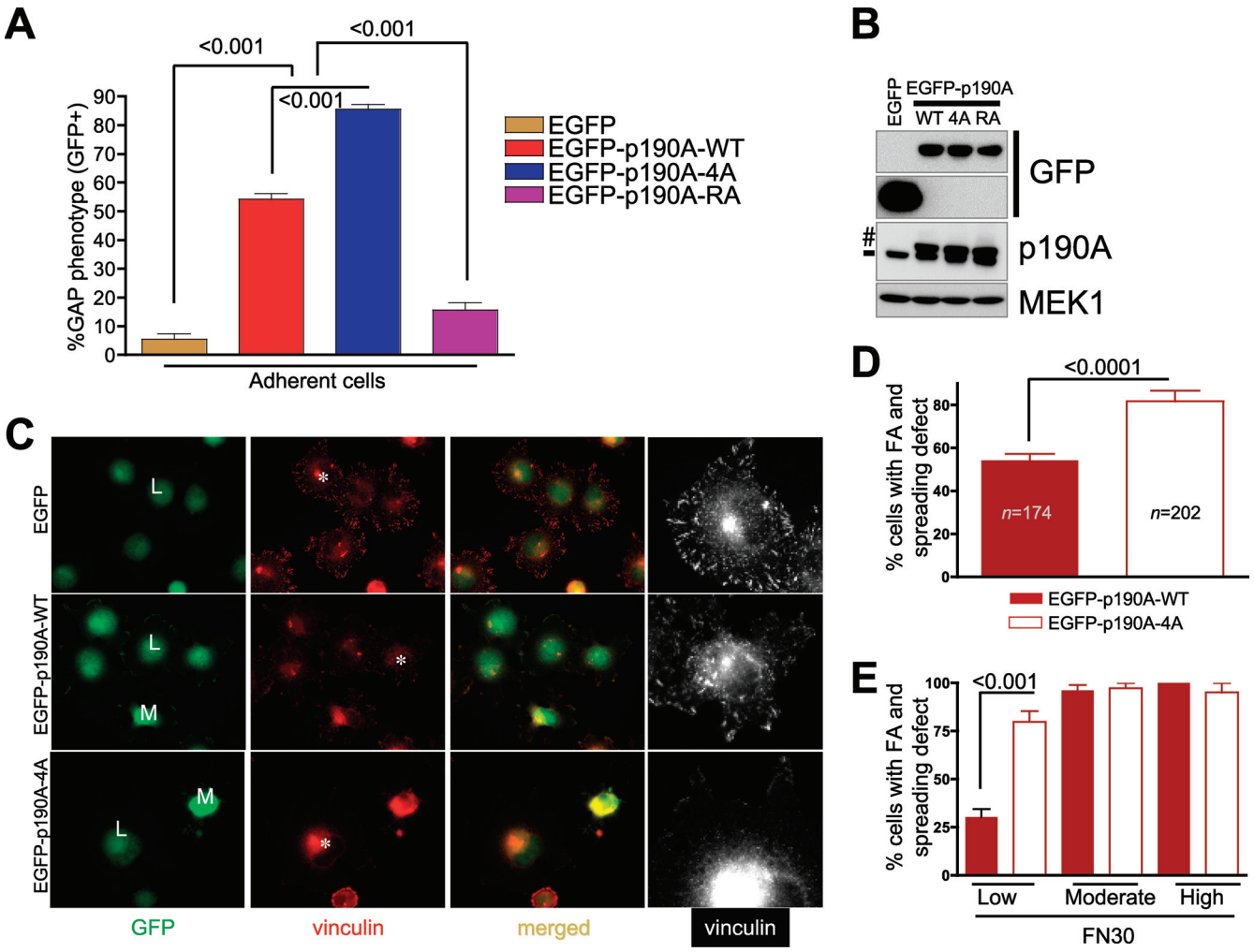


FIG. 7. Mutation of C-terminal ERK phosphorylation sites increases p190A RhoGAP biological activity. REF52 cells were transfected with EGFP vector, EGFP-p190A, EGFP-p190A-4A, or catalytically inactive EGFP-p190A-RA. Twenty-four hours after transfection, cells were scored for the GAP phenotype (see text). (B) Expression of endogenous (—) and exogenous (#) proteins in cells used in panel A. (C to E) Cells transfected with EGFP-p190A constructs were replated on fibronectin-coated coverslips for 30 min, fixed, and imaged for GFP and vinculin. Expression levels of EGFP-p190A were optically determined; low and moderate expressors contained about 2 and 5 times more GFP fluorescence than background, respectively. Gray scale images are magnified images of cells indicated by asterisks. (D to E) Quantitation of focal adhesion (FA) and spreading defects. Data from the entire GFP-positive populations are shown in panel D; further analysis reveals that the frequency of focal adhesion and spreading defects is greatest in a subpopulation expressing low levels of p190A-4A (E).

The mutant construct bound to RhoA-63L approximately 2.5-fold more avidly than wild-type p190A (Fig. 8A and B), consistent with our finding that UO126 stimulated binding of endogenous p190A to RhoA-63L (Fig. 4E and F). FLAG-RhoGTP levels were reduced by approximately 70% in cultures cotransfected with EGFP-p190A as compared to either empty EGFP vector (Fig. 8C and D) or a GAP-deficient mutant (p190A-RA; data not shown). Cotransfection with the EGFP-p190A-4A mutant modestly (~20%) but reproducibly decreased FLAG-RhoGTP further than wild-type p190A (Fig. 8C and D). Thus, the primary consequence of phosphorylation of the p190A C terminus on one or more of the four PXS/TP motifs is inhibition of RhoA binding.

Together the biochemical and functional data strongly support an important role for ERK phosphorylation of the C terminus in inhibiting p190A GAP function *in vivo* in the context of focal adhesion formation.

Localization of p190A is regulated by MEK signaling and the C-terminal ERK phosphorylation sites. p190A function is linked to its localization (4, 17, 40). In particular, previous reports have shown that focal adhesions and stress fibers are formed preferentially in lamellipodia lacking p190A (6, 84). We therefore reasoned that ERK phosphorylation of p190A might function in part to localize p190A away from newly forming focal adhesions and stress fibers during cell spreading on fibronectin. Indeed, endogenous p190A was largely excluded from a zone containing circumferential stress fibers in cells spreading on fibronectin for 30 min, whereas p190A was distinctly colocalized with poorly organized actin at the periphery of UO126-treated cells (Fig. 9A). In good agreement, com-

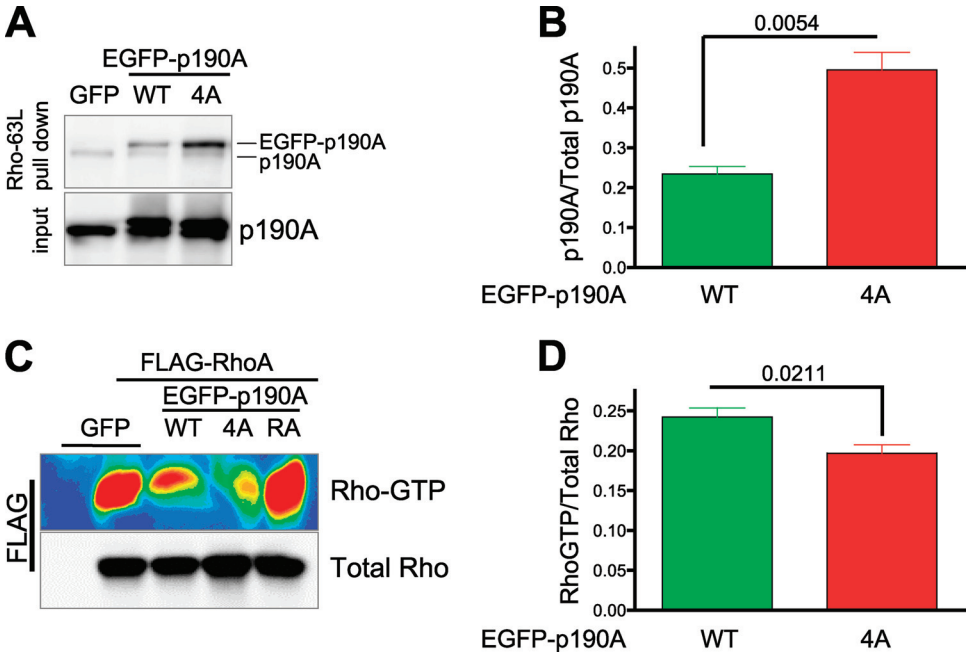


FIG. 8. Mutation of C-terminal ERK phosphorylation sites increases p190A biochemical activity in adherent cells. (A) Mutation of putative C-terminal ERK sites stimulates p190A RhoGTP binding activity. Cells were transfected with either EGFP vector, EGFP-p190A (WT) or EGFP-p190A-4A. Binding of wild-type and mutant p190A RhoGAP to RhoGTP was assessed by pulldown with GST-RhoA63L. (B) Ratio of active p190A in GST-RhoA-63L pulldown to total p190A in the starting extract. Digital images from 10 experiments were statistically analyzed. (C) Mutation of putative C-terminal ERK sites modestly stimulates p190A GAP activity in cells. Cells were cotransfected with FLAG-RhoA and EGFP, EGFP-p190A, EGFP-p190-4A, or EGFP-p190A-RA (GAP-deficient R1258A mutant). FLAG-RhoGTP levels were determined by GST-Rhotekin Rho binding domain pulldown assay. Rhotekin pulldown was blotted with FLAG antibody and pseudocolored for presentation. (D) Digital images from five experiments were statistically analyzed.

bined mutation of the four ERK phosphorylation sites in the C terminus enhanced retention of GFP-p190A-4A in peripheral membranes (Fig. 9B). These observations indicate that MEK-dependent phosphorylation of the C-terminal ERK sites is required for translocation of p190A away from developing focal adhesions and stress fibers.

DISCUSSION

Integrin engagement activates Rac and Cdc42 while simultaneously and transiently inactivating Rho (4, 5, 24, 63, 65). Rac and Cdc42 activation stimulate membrane protrusion and formation of focal complexes (57, 58, 67–69), while suppression of Rho diminishes contractile forces and allows the formation of lamellipodia. Subsequent recovery of RhoGTP promotes focal adhesion maturation and formation of stress fibers. In concert with these morphological changes, integrin engagement also activates ERK signaling (20, 32, 76). ERK in protruding membranes and nascent focal adhesions directly activates myosin light chain kinase that promotes contraction of the actomyosin system by phosphorylating myosin light chain (9, 32, 50). Contraction of the actin cytoskeleton and formation of stress fibers and focal adhesions require Rho activation (22, 55), raising the possibility that integrin-activated ERK may stimulate Rho activation during adhesion and spreading on extracellular matrix.

Here we demonstrate that inhibiting ERK activity with UO126 or with dominant-negative MEK1 abrogates focal adhesion maturation in acutely adhering cells. We further find that fibronectin-stimulated RhoGTP loading and ROCK-dependent phosphorylation of MYPT and cofilin are inhibited by UO126, while the contractile phenotypes induced by expression of constitutively active Rho or ROCK are resistant to the MEK inhibitor. These data support the hypothesis that ERK signaling upstream of Rho is necessary for focal adhesion maturation.

Rho regulation during adhesion and spreading. The opposing actions of GEFs and GAPs likely set the dynamic level of RhoGTP during spreading on fibronectin. p190A, a major GAP for RhoA, is activated by tyrosine phosphorylation in response to integrin signaling (4, 5, 8, 42, 56, 70) and causes a transient decrease in RhoGTP levels following initial adhesion to fibronectin (5, 65). Association of p190A RhoGAP with p120 RasGAP is essential for recruitment of p190A to the cell periphery, where it is active against Rho (8, 61), and disruption of the p190A RhoGAP-p120 RasGAP complex suppresses actin filament reorientation and migration in wound healing assays (27, 51). Thus, p190A RhoGAP function in cell spreading and migration requires tyrosine phosphorylation, association with p120 RasGAP, and recruitment to peripheral sites of action.

The decrease in RhoGTP seen following initial adhesion largely recovers by 30 min (5, 65). Thus, RhoGEF activity increases to exceed p190A RhoGAP activity and/or p190A RhoGAP activity is suppressed at later time points of adhesion to fibronectin. Chen et al. (19) reported that oncogenic Ras decreases p190A RhoGAP abundance and increases RhoGTP

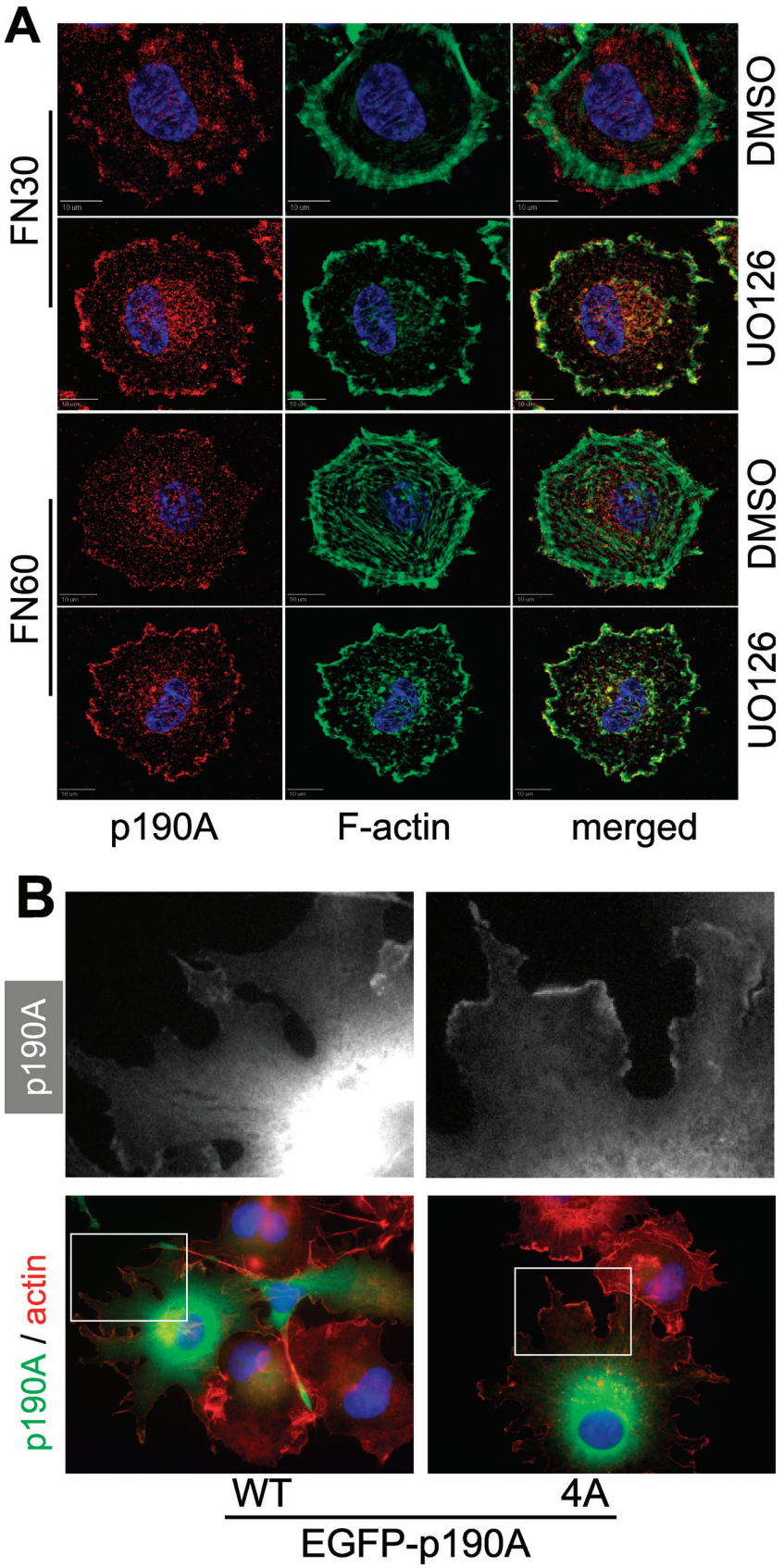


FIG. 9. ERK signaling regulates localization of p190A in spreading cells. (A) REF52 cells were treated with UO126 or vehicle control and replated on fibronectin for 30 or 60 min (FN30 and FN60, respectively). Fixed cells were stained for endogenous p190A and F-actin. (B) COS1 cells transfected with EGFP-p190A or EGFP-p190A-4A were replated on fibronectin for 60 min, fixed, and imaged for F-actin (red) and GFP (green) fluorescence. The gray scale images correspond to GFP fluorescence magnified from the areas indicated by white boxes in the color images.

in a nonionic detergent-soluble fraction. Translocation of p190A RhoGAP (in complex with p120 RasGAP) to the insoluble fraction requires MEK signaling, and activated forms of Raf and MEK are sufficient to cause this translocation (19). These observations are consistent with the view that ERK signaling suppresses p190A activity in part by separating p190A from its RhoGTP substrate.

ERK regulation of p190A RhoGAP. Inspection of the p190A primary sequence reveals four consensus ERK phosphorylation sites clustered in the C-terminal domain of p190A but missing from p190B. ERK substrates typically also contain ERK docking sites that are necessary for efficient substrate recognition (46, 83). Two putative ERK docking sites conforming to the consensus $(R/K)(R/K)(X_{1-5})(L/I)X_{1-3}(L/I)$ (46) (where X represents any amino acid) are present at leucine 341 and leucine 344 of p190A. A computational search using algorithms at Scansite (60) independently identified the two docking sites and S1476 as a potential ERK site (Fig. 6B). Our experiments show that recombinant ERK phosphorylates only the C-terminal sites *in vitro* and that the C terminus is phosphorylated on one or more of these sites in adherent cells. In addition, combined mutation of the four potential C-terminal ERK sites (p190A-4A) prevents the previously reported (19) MEK-dependent translocation of p190A to a detergent-insoluble fraction (data not shown). Mutation of the C-terminal ERK phosphorylation motifs significantly enhances Rho binding and causes a substantial increase in the number of cells exhibiting a "GAP" phenotype. Furthermore, exogenous p190A-4A is a more potent inhibitor of focal adhesion formation than wild-type p190A during acute adhesion of REF cells to fibronectin. Together these biochemical and functional data indicate that phosphorylation of the p190A C terminus inhibits RhoGAP function. These observations extend those of Jiang et al. (47), who recently demonstrated that at least two (S1476 and S1483) of the putative C-terminal phosphorylation sites are phosphorylated following overexpression of a 50-amino-acid C-terminal fragment of p190A and that S1476 and T1480 are direct targets for ERK *in vitro*. Indeed, S1476A and T1480A mutants exhibited ~50% more GAP activity toward RhoA in adherent cells and fail to rescue polarization and migration in p190A-null fibroblasts (47).

Fluorescence-resonance energy transfer (FRET)-based biosensors in living cells reveal that RhoGTP levels are highest at the periphery (54, 62). Previous observations have also indicated that p190A is excluded from maturing lamellipodia (6, 84) and that the protein translocates to a nonionic detergent-insoluble compartment in a MEK-dependent fashion coincident with a decrease in soluble p190A RhoGAP activity (19, 75). These data suggest an important role for ERK signaling in the separation of p190A from its Rho substrate. Indeed, we find that endogenous p190A is excluded from regions containing circumferential stress fibers in cells spreading on fibronectin but colocalizes with poorly organized peripheral filamentous actin in cells adhering in the presence of UO126. Mutation of the four putative ERK sites to alanine causes a similar peripheral localization of exogenous p190A. Together, these observations suggest that MEK-dependent phosphorylation of ERK substrate motifs in the p190A C terminus excludes the RhoGAP from regions in which Rho activity is driving new stress fiber and focal adhesion formation.

p190A RhoGAP: an integrator for polarity and adhesion assembly signals? p190A RhoGAP and associated p120 RasGAP are known to play important roles in polarized cell motility. Rho inactivation (4), p190A (47), and p190A-p120 (53) association are required for reorientation of the Golgi apparatus and formation of stress fibers and focal adhesions prior to migration. It appears likely that mechanistic links exist between polarity signaling and focal adhesion formation during directional cell migration. One possibility is that active ERK localized in nascent focal complexes (32, 76) phosphorylates p190A and primes it for further phosphorylation by the polarity regulator glycogen synthase kinase 3β (47), which is known to accumulate in the leading edge of motile cells (28). Dual phosphorylation at these locales would be predicted to inhibit p190A RhoGAP, allowing polarized maturation of focal complexes and directional migration. Given that at least five C-terminal phosphorylation sites may cooperate to regulate p190A function (this report and see reference 47), the precise phosphorylation mechanisms involved may be complex. Novel phosphorylation site-specific antisera will be needed to discern whether common phosphorylation sites are utilized for both adhesion formation and polarized movement or whether these processes are regulated by signals impinging on independent sites in the p190A RhoGAP C terminus.

Distinct roles for ERK in adhesion assembly and disassembly. ERK can promote disassembly of adhesions by activating calpains (37) that degrade FAK (15), Rho (52), and/or cytoskeletal linkers to adhesions (7, 33). We now demonstrate that ERK signaling also plays a role in adhesion assembly, raising important questions of how these mutually antagonistic outcomes are coordinated in time and space in motile cells. ERK activation in focal adhesions is regulated by a number of scaffolding molecules, including paxillin (44), GIT1 (89), and RACK1 (85). We have previously shown that MP1 (72) is required for full ERK activation during adhesion of REF52 cells to fibronectin (64). Strikingly, siRNA-mediated knockdown of MP1 does not abolish adhesion formation on fibronectin but rather results in the elaboration of large vinculin-containing structures (64). Vomastek et al. (85) have also found that the ERK scaffold RACK1 is required for focal adhesion disassembly. In contrast, here we find that global inhibition of MEK with UO126 blocks adhesion assembly, suggesting that active ERK is selectively coupled to distinct substrates required for adhesion assembly or disassembly in part by the actions of scaffolding molecules. The identification of these substrates and the mechanisms insulating their phosphorylation in time and space are important goals of current experiments.

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We declare that there are no conflicts of interest.

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