MOLECULAR AND CELLULAR BIOLOGY, June 2005, p. 5119–5133 Vol. 25, No. 12
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.12.5119–5133.2005

The MEK1 Scaffolding Protein MP1 Regulates Cell Spreading by Integrating PAK1 and Rho Signals

Ashok Pullikuth,[1] Evangeline McKinnon,[1] Hans-Joerg Schaeffer,[2]† and Andrew D. Catling[1,3]*

Department of Pharmacology[1] and Stanley S. Scott Cancer Center,[3] Louisiana State University Health Sciences Center, 1901 Perdido Street, New Orleans, Louisiana 70112, and Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908[2]

Received 7 July 2004/Returned for modification 19 August 2004/Accepted 17 March 2005

How the extracellular signal-regulated kinase (ERK) cascade regulates diverse cellular functions, including cell proliferation, survival, and motility, in a context-dependent manner remains poorly understood. Compelling evidence indicates that scaffolding molecules function in yeast to channel specific signals through common components to appropriate targets. Although a number of putative ERK scaffolding proteins have been identified in mammalian systems, none has been linked to a specific biological response. Here we show that the putative scaffold protein MEK partner 1 (MP1) and its partner p14 regulate PAK1-dependent ERK activation during adhesion and cell spreading but are not required for ERK activation by platelet-derived growth factor. MP1 associates with active but not inactive PAK1 and controls PAK1 phosphorylation of MEK1. Our data further show that MP1, p14, and MEK1 serve to inhibit Rho/Rho kinase functions necessary for the turnover of adhesion structures and cell spreading and reveal a signal-channeling function for a MEK1/ERK scaffold in orchestrating cytoskeletal rearrangements important for cell motility.

The mitogen-activated protein kinase (MAPK) module containing Raf, MEK, and extracellular signal-regulated kinase (ERK) transduces diverse extracellular signals to regulate a variety of cellular functions, including proliferation, differentiation, and migration, in a context-specific manner. To elicit the appropriate downstream response, this generic cascade is selectively coupled to specific upstream activators and downstream targets. However, the mechanisms permitting simultaneous processing of parallel signals through this core module remain poorly understood.

Flexibility and specificity may be conferred upon the ERK pathway through the assembly of signaling complexes around scaffolding proteins, and compelling evidence supports a critical role for such proteins in specifying the biological function of MAP kinase pathways in yeast (reviewed in references 18 and 68). Disparate evidence supports the contention that putative scaffolds regulate the localization, efficiency, and agonist specificity of ERK signaling (14, 40, 50, 67, 74, 77, 81, 82, 89, 96), but a coherent picture of how an individual scaffold channels a specific extracellular signal to the appropriate downstream response has not emerged. For instance, MEK partner 1 (MP1) (67) may localize ERK signaling to endosomes (80, 94), but the spectra of stimuli transduced and the cellular responses mediated by MP1 are unknown; homozygous disruption of KSR (55) or inhibition of IMP (50) decreases ERK signaling, but it is not clear that KSR and IMP target the ERK cascade to specific activators or targets; MORG (89) facilitates ERK activation by select agonists, but the biological context(s) in which this scaffold functions is unknown. How scaffolds impose and maintain signaling specificity remains a critical question in our understanding of ERK function.

A pool of activated ERK localizes to focal complexes and adhesions in response to extracellular matrix stimulation (20, 65, 73). Focal adhesions serve a dual role as anchors for actin stress fibers that transmit the force of actin-myosin contraction to the substratum during migration and as sites of signal transduction through pathways that control the kinetics of cell spreading and motility (reviewed in reference 58). While focal adhesion kinase (FAK) and tyrosine phosphorylation of focal adhesion proteins are not required for focal adhesion assembly per se (21, 34), FAK and Src control the turnover of adhesion structures (19, 58, 91, 92). Importantly, FAK and Src control the activation of ERK in adhesion structures in response to fibronectin stimulation (20, 73), and ERK stimulates the disassembly of focal adhesion structures (27, 91, 92). These data suggest that localized mechanisms and targets of ERK activation within focal adhesions are critical for dynamic remodeling of adhesion structures during morphogenetic events.

The formation and remodeling of adhesion structures and stress fibers is mediated by the Rho family GTPases (reviewed in reference 78). Thus, Cdc42 activation drives the protrusion of filopodia, while Rac signaling through PAK (p21-activated kinase) is necessary for the formation of membrane ruffles and lamellipodia; the protrusion of these actin-rich membrane structures in turn allows for the formation of new focal complexes. In contrast, Rho stimulates the formation of stress fibers and terminal focal adhesions in part through the activation of Rho kinase (ROCK) (12, 90) and the sequential phosphorylation of LIM kinase (48) and cofilin (2, 95) and thus counteracts membrane protrusion stimulated by Cdc42 and Rac.

The regulated assembly and disassembly of adhesive structures is fundamental to cell motility (34, 58), and decreased

* Corresponding author. Mailing address: Department of Pharmacology and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112. Phone: (504) 568-4740. Fax: (504) 568-2361. E-mail: acatli@lsuhsc.edu.

† Current address: Department of Cell Biology, Max Delbrück Center, Robert-Roessle-Str. 10, Berlin 13092, Germany.

stability of adhesion structures contributes to the enhanced motility and invasiveness of tumor cells (66, 87). Considerable evidence indicates that the integration of Rho family GTPase and ERK signaling is important in the control of cell morphogenesis (27, 36, 38, 56, 66, 75, 87). ERK is also implicated in the coordinate control of Rho and Rac activities in fibroblast models of transformation (66) and human tumor cells (87).

Rho family GTPases are acutely regulated by adhesion to extracellular matrix proteins. Adhesion to fibronectin transiently decreases Rho-GTP levels (62), while Rac and Cdc42 activities are coincidently elevated (61). Although Rac, Cdc42 (41, 76), and their effector PAK1 (49, 98) have been shown to antagonize Rho function, the mechanism(s) by which Rho is transiently inactivated during adhesion and spreading is not clear. A role for ERK in inhibiting Rho function is suggested by the observation that the inhibition of MEK increases stress fiber formation in Swiss 3T3 cells (66), and Webb et al. (91) have recently provided direct evidence that MEK is necessary for the disassembly of focal adhesions. Focal adhesion disassembly may in part be controlled by ERK phosphorylation and activation of M-calpain (7, 27–29) and subsequent proteolysis of Rho and proteins resident in adhesion structures (8, 15, 23, 42).

We recently reported that adhesion controls the assembly and activation of MEK1 signaling complexes through PAK1 phosphorylation of MEK1 in peripheral membrane structures (16, 73). Interestingly, in spite of the conservation of the corresponding serine residue, MEK2 appears not to be phosphorylated by PAK1 (16, 24, 73), indicating that additional factors control the specificity of adhesion signaling to ERK. Circumstantial evidence supports a role for MP1 in the assembly and activation of MEK1-containing signaling complexes in the context of cellular adhesion and morphogenesis. First, MP1 binds selectively to MEK1 but not MEK2 (67). Second, MP1 appears to bind MEK1 through a proline-rich sequence (PRS) that contains serine 298, the site of MEK1 phosphorylation by PAK1 (10, 67). Third, both MP1 (67) and PAK1 phosphorylation of MEK1 (16) stimulate MEK1-ERK association.

Here we report that MP1 associates with PAK1 to selectively couple the activation of MEK1-ERK to upstream adhesion signals. Furthermore, we provide evidence that MP1 is required for the transient suppression of Rho and ROCK functions necessary for focal adhesion turnover and cell spreading, revealing signal-channeling functions for a MEK1/ERK scaffolding complex in the reciprocal regulation of Rac/Cdc42 and Rho and the orchestration of cytoskeletal rearrangements important for cell motility. Our finding that ERK activation in response to platelet-derived growth factor (PDGF) is independent of MP1 reveals a scaffolding mechanism by which signal processing in the mammalian ERK pathway is insulated to allow for parallel and independent regulation of multiple ERK-dependent responses.

MATERIALS AND METHODS

Cells and transfections. REF52 fibroblasts were cultured as described previously (73) and transfected with small interfering RNAs (siRNAs; final concentration, 120 nM) using either calcium phosphate or TransIt TKO reagent (Mirus). COS1, COS7, and CCL39 cells were transfected as described previously (10, 16). ROCK inhibitors Y27632 (5 to 10 μM; Calbiochem) or H1152 (114 nM; Calbiochem) were added to suspended cells 30 min prior to replating or to adherent cells 30 min prior to lysis. UO126 (25 μM; Calbiochem) or dimethyl sulfoxide vehicle were included as indicated in figure legends.

Plasmids and RNAs. The following siRNAs (Dharmacon) were used: nonspecific control VII, ACU CUA UCG CCA GCG UGA CUU; MP1 no. 1, UCG UUU GCC UCU GGU GGU GUU; MP1 no. 2, GCA UCA UCU GCU ACU AUA AUU; and p14, GAC AGU CUC AAA UUU AUC CUU. Maximal depletion of MP1 was seen at 72 to 96 h posttransfection; experiments were performed between these time points. Hemagglutinin (HA)-tagged MEK, glutathione *S*-transferase (GST)-His_6 double-tagged MEK1, and FLAG-tagged MP1 constructs were described previously (9, 10, 67). Additional MEK mutants were made using a Transformer site-directed mutagenesis kit (Clontech). The following plasmids were generous gifts: GST-PAK1 232-544 (24) (M. Cobb), Myc-p14 (94) (L. Huber), V5-PAK1 constructs (1) (S. Alahari), and GST-Rhotekin Rho binding domain (62) (R. Worthylake).

Cell spreading assays. Cells transfected with control, MP1, or p14 siRNA were suspended and replated on fibronectin as described previously (73). Cells were fixed (4% paraformaldehyde, 4% sucrose in phosphate-buffered saline [PBS]) and adhesion and spreading quantitated in at least four random fields (690 by 510 μm). Data shown are means and standard deviations from one experiment representative of two to four trials.

Yeast two-hybrid and protein-protein interaction analyses. Two-hybrid assays were performed according to the manufacturer's instructions (Proquest, Gibco-BRL). MEK1 mutants were fused with the GAL4 DNA binding domain; MP1 and Raf-1 were fused to the GAL4 transactivation domain (67). For pull-down assays, ~2 μg of GST, GST-PAK1 232-544, or GST MEK1 proteins was incubated with 6 μg recombinant MP1/p14 complex in 0.5 ml binding buffer (50 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$, 2 mM EGTA, 0.05% Tween 20) on ice for 2 h. GST fusion proteins were collected on glutathione Sepharose (Amersham) for 30 min and washed three times with binding buffer. Bound proteins were eluted by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. GST-His_6-MEK1 proteins were purified from *Escherichia coli* BL21 by sequential glutathione Sepharose and nickel-chelate chromatography as described previously (9). GST-PAK1 232-544 (24) was similarly purified on glutathione Sepharose except that host *E. coli* JM109 cells were induced for 4 h at 30°C. Purified MP1/p14 complex (45) was a generous gift from M. Cygler and M. Sacher.

Western blotting and immunoprecipitation. Cells were lysed as described previously (67) except that lysis buffer was supplemented with 1 μM microcystin. For detection of phospho-cofilin, cells were lysed in Rho activity buffer (62) (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotinin). FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 agarose (Sigma). HA-tagged proteins were immunoprecipitated and blotted with anti-HA antibody 12CA5 (Berkley Antibody Company). Additional antibodies were obtained from the indicated sources: RhoA 26C4, Myc 9E10, and Raf1 C12 from Santa Cruz; FAK, ROCKII, and anti-phosphotyrosine 4G10 from Upstate Biotechnology; MEK1, ROCKI, and ROCKII from BD Transduction Laboratories; anti-pY397 FAK from Biosource; anti-pS3 cofilin from Cell Signaling Technology; anti-V5 from Invitrogen; anti-pS218/222 MEK1 from Sigma; and anticofilin from Cytoskeleton. ERK2 (B3B9), phospho-ERK (97), and MP1 (67) antisera were kind gifts from M. Weber. Anti-pS298 MEK1 (73) and anti-p14 (94) antisera were kind gifts from M. Marshall and L. Huber, respectively.

Measurement of Rho and Rho kinase activity. Rho-GTP levels were measured essentially as described previously (62). Rho kinase activity was measured using recombinant myosin phosphatase targeting subunit 1 (MYPT1; Upstate Biotechnology) as substrate (51). REF52 cells were lysed in ROCK lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM $Na_4P_2O_7$, 2 mM $CaCl_2$, 0.1 mM sodium orthovanadate, 1% Triton X-100, 0.5% Igepal, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μg/ml of leupeptin and aprotinin), and clarified extract was immunoprecipitated with ROCKII antibody (Upstate Biotechnology) overnight at 4°C. Immunoprecipitates were washed four times with ice-cold wash buffer (20 mM HEPES-KOH, pH 7.4, 10 mM $MgCl_2$), suspended in 50 μl of wash buffer supplemented with 5 mM $MnCl_2$, 10 μM ATP, 0.02% Brij 35, 0.5 μg of MYPT1, and 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham) with or without prior incubation with 20 μM Y27632 (30 min), and incubated for 30 min at 30°C.

Immunofluorescence. Cells were rinsed twice in PBS and fixed in 3.7% paraformaldehyde, 4% sucrose, 0.5% Triton X-100 for 30 min at room temperature (RT). Fixed cells were washed in PBS and blocked with 1% bovine serum albumin in PBS. Vinculin was detected with antivinculin antibody (1:400, clone hVIN-1; Sigma) for 2 h at RT or overnight at 4°C and with Cy3-conjugated goat anti-mouse secondary antibody (1:200; Jackson ImmunoResearch Laboratories)

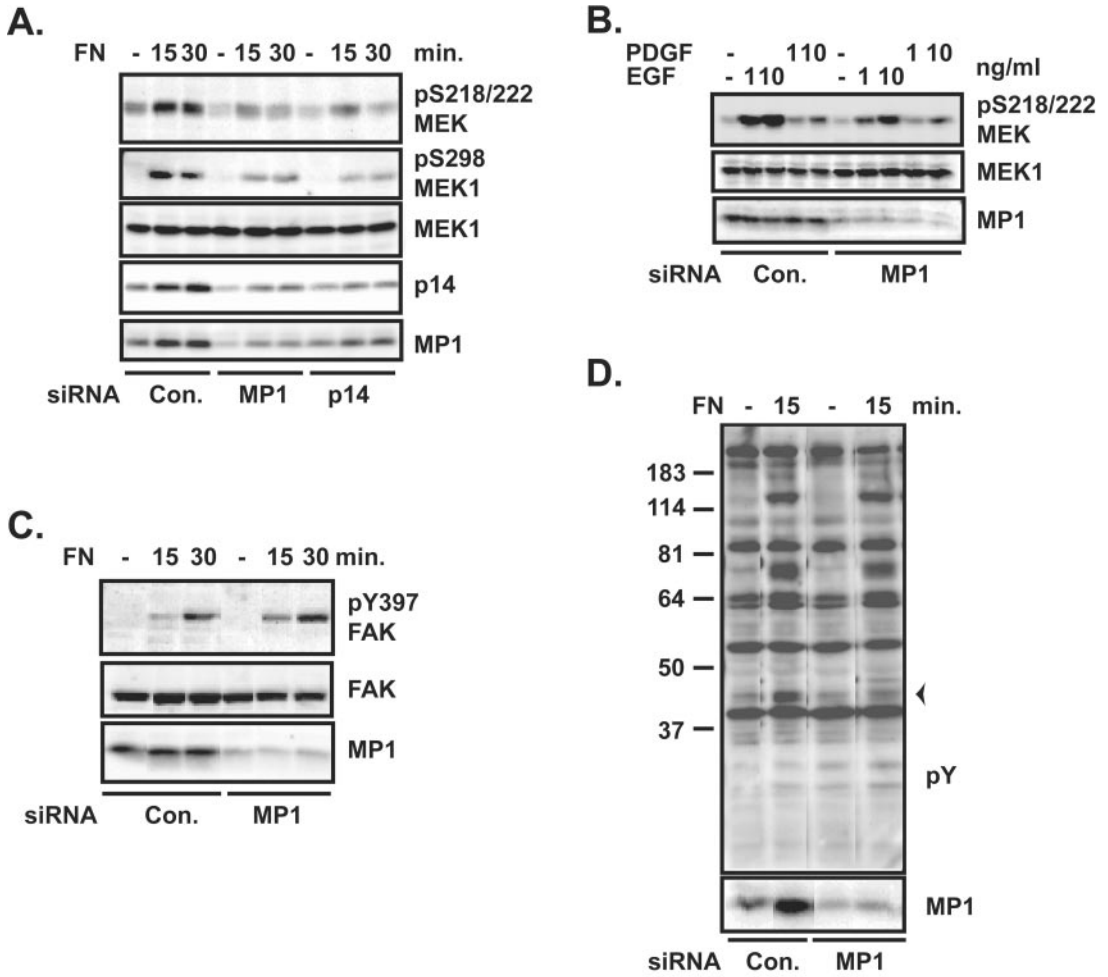


FIG. 1. MP1/p14 regulates PAK1-dependent MEK1 phosphorylation and activation. (A) Depletion of MP1/p14 inhibits fibronectin-stimulated PAK1 phosphorylation (pS298 MEK1) and activation of MEK1 (pS218/222 MEK1). (B) Selective inhibition of MEK activation by depletion of MP1/p14. REF52 cells transfected with MP1 siRNA or control RNA were stimulated with EGF or PDGF, and MEK activation was assessed as described for panel A. (C) FAK autophosphorylation proceeds normally in cells depleted of MP1. Identical results were seen in cells transfected with either an independent MP1 siRNA or p14 siRNA (data not shown). (D) Fibronectin-stimulated tyrosine phosphorylation events are mostly independent of MP1/p14. Lysates prepared from control or MP1 siRNA-transfected cells were blotted with anti-phosphotyrosine antisera. The arrowhead indicates tyrosine-phosphorylated ERK (see the text).

for 1 h at RT. Cells were rinsed three times with PBS, stained for F-actin and DNA with Oregon Green 488-phalloidin (1:25; Molecular Probes) and DAPI (4′,6′-diamidino-2-phenylindole) (0.03 μg/ml; Sigma), respectively, rinsed with PBS–20% glycerol, and mounted in ProLong Anti-Fade (Molecular Probes) or VectaShield (Vector Laboratories). All staining solutions were prepared in PBS containing 0.05% NP-40. Images were obtained using a Leica DM RA2 fluorescence microscope fitted with appropriate filters and controlled with SlideBook software (Intelligent Imaging Innovations). Fluorescent images are single Z planes close to the basal surface of the cell.

RESULTS

MP1 and p14 regulate PAK1-dependent MEK activation. MP1 binds MEK1 but not MEK2, promotes MEK1-ERK complex formation, and sensitizes MEK1 to activation by Raf in vitro (67). Similar functional consequences accompany PAK1 phosphorylation of MEK1 (16, 24, 73), suggesting MP1 as a candidate regulator of PAK1-dependent activation of MEK1 in response to adhesion. To test this hypothesis, we used RNA interference to reduce MP1 expression in REF52 cells. The depletion of MP1 attenuated S298 phosphorylation of MEK1 (pS298 MEK1), MEK activation (pS218/222 MEK) (Fig. 1A), and ERK activation (Fig. 1D; see also Fig. 7C) in response to fibronectin stimulation. Identical results were obtained with a second MP1 siRNA (data not shown), affirming the specificity of this response.

MP1 tightly associates with p14, a protein localized to late endosomal and lysosomal membranes, and functions in part as an MP1/p14 complex (45, 80, 94). The depletion of p14 also inhibited MEK1 S298 phosphorylation and activation of MEK1 during adhesion to fibronectin (Fig. 1A). As previously reported (80), knockdown of MP1 reduces the expression of p14 (Fig. 1A). Similarly, knockdown of p14 results in a corresponding loss of MP1 in REF52 cells, suggesting that the MP1/p14 complex is more stable than either individual subunit (80). The expression of FAK, PAK1, MEK1, ERK, ROCK, and Rho

family GTPases was not affected by the depletion of MP1 and p14 (see below; data not shown).

These data might suggest that MP1/p14 is required for PAK1 phosphorylation of MEK1, which in turn facilitates MEK1 activation during adhesion (73). An alternative interpretation is that MP1/p14 is required for MEK1 activation and subsequent autophosphorylation on serine 298 (64). However, S298 phosphorylation during adhesion to fibronectin is not inhibited in kinase-defective MEK1 (data not shown) or by the MEK inhibitor UO126 (17), ruling out autophosphorylation as a major contributor to MEK1 S298 phosphorylation. Our data therefore indicate that MP1 and/or p14 are necessary for PAK1-dependent activation of MEK1 during acute adhesion to fibronectin.

Scaffolding proteins in yeast couple multifunctional MAP kinase modules to specific upstream stimuli in a context-dependent manner (reviewed in references 18 and 68). To begin to investigate whether MP1 and p14 channel select upstream stimuli to MEK1 and ERK, we determined whether MP1/p14 was also required for MEK1 activation in response to epidermal growth factor (EGF) or PDGF stimulation. While knockdown of MP1/p14 inhibited EGF-stimulated activation of MEK1 and ERK, consistent with published observations (80), depletion was without effect on PDGF stimulation (Fig. 1B). Since Rac potentiates EGF-stimulated ERK activation (44) and PAK1 phosphorylation of MEK1 S298 is necessary for full activation of MEK1 by combined fibronectin and EGF stimulation (73), these data are consistent with MP1/p14 regulating PAK1-dependent activation of MEK1 in response to adhesion signals alone or in response to soluble agonists that cooperate with adhesion signals to induce full MEK1 activation.

MP1 and p14 function downstream or parallel to adhesion-stimulated tyrosine kinase activation. To identify the signaling node requiring MP1, we next asked if MP1 was necessary for activation of upstream regulators of MEK1 S298 phosphorylation. The activation of PAK1 and phosphorylation of MEK1 at S298 during adhesion to fibronectin require the activity of FAK and Src family tyrosine kinases (73). FAK autophosphorylation proceeded normally or was somewhat enhanced in cells transfected with either MP1 or p14 siRNAs (Fig. 1C and data not shown) under conditions where MEK1 S298 phosphorylation was inhibited (Fig. 1A and data not shown). Furthermore, the spectra of tyrosine-phosphorylated proteins following adhesion to fibronectin was largely indistinguishable in control and MP1 siRNA-transfected cells (Fig. 1D); a notable exception was the tyrosine phosphorylation of a ~40-kDa protein, likely ERK1/2, which was attenuated by the depletion of MP1 (Fig. 1D; see also Fig. 7C). These data demonstrate that MP1/p14 functions downstream or parallel to FAK and early adhesion-stimulated tyrosine phosphorylation events in PAK1 phosphorylation of MEK1.

MP1 binds PAK1. Since MP1 specifically associates with MEK1 but not MEK2 (67), and serine 298 phosphorylation appears to be a MEK1-specific event in fibroblasts (24, 73), we reasoned that MP1/p14 might physically interact with PAK1 to integrate adhesion signals into the ERK pathway. Indeed, full-length PAK1 and the PAK1 C terminus (PAK1 CT) were recovered in MP1 immunoprecipitates following cotransfection of MP1, p14, and PAK1 expression constructs, whereas the PAK1 N terminus was unable to bind to MP1/p14 (Fig. 2A). Since the PAK1 catalytic domain (PAK1 CT) coimmunoprecipitates more efficiently with MP1/p14 than full-length PAK1 (Fig. 2A), this might indicate that MP1/p14 associates preferentially with either catalytically active or "open" conformers of PAK1. Consistent with the former possibility, mutationally activated (T423E) PAK1 and kinase-defective (K299R) PAK1 coimmunoprecipitated with MP1/p14 more and less efficiently, respectively, than wild-type PAK1 (Fig. 2B). Furthermore, PAK1 L107F, which is "open" and catalytically active by virtue of a mutation in the N-terminal autoregulatory domain (31), coimmunoprecipitated with MP1/p14, whereas "open" but kinase-defective PAK1 (PAK1 L107F K299R) was unable to bind (Fig. 2B).

To investigate whether MP1/p14 and PAK1 can associate directly, we performed pull-down assays (Fig. 2C). Recombinant PAK1 CT was able to associate with the recombinant MP1/p14 complex, indicating that the MP1/p14-PAK1 interaction seen in coimmunoprecipitation assays (Fig. 2A and B) is likely direct.

Together, these observations indicate that MP1/p14 and PAK1 associate through the PAK1 C terminus in cells, MP1/p14 binds more efficiently to catalytically competent PAK1, and the MP1/p14-PAK1 interaction is likely direct. Taken together with our MP1 and p14 knockdown experiments, these observations are consistent with the hypothesis that MP1/p14 regulates MEK1 S298 phosphorylation and activation through physical interactions with active PAK1.

The MEK1-MP1 interaction may be dispensable for PAK1 phosphorylation of MEK1. Both the MEK1-MP1 and MEK1-Raf interactions require the MEK1 PRS (10, 67). MP1 binding correlates with the MEK1 PRS in MEK1-MEK2 chimeras (H.-J. Schaeffer, A. D. Catling, and M. J. Weber, unpublished data), indicating that the MEK1 PRS is likely a site of interaction between MP1 and MEK1. By aligning the MEK1 PRS with the proline-rich sequences from MEK2 and *Xenopus* MEK (both unable to bind MP1 [67; Schaeffer et al., unpublished data]), we identified nine MEK1 PRS residues potentially important for MP1 binding (Fig. 3A). Individual MEK1 residues were mutated to the corresponding MEK2 residue and mutant proteins tested for their ability to bind MP1 or c-Raf in coimmunoprecipitation and yeast two-hybrid assays. While these substitutions within the MEK1 PRS did not compromise c-Raf binding (Fig. 3B and D), replacement of leucine 274 with serine severely compromised MEK1 binding to MP1 in both assays (Fig. 3B and C). Similar results were seen in the glutamine 278-to-proline mutant, although in this case the disruption of the MEK-MP1 interaction was more apparent in immunoprecipitation assays (Fig. 3B and C); this may indicate that additional binding partners compete with MP1 for MEK1 in mammalian cells. MEK2 and MEK1 ΔPRS, which are unable to bind MP1 (67) and c-Raf (10), respectively, were used as negative controls in the coimmunoprecipitation experiments. Pull-down assays using recombinant MP1/p14 and MEK1 proteins confirmed that wild-type MEK1 is able to bind directly to MP1/p14, whereas MEK1 L274S is compromised for binding (Fig. 2C). These data indicate that L274S and Q278P mutations selectively inhibit MP1 binding and might therefore be useful for dissecting the signaling role of the MEK1-MP1 interaction.

To test the hypothesis that MP1/p14 recruits MEK1 to

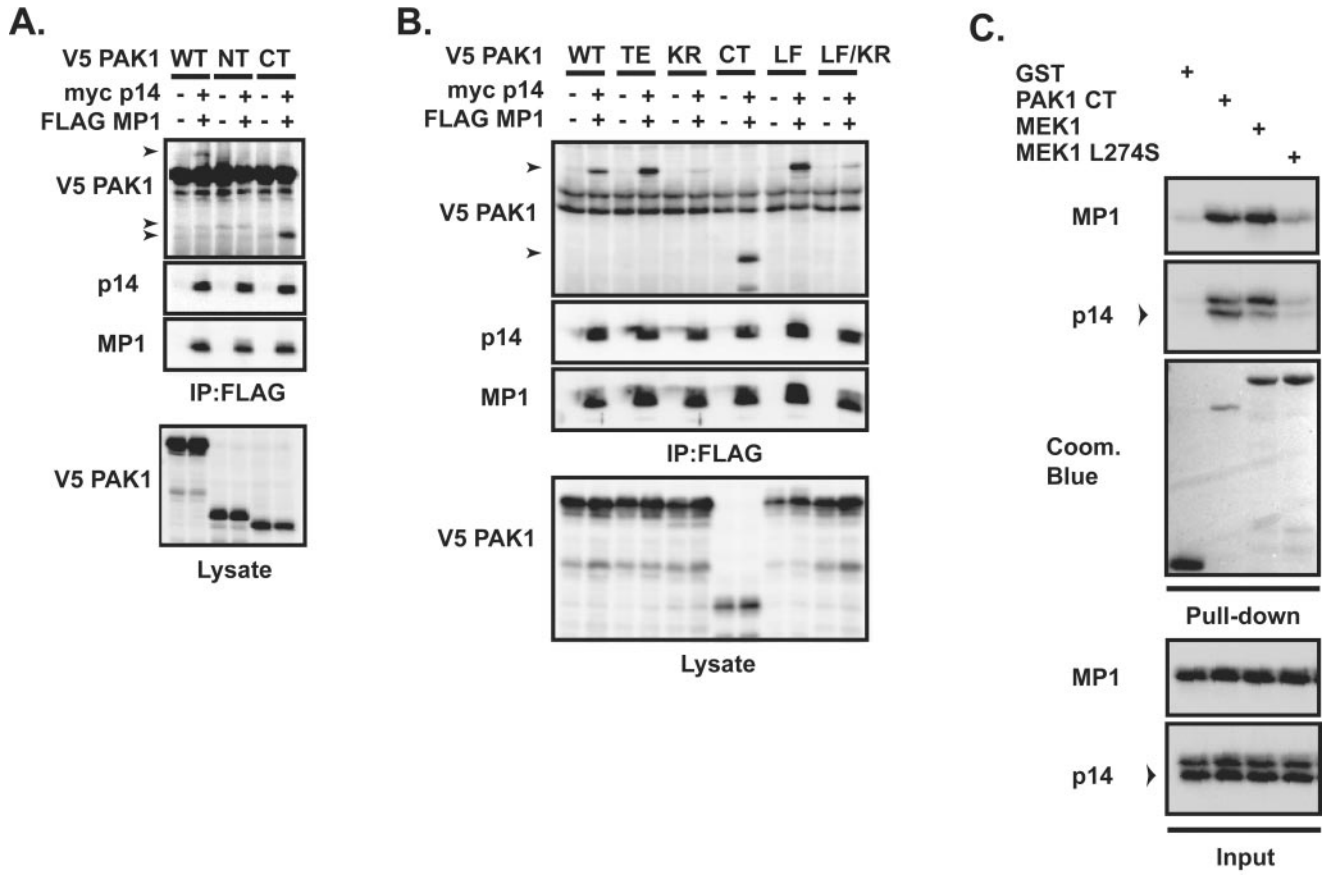
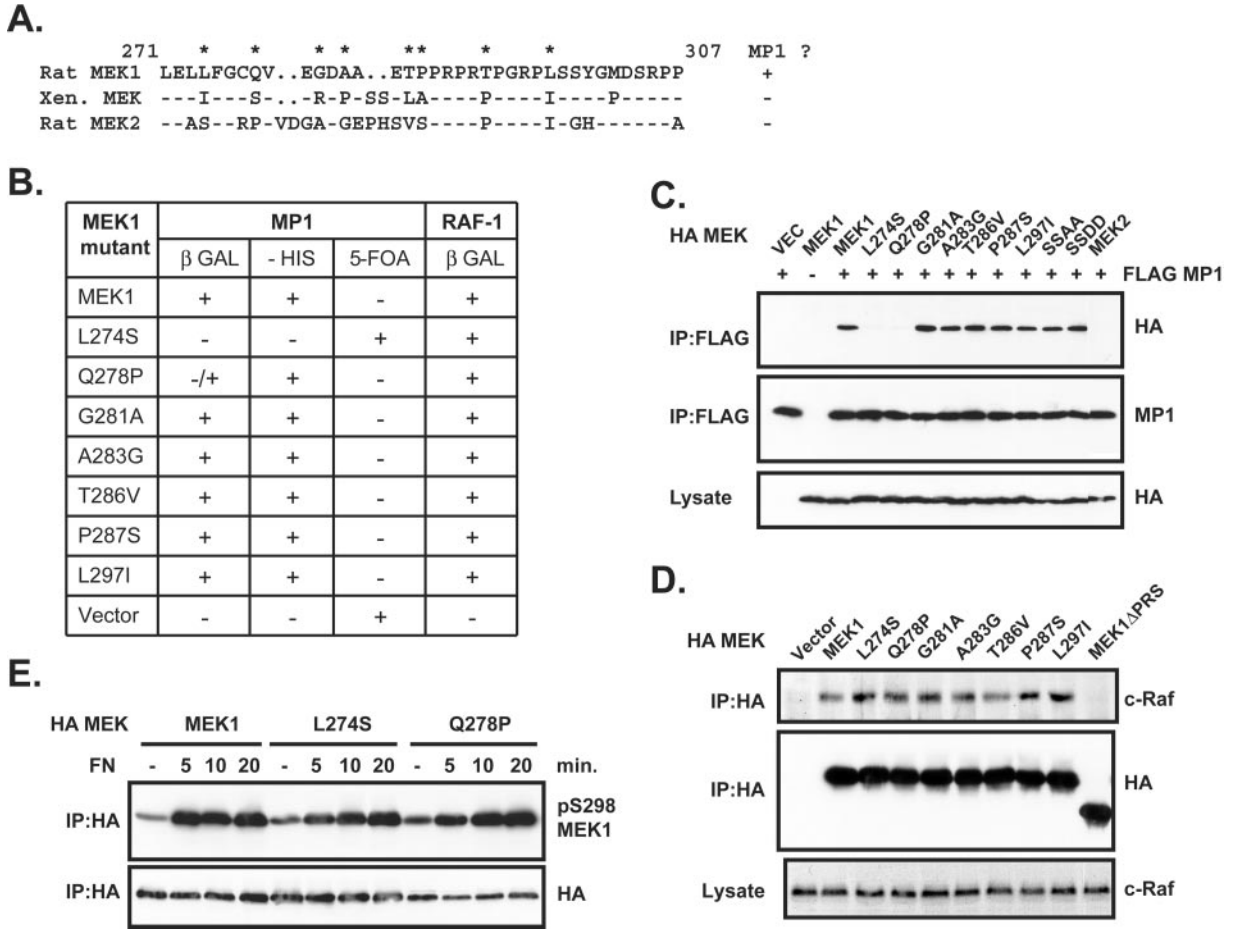


FIG. 2. MP1 associates with PAK1. (A) MP1 associates with the PAK1 C terminus. COS cells were transiently transfected with V5-tagged PAK1 constructs (WT, wild type; NT, amino acids 1 to 248; CT, amino acids 248 to 545) with or without FLAG-tagged MP1 and Myc-tagged p14. FLAG immunoprecipitates were formed and blotted with V5, p14, or MP1 antisera (top three panels). Expression of V5 PAK1 constructs was confirmed by blotting lysates with V5 antisera (bottom panel). The intense band present in all immunoprecipitates is immunoglobulin G heavy chain. (B) MP1 preferentially associates with activated PAK1. COS cells were transiently transfected with the V5-tagged PAK1 constructs indicated with or without FLAG-tagged MP1 and Myc-p14. FLAG immunoprecipitates were formed and blotted with V5, p14, or MP1 antisera (top three panels). The expression of V5 PAK1 constructs was confirmed by blotting lysates with V5 antisera (bottom panel). TE, T423E; KR, K299R; LF, L107F; LF/KR, L107F K399R. (C) The PAK1 C terminus (residues 232 to 544) binds directly to MP1/p14. GST fusion proteins were incubated with recombinant MP1/p14 complex before capture with glutathione Sepharose. MP1/p14 pulled down by the fusion proteins was assessed by blotting (top panels) and the presence of fusion protein verified by Coomassie blue staining (bottom panel). Blotting of input (1%) established that MP1/p14 was present in each reaction. The upper band seen in the p14 blots is residual MP1 signal remaining after stripping.

PAK1 in cells, we asked whether mutants of MEK1 unable to associate with MP1 were phosphorylated by PAK1 during adhesion to fibronectin. As expected, wild-type MEK1 was rapidly and robustly phosphorylated on serine 298 in response to fibronectin stimulation, with maximal phosphorylation observed at 5 min (Fig. 3E). MEK1 L274S and MEK1 Q278P mutants were typically phosphorylated on serine 298 as efficiently and rapidly as wild-type MEK1, although in some trials (Fig. 3E) phosphorylation was somewhat delayed. Since our two-hybrid (Fig. 3B), coimmunoprecipitation (Fig. 3C), and in vitro reconstitution (Fig. 2C and data not shown) experiments indicate that the L274S and Q278P mutants are substantially compromised for binding to MP1, these data argue that the MEK1-MP1 interaction is not essential for PAK phosphorylation of MEK1. Our inability to demonstrate the existence of a ternary complex between MEK1, MP1, and PAK1 (data not shown) is consistent with this view. However, we cannot exclude the possibility that overexpression of MEK1 reduces the requirement for MP1-mediated scaffolding in PAK1 phosphorylation of MEK1 or that such scaffolding complexes are transient in nature or too unstable to be detected in our coimmunoprecipitation assays. These possibilities are considered further in the Discussion.

Our data indicate that MP1/p14 is required for PAK1 phosphorylation of endogenous MEK1 and, consistent with this observation, that MP1/p14 associates with active forms of PAK1. Together, these observations suggest that MP1/p14 may facilitate rapid MEK1 serine 298 phosphorylation in response to fibronectin stimulation. MEK1 phosphorylated on S298 is localized proximal to peripheral adhesion structures and is important for adhesion-stimulated MEK1-ERK complex assembly and activation (16, 73). These data reveal a novel role for the MP1/p14 scaffolding complex in PAK1-dependent assembly and activation of peripherally localized MEK1 signaling complexes during adhesion to fibronectin and suggest an



MEK1 mutant	MP1			RAF-1
	β GAL	- HIS	5-FOA	β GAL
MEK1	+	+	-	+
L274S	-	-	+	+
Q278P	-/+	+	-	+
G281A	+	+	-	+
A283G	+	+	-	+
T286V	+	+	-	+
P287S	+	+	-	+
L297I	+	+	-	+
Vector	-	-	+	-

FIG. 3. Mutations in the MEK1 proline-rich sequence selectively inhibit MEK1-MP1 binding. (A) Residues present in the MEK1 PRS but not conserved in MEK2 and *Xenopus* MEK proline-rich sequences (which do not bind MP1 [67; data not shown]) were mutated to the corresponding MEK2 residue. (B) Yeast two-hybrid assays identify MEK1 mutants selectively defective for binding MP1. Mutants were analyzed for their ability to bind MP1 and Raf-1 in yeast two-hybrid assays. Growth on media lacking histidine and induction of β-galactosidase activity indicates two-hybrid interaction; growth on media lacking uracil and containing 5-fluoroorotic acid (5-FDA) indicates no bait-prey interaction. The expression of all MEK1 mutants, MP1, and Raf-1 was verified by Western blotting (data not shown). (C) MEK1 L274S and MEK1 Q278P are unable to bind MP1. CCL39 cells were cotransfected with HA-tagged MEK mutants and FLAG-tagged MP1. FLAG immunoprecipitates were formed and blotted with HA antiserum (top panel) or MP1 antiserum (middle panel). HA blotting of lysates confirmed expression of all MEK constructs (bottom panel). (D) MEK1 mutants unable to bind MP1 are competent to bind Raf. CCL39 cells were transiently transfected with HA-tagged MEK1 constructs. HA immunoprecipitates were blotted for endogenous Raf-1 (top) or HA-MEK (middle). Lysates were blotted with Raf-1 antiserum (bottom). (E) MEK1 mutants unable to bind MP1 are phosphorylated by PAK1 during adhesion to fibronectin. REF52 cells were transiently transfected with HA-tagged MEK1 constructs, suspended in serum-free medium, and allowed to adhere to fibronectin-coated plates. HA immunoprecipitates were blotted with anti-phospho S298 MEK1 antiserum (top) or HA antiserum (bottom).

important role for this scaffolding complex in the context of cellular adhesion and morphogenesis.

MP1 is required for cell spreading. Strikingly, REF52 cells depleted of MP1/p14 were severely compromised for spreading on fibronectin (Fig. 4) or vitronectin (data not shown): while ~50% of attached control transfected cells had formed lamellipodia and spread at 10 min following replating on fibronectin (Fig. 4A and Fig. 4B, arrows), only ~10% of cells transfected with MP1 or p14 siRNAs had spread at this time point. Indeed, the majority of cells depleted of MP1/p14 remained round and refractile and frequently expressed plasma membrane blebs (Fig. 4B, arrowheads). At 30 min after replating, ~90% of control transfected cells had fully spread, whereas ~50% of cells transfected with either MP1 or p14 siRNAs remained refractile with prominent membrane blebs (Fig. 4A and data not shown).

ERK signaling is implicated in "inside-out" suppression of integrin affinity (32). However, REF52 cells transfected with control, MP1, or p14 siRNAs were equally able to both adhere to fibronectin (Fig. 4A) and stimulate proximal tyrosine phosphorylation events (Fig. 1C and D), suggesting that the affinity and signaling activity of the fibronectin receptor(s) are not substantially altered by depletion of MP1/p14. Since pharmacological inhibition of MEK also inhibits REF52 cell spreading (20), our data suggest that MP1/p14 controls a population of MEK1 and ERK whose activation is acutely regulated by PAK1 and is required for cell spreading on fibronectin.

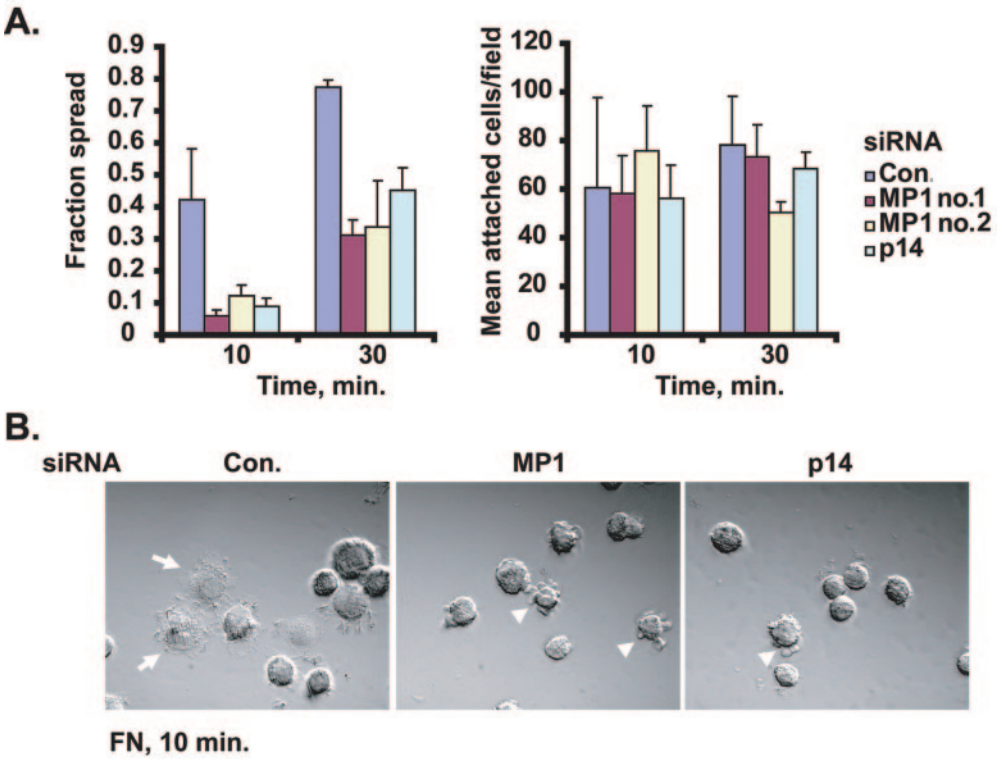


FIG. 4. MP1/p14 regulates cell spreading on fibronectin. (A) REF52 cells transiently transfected with control, MP1, or p14 siRNA were replated on fibronectin-coated petri dishes as described previously (73). Cultures were fixed at the indicated time points and random fields scored for spread cells (left panel) and adherent cells (right panel). Data are representative of three experiments. (B) Typical morphology of cells transfected with siRNA following replating on fibronectin for 10 min. Control transfected cells spread rapidly (arrows), whereas MP1 or p14 siRNA-transfected cells remain round and refractile with multiple membrane blebs (arrowheads). Immunoblots confirmed depletion of MP1 and p14 (not shown).

MP1 and MEK1 regulate focal adhesions. The depletion of MP1/p14 inhibits the spreading of acutely adherent cells on fibronectin, resulting in a round morphology with plasma membrane blebbing (Fig. 4B) similar to that seen in cells expressing active Rho or ROCK (13, 52, 71). We therefore asked if Rho-GTP loading was altered in cells depleted of MP1/p14. While the depletion of MP1/p14 decreased the activation state of ERK1, it did not significantly alter Rho-GTP levels (Fig. 5A) in continuously adherent cells. However, this biochemical assay does not measure the cumulative effects of small changes in Rho GTP loading associated with transient membrane protrusion and retraction or the levels of Rho-GTP at specific locations within the cell (53). We therefore asked whether cells depleted of MP1/p14 exhibited changes in focal adhesion distribution characteristic of a cumulative increase in Rho signaling (12). Notably, focal adhesions in control transfected cells were primarily located at the cell periphery (Fig. 5B, arrows), whereas knockdown of MP1/p14 led to the accumulation of abundant, elongate focal adhesions under the cell body (Fig. 5B, arrowheads). Treatment of continuously adherent REF cells with UO126 also caused the accumulation of abundant, elongated focal adhesions (Fig. 5C, arrowheads). Together these data indicate that MP1/p14 and MEK activity are required to maintain the discrete morphology and peripheral localization of Rho-dependent focal adhesions in continuously adherent fibroblasts, a function previously suggested for ERK (8, 20, 27, 66, 87).

MP1 and MEK1 regulate ROCK. ROCK stimulates focal adhesion and actin stress fiber formation (90), prompting us to test whether elevated ROCK activity causes the altered size and distribution of focal adhesions in cells treated with UO126 or transfected with MP1 siRNA. ROCKII kinase activity was measured in cells treated with UO126 or transfected with MP1 siRNA using a physiological substrate for ROCK (MYPT1) (37) as the in vitro protein substrate (Fig. 5D). ROCKII activity was consistently elevated in MP1 siRNA-transfected cells compared to controls (compare lanes 1 and 2 in Fig. 5D). Importantly, stimulation of ROCKII activity was also seen in control cells treated with the MEK inhibitor UO126 (Fig. 5D, lane 5). Thus, the inhibition of MEK and ERK by either pharmacological means or by depletion of MP1/p14 stimulates ROCKII activity, consistent with the observed changes in focal adhesion morphology (Fig. 5B and C). Interestingly, combined depletion of MP1/p14 and treatment with UO126 further activates Rho kinase activity (Fig. 5D, lane 6). Possible explanations for this observation are outlined in the Discussion.

We next investigated whether phosphorylation of an endogenous ROCK effector was stimulated in cells lacking MP1. ROCK phosphorylates and activates LIM kinase (48), which in turn phosphorylates cofilin and inhibits its F-actin severing

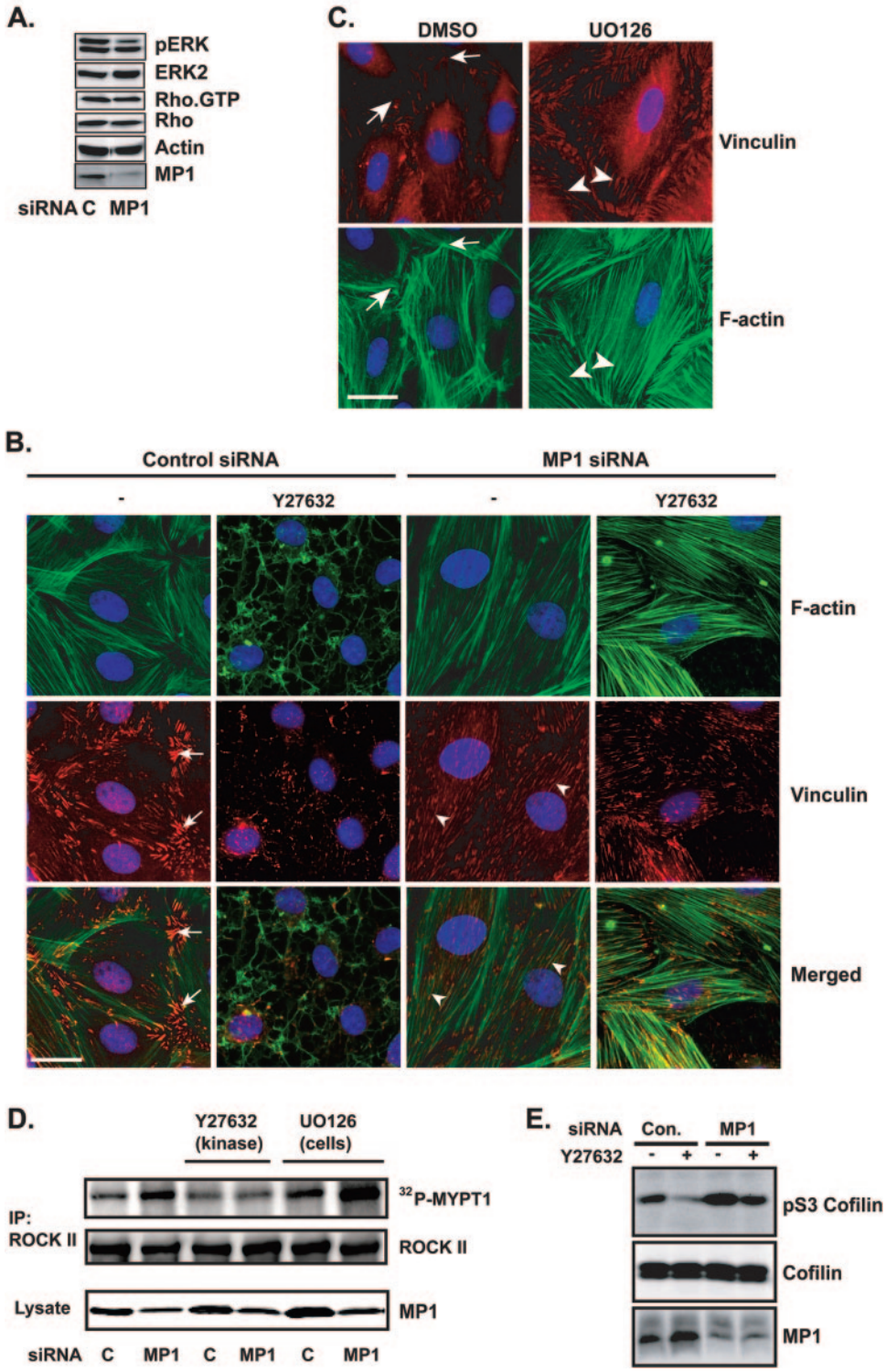


FIG. 5. MP1 and MEK regulate Rho-ROCK signaling in adherent cells. (A) Depletion of MP1 and p14 has no effect on Rho-GTP levels in continuously adherent cells. Rho-GTP loading was measured as described previously (62). (B) Depletion of MP1 and p14 stimulates accumulation of Y27632-resistant focal adhesions and stress fibers in continuously adherent cells. Vinculin and F-actin were detected with antivinculin antisera and phalloidin, respectively. Focal adhesions in control RNA-transfected cells are primarily localized at the cell periphery (arrows); in contrast, abundant adhesions form tracks under the cell body in cells depleted of MP1 (arrowheads). Pretreatment with Y27632 (10 μM, 30 min) disrupted stress fibers and adhesions in control transfected cells but not in cells depleted of MP1. (C) Pharmacological inhibition of MEK signaling leads to accumulation of abundant, elongated focal adhesions under the cell body. REF52 cells were treated with or without UO126, fixed, and stained for vinculin and F-actin. Control cells exhibit mostly peripheral focal adhesions (arrows); in contrast, abundant, elongated adhesions form under the cell body in the presence of UO126 (arrowheads). Scale bars, 25 μm. (D) Depletion of MP1/p14 or inhibition of MEK (25 μM UO126; 30 min) stimulates Rho kinase activity. ROCKII was immunoprecipitated and assayed as described in Materials and Methods. Incubation of immunoprecipitates with Y27632 confirmed substrate phosphorylation by Rho kinase. (E) Depletion of MP1/p14 stimulates phosphorylation of cofilin. Continuously adherent cells transfected with control or MP1 siRNA were incubated with or without Y27632 (10 μM; 30 min) prior to lysis. Phosphorylation of endogenous cofilin was assessed by Western blotting.

function (2, 95). Measurement of cofilin phosphorylation is thus a surrogate measure of ROCK kinase activity in intact cells. Cofilin phosphorylation was evident in control RNA-transfected cells (Fig. 5E) and, in good agreement with the immune-complex ROCK assays, was further stimulated by depletion of MP1/p14 (Fig. 5E). Interestingly, while the ROCK inhibitor Y27632 largely eliminated cofilin phosphorylation in control cells, substantial phosphorylation was maintained in the presence of Y27632 in MP1 siRNA-transfected cells (Fig. 5E). In striking agreement with these biochemical data, focal adhesions and stress fibers were lost in control cells treated with Y27632 but were maintained in MP1 siRNA-transfected cells treated with the drug (Fig. 5B).

A conservative interpretation of these data is that Y27632 treatment incompletely inhibits ROCK activity in MP1 siRNA-transfected cells (Fig. 5D), resulting in residual cofilin phosphorylation (Fig. 5E) and stabilized stress fibers and focal adhesions (Fig. 5B). However, we cannot exclude the more interesting possibility that both ROCK and a novel Y27632-resistant effector are activated by depletion of MP1 and stimulate cofilin phosphorylation and the stabilization of stress fibers and adhesions in continuously adherent cells. This possibility is considered in the Discussion.

Continuously adherent cells depleted of MP1/p14 cover a noticeably larger surface area than control cells (data not shown); the reason(s) for this is not yet clear, although recent reports indicate that inhibition of myosin light chain kinase, a putative ERK substrate (38), and expression of activated ROCKI can increase cell surface area (83, 84).

MP1 regulates Rho function during cell spreading. Since alterations in focal adhesions observed in continuously adherent cells may reflect long-term consequences of depleting MP1/p14 and the associated inhibition of ERK, we next investigated MP1/p14 regulation of Rho and ROCK function in cells acutely adhering to fibronectin. In control RNA-transfected cells, Rho-GTP levels were high in suspension but transiently decreased (Fig. 6A) during adhesion and spreading on fibronectin before recovering to levels similar to those seen in suspended cells. This time course of Rho-GTP loading is typical in fibroblasts adhering to fibronectin (63). In contrast, cells transfected with MP1 siRNA and incubated in suspension contained low levels of Rho-GTP that increased rapidly following adhesion to fibronectin (Fig. 6A). These data demonstrate that MP1/p14 is essential for maintaining Rho in the GTP-bound state in suspended cells and for the transient decrease in Rho-GTP seen during adhesion to fibronectin, parameters previously shown to be important for cell spreading (4, 63). The kinetics of MP1/p14-dependent MEK1 activation during adhesion to fibronectin (Fig. 1A) are consistent with MP1/p14, PAK1, and MEK1 playing a role in the acute control of Rho-GTP loading.

As a functional measure of Rho activation, we asked if Rho-dependent stress fiber and focal adhesion formation (12) were altered by the depletion of MP1/p14 during acute adhesion and spreading on fibronectin. In control RNA-transfected cells allowed to spread on fibronectin, polymerized actin was found in peripheral ruffles at 15 min and became organized into stress fibers that frequently crossed the cell body or localized to one primary lamellipodium at 30 and 60 min (Fig. 6B and data not shown). In contrast, we did not observe actin ruffles in cells depleted of MP1 at any time point, but rather polymerized actin was localized around the nucleus and in peripheral "blebs" at 15 min and became organized into pronounced circumferential stress fibers by 60 min (Fig. 6B). Focal adhesions identified by vinculin staining were discrete and well organized in control transfected cells (Fig. 6B and data not shown). In striking contrast, large patches of vinculin staining were observed in cells depleted of MP1/p14 (Fig. 6B) during acute adhesion to fibronectin. These data are strong evidence of a requirement for MP1/p14 for remodeling of actin and focal adhesion structures during cell spreading.

MP1 is required to suppress ROCK function during cell spreading. To further test the hypothesis that inappropriate activation of Rho and ROCK underlies this acute spreading defect, we analyzed phosphorylation of the ROCK effector cofilin in control and MP1 siRNA-transfected cells acutely adhering to fibronectin. Depletion of MP1 stimulated cofilin phosphorylation above that seen in control transfected cells (Fig. 6C), as seen in continuously adherent cells (Fig. 5E). These morphological and biochemical data suggested that elevated ROCK activity resulting from the depletion of MP1 might underlie the observed spreading defect. To directly test this hypothesis, we asked whether ROCK inhibitors could rescue spreading in cells depleted of MP1. Y27632 substantially inhibited fibronectin-stimulated cofilin phosphorylation (Fig. 6C) and rescued the spreading defect resulting from the depletion of MP1/p14 (Fig. 7A and B); cell spreading was also rescued with a structurally distinct Rho kinase inhibitor, H1152 (33) (Fig. 7A). Spreading rescued by inhibition of ROCK was accompanied by partial restoration of typical focal adhesions and stress fibers that crossed the cell body (Fig. 6B and data not shown). Together, these morphological and biochemical data indicate that ROCK activity is elevated in fibronectin-stimulated cells lacking MP1 and that inhibition of this activity partially restores adhesions and stress fibers and allows cell spreading. ROCKI and ROCKII protein levels are not altered by depletion of MP1 (Fig. 7A, inset), indicating that ROCK-specific activity is elevated in the absence of MP1.

MEK1 S298 phosphorylation and ERK activation were not restored in cells depleted of MP1/p14 when spreading was rescued with the ROCK inhibitor Y27632 (Fig. 7C). Together, these data place Rho-ROCK downstream of MP1/p14-regulated MEK1 phosphorylation and ERK activation and indicate that MP1/p14-regulated ERK suppresses Rho and ROCK function to allow focal adhesion remodeling and membrane protrusion (93) during spreading on fibronectin.

DISCUSSION

The mechanisms by which ERK signaling controls morphogenetic events are poorly understood. Here, we present evidence that MEK1 and ERK controlled by the scaffold proteins MP1 and p14 and the Rac/Cdc42 effector PAK1 acutely suppress Rho function to allow cell spreading. Our data suggest a mechanism for the reciprocal regulation of Rac/Cdc42 and Rho (41, 43) that accommodates the spatial and temporal control of the Rho family GTPases and effectors involved.

MP1 regulates PAK phosphorylation and activation of MEK1. We have previously delineated a FAK- and Src-dependent mechanism for PAK1 phosphorylation of MEK1 but not

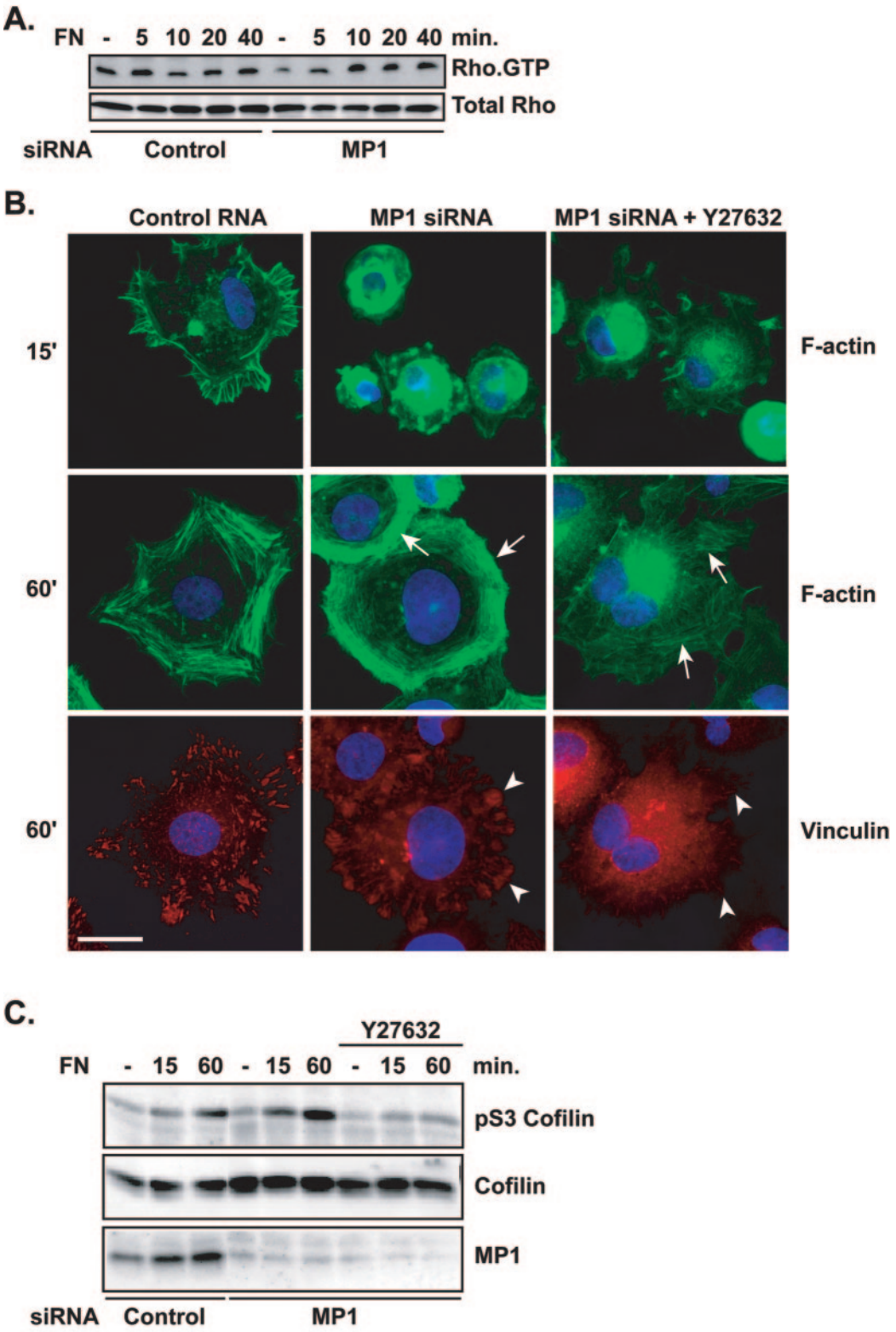


FIG. 6. MP1 regulates Rho-GTP loading and function during acute spreading on fibronectin. (A) MP1 controls Rho-GTP loading. REF52 cells transfected with control or MP1 siRNA were suspended and then allowed to adhere to fibronectin-coated plates for the indicated times. Rho-GTP loading was measured as described previously (62). (B) Rho-dependent focal adhesion and stress fiber formation are controlled by MP1. REF52 cells transfected and suspended as described for panel A were treated with or without the Rho kinase inhibitor Y27632 (10 μM) for 30 min prior to replating on fibronectin for the indicated times. Cells depleted of MP1/p14 exhibit dense circumferential stress fibers (arrows) and large vinculin-containing adhesions (arrowheads). Typical stress fiber (arrows) and vinculin staining (arrowheads) are partially restored by pretreatment with the Rho kinase inhibitor. Scale bar, 25 μm. (C) Depletion of MP1/p14 stimulates ROCK-dependent phosphorylation of cofilin in acutely adhering cells. Cells transfected with control or MP1 siRNA were incubated with or without Y27632 (10 μM; 30 min) prior to replating on fibronectin. Phosphorylation of endogenous cofilin was assessed by Western blotting.

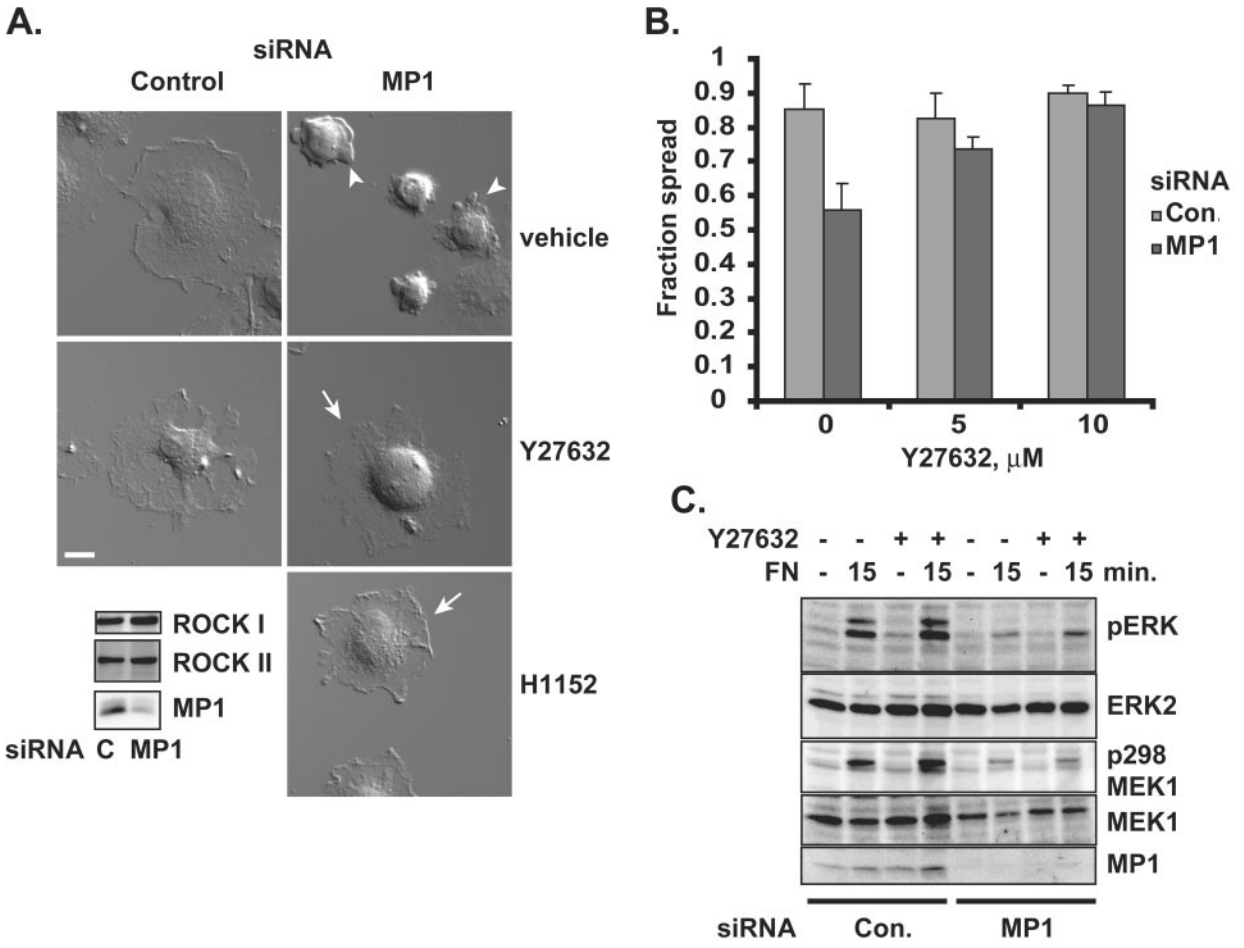


FIG. 7. MP1-regulated ERK activation suppresses ROCK function to allow cell spreading. (A) Inhibition of ROCK rescues the spreading defect of cells depleted of MP1/p14. Cells were allowed to attach and spread on fibronectin for 30 min with or without prior treatment with Y27632. A structurally distinct ROCK inhibitor, H1152, was used to confirm that elevated ROCK function underlies the spreading defect. Lysates prepared from REF cells transfected with control or MP1 siRNA were blotted with ROCKI and II antisera (inset); ROCK expression is unaffected by depletion of MP1/p14. (B) Inhibition of ROCK rescues the spreading defect in cells depleted of MP1. Cultures were fixed at 30 min and random fields scored for spread cells. Data are representative of two experiments. (C) Rho kinase is downstream of MP1/p14-regulated MEK1 phosphorylation and ERK activation. Lysates were blotted for MEK1 phosphorylation and ERK activation following replating on fibronectin (30 min) in the presence or absence of Y27632. Scale bar, 25 μm.

MEK2 in peripheral membrane structures during adhesion and spreading (73). We now show evidence that the MEK1-specific binding protein MP1 (67) and its partner p14 (94) are important for PAK1 phosphorylation and activation of MEK1 in response to fibronectin stimulation. Furthermore, we find that MP1 preferentially associates with active PAK1, consistent with the hypothesis that MP1/p14 facilitates MEK1 S298 phosphorylation through physical interactions with both kinases. However, a direct test of this model using MEK1 mutants (MEK1 L274S and MEK1 Q278P) unable to bind MP1 reveals that while MP1 is required for PAK1 phosphorylation of endogenous MEK1 during adhesion to fibronectin, the MP1-MEK1 interaction is dispensable when MEK1 is overexpressed. While further studies are required to elucidate the mechanism of action of MP1, it is plausible that the overexpression of MEK overcomes a requirement for the MP1-MEK1 interaction for MEK1 S298 phosphorylation in our assays. Indeed, we find that overexpressed MEK2 is appreciably phosphorylated on serine 298 in response to fibronectin stimulation (data not shown), in contrast to endogenous MEK2. Furthermore, MEK1 L274S and MEK1 Q278P mutants did exhibit delayed fibronectin-stimulated MEK1 S298 phosphorylation in some trials, consistent with the hypothesis that MP1/p14-mediated scaffolding is important for rapid PAK1 phosphorylation during adhesion. Interestingly, rapid MEK1 phosphorylation (5 to 15 min) in response to fibronectin stimulation requires FAK and Src activity, whereas phosphorylation at later time points (15 to 45 min) appears independent of these activities (73). It is plausible that the MP1-MEK1 interaction is required only for the early FAK- and Src-dependent phase of MEK1 S298 phosphorylation.

A requirement for simultaneous interactions between MP1, MEK1, and PAK1 for fibronectin-stimulated MEK1 S298 phosphorylation would predict the existence of a ternary complex between MEK1, MP1, and PAK1. We have been unable to detect a complex following transient transfection of MEK1,

MP1, and active PAK1 (data not shown). While we have no evidence that MP1/p14 simultaneously scaffolds MEK1 and PAK1, the small size of MP1 (14 kDa) makes it likely that additional components would be required to stabilize a scaffolding complex. Indeed, it has recently been suggested that the binding activities of an ERK scaffold are distributed among a number of molecules in mammalian cells (89). Additional studies are required to identify and characterize components that may function to scaffold MEK1 and PAK1.

Although the precise mechanism of action of MP1 is not understood, our data demonstrate that MP1 is required for phosphorylation of MEK1 by PAK1 and MEK1 and ERK activation during adhesion to fibronectin. PAK1 phosphorylation of MEK1 occurs in peripheral membrane structures (73) and stimulates localized MEK1-ERK complex formation and activation proximal to newly forming adhesion structures (16, 73), suggesting a mechanism for the focal complex and/or focal adhesion localization of active ERK in response to fibronectin stimulation (20, 73).

MP1 and MEK regulate Rho function. Our data show that MEK1-ERK regulated by MP1/p14 acutely suppresses Rho-dependent contractility and thereby allows the rapid remodeling of actin and adhesion structures necessary for membrane protrusion and cell spreading to occur. In support of this model, FAK-null cells exhibit reduced PAK phosphorylation of MEK1 (73) and activation of ERK during adhesion (69, 70, 73) and spreading defects resulting from Rho-mediated stabilization of circumferential stress fibers and focal adhesions (11, 63). Furthermore, v-Src causes dissolution of focal adhesions (19) and disruption of stress fibers reversible by acute inhibition of MEK (59, 60). Together, these data support the hypothesis that MP1/p14-controlled activation of MEK1-ERK downstream of FAK, Src, and PAK1 couples Rac/Cdc42 activation stimulated by local integrin engagement temporally to the down-regulation of Rho function(s) necessary for focal adhesion turnover and cell spreading. Furthermore, the localization of activated PAK1 (72), PAK1-phosphorylated MEK1 (73), and activated ERK to peripheral adhesion structures (20, 73) during cell spreading on fibronectin allows for spatial regulation of this process. This model may explain why MEK1-null fibroblasts are defective for migration on fibronectin despite continued expression of MEK2 (26): MEK2 is unable to bind MP1/p14 and integrate PAK1 signals in response to integrin ligation (24, 73) and hence may be uncoupled from the downstream turnover of focal adhesion structures.

Integration of ERK and Rho signaling. The balanced assembly and disassembly of adhesion structures and stress fibers are of critical importance for cell spreading and migration, and an abundance of stable focal adhesions and stress fibers is frequently associated with decreased motility (11, 19, 66, 87). Diverse data support a role for the integration of Rho family GTPase and ERK signaling in the control of cell motility (36, 38, 56, 73, 75). Importantly, ERK is implicated in the coordinate control of Rac and Rho activities in human tumor cells (87) and in fibroblast models of transformation (66). In these situations, ERK signaling does not acutely influence Rho function, but rather sustained ERK signaling selects for down-regulation of Rho or Rho effector function (66, 87). In colon carcinoma cells, the loss of Fra1 results in an up-regulation of Rho-GTP levels and ROCK activity as a result of activation of β_1 but not β_3 integrins (87). Since Fra1 expression is controlled by ERK (86), these studies demonstrate that ERK activity promotes the migration of colon carcinoma cells in part by Fra1-dependent suppression of Rho/ROCK activation. In contrast, Ras- and Raf-transformed fibroblasts exhibit increased motility in spite of high Rho-GTP levels (66). In these cases, sustained ERK activation uncouples ROCK from Rho and thereby inhibits stress fiber formation (66).

These mechanisms evolve during cellular transformation and likely function to increase the motile and invasive behavior of tumor cells (66, 87). The mechanism described here is distinct from those selected during cellular transformation in three ways. First, the depletion of MP1/p14 or the acute inhibition of MEK (20) attenuates REF52 cell spreading, indicating that ERK substrates, likely localized proximal to focal adhesions, are direct regulators of this process. Second, the depletion of MP1 and p14 does not detectably change Rho-GTP loading or Rho or ROCK expression in continuously adherent cells but instead alters Rho-GTP loading during acute adhesion to fibronectin. Third, the depletion of MP1 and p14 inhibits spreading on both fibronectin (a β_1-ligand) and vitronectin (β_3-ligand; data not shown), indicating that the suppression of Rho function by MP1 is not a β_1 integrin-specific mechanism in fibroblasts. MP1 and p14 thus control acute Rho-mediated remodeling of actin and adhesion structures required for rapid, spatially coordinated cellular responses to the extracellular matrix.

ERK regulates the assembly and turnover of focal adhesions (8, 20, 27, 66, 75, 87, 91), although the spectrum of substrates phosphorylated in this context is unknown. Our data suggest Rho GTPase-activating proteins (RhoGAPs) and Rho guanine nucleotide exchange fators (RhoGEFs0 or their upstream regulators localized proximal to peripheral membrane structures and adhesions as potential MP1/p14-dependent ERK substrates. Down-regulation of Rho by p190 RhoGAP is required for fibroblast spreading (3), and p190 RhoGAP is localized to sites of membrane protrusion and ruffling (6, 54). However, it is not known if ERK regulates p190 RhoGAP localization or activity. In contrast, the FAK-associated RhoGAP GRAF (30) is known to be phosphorylated by ERK (79), but whether ERK phosphorylation regulates the activity or localization of GRAF remains to be determined.

The biochemical and morphological data presented also strongly support the hypothesis that MP1/p14 and MEK1 control ROCK activity in acutely spreading cells. Specifically, cells lacking MP1 accumulate large and/or stabilized focal adhesions and dense circumferential stress fibers and are markedly defective for spreading on fibronectin. Increased ROCK activity is responsible for these defects in acutely spreading cells, since two highly specific and structurally distinct ROCK inhibitors (Y27632 and H1152) rescue cell spreading and partially restore typical focal adhesions and stress fibers in cells depleted of MP1.

The situation may be more complex in continuously adherent cells. Although ROCK activity is stimulated following treatment with UO126 or depletion of MP1, focal adhesions, stress fibers and phosphorylation of cofilin, an endogenous ROCK effector, are maintained even in the presence of Y27632 in MP1 siRNA-transfected cells. The most conservative explanation for this observation is that residual ROCK

activity remaining after Y27632 treatment is sufficient to stimulate cofilin phosphorylation and maintain adhesions and stress fibers. However, we cannot exclude the more interesting possibility that MP1 controls both ROCK and a distinct Y27632-resistant kinase with overlapping substrate specificity in continuously adherent cells. Rho-regulated kinases (25, 47, 88) relatively insensitive to Y27632 (35) have been described, but to our knowledge none of these is known to stimulate cofilin phosphorylation. Further work is needed to clarify whether MP1 controls focal adhesion and stress fiber dynamics in adherent cells through ROCK alone or through ROCK and additional effectors.

The depletion of MP1/p14 and treatment with the MEK inhibitor UO126 both stimulate the accumulation of enlarged focal adhesions, albeit of subtly different morphologies. It is plausible that UO126 and the loss of MP1/p14 are differentially effective in inhibiting the pool of ERK controlling focal adhesion dynamics. Alternatively, it is possible that MP1/p14 might regulate PAK1 activity towards substrates other than MEK1 important for the remodeling of adhesion structures. Consistent with this notion, we find that combined MP1 knockdown and UO126 treatment enhances ROCK activity above that seen with either MP1 knockdown or the MEK inhibitor alone.

Spatial regulation of MEK1 signaling during adhesion. The mechanism by which MEK1 is recruited to peripheral membrane sites of PAK1 activation is unknown. MP1 and associated p14 localize to late endosomal and lysosomal structures (80, 94), raising the possibility that endosomal MP1/p14 may traffic MEK1 to or from peripheral membrane structures during adhesion. Additional support for this possibility was recently provided by Sacher and colleagues (45), who found that MP1 and p14 are structurally related to SNARE-like proteins involved in membrane trafficking. That MP1 is enriched in lipid rafts (22) is intriguing in this regard given the recent finding that Rho function is intimately linked to FAK-dependent translocation of raft components (57). One might speculate that FAK-dependent localization of MP1 to lipid rafts at the cell periphery allows for subsequent ERK-dependent down-regulation of Rho function in a temporally and spatially controlled manner. Alternatively, localization of the MP1/p14 scaffold complex may be regulated by dynein motor activity, since p14 exhibits sequence homology to the Roadblock/LC7 family of dynein-binding proteins (5, 39) and PAK1 binds to dynein light chain (85).

MP1: a MEK1 scaffolding protein that regulates morphogenesis. Our finding that MP1/p14 regulates the PAK1-dependent activation of MEK1 required for cell spreading while apparently being unnecessary for PDGF-stimulated MEK activation constitutes strong evidence that MP1 and p14 direct MEK and ERK to specific upstream regulators and downstream targets in a context-dependent manner. This is reminiscent of the prototypical MAP kinase scaffolding protein STE5 in yeast, which is thought to "insulate" the pheromone response from functionally distinct pathways sharing common components (reviewed in references 18 and 68). The mechanism we propose here could insulate ERK-dependent morphogenetic events from regulation by other stimuli through scaffold-mediated localization of the shared-component MEK1 and the integration of adhesion signals from PAK1.

To our knowledge, this is the first demonstration of a morphogenetic function for a mammalian ERK pathway scaffold. Interestingly, the PAK-related kinases STE20 and STE11 regulate localized actin remodeling through STE5-mediated activation of the FUS3 MAP kinase in yeast (46), suggesting the existence of an evolutionarily conserved mechanism for the control of cell morphology by MAP kinase scaffolds and PAK family kinases. If, as suggested previously (68) and demonstrated recently by Vomastek et al. (89), the binding activities of the yeast scaffold STE5 are distributed among a number of discrete subunits in mammalian cells, additional MP1 and p14 partners with binding specificity for upstream regulators and downstream effectors remain to be discovered.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants RO1 GM068111 to A.D.C. and P20 RR18766 (project principal investigator, A.D.C.; overall prinicpal investigator, Stephen Lanier) and start-up funds provided by the Department of Pharmacology and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center.

We are grateful to S. Alahari, G. Bokoch, M. Cobb, L. Huber, M. Marshall, J. T. Parsons, M. Weber, S. Weed, and R. Worthylake for plasmids and antisera. We thank Electa Park for providing recombinant PAK1 and Mirek Cygler and Michael Sacher for their kind gift of recombinant MP1/p14 complex. We thank Adel Tarcsafalvi, Dan Santos, and Tomas Vomastek (University of Virginia) and members of the Boulares, Catling, Lanier, and Wu laboratories (Louisiana State University Health Sciences Center) for helpful discussions.

REFERENCES

1. **Alahari, S. K., P. J. Reddig, and R. L. Juliano.** 2004. The integrin-binding protein Nischarin regulates cell migration by inhibiting PAK. EMBO J. **23:**2777–2788.
2. **Arber, S., F. A. Barbayannis, H. Hanser, C. Schneider, C. A. Stanyon, O. Bernard, and P. Caroni.** 1998. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature **393:**805–809.
3. **Arthur, W. T., and K. Burridge.** 2001. RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity. Mol. Biol. Cell **12:**2711–2720.
4. **Bobak, D., J. Moorman, A. Guanzon, L. Gilmer, and C. Hahn.** 1997. Inactivation of the small GTPase Rho disrupts cellular attachment and induces adhesion-dependent and adhesion-independent apoptosis. Oncogene **15:**2179–2189.
5. **Bowman, A. B., R. S. Patel-King, S. E. Benashski, J. M. McCaffery, L. S. Goldstein, and S. M. King.** 1999. Drosophila roadblock and Chlamydomonas LC7: a conserved family of dynein-associated proteins involved in axonal transport, flagellar motility, and mitosis. J. Cell Biol. **146:**165–180.
6. **Brouns, M. R., S. F. Matheson, K. Q. Hu, I. Delalle, V. S. Caviness, J. Silver, R. T. Bronson, and J. Settleman.** 2000. The adhesion signaling molecule p190 RhoGAP is required for morphogenetic processes in neural development. Development **127:**4891–4903.
7. **Carragher, N. O., V. J. Fincham, D. Riley, and M. C. Frame.** 2001. Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J. Biol. Chem. **276:**4270–4275.
8. **Carragher, N. O., M. A. Westhoff, V. J. Fincham, M. D. Schaller, and M. C. Frame.** 2003. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr. Biol. **13:**1442–1450.
9. **Catling, A. D., S. T. Eblen, H. J. Schaeffer, and M. J. Weber.** 2001. Scaffold protein regulation of mitogen-activated protein kinase cascade. Methods Enzymol. **332:**368–387.
10. **Catling, A. D., H. J. Schaeffer, C. W. Reuter, G. R. Reddy, and M. J. Weber.** 1995. A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. Mol. Cell. Biol. **15:**5214–5225.
11. **Chen, B. H., J. T. Tzen, A. R. Bresnick, and H. C. Chen.** 2002. Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells. J. Biol. Chem. **277:** 33857–33863.
12. **Chrzanowska-Wodnicka, M., and K. Burridge.** 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J. Cell Biol. **133:**1403–1415.
13. **Coleman, M. L., E. A. Sahai, M. Yeo, M. Bosch, A. Dewar, and M. F. Olson.** 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat. Cell Biol. **3:**339–345.

14. **Denouel-Galy, A., E. M. Douville, P. H. Warne, C. Papin, D. Laugier, G. Calothy, J. Downward, and A. Eychene.** 1998. Murine Ksr interacts with MEK and inhibits Ras-induced transformation. Curr. Biol. **8:**46–55.
15. **Dourdin, N., A. K. Bhatt, P. Dutt, P. A. Greer, J. S. Arthur, J. S. Elce, and A. Huttenlocher.** 2001. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. J. Biol. Chem. **276:** 48382–48388.
16. **Eblen, S. T., J. K. Slack, M. J. Weber, and A. D. Catling.** 2002. Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. Mol. Cell. Biol. **22:**6023–6033.
17. **Eblen, S. T., J. K. Slack-Davis, A. Tarcsafalvi, J. T. Parsons, M. J. Weber, and A. D. Catling.** 2004. Mitogen-activated protein kinase feedback phosphorylation regulates MEK1 complex formation and activation during cellular adhesion. Mol. Cell. Biol. **24:**2308–2317.
18. **Elion, E. A.** 2001. The Ste5p scaffold. J. Cell Sci. **114:**3967–3978.
19. **Fincham, V. J., and M. C. Frame.** 1998. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. **17:**81–92.
20. **Fincham, V. J., M. James, M. C. Frame, and S. J. Winder.** 2000. Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. EMBO J. **19:**2911–2923.
21. **Fincham, V. J., J. A. Wyke, and M. C. Frame.** 1995. v-Src-induced degradation of focal adhesion kinase during morphological transformation of chicken embryo fibroblasts. Oncogene **10:**2247–2252.
22. **Foster, L. J., C. L. De Hoog, and M. Mann.** 2003. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc. Natl. Acad. Sci. USA **100:**5813–5818.
23. **Franco, S. J., M. A. Rodgers, B. J. Perrin, J. Han, D. A. Bennin, D. R. Critchley, and A. Huttenlocher.** 2004. Calpain-mediated proteolysis of talin regulates adhesion dynamics. Nat. Cell Biol. **6:**977–983.
24. **Frost, J. A., H. Steen, P. Shapiro, T. Lewis, N. Ahn, P. E. Shaw, and M. H. Cobb.** 1997. Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. EMBO J. **16:**6426–6438.
25. **Fujisawa, K., P. Madaule, T. Ishizaki, G. Watanabe, H. Bito, Y. Saito, A. Hall, and S. Narumiya.** 1998. Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules. J. Biol. Chem. **273:**18943–18949.
26. **Giroux, S., M. Tremblay, D. Bernard, J. F. Cardin-Girard, S. Aubry, L. Larouche, J. Huot, J. Landry, L. Jeannotte, and J. Charron.** 1999. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr. Biol. **9:**369–372.
27. **Glading, A., R. J. Bodnar, I. J. Reynolds, H. Shiraha, L. Satish, D. A. Potter, H. C. Blair, and A. Wells.** 2004. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. Mol. Cell. Biol. **24:**2499–2512.
28. **Glading, A., P. Chang, D. A. Lauffenburger, and A. Wells.** 2000. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J. Biol. Chem. **275:** 2390–2398.
29. **Glading, A., F. Uberall, S. M. Keyse, D. A. Lauffenburger, and A. Wells.** 2001. Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. J. Biol. Chem. **276:**23341–23348.
30. **Hildebrand, J. D., J. M. Taylor, and J. T. Parsons.** 1996. An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. Mol. Cell. Biol. **16:**3169–3178.
31. **Howe, A. K., and R. L. Juliano.** 2000. Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase. Nat. Cell Biol. **2:**593–600.
32. **Hughes, P. E., M. W. Renshaw, M. Pfaff, J. Forsyth, V. M. Keivens, M. A. Schwartz, and M. H. Ginsberg.** 1997. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. Cell **88:**521–530.
33. **Ikenoya, M., H. Hidaka, T. Hosoya, M. Suzuki, N. Yamamoto, and Y. Sasaki.** 2002. Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. J. Neurochem. **81:**9–16.
34. **Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto.** 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature **377:**539–544.
35. **Ishizaki, T., M. Uehata, I. Tamechika, J. Keel, K. Nonomura, M. Maekawa, and S. Narumiya.** 2000. Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. Mol. Pharmacol. **57:**976–983.
36. **Jo, M., K. S. Thomas, A. V. Somlyo, A. P. Somlyo, and S. L. Gonias.** 2002. Cooperativity between the Ras-ERK and Rho-Rho kinase pathways in urokinase-type plasminogen activator-stimulated cell migration. J. Biol. Chem. **277:**12479–12485.
37. **Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi.** 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science **273:**245–248.
38. **Klemke, R. L., S. Cai, A. L. Giannini, P. J. Gallagher, P. de Lanerolle, and D. A. Cheresh.** 1997. Regulation of cell motility by mitogen-activated protein kinase. J. Cell Biol. **137:**481–492.
39. **Koonin, E. V., and L. Aravind.** 2000. Dynein light chains of the roadblock/LC7 group belong to an ancient protein superfamily implicated in NTPase regulation. Curr. Biol. **10:**R774–R776.
40. **Kornfeld, K., D. B. Hom, and H. R. Horvitz.** 1995. The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in C. elegans. Cell **83:**903–913.
41. **Kozma, R., S. Sarner, S. Ahmed, and L. Lim.** 1997. Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol. Cell. Biol. **17:**1201–1211.
42. **Kulkarni, S., D. E. Goll, and J. E. Fox.** 2002. Calpain cleaves RhoA generating a dominant-negative form that inhibits integrin-induced actin filament assembly and cell spreading. J. Biol. Chem. **277:**24435–24441.
43. **Leeuwen, F. N., H. E. Kain, R. A. Kammen, F. Michiels, O. W. Kranenburg, and J. G. Collard.** 1997. The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. J. Cell Biol. **139:**797–807.
44. **Leng, J., R. L. Klemke, A. C. Reddy, and D. A. Cheresh.** 1999. Potentiation of cell migration by adhesion-dependent cooperative signals from the GTPase Rac and Raf kinase. J. Biol. Chem. **274:**37855–37861.
45. **Lunin, V. V., C. Munger, J. Wagner, Z. Ye, M. Cygler, and M. Sacher.** 2004. The structure of the MAPK scaffold, MP1, bound to its partner, p14. A complex with a critical role in endosomal MAP kinase signaling. J. Biol. Chem. **279:**23422–23430
46. **Lyons, D. M., S. K. Mahanty, K. Y. Choi, M. Manandhar, and E. A. Elion.** 1996. The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**4095–4106.
47. **Madaule, P., M. Eda, N. Watanabe, K. Fujisawa, T. Matsuoka, H. Bito, T. Ishizaki, and S. Narumiya.** 1998. Role of citron kinase as a target of the small GTPase Rho in cytokinesis. Nature **394:**491–494.
48. **Maekawa, M., T. Ishizaki, S. Boku, N. Watanabe, A. Fujita, A. Iwamatsu, T. Obinata, K. Ohashi, K. Mizuno, and S. Narumiya.** 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science **285:**895–898.
49. **Manser, E., H.-Y. Huang, T.-H. Loo, X.-Q. Chen, J.-M. Dong, T. Leung, and L. Lim.** 1997. Expression of constitutively active alpha PAK reveals effects of the kinase on actin and focal complexes. Mol. Cell. Biol. **17:**1129–1143.
50. **Matheny, S. A., C. Y. Chen, R. L. Kortum, G. L. Razidlo, R. E. Lewis, and M. A. White.** 2004. Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. Nature **427:**256–260.
51. **McBeath, R., D. M. Pirone, C. M. Nelson, K. Bhadriraju, and C. S. Chen.** 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev. Cell **6:**483–495.
52. **Mills, J. C., N. L. Stone, J. Erhardt, and R. N. Pittman.** 1998. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. J. Cell Biol. **140:**627–636.
53. **Miyamoto, S., S. K. Akiyama, and K. M. Yamada.** 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science **267:**883–885.
54. **Nakahara, H., S. C. Mueller, M. Nomizu, Y. Yamada, Y. Yeh, and W. T. Chen.** 1998. Activation of beta1 integrin signaling stimulates tyrosine phosphorylation of p190RhoGAP and membrane-protrusive activities at invadopodia. J. Biol. Chem. **273:**9–12.
55. **Nguyen, A., W. R. Burack, J. L. Stock, R. Kortum, O. V. Chaika, M. Afkarian, W. J. Muller, K. M. Murphy, D. K. Morrison, R. E. Lewis, J. McNeish, and A. S. Shaw.** 2002. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol. Cell. Biol. **22:**3035–3045.
56. **Nguyen, H. D., A. D. Catling, D. J. Webb, M. Sankovic, L. A. Walker, A. V. Somlyo, M. J. Weber, and S. L. Gonias.** 1999. Myosin light chain kinase functions downstream of Ras/ERK to promote migration of urokinase-type plasminogen activator-stimulated cells in an integrin-selective manner. J. Cell Biol. **146:**149–164.
57. **Palazzo, A. F., C. H. Eng, D. D. Schlaepfer, E. E. Marcantonio, and G. G. Gundersen.** 2004. Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. Science **303:**836–839.
58. **Parsons, J. T., K. H. Martin, J. K. Slack, J. M. Taylor, and S. A. Weed.** 2000. Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene **19:**5606–5613.
59. **Pawlak, G., and D. M. Helfman.** 2002. MEK mediates v-Src-induced disruption of the actin cytoskeleton via inactivation of the Rho-ROCK-LIM kinase pathway. J. Biol. Chem. **277:**26927–26933.
60. **Pawlak, G., and D. M. Helfman.** 2002. Post-transcriptional down-regulation of ROCKI/Rho-kinase through an MEK-dependent pathway leads to cytoskeleton disruption in Ras-transformed fibroblasts. Mol. Biol. Cell **13:**336–347.
61. **Price, L. S., J. Leng, M. A. Schwartz, and G. M. Bokoch.** 1998. Activation of

Rac and Cdc42 by integrins mediates cell spreading. Mol. Biol. Cell **9:**1863–1871.
62. **Ren, X. D., W. B. Kiosses, and M. A. Schwartz.** 1999. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. **18:**578–585.
63. **Ren, X. D., W. B. Kiosses, D. J. Sieg, C. A. Otey, D. D. Schlaepfer, and M. A. Schwartz.** 2000. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. J. Cell Sci. **113:**3673–3678.
64. **Resing, K. A., S. J. Mansour, A. S. Hermann, R. S. Johnson, J. M. Candia, K. Fukasawa, W. F. Vande, and N. G. Ahn.** 1995. Determination of v-Mos-catalyzed phosphorylation sites and autophosphorylation sites on MAP kinase kinase by ESI/MS. Biochemistry **34:**2610–2620.
65. **Roberts, M. S., A. J. Woods, P. E. Shaw, and J. C. Norman.** 2003. ERK1 associates with alpha(v)beta 3 integrin and regulates cell spreading on vitronectin. J. Biol. Chem. **278:**1975–1985.
66. **Sahai, E., M. F. Olson, and C. J. Marshall.** 2001. Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increased motility. EMBO J. **20:**755–766.
67. **Schaeffer, H. J., A. D. Catling, S. T. Eblen, L. S. Collier, A. Krauss, and M. J. Weber.** 1998. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science **281:**1668–1671.
68. **Schaeffer, H. J., and M. J. Weber.** 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. **19:**2435–2444.
69. **Schlaepfer, D. D., S. K. Hanks, T. Hunter, and P. van der Geer.** 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature **372:**786–791.
70. **Schlaepfer, D. D., and T. Hunter.** 1996. Signal transduction from the extracellular matrix—a role for the focal adhesion protein-tyrosine kinase FAK. Cell Struct. Funct. **21:**445–450.
71. **Sebbagh, M., C. Renvoize, J. Hamelin, N. Riche, J. Bertoglio, and J. Breard.** 2001. Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. Nat. Cell Biol. **3:**346–352.
72. **Sells, M. A., A. Pfaff, and J. Chernoff.** 2000. Temporal and spatial distribution of activated Pak1 in fibroblasts. J. Cell Biol. **151:**1449–1458.
73. **Slack-Davis, J. K., S. T. Eblen, M. Zecevic, S. A. Boerner, A. Tarcsafalvi, H. B. Diaz, M. S. Marshall, M. J. Weber, J. T. Parsons, and A. D. Catling.** 2003. PAKI phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. J. Cell Biol. **162:**281–291.
74. **Spence, H. J., A. S. Dhillon, M. James, and S. J. Winder.** 2004. Dystroglycan, a scaffold for the ERK-MAP kinase cascade. EMBO Rep. **5:**484–489.
75. **Stahle, M., C. Veit, U. Bachfischer, K. Schierling, B. Skripczynski, A. Hall, P. Gierschik, and K. Giehl.** 2003. Mechanisms in LPA-induced tumor cell migration: critical role of phosphorylated ERK. J. Cell Sci. **116:**3835–3846.
76. **Stam, J. C., E. E. Sander, F. Michiels, F. N. van Leeuwen, H. E. Kain, R. A. van der Kammen, and J. G. Collard.** 1997. Targeting of Tiam1 to the plasma membrane requires the cooperative function of the N-terminal pleckstrin homology domain and an adjacent protein interaction domain. J. Biol. Chem. **272:**28447–28454.
77. **Sundaram, M., and M. Han.** 1995. The C. elegans ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell **83:**889–901.
78. **Tapon, N., and A. Hall.** 1997. Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr. Opin. Cell Biol. **9:**86–92.
79. **Taylor, J. M., J. D. Hildebrand, C. P. Mack, M. E. Cox, and J. T. Parsons.** 1998. Characterization of graf, the GTPase-activating protein for rho associated with focal adhesion kinase. Phosphorylation and possible regulation by mitogen-activated protein kinase. J. Biol. Chem. **273:**8063–8070.
80. **Teis, D., W. Wunderlich, and L. A. Huber.** 2002. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev. Cell **3:**803–814.
81. **Therrien, M., H. C. Chang, N. M. Solomon, F. D. Karim, D. A. Wassarman, and G. M. Rubin.** 1995. KSR, a novel protein kinase required for RAS signal transduction. Cell **83:**879–888.
82. **Torii, S., M. Kusakabe, T. Yamamoto, M. Maekawa, and E. Nishida.** 2004. Sef is a spatial regulator for Ras/MAP kinase signaling. Dev. Cell **7:**33–44.
83. **Totsukawa, G., Y. Wu, Y. Sasaki, D. J. Hartshorne, Y. Yamakita, S. Yamashiro, and F. Matsumura.** 2004. Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. J. Cell Biol. **164:**427–439.
84. **Ueda, H., R. Morishita, H. Itoh, S. Narumiya, K. Mikoshiba, K. Kato, and T. Asano.** 2001. Galpha11 induces caspase-mediated proteolytic activation of Rho-associated kinase, ROCK-I, in HeLa cells. J. Biol. Chem. **276:**42527–42533.
85. **Vadlamudi, R. K., R. Bagheri-Yarmand, Z. Yang, S. Balasenthil, D. Nguyen, A. A. Sahin, P. den Hollander, and R. Kumar.** 2004. Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. Cancer Cell **5:**575–585.
86. **Vial, E., and C. J. Marshall.** 2003. Elevated ERK-MAP kinase activity protects the FOS family member FRA-1 against proteasomal degradation in colon carcinoma cells. J. Cell Sci. **116:**4957–4963.
87. **Vial, E., E. Sahai, and C. J. Marshall.** 2003. ERK-MAPK signaling coordinately regulates activity of Rac1 and RhoA for tumor cell motility. Cancer Cell **4:**67–79.
88. **Vincent, S., and J. Settleman.** 1997. The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. Mol. Cell. Biol. **17:**2247–2256.
89. **Vomastek, T., H. J. Schaeffer, A. Tarcsafalvi, M. E. Smolkin, E. A. Bissonette, and M. J. Weber.** 2004. Modular construction of a signaling scaffold: MORG1 interacts with components of the ERK cascade and links ERK signaling to specific agonists. Proc. Natl. Acad. Sci. USA **101:**6981–6986.
90. **Watanabe, N., T. Kato, A. Fujita, T. Ishizaki, and S. Narumiya.** 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat. Cell Biol. **1:**136–143.
91. **Webb, D. J., K. Donais, L. A. Whitmore, S. M. Thomas, C. E. Turner, J. T. Parsons, and A. F. Horwitz.** 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat. Cell Biol. **6:**154–161.
92. **Webb, D. J., J. T. Parsons, and A. F. Horwitz.** 2002. Adhesion assembly, disassembly and turnover in migrating cells—over and over and over again. Nat. Cell Biol. **4:**E97–E100.
93. **Worthylake, R. A., and K. Burridge.** 2003. RhoA and ROCK promote migration by limiting membrane protrusions. J. Biol. Chem. **278:**13578–13584.
94. **Wunderlich, W., I. Fialka, D. Teis, A. Alpi, A. Pfeifer, R. G. Parton, F. Lottspeich, and L. A. Huber.** 2001. A novel 14-kilodalton protein interacts with the mitogen-activated protein kinase scaffold mp1 on a late endosomal/lysosomal compartment. J. Cell Biol. **152:**765–776.
95. **Yang, N., O. Higuchi, K. Ohashi, K. Nagata, A. Wada, K. Kangawa, E. Nishida, and K. Mizuno.** 1998. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature **393:**809–812.
96. **Yeung, K., T. Seitz, S. Li, P. Janosch, B. McFerran, C. Kaiser, F. Fee, K. D. Katsanakis, D. W. Rose, H. Mischak, J. M. Sedivy, and W. Kolch.** 1999. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature **401:**173–177.
97. **Zecevic, M., A. D. Catling, S. T. Eblen, L. Renzi, J. C. Hittle, T. J. Yen, G. J. Gorbsky, and M. J. Weber.** 1998. Active MAP kinase in mitosis—localization at kinetochores and association with the motor protein CENP-E. J. Cell Biol. **142:**1547–1558.
98. **Zhao, Z. S., E. Manser, T. H. Loo, and L. Lim.** 2000. Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. Mol. Cell. Biol. **20:**6354–6363.