Constitutive p21-activated Kinase (PAK) Activation in Breast Cancer Cells as a Result of Mislocalization of PAK to Focal Adhesions^D

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Cytoskeletal remodeling is critical for cell adhesion, spreading, and motility. p21-activated kinase (PAK), an effector molecule of the Rho GTPases Rac and Cdc42, has been implicated in cytoskeletal remodeling and cell motility. PAK kinase activity and subcellular distribution are tightly regulated by rapid and transient localized Rac and Cdc42 activation, and by interactions mediated by adapter proteins. Here, we show that endogenous PAK is constitutively activated in certain breast cancer cell lines and that this active PAK is mislocalized to atypical focal adhesions in the absence of high levels of activated Rho GTPases. PAK localization to focal adhesions in these cells is independent of PAK kinase activity, NCK binding, or GTPase binding, but requires the association of PAK with PIX. Disruption of the PAK–PIX interaction with competitive peptides displaces PAK from focal adhesions and results in a substantial reduction in PAK hyperactivity. Moreover, disruption of the PAK–PIX interaction is associated with a dramatic decrease of PIX and paxillin in focal adhesions, indicating that PAK localization to these structures via PIX is required for the maintenance of paxillin- and PIX-containing focal adhesions. Abnormal regulation of PAK localization and activity may contribute to the tumorigenic properties of certain breast cancer cells.

INTRODUCTION

Regulated cell migration is required for a variety of physiological processes, including immune cell function, tissue remodeling, neurogenesis, and wound healing. Improper regulation of cell migration can contribute to pathological processes, including tumor cell invasion and metastasis. Cell migration is a coordinated process involving directed protrusion of the cell membrane, attachment of the membrane to the substratum, contraction of the cell body, and detachment of the cell rear (Lauffenburger and Horwitz, 1996). The dynamic reorganization of the actin cytoskeleton by the Rho GTPases Rac, Cdc42, and Rho orchestrates cell motility (Ridley, 2001; Etienne-Manneville and Hall, 2002). Activation of Rac and Cdc42 promote the polymerization of actin filaments and membrane protrusion via activation of the Arp 2/3 complex (Ma *et al.*, 1998; Machesky *et al.*, 1999; Rohatgi *et al.*, 1999; Miki *et al.*, 2000). Rac also promotes the formation of transient, nascent sites of adhesion at the tips of the cell membrane protrusions known as focal complexes (Nobes and Hall, 1999; Rottner *et al.*, 1999). Rho promotes cell contractility, formation of actin stress fibers, and maturation of focal complexes into larger, more stable adhesive structures known as focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Clark *et al.*, 1998; Nobes and Hall, 1999; Rottner *et al.*, 1999). More than 50 proteins have been shown

to localize to focal adhesions, including the integrins, transmembrane receptors for extracellular matrix proteins, the protein kinases src and FAK, and the adaptor proteins paxillin, α actinin, and vinculin, which link integrins and protein kinases to the actin cytoskeleton (Geiger *et al.*, 2001; Zamir and Geiger, 2001). Focal adhesions not only serve as the link between the extracellular matrix, integrins, and the actin cytoskeleton but also function as signal transduction centers important in the regulation of cell growth and cell death responses.

Recently, the p21-activated kinase (PAK)1 has been shown to localize to focal complexes at the tips of membrane protrusions in response to stimuli that activate Rac and Cdc42 and promote cell migration (Dharmawardhane *et al.*, 1997; Sells *et al.*, 2000). The PAK family of serine/threonine kinases are important effectors of Rac and Cdc42 (Bokoch, 2003). PAKs 1–3 are activated by binding of active Rac and Cdc42 to PAK homodimers at an N-terminal regulatory domain (Parrini *et al.*, 2002). GTPase binding disrupts an inhibitory interaction between the PAK kinase domain and the PAK autoinhibitory domain, resulting in PAK phosphorylation and activation (Zhao *et al.*, 1998; Zenke *et al.*, 1999). PAK1 has been shown to modulate dynamics of the actin and microtubule cytoskeletons to regulate cell migration. Overexpression of constitutively activated PAK1 mutants induces dissolution of actin stress fibers and focal adhesions and increases membrane protrusions, cell polarization, and cell motility (Manser *et al.*, 1997; Sells *et al.*, 1997, 1999; Frost *et al.*, 1998). Conversely, in endothelial cells, overexpression of active PAK1 results in decreased cell migration and stabilization of actin stress fibers and focal adhesions (Kiosses *et al.*, 1999). These effects are mediated through the action of

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PAK on cytoskeletal regulatory proteins such as LIM kinase (Edwards *et al.*, 1999), myosin light chain kinase (Sanders *et al.*, 1999), filamin A (Vadlamudi *et al.*, 2002), and Op18/Stathmin (Daub *et al.*, 2001; Wittmann *et al.*, 2003). Increased PAK activity has been correlated with enhanced motility and invasiveness of human breast cancer cell lines (Vadlamudi *et al.*, 2000).

The molecular mechanism(s) that localize PAK1 to focal adhesions and focal complexes are beginning to be investigated. Ectopic expression of kinase-dead PAK1 or a kinase domain truncation mutant of PAK1, but not wild-type or constitutively active PAK1, results in the localization of PAK to focal adhesions in HeLa cells (Manser *et al.*, 1997). Wild-type PAK1 localizes to focal adhesions both in the presence of the autoinhibitory domain of PAK (Zhao *et al.*, 2000a) and upon expression of activated forms of Rac or Cdc42 (Manser *et al.*, 1997), leading to the apparently contradictory results that both inactivating PAK with the autoinhibitory domain or activating PAK kinase with active GTPases can induce focal adhesion localization. The regions of PAK1 required for focal adhesion targeting have been examined. The N terminus of PAK1 contains multiple proline-rich sites that bind to src homology (SH)3 domain-containing proteins, including the adaptor proteins NCK (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996) and Grb2 (Puto *et al.*, 2003) and the guanine nucleotide exchange factor PIX (Manser *et al.*, 1998). Both PIX and NCK have been implicated in localization of PAK to focal adhesions, because disruption of either the NCK- (Zhao *et al.*, 2000a) or PIX-binding (Manser *et al.*, 1998) proline-rich regions abrogated the ability of ectopically expressed PAK1 to localize to focal adhesions. Recently, it has been demonstrated that the highly homologous ARF GAP proteins PKL (Turner *et al.*, 1999) and GIT1 (Premont *et al.*, 1998) play important roles in targeting PAK to focal adhesions (Brown *et al.*, 2002; Manabe *et al.*, 2002). PKL and GIT1 serve as adaptor proteins, able to bind the focal adhesion protein paxillin and PAK-associated PIX (Turner *et al.*, 1999; Zhao *et al.*, 2000b). Overexpression of paxillin mutants that no longer bind PKL prevented localization of the N terminus of PAK to focal adhesions (Brown *et al.*, 2002).

In this study, we show that PAK1 and/or PAK2 are constitutively activated in SK-BR-3 and ZR-75-1 human breast cancer cell lines in the absence of high levels of activated Rac1 or Cdc42. Moreover, endogenous, activated PAK is constitutively localized to large, atypical focal adhesion structures. The binding of PIX, but not Rho GTPases or NCK, to PAK targets PAK to these focal adhesions. The displacement of PAK from focal adhesions abrogates constitutive PAK activity and leads to the increased turnover of these paxillin- and PIX-containing adhesion structures. These results indicate an unexpected role for PAK/PIX association in the maintenance of paxillin-containing focal adhesions.

MATERIALS AND METHODS

Tissue Culture

Human breast cancer cell lines MDA-MB 231, SK-BR-3, and ZR-75-1 were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 1 mM glutamine, 10 mM HEPES, 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The hamster fibroblast cell line BHK-21 was grown in Glasgow MEM containing 5% heat-inactivated fetal bovine serum, 1 mM glutamine, 10 mM HEPES, 100 U/ml penicillin-streptomycin, 5% tryptone phosphate buffer at 37°C in a humidified atmosphere of 5% CO₂.

PAK Kinase Assay

PAK in-gel kinase assays were performed as described previously (Ding *et al.*, 1996). In brief, cells were lysed in radioimmunoprecipitation (RIPA) buffer

(100 mM Tris, 0.15 M NaCl, 1% cholate, 1% NP-40, 0.1% SDS) containing the following inhibitors: 2 mM vanadate, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 20 µg/ml leupeptin. Cell lysates were made to 1× SDS-PAGE buffer and heated at 100°C for 5 min. Approximately 40 µg of protein was loaded per lane. PAK activity was measured in a 7% SDS-PAGE gel containing p47^{phox} peptide substrate (CRRSSIKRLSYRRNS) and subjected to autoradiography for visualization. PAK in vitro kinase assays were performed as described previously (Daniels *et al.*, 1999), except that endogenous PAK was immunoprecipitated from RIPA lysates.

Immunoblots and GTPase Activity Assay

Immunoblots were performed as described previously (Puto *et al.*, 2003). In brief, cells were lysed in RIPA buffer (as described above), and ~40 µg of protein was loaded per lane onto a 7% SDS-PAGE gel or a 12% SDS-PAGE gel to separate Rho GTPases. The gel was transferred to nitrocellulose membrane (Millipore, Billerica, MA) by using a transfer apparatus (Bio-Rad, Hercules, CA). PAK was detected using polyclonal antisera R2124 (Knaus *et al.*, 1995) at a dilution of 1:2000. Rho GTPases were detected using polyclonal αCdc42 at a dilution of 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal αRac1(UBI) at a dilution of 1:2000, and monoclonal αRhoA (Santa Cruz Biotechnology) at a dilution of 1:1000. Polyclonal αPIX antisera were generated against GST-αPIX fusion protein containing the SH3 domain of αPIX. α/βPIX was detected with polyclonal αPIX antibody at a concentration of 1:10,000. For polyclonal antibodies, immunoblots were incubated with protein A horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) at a dilution of 1:5000; for monoclonal antibodies, immunoblots were incubated with goat mouse horseradish peroxidase (Amersham Biosciences) at a dilution of 1:5000. Immunoreactive proteins were visualized using chemiluminescence (Pierce Chemical, Rockford, IL). For immunoprecipitations, cell lysates were incubated with monoclonal αmyc antibodies (9E10) at a dilution of 1:100 or αPAK (R2124) at a dilution of 1:100 for 2–3 h at 4°C and were incubated with bovine serum albumin (BSA)-coated protein A beads (Repligen, Waltham, MA) for αPAK immunoprecipitates or BSA-coated protein G beads (Amersham Biosciences) for αmyc immunoprecipitates for 1 h at 4°C. Immunoprecipitated proteins were washed three to four times with lysis buffer, made to 1× SDS-PAGE buffer, and heated at 100°C for 5 min before separation by SDS-PAGE. GTP-bound Rac1, Cdc42, or RhoA were determined using the affinity based pull-down assays as described previously (Benard *et al.*, 1999; Ren *et al.*, 1999). For activation of endogenous G proteins, cell lysates were treated with 10 mM MgCl₂, 10 mM NaF, and 10 µM AlCl₃ to generate AlF₄⁻.

Immunofluorescence and F-Actin Staining

Cells grown on coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, permeabilized in PBS containing 0.5% Triton X-100 for 20 min, and blocked with 3% BSA in PBS for 1 h. Coverslips were incubated for 1 h with one of the following primary antibodies: polyclonal αpPAK raised against the phosphorylated peptide SKRST(P)MVGTPYC corresponding to amino acids 419–429 of PAK1 at 1:200 (Sells *et al.*, 2000), polyclonal αpPAK raised against the phosphorylated peptide PEHTK-S(P)VYTRS(P)VIEP corresponding to amino acids 193–207 of PAK1 at 1:500 (Shamah *et al.*, 2001), monoclonal αvinculin (Sigma-Aldrich, St. Louis, MO) at 1:1000 and monoclonal αpaxillin at 1:500 (BD Transduction Laboratories, Lexington, KY), polyclonal αPIX at 1:2000, polyclonal αzyxin B71 at 1:500 (Hoffman *et al.*, 2003), and polyclonal αNCK (NeoMarkers) at 1:200. For αPIX staining of microinjected cells, aliquots of αPIX antisera were incubated with glutathione S-transferase (GST) fusion protein overnight at 4°C to remove αGST antibodies. Coverslips were then washed three times in PBS for 1 h and incubated with one of the following secondary antibodies for 1 h at a dilution of 1:500: for cells stained with a monoclonal primary antibody, Alexa Fluor 488 or 568-conjugated goat mouse antibody (Molecular Probes, Eugene, OR); for cells stained with a polyclonal primary antibody, Alexa Fluor 488 or 568 goat rabbit antibody (Molecular Probes). F-actin was detected using Alexa Fluor 568-conjugated phalloidin at 1:500 (Molecular Probes). Cells were washed three times in PBS for 1 h and mounted with ProLong mounting media (Molecular Probes). Micrographs were taken using an Olympus IX 70 inverted microscope system equipped with ISEE analytical imaging software by using 60× and 100× objectives.

Plasmids and Transient Transfection

pGEX-KG encoding PAK 1-74 and PAK 147-231 were kind gifts of M. Cobb (University of Texas, Dallas, TX). pGEX4T encoding PAK1 67-150 has been described previously (Benard *et al.*, 1999). GST proteins were purified as described previously (Zenke *et al.*, 1999), except that proteins were dialyzed against 25 mM Tris, pH 7.5, 50 mM NaCl. Semliki Forest virus (SFV) constructs encoding myc-tagged PAK1, PAK1 K299R, PAK1 T423E, PAK1 83,86L, PAK1 83,86L K299R, PAK1 67-150, PAK1 83-149, and PAK1 83-149 L107F have been described previously (Edwards *et al.*, 1999; Sanders *et al.*, 1999; Mira *et al.*, 2000). Myc-tagged PAK1 1-74, PAK1 R193A, P194A, and PAK1 P13A, and myc tagged RhoAT19N, Rac1T17N, and Cdc42T17N were subcloned into the pSFV3 SFV vector (Invitrogen, Carlsbad, CA) by using standard cloning techniques. PAK1 1-205-CAAX in the mammalian expression vector pCMV6 has been described previously (Daniels *et al.*, 1998). Enhanced green fluores-

cent protein (EGFP) PAK1 K299R was subcloned into pEGFPN1 vector (BD Biosciences Clontech, Palo Alto, CA) by using standard cloning techniques. Transient transfection of PAK1 constructs into cells was achieved by using the SFV gene expression system (Invitrogen) as described previously (Sanders *et al.*, 1999) or the Geneporter reagent (Gene Therapy Systems) following manufacturer's suggestions.

Microinjections

GST fusion proteins containing the PIX binding domain of PAK1 (aa 147–231), the NCK binding domain of PAK1 (aa 1–74), or the GTPase binding site of PAK1 (aa 67–150) were microinjected with rhodamine dextran into SK-BR-3 or ZR-75-1 cells at final concentrations of 2.5–3.0 $\mu\text{g}/\mu\text{l}$. Microinjections into the cytoplasm of cells were done with an Eppendorf 5242 microinjector and an Eppendorf 5171 micromanipulator. Viability was assessed on microinjections of ~200 cells from several coverslips by using propidium iodide, and viability was consistently >96% after microinjection on any given coverslip. Cells were placed in a humidified 5% CO_2 incubator for 30 min, and then fixed and stained for paxillin, vinculin, PIX, or pPAK as described previously. Microinjected cells were scored as having reduced amounts of PIX, paxillin, or pPAK-containing focal adhesions if there was a complete loss of these proteins from focal adhesions or a conversion of large focal adhesions to smaller focal complexes on the cell periphery, which contained PIX, pPAK, or paxillin. For live cell imaging experiments, cells were transfected with C-terminally tagged EGFP PAK1 K299R or EGFP PAK1 WT. Transfected cells were versene-treated and replated onto glass coverslips for microinjection. Replated cells were allowed to adhere and spread for ~6 h and then microinjected with the indicated peptide on a heated stage. Fluorescent images were taken every 30 s for a maximum of 1 h (or until PAK1 was displaced from focal adhesions) after peptide microinjection by using an Olympus IX 70 inverted microscope system equipped with a cooled 12 bit Micromax charge-coupled device camera (Princeton Scientific Instruments, Monmouth Junction, NJ) and ISEE analytical imaging software by using 60 $\times$ objectives. The area of focal adhesions as a percentage of total cell area was determined using the integrated morphometry analysis function of MetaMorph analytical imaging software.

RESULTS

Hyperactive PAK in Focal Contacts of SK-BR-3 and ZR-75-1 Breast Cancer Cell Lines

The study of Mira *et al.* (2000) demonstrated that some breast cancer cell lines have high endogenous levels of PAK1 and PAK2 activity due to the presence of an activated Rac3 GTPase. In the present study, we examined two breast cancer cell lines, SK-BR-3 and ZR-75-1, that have endogenous hyperactive PAK but no detectable hyperactive Rho GTPase. As shown in Figure 1, both PAK1 and PAK2 activity was constitutively elevated in ZR-75-1 cell lysates, and PAK2 activity was elevated in SK-BR-3 lysates compared with cell lysates from the breast cancer cell line MDA-231 (Figure 1) or a fibroblast cell line BHK-21 (our unpublished data). Immunoblot analysis demonstrates that comparable PAK2 protein levels were present in all cell types, although PAK1 expression differed (Figure 1A). The presence of activated PAK1 and/or PAK2 was confirmed by immunoprecipitation with an antibody that recognizes PAK1 and PAK2 (Knaus *et al.*, 1995) followed by in gel kinase assay (Figure 1B). Similar results showing that PAK kinase activity is elevated in SK-BR-3 and ZR-75-1, but not MDA 231 cells were obtained using an in vitro kinase assay with myelin basic protein as substrate (see Supplemental Fig. 1).

Because PAK activity has been linked to cytoskeletal rearrangements (Manser *et al.*, 1997; Sells *et al.*, 1997; Frost *et al.*, 1998), we examined the actin cytoskeleton with phalloidin staining and focal adhesions by vinculin staining. Interestingly, both SK-BR-3 and ZR-75-1 cells exhibited dramatic thickening of existing actin filaments and large atypical focal adhesions compared with MDA-MB-231 cells (Figure 2) or BHK-21 cells (our unpublished data). We used an antibody that detects phosphorylated S199/204 (Shamah *et al.*, 2001) present in active PAK1 or S192/197 present in active PAK2, to localize activated PAK in these cell lines (Figure 3). In both the SK-BR-3 and ZR-75-1 cells, the anti-phospho-PAK

Figure 1. SK-BR-3 and ZR-75-1, but not MDA-MB-231, human breast cancer cells contain constitutively activated PAK1 and/or PAK2. (A) Breast cancer cell lysates were subjected to an in-gel kinase assay by using p47 phox peptide as substrate (see MATERIALS AND METHODS). Western blot analysis of lysates with anti-PAK antiserum demonstrates equal levels of PAK2 in all breast cancer cell lines, with differing levels of PAK1. (B) Cell lysates were immunoprecipitated with anti-PAK antiserum and subjected to an in-gel kinase assay using p47 phox peptide as substrate.

antibody specifically stained the focal adhesions, suggesting that the majority of activated PAK was associated with these structures. Notably, the pPAK antibody did not stain the focal adhesions in MDA-MB-231 cells. To confirm the specificity of this pPAK antibody for endogenous active PAK in focal adhesions, SK-BR-3 cells were transfected with the kinase inhibitory fragment of PAK 83–149 (PID) (Zhao *et al.*, 1998) to specifically inactivate PAK. As a control, cells were transfected with the inactive mutant PID 83–149 L107F (Zhao *et al.*, 1998). Transfection of the PAK1 inhibitory domain (PID) abolished pPAK staining in focal adhesions in 98% of cells ($n = 174$), confirming the specificity of this pPAK antibody for active PAK (see Supplementary Fig. 2). In contrast, transfection of the PIDL107F abolished pPAK staining in 3% of cells ($n = 102$) (see Supplementary Figure 2). To further confirm the localization of endogenous activated PAK in focal adhesions, cells were stained with antisera that detects phosphorylated T423/403 in PAK1/2 (Sells *et al.*, 2000). Consistent with the results obtained with the S199/204 antibody, the T423/403 antibody showed the presence of active PAK1/2 in the focal adhesions of SK-BR-3 and ZR-75-1, but not MDA-MB 231 cells (our unpublished data).

In contrast to previous studies in which hyperactive PAK1 and PAK2 activity in breast cancer cells required constitutive Rac3 activation, we did not detect high levels of activated Rac1, Cdc42, RhoA, or Rac3 (Mira and Knaus, unpublished data) in these cell lines, as determined by the respective affinity-based assays (Figure 4, A–C). Although we were able to detect an increase in the level of Rac1 GTP in response to growth factors such as heregulin β 1 (Figure 4D), we cannot rule out the possibility that the basal endogenous RhoGTPase activation under the conditions used is below the limits of detection of the assays. In support of a requirement for RhoGTPase activity for elevated PAK ki-

Figure 2. Breast cancer cells with high endogenous PAK activity have large focal adhesions and thick bundles of actin filaments. MDA-MB-231, SK-BR-3, and ZR-75-1 cells were fixed and stained with Alexa Fluor 568-labeled phalloidin and anti-vinculin/anti-mouse Alexa Fluor 488 to visualize actin filaments and focal adhesions, respectively, as described in MATERIALS AND METHODS. Bar, 10 μ m.

nase activity, treatment of cells with *Clostridium difficile* Toxin B, which inhibits Rac, Cdc42, and Rho activity, substantially reduces PAK kinase activity in the SK-BR-3 and ZR-75-1 cells (Figure 5A). Furthermore, ectopic expression of dominant negative Rac1 or dominant negative RhoA dramatically decreases pPAK staining in focal adhesions in 76% ($n = 215$) and 83% ($n = 111$) of transfected cells, whereas expression of dominant negative Cdc42 affected pPAK staining in focal adhesions in only 26% ($n = 238$) of transfected cells (Figure 5B). However, use of the dominant negative RhoGTPases and Toxin B treatment was accompanied by a dramatic reduction in actin stress fibers and focal adhesions in the treated cells (our unpublished data), making it impossible to conclude that the effects seen on pPAK staining were due to the loss of Rho GTPase activity (see following sections).

To test whether constitutive endogenous PAK activity required intact focal adhesion structures and/or an intact actin cytoskeleton, we induced loss of focal adhesions in two ways. First, the cells were treated with cytochalasin D at concentrations sufficient to disrupt the organized actin cytoskeleton and focal adhesions. Within 10 min of cytochalasin D treatment, PAK activity was noticeably decreased, and by 1 h constitutive PAK activity was abolished (Figure 6A). This correlated very well with loss of focal adhesion structures and actin stress fibers. Second, focal adhesions were disrupted by placing the cells in suspension. There was a dramatic decrease in constitutive PAK activity in the cells placed in suspension as opposed to attached cells, similar to the reduction of elevated PAK activity after cytochalasin D treatment (Figure 6B). Replating the cells restored the large focal adhesion structures, concomitant with relocalization of PAK to these sites and an increase in PAK activity. These

effects were observed in both the ZR-75-1 and SK-BR-3 cells, and were not mimicked by serum starvation (our unpublished data).

Localization of PAK to Focal Adhesions Is Independent of Kinase Activity

To examine the molecular basis for the localization of PAK to focal adhesions in these breast cancer cells, we transfected SK-BR-3, ZR-75-1, and MDA-MB-231 cells with a myc-tagged PAK1 wild-type (PAK1 WT) construct. Myc staining revealed that PAK1 WT localized to focal adhesions only in the SK-BR-3 and ZR-75-1 cells, and not in MDA-MB-231 cells (Figure 7 and Table 1). The inability of ectopically expressed PAK1 WT to localize to focal adhesion in MDA-MB-231 cells was not due to decreased levels of focal adhesion proteins thought to be important for PAK localization to focal contacts, such as paxillin and PKL (see Supplemental Figure 3). A constitutively active PAK1 T423E also localized to focal adhesions in SK-BR-3 and ZR-75-1 cells, but localization was not kinase dependent, because expression of a kinase-dead PAK1 K299R or the PAK1 N terminus (which lacks the C-terminal PAK kinase domain) in SK-BR-3 or ZR-75-1 cells also resulted in localization to the focal adhesions (Table 1). These data suggest that ectopically expressed PAK1 is abnormally recruited to focal adhesions in SK-BR-3 and ZR-75-1 breast cancer cells, and that this recruitment is independent of PAK kinase activity. In contrast to other cell lines, ectopically expressed active PAK1 did not disrupt focal adhesions in either the SK-BR-3 or ZR-75-1 cells, as measured by colocalization of active PAK1 with paxillin and vinculin (our unpublished data).

PAK Localization to Focal Adhesions Is Mediated via PIX

PAK has been shown in previous studies to have many binding partners, some of which have been reported to localize ectopically expressed PAK1 to focal adhesions in HeLa, Chinese hamster ovary, and NIH3T3 cells (Manser *et al.*, 1998; Zhao *et al.*, 2000a; Brown *et al.*, 2002; Manabe Ri *et al.*, 2002). Two such PAK binding partners are PIX (PAK-interacting guanine nucleotide exchange factor) (Manser *et al.*, 1998) and the adapter protein NCK (Bokoch *et al.*, 1996). Immunocytochemistry revealed that both PIX and NCK localized with active PAK in focal adhesions of both SK-BR-3 and ZR-75-1 cells (Figure 8). We therefore examined whether the binding of PAK to either of these proteins was necessary for focal adhesion targeting. Expression of full-length PAK1 (R193A, P194A) mutant, which can no longer bind PIX, or truncated forms of PAK1 (aa 67–150, aa 83–149), which do not contain the PIX binding domain, resulted in a diffuse, cytosolic localization in the SK-BR-3 or ZR-75-1 cells (Table 1). The localization of PAK1 to focal adhesions is not due to binding Rac or Cdc42, because a PAK1 mutant defective in Rho GTPase binding, PAK1 (H83, 86L), effectively localizes to focal adhesions. Expression of full-length PAK1 (P13A) mutant that does not bind NCK also resulted in localization to focal adhesions in SK-BR-3 and ZR-75-1 cells. These data suggest that the ability to bind to PIX is the major determinant for PAK localization to focal adhesions.

To establish the importance of the PIX-PAK interaction in focal adhesion localization, we determined whether introduction of a PAK1 fragment containing the PIX binding domain could displace PAK from focal adhesions. The peptide region of PAK used to disrupt PAK-PIX interaction contains the noncanonical, high-affinity proline-rich region of PAK, which binds to the SH3 domain of PIX (Manser *et al.*, 1998). This site is present only in PAK1, PAK2, and

Figure 3. Endogenous, activated PAK1/2 localizes to focal adhesions in SK-BR-3 and ZR-75-1 breast cancer cells. MDA-MB-231, SK-BR-3, and ZR-75-1 cells were fixed and stained with anti-phospho-PAK (raised against phosphorylated S199/S204 in PAK1) (anti-pPAK)/anti-rabbit Alexa Fluor 488 and anti-paxillin/anti-mouse Alexa Fluor 568 to visualize phosphorylated, active PAK1/2 and focal adhesions, respectively, as described in MATERIALS AND METHODS.

PAK3, and a peptide encompassing this region has been previously shown to disrupt endogenous PAK-PIX interaction in cell lines (Obermeier *et al.*, 1998; He *et al.*, 2001). EGFP-PAK1 (K299R) was transiently expressed in SK-BR-3 cells, where it localized to focal adhesions (Table 1). The EGFP PAK1 K299R-expressing cells were then microinjected with GST fusion proteins containing the PAK1 NCK and Grb2 binding domains (GST-PAK, aa 1–74), followed by the PIX binding domain of PAK1 (GST-PAK, aa 147–231). After ~5 min, there was a significant decrease in the amount of EGFP-PAK protein found in the focal adhesions of cells microinjected with PIX binding domain compared with cells microinjected with the NCK and Grb2 binding domains (Figure 9). These data indicate a critical role for PIX in the localization of PAK to focal adhesions in SK-BR-3 breast cancer cells.

Displacement of PAK Disrupts Focal Adhesions

We examined whether loss of EGFP PAK1 from focal adhesions was a consequence of the disruption of focal adhesions, or whether it was due to a specific displacement of PAK from intact focal adhesions. SK-BR-3 cells were microinjected with the PIX binding fragment of PAK1, the NCK and Grb2 binding fragment of PAK1, or the GTPase binding domain of PAK1, and the presence of PIX and paxillin in the focal adhesions was determined by immunocytochemistry. SK-BR-3 cells were fixed and stained 25–30 min after microinjection, a time sufficient for loss of EGFP PAK1 from focal

adhesions. As shown in Figure 10, microinjection of the PIX-binding polypeptide resulted in a dramatic decrease in the number and intensity of PIX-containing focal adhesions in 88% of the cells ($n = 140$). In contrast to the PIX-binding peptide, microinjection of the NCK or GTPase PAK peptides reduced PIX in focal adhesions in only 16% ($n = 155$) and 10% ($n = 182$) of SK-BR-3 cells, respectively.

To determine whether there was a selective loss of PIX from focal adhesions or a general disruption of focal adhesion structures, we examined the effects of peptide microinjection on paxillin and vinculin, two characteristic components of focal adhesions. Notably, injection of the PIX binding fragment of PAK1 resulted in a dramatic reduction in endogenous paxillin staining in 73% of the cells ($n = 295$) (Figure 10). In contrast, injection of the NCK and Grb2 binding fragment of PAK1 resulted in only 28% ($n = 336$) of the injected cells showing a reduction in paxillin staining (Figure 10). The inability of the NCK and Grb2 binding peptide to decrease the paxillin staining in the cells was not due to a delayed time course of focal adhesion turnover, because the paxillin-containing focal adhesions remained intact for up to 6 h after microinjection. Similar to the results with the NCK and Grb2 binding peptide microinjection, microinjection of the GTPase binding domain of PAK1 (aa 67–150) resulted in only 21% ($n = 171$) of the cells displaying reduced focal adhesion staining of paxillin. In contrast, microinjection of the PIX-binding peptide in BHK fibroblasts or MDA-MB-231 cells, which both do not contain endogenous

Figure 4. SK-BR-3 and ZR-75-1 cell lines with activated PAK do not contain detectable levels of active RhoGTPases. Cell lysates with GDP or guanosine 5'-O-(3-thio) triphosphate loading or without nucleotide loading were incubated with GST fusion protein containing the p21 binding domain of PAK1 (GST-PBD). Activated Rac1 (A) or Cdc42 (B) was detected by Western blot analysis of precipitated proteins with monoclonal anti-Rac1 antibody or polyclonal anti-Cdc42 antiserum, respectively. Similar amounts of Rac1 or Cdc42 were detected in cell lysates by Western blot analysis with anti-Rac1 or anti-Cdc42 antibodies. (C) Cell lysates were incubated in the presence or absence of AIF₄⁻ and incubated with GST fusion protein containing the Rho binding domain of Rho-tekkin (GST-RBD). Activated RhoA was detected by Western blot analysis of precipitated proteins with monoclonal RhoA antibody. Similar amounts of RhoA in SK-BR-3 and ZR-75-1 cell lysates were detected by Western blotting with anti-RhoA antibody. (D) SK-BR-3 cells were stimulated with 50 ng/ml Heregulin β1 for the indicated times and cell lysates were incubated with GST-PBD and precipitated proteins were detected with anti-Rac1 monoclonal antibody. Equivalent amounts of Rac1 in cell lysates were detected by Western blotting with anti-Rac1.

PIX and active PAK in focal adhesions, had no effect on the level of paxillin in focal adhesions (our unpublished data). As with the effects of the PIX peptide on paxillin-containing focal adhesions in SK-BR-3 cells, microinjection of the PIX peptide in ZR-75-1 cells resulted in a reduction of paxillin-containing focal adhesions in 57% ($n = 60$) of microinjected cells. These results suggest that PAK/PIX association and localization may regulate the turnover of paxillin-containing focal contacts in SK-BR-3 and ZR-75-1 breast cancer cells.

In contrast to the dramatic reduction in the size and number of paxillin-containing focal adhesions in SK-BR-3 cells after microinjection of PAK1, aa 147–231, the size and number of vinculin and zyxin-containing focal adhesions was not dramatically altered (see Supplementary Figure 4; our unpublished data). Microinjection of the PIX peptide resulted in only 13% of cells ($n = 110$) and 26% of cells ($n = 137$) displaying decreased focal contact staining of vinculin and zyxin, respectively. Similarly, microinjection of the NCK and Grb2 binding peptide resulted in 16% of cells ($n = 108$) and 13% of cells ($n = 114$) displaying decreased vinculin and zyxin staining, respectively. Quantitation of paxillin, PIX, and vinculin staining in cells microinjected with the PIX peptide demonstrated that the PIX peptide significantly re-

duces the total area of paxillin- and PIX-containing focal adhesions in SK-BR-3 cells, compared with noninjected cells or cells injected with the NCK and Grb2 peptide (see Supplementary Figure 4). Notably, microinjection of the PIX peptide did not significantly reduce the area of vinculin-containing focal adhesions. Together, these results suggest that blocking the PAK/PIX interaction in SK-BR-3 cells specifically affects the localization of paxillin and PIX to focal adhesions without dissolving focal adhesions per se.

PAK Localization to Focal Adhesions Is Required for Abnormal PAK Activation

The localization of constitutively activated PAK to focal adhesions as evaluated by pPAK immunofluorescence, and the loss of PAK activity after disruption of these structures by cytochalasin D or suspension, strongly suggested that constitutive PAK activity was induced by its localization to the focal adhesions. We determined whether this localization was required for PAK activation. Cells were microinjected with the PIX binding polypeptide to disrupt PAK/PIX interaction, and cells were stained with antibody that detects phosphorylated S199/204 in PAK1 and S192/197 in PAK2. As shown in Figure 11A,

Figure 5. Inhibition of RhoGTPases decreases PAK activation in SK-BR-3 and ZR-75-1 cells. (A) SK-BR-3 and ZR-75-1 cells were treated with the indicated amounts of *C. difficile* Toxin B (ToxB) overnight, and cell lysates were subjected to an in-gel kinase assay. (B) SK-BR-3 cells were infected with recombinant Semliki Forest virus constructs encoding myc-tagged RacT17N, Cdc42 T17N, or RhoA T19N. Cells were fixed and immunostained with anti-myc/Alexa Fluor 568 and anti-pPAK/Alexa Fluor 488. The percentage of cells with pPAK present in large atypical focal contacts was determined by counting cells in the field visually.

microinjection of the 147-231 peptide resulted in a dramatic decrease in the amount of pPAK staining (S199/204, S192/197) in focal adhesions in 79% (n = 128) of the cells. In contrast,

microinjection of the NCK and Grb2 polypeptide resulted in a decrease of pPAK staining in focal adhesions in only 28% (n = 197) of the cells. These results suggest that PAK localization to focal adhesions is required for PAK activation.

To confirm that elevated PAK kinase activity requires localization to focal adhesions, wild-type PAK1, active PAK1, or a PAK1 mutant, which cannot bind PIX, PAK1 (R193A, P194A), was overexpressed in SK-BR-3 cells, and kinase activity was assessed. In comparison with wild-type PAK1, PAK1 (R193A, P194A) displayed greatly reduced kinase activity (Figure 11B). These results are consistent with a requirement for focal adhesion localization for constitutive PAK kinase activity. An alternative explanation is that preventing PAK/PIX interaction per se blocks PAK activation. However, we could detect no change in the amount of PAK associated with PIX in immunoprecipitates from cytochalasin D-treated cells or cells placed in suspension, two conditions that result in PAK inactivation (our unpublished data). Furthermore, in BHK or MDA-MB-231 cells, which do not have active PAK kinase, PAK and PIX were constitutively associated as determined by immunoprecipitation and Western blot.

DISCUSSION

In the current study, we demonstrate that the breast cancer cell lines SK-BR-3 and ZR-75-1 contain high endogenous levels of activated PAK1 and/or PAK2 in the absence of detectable levels of active Rho GTPases. These results are in contrast to a previous study that showed

Figure 6. Disruption of the actin cytoskeleton and focal contacts abrogates PAK kinase activity in breast cancer cells with active endogenous PAK1/2. (A) SK-BR-3 and ZR-75-1 cells were treated with dimethyl sulfoxide (0) or 2 μ M cytochalasin D (cyto D) for 10 and 60 min. Cells were lysed and subjected to an in-gel kinase assay by using p47 phox peptide as substrate. (B) SK-BR-3 and ZR-75-1 cells were untreated (att), treated with 2 μ M cytochalasin D (cyto D) for 60 min, or trypsinized and placed in suspension for 30 min (susp) in PBS before lysis and in gel kinase assay. Levels of PAK did not change with treatment, as detected by immunoblot with anti-PAK antisera.

Figure 7. Ectopically expressed PAK1 localizes to focal adhesions in SK-BR-3 and ZR-75-1 cells. Breast cancer cell lines were infected with recombinant Semliki Forest virus constructs encoding myc-tagged PAK1. Cells were fixed and stained with anti-myc/anti-mouse Alexa Fluor 488 to visualize the subcellular localization of PAK1 as described in MATERIALS AND METHODS. Bar, 10 μ m.

that highly proliferative breast cancer cell lines such as MCF-7, T47D, and MDA-MB-435 contained high levels of activated PAK1 and PAK2 as a consequence of constitutive activation of endogenous Rac3 (Mira *et al.*, 2000). Our results indicate that endogenous, active PAK1 and PAK2 constitutively localize to large, atypical focal adhesion structures in SK-BR-3 and ZR-75-1 breast cancer cell lines. In contrast, active PAK localizes to membrane ruffles in the MCF-7 breast cancer cell line (Vadlamudi *et al.*, 2002). Similarly, activation of ectopically expressed PAK1 by growth factor or induced wound healing results in localization of active PAK1 throughout lamellipodia and focal

Table 1. PAK localization to focal adhesions is dependent on PIX binding

PAK1 Construct	Localization to Focal Adhesions in SK-BR-3 and/or ZR-75-1 Cells	
PAK1 WT	YES	++
PAK1 K299R	YES	++
PAK1 T423E	YES	++
PAK1 H83,86L	YES	+
PAK1 H83,86L K299R	YES	++
PAK1 P13A	YES	+
PAK1 R193A,P194A	NO	–
PAK1 P13A H83,86L	YES	+
PAK1 1–205 CAAX	YES	++
PAK1 67–150	NO	–
PAK1 83–149	NO	–
PAK1 83–149 L107F	NO	–
PAK 1–74	NO	–

SK-BR-3 and/or ZR-75-1 cells were transfected with the indicated PAK1 constructs as described in Materials and Methods. Cells were scored as follows: (–) indicates diffuse cytosolic localization; (+) indicates cytosolic and weak focal adhesion localization; (++) indicates higher focal adhesion localization versus cytosolic localization.

complexes at the tips of the lamellipodia (Sells *et al.*, 2000). To our knowledge, our data are the first demonstration of focal adhesion localization of endogenous, hyperactive PAK1 and PAK2.

Localization of PAK1/2 to Focal Adhesions

The molecular mechanism by which PAK, PIX and paxillin are recruited to focal adhesions is incompletely understood. It has been proposed (Brown *et al.*, 2002) that PAK and PKL recruitment to focal adhesions by active Rac or Cdc42 involves a pathway in which PAK activation stimulated by active Rho GTPase binding induces a conformational change permissive for the “scaffolding function” of PAK, such that PAK can interact with adaptor proteins such as NCK and the guanine nucleotide exchange factor PIX via the proline-rich N terminus of PAK. The PAK/PIX complex is recruited to PKL via the interaction of PIX with PKL. The PIX and PKL interaction exposes the paxillin-binding domain of PKL, resulting in recruitment of the PAK–PIX–PKL complex to paxillin-containing focal adhesions. Binding of PIX to GIT1, which is highly homologous to PKL, has been shown to enhance GIT1 binding to paxillin (Zhao *et al.*, 2000b), but this has not been formally proven for PKL. PAK activation and phosphorylation of serine residues adjacent to the PIX binding site in PAK has been reported to dissociate PAK from PIX (Zhao *et al.*, 2000a), and this dissociation has been hypothesized to cause a loss of PAK from focal adhesions. However, the recruitment of PAK, PIX, and PKL to focal adhesions in the SK-BR-3, ZR-75-1 and MDA-MB-231 cells seems to be more complex than suggested by this model. First, PAK, PIX, and PKL constitutively interact in all cell lines examined in this study without high levels of active Rho GTPases being present. Second, active endogenous PAK and ectopically expressed, constitutively active PAK localize to large focal adhesions in the SK-BR-3 and ZR-75-1 cells. Finally, PAK, PIX, and PKL interact in MDA-MB-231

Figure 8. Both PIX and NCK localize to focal adhesions in breast cancer cell lines with active PAK1/2. SK-BR-3 and ZR-75-1 cells were fixed and stained with anti-PIX/anti-rabbit Alexa Fluor 568 or anti-NCK/anti-rabbit Alexa Fluor 488 as described in MATERIALS AND METHODS. Bar, 10 μ m.

cells but this complex does not localize to focal adhesions (Stofega and Bokoch, unpublished observations) as would be predicted by the above-described model. Together, these data suggest that the regulation of the activity and localiza-

tion of PAK in the breast cancer cells used in this study is atypical.

The results presented here suggest that the PAK-PIX interaction in SK-BR-3 and ZR-75-1 breast carcinoma cells

Figure 9. Microinjection of a PIX-competing peptide displaces ectopically expressed PAK1 from focal adhesions. SK-BR-3 cells were transfected with EGFP-tagged PAK1 K299R and microinjected with either a PIX competing peptide (PAK1, aa 147-231) or a NCK competing peptide (PAK1, aa 1-74). Fluorescent images were taken every 15 s for 1 h after peptide microinjection or until EGFP PAK was displaced from focal adhesions. Microinjected cells are indicated by an asterisk.

Figure 10. Microinjection of a PIX competing peptide disrupts PIX and paxillin localization to focal adhesions. SK-BR-3 cells were microinjected with either a PIX-competing peptide (PAK1, aa 147–231), a NCK-competing peptide (PAK1, aa 1–74), or a small GTPase-competing peptide (PAK1, aa 67–150). Thirty minutes after microinjection, cells were fixed and stained with either anti-PIX/anti-rabbit Alexa Fluor 488 or anti-Paxillin/anti-mouse Alexa Fluor 488 as described in MATERIALS AND METHODS. Cells were scored as described in MATERIALS AND METHODS. Microinjected cells are indicated by an asterisk.

plays a role in the maintenance of active PAK, PIX and paxillin in focal adhesions. Disruption of the PAK–PIX interaction could potentially decrease PIX association with PKL, resulting in disruption of the paxillin–PKL interaction and a loss of paxillin from focal adhesions. How the interaction of the PAK–PIX–PKL complex with paxillin regulates paxillin stability in focal adhesions is unclear. The regions of paxillin required for localization to focal adhesions do not overlap with PKL binding site on paxillin (Brown *et al.*, 1996). Recently, it has been shown that the PIX, GIT2, and paxillin interaction is regulated and that this interaction increases when paxillin is localized to focal contacts by ectopically expressed CrkII (Lamorte *et al.*, 2003). Whether enhanced association of PIX and GIT with paxillin regulates paxillin redistribution from the cytosol to focal adhesions remains to be determined.

Paxillin localization to focal adhesions has been reported to be regulated by its serine/threonine phosphorylation by an as yet unidentified protein kinase (Brown *et al.*, 1998). The interaction between paxillin and PKL, as well as the interaction between PKL and PIX, could be regulated by phosphorylation of PIX, PKL, or paxillin by PAK1 or an as yet unidentified protein kinase. PAK1 phosphorylates PIX and PKL in vitro (Manser *et al.*, 1998; Chong *et al.*, 2001) and PAK3 has been reported to phosphorylate paxillin (Hashi-

Figure 11. PAK association with PIX and localization to focal adhesions is required for PAK kinase activity. (A) SK-BR-3 cells were microinjected with either a PIX-competing peptide (PAK1, aa 147–231) or a NCK-competing peptide (PAK1, aa 1–74). Thirty minutes after microinjection, cells were fixed and stained with anti-phospho-PAK (anti-pPAK; raised against S199/204 of PAK1)/anti-rabbit Alexa Fluor 488. Cells were scored as described in MATERIALS AND METHODS. Microinjected cells are indicated by an asterisk. (B) SK-BR-3 cells were infected with recombinant Semliki Forest virus constructs encoding myc-tagged PAK1, kinase-dead (K299R), kinase active (T423E), or mutant PAK1 incapable of binding to PIX (R193, P194A). Cell lysates were immunoprecipitated with anti-myc antibodies and subjected to an in-gel kinase assay by using p47 phox peptide as substrate. Immunoprecipitated proteins were subjected to an anti-PAK Western blot to ensure equal loading.

moto *et al.*, 2001). It is possible that in the breast cancer cells used in this study, PAK-mediated phosphorylation of GIT or PKL enhances the association of PKL with paxillin, and plays a role in the constitutive recruitment of PAK, PIX and PKL to focal adhesions. Alternatively, paxillin association with PKL could regulate paxillin localization by modulation of the ARF GAP activity of GIT1 and/or PKL. Overexpression of ARF GAPs such as ASAP-1, PAG-3, and GIT1/2 induce the loss of paxillin from focal adhesions in fibroblasts (Kondo *et al.*, 2000; Randazzo *et al.*, 2000; Zhao *et al.*, 2000b; Mazaki *et al.*, 2001; Furman *et al.*, 2002; Liu *et al.*, 2002). The mechanism by which overexpression of ARF GAPs affects paxillin localization has not been determined, although it has been suggested that modulation of the activation state of ARF1 could play a role. The effects of PAK-mediated phosphorylation of GIT1 on ARF GAP activity and the effects of PIX and paxillin binding to GIT1 on ARF GAP activity and endogenous ARF1 GTP levels have not been determined.

Activation of PAK1/2 in Focal Adhesions

Our results show that in SK-BR-3 and ZR-75-1 breast cancer cells, PAK1 and/or PAK2 are constitutively activated in the absence of high levels of RhoGTPases, coincident with their abnormal localization to focal adhesion structures. We established that PAK activation is dependent on association of PAK with PIX, and the PIX-mediated localization of PAK to focal adhesions. The requirement for PAK/PIX interaction for PAK activation in the breast cancer cell lines used in this

study most likely reflects the requirement for PIX-mediated focal adhesion localization of PAK for activation. In support of this, treatments that result in the loss of focal adhesions (cytochalasin D and cell suspension) abrogate PAK kinase activity but do not decrease the PAK-PIX interaction (Stofega and Bokoch, unpublished data). Alternatively, the loss of PAK activation in suspension cells could result from mislocalization of a small pool of active Rac1GTP (del Pozo *et al.*, 2000). The local activation of endogenous PAK in focal adhesions in SK-BR-3 and ZR-75-1 cells may result from local activation of PAK via PIX, local activation of a Rho GTPase, an exclusion of PAK phosphatases, such as POPX (Koh *et al.*, 2002) from PAK/PIX/PKL-containing focal adhesions, or a combination of all three. Alternatively, PAK may be activated by a RhoGTPase independent mechanism involving sphingosine signaling (Bokoch *et al.*, 1998). Because the sphingosine inhibitor *N,N*-dimethyl sphingosine blocks PAK activation by sphingolipids and RhoGTPases (Bokoch *et al.*, 1998), it was not possible to rule out a potential sphingolipid signaling contribution to PAK activation. Simply targeting PAK to focal adhesions does not result in PAK activation, because the ectopic expression of a PAK construct that contains the FAK focal adhesion targeting sequence does not result in activation, as measured by PAK-dependent ERK activation (Lu *et al.*, 1997).

We cannot rule out the possibility that, in the breast cancer cell lines used in this study, there is a small pool of undetectable focal adhesion-localized activated GTPase that could stimulate PAK. Although we did not detect active Rac or Cdc42 in the p21 binding domain (PBD) affinity assay, this assay may not have been sensitive enough or have access to this pool of active Rac or Cdc42. Rho GTPases do contribute to PAK activation because treatment of SK-BR-3 and ZR-75-1 cells with Toxin B results in a decrease in PAK kinase activity. However, it is difficult to determine if the loss of PAK activity was a due to a specific RhoGTPase-dependent signaling event or a global disruption of focal adhesions and the actin cytoskeleton. Similarly, ectopic expression of dominant negative Rac or Rho (but not Cdc42) resulted in a decrease of PAK activation as measured by pPAK staining, but this loss of PAK activation was accompanied by a loss of stress fibers and PIX and/or paxillin form focal adhesions. To attempt to address the contribution of Rho GTPases to the activation of PAK in focal adhesions without disrupting the focal adhesions and actin cytoskeleton, SK-BR-3 cells were transfected with KD PAK or KD + GTPase binding-deficient PAK (KD PAK H83, 86L). KD PAK and KD PAK H83, 86L localize to focal adhesions (Table 1), but only KD PAK abrogates pPAK staining in focal adhesions (see Supplemental Figure 5). KD PAK abolishes pPAK staining in 89% of transfected cells ($n = 72$), whereas KD + H83, 86L PAK only abolishes pPAK staining in 5% of cells ($n = 102$). Neither PAK construct perturbed focal adhesions as measured by the retention of paxillin in focal adhesions. The simplest explanation is that KD PAK, but not KD (H83, H86L) PAK sequesters locally active Rac or Cdc42 in the focal adhesions. Alternatively, KD PAK could be more effective at displacing endogenous, active PAK from focal adhesions, although both KD PAK and KD (H83, H86L) PAK seem to localize to focal adhesions (Table 1). Recent evidence suggests that active Rac can be found at sites of integrin clustering (Del Pozo *et al.*, 2002). Both SK-BR-3 and ZR-75-1 breast carcinoma cells have $\alpha v\beta 5$ and $\beta 1$ integrins in the large focal adhesion structures (Sanders and Bokoch, unpublished observations), and ligation and/or constitutive activation of these integrins could locally activate Rac and PAK.

PAK and Breast Cancer

Elevated PAK kinase activity and expression may play a role in breast cancer progression. PAK1 and/or PAK2 have been demonstrated to modulate the apoptotic response, cell proliferation, cell migration, and angiogenesis (Bokoch, 2003), all processes that are critical for tumor progression. PAK1 protein levels and kinase activity are elevated in breast tumor samples, but not in adjacent normal breast tissue (Salh *et al.*, 2002). Moreover, there is a correlation between higher grade breast tumors and increased protein levels and kinase activity of PAK1 (Vadlamudi *et al.*, 2000). There may be a functional link between enhanced PAK levels and PAK kinase activity and breast cancer. Targeted overexpression of PAK1 in mice leads to mammary gland hyperplasia (Wang *et al.*, 2002). In multiple human breast carcinoma cell lines, PAK1 and PAK2 kinase activity is constitutively elevated, and inhibiting PAK kinase activity in these cell lines decreases cell proliferation (Mira *et al.*, 2000). It has been proposed that there is a correlation between increased PAK activity and increased breast carcinoma cell motility and invasion (Vadlamudi *et al.*, 2000). Ectopic expression of constitutively activated PAK1 in nonmetastatic MCF-7 breast carcinoma cells increased motility (Vadlamudi *et al.*, 2000). Conversely, overexpression of kinase-dead PAK1 in the highly metastatic MDA-MB-435 breast carcinoma cell line decreased cell motility and invasion, concomitant with an increase in focal contacts and stress fiber formation (Adam *et al.*, 2000). In contrast, in the SK-BR-3 and ZR-75-1 cells used in this study, there are large adhesions and large bundles of stress fibers in the presence of high levels of endogenous, activated PAK. The effects of PAK on stress fiber and focal adhesion formation are highly cell-type specific and may not depend on PAK kinase activity, because kinase-dead and kinase active PAK stabilize focal adhesions and stress fibers in endothelial cells (Kiosses *et al.*, 1999).

Our data also indicate that there is not a simple correlation between increased PAK kinase activity and increased breast carcinoma cell motility and invasion. The SK-BR-3 and ZR-75-1 breast cancer cell lines are nonmotile, noninvasive, and nonmetastatic in nude mice (Thompson *et al.*, 1992), although these cell lines have high levels of active PAK1 and/or PAK2. In contrast, the highly invasive and metastatic MDA-MB-231 cells do not have high levels of PAK activity. The cellular context in which PAK is activated may play a role in increased cell motility. For example, in the highly invasive MDA-MB-435 cells, hyperactive Rac3 contributes to constitutive activation of PAK1/2 and c-Jun NH₂-terminal kinase (JNK) (Mira *et al.*, 2000). Hyperactivation of Rac3 may act apart from or in conjunction with PAK to stimulate signaling pathways required for increased breast carcinoma cell motility and invasion. Recently, phosphorylation of paxillin by JNK has been demonstrated to increase cell motility (Huang *et al.*, 2003). In the absence of activated Rac3, JNK, or other signaling pathways, increased PAK kinase activity may not be sufficient for, nor conducive to, increased motility and invasion.

Although the SK-BR-3 and ZR-75-1 lines are not metastatic in nude mice (Thompson *et al.*, 1992), both cell lines were derived from breast carcinoma cells isolated from metastatic sites in breast cancer patients. Moreover, these cell lines will migrate and/or invade Matrigel in response to chemokines (Youngs *et al.*, 1997), cytokines (Tamm *et al.*, 1989), or members of the epidermal growth factor family of growth factors (Watabe *et al.*, 1998; Yao *et al.*, 2001). It is tempting to speculate that in the proper environment, the SK-BR-3 and ZR-75-1 cells migrate, invade blood vessels,

and metastasize to distant sites. Once the cells have reached the metastatic site, the formation of large focal contacts could firmly attach the tumor cell to the tissue and result in constitutive hyperactivation of PAK. On activation, PAK could promote angiogenesis, resistance to apoptosis, and cell proliferation and thereby promote tumor cell growth. The role of PAK in breast cancer cell invasion, metastasis, and maintenance merits further study.

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REFERENCES

- Adam, L., Vadlamudi, R., Mandal, M., Chernoff, J., and Kumar, R. (2000). Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. *J. Biol. Chem.* 275, 12041–12050.
- Benard, V., Bohl, B.P., and Bokoch, G.M. (1999). Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J. Biol. Chem.* 274, 13198–13204.
- Bokoch, G.M. (2003). Biology of the p21-Activated Kinases. *Annu. Rev. Biochem.* 72, 743–781.
- Bokoch, G.M., Reilly, A.M., Daniels, R.H., King, C.C., Olivera, A., Spiegel, S., and Knaus, U.G. (1998). A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. *J. Biol. Chem.* 273, 8137–8144.
- Bokoch, G.M., Wang, Y., Bohl, B.P., Sells, M.A., Quilliam, L.A., and Knaus, U.G. (1996). Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J. Biol. Chem.* 271, 25746–25749.
- Brown, M.C., Perrotta, J.A., and Turner, C.E. (1996). Identification of LIM3 as the principal determinant of paxillin LIM domains regulates paxillin focal adhesion kinase binding. *J. Cell Biol.* 135, 1109–1123.
- Brown, M.C., Perrotta, J.A., and Turner, C.E. (1998). Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin. *Mol. Biol. Cell* 9, 1803–1816.
- Brown, M.C., West, K.A., and Turner, C.E. (2002). Paxillin-dependent paxillin kinase linker and p21-activated kinase localization to focal adhesions involves a multistep activation pathway. *Mol. Biol. Cell* 13, 1550–1565.
- Chong, C., Tan, L., Lim, L., and Manser, E. (2001). The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity. *J. Biol. Chem.* 276, 17347–17353.
- Chrzanowska-Wodnicka, M., and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J. Cell Biol.* 133, 1403–1415.
- Clark, E.A., King, W.G., Brugge, J.S., Symons, M., and Hynes, R.O. (1998). Integrin-mediated signals regulated by members of the rho family of GTPases. *J. Cell Biol.* 142, 573–586.
- Daniels, R.H., Hall, P.S., and Bokoch, G.M. (1998). Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. *EMBO J* 17, 754–764.
- Daniels, R.H., Zenke, F.T., and Bokoch, G.M. (1999). alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. *J. Biol. Chem.* 274, 6047–6050.
- Daub, H., Gevaert, K., Vandekerckhove, J., Sobel, A., and Hall, A. (2001). Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16. *J. Biol. Chem.* 276, 1677–1680.
- Del Pozo, M.A., Kiesses, W.B., Alderson, N.B., Meller, N., Hahn, K.M., and Schwartz, M.A. (2002). Integrins regulate GTP-Rac localized effector interactions through dissociation of Rho-GDI. *Nat. Cell Biol.* 4, 232–239.
- Del Pozo, M.A., Price, L.S., Alderson, N.B., Ren, X.D., and Schwartz, M.A. (2000). Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. *EMBO J.* 19, 2008–2014.
- Dharmawardhane, S., Sanders, L.C., Martin, S.S., Daniels, R.H., and Bokoch, G.M. (1997). Localization of p21-activated kinase 1 (PAK1) to pinocytotic vesicles and cortical actin structures in stimulated cells. *J. Cell Biol.* 138, 1265–1278.
- Ding, J., Knaus, U.G., Lian, J.P., Bokoch, G.M., and Badwey, J.A. (1996). The renaturable 69- and 63-kDa protein kinases that undergo rapid activation in chemoattractant-stimulated guinea pig neutrophils are p21-activated kinases. *J. Biol. Chem.* 271, 24869–24873.
- Edwards, D.C., Sanders, L.C., Bokoch, G.M., and Gill, G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat. Cell Biol.* 1, 253–259.
- Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629–635.
- Frost, J.A., Khokhlatchev, A., Stippes, S., White, M.A., and Cobb, M.H. (1998). Differential effects of PAK1-activating mutations reveal activity-dependent and -independent effects on cytoskeletal regulation. *J. Biol. Chem.* 273, 28191–28198.
- Furman, C., Short, S.M., Subramanian, R.R., Zetter, B.R., and Roberts, T.M. (2002). DEF-1/ASAP1 is a GTPase-activating protein (GAP) for ARF1 that enhances cell motility through a GAP-dependent mechanism. *J. Biol. Chem.* 277, 7962–7969.
- Galisteo, M.L., Chernoff, J., Su, Y.C., Skolnik, E.Y., and Schlessinger, J. (1996). The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. *J. Biol. Chem.* 271, 20997–21000.
- Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K.M. (2001). Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. *Nat. Rev. Mol. Cell Biol.* 2, 793–805.
- Hashimoto, S., Tsubouchi, A., Mazaki, Y., and Sabe, H. (2001). Interaction of paxillin with p21-activated Kinase (PAK). Association of paxillin alpha with the kinase-inactive and the Cdc42-activated forms of PAK3. *J. Biol. Chem.* 276, 6037–6045.
- He, H., Hirokawa, Y., Manser, E., Lim, L., Levitzki, A., and Maruta, H. (2001). Signal therapy for RAS-induced cancers in combination of AG 879 and PP1, specific inhibitors for ErbB2 and Src family kinases, that block PAK activation. *Cancer J.* 7, 191–202.
- Hoffman, L.M., *et al.* (2003). Targeted disruption of the murine zyxin gene. *Mol. Cell Biol.* 23, 70–79.
- Huang, C., Rajfur, Z., Borchers, C., Schaller, M.D., and Jacobson, K. (2003). JNK phosphorylates paxillin and regulates cell migration. *Nature* 424, 219–223.
- Kiosses, W.B., Daniels, R.H., Otey, C., Bokoch, G.M., and Schwartz, M.A. (1999). A role for p21-activated kinase in endothelial cell migration. *J. Cell Biol.* 147, 831–844.
- Knaus, U.G., Morris, S., Dong, H.J., Chernoff, J., and Bokoch, G.M. (1995). Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. *Science* 269, 221–223.
- Koh, C.G., Tan, E.J., Manser, E., and Lim, L. (2002). The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. *Curr. Biol.* 12, 317–321.
- Kondo, A., Hashimoto, S., Yano, H., Nagayama, K., Mazaki, Y., and Sabe, H. (2000). A new paxillin-binding protein, PAG3/Papalpa/KIAA0400, bearing an ADP-ribosylation factor GTPase-activating protein activity, is involved in paxillin recruitment to focal adhesions and cell migration. *Mol. Biol. Cell* 11, 1315–1327.
- Lamorte, L., Rodrigues, S., Sangwan, V., Turner, C.E., and Park, M. (2003). Crk Associates with a Multimolecular Paxillin/GIT2/[beta]-PIX Complex and Promotes Rac-dependent Relocalization of Paxillin to Focal Contacts. *Mol. Biol. Cell* 14, 2818–2831.
- Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* 84, 359–369.
- Liu, Y., Lojens, J.C., Martin, K.H., Karginov, A.V., and Parsons, J.T. (2002). The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. *Mol. Biol. Cell* 13, 2147–2156.
- Lu, W., Katz, S., Gupta, R., and Mayer, B.J. (1997). Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr. Biol.* 7, 85–94.
- Ma, L., Rohatgi, R., and Kirschner, M.W. (1998). The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. *Proc. Natl. Acad. Sci. USA* 95, 15362–15367.
- Machesky, L.M., Mullins, R.D., Higgs, H.N., Kaiser, D.A., Blanchoin, L., May, R.C., Hall, M.E., and Pollard, T.D. (1999). Scar, a WASP-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc. Natl. Acad. Sci. USA* 96, 3739–3744.

- Manabe Ri, R., Kovalenko, M., Webb, D.J., and Horwitz, A.R. (2002). GIT1 functions in a motile, multi-molecular signaling complex that regulates protrusive activity and cell migration. *J. Cell Sci.* 115, 1497–1510.
- Manser, E., Huang, H.Y., Loo, T.H., Chen, X.Q., Dong, J.M., Leung, T., and Lim, L. (1997). Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Mol. Cell. Biol.* 17, 1129–1143.
- Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol. Cell* 1, 183–192.
- Mazaki, Y., Hashimoto, S., Okawa, K., Tsubouchi, A., Nakamura, K., Yagi, R., Yano, H., Kondo, A., Iwamatsu, A., Mizoguchi, A., and Sabe, H. (2001). An ADP-ribosylation factor GTPase-activating protein Git2-short/KIAA0148 is involved in subcellular localization of paxillin and actin cytoskeletal organization. *Mol. Biol. Cell* 12, 645–662.
- Miki, H., Yamaguchi, H., Suetsugu, S., and Takenawa, T. (2000). IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature* 408, 732–735.
- Mira, J.P., Benard, V., Groffen, J., Sanders, L.C., and Knaus, U.G. (2000). Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. *Proc. Natl. Acad. Sci. USA* 97, 185–189.
- Nobes, C.D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol.* 144, 1235–1244.
- Obermeier, A., Ahmed, S., Manser, E., Yen, S.C., Hall, C., and Lim, L. (1998). PAK promotes morphological changes by acting upstream of Rac. *EMBO J.* 17, 4328–4339.
- Parrini, M.C., Lei, M., Harrison, S.C., and Mayer, B.J. (2002). Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. *Mol. Cell* 9, 73–83.
- Premont, R.T., Claing, A., Vitale, N., Freeman, J.L., Pitcher, J.A., Patton, W.A., Moss, J., Vaughan, M., and Lefkowitz, R.J. (1998). beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein. *Proc. Natl. Acad. Sci. USA* 95, 14082–14087.
- Puto, L.A., Pestonjamas, K., King, C.C., and Bokoch, G.M. (2003). p21-activated kinase 1 (PAK1) interacts with the Grb2 adapter protein to couple to growth factor signaling. *J. Biol. Chem.* 278, 9388–9393.
- Randazzo, P.A., Andrade, J., Miura, K., Brown, M.T., Long, Y.Q., Stauffer, S., Roller, P., and Cooper, J.A. (2000). The Arf GTPase-activating protein ASAP1 regulates the actin cytoskeleton. *Proc. Natl. Acad. Sci. USA* 97, 4011–4016.
- Ren, X.D., Kiosses, W.B., and Schwartz, M.A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* 18, 578–585.
- Ridley, A.J. (2001). Rho GTPases and cell migration. *J. Cell Sci.* 114, 2713–2722.
- Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M.W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97, 221–231.
- Rottner, K., Hall, A., and Small, J.V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr. Biol.* 9, 640–648.
- Salh, B., Marotta, A., Wagey, R., Sayed, M., and Pelech, S. (2002). Dysregulation of phosphatidylinositol 3-kinase and downstream effectors in human breast cancer. *Int. J. Cancer* 98, 148–154.
- Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* 283, 2083–2085.
- Sells, M.A., Boyd, J.T., and Chernoff, J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. *J. Cell Biol.* 145, 837–849.
- Sells, M.A., Knaus, U.G., Bagrodia, S., Ambrose, D.M., Bokoch, G.M., and Chernoff, J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr. Biol.* 7, 202–210.
- Sells, M.A., Pfaff, A., and Chernoff, J. (2000). Temporal and spatial distribution of activated Pak1 in fibroblasts. *J. Cell Biol.* 151, 1449–1458.
- Shamah, S.M., et al. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell* 105, 233–244.
- Tamm, I., Cardinale, I., Krueger, J., Murphy, J.S., May, L.T., and Sehgal, P.B. (1989). Interleukin 6 decreases cell-cell association and increases motility of ductal breast carcinoma cells. *J. Exp. Med.* 170, 1649–1669.
- Thompson, E.W., et al. (1992). Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J. Cell Physiol.* 150, 534–544.
- Turner, C.E., Brown, M.C., Perrotta, J.A., Riedy, M.C., Nikolopoulos, S.N., McDonald, A.R., Bagrodia, S., Thomas, S., and Leventhal, P.S. (1999). Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling. *J. Cell Biol.* 145, 851–863.
- Vadlamudi, R.K., Adam, L., Wang, R.A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M.C., and Kumar, R. (2000). Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. *J. Biol. Chem.* 275, 36238–36244.
- Vadlamudi, R.K., Li, F., Adam, L., Nguyen, D., Ohta, Y., Stossel, T.P., and Kumar, R. (2002). Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat. Cell Biol.* 4, 681–690.
- Wang, R.A., Mazumdar, A., Vadlamudi, R.K., and Kumar, R. (2002). P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. *EMBO J.* 21, 5437–5447.
- Watabe, T., Yoshida, K., Shindoh, M., Kaya, M., Fujikawa, K., Sato, H., Seiki, M., Ishii, S., and Fujinaga, K. (1998). The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. *Int. J. Cancer* 77, 128–137.
- Wittmann, T., Bokoch, G.M., and Waterman-Storer, C.M. (2003). Regulation of leading edge microtubule and actin dynamics downstream of Rac1. *J. Cell Biol.* 161, 845–851.
- Yao, J., Xiong, S., Klos, K., Nguyen, N., Grijalva, R., Li, P., and Yu, D. (2001). Multiple signaling pathways involved in activation of matrix metalloproteinase-9 (MMP-9) by heregulin-beta1 in human breast cancer cells. *Oncogene* 20, 8066–8074.
- Youngs, S.J., Ali, S.A., Taub, D.D., and Rees, R.C. (1997). Chemokines induce migrational responses in human breast carcinoma cell lines. *Int. J. Cancer* 71, 257–266.
- Zamir, E., and Geiger, B. (2001). Components of cell-matrix adhesions. *J. Cell Sci.* 114, 3577–3579.
- Zenke, F.T., King, C.C., Bohl, B.P., and Bokoch, G.M. (1999). Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. *J. Biol. Chem.* 274, 32565–32573.
- Zhao, Z.S., Manser, E., Chen, X.Q., Chong, C., Leung, T., and Lim, L. (1998). A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol. Cell. Biol.* 18, 2153–2163.
- Zhao, Z.S., Manser, E., and Lim, L. (2000a). Interaction between PAK and nck: a template for Nck targets and role of PAK autophosphorylation. *Mol. Cell. Biol.* 20, 3906–3917.
- Zhao, Z.S., Manser, E., Loo, T.H., and Lim, L. (2000b). Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. *Mol. Cell. Biol.* 20, 6354–6363.