Temporal and Spatial Distribution of Activated Pak1 in Fibroblasts

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Abstract. p21-activated kinases (Paks) are effectors of the small GTPases Cdc42 and Rac, and are thought to mediate some of the cytoskeletal and transcriptional activities of these proteins. To localize activated Pak1 in cells, we developed an antibody directed against a phosphopeptide that is contained within the activation loop of Pak1. This antibody specifically recognizes the activated form of Pak1. Immunofluorescence analysis of NIH-3T3 cells coexpressing activated Cdc42 or Rac1 plus wild-type Pak1 shows that activated Pak1 accumulates at sites of focal adhesion, throughout filopodia and within the body and edges of lamellipodia. Platelet-derived growth factor stimulation of NIH-3T3 cells shows a pattern of Pak1 activation similar to that observed with Rac1. During closure of a fibroblast monolayer wound, Pak1 is rapidly activated and localizes to the leading edge of motile cells, then gradually tapers off as the wound closes. The activation of Pak1 by wounding is blocked by inhibitors of phosphatidylinositol 3-kinase, and Src family kinases, but not by an inhibitor of the epidermal growth factor receptor. These findings indicate that activated Pak1, and by extension, probably activated Cdc42 or Rac, accumulates at sites of cortical actin remodeling in motile fibroblasts.

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

Introduction

Cell motility is a complex and dynamic process. Portions of the molecular mechanisms that link extracellular stimuli to the remodeling of actin and to changes in cell motility have recently come into view. Small GTPases of the Rho family, such as Cdc42, Rac, and Rho, are key regulators of actin assembly and control the formation of filopodia, lamellipodia, and stress fibers, respectively (Hall, 1998). The proximal means by which Cdc42, Rac, and Rho small GTPases regulate actin dynamics and cell motility are presumably related to the suite of effector proteins recruited by these GTPases. Among these effectors, the p21-activated protein kinases (Paks)[1] have come under particular scrutiny due to a growing body of genetic and biochemical data implicating Paks in regulating the structure of the actin cytoskeleton.

Paks are a highly conserved group of enzymes that act as effectors for the Rho family GTPases Cdc42 and Rac (Lim et al., 1996; Sells and Chernoff, 1997; Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999). Paks specifically associate with the activated (GTP-bound) forms of Cdc42 and Rac and are thought to mediate some of their effects on actin organization, motility, adhesion, and gene transcription. Upon binding to these GTPases, Pak undergoes a conformational change, accompanied by autophosphorylation at several sites and a marked increase in kinase activity towards exogenous substrates. In mammalian cells, these substrates include LIM kinase (Edwards et al., 1999), myosin light chain kinase (Sanders et al., 1999), Bad (Schurmann et al., 2000), Raf (King et al., 1998), and Mek (Frost et al., 1997), and undoubtedly other proteins that remain to be discovered.

In resting cells, Pak1 is localized to membranous structures within the cytoplasm (Dharmawardhane et al., 1997). Upon stimulation of cells by growth factors, Pak is recruited to focal adhesions and sites of membrane ruffles, although the activity of Pak in any of these locations is not known (Dharmawardhane et al., 1997; Manser et al., 1997). As Pak1 itself lacks a focal adhesion targeting motif, it is likely that Pak1 is brought to these locations by its binding partners. These proteins include the guanine nucleotide exchange factor PIX (Manser et al., 1998), which binds to a linker protein known as $p95^{Pkl}$ that in turn binds to the focal adhesion protein paxillin (Turner et al., 1999). In addition, Pak1 may be brought to the plasma membrane and to focal adhesion complexes via its association with the adaptor protein Nck (Bagrodia et al., 1995; Bokoch et al., 1996; Galisteo et al., 1996; Hing et al., 1999), which binds to activated receptor tyrosine kinases as well as to tyrosine phosphorylated focal adhesion proteins such as Fak, $p130^{Cas}$, and HEF1 (Minegishi et al., 1996; Law et al., 1996; Schlaepfer et al., 1997; Tu et al., 1999).

We have developed an antibody that reacts specifically with an autophosphorylated, activated form of Pak1, and here report the use of this reagent to reveal the spatial and

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[1]*Abbreviations used in this paper:* CA, constitutive-active; Erk, extracellular signal–regulated kinase; GC, germinal center; HA, hemagglutinin; KD, kinase-dead; Pak, p21-activated kinase; PI3 kinase, phosphatidylinositol 3-kinase; RMLC, regulatory myosin light chain.

temporal distribution of activated Pak1 in cells. We show that activated Pak1 accumulates at focal adhesions, throughout filopodia, and in the body and edges of lamellipodia. Further, we are able to document the dynamic behavior of Pak1 activation during cell movement after wounding of a confluent fibroblast monolayer, and to dissect the signaling elements that mediate Pak1 activation by wound healing.

Materials and Methods

Materials

Polyclonal antibodies against Pak1 (C19) were obtained from Santa Cruz Biotechnology, Inc. Monoclonal antibodies against Myc (9E10) and hemagglutinin (HA; 12CA5) were from Santa Cruz Biotechnology, Inc. and BabCo, respectively, antipaxillin was from Transduction Laboratories, and LY294002, tyrphostin AG1478, and PP1 were obtained from Calbiochem.

Phospho-specific Antibodies

The phosphopeptide SKRST(P)MVGTPYC, derived from amino acid residues 419–429 in human Pak1 (Sells et al., 1997), was synthesized and coupled to keyhole limpet hemocyanin via a COOH-terminal linkage. The coupled peptide was injected into rabbits and the resulting antisera purified by protein A–Sepharose chromatography. The sera were then affinity purified, first by passage over a nonphosphorylated peptide (EQSKRST-MVGTPYWMAPEVVTRK) column to remove antibodies that react with nonphosphorylated epitopes, then by passage over a phosphopeptide column. The sera were eluted in 1-ml fractions from this column with 0.1 M glycine, pH 2.5, immediately neutralized by the addition of 50 μl 1 M Tris, pH 9.5, then extensively dialyzed against PBS.

Protein Production

His-tagged, activated (L107F) and inactive (K299A) human Pak1 (a gift from P. Diamond, Harvard University, Cambridge, MA) were produced via baculovirus infection of Sf9 cells and purified by metal chelate chromatography. The purified proteins were dialyzed against PBS and stored at a concentration of 1 mg/ml in 10-μl aliquots at −80°C. Mst2 was similarly produced, but not purified, from a baculovirus-infected Sf9 cell lysate (a gift from A. Abo, Onyx Pharmaceuticals, Richmond, CA).

Cell Culture

NIH-3T3 S2-6 cells (Shockett et al., 1995) expressing wild-type or mutant forms of Pak1 under a tetracycline-regulated promoter were maintained in DME plus 10% calf serum, 2.5 mM histidinol, 2 μg/ml puromycin, and 1 mg/ml tetracycline (Sells et al., 1999). COS1 cells were grown in DME, 10% fetal bovine serum containing 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml kanamycin. Where indicated, cells were transfected using lipofectamine (GIBCO BRL) according to the manufacturer's protocol.

PDGF Stimulation

NIH-3T3 S2-6 cells (Shockett et al., 1995) expressing wild-type Pak1 under a tetracycline-regulated promoter were induced for Pak1 expression, starved in 0.2% calf serum for 16 h, and then stimulated with 30 ng/ml PDGF for 5 or 10 min. The cells were then fixed and stained as indicated below.

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-glycerol-phosphate) 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 chemiluminescence system (Dupont/New England Nuclear).

Protein Kinase Assays

0.5 μg recombinant Pak1 or Mst2, purified from baculovirus-infected Sf9 cells, was incubated in 25 μl protein kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) containing 25 μM ATP plus 5 μCi [γ-^{32}P]ATP at 30°C for 20 min. The reactions were stopped by the addition of 5 μl 6× SDS-PAGE sample buffer, boiled for 5 min, then separated by 10% SDS-PAGE. The dried gel was then autoradiographed.

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. After incubation with primary antibodies, the cells were stained with Rhodamine X–conjugated goat anti–rabbit antibodies along with AMCA-conjugated and Cy5-conjugated goat anti–mouse antibodies. For staining of F-actin, 0.05 μM Oregon green–conjugated phalloidin was included along with secondary antibodies. Confocal microscopy was performed using a Bio-Rad Laboratories MRC 600 laser scanning confocal microscope. Other photomicrographs were obtained using a Quantix CCD and the Isee™ imaging program from Inovision. This same system was used for gray scale quantitation.

Cell Wounding Assays

Marginally subconfluent cells were induced for 36 h and starved for 24 h in DME plus 0.5% calf serum. After these treatments, the confluent monolayer of cells was wounded by scraping a narrow 200 μl Rainin Pipetman tip across a 100-mm plate 4 times (in experiments where cells were plated onto coverslips) or 60 times (for cells plated onto 100-mm plates for Pak1 autophosphorylation assay). For inhibitor studies, LY294002 (25 μM), PP1 (10 μM), or tyrphostin AG1478 (10 μM) was added 10 min before wounding. For activity assays, cells were lysed at appropriate times thereafter in NP-40 lysis buffer and assayed for the presence of autophosphorylated Pak1 by immunoblot.

Results

Production and Specificity of Phospho-specific Anti-Pak Sera

Upon activation by Cdc42, Rac, or sphingolipids, Pak1 is known to autophosphorylate at several sites, including threonine 423 within the activation loop of the kinase (Manser et al., 1997; Zenke et al., 1999). Phosphorylation at threonine 423 is strongly correlated with activation, and substitution of the acidic residue glutamic acid at this site yields a constitutively active enzyme (Manser et al., 1997; Sells et al., 1997). To create a reagent that could be used to identify only activated Pak, we raised antibodies against a phosphorylated peptide that encompassed the activation loop of human Pak1 (Fig. 1 A).

To demonstrate the specificity of this reagent, we incubated purified constitutive-active (CA) or inactive, kinase-dead (KD) Pak1 in protein kinase buffer in the presence of ATP, then performed immunoblots using anti-Pak1 and antiphospho-Pak, as well as autoradiography to detect phosphorylated proteins (Fig. 1 B). While both forms of the protein were detected with anti-Pak1, only activated Pak1 was detected using the phospho-specific antibody, despite the large amount of recombinant protein used in this assay. Similar results were obtained when purified wild-type Pak1 was activated in vitro with GTP-Cdc42 (Fig. 1 C). These results show that the phospho-Pak antisera recognize active, autophosphorylated Pakl, but are not able to recognize inactive, nonautophosphorylated Pak1.

The sensitivity of the phospho-Pak sera in immunoblots compares favorably with a widely used commercial anti-Pak1 reagent. Decreasing amounts of phosphorylated active and nonphosphorylated inactive Pak1 were probed with both anti-Pak1 and antiphospho-Pak. The results show that antiphospho-Pak detects as little as 20 ng of activated Pak1 protein in immunoblots (Fig. 1 D).

Because members of the Pak and germinal center (GC) kinase family share a nearly identical activation loop se-

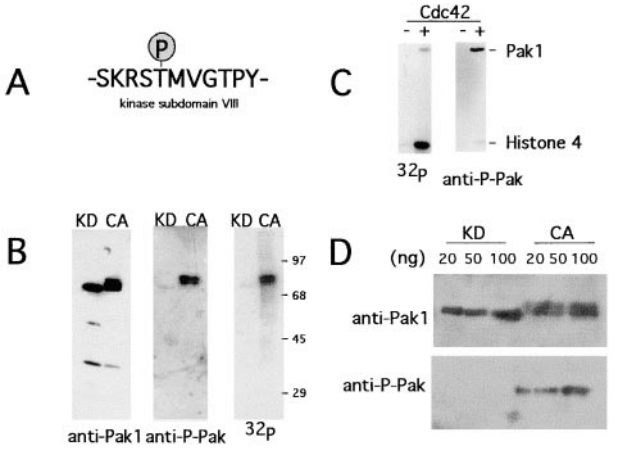


Figure 1. Antiphospho-Pak1 antibody specifically recognizes activated Pak1. (A) Activation loop sequence of the phosphorylated peptide used for production of polyclonal antiphospho-Pak1 antisera. (B) Immunoblots (lanes 1–4) and radiolabeled kinase reaction products (lanes 5 and 6) of baculovirus-produced Pak1. Purified KD or CA Pak1 was incubated in protein kinase buffer in the presence of ATP, then immunoblotted using anti-Pak1 (lanes 1 and 2) and antiphospho-Pak antisera (lanes 3 and 4) autoradiographed to detect antibody-bound proteins. Although both forms of the protein were detected with anti-Pak1, only activated Pak1 was detected using the phospho-specific antibody, despite the large amount of recombinant protein used in this assay. Lanes 5 and 6 show autoradiographed PAGE gel of reaction products from a kinase assay performed in the presence of [γ-^{32}P]ATP. Molecular mass in kilodaltons is shown to the right of the blot. (C) Comparison between immunoblot and radiolabeled kinase reaction products of Pak1 activated by Cdc42. Purified baculovirally produced Pak1 was incubated with or without activated Cdc42, as indicated. The kinase was then incubated in protein kinase buffer, containing histone 4 as substrate, in the presence of [γ-^{32}P]ATP. An immunoblot and autoradiogram are shown. (D) Sensitivity of antiphospho-Pak sera. 20–100 ng of KD and CA Pak1 were probed with both anti-Pak1 and antiphospho-Pak.

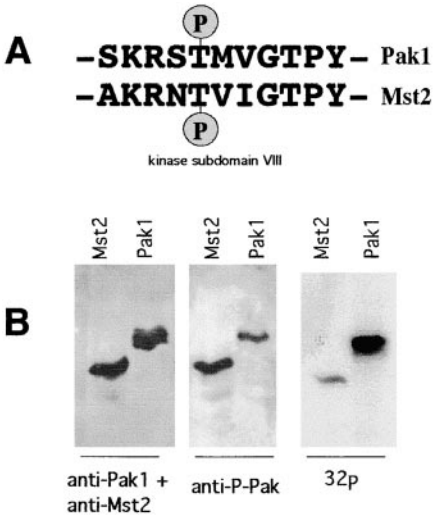


Figure 2. Cross-reactivity with Pak-related kinases. (A) Comparison of activation loop sequences in Pak1 and Mst2. (B) Purified, autophosphorylated recombinant Pak1 and Mst2 were assayed and probed as in the legend to Fig. 1.

quence (Fig. 2 A), we asked whether the phospho-Pak sera might cross-react with activated forms of these kinases (Fig. 2 B). Autophosphorylated Mst2 (a member of the GC kinase group) (Creasy and Chernoff, 1995; Sells and Chernoff, 1997) was recognized by the antiphospho-Pak sera, demonstrating that this antibody is not absolutely specific for Pak1. As expected, these sera also cross-reacted with Pak2, which has an identical activation loop, but not with protein kinase D–activated Akt, which bears a similar, but not identical sequence at this site (data not shown).

Antiphospho-Pak Sera Detects Activated Pak1 in Cell Lysates

We next asked if the antiphospho-Pak sera could be used to detect activated Pak1 in cell lysates. COS cells were cotransfected with Myc-tagged Pak1 plus HA-tagged wild-type or activated Cdc42, activated Rac1, or activated RhoA, and lysates were probed by immunoblot with anti-Myc (to detect exogenous Pak1), antiphospho-Pak, and anti-HA (to detect the GTPases) (Fig. 3, A and B). In cells expressing Myc-tagged Pak1 but lacking activated exogenous GTPases, faint bands of ~47 and 53 kD are detected upon blotting with antiphospho-Pak (Fig. 3 A, lanes 1, 3, and 5). The intensity of these bands does not change upon coexpression of activated GTPases (lanes 2, 4, and 6), nor is the intensity decreased in cells not expressing exogenous Pak1 (not shown), indicating that they are unlikely to represent Paks. The antiphospho-Pak blot clearly detects an ~70-kD band in cells transfected with activated (L61) forms of Cdc42 (Fig. 3 A, lane 2) and Rac1 (Fig. 3 A, lane 4), but not with an activated (L63) form of RhoA (Fig. 3 A, lane 6). Anti-Myc blots reveal upshifted bands in the same lanes, consistent with autophosphorylation of Pak1. These data are consistent with the known properties of Pak1: it binds to, and is activated by, Cdc42 and Rac but not RhoA (Manser et al., 1994). In cells transfected with activated Cdc42, but not Myc-tagged Pak1, only the background 47- and 53-kD signals are seen with antiphospho-Pak (data not shown). The amount of ~70-kD band detected by the antiphospho-Pak in lysates from COS cells cotransfected with Pak1 plus wild-type Cdc42 (Fig. 3 B, lane 1) is dramatically less than that detected from cells cotransfected with Pak1 and activated Cdc42 (Fig. 3 B, lane 2), consistent with the well-established fact that Cdc42 must be activated in order to activate Pak1.

These results show that antiphospho-Pak can recognize exogenous activated Pak1 in cell lysates. Although the antiphospho-Pak antibody is not completely specific for activated Pak1, our results also show that, under basal conditions, endogenous Pak1, as well as other Paks, is either inactive or not present in sufficient quantity in cell lysates to be recognized by these sera. The 47- and 53-kD proteins detected by the phospho-Pak sera might represent members of the related GC kinase family. However, these signals do not change in response to GTPase expression (Fig. 3 A) or other stimuli that we tested, such as growth factor stimulation or monolayer wounding (see below). Therefore, in lysates from transfected COS cells, positive signals can be attributed to the transgenic Pak1 rather than to other endogenous protein kinases in the Pak/GC kinase family.

Cotransfection of Wild-Type Pak1 and Activated Cdc42 or Rac Stimulates Pak1 Phosphorylation in Cortical Actin-based Structures

We have previously developed tetracycline-regulated NIH-3T3 cells lines that inducibly express Pak1 (Sells et

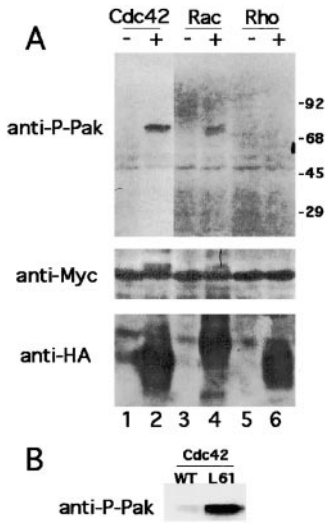


Figure 3. Antiphospho-Pak sera detects activated Pak1 in cell lysates. (A) COS1 cells were transfected with wild-type Pak1 alone (lanes 1, 3, and 5), or wild-type Pak1 plus activated Cdc42 (lane 2), Rac1 (lane 4), or RhoA (lane 6). Lysates were probed with anti-Myc (to detect exogenous Pak1), antiphospho-Pak, and anti-HA (to detect the GTPases). (B) COS1 cells were cotransfected with wild-type Pak1 in combination with wild-type (WT) (lane 1) or activated (L61) Cdc42 (lane 2). An immunoblot of cell lysates was probed with antiphospho-Pak.

al., 1999). These cells, as well as cells that inducibly express wild-type Pak1 plus activated Cdc42 or Rac1, were used to determine the subcellular location of activated Pak1. In control cells, little phospho-Pak1 is detected by immunofluorescence, consistent with the low basal activity of this enzyme (Fig. 4, A, D, and G). Cells coexpressing Pak1 plus activated Rac1 develop extensive lamellipodia and membrane ruffles, with activated Pak1 concentrated primarily within the edges and body of these structures (Fig. 4, B, E, and H). Similar experiments with cells expressing Pak1 plus activated Cdc42 develop microspikes and filopodia, with phospho-Pak1 throughout the filopodia (Fig. 4, C, F, and I). Pseudocoloration based on fluorescence intensity of sagittal confocal images of these cells defines the highest concentrations of phospho-Pak1 to be in sites of dynamic cortical actin remodeling (Fig. 4, G–I).

PDGF Stimulates Pak1 Phosphorylation within Lamellipodia and Focal Adhesions

PDGF is known to activate Pak1 (Dharmawardhane et al., 1997). Using the phospho-Pak antibody, we confirmed this result in NIH-3T3 S2-6 cells expressing wild-type Pak1, showing that maximal activation of an ~70-kD band, comigrating with Myc-Pak1, occurs at ~5 min after treatment (Fig. 5 A). No other PDGF-stimulated signals were detected by the antiphospho-Pak sera (not shown). To assess the distribution of activated Pak1 in response to growth factors, we treated starved NIH-3T3 S2-6 cells expressing wild-type Pak1 with PDGF for 5 min, then determined the location of total and activated Pak1 by immunofluorescence (Fig. 5 B). PDGF induces the formation of lamellipodia. Whereas total Pak is expressed throughout the cytoplasm (Fig. 5 B, panels c and g), the highest levels of activated Pak1 (Fig. 5 B, panel f) are found within lamellipodia. These data indicate that PDGF induces activation of a fraction of the total Pak1 pool, and that acti-

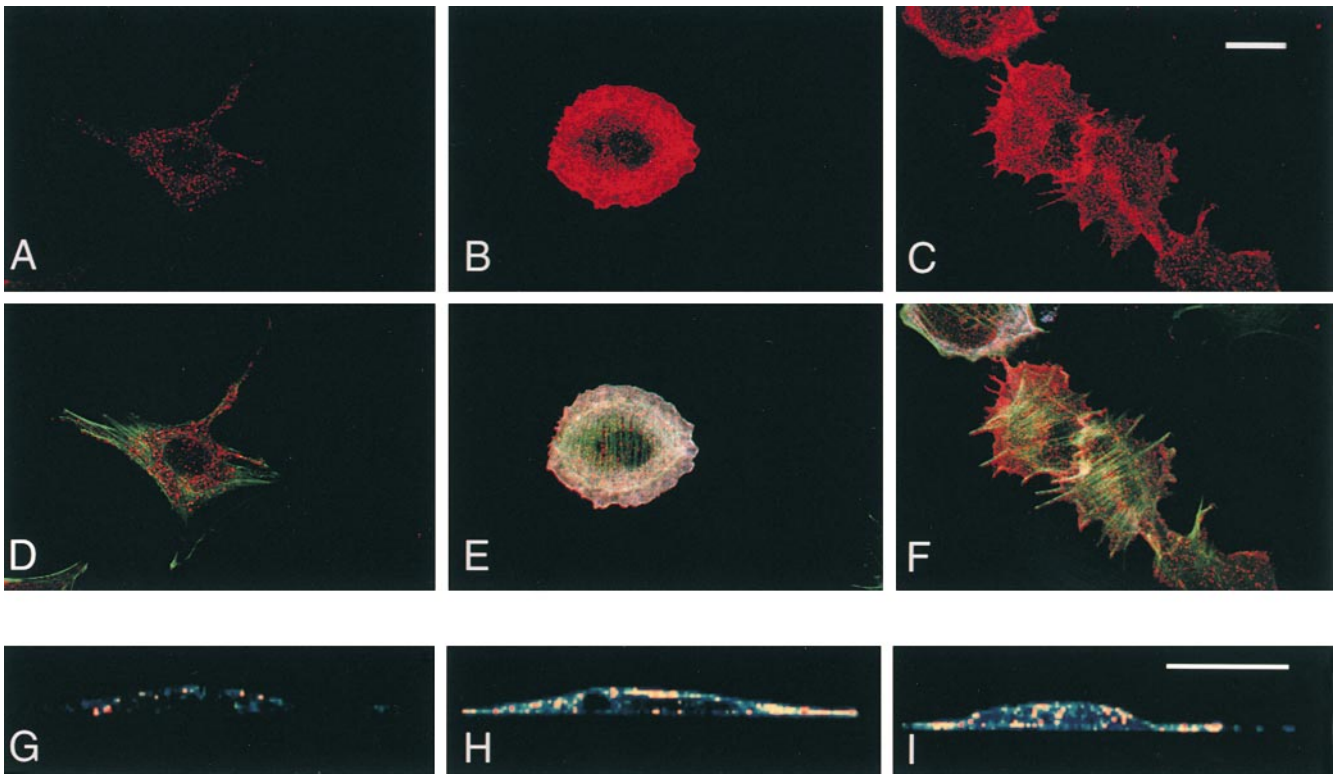


Figure 4. Localization of GTPase-activated Pak1 in fibroblasts. Tetracycline-regulated NIH-3T3 cell lines that inducibly express Pak1 (A, D, and G), as well as cells that inducibly express wild-type Pak1 plus activated Rac1 (B, E, and H) or activated Cdc42 (C, F, and I), were used to determine the subcellular location of activated Pak1. (A–C) Antiphospho-Pak1 staining (red); (D–F) merged phalloidin (green) and antiphospho-Pak1 (red) and GTPase (blue). Colocalization of phospho-Pak1 and Cdc42 or Rac1 produces white-yellow; (G–I) pseudocolored sagittal scans of these Z scanned images. Red, high intensity fluorescence; yellow, medium intensity fluorescence; and blue, low intensity fluorescence. Bars, 25 μm.

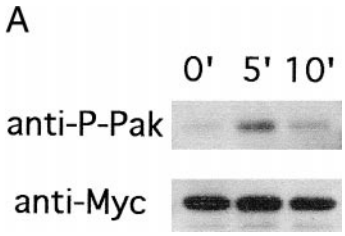
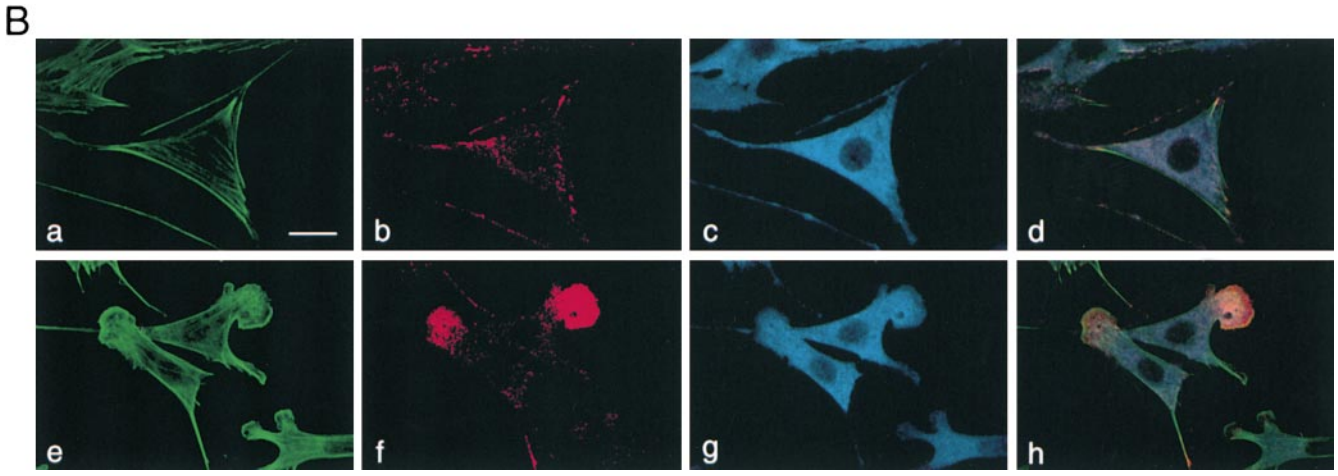


Figure 5. Activation of Pak1 by PDGF. (A) A tetracycline-regulated NIH-3T3 cell line expressing wild-type Pak1 was treated with PDGF. At the indicated times, the cells were lysed and analyzed by immunoblot using antiphospho-Pak antisera. The blot is representative of results obtained from three independent experiments. (B) The cells were treated for 5 min with vehicle alone (A–D) or 30 ng/nl PDGF (E–H), then fixed and stained for actin (A and E), phospho-Pak (B and F), and Pak1 (C and G). These confocal images were then merged (D and H). Bar, 25 μm.



vated Pak1 accumulates preferentially in growth factor–induced, actin-based structures.

To quantitate these changes, we assessed the staining intensity of total Pak1 and phospho-Pak in 20–30 control or PDGF-treated cells. For Pak1, the staining intensity was unchanged by PDGF treatment (mean average gray scale pixel intensity 2,184.4 ± 135.5 (arbitrary units) before PDGF treatment, and 2,127.1 ± 192.7 5 min after PDGF treatment), whereas phospho-Pak1 levels increased by 28% (735.1 ± 24.9 to 942.9 ± 63.6). For these measurements, staining intensity across the entire cell was surveyed, rather than just areas of membrane ruffles, and localized increases in phospho-Pak staining are in fact much higher than 28%.

Pak1 Is Activated upon Fibroblast Monolayer Wounding and Localizes to the Leading Edge of Wounded Fibroblasts

To assess Pak1 activation kinetics in a cell culture system, we wounded confluent monolayers of NIH-3T3 S2-6 cells expressing wild-type Pak1 by scraping away a 200–250-μm row of cells, then stained for phospho-Pak at defined time intervals (Fig. 6). Before wounding, cells contained little active Pak1. 5 min after wounding, cells expressing wild-type Pak1 contained markedly more phospho-Pak1, particularly in cells near the wound edge (Fig. 6 A). Maximal increase in activity (~2.5 fold) is seen by 15 min, followed by a gradual decline (Fig. 6 B). The activation of Pak1 occurs not only in the cells immediately lining the wound, but also appears to extend back from the wound edge at least several cell layers. Similar data have been reported for the activation of extracellular signal–regulated kinase (Erk) by monolayer wounding (Nobes and Hall, 1999). At the wound edge, activated Pak1 colocalized with F-actin (not shown). These data show that Pak1 is rapidly and specifically activated by monolayer wounding, accumulating at localized sites of dynamic actin remodelling.

In cells closest to the wound edge, activated Pak1 accumulates in leading edge lamellipodia that extend into the wounded region (Fig. 7 A). A substantial portion of the activated fraction of Pak1 also colocalizes with paxillin, a marker of focal adhesions (Fig. 7, B and D). Thus, in a wounded monolayer model, Pak1 is activated in cells flanking the wound edge and accumulates in actin-based structures that extend into the wounded region.

Signaling Elements Required for Pak1 Activation by Wounding

The mechanisms by which monolayer wounding initiates changes in cell shape and motility are not understood. To determine the outlines of the signal transduction pathways that mediate Pak1 activation by wounding, we examined the effects of chemical inhibitors of the EGF receptor, Src family kinases, and phosphatidylinositol 3-kinases (PI3 kinases) on Pak stimulation during this process. The EGF receptor pathway was specifically targeted because this receptor kinase is frequently transactivated by non-growth factor extracellular stimuli or stresses (e.g., cell–matrix adhesion, stimulation of G protein–coupled receptors, and UV irradiation) (for review see Weiss et al., 1997). Src is similarly activated by a wide variety of stimuli, and PI3 kinases are known to activate various Rac- and Cdc42-specific guanine nucleotide exchange factors. Cells expressing wild-type Pak1 were therefore incubated with vehicle alone, tyrphostin AG1478 (an EGF receptor inhibitor), PP1 (a Src family kinase inhibitor), or LY294002 (a PI3 kinase inhibitor) 10 min before extensive wounding. Monolayers were then either lysed, analyzed, and quantitated by immunoblot (Fig. 8) or stained with antiphospho-Pak1 15 min after wounding and the staining intensity of phospho-Pak was quantified (not shown). These experiments show that the Src inhibitor PP1 and the PI3 kinase inhibitor LY294002 effectively block Pak1 activation by wounding.

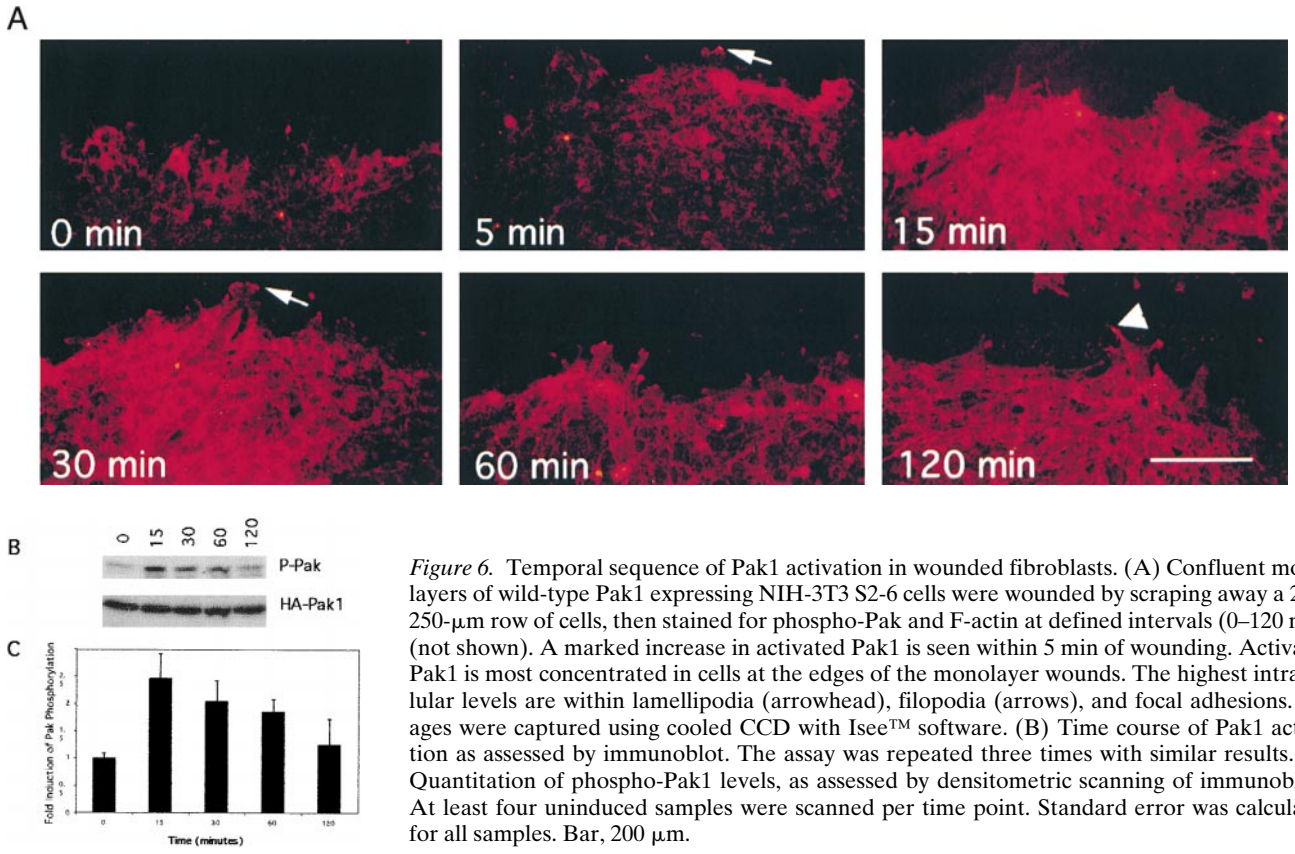


Figure 6. Temporal sequence of Pak1 activation in wounded fibroblasts. (A) Confluent monolayers of wild-type Pak1 expressing NIH-3T3 S2-6 cells were wounded by scraping away a 200–250-μm row of cells, then stained for phospho-Pak and F-actin at defined intervals (0–120 min) (not shown). A marked increase in activated Pak1 is seen within 5 min of wounding. Activated Pak1 is most concentrated in cells at the edges of the monolayer wounds. The highest intracellular levels are within lamellipodia (arrowhead), filopodia (arrows), and focal adhesions. Images were captured using cooled CCD with Isee™ software. (B) Time course of Pak1 activation as assessed by immunoblot. The assay was repeated three times with similar results. (C) Quantitation of phospho-Pak1 levels, as assessed by densitometric scanning of immunoblots. At least four uninduced samples were scanned per time point. Standard error was calculated for all samples. Bar, 200 μm.

In contrast, the EGF inhibitor, tyrphostin AG1478, does not affect Pak phosphorylation induced by wounding. When examined by phase–contrast and by immunofluorescence (rhodamine-phalloidin) microscopy, we found that both PP1 and LY294002 inhibited ruffling in cells at the wound edge, whereas tyrphostin AG1478 did not have this effect (data not shown). Thus, there is a concordance between the effects of these drugs on Pak1 activity and ruffling in wounded fibroblasts.

Discussion

We show here for the first time the location of activated Pak1 in cells. It was previously determined that a portion of Pak1 relocalizes from membranous cytoplasmic structures to focal adhesion sites upon stimulation with growth factors, but the activity of Pak in either location could not be assessed (Dharmawardhane et al., 1997; Manser et al., 1997). Using our phospho-specific antibody, we are now able to determine when and where Pak1 is activated by extracellular stimuli or by coexpression of constitutively activated GTPases. As Pakl stably binds to activated Rac1 and Cdc42 (Manser et al., 1994), the sites of phospho-Pak1 accumulation may also indirectly reveal the location of the activated forms of these GTPases in fibroblasts.

The site of Pak activation is presumed to be at the plasma membrane and/or sites of focal adhesion. This assumption is supported by the finding that Pak binds to several proteins that are known to localize to these structures. For example, Pak1 directly binds to the adaptor protein Nck, which may shuttle Pak to activated receptor protein tyrosine kinases at the plasma membrane or to focal adhesions (Minegishi et al., 1996; Lu et al., 1997; Schlaepfer et al., 1997). In addition, Pak, through a series of intermediary proteins, indirectly binds to the focal adhesion protein paxillin (Turner et al., 1999). Our results show that, in cells overexpressing activated Cdc42 or Rac1, activated Pak1 accumulates throughout filopodia and at the edges and within the body of lamellipodia, respectively. These locations coincide with sites of active actin polymerization in fibroblasts, suggesting that Pak1 plays a role in this process (Symons and Mitchison, 1991; Welch et al., 1997; Chan et al., 1998; Schafer et al., 1998; Bailly et al., 1999; Chan et al., 2000).

We and others have previously shown that Pak1 affects the organization of the actin cytoskeleton (Manser et al., 1997; Sells et al., 1997; Frost et al., 1998; Obermeier et al., 1998). Although the mechanisms by which Pak1 and other members of this enzyme family regulate actin dynamics are not fully known, a portion of these effects may be mediated by phosphorylation of LIM kinase, which inactivates the actin depolymerizing protein cofilin by phosphorylating it at an NH_2-terminal serine residue (Edwards et al., 1999). Pak1 also alters the phosphorylation state of the regulatory myosin light chain (RMLC), indirectly, through phosphorylation and inhibition of the myosin light chain kinase (Sanders et al., 1999), and directly, by phosphorylation of serine 19 in the RMLC (Ramos et al., 1997; Chew et al., 1998; Sells et al., 1999; Zeng et al., 2000). Both these activities are more than likely relevant to Pak's effects on cell morphology and motility. Finally, Pak also directly influences activity of Raf and Mek, and thereby Erk, which might contribute to changes in actin structure and cell motility through activation of RMLC (Klemke et al., 1997; Cheresh et al., 1999; Nguyen et al., 1999).

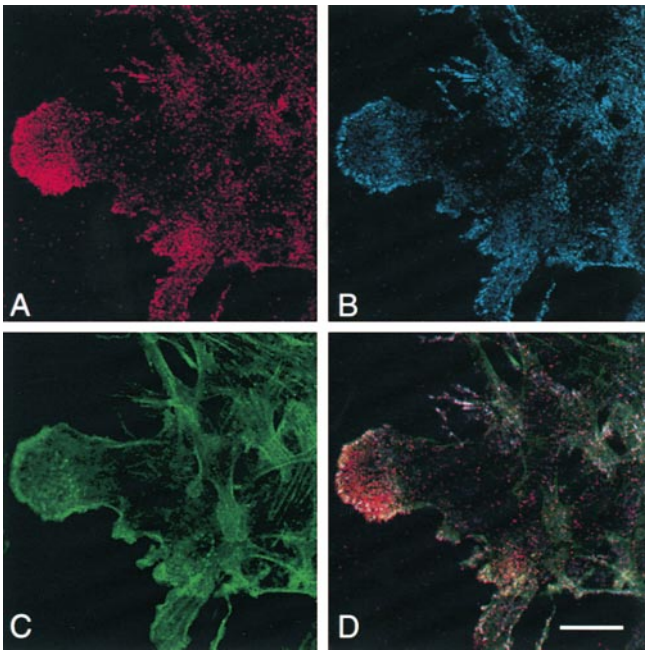


Figure 7. Colocalization of activated Pak1 and paxillin at the leading edge of motile fibroblasts. A monolayer of wild-type Pak1 expressing NIH-3T3 S2-6 cells were wounded as in the legend to Fig. 6. The leading edge of the wounded monolayer is depicted. Cells were stained with (A) antiphospho-Pak1 (red); (B) antipaxillin (blue); and (C) phalloidin (green). A merged image (D) is also shown. Bar, 25 μm.

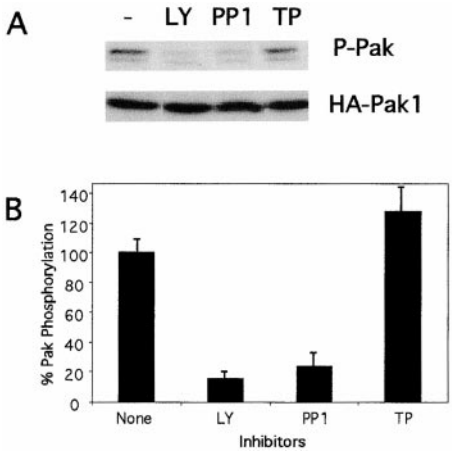


Figure 8. Signaling pathways required for Pak1 activation by wounding. Monolayers of wild-type Pak1 expressing NIH-3T3 S2-6 cells were pretreated for 10 min with the indicated compounds, then wounded as in the legend to Fig. 6. (A) 15 min after wounding, the cells were lysed and immunoblotted with antiphospho-Pak. All assays were carried out in triplicate. (B) These data were also quantified by PhosphorImager (Fuji) analysis. LY, LY294002; TP, tyrphostin AG1478.

Our data show that active Pak1 accumulates at the leading edge of motile fibroblasts. Consistent with the proposed role of this kinase in cell movement, expression of activated forms of Pak1 in fibroblasts increases the persistence of movement, while expression of a kinase-defective form of Pak1 is associated with increased nondirected, random movement (Sells et al., 1999). In *Dictyostelium*, Chung and Firtel (1999) have recently shown that Pak-a is activated by chemoattractants and is required for chemotaxis and for cytokinesis. As in mammalian fibroblasts, loss of Pak function is associated with random pseudopod extension, whereas increase of Pak function is associated with suppression of lateral pseudopod extension and increased persistence of movement. However, in contrast to our results in mammalian cells, in *Dictyostelium*, Pak-a predominantly localizes to the posterior of polarized, chemotaxing cells. It should be noted that localization of activated, as opposed to total, Pak-a could not be assessed in these experiments. It is possible that Pak-a is activated, as in mammalian fibroblasts, at the leading edge of locomoting *Dictyostelium* cells, but that the amount of activated Pak-a is small compared with the total pool of this enzyme. As the activation loop sequence in *Dictyostelium* Pak-a is similar to that of mammalian Pak1, it might be possible to address this question using the phospho-specific antibody described here.

We have shown that Pak1 is activated by wounding of a confluent monolayer. The signaling mechanisms that induce cell shape changes and movement after wounding are poorly understood. It is thought that wounding induces localized release of growth factors and cytokines which then act upon adjacent cells to induce changes in gene expression, actin reorganization, polarization, and motogenesis. Unlike integrin engagement (Moro et al., 1998; Li et al., 1999), activation of signaling by monolayer wounding does not appear to involve the EGF receptor, as the potent EGF receptor inhibitor, tyrphostin AG1478, did not affect Pak1 activation by wounding. However, our results do support a role for PI3 kinase and Src family kinases in this signaling pathway, as both LY294002 and PP1 effectively blocked Pak1 activation by wounding.

Nobes and Hall (1999) showed that the wounding response in Swiss 3T3 cells is likely to involve the small GTPases Ras, Cdc42, Rac, and Rho. Based on experiments using dominant-negative proteins, these authors showed that Cdc42 plays an important role in cell polarization and that Rac is essential for movement during wound closure. Based on our results and on these data, it is reasonable to assume that wounding activates, at a minimum, a Src family kinase, PI3 kinase, and Cdc42 and/or Rac. Activation of kinases of the Src family has been reported in fibroblasts treated with thrombin (a likely mediator of at least part of the wounding response) (Chen et al., 1994; Vaingankar and Martins-Green, 1998). Our results with the Src inhibitor PP1 are consistent with these data, and suggest that a Src family kinase lies upstream of Rac/Cdc42 in this response pathway. The production of phosphatidylinositol 3, 4, 5-triphosphate by PI3 kinase has been linked to activation of Rho guanine nucleotide exchange factors such as Sos, Vav, and Tiam1 (Han et al., 1998; Nimnual et al., 1998; Sander et al., 1998), and therefore presumably to Rac or Cdc42 and its effectors such as Pak. It should be noted, however, that measurements of Rac activity after monolayer wounding have not been reported. The indirect data presented here support this model of wound-initiated signaling, suggesting that Cdc42, Rac, or both proteins are activated by this event, and that activation takes place in areas of dynamic actin reorganization. It should be possible to further dissect this proposed signaling pathway by a combination of additional pharmacological, biochemical, and genetic approaches.

Monolayer wounding has also been shown to stimulate Erk activity (Nobes and Hall, 1999). As Pak has been implicated in stimulating Erk in growth factor signaling transduction, through phosphorylation of Raf and Mek (Frost et al., 1997; King et al., 1998), it is possible that Pak also is required for wound-initiated Erk activation. We are currently testing this hypothesis by use of specific Pak inhibitors such as the Pak inhibitory domain (Zhao et al., 1998).

This report defines, for the first time, the subcellular localization of activated Pak1. In response to extracellular stimuli such as growth factor stimulation or wounding, Pak1 phosphorylation, an indicator of enzymatic activation, can be detected by the antiphospho-Pak antibody described herein. Irrespective of stimulus used, it appears that the bulk of phospho-Pak accumulates in areas of cortical actin polymerization. This pattern of activation suggests a direct role for Pak1 in regulating actin dynamics at these sites. The exact mechanism of Pak1's role in actin remodeling, however, remains to be defined.

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