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MEK1 activation by PAK: A novel mechanism

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Abstract

Extracellular signal-Regulated Kinase (ERK) controls a variety of cellular processes, including cell proliferation and cell motility. While oncogenic mutations in Ras and B-Raf result in deregulated ERK activity and proliferation and migration in some tumor cells, other tumors exhibit elevated ERK signaling in the absence of these mutations. Here we provide evidence that PAK can directly activate MEK1 by a mechanism distinct from conventional Ras/Raf mediated activation. We find that PAK phosphorylation of MEK1 serine 298 stimulates MEK1 autophosphorylation on the activation loop, and activation of MEK1 activity towards ERK in in vitro reconstitution experiments. Serines 218 and/or 222 in the MEK1 activation loop are required for PAK-stimulated MEK1 activity towards ERK. MEK2, which is a poor target for PAK phosphorylation in cells, is not activated in this manner. Tissue culture experiments verify that this mechanism is used in suspended fibroblasts expressing mutationally activated PAK1. We speculate that aberrant signaling through PAK may directly induce anchorage-independent MEK1 activation in tumor cells lacking oncogenic Ras or Raf mutations, and that this mechanism may contribute to localized MEK signaling in focal contacts and adhesions during cell adhesion or migration.

Keywords

Adhesion; PAK; MEK; ERK; Raf-1; Phosphorylation; Signaling

1. Introduction

The ERK pathway regulates many cellular processes, including proliferation, adhesion, and migration. Serum growth factors activate the small GTPase Ras which signals through Raf and the dual-specificity kinase MEK to phosphorylate and activate ERK. While this mechanism is well established, it is becoming increasingly clear that other regulatory inputs also control the signaling activity and localization of the ERK pathway. Importantly, while serum-stimulated Ras activation proceeds normally in fibroblasts removed from the extracellular matrix, activation of ERK is blocked at the level of Raf [1] or MEK [2]; adhesion of stimulated cells to fibronectin restores ERK activation. Thus, adhesion to the extracellular matrix permits functional coupling between Raf, MEK and ERK. While both Raf and MEK are well placed

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to serve as anchorage-sensors for growth factor stimulation of ERK, the mechanisms by which cellular adhesion signals are received by Raf and MEK are incompletely understood.

Attachment to the extracellular matrix stimulates activation of the small GTPase Rac and p21-activated kinase 1 (PAK1) [3]. Rac and PAK signaling contributes to oncogenic transformation in model systems and in human tumors. Mutationally activated Rac1 weakly stimulates anchorage-independent growth in fibroblasts [4,5], and PAK1 is required for oncogenic transformation of some fibroblasts by Ras [6]. Exogenous expression of a mutationally active PAK1 or PAK4 leads to anchorage-independent growth in MCF7 cells [7] and fibroblasts [8] respectively. Consistent with these observations, endogenous PAK1 activity is elevated in some metastatic breast cancer cell lines [7,9], and enhanced proliferation of some breast cancer cells lacking oncogenic Ras has been shown to result from hyperactivity of Rac3 and PAK [10].

The ERK and JNK mitogen-activated protein kinase (MAPK) cascades are important regulators of cell proliferation. While increased Rac and PAK signaling can activate the JNK pathway in some systems [7,11–13], Rac3- and PAK-dependent DNA synthesis in MDA-MB 435, T47D and MCF7 breast cancer cells is independent of JNK [10]. Indeed, anchorage-independent growth of MCF7 cells stimulated by expression of mutationally activated PAK1 requires activation of the ERK pathway [7]. Significantly, activated Rac synergizes with Raf to dramatically promote soft agar growth and focus formation [4,5], and activated Rac and Cdc42 and their effector PAK1 also synergize with Raf to activate ERK signaling [14,15]. Furthermore, expression of dominant-negative forms of PAK or the PAK1 autoinhibitory domain inhibits growth factor and adhesion-stimulated ERK activation [16–18]. PAK family kinases can directly phosphorylate Raf on serine 338 [19], and render Raf sensitive to activation by Ras [19–21]. Juliano and colleagues have recently reported that Raf S338 phosphorylation is inhibited in suspended NIH 3T3 fibroblasts, and that restoration of phosphorylation through expression of activated PAK1 or the phosphomimetic Raf S338D mutant is sufficient to permit ERK signaling in response to EGF stimulation [17,22]. However, other reports suggest that serine 338 phosphorylation is either not required for Raf activation in response to EGF in adherent cells [16], or that PAK is not the relevant Raf S338 kinase [23].

PAK also directly phosphorylates MEK1 on serine 298 [24]. MEK1 S298 phosphorylation is proposed to sensitize MEK1 to activation by Raf [14,15], but evidence for a direct priming effect is lacking [15]. MEK1 S298 and Raf S338 phosphorylation may increase the efficiency of ERK signaling in response to low-level inputs by promoting protein-protein interactions between Raf and MEK [14,25] and/or MEK1 and ERK [18,26]. Consistent with this view, neither Raf S338 nor MEK1 S298 phosphorylation are required for activation of ERK signaling by fully active Ras or Raf [15].

MEK1 S298 phosphorylation is not regulated by serum growth factors [24,27] and is not essential for EGF- and PDGF-stimulated MEK1 activation in adherent cells [16]. However, MEK1 S298 phosphorylation is robustly stimulated during cellular adhesion to extracellular matrix proteins [24] and is required for MEK1 activation in response to fibronectin stimulation [18,24]. Together these observations suggest the hypothesis that PAK-Raf and PAK-MEK phosphorylation events control different ERK activation mechanisms in distinct cellular contexts: PAK phosphorylation of Raf permits functional coupling between Ras and Raf in response to growth factors signals, whereas PAK phosphorylation of MEK1 is required for ERK activation during adhesion to the extracellular matrix.

While it is well established that Ras-Raf signaling is required for ERK activation by growth factors, the requirement for Ras-Raf signaling to activate ERK during cellular adhesion is less clear. Some reports indicate that Ras is neither activated by fibronectin stimulation nor required

for ERK activation in response to fibronectin stimulation of NIH 3T3 cells [28], whereas others report that dominant-negative Ras inhibits fibronectin-stimulated ERK activation in both NIH 3T3 cells [29] and COS cells [30]. Surprisingly, the requirement for Raf for ERK activation during adhesion has not been thoroughly defined. Thus, while ERK activation in response to growth factors and phorbol esters is primarily dependent upon B-Raf [31,32], to our knowledge cells derived from A, B or c-Raf deficient mice [32–35] have not been analyzed for ERK activation during adhesion to extracellular matrix components. Hence, novel Ras- and Raf-independent mechanisms distinct from the conventional growth factor pathway may contribute to ERK activation in response to integrin ligation.

Here we provide evidence that PAK can directly activate MEK1 by a mechanism distinct from conventional Ras/Raf-mediated activation. PAK phosphorylation of MEK1 serine 298 stimulates MEK1 autophosphorylation on the conventional activation loop sites (serines 218/222), and activation of MEK1 activity towards ERK in in vitro reconstitution experiments. MEK2, which is believed not to be a target for PAK phosphorylation [14,24], is not activated in this manner. Tissue culture experiments verify that this mechanism is used in suspended fibroblasts expressing mutationally activated PAK1. These data suggest the possibility that aberrant adhesion signaling through PAK may directly induce MEK1 and ERK activation in tumor cells lacking oncogenic Ras or Raf mutations.

2. Experimental procedures

2.1. Reagents

REF52 (REF) cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (Invitrogen) with or without antibiotic-antimycotics (final concentrations penicillin G 100 U/mL, streptomycin 100 µg/mL, amphotericin B 250 ng/mL; Invitrogen). Recombinant human EGF (Sigma) was dissolved in DMEM at 100 µg/mL and stored at –20 °C. UO126 (Calbiochem) was reconstituted in DMSO at 25 mM and stored at –20 °C. Fibronectin-coated dishes were prepared as described previously [24].

2.2. Plasmids

Expression constructs for HA-tagged MEK1 and MEK2 were described previously [27]; pRK5 myc PAK1 T423E was a gift from Scott Weed (West Virginia University, Morgantown, WV) [36]; HA-tagged Ras G12V was a gift from Channing Der (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC).

2.3. Transfection assays—DNA

To analyze PAK activation of MEK, REF cells (2.0×10^6 /150 mm dish) were transfected with 1 µg MEK and 4 µg PAK1 T423E or pRK5 vector control using 67.5 µL Superfect (Qiagen). To compare Ras and PAK mediated activation of MEK1, cells ($8.0\text{--}8.9 \times 10^5$ /100 mm dish) were transfected with 1 µg MEK and 2.5 µg PAK1 T423E or 1 µg MEK, 0.5 µg Ras V12 and 2.0 µg empty pRK5 vector using 30 µL Superfect (Qiagen). Eighteen to 24 h post-transfection cells were placed in suspension in serum-free DMEM as described previously [24] for 1 h before analysis. Continuously adherent cultures were serum-starved in parallel, then stimulated with EGF for 5–10 min. Cells were washed in ice cold PBS before lysis. RNA: REF52 cells were plated at 3.0×10^5 /100 mm dish the day before transfection. Cells were transfected with 100 nM control or Raf1 siRNA (SMARTpool, Dharmacon) using 30 µL Lipofectamine 2000 (Invitrogen). Seventy-two hours post-transfection, cells were placed in suspension in serum-free media or serum-starved for 2 h before plating on fibronectin-coated dishes for indicated times or stimulation with EGF.

2.4. Recombinant proteins

GST MEK1 S218/222AA His₆ was generated using the Transformer Site-directed mutagenesis kit (Clontech). Recombinant GST-MEK His₆, GST B-Raf and His₆-ERK2 K52R proteins were purified as described [37,38]. Recombinant PAK3 was purified from baculovirus-infected Sf9 cells and was a kind gift from Mark Marshall (Department of Medicine, Indiana University, Indianapolis, IN). Recombinant, polyhistidine-tagged PAK2 was expressed in BL-21 *E. coli* under the control of the lac Z promoter using pET28-PAK2 provided by Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). Cultures at A₆₀₀ ~0.5 were induced with 30 μM IPTG for 4 h at 30 °C, pelleted and lysed by sonication on ice in TN buffer [50 mM Tris-HCl, 50 mM NaCl, 1 mM PMSF, 0.1% (v/v) 2-Mercaptoethanol; pH 8.0 at 4 °C] supplemented with 0.1% Triton X-100, 1 μg/mL Leupeptin, 3 mM Benzamidine, and 5 mM sodium pyrophosphate. Clarified lysates (20,000 ×g, 20 min at 4 °C) were filtered through a 0.2 μm filter and loaded (0.5 mL/min) onto a nickel-NTA agarose (Qiagen) column (2 mL bed volume) equilibrated in TN buffer. The column was sequentially washed with 20 mL TN/0.1% Triton X-100, 20 mL TN/0.5 M NaCl, and 10 mL TN at a flow rate of 1 mL/min. Protein was eluted using a 40 mL linear gradient from 0–250 mM imidazole in TN buffer. Fractions containing PAK2 (eluting at 37.5 mM to 112.5 mM imidazole) were pooled and dialyzed into TN/50% glycerol (lacking 2-Mercaptoethanol but containing 0.1 mM PMSF and 1 mM DTT) and stored at –20 °C. Typical yield of full-length PAK2 was ~0.5 mg from a 100 mL culture.

2.5. In vitro kinase assays

One microgram of recombinant MEK1 was mixed with the indicated concentration of recombinant PAK or B-Raf in kinase buffer (25 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP). The reactions were incubated at 30 °C for 40 min, at which time 1 μg of recombinant ERK2 K52M was added. The reaction was allowed to proceed for 15 min and stopped by the addition of SDS-PAGE loading solution and boiling.

2.6. Western blotting and immunoprecipitation

For immunoprecipitation, cells were lysed in FLAG lysis buffer supplemented with protease and phosphatase inhibitors as previously described [37,39]. Immunoprecipitations were carried out using anti-HA antibody 12CA5 (Covance) as described previously [27,39]. Proteins were detected by western blotting using the following primary antibodies: MEK1 phospho-S298 [24]; MEK1 phospho-S218/S222 (Sigma); ERK1/2 phospho-T202/Y204 (Cell Signaling); HA 12CA5; myc 9E10 (Santa Cruz). Bound secondary antibodies or protein A conjugated to horseradish peroxidase (GE Health Sciences) were detected using ECL substrate (GE Health Sciences) and digitally imaged using a Fuji Intelligent Dark Box.

3. Results

We first asked whether MEK1 serine 298 phosphorylation is required for MEK1 activation by PAK1 in suspended cells. Rat embryo fibroblasts (REF52) were transiently co-transfected with HA-tagged MEK1 and constitutively active PAK1 (myc PAK1 T423E) constructs, and placed in suspension [24] to suppress endogenous adhesion and Raf signaling. HA-MEK1 was immunoprecipitated and analyzed for phosphorylation on the PAK site (serine 298) and activation loop sites (serines 218/222) by western blotting with appropriate phospho-specific antisera. Phosphorylation of serines 218 and/or 222 is necessary and sufficient for activation of MEK [40,41]; phosphorylation of these sites is a surrogate measure of MEK1 activity. When transfected alone, wild-type MEK1 was not phosphorylated on S298 or the activation loop sites in suspended cells, but was phosphorylated on both S298 and the S218/S222 activating sites in suspended cells co-expressing activated PAK1 (Fig. 1). In contrast, MEK1 S298A was inactive even in the presence of active PAK1. Thus, active PAK1 is sufficient to stimulate

MEK1 activation in suspended fibroblasts and serine 298, the site of PAK1 phosphorylation on MEK1, is necessary for MEK1 activation.

We next asked whether the MEK inhibitor UO126 [42] was able to inhibit PAK-mediated activation of MEK1 in suspended cells. While UO126 was without effect on PAK1 phosphorylation of serine 298, the drug was effective in inhibiting PAK1-stimulated phosphorylation of the serine 218/222 activation loop sites of wild-type MEK1 (Fig. 2a). These data might suggest that PAK1 phosphorylation primes MEK1 for UO126-sensitive phosphorylation on S218/222 by Raf [14,15]. Alternatively, since UO126 is an inhibitor of MEK catalytic activity [42], the data are also consistent with the possibility that PAK1 stimulates MEK1 autophosphorylation on serines 218 and/or 222. Autophosphorylation of these sites has been reported previously [43]. To distinguish between these mechanisms we asked whether kinase-defective MEK1 (MEK1 K97A) could be phosphorylated on S218/222 in response to PAK1 in suspended REF cells. While kinase-defective MEK1 was robustly phosphorylated by PAK1 on serine 298, phosphorylation on the S218/S222 activating sites was almost undetectable (Fig. 2a). This result does not support a priming role for serine 298 phosphorylation for phosphorylation by Raf, but rather suggested that MEK1 autocatalytic activity was required for activation of MEK1 by PAK1.

As PAK-mediated MEK1 activation appeared to require both MEK1 kinase activity and S298 phosphorylation, we next asked whether Ras/Raf activation of MEK1 carried these same requirements. Importantly, Raf mediated phosphorylation of MEK1 on serines 218/222 by either EGF stimulation of adherent REF cells or expression of mutationally activated Ras in suspended REF cells was independent of both MEK1 catalytic activity and serine 298 phosphorylation (Fig. 2b). These data reveal mechanistic differences between PAK1- and Ras/Raf-mediated activation of MEK1 and suggest that Raf may be dispensable for activation of MEK1 by PAK1 in suspended REF cells.

To begin to test the possibility of Raf-independent activation of MEK1 in a more physiological context, we used small interfering RNA (siRNA) to knock down Raf-1 protein in REF cells and evaluated the ability of these cells to activate endogenous PAK, MEK, and ERK during adhesion to an extracellular matrix. Raf-1 was chosen because it may serve as an adhesion sensor in ERK signaling via PAK phosphorylation of S338 [22]. We chose to test the requirement for Raf-1 during adhesion to fibronectin, as PAK1 is known to be activated in this context, as are MEK and ERK, while in our hands Raf-1 activity is very low and not induced by fibronectin [24]. While Raf-1 protein was efficiently depleted by transfection with targeted but not control siRNA, activation of ERK was not inhibited in response to cellular adhesion to fibronectin or stimulation with EGF (Fig. 3). Activation of MEK in response to fibronectin stimulation also proceeded normally in cells depleted of Raf-1 (Fig. 3). In contrast, depletion of Raf-1 caused a modest inhibition of MEK activation in response to EGF stimulation. These data are consistent with, but do not prove, the hypothesis that activation of MEK and ERK during adhesion to fibronectin is Raf-1 independent.

To more definitively test the hypothesis that PAK can activate MEK1 directly, we used in vitro reconstitution experiments. Recombinant wild-type, kinase-defective (K97A), or S298A MEK1 were incubated with recombinant PAK3 and Mg^{2+} /ATP as described in Experimental procedures. Recombinant, kinase-defective (K52R) ERK2 was then added to measure MEK catalytic activity. As shown in Fig. 4a, wild-type MEK1 is phosphorylated by PAK3 on S298, and is activated as evidenced by phosphorylation of MEK1 serines 218/S222 and by phosphorylation of ERK2. In agreement with the data obtained in suspended fibroblasts, MEK1 S298A is not activated by PAK3. Furthermore, while kinase-deficient MEK1 is phosphorylated by PAK3 on S298, it is not efficiently phosphorylated on the S218/222 activation loop sites.

These data support the hypothesis that PAK phosphorylation of MEK1 serine 298 induces MEK1 autophosphorylation of S218/S222 and kinase activity towards ERK. However, we could not rule out the possibility that a small amount of Raf or other MEK activator contaminated the PAK3 preparation purified from insect cells. To exclude this possibility we purified recombinant histidine-tagged PAK2 protein from *E. coli* and tested its activity towards the MEK1 variants. Recombinant PAK2 also stimulated activation of MEK1 in a serine 298- and MEK1 kinase activity-dependent manner (Fig. 4b), and the MEK inhibitor UO126 inhibited phosphorylation on serines 218/222 without inhibiting PAK phosphorylation of MEK1 S298 (Fig. 5a). In striking contrast, direct serine S218/222 phosphorylation and activation of MEK1 by recombinant B-Raf does not require serine 298 phosphorylation or MEK1 catalytic activity (Fig. 5b). These data strongly support the hypothesis that PAK phosphorylation of serine 298 can stimulate MEK1 autophosphorylation of the activation loop serines 218/222 and activity towards ERK in the absence of Raf family kinases.

While serine 298 and contiguous sequences are partially conserved in MEK2, MEK2 appears to be a poor substrate for PAK in cells [14,24]. We therefore considered MEK2 a useful tool to test the specificity of the *in vitro* mechanism. MEK2 was not activated by recombinant PAK2 in our reconstitution assay (Fig. 4b) but was activated by B-Raf (data not shown). As an additional measure of specificity, we asked whether threonine 292, a site of phosphorylation in the MEK1 PRS [15,26,27,44] not used by PAK [26], was required for activation by PAK2 *in vitro*. Threonine 292 was dispensable for activation by PAK2 (Fig. 4a,b). Thus, the novel mechanism we have elucidated *in vitro* has a similar isozyme and phosphorylation-site specificity to the PAK-dependent mechanism(s) of MEK activation reported in cells [14,15,18,24].

These experiments do not determine whether phosphorylation of the MEK1 activation loop (S218/222) is required for MEK1 activity towards ERK or is alternatively a consequence of PAK-stimulated MEK1 activity. To distinguish between these possibilities, we tested MEK1 S218A/S222A for its ability to phosphorylate ERK in response to PAK phosphorylation. While MEK1 S218/222A is robustly phosphorylated by PAK2 on serine 298, it has essentially undetectable activity towards ERK (Fig. 5b). As expected, MEK1 S218/222A cannot be activated by B-Raf (Fig. 5b). These data demonstrate that PAK-stimulated MEK1 autophosphorylation on S218/S222 is required for phosphorylation of ERK.

These reconstitution experiments with bacterially produced PAK, MEK and ERK proteins demonstrate that PAK is sufficient to activate MEK1 in a Raf-independent manner *in vitro*.

4. Discussion

The ERK pathway serves important roles in many cellular processes, including cell proliferation, motility, secretion and survival. How these outcomes are simultaneously and independently controlled by a shared signaling pathway is unclear, but must require that functionally distinct pools of ERK be regulated independently of one another. One possibility is that scaffold proteins localize the generic Raf-MEK-ERK module to outcome-specific stimuli and effectors, and thereby confer signaling specificity [45–48]. Alternatively, distinct activation and inactivation mechanisms in addition to the conventional tripartite cascade may control MEK and ERK function at distinct subcellular locales.

We previously demonstrated that MEK1 and ERK activation during cellular adhesion to fibronectin required FAK and PAK activation, and MEK1 serine 298 phosphorylation, but occurred in the absence of detectable Raf-1 activity [18,24]. Interestingly, while the Src family kinase inhibitor PP2 caused a substantial and paradoxical increase in fibronectin-stimulated Raf-1 activity, MEK1 and ERK were not activated in this context [24]. These data suggested

that MEK/ERK activation is uncoupled from Raf-1 activity during adhesion of REF cells to fibronectin. Consistent with this view, we find that efficient siRNA-mediated depletion of c-Raf-1 is without effect on fibronectin-stimulated MEK and ERK activation while it modestly inhibits EGF-stimulated MEK activation. These data indicate that c-Raf-1 is not absolutely required for adhesion-stimulated MEK activation. While B-Raf activation by PAK has not been described, we cannot rule out the possibility that B-Raf may be responsible for the activation seen in cells stimulated with fibronectin. We were unable to reliably and efficiently knock-down B-Raf in REF cells, precluding a direct test of this possibility (data not shown).

Here we present evidence for a novel mechanism of MEK1 activation by PAK family kinases. We find that mutationally activated PAK1 is sufficient to activate MEK1 in suspended REF cells, and that activation requires a site of PAK phosphorylation (serine 298) within the MEK1 proline-rich sequence. To our surprise, we found that MEK1 catalytic activity is also required for activation by PAK, whereas neither serine 298 nor MEK1 catalytic activity are required for activation by EGF or active Ras. The finding that kinase-defective MEK1 is not phosphorylated on the activation loop sites in response to PAK appears to rule out the possibility that PAK phosphorylation primes MEK1 for phosphorylation by low levels of Raf activity in this context [14,15], but rather indicates that mutationally active PAK is able to activate MEK1 by a mechanism distinct from conventional Ras/Raf signaling.

This hypothesis was confirmed in in vitro reconstitution assays using recombinant proteins produced in *E. coli*. These experiments demonstrated that PAK is sufficient to activate MEK1 in the absence of Raf, by stimulating MEK1 autophosphorylation of the same activation loop sites (serine 218/222) used by Raf. MEK2, which is not believed to be a good substrate for PAK in cells, was not activated by PAK in vitro. Thus, the data obtained in both REF cells and in vitro reconstitution experiments reveal a novel mechanism of PAK-stimulated MEK1 autoactivation with regulatory features distinct from conventional Ras/Raf signaling.

The extent of MEK1 activation in REF cells in response to active PAK1 is considerably less than that seen with active Ras or EGF. Furthermore, MEK1 is largely inactive, as measured by phosphorylation of serines 218 and 222, in serum-starved continuously adherent cultures, or following greater than ~30 min of adhesion to fibronectin, in spite of continued robust Serine 298 phosphorylation. These observations suggest that PAK-stimulated activation is limited by additional regulatory events following prolonged adhesion. It is conceivable that PAK-stimulated MEK1 autophosphorylation on serines 218 and 222 is rapidly reversed by phosphatase activity, such that a minority of the serine 298-phosphorylated MEK1 is phosphorylated on the activation loop in serum-starved adherent cells. This model is consistent with the observations that MEK and ERK activation in response to fibronectin stimulation is both quantitatively modest and transient compared to that seen in response to growth factors (Fig. 3, Control lanes). Alternatively, negative regulatory phosphorylation events might counteract the activating effect of serine 298 phosphorylation. For instance, we have found that ERK phosphorylation of MEK1 on threonine 292 follows serine 298 phosphorylation during adhesion, in concert with loss of MEK1 activation [26]. Importantly, mutation of threonine 292 to alanine prolonged MEK1 activation during adhesion to fibronectin [26]. These data suggest that MEK1 diphosphorylated on threonine 292 and serine 298 has a lower potential for autoactivation or is particularly sensitive to phosphatases that remove phosphate from the activating serines 218 and 222. Our previous phosphopeptide mapping studies confirmed that a pool of threonine 292/serine 298 diphosphorylated MEK1 exists in serum-starved adherent cells [27].

Molecular scaffolding could also be important for regulating PAK-induced MEK1 activation. We recently reported that the MEK1-specific binding protein MEK Partner 1 (MP1) associates preferentially with catalytically active forms of PAK1 [46], although MEK1 mutants

compromised for binding to MP1 were nonetheless able to be phosphorylated by PAK1 during cellular adhesion to fibronectin. The potential role of MP1 in direct activation of MEK1 by PAK remains to be thoroughly investigated. Similarly, Berk and colleagues have recently reported that GIT1 functions as a scaffold to bind MEK and ERK, and to stimulate MEK and ERK activation in focal adhesions in response to EGF [49,50]. While GIT1 associates with PAK1 [51], we find that GIT1 is not required for PAK1 phosphorylation of MEK1 (T. Milosavljevic, E.R.P. and A.D.C., data not shown).

Recombinant MEK1 and MEK2 exhibit slow activation loop autophosphorylation and autoactivation following purification from *E. coli* [43,52]. Our data indicates that PAK phosphorylation of serine 298 within the MEK1 proline-rich sequence increases the rate of MEK1 autophosphorylation and auto-activation. In contrast, phosphorylation of threonines 286 and 292 within the proline-rich sequence inhibits MEK1 catalytic activity [44,53] or PAK-stimulated phosphorylation of the MEK1 activation loop [26]. The proline-rich sequence containing these regulatory sites is not well ordered in the available crystal structure of recombinant MEK1 [54], suggesting that this sequence is highly flexible in its unphosphorylated state. Precisely how phosphorylation of the MEK1 PRS influences activation loop phosphorylation and/or position within the MEK1 structure remains an important question given the promising therapeutic utility of MEK inhibitors [55].

By mixing catalytically active and inactive mutants of MEK1, Ahn and colleagues determined that basal autophosphorylation of recombinant MEK1 is intramolecular [43]. However, MEK1 and MEK2 crystals are composed of dimeric units, and MEK dimers could also be detected in solution [54]. It is possible that PAK phosphorylation might facilitate transphosphorylation of activation loops within such dimeric structures.

We speculate that direct activation of MEK1 by PAK may be physiologically relevant in three ways. First, this novel mechanism may confer spatial regulation of MEK1 activation and function. Active PAK1 localizes proximal to peripheral membranes and in sites of cell-substrate contact (focal contacts) [56–58], where it functions in part to stimulate focal complex disassembly, and actin filament assembly important for cell motility [59]. MEK1 phosphorylated on serine 298 [24] and active ERK are found in membrane proximal sites and focal contacts [24,49,60], and importantly, MEK signaling is required for focal adhesion disassembly [61]. These observations are consistent with PAK activating a population of MEK1 and ERK in focal complexes to regulate focal contact turnover during motility. These observations also suggest a way in which PAK and Ras/Raf signaling might simultaneously but independently control adhesion and growth factor regulated populations of ERK.

Second, while MEK1 and MEK2 are highly conserved (84% identity, rat enzymes) and are both activated by Ras/Raf signaling, gene knock-out experiments demonstrate that they have distinct functions during development [62,63]. MEK1 and MEK2 do differ significantly in their proline-rich sequences (~50% identical), and in their regulation by phosphorylation of this sequence [14,15,24,26,27,53]. In particular, MEK2 is not thought to be a good substrate for PAK [14], suggesting that some MEK1-specific functions are controlled by direct activation by PAK family kinases. The finding that MEK1-null fibroblasts exhibit motility defects [63] is consistent with both a role for localized activation of MEK1 by PAK in adhesion structures, and isozyme-specific activation of MEK1 but not MEK2 by PAK.

Third, direct activation of MEK by PAK may contribute to ERK-dependent proliferation and invasion/motility in tumor cells lacking oncogenic Ras and Raf mutations. Tumor cells frequently express elevated PAK activity (reviewed in [64]), or in other cases where PAK activity was not reported, elevated FAK/Src signaling [65,66] required for PAK phosphorylation of MEK1 [24]. Indeed, mutationally activated PAK1 is sufficient to stimulate

ERK signaling required for anchorage-independent growth in MCF-7 breast cancer cells [7], although the mechanism of ERK activation in this context was not determined. Our preliminary data indicate that MEK1 S298 phosphorylation and activation is anchorage-independent in highly metastatic DU145 prostate tumor cells, but anchorage-dependent in weakly metastatic LNCaP cells (data not shown). Future experiments will test whether the novel mechanism of MEK1 activation we have described contributes to anchorage-independent proliferation and/or invasion and motility in tumor cells lacking oncogenic Ras and Raf.

ERK controls many cellular processes in a context-specific manner. This regulatory flexibility likely results from fine-tuning of the localization and activation state of the canonical Raf–MEK–ERK cascade by both scaffolding components and regulatory phosphorylation events outside of the Raf and MEK activation loops [26,48,67,68]. While these mechanisms probably are the predominate ways in which ERK activity and function is regulated, the data presented here suggest that non-canonical activation of MEK1 by PAK family kinases may allow for additional regulatory flexibility of this multifunctional pathway.

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Abbreviations

EGF	epidermal growth factor
ERK	extracellular-signal regulated kinase
GIT1	G-protein-coupled receptor kinase interacting protein 1
IPTG	isopropyl- β -D-thiogalactopyranoside
JNK	Jun N-terminal kinase
MAP kinase	Mitogen-activated protein kinase
MEK	MAP kinase or ERK kinase
MP1	MEK Partner-1
PAK	p21-activated kinase
PDGF	platelet-derived growth factor

siRNA

small interfering RNA

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Fig. 1.

PAK1-stimulated activation of MEK1 in suspended fibroblasts requires serine 298 phosphorylation. REF52 cells co-transfected with the indicated constructs were placed in suspension in serum-free media for 1 h, then collected for analysis. MEK1 proteins were immunoprecipitated using anti-HA antibody and phosphorylation state assessed by western blotting with phospho-specific antibodies. Equal loading was subsequently verified by blotting with anti-HA antibody. Expression of mutationally active PAK1 was assessed by blotting whole cell lysates (WCL) with an antibody against the myc tag.

Fig. 2.

PAK1 and Ras/Raf use distinct mechanisms to stimulate activation of MEK1 in suspended fibroblasts. **a**, REF52 cells co-transfected with HA tagged-MEK1, MEK1 K97A (kinase-defective), or MEK1 S298A and vector or myc-tagged PAK1 T423E were harvested 24 h post-transfection, placed in suspension in serum-free media for 1 h, then treated with 25 μ M UO126 or vehicle control. Cells were collected 30 min post-treatment. MEK1 proteins were immunoprecipitated using anti-HA antibody and phosphorylation state assessed by western blotting with phospho-specific antibodies. Expression of PAK was assessed by blotting whole cell lysates (WCL) with an antibody against the myc tag. **b**, REF52 cells co-transfected with HA-tagged MEK1 variants and either myc-PAK1 T423E, HA-Ras G12V or control vector were serum-starved (adherent cells) or placed in suspension in serum-free media for one hour 24 h post transfection. Where indicated, cells were stimulated with EGF (1 ng/ml) for 10 min. HA-MEK1 was immunoprecipitated and analyzed as described in **a**. PAK1 T423E and Ras G12V expression were assessed by blotting whole cell lysates (WCL) or HA immunoprecipitates respectively with antisera against their respective epitope tags. W, wild type MEK1; K, MEK1 K97A; S, MEK1 S298A; * shorter exposure of pS218/S222 MEK blot.

Fig. 3.

Activation of MEK1 and ERK during adhesion to fibronectin is Raf-1-independent. REF52 cells were placed in suspension, or adherent cultures were serum-starved for two hours seventy-two hours post transfection with control or Raf1 siRNA. Adherent cultures were then stimulated with EGF (1 ng/mL for 5 min) or plated on fibronectin (FN)-coated dishes for the indicated times. Cell lysates were analyzed by western blot with the indicated antisera.

Fig. 4.

PAK directly activates MEK1 in vitro in a serine 298- and MEK1 kinase activity-dependent manner. Recombinant MEK and ERK proteins were incubated in kinase buffer as described in Experimental procedures with a, 90 ng recombinant PAK3 purified from baculovirus-infected Sf9 cells, or b, 100 ng recombinant PAK2 protein purified from *E.coli*. The phosphorylation state of MEK and ERK was assessed by western blotting with the indicated phosphospecific antibodies.

Fig. 5.

Activation of MEK1 by PAK requires autophosphorylation of the activation loop serines 218 and/or 222. **a**, Recombinant MEK1 and 40 ng PAK2 were incubated in kinase buffer in the presence of varying concentrations of UO126. **b**, Recombinant MEK and ERK proteins were incubated in kinase buffer as described in Experimental procedures with 40 ng recombinant PAK2 or 4.2 ng recombinant BRaf. The phosphorylation state of MEK and ERK was assessed by western blotting with the indicated phosphospecific antibodies.