**p21-Activated Kinase 1 (Pak1) Regulates Cell Motility in Mammalian Fibroblasts**

Mary Ann Sells, Jonathan T. Boyd, and Jonathan Chernoff
Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

**Abstract.** The p21 (Cdc42/Rac) activated kinase Pak1 regulates cell morphology and polarity in most, if not all, eukaryotic cells. We and others have established that Pak’s effects on these parameters are mediated by changes in the organization of cortical actin. Because cell motility requires polarized rearrangements of the actin/myosin cytoskeleton, we examined the role of Pak1 in regulating cell movement. We established clonal tetracycline-regulated NIH-3T3 cell lines that inducibly express either wild-type Pak1, a kinase-dead, or constitutively-active forms of this enzyme, and examined the morphology, F-actin organization, and motility of these cells. Expression of any of these forms of Pak1 induced dramatic changes in actin organization which were not inhibited by coexpression of a dominant-negative form of Rac1. Cells inducibly expressing wild-type or constitutively-active Pak1 had large, polarized lamellipodia at the leading edge, were more motile than their normal counterparts when plated on a fibronectin-coated surface, and displayed enhanced directional movement in response to an immobilized collagen gradient. In contrast, cells expressing a kinase-dead form of Pak1 projected multiple lamellipodia emerging from different parts of the cell simultaneously. These cells, though highly motile, displayed reduced persistence of movement when plated on a fibronectin-coated surface and had defects in directed motility toward immobilized collagen. Expression of constitutively activated Pak1 was accompanied by increased myosin light chain (MLC) phosphorylation, whereas expression of kinase-dead Pak1 had no effect on MLC. These results suggest that Pak1 affects the phosphorylation state of MLC, thus linking this kinase to a molecule that directly affects cell movement.

**Key words:** p21-activated kinase • motility • GTPases • fibroblasts • phosphorylation

Many cell types are induced to move in response to external factors. Cell migration is essential during embryonic development, in immune response, and wound healing, and aberrant motility is a hallmark of metastatic cells. The migration of fibroblasts in vitro requires acquisition of spacial asymmetry with the extension of lamellipodia, attachment of the cytoskeleton to the extracellular matrix, and generation of force that propels the cell forward while pulling the attachments rearward (Sheetz, 1994). Actin polymerization is thought to drive the formation and extension of the lamellipodial leading edge, while the activation of myosin, particularly myosin II, is thought to be at least partially responsible for creating the traction force needed for cell movement (Condeelis, 1993). In addition, the strength and dynamics of focal adhesion formation play a major role in cell locomotion. The molecular basis of the signaling steps that regulate motile responses are poorly understood; however, Ras and related GTPases of the Rho family clearly play important roles in this process. For example, the scattering response of MDCK cells in response to HGF can be abolished by dominant negative Ras, Rac1, as well as constitutively active RhoA (Ridley et al., 1995), and activated forms of Rac and Cdc42 can increase cell motility and the invasive potential of epithelial cells (Keeley et al., 1997; Sander et al., 1998). In addition, Rac1 modulates growth factor-driven chemotaxis in fibroblasts and macrophages (A nand-Apte et al., 1997; A llen et al., 1998).

Reorganization of the actin cytoskeleton is a prerequisite for cell motility. The Rho GTPases, Cdc42, Rac1, and RhoA are well known for their ability to induce rearrangements of filamentous actin. In the case of Cdc42 and Rac, several candidate effectors have been identified that might link these GTPases to the actin cytoskeleton. In mammalian cells, these candidate effectors include (a) protein...
kinases, such as p21-activated kinases (Paks), myotonic-dystrophy-related kinases, MEKKS, and mixed-lineage kinases, (b) lipid kinases, such as PI3 kinase and P15 kinase, (c) formins, such as the mouse homologue of Diaphanous (p140Di), (d) actin binding proteins, such as IQGAPs, and (e) other proteins that affect actin structure, such as Wiskott-Aldrich protein and POR1 (reviewed in Van Aelst and D’Souza-Schorey, 1997). A major point of controversy in the literature is whether Paks have a role in mediating F-actin assembly by these GTPases in mammalian cells. On the one hand, mutants of Rac1 and Cdc42 that do not efficiently bind the three known mammalian Paks are nonetheless able to induce characteristic actin reorganization in fibroblasts (J oneson et al., 1996; L amarche et al., 1996; W estwick et al., 1997). On the other hand, expression of activated forms of Pak1 induces changes in actin and focal adhesion structure similar to those seen with activated GTPases (M anser et al., 1997; S ells et al., 1997).

A possible resolution to these two seemingly conflicting results has been provided recently by M anser et al., who showed that Pak1 can be activated even in the absence of direct binding to Cdc42 through interaction with the guanine-nucleotide exchange factor PIX (M anser et al., 1998).

In lower eukaryotes, Paks clearly play a role in regulating actin-based morphogenic events. Deletion of the Pak homologue STF 20 in budding yeast results in sterile cells, unable to form mating projections or activate the transcription program required for mating (L ebere r et al., 1992). Overexpression of STF 20 is sufficient to relieve the morphological defects associated with loss of Cdc42p function, consistent with the postulated effector role for Ste20p in regulating cortical actin assembly (E by et al., 1998).

In this system, myosin I has been identified as a potential Pak target (W u et al., 1996, 1997), providing a possible explanation for Pak’s morphogenic effects. In fission yeast, deletion and overexpression studies have shown that both Pak1p and -2p regulate cell morphogenesis (M arcus et al., 1995; O ttilie et al., 1995; S ells et al., 1998; Y ang et al., 1998). Interestingly, a temperature-sensitive mutation of pak1 was isolated recently during a screen for rounded (orb) mutants of S. pombe (V erde et al., 1995, 1998), providing independent evidence for a role for this protein in morphogenesis. Genetic analysis has placed this gene (orb2) upstream of another orb gene (orb6), which encodes a homologue of R ok, a R ho-activated kinase (V erde et al., 1998). In vertebrates (K imura et al., 1996) and probably in C. elegans (W issmann et al., 1997), R ok inactivates myosin light chain (M LC) phosphatase, resulting in increased M LC phosphorylation. Therefore, the observed effects of S. pombe Pak1 on cell morphology may involve activation of M LC via Orb6p. Whether these findings will extend to the mammalian system remains to be seen.

In a previous analysis, we noted that a majority of the Swiss 3T3 cells transiently expressing an activated form of Pak1 developed large, polarized lamellipodia numerous small focal complexes (S ells et al., 1997). Two regions of Pak1 were found to be important for these cytoskeletal effects; proline-rich sequences in the NH2 terminus, which bind to src-homology 3 (SH3) proteins such as the adaptor Nck (G alisteo et al., 1996) and the guanine-nucleotide exchange factor PIX (M anser et al., 1998), and the COOH-terminal protein kinase domain (M anser et al., 1997; S ells et al., 1997). The NH2-terminal proline-rich sequences appear to be important for Pak localization and are required for the development of polarized lamellipodia. The function of the kinase domain is less certain. Inactivating mutations in the kinase domain do not abolish the ability of Pak1 to reorganize actin, but cells expressing such mutants have multiple protrusions and no apparent leading edge (S ells et al., 1997). Therefore, the kinase domain of Pak1 may be important for the disposition of lamellipodia.

The polarized phenotype induced by activated Pak1 is reminiscent of that seen in motile fibroblasts. In this study, we have analyzed the effects of Pak1 on cell motility, using cell lines that indubitably express various forms of Pak1. Here, we show that Pak1 induces the formation of leading edge lamellipodia. These structures are not affected by coexpression of a dominant-negative form of Rac1. Consistent with this morphology, Pak1 overexpression stimulates cell movement. Pak1 overexpression increases random cell movement on fibronectin-coated surfaces, irrespective of its protein kinase activity. In contrast, Pak1 also affects directional cell movement toward an immobilized collagen gradient, but this effect requires its kinase activity. Activated forms of Pak1 induce MLC phosphorylation, whereas a kinase-deficient mutant is without effect. These results suggest that Pak1 plays a role in regulating directional cell motility through its effects on M LC.

Materials and Methods

Materials

Monoclonal anti-hemagglutinin (HA) antibody (12CA5) was obtained from BabCo, polyclonal anti-Myc was obtained from Santa Cruz Biotechnology, Inc., and monoclonal anti-Myc antibody (9E10) was a gift from Phil Tischkis (Fox Chase Cancer Center, Philadelphia, PA). Antibodies against activated Erk and Jnk were obtained from Promega Corp., anti-vinculin was from Sigma Chemical Co., and anti-phospho-M LC (M atsumura et al., 1998) was a kind gift from Fumio M atsumura (Rutgers University, New Brunswick, N J). Fluorochrome-conjugated goat anti-mouse and anti-rabbit antibodies were from Jackson Immunoresearch Laboratories, Inc.

Expression Plasmids

Wild-type and mutant forms of Pak1 (S ells et al., 1997) were subcloned from pJ3H (S ells and C hernoff, 1995) as SalI/EcoRI→ blunt) fragments into SalI/EcoR V-cut pTet-S plice (S hockett et al., 1999). These plasmids express NH2-terminal HA-tagged Pak1 when cells are grown in the absence of tetracycline. The tetracycline-regulated expression vectors pU M-Rac1 V12 and pU M-Rac1 N17 (both M yc-tagged; A nd A-pte et al., 1997) were kindly donated by M ark Symons (O nyx Corp., Richmond, CA).

Cell Culture

NIH-3T3 cells and derivatives were maintained in DME plus 10% calf serum (CS). The NIH-3T3 variant S2-6 (S hockett et al., 1995), bearing a tetracycline-regulated transactivator, was used to construct cell lines that indubitably express various forms of Pak1 or Rac1. To prepare stable, regulated clonal cell lines, S2-6 cells were cotransfected with either pTet-
Splice-Pak1 or pU M. R-ac1 plasmids plus a plasmid encoding a puromycin resistance gene using a calcium phosphate precipitation method (Chen and O'kayama, 1987). 48 h after transfection, the cells were selected in media containing 2.5 mM histidino (to retain the tetracycline-V-F16 transactivator), 2 μg/ml puromycin (to select for the tetracycline-regulated expression vector), and 1 μg/ml tetracycline (to repress transgene expression during selection). Clonal cells lines were isolated and expanded and at least 24 lines for each form of Pak1 or R-ac1 were examined for inducible transgene expression by anti-HA or anti-Myc immunoblot.

**Immunoblot**

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 50 mM NaF, 10 mM β-glycerophosphate, containing 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg per ml aprotinin. 10 μg extract was fractionated by 10% SDS/PAGE and transferred to polyvinyl difluoride (Immobilon) membranes. Membranes were blocked using fat-free milk, probed with antibodies, and developed using an alkaline-phosphatase-based chemiluminescent system (Duoset/New England Nuclear).

**Protein Kinase Assays**

HA-tagged Pak1 was immuno precipitated using mAbs 12CA5 (Babco) from 35-mm wells of S2-6 cells grown in tetracycline-free DME. The immunoprecipitates were washed three times in NP-40 lysis buffer, once in 0.5 M LiCl, and then once in protein kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT). The immunoprecipitates were then incubated at 30°C for 20 min in 25 μl protein kinase buffer containing 25 μM ATP plus 5 μCi γ-32P-ATP. The reactions were stopped by the addition of 5 μl 6x SDS/PAGE sample buffer, boiled for 5 min, then separated by 10% SDS/PAGE G E. The dried gel was then autoradiographed.

**Transient Transfection**

Cells were plated at 3.2 × 105 cells/35-mm culture dish in DME plus 10% CS and transfected with expression plasmids using a calcium phosphate precipitation method (Chen and O'kayama, 1987).

**Immunofluorescence Detection**

Cells cultured on coverslips were fixed in 3.5% formaldehyde, permeabilized in 0.1% Tween-20 and blocked with 3% BSA in PBS. A primary incubation with primary antibodies, the cells were stained with rhodamine X-conjugated goat anti-rabbit antibodies along with AMCA-conjugated and Cy3-conjugated anti-mouse antibodies. For staining of filamentous actin, 0.1 μM Oregon Green or FITC-conjugated phalloidin was included during incubation with the secondary antibodies. Confocal microscopy was performed using a Bio-Rad MRC 600 laser scanning confocal microscope.

**Tetracycline Titration**

Cells from one inducibly expressing clonal line for each of two constitutive active mutant forms of Pak were plated at 30% confluence into 35-mm wells with and without coverslips. 24 h later the cells were washed one time in PBS and re-fed with DME plus 10% CS alone or with one of the following: 1, 0.2, 0.1, 0.04, or 0.02 μg/ml tetracycline and incubated for another 8 h. At that time, the cells were again washed and re-fed with DME plus 0.2% CS, retaining the same tetracycline concentrations as 8 h earlier. 16 h later, HA-tagged Pak was detected in the cells on coverslips by indirect immunofluorescent staining, while the remaining cells were lysed in NP-40 lysis buffer and immunoblotted as described above.

**Motility Assays**

Pak1 expression was induced by growth in tetracycline-free DME plus 10% CS for 16 h. A filter protein induction, the cells were treated with trypsin, washed in the presence of soybean trypsin inhibitor and re-plated in tetracycline-free DME plus 0.1% BSA onto plates coated with 1% BSA for 30 min. Uninduced cells were treated in the same manner. The cells were then transferred to a plate coated with 50 μg/ml fibronectin and allowed to attach for 40 min. Phase contrast images were captured thereafter at 30-s intervals for calculation of cell speed and at 2-min intervals for long term analysis (4 h sampling). The cells were tracked with the aid of Image™ imaging software and the results were tabulated using Excel™.

**Results**

**Establishment of Stable Cell Lines That Inducibly Express Pak1 and Rac1 Mutants**

To assess the effects of Pak1 on motility, we established stable cell lines in which Pak1 expression is regulated by tetracycline. These cell lines included HA-tagged wild-type, constitutive-active, and kinase-dead mutants of Pak1, as well as Myc-tagged activated and dominant-negative forms of R-ac1 (Fig. 1 a). Two constitutive active forms of Pak1, one that binds Cdc42/Rac normally (Pak1E1423), and one that does not (Pak1E183,L86), were included in order to assess the role of p21 binding in any phenotypes induced by Pak1 expression (Sells et al., 1997). Transgene expression was tightly controlled by tetracycline, displaying 5-10-fold induction, as assessed by densitometry of anti-HA A immunoblot (Fig. 1 b and data not shown). Importantly, transgene expression was barely detectable in cells grown in tetracycline-containing media, even on prolonged exposure of immunoblot. As expected, immunoprecipitated Pak1E1423 displayed the most protein kinase activity, followed by Pak1E183,L86 (Fig 1 c). Neither wild-type nor kinase-dead forms of Pak1 displayed detectable kinase activity, consistent with previous results (Manter et al., 1997; Sells et al., 1997).

**Expression of Mutant Forms of Pak1 Modulates Cell Morphology, F-Actin Distribution, and Focal Complex Formation in NIH-3T3 Clonal Cell Lines**

We and others have previously shown that transient expression of Pak1 affects the structure of the actin cytoskeleton in Swiss 3T3 (Sells et al., 1997), HEla (Manter et al., 1997), and PC12 cells (Daniels et al., 1998). However, the effects of stable Pak1 expression on populations of cells have not been reported. Our tetracycline-regulatable cell lines permit large numbers of cells to be examined, each expressing similar levels of transgenic Pak1. In addition, we can titrate the level of Pak1 expression by varying the concentration of tetracycline. In induced NIH-3T3 cultures, wild-type Pak1 has only modest effects on F-actin distribution as we previously reported in transiently transfected Swiss 3T3 cells (Sells et al., 1997), although dorsal ruffles develop in a few (<20%) cells (Fig. 2 b). Expression of an activated Pak1 mutant, Pak1E183,L86 resulted in formation of not only dorsal ruffles but also of large, asymmetric lamellipodial structures characteristic of the leading edge of the cells (Fig. 2 c). These latter structures resemble those found in motile fibroblasts. Interestingly, asymmetric lamellipodia were seen in >50% of cells ex-
pressing another activated mutant, Pak1E423 (Fig. 2 d); the remaining cells have a morphology characterized by a marked loss of stress fibers and few lamellipodia similar to those observed upon transient transfection (Manser et al., 1997; Sells et al., 1997). That these differences are due to the different cell types used for these studies is unlikely, given that transient transfection of S2-6 cells with pCMV6M-Pak1E423 (the same vector used in previous studies with Swiss 3T3 cells; Sells et al., 1997), results in a flattened, stress fiber-free cell phenotype (data not shown). A more likely explanation for differences in cell morphology may relate to the very high expression levels achieved in transient transfection versus the more moderate, but stable levels attained in the tetracycline-regulated cells. In support of this latter hypothesis, when a small amount of tetracycline (0.04 μg/ml) was added to the medium, the population of cells as a whole produced lower levels of Pak1E423, with concomitantly higher percentage of the cells having asymmetric lamellipodia as Pak1 production decreased (50.4% lamellipodia in tetracycline-free medium; 76.1% lamellipodia in medium containing 0.04 μg/ml tetracycline; 800 cells counted in total).

Expression of a kinase-dead form of Pak1 also induced formation of lamellipodia, but, compared with cells expressing activated Pak1, these cells tended to have increased numbers of such structures dispersed throughout the cell body, giving the cells a multi-lobed appearance (Fig. 2 e). Often, lamellipodia accumulated on multiple sides of the cell, a phenotype that is virtually never observed in cells expressing activated forms of Pak1.

To ensure that the NIH-3T3 derivatives used in these studies respond to Rho-family GTPases like other commonly studied fibroblasts, we examined the cytoskeletal effects of Rac1 in the parental S2-6 NIH-3T3 cells. As in Swiss 3T3 cells (Ridley et al., 1992) and REF52 cells (Joneson et al., 1996), activated Rac1 induced cell flattening and circumferential lamellipodia, formation of cortical focal complexes, and a loss of central stress fibers (Fig. 2 f), while the dominant-negative version induced compact but elongated cells (data not shown). Thus, Rac1 expression gave rise to phenotypes similar to those described previously in Swiss 3T3 and REF52 cells, indicating that the S2-6 cells behave in a similar manner as these well-described cell systems.

The Morphologic Effects Produced by Overexpression of Pak1 in Fibroblasts Are Not Mediated by Rac1

We have previously argued that, in Swiss 3T3 cells, the effects of Pak1L83,L86 on F actin organization are independent of Rac1 (Sells et al., 1997). However, Obermeier et al. (1998) showed that Pak1L83,L86 induces neurite outgrowth
in PC12 cells, and that ~40% of these effects are inhibited by coexpression of a dominant-negative form of Rac1, implying that Pak1 may operate upstream of Rac1. To reexamine this issue in fibroblasts, we transiently transfected Rac1\textsuperscript{N17} into the tetracycline regulated cell line expressing Pak1\textsuperscript{L83,L86}. Cell expressing Pak1\textsuperscript{L83,L86} alone have, as expected, large asymmetric lamellipodia (Fig. 3 a, lower cell). Coexpression of Rac1\textsuperscript{N17} has no apparent effect on this phenotype (Fig. 3 a, upper cell). In cells that lack Pak1\textsuperscript{L83,L86} expression (i.e., those grown in the presence of tetracycline), Rac1\textsuperscript{N17} induces cell shrinkage, indicating that this protein is biologically active (data not shown). To quantitate these effects, we scored the phenotypes of 400 cells per group grown in the presence or absence of tetracycline with or without coexpressed Rac1\textsuperscript{N17}. As expected, when grown in the presence of tetracycline, few cells display lamellipodia. Upon induction, ~91% of cells expressing Pak1\textsuperscript{L83,L86} alone, and 90% of cells coexpressing Rac1\textsuperscript{N17} display characteristic lamellipodial structures (Fig. 3 b). A second activated Pak1 mutant, Pak1\textsuperscript{E423}, was less potent in inducing lamellipodia (56% of cells), but, as with Pak1\textsuperscript{L83,L86}, coexpression of Rac1\textsuperscript{N17} did not inhibit formation of these structures. The results of these studies show that Rac1\textsuperscript{N17} has very little effect on the phenotype elicited by Pak1\textsuperscript{L83,L86} or Pak1\textsuperscript{E423}, and therefore, in this cell type, the effects of these activated Pak1 mutants on F-actin organization are independent of Rac1.

**Expression of Pak1 Regulates Random Cell Locomotion**

Because the morphology induced by activated Pak1 mimics that seen in motile fibroblasts, we investigated the role of this enzyme in regulating cell movement. A thorough control of cell motility at the molecular level is poorly understood, it has been shown in a number of systems that Ras, and related GTPases of the Rho family clearly play important roles in this process (Ridley et al., 1995; Keely et al., 1997; Sander et al., 1998). We assessed the effect of Pak1 expression on cell motility by tracking the movement of individual cells at 30-s intervals over a 1-h time course after their attachment to fibronectin-coated dishes (Fig. 4). In the presence of tetracycline, the cell lines showed minor differences in basal movement rate (16.51 ± 0.85 nm/s for control cells, 12.49 ± 0.66, 12.08 ± 0.56, 14.67 ± 0.75, and 13.86 ± 0.60 nm/s for wild-type Pak1, Pak1\textsuperscript{L83,L86}, Pak1\textsuperscript{E423}, and Pak1\textsuperscript{R299} expressing cell lines, respectively; Fig. 4). These differences probably reflect intrinsic clonal variation. However, in the absence of tetracycline, the average control cell speed increased by only 0.24 nm/s, never attaining the speed of Pak1-expressing cells, even though these latter cells have a lower basal speed. In fact, the average speed of cells expressing wild-type Pak1, Pak1\textsuperscript{L83,L86}, Pak1\textsuperscript{E423}, and Pak1\textsuperscript{R299} expressing cell lines, respectively: Fig. 4). These differences probably reflect intrinsic clonal variation. However, in the absence of tetracycline, the average control cell speed increased by only 0.24 nm/s, never attaining the speed of Pak1-expressing cells, even though these latter cells have a lower basal speed. In fact, the average speed of cells expressing wild-type Pak1, Pak1\textsuperscript{L83,L86}, Pak1\textsuperscript{E423}, and Pak1\textsuperscript{R299} increased to 17.03 ± 0.91, 17.58 ± 0.83, 21.06 ± 0.88, and 23.25 ± 1.07, respectively, when assayed in the absence of tetracycline. These data show that increased Pak1 levels cause an increase in cell movement, and that this increase is independent of protein kinase activity.

**Directional Cell Movement Is Affected by Pak1 Kinase Activity**

Interestingly, although random cell motility is independent of Pak1’s catalytic function, directional movement strongly correlates with kinase activity. 4-h cell tracking...
experiments indicate that cells expressing kinase-dead Pak1, though highly motile, display poor persistence of movement compared with wild-type or activated forms of Pak1 (Fig. 5). In contrast to cells expressing activated Pak1 move in relatively straight paths (Fig. 6 b and d), whereas those expressing kinase-dead Pak1 move randomly (Fig 6 f). We speculated that these different types of movement, which are related to Pak1’s protein kinase activity, might be reflected in other measurements of directional motility. In agreement with this prediction, cells expressing activated forms of Pak1 display increased haptotactic movement in response to an immobilized collagen gradient, whereas cells expressing kinase-inactive Pak1 show slightly decreased haptotaxis (Fig. 7). As a control, we also assessed haptotaxis in Rac1-expressing cells. Activated Rac1 enhances haptotactic movement, whereas a dominant-negative form inhibits this directional movement (data not shown). Interestingly, neither Pak1 nor Rac1 affect chemotaxis towards soluble fibronectin (data not shown and Anand-Apte et al., 1997), suggesting that this signaling pathway is regulated by a different set of proteins.

Pak1 Induces Phosphorylation of MLC at Serine 19

The activation of MLC by phosphorylation has been correlated with cell motility (Wilson et al., 1991, 1992; Matsumura et al., 1998), perhaps due to its role in retracting nondominant pseudopodia (Bailly et al., 1998b). Pak1 has been reported to affect myosin function in a variety of organisms (Brzeska et al., 1996, 1997; Lee et al., 1996; Wu et al., 1996), and mammalian Paks have been shown to phosphorylate MLC at serine 19 in vitro (Ramos et al., 1997; Chew et al., 1998; Van Eyk et al., 1998). We therefore asked whether Pak1 expression in fibroblasts results in increased MLC phosphorylation. For these studies we used an antibody that specifically recognizes the serine-19 phosphorylated form of MLC (Matsumura et al., 1998).

Lysates from control cells, or cells expressing WT, activated, or kinase-dead forms of Pak1 were examined by immunoblot for protein expression, MAPK and SAPK activity, and phospho-MLC content. These studies show that activated forms of Pak1, like Rac1, induce activation of a SAPK (Jnk) but not a MAPK (Erk1) pathway (Fig. 8). These results are in agreement with most published studies, which show modest Jnk and p38 activation by activated forms of Pak (reviewed in Sells and Chernoff, 1997). As expected, control cells treated with growth-stimuli such as PDGF or LPA show Erk1 activation, while cells exposed to a hyperosmotic shock show Jnk activation, demonstrating that signaling pathways in the S2-6 cell lines are generally similar to those observed in most other fibroblasts. Phospho-MLC is apparent in growth-factor treated cells, as well as in cells expressing activated forms of Rac1 or Pak1, but not in control cells or cells expressing a kinase-dead form of Pak1. These results indicate that activated Pak1 induces MLC phosphorylation and that this phosphorylation is not mediated via downstream activation of Erk1.

Figure 3. Pak1-induced cytoskeletal changes are not mediated by activation of Rac1. (a) Cells conditionally expressing activated Pak1 were transfected with an constitutive expression vector for a dominant-negative (N17) form of Rac1. Pak1 expression was induced by growth in tetracycline-free DME plus 10% CS for 8 h, followed by 16 h starvation in tetracycline-free DME plus 0.5% CS. The cells were then fixed and stained for immunofluorescence. HA-tagged Pak1L83,L86 (blue), Myc-tagged Rac1N17 (red), F-actin (green). Bar, 25 μm. (b) At least 400 cells expressing Pak1 or Pak1 plus Rac1 were assessed for presence of lamellipodia. Values represent combined data from three separate experiments.

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We also used the phospho-MLC antibody to localize activated MLC by immunofluorescence. Cells inducibly expressing an activated form of Pak1 were grown in the presence or absence of tetracycline for 16 h before fixation. These experiments show that cells expressing activated forms of Pak1 induce an accumulation and localization of phospho-MLC to the lamellipodia (Fig. 9). In nonexpressing cells, phospho-MLC levels are low and are found mainly in a perinuclear location (Fig. 9 a, left and right panels). In Pak1-expressing cells, the amount of total phospho-MLC is apparently significantly increased and a substantial fraction of this protein now localizes just beneath the leading edge of lamellipodia (Fig. 9 b, left and right panels). These results are consistent with a previous study which showed that phospho-MLC accumulates in lamellipodia in locomoting cells (Matsumura et al., 1998).

Discussion

The p21-activated kinases comprise a family of 62–68-kD serine/threonine kinases. These enzymes are catalytically activated by binding specifically to activated forms of Cdc42 and Rac1, two GTPases of the Rho subfamily, thus making Paks good candidate effectors for these signaling molecules (Lim et al., 1996; Sells and Chernoff, 1997). In fact, a number of the Paks have been implicated as the downstream effectors for several Cdc42 and Rac1 regulated signaling pathways including modulation of the actin cytoskeleton and establishment of cell polarity (Cvrckova et al., 1995; Marcus et al., 1995; Ottilie et al., 1995; Manser et al., 1997; Sells et al., 1997), processes that are required for cell motility. Although it has been shown recently that both Cdc42 and Rac1 regulate motility and invasiveness of epithelial cells (Hordijk et al., 1997; Keely et al., 1997), and that Rac1 modulates growth factor–driven chemotaxis in fibroblasts and macrophages (Anand-Apte et al., 1997; Allen et al., 1998) whether any of the Paks play a role in regulating these processes has not been established.

Pak1 has two types of effects on cell morphology, one related to its protein kinase activity and one that is kinase independent (Manser et al., 1997; Sells et al., 1997). We and others have previously shown that at least some of Pak’s kinase-independent effects on the actin cytoskeleton are mediated by one or more SH3-containing proteins that associate with proline-rich regions located in the NH₂-terminal half of Pak1 (Sells et al., 1997, Daniels et al., 1998; Obermeier et al., 1998). These SH3-containing proteins include, but are not necessarily limited to, the adaptor Nck and the guanine-nucleotide exchange factor PIX and its relatives (Bokoch et al., 1996; Galisteo et al., 1996; Lu et al., 1996).
Figure 5. Pak1 kinase activity affects character of movement. Pak1 expression was induced by growth in tetracycline-free DME as in Fig. 4. After attachment to fibronectin-coated plates, individual cells were tracked. Phase contrast micrographs shown were taken at 30-min intervals. (a) control cells; (b) wild-type Pak1, (c) Pak1<sup>L83,L86</sup>, (d) Pak1<sup>E423</sup>, (e) Pak1<sup>R299</sup> (the cell denoted by an arrow displayed from two to four lamellipodia throughout the course of the experiment), (f) Rac1<sup>V12</sup>, and (g) Rac1<sup>N17</sup>. Arrows mark the track of a single motile cell for each sample. Bar, 50 μm.
Because PIX is an activator of Rac1, Obermeier et al., have proposed that the kinase-independent activity of Paks on actin polymerization is mediated by Rac1 (Obermeier et al., 1998). In support of this model, they have shown that the kinase-independent effects of Pak3 on neurite extension in PC12 cells are partially inhibited by a dominant-negative from of Rac1. In contrast, we have argued that, in microinjected Swiss 3T3 cells, the kinase-independent effects of Pak1 are not inhibited by dominant-negative Rac1 (Sells et al., 1997). Possibly, cell-type differences, or the isoforms of Paks studied, account for these apparent discrepancies. However, it should be noted that less than half of Pak3’s effects in PC12 cells are blocked by dominant-negative.
Rac1, that a nearly identical degree of inhibition is seen in control cells, and that dominant-negative Cdc42 has an even more inhibitory effect (Obermeier et al., 1998). Therefore, it is not clear if or how Pak3 functionally links Cdc42 to Rac1. Whatever the situation in PC12 cells, our present data show that dominant-negative Rac1 has no measurable effect on Pak1-driven actin reorganization in NIH-3T3 cells. Therefore, at least in cells of mesenchymal origin, the kinase-independent effects of Pak1 do not require Rac1.

As with cell morphology, the effects of Pak1 on cell movement also have a kinase-dependent and independent component. Overexpression of wild-type or mutant forms of Pak1 all increase cell movement, but protein kinase activity affects the persistence of these movements. Cells expressing kinase-dead Pak1 are characterized by multiple, randomly arrayed, lamellipodia, that continuously form and recycle. These cells move more quickly than controls, but do so in a haphazard manner, frequently and abruptly switching direction. In contrast, cells expressing wild-type or activated forms of Pak1 are characterized by a single lamellipodium at the leading edge. These cells move more quickly than controls, and also display increased persistence. As mentioned above, these effects are likely to be mediated by interactions between the NH2-terminal, non-catalytic, domain of Pak1 and other proteins that affect actin polymerization. Although our data argue against the possibility that Rac1 acts downstream of Pak1, it is possible that other GTPases that affect actin organization, such as Arf6, might be activated by Pak1 (Radhakrishna et al., 1999). Activation of this or a similar GTPase might account for the kinase-independent ruffling induced by Pak1. A infinity to Pak1 by itself, however, is insufficient for directed cell movement. In gradient-directed cell movement, one or more pseudopodia become dominant, while the others are suppressed, and these dominant pseudopodia are stabilized as leading edge lamellipodia, whose firm attachment to the substratum are thought to provide an anchorage point for contractile forces. The process of selection, generation, and propagation of a leading edge lamellipodium from a group of random pseudopodia is poorly understood, but may involve stabilization of filaments through the formation of strong focal contacts. In addition, nonadherent extensions may be actively suppressed once a leading edge lamellipodium is established (Bailly et al., 1998a; Wyckoff et al., 1998). In NIH-3T3 cells, expression of activated, but not kinase-dead, Pak1 results in large, polarized lamellipodia and persistent cell movement. Phospho-MLC is increased, both in the trailing edge and at the leading edge of the motile cells. A similar distribution has been noted previously in other motile cells (DeBiasio et al., 1996; Matsumura et al., 1998). Phosphorylation at the tail is thought to be responsible for pushing the cell body forward by tail contraction. The function of phosphorylated MLC at the leading edge is less clear, but may contribute to the membrane extensions at the leading edges, either by translocating actin filaments to this location or by suppressing lamellipodia formation in the absence of the reinforcement provided by stable focal contact (Goeckeleer...
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According to this scenario, active Pak1 may concentrate at the incipient leading edge in resting cells, perhaps directed there by the exchange factor PIX, where it acts upon myosin II, augmenting the polarity of the cell. This might explain the effects of active Pak1 on directional cell movement. Whatever the exact function of myosin II in locomoting cells, our data are consistent with the notion that Pak1 has two types of effects on cell morphology and motility; a kinase-independent activity that affects actin polymerization, and a kinase-dependent function that increases phospho-MLC levels and stabilizes leading edge lamellipodia.

How does Pak1 affect the phosphorylation state of the MLC? The effect of Pak1 on MLC may be direct, as Pak1 has been shown to phosphorylate serine 19 of the MLC in vitro (Ramos et al., 1997; Chew et al., 1998; Van Eyk et al., 1998). Alternatively, Pak1 might induce MLC phosphorylation indirectly, via activation of MAPK or Rho kinase, both of which affect MLC kinase activity (Amano et al., 1996; Klemke et al., 1997). Although Paks are usually considered to be activators of SAKs such as Jnk and p38, rather than of MAPKs such as Erk1 and -2 (Kiriakis and Alve, 1996; Sells and Chernoff, 1997), Pak2 has recently been shown to phosphorylate Raf-1 (King et al., 1998). (The Pak isoform that phosphorylates Raf is actually Pak2, not Pak3 as stated in King et al., 1998. This error in nomenclature can be traced to mistakes in the GenBank annotations for γ-Pak, which is also known as Pak2 (see Sells and Chernoff, 1997). This phosphorylation is required for efficient Raf-1 activation. In addition, Pak1 phosphorylates MEK on serine 298, increasing its affinity for its activator, Raf-1 (Frost et al., 1997). These data suggest that Pak1 activity positively regulates the MAPK pathway. A s activation of MAPK is associated with increased MLC phosphorylation and cell movement (Klemke et al., 1997), this pathway might explain Pak1’s effects on motility. However, in our inducible cell lines, neither wild-type nor mutant forms of Pak1 appreciably stimulate Erk1 or -2, while activated forms do modestly activate Jnk. Therefore, activation of MLC via MAPK is cannot explain our findings. Alternatively, since Pak1 is associated with PIX, a guanine-nucleotide exchange factor for Rac1 (Maner et al., 1998), Pak1 might activate Rho kinase through a GTPases cascade (Cdc42 to Rac1 to RhoA) (Nobes and Hall, 1995). However, coexpression of a dominant-negative form of Rac1 did not interfere with Pak1-induced cell polarity changes, making it unlikely that its motility effects are mediated through a known GTPase cascade. Therefore, if Pak2 activates Rok, it is not likely via downstream activation of Rac1 and RhoA. In this regard, it is interesting to note that fission yeast Pak1p has recently been shown to operate upstream of the Rok homologue Orb6p (Verde et al., 1998). Whether fission yeast Rho1p is interposed between Pak1p and Orb6p is not known, but these data do reinforce the notion that some of Pak’s effects on myosin phosphorylation might be mediated by Rok. Therefore, at present we are uncertain if Pak1 directly phosphorylates MLC or does so indirectly through Rho, Rok, and/or MLC. These possibilities may be distinguishable through the use of C3 toxin or dominant-negative forms of RhoA or Rok.

We thank Fumio Matsumura for his kind gift of anti-phospho-MLC antibodies, Sybil Genther and Amanda Pfaff for technical assistance, Joshua Figure 9. Pak1-induced phosphorylated MLC localizes to leading edge lamellipodia and to stress fibers. Immunofluorescent staining of a stable clonal line expressing a constitutively active form of Pak1 (Pak1<sub>L83,L86</sub>) in the presence (+tet; row a) or absence (−tet; row b) of tetracycline was performed after starvation overnight in media containing 0.5% CS. A row indicates punctate staining in edge ruffle. MLC phosphorylation (P-MLC) was detected by a polyclonal antibody specific for phospho-MLC (left and right panels, blue stain). Phalloidin stained F-actin is shown in the middle and right panels (green stain) whereas Pak1 expression is shown in the right panel (red stain). Bar, 25 μm.
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