Differential Effects of PAK1-activating Mutations Reveal Activity-dependent and -independent Effects on Cytoskeletal Regulation

(Received for publication, June 3, 1998, and in revised form, August 20, 1998)

Jeffrey A. Frost‡, Andrei Khokhlatchev§§, Steve Stippec‡, Michael A. White¶, and Melanie H. Cobb‡

From the University of Texas Southwestern Medical Center, ‡Departments of Pharmacology and ¶Cell Biology and Neuroscience, Dallas, Texas 75235-9041

PAKs are serine/threonine protein kinases that are activated by binding to Rac or Cdc42hs. Different forms of activated PAK1 have been reported to either promote membrane ruffling and focal adhesion assembly or cause focal adhesion disassembly and stress fiber dissolution. To understand the basis for these distinct morphological effects, we have examined the mechanism of mutational activation of PAK1, and characterized the effects of different active PAK1 proteins on cytoskeletal structure in vivo. We find that PAK1 contains an autoinhibitory domain that overlaps with its small G protein binding domain and that two separate activating mutations within this regulatory region each decrease autoinhibitory activity. Because only one of these mutations affects Cdc42hs binding activity, this indicates that activation of PAK1 by these mutations results from interference with the function of the autoinhibitory domain and not with small G protein binding activity. When we examined the morphological effects of these different forms of PAK1 in vivo, we found that PAK1 kinase activity was associated with disassembly of focal adhesions and actin stress fibers and that this may require interaction with potential SH3 domain-containing proteins. Lamellipodia formation and membrane ruffling caused by active PAK1 expression, however, was independent of PAK1 catalytic activity and likely requires interaction among multiple proteins binding to the PAK1 regulatory domain.

Rho family small G proteins are key regulators of cytoskeletal organization and gene expression, but the mechanisms by which they control these processes are unclear. In their GTP-bound state, these small G proteins bind to a number of enzymes that are believed to act as downstream effectors. Among these are a family of serine/threonine kinases known as p21-activated kinases, or PAKs (1–5). PAKs are activated by binding to Rac or Cdc42hs. Small G protein binding is accomplished by autophosphorylation on multiple residues both in vivo and in vitro, and this is thought to be crucial for activation (6). Once activated, PAKs may be targeted to particular intracellular compartments through sustained binding to Rac and Cdc42hs, as well as through interaction with PAK1-binding proteins such as Nck and PIX (2, 3, 5, 7–9). A number of responses have been associated with PAK activity, including regulation of the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAK) mitogen-activated protein kinase pathways (10–13), promotion of apoptosis (PAK2 only) (14, 15), and alteration of cytoskeletal organization (6, 16, 17). The mechanisms by which PAKs control these events are for the most part undefined.

To date, three PAK isoforms have been cloned (PAK1, 2, and 3). Each contains a C-terminal catalytic domain and an N-terminal regulatory domain that binds Rac and Cdc42hs. The catalytic domains share greater than 90% identity. The regulatory domains are less well conserved with an overall identity of 55%. Within this regulatory, noncatalytic region of approximately 250 amino acids, however, is a stretch of 66 residues that is over 90% identical among PAK isoforms (amino acids 67–132 in PAK1). PAK1 also contains five potential SH3-binding sites, as defined by the amino acid sequence (18). The most N-terminal of these, starting at proline 13, binds the SH2-SH3-containing adaptor protein Nck constitutively (7, 8). PAK1 has also been shown to bind the Rho family exchange factor PIX within a polyproline sequence between amino acids 182 and 203 (9). Ligands for the other potential SH3-binding sites have not been identified.

A role for PAK1 in cytoskeletal regulation has been demonstrated. By transient transfection of an activated PAK1 mutant unable to bind small G proteins (PAK1 H83/86L), one group has shown in Swiss 3T3 cells that prolonged expression of PAK1 H83/86L promotes the formation of polarized actin ruffles that contain vinculin-associated focal adhesions (16). In contrast, another group has shown in HeLa cells that PAK1 itself associates with focal adhesions and that expression of three distinct forms of active PAK1 promotes stress fiber disassembly (6, 17). These apparently conflicting results could be due to differences in the activating mutations used, as well as the distinct cell types examined. To define more precisely the effects of PAK1 activation on cell morphology, we have characterized three commonly used activating mutations in terms of their mechanism of activation and ability to interact with Cdc42hs. In short term microinjection assays we have also examined the effects of these PAK1 molecules on cytoskeletal structure. The results highlight key features involved in the regulation of PAK1 activity and the effects of PAK1 on cytoskeletal organization.

MATERIALS AND METHODS

Cell Culture—REF52 and 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 100 units/ml penicillin/streptomycin (Life Technologies, Inc.). For microinjection, REF52 cells were plated on acid-washed
Effects of PAK1 Mutations on Cytoskeletal Organization

were expressed in the bacterial strain BL21DE3. Cells were grown at 37 °C in A-Sepharose (Amersham Pharmacia Biotech) using either Mono Q or Mono S ion exchange matrices. Proteins were purified by sequential glutathione-agarose affinity chromatography for 4 h, and harvested. Expressed GST-PAK1 proteins were detected with either mouse anti-Myc or rabbit anti-Myc (Santa Cruz Biotech, 1:100 dilution). PAK1 was detected with mouse anti-paxillin (Transduction Laboratories, 1:100 dilution). β-Galactosidase was detected with mouse anti-β-galactosidase (Sigma, 1:50 dilution). All primary antibodies were incubated with cells for 1 h at 37 °C. After washing with 3 × 5 min in PBS + 0.05% Tween 20, the cells were incubated with one or more of the following as indicated: fluorescein isothiocyanate-conjugated phalloidin (Sigma), fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody (Jackson Laboratories), or tetramethyl rhodamine conjugated donkey anti-mouse antibody (Jackson Laboratories). Immunofluorescence was detected with a Zeiss Axiosvert 20 inverted microscope, and images were recorded with a Quantix photomager (Photometrics).

RESULTS

Identification of an Autoinhibitory Domain within PAK1—PAK1 has been reported by different groups to either promote membrane ruffling and focal adhesion assembly (16) or cause a dissolution of focal adhesions and actin stress fibers (6, 17). Much of the work showing membrane ruffling had been obtained by transfecting Swiss 3T3 cells with an active PAK1 mutant unable to bind Rac or Cdc42 (PAK1 H83/86L) (16). PAK1-mediated disruption of the actin cytoskeleton had been demonstrated by microinjecting HeLa cells with plasmids encoding either PAK1 fused to active Cdc42 lacking a C-terminal CAAX box or a PAK1 molecule containing an activating point mutation (PAK1 L107F) (6, 17). Discrepancies in these results could be due to differences in the level of activity between these different PAK1 molecules, differences in their intracellular mode of action, or physiological differences between the cell types examined. To understand the actions of distinct PAK1 mutants, we examined the mechanism of mutational activation of PAK1 and monitored effects of the mutants on cytoskeletal structure in short term microinjection assays. We chose three commonly used forms of PAK1 for study, PAK1 H83/86L (16), PAK1 L107F (13, 17), and PAK1-(165-544) (PAK1-(165)), an N-terminal deletion mutant lacking the first 164 residues of PAK1 (11).

Wild type PAK1 is activated by binding to active Rac or Cdc42 (1). Because most PAK1-activating mutations occur within its small G protein binding domain (Fig. 1, panel A), we first determined their effects on Cdc42 binding in vitro. Both GST-wild type PAK1 (wt PAK1) and the N-terminal fragment 1–231 efficiently bound Val12Cdc42 (Fig. 1, panel B). A smaller portion of the N terminus, residues 52–122, was also fully capable of binding to Val12Cdc42. However, residues 75–132, corresponding to the N-terminal region of almost complete identity among PAKs, was unable to bind. This indicates that residues between 52 and 75 are required for small G protein binding, consistent with the finding that a fusion protein containing amino acids 20–90 of PAK1 binds GTP-ligated Rac in vitro (20). Also consistent with these results, the N-terminal deletion mutant PAK1-(165) did not bind Val12Cdc42. Thus, the fact that the most conserved part of the N-terminal domain is not involved in binding to small G proteins indicates that this region has another function. Previously it has been shown that mutation of histidine 83 and 86 to leucine (H83/86L) abolishes Rac binding in vitro (16, 17). On the other hand, the effect of the L107F mutation on small G protein binding has not been tested. When PAK1(1–231) H83/86L was incubated with Cdc42hs, no binding was detected (Fig. 1, panel B). The L107F mutation, however, had little to no effect on Val12Cdc42 binding. Because this muta-

1 The abbreviations used are: GST, glutathione S-transferase; MBP, myelin basic protein; wt, wild type; PBS, phosphate-buffered saline.
Effects of PAK1 Mutations on Cytoskeletal Organization

Panels A, B, and C of the figure show the schematic of PAK1 fragments. Panel A illustrates the functional domains and activating mutations. Panel B displays the autoinhibition of PAK1 N-terminal fusion proteins by N-terminal GST-PAK1. Panel C represents the kinase activity of PAK1 proteins.

The kinase-inactivating mutation K298A. The region coding for PAK1-(232–544) alone and are the average of at least three independent experiments. The N-terminal truncation mutant PAK1-(165) was maximally active (270-fold, on average) and largely unresponsive to small G protein expression, exhibiting less than 2-fold greater activity in the presence of active Rac or Cdc42hs. This was expected, since this clone lacks both the small G protein binding domain (52–122) and the autoinhibitory domain (minimally 75–132). PAK1-(1–231) L107F did not inhibit PAK1 catalytic activity, but this required higher concentrations of protein than observed for PAK1-(1–231). The minimal PAK1 fragment competent to bind Cdc42hs, PAK1-(52–122), was much less effective at inhibiting PAK1 activity. These results suggest that the activating point mutations H83/86L and L107F increase PAK1 activity not because they affect small G protein binding but because they impair the function of the autoinhibitory domain. Because the L107F mutation is more effective at blocking autoinhibitory activity than the H83/86L mutations, this suggests that the L107F mutation is more activating than the H83/86L mutations.

To test the relative kinase activities of the activated PAK1 mutants, we transiently cotransfected 293 cells with either wild type PAK1, PAK1-(165), PAK1 H83/86L, or PAK1 L107F, along with empty vector or various small G proteins. PAK1 proteins were then tested for their respective MBP kinase activities following immunoprecipitation with an antibody directed against an N-terminal Myc epitope tag. A representative experiment is shown in Fig. 2. Coexpression of either Val12Rac2 or Val12Cdc42hs led to a significant increase in wt PAK1 kinase activity (12- and 8-fold, respectively), whereas Val12Ha-Ras and Leu63RhoA coexpression were without effect. The N-terminal truncation mutant PAK1-(165) was maximally active whether or not a small G protein was coexpressed (260- and 270-fold, on average). This was expected, since this clone lacks both the small G protein binding domain (52–122) and the autoinhibitory domain (minimally 75–132). PAK1 L107F also was maximal (270-fold, on average) and largely unresponsive to small G protein expression, exhibiting less than 2-fold greater activity in the presence of active Rac or Cdc42hs. Because coexpression of either Rac or Cdc42hs caused a slight increase in PAK1 L107F activity along with a decreased mobility in the gel (Fig. 2), this suggests that PAK1 L107F can...
still interact with small G proteins in vivo, consistent with the binding activity of its N terminus in vitro. The near maximal kinase activity of PAK1 L107F also fits well with the complete loss of autoinhibitory activity in vitro. It should be noted that PAK1 H83/86L may also be slightly responsive to coexpression of Val12Rac2 in vivo, although we detected no binding to small G proteins in vitro. This may reflect either a substantial decrease in the density of staining of actin stress fibers that colocalize with vinculin-containing focal adhesions (PAK1 H83/86L) (16). In other experiments, microinjection of either a PAK1-Val12Cdc42hs fusion protein or PAK1 L107F into cells (6, 17). To resolve these opposing findings, we compared the effects of activated PAK1 mutants on cytoskeletal structure in short term microinjection assays. In these assays, expression of PAK1 cDNAs could be detected 2–3 h after injection. As shown in Fig. 3, 4 h after injection of plasmids encoding either wt PAK1 or PAK1 H83/86L, cell morphology was not noticeably affected. Both of these proteins appeared to be distributed throughout the cytoplasm, with PAK1 H83/86L often staining more distinctly in a punctate pattern. Expression of PAK1 L107F in some cells caused the extension of small lamellipodia with a slight decrease in the density of staining of actin stress fibers (Fig. 3). PAK1 L107F also exhibited largely punctate staining. Expression of PAK1 (165), on the other hand, caused a dramatic collapse of the cell periphery that began within 2 h after injection. By 4 h, these cells were devoid of actin stress fibers (Fig. 3) and lacked paxillin-containing focal adhesions (data not shown). This phenotype was dependent on PAK1 kinase activity, since expression of catalytically inactive PAK1-(165) K298A was without effect (data not shown). This phenotype is most similar to that observed upon expression of either a PAK1-Val12Cdc42hs fusion protein or PAK1 L107F in HeLa cells (6, 17).

Longer term expression of active PAK1 (16–18 h) altered many of the phenotypes observed at 4 h. Compared with the surrounding uninjected cells, as well as cells injected with a vector encoding β-galactosidase (Fig. 4, panels A and B), wt PAK1 overexpression caused a flattening of the cell that was accompanied by the formation of lamellipodia and occasional ruffling of the cell membrane. There was no apparent change in actin stress fiber organization (Fig. 4, panels C and D) or in the number or size of paxillin-containing focal adhesions (data not shown). After 18 h, PAK1 H83/86L also promoted lamellipodia. Effects of PAK1 Mutations on Cytoskeletal Structure—Past results disagree as to the effects of PAK1 expression on cytoskeletal structure. Studies in transfected cells have shown that PAK1 has the potential to localize to focal adhesions (both catalytically inactive PAK1-K298A and PAK1-CAAX (6)) and can promote the formation of polarized actin ruffles that colocalize with vinculin-containing focal adhesions (PAK1 H83/86L) (16). In other experiments, microinjection of either a PAK1-Val12Cdc42hs fusion protein or PAK1 L107F have been shown to cause retraction of the cell periphery with a loss of paxillin-containing focal adhesions and actin stress fibers (6, 17). To resolve these opposing findings, we compared the effects of activated PAK1 mutants on cytoskeletal structure in short term microinjection assays. In these assays, expression of PAK1 cDNAs could be detected 2–3 h after injection. As shown in Fig. 3, 4 h after injection of plasmids encoding either wt PAK1 or PAK1 H83/86L, cell morphology was not noticeably affected. Both of these proteins appeared to be distributed throughout the cytoplasm, with PAK1 H83/86L often staining more distinctly in a punctate pattern. Expression of PAK1 L107F in some cells caused the extension of small lamellipodia with a slight decrease in the density of staining of actin stress fibers (Fig. 3). PAK1 L107F also exhibited largely punctate staining. Expression of PAK1-(165), on the other hand, caused a dramatic collapse of the cell periphery that began within 2 h after injection. By 4 h, these cells were devoid of actin stress fibers (Fig. 3) and lacked paxillin-containing focal adhesions (data not shown). This phenotype was dependent on PAK1 kinase activity, since expression of catalytically inactive PAK1-(165) K298A was without effect (data not shown). This phenotype is most similar to that observed upon expression of either a PAK1-Val12Cdc42hs fusion protein or PAK1 L107F in HeLa cells (6, 17).
RhoA (Asn19RhoA) did not inhibit cell collapse. Coexpression of Asn17Cdc42hs, in fact, exacerbated the effect. Coexpression of nontype observed at 4 h (data not shown). PAK1-(165)-expressing cells were still viable minor compared with that caused by expression of active Rac1 formation, and these structures sometimes contained one or more filopodia on their ends (panels G and H). These cells also occasionally contained actin-staining membrane ruffles on one or more of their edges. Overall, the degree of membrane ruffling caused by wild type or H83/86L PAK1 expression was minor compared with that caused by expression of active Rac1 (data not shown). PAK1-(165)-expressing cells were still viable at this time and exhibited the same collapsed, arborized phe-
notype observed at 4 h (panels G and H). Cells expressing catalytically inactive PAK1-(165) still appeared phenotypically normal (data not shown).

Longer term expression of PAK1 L107F dramatically altered its resultant phenotype. By 18 h, cells expressing this mutant had assumed a morphology nearly identical to that caused by PAK1-(165) (Fig. 4, panels I and J). Because the catalytic activities of PAK1 L107F and PAK1-(165) are virtually identical (Fig. 2), it is unclear why the rate of cell collapse was so much slower for PAK1 L107F than PAK1-(165). A plausible explanation is that PAK1 L107F interacts with other proteins through N-terminal sequences that PAK1-(165) lacks. These interactions may sequester active PAK1 from sites where its activity can cause focal adhesion disassembly and stress fiber dissolution. Interactions with Rac and Cdc42hs are unlikely to mediate the slower action of PAK1 L107F relative to PAK1-(165) because expression of a PAK1 L107F molecule unable to bind small G proteins (PAK1 H83/86L, L107F) resulted in the same rate of cell collapse as PAK1 L107F (data not shown).

Cell collapse caused by PAK1-(165) expression apparently does not depend on changes in Rho family small G protein activity. As shown in Fig. 5, coinjection of plasmids for dominant negative Rac1 (Asn17Rac1), Cdc42hs (Asn17Cdc42hs), or RhoA (Asn19RhoA) did not inhibit cell collapse. Coexpression of Asn17Cdc42hs, in fact, exacerbated the effect. Coexpression of catalytically inactive PAK1-(165), on the other hand, reduced the cytoskeletal actions of active PAK1-(165). Thus, these data suggest that PAK1-(165) can regulate cell morphology independently of endogenous Rho family small G protein activity, either through its direct actions as a Rac and Cdc42hs effector or by modulation of the activity of other small G protein effectors.

**Effects of Catalytically Inactive, Full-length PAK1 on Cytoskeletal Structure**—The effects of PAK1-(165) on cell morphology are clearly dependent on its catalytic activity. This, however, was not the case for full-length PAK1 molecules. As shown in Fig. 6, overnight expression of catalytically inactive, full-length PAK1 K298A, PAK1 H83/86L K298A, or PAK1 L107F K298A caused cells to spread out and extend numerous lamellipodia. These lamellipodia contained numerous paxillin-staining focal adhesions (Fig. 6) and exhibited essentially normal staining for actin stress fibers (data not shown). Extensive membrane ruffling was also observed (data not shown). These effects were dependent upon the presence of the PAK1 N terminus, since expression of catalytically inactive PAK1-(165) did not cause these changes (Fig. 6). Interestingly, the degree of morphological change was highest following expression of catalytically inactive PAK1 L107F. This may be due to an increased accessibility of the PAK1 L107F N terminus to other interacting proteins, since this mutation strongly inhibits the ability of the N terminus to interact with and inhibit the PAK1 catalytic domain. These findings are consistent with the hypothesis that PAK1-stimulated lamellipodia formation is independent of its catalytic activity. This idea fits with results reported by others (21), in which expression of catalytically inactive PAK1 H83/86L promotes morphological differentiation of PC12 cells.

**Coexpression of the PAK1 Autoinhibitory Domain Blocks**

**FIG. 5.** **Dominant negative Rho family small G proteins do not inhibit cell collapse caused by PAK1-(165).** Serum-starved REF52 cells were injected with PAK1-(165) (0.2 mg/ml in the needle) and either empty vector (panels A and B), Asn17Rac1 (panels C and D), Asn17Cdc42hs (panels E and F), Asn19RhoA (panels G and H), or PAK1-(165) K298A (panels I and J). Coinjected vectors were injected at 0.5 mg/ml in the needle. 4 h after injection, the cells were fixed and stained for expression of Myc-tagged proteins (panels A, C, E, G, and I) and F-actin (panels B, D, F, H, and J). Myc-tagged dominant negative small G proteins alone were without effect in this assay (data not shown). PAK1-(165) Action in Vivo—PAK1-(75–132) can act as a minimal inhibitory domain when combined with the PAK1 catalytic domain in vitro. Zhao et al. (17) have shown that a larger PAK1 fragment, 83–149, can inhibit PAK1 kinase activity and prevent cell collapse caused by PAK1 L107F expression in vitro (17). To test if this autoinhibitory domain can inhibit PAK1-(165) effects in vivo, we coinjected PAK1-(165) with plasmids encoding either the minimal inhibitory fragment PAK1-(75–132), PAK1-(83–149), or PAK1-(83–149) L107F. The L107F point mutation abolishes the ability of larger N-terminal fragments to inhibit PAK1 catalytic activity in vitro (Fig. 1, panel C). Four hours after injection, the cells were fixed, stained, and examined for cytoskeletal integrity. As shown in Fig. 7, coexpression of PAK1-(75–132) did not block the ability of PAK1-(165) to cause stress fiber dissolution. On the other hand, coexpression of PAK1-(83–149) almost completely blocked cell collapse. PAK1-(83–149) L107F was unable to block PAK1-(165)-mediated cell collapse. These data suggest that residues between 132 and 149 are required for inhibition of PAK1 activity in vivo. Interestingly, these residues are not conserved among PAK family members, suggesting that they may either confer specificity for autoinhibition between PAK isoforms or may play a structural role in promoting correct folding of the autoinhibitory domain.
Myc-tagged PAK1 proteins (Serum-starved REF52 cells were injected with plasmids encoding kinase-inactive (K298A) forms of full-length PAK1 (panels A and B), PAK1 H83/86L (panels C and D), PAK1 L107F (panels E and F), or PAK1-(165) (panels G and H). 18 h after injection the cells were fixed and stained for Myc-tagged PAK1 proteins (panels A, C, E, and G) and paxillin (panels B, D, F, and H).

**Fig. 6.** Overnight expression of kinase-inactive, full-length PAK1 proteins cause distinct morphological changes. Serum-starved REF52 cells were injected with plasmids encoding kinase-inactive (K298A) forms of full-length PAK1 (panels A and B), PAK1 H83/86L (panels C and D), PAK1 L107F (panels E and F), or PAK1-(165) (panels G and H). 18 h after injection the cells were fixed and stained for Myc-tagged PAK1 proteins (panels A, C, E, and G) and paxillin (panels B, D, F, and H).

**Mutation of the Potential SH3-binding Sites at Residues 209–212 and 220–223 Reduces the Efficiency of Cytoskeletal Regulation by PAK1-(165) and PAK1 L107F—**PAK1-(165) contains three potential SH3-binding sites, as defined by the sequence PXXP (Fig. 1, panel A). We tested whether the two most C-terminal sites were required for effects on the cytoskeleton by mutating the first proline to an alanine in either the second (P209A), the third (P220A), or both the second and third (P209/220A) potential SH3-binding sites. Mutation of the first proline in an SH3-binding site abolishes SH3-domain binding (18). Initially, these constructs were tested for their respective abilities to promote cell collapse in short term assays. In these experiments mutation of prolines 209 and 220 to alanine appeared to reduce the severity of cell collapse, such that many cells had lost actin stress fiber staining but still maintained a partially extended cytoplasm (data not shown). This was not the case for the single proline mutants. This suggested that these prolines may mediate interaction with one or more potential SH3 domain-containing proteins that are important for PAK1 cytoskeletal effects. To both quantify this effect and determine whether these prolines were important for PAK1 kinase activity, we scored the percent-
age of cells displaying a collapsed phenotype after overnight expression of PAK1-(165) and PAK1 L107F proline mutants. As shown in Fig. 8, panel A, mutation of prolines 209 and 220 to alanine in either PAK1-(165) or PAK1 L107F caused a small but statistically significant decrease in the percentage of cells displaying morphological change. This decrease was more pronounced for PAK1 L107F, reducing the percentage of collapsed cells from 75 to 50%. Mutation of these prolines did not reproducibly affect the kinase activity of either PAK1-(165) of PAK1 L107F (Fig. 8, panel B). These results suggest that prolines 209 and 220 may be important for the interaction of PAK1 with other proteins that allow it to cause focal adhesion disassembly and stress fiber dissolution.

Recently it has been shown that PAK1 interacts with a Rho family guanine nucleotide exchange factor called PIX (9). PIX has been shown to bind to a proline-rich sequence between residues 186–203. Nevertheless, we tested whether mutation of prolines 209 and 220 affect PIX binding. In a yeast two-hybrid interaction assay, mutation of either P209 alone, or P209 and P220 to alanine together did not affect interaction with the SH3 domain of PIX (data not shown). Thus, prolines 209 and 220 may mediate interaction with proteins other than PIX that are important for modulation of cytoskeletal structure by PAK1.

**DISCUSSION**

As a first step in describing the mechanism by which PAK1 modulates cytoskeletal structure, we have characterized several commonly used active PAK1 mutants, and we identified an autoinhibitory domain that regulates PAK1 activation. We demonstrated that residues 75–132 function as a minimal autoinhibitory domain in vitro and that the H83/86L and L107F mutations reduce the ability of the autoinhibitory domain to function. Because these mutants occur in or near the small G protein binding domain, it had been inferred that altered small G protein binding caused the increase in activity. We show here that these mutations activate PAK1 because they have reduced autoinhibitory activity. The degree to which these mutations block autoinhibition correlates with the degree of activation of PAK1 kinase activity. Thus, the double mutation H83/86L only partially blocks the autoinhibitory domain and results in only a slight activation of PAK1 kinase activity (5–10-fold). On the other hand, the L107F mutation completely prevents autoinhi-
Effects of PAK1 Mutations on Cytoskeletal Organization

PAK1-(165) and PAK1-(165) P209/220A, PAK1 L107F, or PAK1 L107F P209/220A. Cells were injected as described and fixed 18 h later. Results are the average of at least four independent experiments, and error bars correspond to the standard deviation. Significance of the results was analyzed by a standard t test. Panel B, mutation of prolines 209 and 220 to alanine does not affect the catalytic activity of PAK1-(165) or PAK1 L107F. 293 cells were transfected with plasmids encoding the PAK1 proteins shown. Soluble PAK1 proteins were assayed for their MBP kinase activities as described. Shown is a representative experiment. MBP phosphorylation is pictured in the top panel. A Western blot for Myc-tagged PAK1 expression is shown in the bottom panel.

Our characterization of these mutants allows us to ascertain PAK1 effects in vivo. We find that PAK1 kinase activity is essential for promotion of focal adhesion disassembly, since only PAK1-(165) and PAK1 L107F but not PAK1 H83/86L or kinase-inactive mutants are active in this regard. Thus, PAK1 probably phosphorylates proteins that either regulate cytoskeletal assembly or the cytoskeletal elements themselves. Interestingly, kinase-inactive, full-length versions of PAK1 do promote other morphological changes. Specifically, they cause extensive lamellipodia formation and membrane ruffling (9). Since kinase-inactive PAK1-(165) can still bind to Pix but does not promote lamellipodia formation or membrane ruffling, it is possible that endogenous Pix bound to PAK must interact with other proteins associated with the PAK1 N terminus to have these effects. Thus, the PAK1 regulatory domain may act as a nucleating factor that brings together proteins involved in cytoskeletal regulation. It is important to note that the effects of PAK1 H83/86L expression on cell morphology may largely be due to this sort of scaffolding role. This idea is supported by the observation that overnight expression of either active PAK1 H83/86L or its kinase-inactive form both promote lamellipodia formation and membrane ruffling. The reduced efficiency of kinase-active PAK1 H83/86L in this regard (compare Figs. 4 and 6) probably reflects a certain degree of focal adhesion disassembly resulting from its modestly activated kinase activity.

The disassembly of focal adhesions and actin stress fibers caused by PAK1 kinase activity may also require sustained interaction with other proteins. This notion is supported by the finding that mutation of prolines 209 and 220 to alanine, which disrupts two potential SH3-binding sites, reduces the efficiency of cytoskeletal disassembly mediated by PAK1. These interactions are likely to be specific for PAK1, since the two potential SH3-binding sites containing prolines 209 and 220 are not conserved among PAK isoforms. The identity of the interacting protein (or proteins) is presently unknown.

The ability of PAK1 to cause remodeling of cytoskeletal structures points to a role in regulating processes such as cell motility. A migrating cell must be able to form filopodia and lamellipodia at its leading edge, as well as disassemble cell-substrate contacts at the retracting edge. This requires both localized assembly and disassembly of focal adhesions. The ability of PAK1 itself to promote focal adhesion disassembly as well as PAK1-associated proteins (such as PIX) to promote lamellipodia formation is likely to be crucial to these processes. As an effector of Rac and Cdc42h2a, PAK1 activity probably counteracts the actions of RhoA effectors such as ROCK (22-25). Interestingly, coexpression of constitutively active ROCK, which itself promotes focal adhesion assembly and stress fiber formation, can partially inhibit cell collapse caused by overexpression of active PAK1 (data not shown). Future studies will be needed to determine the precise nature of interplay between Rac, Cdc42hs, and Rock effectors in these processes.

Acknowledgments—We thank Marife Dy for help with two-hybrid analysis. We also thank Megan Robinson for helpful suggestions and Tina Arikan for assistance with manuscript preparation.

REFERENCES
1. Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S., and Lim, L. (1994) Nature 367, 40–46
2. Manser, E., Chong, C., Zhao, Z.-S., Leung, T., Michael, G., Hall, C., and Lim, L. (1995) J. Biol. Chem. 270, 25070–25078
3. Teo, M., Manser, E., and Lim, L. (1995) J. Biol. Chem. 270, 25090–25097
4. Martin, G. A., Bolag, G., McCormick, F., and Abe, A. (1995) EMBO J. 14, 1970–1978
5. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) J. Biol. Chem. 270, 22731–22737
6. Manser, E., Huang, H. Y., Loo, T. H., Chen, Q., Dong, J. M., Leung, T., and Lim, L. (1997) Mol. Cell. Biol. 17, 1129–1143
7. Galislo, M. L., Chernoff, J., Su, Y.-C., Seshchinsky, R., and Schlessinger, J. (1996) J. Biol. Chem. 271, 21007–21010
8. Bokoch, G. M., Wang, Y., Bohl, B. P., Sellers, M. A., Quilliam, L. A., and Knaus, U. G. (1996) J. Biol. Chem. 271, 25746–25754
9. Manser, E., Lee, T. H., Koh, C. G., Zhao, Z.-S., Chen, Q., Tan, L., Tan, L., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 183–192
10. Frost, J., Xu, S., Hutchison, M., Marcus, S., and Cobb, M. H. (1996) Mol. Cell. Biol. 16, 3707–3713
11. Frost, J. A., Steen, H., Shapiro, P. S., Lewis, T., Shaw, P., Ahn, N., and Cobb, M. H. (1997) EMBO J. 16, 6426–6438
12. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. (1995) J. Biol. Chem. 270, 25796–25798
13. Brown, J., Stowers, L., Baer, M., Trejo, J., Coulighin, S., and Chant, J. (1996) Curr. Biol. 6, 598–605
14. Rudel, T., and Bokoch, G. M. (1997) Science 276, 1571–1574
Effects of PAK1 Mutations on Cytoskeletal Organization

15. Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., and Williams, L. T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13642–13647
16. Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, J. (1997) Curr. Biol. 7, 202–210
17. Zhao, Z.-S., Manser, E., Chen, Q., Chong, C., Leung, T., and Lim, L. (1998) Mol. Cell. Biol. 18, 2153–2163
18. Pawson, T., and Scott, J. D. (1997) Science 278, 2075–2080
19. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
20. Burbelo, P. D., Drechsel, D., and Hall, A. (1995) J. Biol. Chem. 270, 29071–29074
21. Daniels, R. H., Hall, P. S., and Bokoch, G. M. (1996) EMBO J. 15, 1885–1893
22. Leung, T., Chen, Q., Manser, E., and Lim, L. (1996) Mol. Cell. Biol. 16, 5313–5327
23. Amano, M., Chihara, K., Kimura, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K. (1997) Science 275, 1308–1311
24. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) EMBO J. 15, 1885–1893
25. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) EMBO J. 15, 2298–2306